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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO INVENTARIO

DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

PCT

SOLICITUD

PATENTE DE INVENCION

ENTRADA EN FASE NACIONAL

21. **EXPEDIENTE No.** 07-133662

54. **TITULO** *USO DE VEGF PARA CICATRIZACIÓN DE HERIDAS*

51. **CLASIFICACION INTERNACIONAL**

71. **SOLICITANTE** GENENTECH, INC.

DOMICILIO SOUTH SAN FRANCISCO, CA, ESTADOS UNIDOS DE AMÉRICA

74. **APODERADO** JUAN PABLO CADENA SARMIENTO

22. **SANTAFE DE BOGOTA, D.C.** 507 31/02/08

33

(FORMA P 10)

Oficina de Comunicaciones

PETITORIO

AS: 105.782

Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 07-133662- -00000-0000
Fecha: 2007-12-18 16:45:49 Dep. 2020 NUECREACIONI
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 296

FORMULARIO ÚNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

17 ene 2008

1 SOLICITUD DE:

☒ Patente de Invención. ☐ Patente de Modelo de Utilidad.

☒ Capítulo I. ☐ Capítulo II.

2 **SOLICITANTE (71)**	Nombre: **GENENTECH, INC.** Dirección: 1 DNA Way, South San Francisco, California 94080-4990, Estados Unidos de América Nacionalidad o Domicilio: Estados Unidos de América Lugar de Constitución: Estados Unidos de América Teléfono: Fax: E-mail	**IDENTIFICACIÓN** C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Cual_____ Número _____
3 **REPRESENTANTE O APODERADO**	Nombre: JUAN PABLO CADENA SARMIENTO Dirección: Cra. 7 No. 71-21 Torre B – Piso 4, Bogotá, Colombia. Teléfono: 744-2200 Fax: 742-2200 E-mail:	**IDENTIFICACIÓN** C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Cual _____ Número 80.410.337 TP 57492
4 **INVENTOR (ES) (72)**	**Nombre:** Timothy J. BREEN, Stuart BUNTING, Charles P. SEMBA. **Dirección:** 432 Coronado Avenue, Half Moon Bay, California 94019, US; 220 Miramontes Avenue, Half Moon Bay, California 94019, US; 1925 Byron Street, Palo Alto, California 94301, US (Respectivamente). **Nacionalidad o Domicilio:** Estados Unidos de América (Respectivamente).	

5 PCT (86)
Solicitud Internacional No. PCT/US2006/023318 Fecha 16-06-2006
Publicación Internacional No WO 2006/138468 A3 Fecha 28-12-2006

6 Título (54): USO DE VEGF PARA CICATRIZACIÓN DE HERIDAS

7 Clasificación Internacional (51): A61K38/19; A61P17/02

8 Prioridad

SI ☒ NO ☐

(33) País de Origen	(32) Fecha	(31) Número de Solicitud
US	17-06-2005	60/691,909
US	21-04-2006	60/794,008

9 Comprobante de pago No. 07- 87,780 **Fecha** 18-12-2007

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

2

10 **ANEXOS**

- [x] Comprobante de pago de la tasa de presentación de la solicitud. $482.000
- [x] Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- [x] Poderes, si fuere el caso. **Fotocopia autenticada**
- [ ] Documento de cesión del inventor al solicitante o a su causante.
- [ ] Declaraciones.
- [x] Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- [x] Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- [ ] Copia del examen preliminar efectuado por la IPEA.
- [ ] Traducción del examen preliminar efectuado por la IPEA.
- [ ] De ser el caso, copia del contrato de acceso.
- [ ] De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- [ ] De ser el caso, certificado de depósito del material biológico.
- [ ] Arte final 12x12.
- [ ] De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- [x] Otros: Tarjeta de Propietarios
  Documentos PCT

11 FIGURA CARACTERÍSTICA:

12 Solicito la concesión de la patente,

NOMBRE: JUAN PABLO CADENA SARMIENTO

FIRMA: Juan Pablo Cad

C.C. 80.410.337 de Usaquén TP 57492 del C.S.J.

Instrucciones para la presentación de los anexos, ver reverso de esta página.

As. 105782

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 07 - 87,780
FECHA : OCTUBRE 25 DE 2007

SUPERINTENDENCIA DE INDUSTRIA Y COMERC O

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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 296

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
BRIGARD & CASTRO LTDA	CONSIGNACION	BANCO POPULAR	050-00110-6	64224566	25/10/2007	28,796,000.00

***** C O N C E P T O *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	482,000.00
		TOTAL : $	482,000.00

SON: CUATROCIENTOS OCHENTA Y DOS MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No._____

105782

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(51) International Patent Classification:
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(21) International Application Number:
PCT/US2006/023318

(22) International Filing Date: 16 June 2006 (16.06.2006)

(25) Filing Language: English

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(30) Priority Data:

60/691,909	17 June 2005 (17.06.2005)	US
60/794,008	21 April 2006 (21.04.2006)	US

(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 Dna Way, Ms 49, South San Francisco, California 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BREEN, Timothy J.** [US/US]; 432 Coronado Avenue, Half Moon Bay, California 94019 (US). **BUNTING, Stuart** [GB/US]; 220 Miramontes Avenue, Half Moon Bay, California 94019 (US). **SEMBA, Charles P.** [US/US]; 1925 Byron Street, Palo Alto, California 94301 (US).

(74) Agents: **PLEASURE, Irene** et al.; c/o GENENTECH, INC., 1 Dna Way, Ms 49, South San Francisco, California 94080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
12 July 2007

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/138468 A3

(54) Title: USE OF VEGF FOR WOUND HEALING

(57) Abstract: Methods for accelerating and/or improving wound healing in a subject by administering vascular endothelial growth factor (VEGF) are provided.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/023318

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/19 A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENENTECH WEB PAGE: "PRO-128115 diabetic foot ulcer" 18 May 2004 (2004-05-18), XP002404950 Retrieved from the Internet: URL:www.prous.com> the whole document	1-25
X	KR 2002 020 564 A (GOODGENE INC [KR]) 15 March 2002 (2002-03-15) abstract & DATABASE HCAPLUS abstract	1-7,12, 15,16, 18,19,24

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 2007	18/05/2007
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Fayos, Cécile

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/023318

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/46249 A (RADIX ORGANIZATION INC [US]; CHEMO PATHOLOGY RESOUR CENTER [US]) 22 October 1998 (1998-10-22) claims 1-15	1-6,8, 10, 12-18, 24,25
Y	the whole document examples 1-3	1-25
X	US 2003/180259 A1 (BREM HAROLD [US]) 25 September 2003 (2003-09-25) claims 1,12,13 page 1, paragraph 9 page 4, paragraph 42-44	1-6, 8-19,24, 25
Y	the whole document	1-25
X	"TOP 10 BIOPHARMA" July 2004 (2004-07), pages 110-112, XP002404951 Retrieved from the Internet: URL:www.contractpharma.com> [retrieved on 2006-10-27]	1-6, 10-14, 24,25
Y	page 110, column 2	1-25
X	US 6 479 654 B1 (BAIRD ANDREW [US] ET AL) 12 November 2002 (2002-11-12) column 5, line 34 - line 46 column 9, line 34 - line 39 figure 1	1-6, 12-16,25
Y	the whole document	1-25

2

International application No.
PCT/US2006/023318

INTERNATIONAL SEARCH REPORT

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/023318

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 2002020564 A		NONE	
WO 9846249 A	22-10-1998	EP 1005355 A1	07-06-2000
US 2003180259 A1	25-09-2003	NONE	
US 6479654 B1	12-11-2002	US 2003144200 A1	31-07-2003

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WO 2006/138468 A2

(51) International Patent Classification:
A61K 38/19 (2006.01) *A61P 17/02* (2006.01)

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(25) Filing Language: English

(26) Publication Language: English

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60/691,909 17 June 2005 (17.06.2005) US
60/794,008 21 April 2006 (21.04.2006) US

(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 Dna Way, Ms 49, South San Francisco, California 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BREEN, Timothy J.** [US/US]; 432 Coronado Avenue, Half Moon Bay, California 94019 (US). **BUNTING, Stuart** [GB/US]; 220 Miramontes Avenue, Half Moon Bay, California 94019 (US). **SEMBA, Charles P.** [US/US]; 1925 Byron Street, Palo Alto, California 94301 (US).

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Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/138468 A2

(54) Title: WOUND HEALING

(57) Abstract: Methods for accelerating and/or improving wound healing in a subject by administering vascular endothelial growth factor (VEGF) are provided.

Wound Healing

RELATED APPLICATION

[0001] This application claims priority to and the benefit of United States Provisional Application Serial No. 60/691,909, filed June 17, 2005, and United States Provisional Application Serial No. 60/794,008, filed April 21, 2006, the specifications of which are incorporated herein in their entirety.

FIELD OF THE INVENTION

[0002] The invention relates to methods of accelerating or improving wound healing by administering vascular endothelial growth factor (VEGF).

BACKGROUND

[0003] Wound healing is a complex process, involving an inflammation phase, a granulation tissue formation phase, and a tissue remodeling phase. Singer and Clark, Cutaneous Wound Healing, *N. Engl. J. Med.* 341:738-46 (1999). These events are triggered by cytokines and growth factors that are released at the site of injury. Many factors can complicate or interfere with normal adequate wound healing. For example, such factors include age, infection, poor nutrition, immunosuppression, medications, radiation, diabetes, peripheral vascular disease, systemic illness, smoking, stress, etc.

[0004] For patients with diabetes, which is a chronic, debilitating disease that will affect approximately 20 million people in the United States in 2005, development of a diabetic foot ulcer (also referred to as a wound) is a common complication. A chronic ulcer is defined as a wound that does not proceed through an orderly and timely repair process to produce anatomic and functional integrity (*see, e.g.*, Lazarus et al., Definitions and guidelines for assessment of wounds and evaluation of healing, *Arch. Dermatol.* 130:489-93 (1994)). By its nature, the diabetic foot ulcer is a chronic wound (American Diabetes Association, Consensus development conference on diabetic foot wound care, *Diabetes Care*, 22(8):1354-60 (1999)). Because the skin serves as the primary barrier again the environment, an open refractory wound can be catastrophic; a major disability (including limb loss) and even death can result. Foot ulceration is the precursor to about 85% of lower extremity amputations in persons with diabetes. *See, e.g.*, Apelqvist, et al., What is the most effective way to reduce

incidence of amputation in the diabetic foot? *Diabetes Metab Res. Rev.*, 16(1 Suppl.): S75-S83 (2000).

[0005] It has been reported that there are over thirty-five million cutaneous wounds requiring intervention annually in the US. *See, e.g.*, Tonnesen et al., Angiogenesis in Wound Healing *JID Symposium Proceedings* 5(1):40-46 (2000). Current wound care therapies have not been very successful due to their disappointing efficacy and to their cost. Thus, there is a need to enhance and optimize wound healing therapies for subjects. The present invention addresses these and other needs, as will be apparent upon review of the following disclosure.

SUMMARY

[0006] Methods for accelerating the healing of wounds, e.g., acute (e.g., burn, surgical wound, etc.) or chronic (e.g., diabetic ulcer, pressure ulcer, a decubitus ulcer, a venous ulcer, etc.), or normal, are provided. Methods for improving wound healing along and reducing the amount of recurrences of ulcers with the administration of vascular endothelial growth factor (VEGF) are also provided. Methods include, e.g., a method of accelerating wound healing in a subject, where a method comprises administering an effective amount of VEGF to a wound, where the administration of the effective amount of VEGF accelerates wound healing greater than 50%, or equal to or greater than 60%, equal to or greater than 70%, equal to or greater than 74%, equal to or greater than 75%, equal to or greater than 80%, equal to or greater than 85%, equal to or greater than 90%, equal to or greater than 95%, equal to or greater than 100%, equal to or greater than 110% or more, when compared to a control. A control includes, but is not limited to, e.g., a subject who is not administered treatment, or a subject who is administered sub-therapeutic amount of VEGF, or a subject who is administered another wound treatment, or a subject who is administered a placebo, either with or without Good Wound Care (GWC), or a subject who is administered GWC alone. GWC can include, but is not limited to, e.g., debridement, cleaning/dressings, pressure relief, infection control, and/or combinations thereof. In one embodiment, a method of accelerating wound healing in a human subject includes administering an effective amount of VEGF to a wound, wherein the administration of the effective amount of VEGF accelerates wound healing greater than 60% when compared a control and wherein the wound is present on the subject for about 4 weeks or more before administering the effective amount of VEGF. In one embodiment, a method of accelerating wound healing in a human subject includes administering an effective amount of rhVEGF165 to a diabetic wound, where the administration of the effective amount of rhVEGF165 accelerates wound healing

greater than 60% when compared a control. In certain embodiments, a VEGFR agonist can be used in place of or with VEGF in the methods.

[0007] Assessment of wound healing can be determined, e.g., by the % reduction in the wound area, or complete wound closure. The wound area can be determined by quantitative analysis, e.g., area measurements of the wound, planimetric tracings of the wound, etc. Complete wound closure can be determined by, e.g., skin closure without drainage or dressing requirements. Photographs of the wound, physical examinations of the wound, etc. can also be used to assess wound healing. Acceleration of wound healing can be expressed in terms of % acceleration or expressed in terms of a Hazard ratio as a time to healing (e.g., VEGF verses a control, e.g., a placebo), etc. In certain embodiments of the invention, the Hazard ratio (HR) is greater than or equal to 1.75, or greater than or equal to 1.8, or greater than or equal to 1.85, or greater than or equal to 1.87, or greater than or equal to 1.9, or greater than or equal to 1.95, or greater than or equal to 1.98, or greater than or equal to 2.0, or greater than or equal to 2.1 or more.

[0008] In one embodiment, the wound further comprises an infection. In another embodiment, the wound is an ischemic wound. In one embodiment, the wound area before treatment is about 0.4 cm^2 or more, or about 1.0 cm^2 or more, or between about 1.0 cm^2 and about 10.0 cm^2, or between about 1.0 cm^2 and about 6.5 cm^2, or between about 1.0 cm^2 and about 5.0 cm^2. In a further embodiment, the wound area is determined before treatment with VEGF but after debridement. In one embodiment, the wound is present on the subject for about 4 weeks or more, or about 6 weeks or more, before administering the VEGF. In certain aspects of the invention, the subject is or has undergone a treatment, where the treatment delays or provides ineffective wound healing. In another embodiment, the subject has a secondary condition, wherein the secondary condition delays or provides ineffective wound healing. In a further embodiment, the secondary condition is diabetes.

[0009] In one embodiment, the VEGF administered is $VEGF_{165}$ (e.g., recombinant human VEGF (e.g., human $VEGF_{165}$)). In one embodiment, the VEGF is administered topically. In certain embodiments, VEGF is administered in combination with other factors that accelerate wound healing (e.g., angiogenesis factor or agent, wound healing agent or procedure, growth factor, etc.). The VEGF can be formulated in, e.g., a slow-release formulation, a gel formulation, a bandage or dressing, etc. In certain embodiments, the subject is a human. In one embodiment, the effective amount of VEGF administered is about 20 $\mu g/cm^2$ to about 250 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 24 $\mu g/cm^2$, or 24 $\mu g/cm^2$. In certain embodiments, the effective amount administered

is about 72 $\mu g/cm^2$, or 72 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 216 $\mu g/cm^2$, or 216 $\mu g/cm^2$. In one embodiment, the effective amount of VEGF administered is 20 $\mu g/cm^2$ to 250 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 24 $\mu g/cm^2$ to about 216 $\mu g/cm^2$, or 24 $\mu g/cm^2$ to 216 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 24 $\mu g/cm^2$ to about 72 $\mu g/cm^2$, or 24 $\mu g/cm^2$ to 72 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 72 $\mu g/cm^2$ to about 216 $\mu g/cm^2$, or 72 $\mu g/cm^2$ to 216 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 216 $\mu g/cm^2$ to about 250 $\mu g/cm^2$, or 216 $\mu g/cm^2$ to 250 $\mu g/cm^2$.

[0010] The administration of the effective amount of VEGF can be daily or optionally a few times a week, e.g., at least twice a week, or at least three times a week, or at least four times a week, or at least five times a week, or at least six times a week. In one embodiment, VEGF is administered for at least six weeks, or greater than six weeks, or at least about twelve weeks, or until complete wound closure (e.g., which can be determined by skin closure without drainage or dressing requirements). In one embodiment, VEGF is administered for less than 20 weeks for one treatment course.

[0011] Methods of the invention also include a method of improving wound healing in a population of subjects. For example, a method comprises administering an effective amount of VEGF to a wound of a subject of the population, where the administration of the effective amount of VEGF results in greater than 10% (or greater than 12%, or 14%, or 15%, or 17%, or 20%, or 25%, or 30%, or 33%, or 35%, or 40%, or 45%, or 50% or more) improvement in wound healing in the population compared to a control population. For example, a control population includes, but is not limited to, e.g., subjects who are not administered treatment, or subjects who are administered sub-therapeutic amount of VEGF, or subjects who are administered another wound treatment, or subjects who are administered a placebo, either with or without Good Wound Care (GWC), or subjects who are administered GWC alone. In one embodiment, improved wound healing is assessed by complete wound healing. In certain embodiments of the invention, the population includes subjects with impaired wound healing. In one embodiment, the population is diabetic patients with chronic wounds, e.g., for about 4 weeks or more before treatment.

[0012] Methods for reducing the recurrence of ulcers are also provided by the invention. For example, a method comprises administering an effective amount of VEGF to an ulcer, where the incidence of ulcer formation is reduced with VEGF administration compared to a control. For example, a control includes, but is not limited to, e.g., a subject

who is not administered treatment, or a subject who is administered sub-therapeutic amount of VEGF, or a subject who is administered another wound treatment, or a subject who is administered a placebo, either with or without Good Wound Care (GWC), or a subject who is administered GWC alone.

BRIEF DESCRIPTION OF THE FIGURES

[0013] **Fig. 1** illustrates a study design, e.g., VGF 2763g, for administering rhVEGF for the treatment of diabetic wounds.

[0014] **Fig. 2** illustrates dose-response curve of the addition of rhVEGF in a rabbit ischemic ear wound model at day 14.

[0015] **Fig. 3** illustrates a does-response curve of the addition of rhVEGF in a diabetic mouse model at day 8.

DETAILED DESCRIPTION

[0016] **Definitions**

[0017] Before describing the invention in detail, it is to be understood that this invention is not limited to particular compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a molecule" optionally includes a combination of two or more such molecules, and the like.

[0018] The term " VEGF" (also referred to as VEGF-A) as used herein refers to vascular endothelial cell growth factor protein. The term "human VEGF" (also referred to as human VEGF-A) as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 145-, 183- 189-, and 206-, (and other isoforms) amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

[0019] A "native sequence" polypeptide comprises a polypeptide having the same amino acid sequence as a polypeptide derived from nature. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring polypeptide from any mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically

encompasses naturally occurring truncated or secreted forms of the polypeptide (e.g., an extracellular domain sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally occurring allelic variants of the polypeptide.

[0020] A polypeptide "variant" means a biologically active polypeptide having at least about 80% amino acid sequence identity with the corresponding native sequence polypeptide, or fragment thereof. Such variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- and/or C-terminus of the polypeptide. Ordinarily, a variant will have at least about 80% amino acid sequence identity, or at least about 90% amino acid sequence identity, or at least about 95% or more amino acid sequence identity with the native sequence polypeptide, or fragment thereof. Analogues or variants are defined as molecules in which the amino acid sequence, glycosylation, or other feature of native VEGF has been modified covalently or noncovalently.

[0021] The term "VEGF variant" as used herein refers to a variant as described above and/or an VEGF which includes one or more amino acid mutations in the native VEGF sequence. Optionally, the one or more amino acid mutations include amino acid substitution(s). VEGF and variants thereof for use in the invention can be prepared by a variety of methods well known in the art. Amino acid sequence variants of VEGF can be prepared by mutations in the VEGF DNA. Such variants include, for example, deletions from, insertions into or substitutions of residues within the amino acid sequence of VEGF, e.g., a human amino acid sequence encoded by the nucleic acid shown in 5,332,671; 5,194,596; or 5,240,848. Any combination of deletion, insertion, and substitution may be made to arrive at the final construct having the desired activity, e.g., VEGF activity, e.g., accelerating wound healing. The mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. EP 75,444A. VEGF variants can be assessed for VEGF activity, e.g., by a cell proliferation assay. For example, a cell proliferation assay includes increasing the extent of growth and/or reproduction of the cell relative to an untreated cell or a reduced treated cell either in vitro or in vivo. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to a molecule of interest. The extent of proliferation can be quantified via microscopic examination of the degree of confluence. Cell proliferation can also be quantified using the thymidine incorporation assay.

[0022] The VEGF variants optionally are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the native VEGF or phage display techniques, thereby

producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture.

[0023] While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed VEGF variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well-known, such as, for example, site-specific mutagenesis. Preparation of the VEGF variants described herein can be achieved by phage display techniques, such as those described in the PCT publication WO 00/63380.

[0024] After such a clone is selected, the mutated protein region may be removed and placed in an appropriate vector for protein production, generally an expression vector of the type that may be employed for transformation of an appropriate host.

[0025] Amino acid sequence deletions generally range from about 1 to 30 residues, optionally 1 to 10 residues, optionally 1 to 5 or less, and typically are contiguous.

[0026] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions of from one residue to polypeptides of essentially unrestricted length as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the native VEGF sequence) may range generally from about 1 to 10 residues, optionally 1 to 5, or optionally 1 to 3. An example of a terminal insertion includes a fusion of a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus to facilitate the secretion from recombinant hosts.

[0027] Additional VEGF variants are those in which at least one amino acid residue in the native VEGF has been removed and a different residue inserted in its place. Such substitutions may be made in accordance with those shown in Table 1. VEGF variants can also comprise unnatural amino acids as described herein.

[0028] Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in *Biochemistry*, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

[0029] Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Table 1

Original Residue	**Exemplary Substitutions**	**Preferred Substitutions**
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe

Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

[0030] "Naturally occurring amino acid residues" (i.e. amino acid residues encoded by the genetic code) may be selected from the group consisting of: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); isoleucine (Ile): leucine (Leu); lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val). A "non-naturally occurring amino acid residue" refers to a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues(s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include, e.g., norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman et al. *Meth. Enzym.* 202:301-336 (1991) & US Patent application publications 20030108885 and 20030082575. Briefly, these procedures involve activating a suppressor tRNA with a non-naturally occurring amino acid residue followed by in vitro or in vivo transcription and translation of the RNA. *See, e.g.,* US Patent application publications 20030108885 and 20030082575; Noren et al. *Science* 244:182 (1989); and, Ellman et al., *supra.*

[0031] "Percent (%) amino acid sequence identity" herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a selected sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are obtained as described below by using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087, and is publicly available

through Genentech, Inc., South San Francisco, California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

[0032] For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

[0033] The term "VEGF receptor" or "VEGFR" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind VEGF.

[0034] The term "VEGFR agonist" refers to a molecule that can activate a VEGF receptor or increase its expression. VEGFR agonists include, but are not limited to, e.g., ligand agonists of a VEGFR, VEGF variants, antibodies and active fragments. VEGF is a VEGFR agonist, but herein it is separately listed and referred to. The term "Anti-VEGFR antibody" is an antibody that binds to VEGFR with sufficient affinity and specificity. In one embodiment, the anti-VEGFR agonist antibody of the invention can be used as a therapeutic agent in treating wounds. In another embodiment, a VEGF variant can be used as a therapeutic agent in treating wounds.

[0035] The term "antibody" is used in the broadest sense and includes monoclonal antibodies (including full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

[0036] Unless indicated otherwise, the expression "multivalent antibody" is used to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is typically engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

[0037] "Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind

antigen. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (e.g. single chain Fv; scFv) (Bird et al., *Science* 242:423-426 (1988); and Huston et al., *PNAS (USA)* 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); and US Patent No. 5,641,870).

[0038] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature* 352:624-628 (1991) or Marks et al., *J. Mol. Biol.* 222:581-597 (1991), for example.

[0039] The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a

particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

[0040] "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

[0041] A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *PNAS (USA)* 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely

inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized *in vitro*). *See, e.g.,* Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.*, 147 (1):86-95 (1991); and US Pat No. 5,750,373.

[0042] Diabetes is a chronic disorder affecting carbohydrate, fat and protein metabolism in animals. Diabetes is the leading cause of blindness, renal failure, and lower limb amputations in adults and is a major risk factor for cardiovascular disease and stroke.

[0043] Type I diabetes mellitus (or insulin-dependent diabetes mellitus ("IDDM") or juvenile-onset diabetes) comprises approximately 10% of all diabetes cases. The disease is characterized by a progressive loss of insulin secretory function by beta cells of the pancreas. This characteristic is also shared by non-idiopathic, or "secondary", diabetes having its origins in pancreatic disease. Type I diabetes mellitus is associated with the following clinical signs or symptoms, e.g., persistently elevated plasma glucose concentration or hyperglycemia; polyuria; polydipsia and/or hyperphagia; chronic microvascular complications such as retinopathy, nephropathy and neuropathy; and macrovascular complications such as hyperlipidemia and hypertension which can lead to blindness, end-stage renal disease, limb amputation and myocardial infarction.

[0044] Type II diabetes mellitus (non-insulin-dependent diabetes mellitus or NIDDM) is a metabolic disorder involving the dysregulation of glucose metabolism and impaired insulin sensitivity. Type II diabetes mellitus usually develops in adulthood and is associated with the body's inability to utilize or make sufficient insulin. In addition to the insulin resistance observed in the target tissues, patients suffering from type II diabetes mellitus have a relative insulin deficiency--that is, patients have lower than predicted insulin levels for a given plasma glucose concentration. Type II diabetes mellitus is characterized by the following clinical signs or symptoms, e.g., persistently elevated plasma glucose concentration

or hyperglycemia; polyuria; polydipsia and/or hyperphagia; chronic microvascular complications such as retinopathy, nephropathy and neuropathy; and macrovascular complications such as hyperlipidemia and hypertension which can lead to blindness, end-stage renal disease, limb amputation and myocardial infarction.

[0045] "Subject" for purposes of the invention refers to any animal. Generally, the animal is a mammal. "Mammal" for purposes of invention refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, sheep, pigs, etc. Typically, the mammal is a human.

[0046] The term "accelerating wound healing" or "acceleration of wound healing" refers to the increase in the rate of healing, e.g., a reduction in time until complete wound closure occurs or a reduction in time until a % reduction in wound area occurs.

[0047] The term "effective amount" or "therapeutically effective amount" refers to an amount of a drug effective to accelerate or improve wound healing in a subject or prevent recurrence of an ulcer in a subject. A therapeutic dose is a dose which exhibits a therapeutic effect on the subject and a sub-therapeutic dose is a dose which does not exhibit a therapeutic effect on the subject treated.

[0048] Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and/or consecutive administration in any order.

[0049] "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

[0050] "Wound healing" refers a condition that would benefit from treatment with a molecule of the invention.

[0051] A "chronic wound" refers a wound that does not heal. *See, e.g.,* Lazarus et al., Definitions and guidelines for assessment of wounds and evaluation of healing, *Arch. Dermatol.* 130:489-93 (1994). Chronic wounds include, but are not limited to, e.g., arterial ulcers, diabetic ulcers, pressure ulcers, venous ulcers, etc. An acute wound can develop into a chronic wound. Acute wounds include, but are not limited to, wounds caused by, e.g., thermal injury, trauma, surgery, excision of extensive skin cancer, deep fungal and bacterial infections, vasculitis, scleroderma, pemphigus, toxic epidermal necrolysis, etc. *See, e.g.,* Buford, Wound Healing and Pressure Sores, *HealingWell.com*, published on: October 24, 2001. A "normal wound" refers a wound that undergoes normal wound healing repair.

[0052] "Good Wound Care (GWC)"refers to the steps to take care of a wound. For example, good wound care practices include, but are not limited to, one or more of the

following, debridement (e.g., surgical/sharp, mechanical, autolytic or chemical/enzymatic), cleaning (e.g., routine wound cleansing with, e.g., saline), dressings, pressure relief (e.g., off-loading pressure to the foot), maintenance of moist wound environment, and/or infection control (e.g., antibiotic ointment or pills). Other steps optionally include fitting subject with comfortable, cushioned footwear, nutritional support, maintaining blood glucose control, management of other risk factors (e.g., weight, smoking), etc. GWC can include one or more of the practices.

[0053] The expression "trauma affecting the vascular endothelium" refers to trauma, such as injuries, to the blood vessels or heart, including the vascular network of organs, to which an animal or human, preferably a mammal and most preferably a human, is subjected. Examples of such trauma include wounds, incisions, and ulcers, or lacerations of the blood vessels or heart. Trauma includes conditions caused by internal events as well as those that are imposed by an extrinsic agent that can be improved by promotion of vascular endothelial cell growth.

[0054] An "angiogenic factor or agent" is a growth factor which stimulates the development of blood vessels, e.g., promotes angiogenesis, endothelial cell growth, stability of blood vessels, and/or vasculogenesis, etc. For example, angiogenic factors, include, but are not limited to, e.g., VEGF and members of the VEGF family, PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands (Angiopoietins), ephrins, ANGPTL3, ANGPTL4, etc. It also includes factors, such as growth hormone, insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), CTGF and members of its family, and TGF-α and TGF-β. *See, e.g.,* Klagsbrun and D'Amore, *Annu. Rev. Physiol.*, 53:217-39 (1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003); Ferrara & Alitalo, *Nature Medicine* 5(12):1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 1 listing angiogenic factors); and, Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003).

[0055] **Wound Healing**

[0056] The healing a wound is a complex process that involves three major phases: inflammation, granulation tissue, and tissue remodeling. At the site of the wound there is many processes occurring, e.g., migration/contraction, matrix metalloproteases (MMP) production, proliferation, and angiogenesis. There is contraction (downsizing of the wound), epithelialization (creation of new epithelial cells) and deposition of connective tissue in order to heal the wound. *See* Singer & Clark, Cutaneous Wound Healing *N. Engl. J. Med.*, 341:738-46 (1999). One of the goals of wound therapy is to promote the granulation matrix,

where an adequate blood supply is needed. However, risk factors, often associated with diseases states, (e.g., include, but are not limited to, age, infection, poor nutrition, immunosuppression, medications, radiation, diabetes, peripheral vascular disease, systemic illness, smoking, stress, etc.) create challenges for wound healing.

[0057] Examples of some of the risk factors for diabetic foot ulcers include peripheral neuropathy, which affects both motor and sensory functions of the foot, limited joint mobility, foot deformities, abnormal distribution of foot pressure, repetitive minor trauma, and impaired visual acuity. *See, e.g.*, Boyko et al., A prospective study of risk factors for diabetic foot ulcer, The Seattle Diabetic Foot Study, *Diabetes Care*, 22:1036-42 (1999); and Apelqvist et al., International consensus and practical guidelines on the management and the prevention of the diabetic foot, International Working Group on the Diabetic Foot., *Diabetes Metab Res. Rev* 16(1 Suppl): S84-S92 (2000). Peripheral sensory neuropathy is a primary factor. Approximately 45%-60% of all diabetic ulcerations are neuropathic, while up to 45% have both neuropathic and ischemic components. With an insensate foot, the patient is unable to perceive repetitive injury to the foot caused by, e.g., poor-fitting footwear during ambulation and activities of daily living. Neuropathy, combined with altered biomechanics of walking, leads to repetitive blunt trauma and distribution of abnormally high stress loads to vulnerable portions of the foot, resulting in callus formation and cutaneous erosion. Once an ulcer is formed, it is often slow to heal, can continue to enlarge, provides an opportunity for local or systemic infection, and requires comprehensive medical and surgical care to promote healing.

[0058] There are also challenges to creating protein therapies to accelerate wound healing, e.g., accelerating healing of chronic, e.g., diabetic foot ulcers, wounds. The wound area is a hostile environment (proteolytic enzymes, naturally produced inhibitors of protein activity along with superimposed infection), where often in disease states (e.g., in diabetes) the host factors are altered (e.g., in diabetes there is suppressed VEGF expression, impaired VEGF response to hypoxia, altered cellular metabolism, suppressed immune/inflammatory response, etc.). For example, based upon in vitro studies, keratinocytes and fibroblasts from diabetic (db/db) mice exhibit selective impairment of cellular processes essential for normal tissue repair, and db/db fibroblasts show significantly decreased cellular migration and growth factor alterations. *See, e.g.*, Frank et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes, Implications for normal and impaired wound healing, *J. Biol. Chem.*, 270:12607-13 (1995); and, Lerman et al, Cellular dysfunction in the

diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia, *Am. J. Pathol.*, 162:303-12 (2003).

[0059] Provided herein are methods for accelerating and/or improving healing of wounds, by administering effective amount of VEGF or a VEGF agonist. For example, a method comprises administering an effective amount of VEGF to a wound of a subject, where the administration of the effective amount of VEGF accelerates wound healing. Methods also include a method of improving wound healing in a population of subjects. For example, a method comprises administering an effective amount of VEGF to a wound of a subject of the population, wherein the administration of the effective amount of VEGF results in greater than 10% (or greater than 12%, or 14%, or 15%, or 17%, or 20%, or 25%, or 30%, or 33%, or 35%, or 40%, or 45%, or 50% or more) improvement in wound healing in the population compared to a control. Methods for reducing the recurrence of ulcers are also provided. For example, a method comprises administering an effective amount of VEGF to an ulcer, wherein the incidence of ulcer recurrence is reduced with VEGF administration compared to a control.

[0060] Methods are also applicable to subjects who are undergoing or have undergone a treatment, where the treatment delays or provides ineffective wound healing. Treatments can include, but are not limited to, medications, radiation, treatments that results in suppressed immune systems, etc. Optionally, a subject of the invention has a secondary condition, wherein the secondary conditions delays or provides ineffective wound healing. Secondary conditions, include, but are not limited to, e.g., diabetes, peripheral vascular disease, infection, autoimmune or collagen vascular disorders, disease states that result in suppressed immune systems, etc.

[0061] Acceleration of wound healing can be described by % acceleration of wound healing and/or a Hazard ratio. In certain embodiments, the administration of the effective amount of VEGF accelerates wound healing greater than 50%, or equal to or greater than 60%, equal to or greater than 70%, equal to or greater than 74%, equal to or greater than 75%, equal to or greater than 80%, equal to or greater than 85%, equal to or greater than 90%, equal to or greater than 95%, equal to or greater than 100%, equal to or greater than 110% or more, when compared to a control. In certain embodiments, the administration of the effective amount of VEGF accelerates wound healing between greater than 60% and 110%, when compared to a control. In certain embodiments, acceleration of wound healing is described by a Hazard ration, which is equal to or greater than 1.75, or is equal to or greater than 1.80, or is equal to or greater than 1.85, or is equal to or greater than 1.95, or is equal to

or greater than 2.0, or is equal to or greater than 2.1, or is equal to or greater than 2.2, or is equal to or greater than 2.3 or more. In certain embodiments, acceleration of wound healing is described by a Hazard ration, which is between 1.75 and 2.3.

[0062] Subjects of the invention has at least one wound. The wound can be a chronic, acute or normal wound. In one embodiment, the wound being treated is a stage 1A wound. See Stages of Wounds in Table 2. A wound of the invention can optionally include an infection or ischemia, or include both an infection and ischemia. In one embodiment, the wound is a diabetic foot ulcer. In one embodiment, the wound is present on the subject for about 4 weeks or more, or about 6 weeks or more before administering the VEGF.

Table 2

	Wound Grade/Depth			
Stage/Comorbidities	**0**	**1**	**2**	**3**
A	Pre- or post-ulcerative lesion completely epithelialized	Superficial ulcer not involving tendon, capsule, or bone	Ulcer penetrating to tendon or capsule	Ulcer penetrating to bone or joint
B	With Infection	With Infection	With Infection	With Infection
C	With Ischemia	With Ischemia	With Ischemia	With Ischemia
D	With Infection and Ischemia	With Infection and Ischemia	With Infection and Ischemia	With Infection and Ischemia

[0063] Quantitative analysis can be used to assess wound healing, e.g., determining the % reduction in the wound area, or complete wound closure (e.g., measured by skin closure without drainage or dressing requirements). Wound area is assessed before, during, and after treatment by methods known to those in the art. For example, assessment can be determined by, e.g., quantitative planimetry (*see, e.g.,* Robson et al., Arch. Surg 135:773-77 (2000)), photographs, physical examinations, etc. The wound area can be determined before,

during and after treatment. In one embodiment, the wound area can be estimated by measuring the length, L, of the wound, the longest edge-to-edge length in, e.g., cm, and the width, W, the longest edge-to-edge width perpendicular to L in, e.g., cm, and multiplying the LxW to get the estimated surface area (cm^2). The size of the wound for treatment can vary. In one embodiment of the invention, the wound area before treatment is about 0.4 cm^2 or more, or about 1.0 cm^2 or more, or between about 0.4 cm^2 and about 10 cm^2, or between about 1 cm^2 and about 10 cm^2, or between about 1 cm^2 and about 6.5 cm^2, or between about 1 cm^2 and about 5 cm^2, or more than 4.0 cm^2. The area can be measured before or after debridement.

[0064] **VEGF**

[0065] An effective amount of VEGF is administered in the methods provided herein to promote accelerated or improve wound healing. The VEGF gene family, for which VEGF is a member, is one of the key regulators of the development of the vascular system. The VEGF gene family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF). *See , e.g.*, Ferrara, Role of Vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications, *Sem Oncol.*, 29(suppl 16): 10-14 (2002); and, Veikkola and Alitalo, VEGFs receptors and angiogenesis *Semin Cancer Biol.* 9:211-220 (1999). VEGF-A, also known as VEGF, is a major regulator of normal angiogenesis including normal wound healing and bone healing and abnormal angiogenesis, such as vascular proliferation in tumors and ophthalmologic disorders (e.g., age-related degeneration, diabetic retinopathy). *See, e.g.*, Ferrara, Vascular Endothelial Growth Factor: Basic Science and Clinical Progress, *Endocrine Reviews* 25(4): 581-611 (2004); Ferrara and Henzel, Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells, *Biochem, Biophys Res Comm* 161:851-58 (1989); and Leung et al., Vascular endothelial growth factor is a secreted angiogenic mitogen, *Science* 246:1306-9 (1989). It is within the scope of the invention to also use VEGF variants having VEGF activity and agonist of the VEGF receptors, e.g., VEGFR1 and/or VEGFR2 agonists, in place of or in addition to VEGF.

[0066] Human VEGF exists as at least six isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$) that arise from alternative splicing of mRNA of a single gene organized into 8 exons located on chromosome 6 (*see, e.g.*, Ferrara N, Davis Smyth T. *Endocr Rev* 18:1–22 (1997); and, Henry and Abraham, Review of Preclinical and Clinical Results with Vascular Endothelial Growth Factors for Therapeutic Angiogenesis, *Current Interventional Cardiology Reports*, 2:228-241 (2000)). *See also*, US Pat. No. 5,332,671 and

6,899,882. In one embodiment, $VEGF_{165}$ is administered in the methods of the invention. Typically, human $VEGF_{165}$ is used (e.g., recombinant human $VEGF_{165}$). $VEGF_{165}$, the most abundant isoform, is a basic, heparin binding, dimeric covalent glycoprotein with a molecular mass of ~45,000 daltons (*Id*). $VEGF_{165}$ homodimer consists of two 165 amino acid chains. The protein has two distinct domains: a receptor binding domain (residues 1-110) and a heparin binding domain (residues 110-165). The domains are stabilized by seven intramolecular disulfide bonds, and the monomers are linked by two interchain disulfide bonds to form the native homodimer. $VEGF_{121}$ lacks the heparin binding domain (*see, e.g.*, US Pat. No. 5,194,596), whereas $VEGF_{189}$ (*see, e.g.*, U.S. Pat. Nos. 5,008,196; 5,036,003; and, 5,240,848) and $VEGF_{206}$ are sequestered in the extracellular matrix. *See, e.g.*, Ferrara VEGF and the quest for tumor angiogenesis factors, *Nature Rev. Cancer* 2:795-803 (2002).

[0067] The biological effects of VEGF are mediated through high affinity tyrosine kinase receptors. Agonists of the VEGF receptors can also be used in the methods of the invention. Two VEGF receptor tyrosine kinases, VEGFR1and VEGFR2, have been identified (Shibuya et al. *Oncogene* 5:519–24 (1990); Matthews et al., *Proc Natl Acad Sci U S A* 88:9026–30 (1991); Terman et al., *Oncogene* 6:1677–83 (1991); Terman et al. *Biochem Biophys Res Commun* 187:1579–86 (1992); de Vries et al., *Science* 255:989–91 (1992); Millauer et al. *Cell* 72:835–46 (1993); and, Quinn et al. *Proc Natl Acad Sci USA* 90:7533–7 (1993)). VEGFR1 has the highest affinity for VEGF, with a Kd of ~10–20 pM (de Vries et al., *Science* 255:989–91 (1992)), and VEGFR2 has a somewhat lower affinity for VEGF, with a Kd of ~75–125 pM (Terman et al., *Oncogene* 6:1677–83 (1991); Millauer et al. *Cell* 72:835–46 (1993); and, Quinn et al. *Proc Natl Acad Sci USA* 90:7533–7 (1993)). A third tyrosine kinase receptor, VEGFR3 has been identified, which is involved in the regulation of lymphatic angiogenesis. VEGFR3 is a receptor for VEGF-C and VEGF-D, which can also bind VEGFR2. These receptors consist of an extracellular domain (including seven immunoglobulin-like regions, a transmembrane region) and an intracellular domain that contains elements related to the tyrosine kinase pathways. VEGF-B and PlGF binds to VEGFR1 but not VEGFR2. $VEGF_{165}$ also binds to neuropilin-1 a receptor that regulates neuronal cell guidance. When co-expressed with VEGFR2, neuropilin-1 enhances the binding of $VEGF_{165}$ to VEGFR2 and VEGF-mediated chemotaxis. Other studies have linked neuropilin 2 (NP2) to lymphatic vessel development. *See, e.g.*, Ferrara, Vascular Endothelial Growth Factor: Basic Science and Clinical Progress, *Endocrine Reviews*, 254(4):581-611. After binding to VEGF-A, VEGFR2 undergoes tyrosine autophosphorylation that leads to subsequent angiogenesis, increased vascular permeability, mitogenesis, and chemotaxis.

[0068] VEGF has several biologic functions, including regulation of VEGF gene expression under hypoxic conditions (Ferrara N, Davis Smyth T. *Endocr Rev* 18:1–22 (1997)), mitogenic activity for micro and macrovascular endothelial cells (Ferrara N, Henzel WJ. *Biochem Biophys Res Commun* 161:851–8 (1989); Leung et al., *Science* 246:1306–9 (1989); Connolly et al. *J Clin Invest* 84:1470–8 (1989a); Keck et al. *Science* 246:1309–12 (1989); Plouet et al., *EMBO J* 8:3801–6 (1989); Conn et al. *Proc Natl Acad Sci USA* 87:2628–32 (1990); and, Pepper et al., *Exp Cell Res* 210:298–305 (1994)), and induction of expression of plasminogen activators and collagenase (Pepper et al., *Biochem Biophys Res Commun* 181:902–6 (1991)). During hypoxia, hypoxia-inducing factor -1 (HIP-1) is upregulated and binds to the promoter region of the VEGF gene and activates transcription (see, e.g., Wang et al., Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular oxygen tension, *PNAS USA* 92:5510 (1995)). Other agonists that can up-regulate VEGF include cytokines (IL-6) and other growth factors- including EGF, PDGF, bFGF.

[0069] VEGF is produced by a wide variety of normal cell types (e.g., keratinocytes, platelets, macrophages, fibroblasts, retinal cells, ovarian cells) throughout the body in addition to various types of solid tumors. Work done over the last several years has established a key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). VEGF is a necessary growth factor for normal embryonic vasculogenesis, cardiac myocyte development (*see, e.g.,* Ferrara et al., Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene, *Nature* 380:439-42 (1996)), normal enchondral bone formation (*see, e.g.,* Gerber et al., VEGF couples hypertrophic cartilage remodeling, ossification, and angiogenesis during enchondral bone formation, *Nat. Med*, 5:623-28 (1999)), tissue repair, and in the physiology of the female reproductive tract—follicular growth and the endocrine function of the corpus luteum are dependent on proliferation of new capillaries (*see, e.g.,* Phillips et al., Vascular endothelial growth factor is expressed in rat corpus luteum, *Endocrinology*, 127:965-67 (1990)). Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol.*. 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)).

[0070] VEGF is also known as vascular permeability factor, based on its ability to induce vascular leakage in animal models. *See, e.g.,* Senger et al., Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid, *Science* 219: 983-89 (1983). Senger and colleagues proposed that an increase in microvascular permeability to proteins is a crucial step in angiogenesis. For example, induced leakage of plasma proteins and formation of extracellular fibrin gel can be sufficient matrix for endothelial cell growth; the role of mitogenic growth factors can be to boost this process. Angiogenesis is also required to allow migration of leukocytes, growth factors, and oxygen during granulation tissue formation during wound healing.

[0071] In wound healing, VEGF plays a pivotal role in the induction of angiogenesis during cutaneous wound healing. It is a potent mitogen for dermal microvascular endothelial cells and is expressed by keratinocytes of healing wounds (*See, e.g.,* Nissen et al., Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing, *Am. J. Pathol.*, 152:1445-52 (1998); Corral et al., Vascular endothelial growth factor is more important than basic fibroblast growth factor during ischemic wound healing, *Arch Surg.*, 134:200-5 (1999); Frank et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes, Implications for normal and impaired wound healing. *J. Biol. Chem.,* 270:12607-13 (1995); Ballaun et al., Human keratinocytes express the three major splice forms of vascular endothelial growth factor, *J. Invest Dermatol.,* 104:7-10 (1995). It also acts in a paracrine manner on dermal microvessels, leading to increased skin vascularity and granulation matrix formation. *See, e.g.,* Corral et al., *supra*; and, Romano de Peppe, et al., Adenovirus-mediated VEGF165 gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice, *Gen Ther.,* 9:1271-7 (2002). Deficiencies in tissue repair, e.g., wound healing, etc., are seen when VEGF levels and other angiogenic factors are altered. *See, e.g.,* Howdieshell, et al., Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation, *J. Surg. Res.*96:173-82 (2001); Street et al., Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover, *PNAS USA,* 99:9656-61 (2002); Tsou et al., Retroviral delivery of dominant-negative vascular endothelial growth factor receptor type 3 to murine wounds inhibits wound angiogenesis, *Wound Repair Regen.,* 10:222-9 (2002); Frank et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes, Implications for normal and impaired wound healing. *J. Biol. Chem.,* 270:12607-13 (1995); and, Lerman et al., Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to

hypoxia, *Am. J. Pathol.,* 162:303-12 (2003). In some animal models, exogenous VEGF promoted wound healing. *See, e.g.,* Galliano et al., Topical Vascular Endothelial Growth Factor Accelerates Diabetic Wound Healing through increased angiogenesis and by Mobilizing and recruiting bone marrow-derived cells, *American Journal of Pathology*, 164 (6):1935-1947 (2004); Romano de Peppe S et al., Adenovirus-mediated VEGF165 gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice, *Gen Ther* 9:1271-7 (2002); and, US Pat. Appl. No. US20030180259.

[0072] As described above, there are challenges to creating protein therapies to accelerating wound healing. We describe herein a clinical trial of treating ulcers with VEGF recombinant protein therapy that results in accelerated wound healing. See example 1, herein.

[0073] **Additional Agents**

[0074] It is within the scope hereof to combine VEGF therapy with one or more of, e.g., good wound care therapy (e.g., GWC), other novel or conventional therapies (e.g., other members of the VEGF family, growth factors such as listed herein, nerve growth factor (NGF), positive angiogenesis factors or agents or activators, anabolic steroids, bioengineered tissue replacements (e.g., Apligraph®, Dermagraft™, etc.) hyperbaric oxygen, vacuum therapy) for enhancing the activity of VEGF, in accelerating and/or improving wound healing. *See, e.g.,* Meier and Nanney, Emerging New Drugs for Wound Repair, *Expert Opin. Emerging Drugs* (2006) 11(1):23-37.

[0075] Six major growth factor families (EGF, FGF, IGF, PDGF, TGF, and VEGF) are associated with wound healing. See, e.g., Nagai and Embil, Becaplermin: recombinant platelet derived growth factor, a new treatment for healing diabetic foot ulcers, *Exprt Opin Biol. Ther* 2:211-18 (2002). Examples of such growth factors include platelet derived growth factor (PDGF-A, PDGF-B, PDGF-C, and PDGF-D), insulin-like growth factor I and II (IGF-I and IGF-II), acidic and basic fibroblast growth factor (aFGF and bFGF), alpha and beta transforming growth factor (TGF-α and TGF-β (e.g., TGF-beta 1, TGF beta 2, TGF beta 3)), epidermal growth factor (EGF), and others. See *Id.* These growth factors stimulate mitosis of one or more of the cells involved in wound healing and can be combined with VEGF.

[0076] Other positive angiogenesis agents that can be combined with VEGF include, but are not limited to, e.g., HGF, TNF-α, angiogenin, IL-8, etc. (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991)), angiogenesis activators in Table 3, angiogenesis factors and agents described herein, etc.

Table 3 Examples of Angiogenesis Activators

Angiogenesis
Angiopoietins 1 and 2
Tie-2
Alpha-5 integrins
Matrix metalloproteinases
Nitric oxide (NO)
COX-2
TGFbeta and receptors
VEGF and receptors

[0077] In addition, the following agents can also be combined with VEGF wound healing treatments, e.g., Platelet-derived growth factor (PDGF) (e.g., Becaplermin (rhPDGF-BB) such as Regranex ®; Johnson & Johnson (*see, e.g.,* U.S. Patent 5,457,093; 5,705,485; and, 5,427,778; Perry, BH et al., A meta-analytic approach to an integrated summary of efficacy: a case study of becamplemin gel., *Cont. Clin. Trials* 23:389-408 (2002)), adenosine-A2A receptor agonists (e.g., MRE0094 (King Pharmaceuticals)); keratinocyte growth factor (KGF-2, repifermin (Human Genome Sciences)); lactoferrin (LF) (Agennix, Inc.,); thymosine beta-4 (Tβ4 (ReGeneRx Biopharmaceuticals)); thrombin-derived activating receptor peptide (TP508; Chrysalin ® (Chrysalis Biotechnology, Inc.)); adenoviral vector encoding platelet-derived growth factor (PDGF-B) (GAM501) (Selective Genetics); autologous bone marrow stem cells (BMSC) (*see, e.g.,* Badiavas & Falanga, Treatment of chronic wounds with bone marrow-derived cells, *Arch Dermatol,* 139:510-16 (2003); and, engineered living tissue grafts (e.g., Apligraf, etc.). Antibiotic and antiseptic ulcer agents can also be combined with VEGF administration. VEGF administration can also be administered along with immunosuppressive treatment (e.g., corticosteroids, radiation therapy, chemotherapy) or cancer treatment.

[0078] It is not necessary that such cotreatment agents or procedures be included per se in the compositions of this invention, although this will be convenient where such agents are proteinaceous. Such admixtures are suitably administered in the same manner and for the same purposes as the VEGF used alone. The useful molar ratio of VEGF to such secondary therapeutic factors is typically 1:0.1-10, with about equimolar amounts in one embodiment of the invention being used.

[0079] **Dosage and Administration**

[0080] Dosages and desired drug concentrations of pharmaceutical compositions of the invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary physician. Animal experiments can provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. The use of interspecies scaling in toxicokinetics *In Toxicokinetics and New Drug Development,* Yacobi et al., Eds., Pergamon Press, New York 1989, pp. 42-96. Examples of dose-response curves for VEGF administered animal wound models can be see in **Fig. 2**, which is a dose response curve for VEGF administered to rabbit ischemic ear wounds. **Fig. 3** is a dose-response curve for VEGF administered to diabetic mouse wound. For the prevention or treatment of disease or type of wound, the appropriate dosage of VEGF will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the agent is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the agent, and the discretion of the attending physician. VEGF will be formulated and dosed in a fashion consistent with good medical practice taking into account the specific disorder to be treated, the condition of the individual patient, the site of delivery of the VEGF, the method of administration, and other factors known to practitioner.

[0081] The dosage to be employed is dependent upon the factors described herein. In certain embodiments of the invention, depending on the type and severity of the condition of the subject, about 1 μg/kg to 50 mg/kg (*e.g.* 0.1-20mg/kg) of VEGF and/or an additional agent, is a candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous application. Guidance as to particular dosages and methods of delivery is provided in the literature. In one embodiment, the effective amount of VEGF administered is about 20 $\mu g/cm^2$ to about 250 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 24 $\mu g/cm^2$, or 24 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 72 $\mu g/cm^2$, or 72 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 216 $\mu g/cm^2$, or 216 $\mu g/cm^2$. In one embodiment, the effective amount of VEGF administered is 20 $\mu g/cm^2$ to 250 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 24 $\mu g/cm^2$ to about 216 $\mu g/cm^2$, or 24 $\mu g/cm^2$ to 216 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 24 $\mu g/cm^2$ to about 72 $\mu g/cm^2$, or 24 $\mu g/cm^2$ to 72 $\mu g/cm^2$. In certain embodiments, the effective amount administered is about 72 $\mu g/cm^2$ to about 216 $\mu g/cm^2$, or

72 μg/cm^2 to 216 μg/cm^2. In certain embodiments, the effective amount administered is about 216 μg/cm^2 to about 250 μg/cm^2, or 216 μg/cm^2 to 250 μg/cm^2. The agent is suitably administered to the subject over a series of treatments or at one time.

[0082] For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs, e.g., complete closure of the wound, or reduction in wound area. However, other dosage regimens may be useful. Typically, the clinician will administered a molecule(s) of the invention until a dosage(s) is reached that provides the required biological effect. The administration of the effective amount of VEGF can be daily or optionally a few times a week, e.g., at least twice a week, or at least three times a week, or at least four times a week, or at least five times a week, or at least six times a week. In one embodiment, the VEGF is administered at least for six weeks, or at least about twelve weeks or until complete wound closure (e.g., which can be determined by skin closure without drainage or dressing requirements). In certain aspects of the invention, the VEGF is administered for less than 20 weeks. The progress of the therapy of the invention is easily monitored by conventional techniques and assays.

[0083] The therapeutic composition of the invention is typically administered topically to the subject. In one embodiment of the invention, the VEGF is in a formulation of a topical gel, e.g., in a pre-filed syringe or container. In certain embodiments, an additional therapeutic agent is also administered topically. Other routes of administration of VEGF and/or additional therapeutic agents, can also be optionally used, e.g., administered by any suitable means, including but not limited to, parenteral, subcutaneous, intraperitoneal, intrapulmonary, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, and intranasal administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration.

[0084] As described herein, VEGF can be combined with one or more additional therapeutic agents or procedures. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order. Use of multiple agents is also included in the invention. For example, VEGF may precede, follow, alternate with administration of the additional therapeutic agent, or may be given simultaneously therewith. In one embodiment, there is a time period while both (or all) active agents simultaneously exert their biological activities. In a combination therapy regimen, the compositions of the invention are administered in a therapeutically effective amount or a therapeutically synergistic amount. As used herein, a

therapeutically effective amount is such that co-administration of VEGF and one or more other therapeutic agents, or administration of a procedure, results in reduction or inhibition of the targeting disease or condition. A therapeutically synergistic amount is that amount of VEGF and one or more other therapeutic agents, e.g., described herein, necessary to synergistically or significantly accelerate and/or improve wound healing.

[0085] **Pharmaceutical Compositions**

[0086] Therapeutic formulations of molecules of the invention, e.g., VEGF or additional therapeutic agents combined with VEGF, used in accordance with the invention are prepared for storage by mixing a molecule, e.g., a polypeptide, having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[0087] In certain embodiments, the formulations to be used for *in vivo* administration are sterile. This is readily accomplished by filtration through sterile filtration membranes. The VEGF can be stored in lyophilized form or as an aqueous solution or gel form. The pH of the VEGF preparations can be about from 5 to 9, although higher or lower pH values may also be appropriate in certain instances. It will be understood that use of certain of the excipients, carriers, or stabilizers can result in the formation of salts of the VEGF.

[0088] The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate)

microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980). *See also* Johnson et al., *Nat. Med.*, 2:795-799 (1996); Yasuda, *Biomed. Ther.*, 27:1221-1223 (1993); Hora et al., *Bio/Technology*, 8:755-758 (1990); Cleland, Design and Production of Single Immunization Vaccines Using Polylactide Polyglycolide Microsphere Systems, in *Vaccine Design: The Subunit and Adjuvant Approach*, Powell and Newman, eds, (Plenum Press: New York, 1995), pp. 439-462; WO 97/03692, WO 96/40072, WO 96/07399; and U.S. Pat. No. 5,654,010.

[0089] Typically for wound healing, VEGF is formulated for site-specific delivery. When applied topically, the VEGF is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, sprays, or suspensions, with or without purified collagen. The compositions also may be impregnated into sterile dressings, transdermal patches, plasters, and bandages, optionally in liquid or semi-liquid form. An oxidized regenerated cellulose/collagen matrices can also be used, e.g., Promogran™ Matrix Wound Dressing or Promogran Prisma Matrix™.

[0090] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing a polypeptide of the invention, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), poly-lactic-coglycolic acid (PLGA) polymer, and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization

depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0091] For obtaining a gel formulation, the VEGF formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as polyethylene glycol to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrins; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. In one embodiment of the invention, the gelling agent herein is one that is, e.g., inert to biological systems, nontoxic, simple to prepare, and/or not too runny or viscous, and will not destabilize the VEGF held within it.

[0092] In certain embodiments of the invention, the polysaccharide is an etherified cellulose derivative, in another embodiment one that is well defined, purified, and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. In one embodiment, methylcellulose is the polysaccharide.

[0093] The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight polyethylene glycols to obtain the proper viscosity. For example, a mixture of a polyethylene glycol of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

[0094] The term "water soluble" as applied to the polysaccharides and polyethylene glycols is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally

sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

[0095] If methylcellulose is employed in the gel, e.g., it comprises about 2-5%, or about 3%, or about 4% or about 5%, of the gel and the VEGF is present in an amount of about 100-2000 μg per ml of gel.

[0096] VEGF and/or an additional agent can also be administered to the wound by gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In gene therapy applications, genes are introduced into cells in order to achieve in vivo synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. The oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. For general reviews of the methods of gene therapy, see, for example, Goldspiel et al. *Clinical Pharmacy* 12:488-505 (1993); Wu and Wu *Biotherapy* 3:87-95 (1991); Tolstoshev *Ann. Rev. Pharmacol. Toxicol.* 32:573-596 (1993); Mulligan *Science* 260:926-932 (1993); Morgan and Anderson *Ann. Rev. Biochem.* 62:191-217 (1993); and May *TIBTECH* 11:155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. eds. (1993) *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; and Kriegler (1990) *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY.

[0097] There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred in vivo gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau et al., *Trends in Biotechnology* 11, 205-210 (1993)). For example, in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, lentivirus, retrovirus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). Examples of using viral vectors in gene therapy can be found in Clowes

et al. *J. Clin. Invest.* 93:644-651 (1994); Kiem et al. *Blood* 83:1467-1473 (1994); Salmons and Gunzberg *Human Gene Therapy* 4:129-141 (1993); Grossman and Wilson *Curr. Opin. in Genetics and Devel.* 3:110-114 (1993); Bout et al. *Human Gene Therapy* 5:3 -10 (1994); Rosenfeld et al. *Science* 252:431-434 (1991); Rosenfeld et al. *Cell* 68:143-155 (1992); Mastrangeli et al. *J. Clin. Invest.* 91:225-234 (1993); and Walsh et al. *Proc. Soc. Exp. Biol. Med.* 204:289-300 (1993).

[0098] In some situations it is desirable to provide the nucleic acid source with an agent that targets the cells of a wound, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., *J. Biol. Chem.* 262, 4429-4432 (1987); and Wagner et al., *Proc. Natl. Acad. Sci. USA* 87, 3410-3414 (1990). For review of gene marking and gene therapy protocols see Anderson et al., *Science* 256, 808-813 (1992).

[0099] **Covalent Modifications to Polypeptides of the Invention**

[0100] Covalent modifications of a polypeptide of the invention, e.g., VEGF or other additional therapeutic polypeptide agents combined with VEGF, are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the polypeptide, if applicable. Other types of covalent modifications of the polypeptide are introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues, or by incorporating a modified amino acid or unnatural amino acid into the growing polypeptide chain, e.g., Ellman et al. *Meth. Enzym.* 202:301-336 (1991); Noren et al. *Science* 244:182 (1989); and, & US Patent applications 20030108885 and 20030082575.

[0101] Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-

chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[0102] Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is typically performed in 0.1 M sodium cacodylate at pH 6.0.

[0103] Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

[0104] Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

[0105] The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

[0106] Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[0107] Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

[0108] Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino

groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[0109] Another type of covalent modification involves chemically or enzymatically coupling glycosides to a polypeptide of the invention. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

[0110] Removal of any carbohydrate moieties present on a polypeptide of the invention may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al. Arch. Biochem. Biophys.* 259:52 (1987) and by Edge *et al. Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties, e.g., on antibodies, can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al. Meth. Enzymol.* 138:350 (1987).

[0111] Another type of covalent modification of a polypeptide of the invention comprises linking the polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

[0112] **Vectors, Host Cells and Recombinant Methods**

[0113] The polypeptides of the invention can be produced recombinantly, using techniques and materials readily obtainable.

[0114] For recombinant production of a polypeptide of the invention, e.g., VEGF or additional therapeutic polypeptide agents combined with VEGF, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: control sequences, a signal sequence, an

origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[0115] The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

[0116] Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[0117] DNA encoding the polypeptide of the invention is readily isolated and/or sequenced using conventional procedures. For example, a DNA encoding VEGF is isolated and sequenced, *e.g.*, by using oligonucleotide probes that are capable of binding specifically to the gene encoding VEGF. An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the polypeptide nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[0118]*Signal Sequence Component*

[0119] Polypeptides of the invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is typically a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the

mature protein or polypeptide. The heterologous signal sequence selected typically is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

[0120] The DNA for such precursor region is ligated in reading frame to DNA encoding the polypeptide of the invention.

[0121]Origin of Replication Component

[0122] Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

[0123]Selection Gene Component

[0124] Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

[0125] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a

protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[0126] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, typically primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

[0127] For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

[0128] Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding a polypeptide of the invention, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

[0129] A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid Yrp7 (Stinchcomb *et al., Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

[0130] In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al., Bio/Technology*, 9:968-975 (1991).

[0131]Promotor Component

[0132] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to a nucleic acid encoding a polypeptide of the

invention. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter, β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the polypeptide of the invention.

[0133] Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

[0134] Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldyhyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0135] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldyhyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

[0136] Transcription of polypeptides of the invention from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and typically Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[0137] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The

immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

[0138]Enhancer Element Component

[0139] Transcription of a DNA encoding a polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the polypeptide-encoding sequence, but is typically located at a site 5' from the promoter.

[0140]*Transcription Termination Component*

[0141] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the polypeptide of the invention. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

[0142]Selection and Transformation of Host Cells

[0143] Suitable host cells for cloning or expressing DNA encoding the polypeptides of the invention in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-

negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Typically, the *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

[0144] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide of the invention-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, *e.g., K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g., Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

[0145] Suitable host cells for the expression of glycosylated polypeptides of the invention are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

[0146] However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in

suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al., Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[0147] Host cells are transformed with the above-described expression or cloning vectors for polypeptide of the invention production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

[0148]Culturing the Host Cells

[0149] The host cells used to produce polypeptides of the invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium ((DMEM), Sigma), normal growth media for hepatocytes (Cambrex), growth media for pre-adipocytes (Cambrex), etc. are suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[0150] **Polypeptide Purification**

[0151] When using recombinant techniques, a polypeptide of the invention, e.g., VEGF or additional therapeutic polypeptide agent that is combined with VEGF, can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. Polypeptides of the invention may be recovered and/or isolated from culture medium or from host cell lysates. An "isolated" polypeptide is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In certain embodiments, the polypeptide will be purified (1) to greater than 95% by weight of polypeptide as determined by the Lowry method, or more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue, or silver stain. Isolated polypeptide includes the polypeptide in situ within recombinant cells since at least one component of the polypeptide's natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

[0152] Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, *Methods in Enzymology*, 182 (1990); Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular polypeptide of the invention produced. If membrane-bound, polypeptides of the invention can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or by enzymatic cleavage. Cells employed in expression of a polypeptide of the invention can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column, DEAE, etc.); chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of polypeptides of the invention.

[0153] For example, a VEGF composition prepared from the cells can be purified using, for example, heparin chromatography, gel electrophoresis, and dialysis. Other techniques for protein purification are also available.

[0154] **Articles of Manufacture**

[0155] In another embodiment of the invention, an article of manufacture containing materials useful for the methods and treatment of wounds described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, dressings, bandages etc. The containers may be formed from a variety of materials such as glass, plastic, nylon, cotton, polyester, etc. The container holds a composition which is effective for treating the condition and may have a sterile access port or may be a tube with multiple dosages or may be a syringe with indications of measured amounts of active agent. At least one active agent in the composition is included in the container. The label on, or associated with, the container indicates that the composition is used for accelerating or improving wound healing. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as normal saline, phosphate-buffered saline, Ringer's solution and dextrose solution, or gel solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, dressings, bandages, applicators, gauze, barriers, semi-permeable barriers, tongue depressors, needles, and syringes. Optionally, a set of instructions, generally written instructions, is included, which relates to the use and dosage of VEGF for administering to the wound described herein. The instructions included with the kit generally include information as to dosage, dosing schedule, and route of administration for the treatment the disorder. The containers of VEGF may be unit doses, bulk packages (e.g., multi-dose packages), or sub-unit doses.

[0156] The specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

EXAMPLES

[0157] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be

suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

[0158] **Example 1: Topical VEGF in wound healing VGF2763g Clinical Trial**

[0159] A double-blind (e.g., pharmacist unblinded, MD blinded and patient blinded) clinical trial was performed to determine if application of topical VEGF could promote wound healing in human subjects with diabetic ulcerations. See Table 4 for a chart of baseline disease characteristics of the patients in the study for administering rhVEGF (as referred herein as "Telbermin") for the treatment of diabetic wounds. The design of the study is indicated in **Fig. 1**.

Table 4 Baseline Disease Characteristics

	Placebo (N=26)	Telbermin (N=29)
Mean Age, y (range)	59.3 (38-81)	59.5 (42-74)
Mean Glucose, mg/dL (range)*	225.8 (77-465)	179.1 (29-593)
Mean HbA1C, % (range)†	8.4 (5.5-13.6)	8.3 (5.6-13.6)
Ulcer Debrided at Screening, N (%)		
Yes	21 (80.8)	27 (93.1)
No	5 (19.2)	2 (6.9)
Mean Ulcer Area, cm^2 (range)		
Length x Width at Screening	1.85 (1.08-2.90)	1.92 (0.96-4.08)
Planimetry at Screening ‡	1.14 (0.50-2.24)	1.35 (0.59-3.51)
Planimetry at Day 1	1.05 (0.62-2.34)	1.15 (0.44-2.97)

* One placebo-treated subject was excluded from summary because of a missing glucose value. †One telbermin-treated subject was excluded from summary because of a missing HbA1Cvalue. ‡Two placebo-treated subjects did not have baseline planimetry assessments.

[0160] 24 of the placebo treated patients completed the treatment phase and 22 completed the observation phase. 27 of the telbermin (topical recombinant VEGF) treated patients completed the treatment phase and 22 completed the observation phase. Patients with diabetes mellitus I or II (controlled, glycosylated hemoglobin Alc (HbA1c) ≤ 12%) with debrided ulcer area of ≥0.4 cm^2 and ≤ 4.0 cm^2 at day 1 that were superficial wounds (e.g., at UT stage 1a (no bone, muscle, tendon), see Table 2) were treated with either VEGF or a placebo 3 times a week for 6 weeks (18 doses total). Treatment was every 48 hours (+/- 24

hours) but no more than 3 doses per week. The amount of VEGF per treatment was 72 $\mu g/cm^2$. The VEGF was prepared on site. 0.22 ml of VEGF (5mg/ml) or the Placebo (buffer vehicle) was removed from the vial and added to about a 5% methylcellulose (e.g., 4.7%) (e.g., Methocel A4M premium methylcellulose (The Dow Chemical Company; Midland, MI) formulation in 5mM succinate buffer, pH 5.0. The VEGF or Placebo and the gel were mixed for 2 hours, which increased viscosity and reduced the loss of dosing material when applied. A final 0.06% VEGF gel (final gel 3% methylcellulose) in 5 mM, pH 5.0 succinate buffer (with, e.g., at VEGF 1.8 mg/ml, 0.0036% polysorbate 20 and 100 mM trehalose dehydrate) was the final dosing material. The final dosing material was applied with a 1.0 ml tuberculin syringe, e.g., filled with 0.6 mL of final dosing material.

[0161] Typically, the ulcer was a chronic ulcer. The ulcer duration was greater than or equal to 4 weeks to less than 6 months before treatment. There was no active infection and the subject had a perfused limb: ankle-brachial index (ABI) ≥ 0.6 and less than or equal to 1.2 on the study foot. During the treatment the two groups VEGF or placebo had good wound care practice and weekly assessments, e.g., physical examinations, planimetric tracings and/or 35-mm photographs (e.g., Food and Drug Administration (FDA) Guidance for Industry 2000, Chronic Cutaneous Ulcer and Burn Wounds-Developing Products for Treatment, June 2000).

[0162] The endpoints to be addressed were incidence of complete wound closure, which included skin closure without drainage or dressing requirements, (e.g., assessed, e.g., 3 months following closure) and accelerated wound healing, where the rate reflects a clinically meaningful diminution of time until, e.g., complete closure occurs, and a time to event analysis (e.g., time to complete closure). Primary efficacy endpoint was percent reduction in total ulcer surface area at day 43 (up to Day 49) from baseline was determined by quantitative analysis of planimetric tracings of the ulcer. Secondary efficacy endpoints included: percent reduction in total ulcer surface area at Day 29 and Day 84 compared with baseline (e.g., Day 1 value), incidence of complete ulcer healings at Days 29, 43, and 84, time (e.g., days) to complete healing of ulcer, time (e.g., days) to recurrence of ulcer formation for subject with complete ulcer healing prior to end of treatment, incidence of increased total ulcer surface area (> 15%) compared with baseline, incidence of advancing ulcer stage (e.g., > UT 1a), and microcirculatory perfusion of the ulcer bed at Days 1, 8, 22, and 43.

[0163] Safety issues that were monitored involved clinically-significant hypotension (e.g., defined as a drop of ≥ 35 mmHg in systolic blood pressure relative to predose at 60

minutes after the application of each dose of study drug during the first treatment week (days 1, 3 and 5), clinically-significant ulcer infection (e.g., defined by increased discharge and malodorous exudates from the ulcer, fever (temperature of ≥ 38.6°C), and a white blood cell (WBC) count of > 10,000μL), production of anti-VEGF antibodies, etc. Blood pressure was measured prior to each does and 60 minutes after each dose during the first week.

[0164] Total volume of gel applied for each treatment is 0.12-0.48 mL (72 μg-288 μg VEGF). The amount of gel applied was based upon wound measurements (LxW), where L is the longest edge-to-edge length in cm and W is the longest edge-to-edge width perpendicular to L in cm (LxW=estimated surface area (cm^2)). For example, the gel is applied by using a sterile tongue depressor, where the total amount of gel applied over entire surface of ulcer, was at a thickness of 1/16''. The wound is covered with sterile, semipermeable barrier (e.g., adaptec film dressing) and wrapped with cotton gauze (e.g., Kerlix) wrap. At the next treatment, the dressing is removed and the ulcer gently irrigated with sterile normal saline. Ulcer surface is measured again, the appropriate dose of gel is applied and the ulcer is redressed.

[0165] Results: Topical VEGF appears to be safe and well-tolerated. Incidence of adverse events was comparable between treatment groups (telbermin and placebo groups). None of the adverse events or serious adverse events observed were attributed to the study drug. Two patients discontinued the study due to serious adverse events (1 in the telbermin group→ infected skin ulcer; 1 in the placebo group→localized infection). There was one patient in the telbermin group who died 4 days following the last treatment, but the death was not attributed to the study drug. No cases of clinically significant hypotension were observed in either treatment group.

[0166] Data suggests evidence of biological activity. No safety signals were observed in a trial that had small ulcer sizes that were UT stage 1a. See Table 5 for a summary of the results for median % reduction in wound area, % of subjects with complete healing and time to healing. In diabetic subjects treated with VEGF for 6 weeks at 3 times per week, the population of subjects showed a 14-25% improvement in complete wound healing after 6 weeks with VEGF compared to placebo. The trial showed that VEGF had acceleration of healing of ~75-100% faster than placebo. See Table 6, which illustrates the time to first complete ulcer healing in patients treated with Telbermin (rhVEGF) or placebo. Time for complete healing of the ulcer was accelerated in patients treated with VEGF, e.g., time to complete healing (25th percentile) was 32.5 days verses 43.0 days.

Table 5

		Median % Reduction in Wound Area (total ulcer surface) (VEGF vs. Placebo)[2*]	**% Subjects with Complete Healing (VEGF vs. Placebo)[3*]**	**Time to Healing (VEGF vs. Placebo)[4*]**
Day 43	Safety	95% vs. 85%	41% vs. 27%	HR 1.75 (p=0.18)
(Week 6)	Evaluable	(p=0.67)	(p=0.39)	
	Efficacy[1]	100% vs. 88%	52% vs. 27%	HR 1.98 (p=0.12)
	Evaluable	(p=0.17)	(p=0.13)	
Day 84	Safety	100% vs. 92%	52% vs. 35%	HR 1.87 (p=0.13)
(WK 12)	Evaluable	(p=0.49)	(p=0.28)	
	Efficacy[1]	100% vs. 93%	71% vs. 38%	HR 2.10 (p=0.08)
	Evaluable	(p=0.05)	(p=0.06)	

[1] Efficacy evaluable subjects (specified prior to unblinding):
Major protocol violators removed
Subjects missing 3 consecutive doses-censored at last available dosing
No LOCF (missing data not imputed)
[2] p-value: Wilcoxon rank-sum test
[3] p-value: Fisher's exact test.
[4] p-value: Log-Rank test
*assessment method-quantitative planimetric analysis

Table 6

Time to Complete Healing*	Placebo(N=26)	Telbermin (N=29)
25th percentile, day	43.0	32.5
50th percentile, day	ND	58.0

ND = Not detectable

* Estimated using the Kaplan Meier method.

[0167] For subjects who achieved complete ulcer healing, recurrence of ulcer formation was assessed between the time of first complete ulcer healing and the time of study completion or discontinuation. Of the safety evaluable subjects who achieved complete ulcer healing, 26.7% of the telbermin-treated subjects (4 of 15) and 33.3% of the placebo-treated subjects (3 of 9) had a recurrence of ulcer formation (log-rank p-value = 0.57). The hazard ratio for recurrence of ulcer formation for telbermin-treated subjects compared with placebo-treated subjects was 0.63 (95% CI: 0.13, 3.15).

[0168] **Example 2: Topical VEGF in wound healing**

[0169] Subjects, e.g., patients with diabetes mellitus I or II, with an estimated ulcer area after sharp debridement of, e.g., ≥ 1.0 cm^2 and ≤ 6.5 cm^2 at the start of treatment, are treated with topical recombinant VEGF (e.g., gel formulation) daily for 12 weeks (for a total of up to 84 doses) total or until complete wound closure (e.g., skin closure without drainage or dressing requirements), which ever comes earlier. Subjects can be observed for 12 weeks or more after the treatment phase. Subjects receive either 24 μg/cm^2, 72 μg/cm^2, or 216 μg/cm^2 VEGF in each daily treatment. The ulcer surface area (cm^2) is estimated, e.g., by the length (L(cm)) is the longest edge-to-edge measurement of the ulcer and the width (W(cm)) is taken from a perpendicular axis to the length at the longest edge-to-edge measurement. The estimated surface area is then LxW. Treatment can be assessed by measurement of the perimeter of the ulcer area via tracings, planimetric analysis tracings of the ulcer margin, photographs, physical examinations, etc. The VEGF applied will be 1.8, 0.6 and 0.2 mg/ml of VEGF, 3% methylcellulose (e.g., Methocel A4M premium methylcellulose (The Dow Chemical Company; Midland, MI), in 5 mM, pH 5.0 succinate buffer (with, e.g., at VEGF 1.8 mg/ml, 0.0036% polysorbate 20 and 100 mM trehalose dehydrate).

[0170] The specification is considered to be sufficient to enable one skilled in the art to practice the invention. It is understood that the examples and embodiments described

herein are for illustrative purposes only. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

CLAIMS

We claim:

1. A method of accelerating wound healing in a subject, the method comprising: administering an effective amount of VEGF to a wound, wherein the administration of the effective amount of VEGF accelerates wound healing greater than 60% when compared a control.

2. The method of claim 1, wherein the acceleration of wound healing is equal to or greater than 74% when compared to the control.

3. The method of claim 1, wherein the acceleration of wound healing is assessed by % reduction in wound area.

4. The method of claim 3, wherein the wound area is about 0.4 cm^2 or more before treatment.

5. The method of claim 3, wherein the wound area is about 1.0 cm^2 or more before treatment.

6. The method of claim 1, wherein the acceleration of wound healing is assessed by rate of complete wound healing

7. The method of claim 1, wherein the wound is a diabetic foot ulcer.

8. The method of claim 1, wherein the effective amount of VEGF is administered at least three times a week.

9. The method of claim 1, wherein the effective amount of VEGF is administered at least for six weeks.

10. The method of claim 1, wherein the effective amount of VEGF is administered until there is complete wound closure.

11. The method of claim 1, wherein the VEGF is $VEGF_{165}$.

12. The method of claim 1 or 11, wherein the VEGF is recombinant human VEGF.

13. The method of claim 1, wherein the administration is topical.

14. The method of claim 1, wherein the VEGF is in a formulation for topical administration.

15. The method of claim 1, wherein the wound is a chronic wound.

16. The method of claim 1, wherein the wound is a pressure ulcer, a decubitus ulcer, a venous ulcer, a burn, a surgical wound, or a normal wound.

17. The method of claim 1, wherein the subject is undergoing or has undergone a treatment, wherein the treatment delays or provides ineffective wound healing.

18. The method of claim 1, wherein the subject has a secondary condition, wherein the secondary conditions delays or provides ineffective wound healing.

19. The method of claim 18, wherein the secondary condition is diabetes.

20. The method of claim 1, wherein the effective amount of VEGF is about 20 $\mu g/cm^2$ to about 250 $\mu g/cm^2$.

21. The method of claim 20, wherein the effective amount of VEGF is about 24 $\mu g/cm^2$.

22. The method of claim 20, wherein the effective amount VEGF is about 72 $\mu g/cm^2$.

23. The method of claim 20, wherein the effective amount VEGF is about 216 $\mu g/cm^2$.

24. The method of claim 1, wherein the subject is human.

25. A method of accelerating wound healing in a human subject, the method comprising administering an effective amount of VEGF to a wound, wherein the administration of the effective amount of VEGF accelerates wound healing greater than 60% when compared a control and wherein the wound is present on the subject for about 4 weeks or more before administering the effective amount of VEGF.

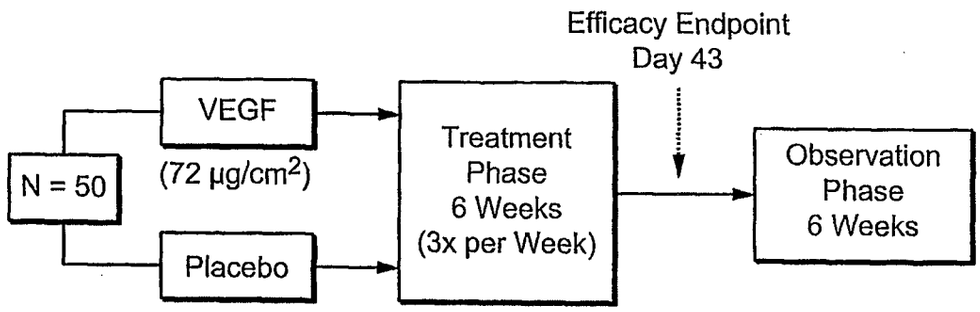

FIG. 1

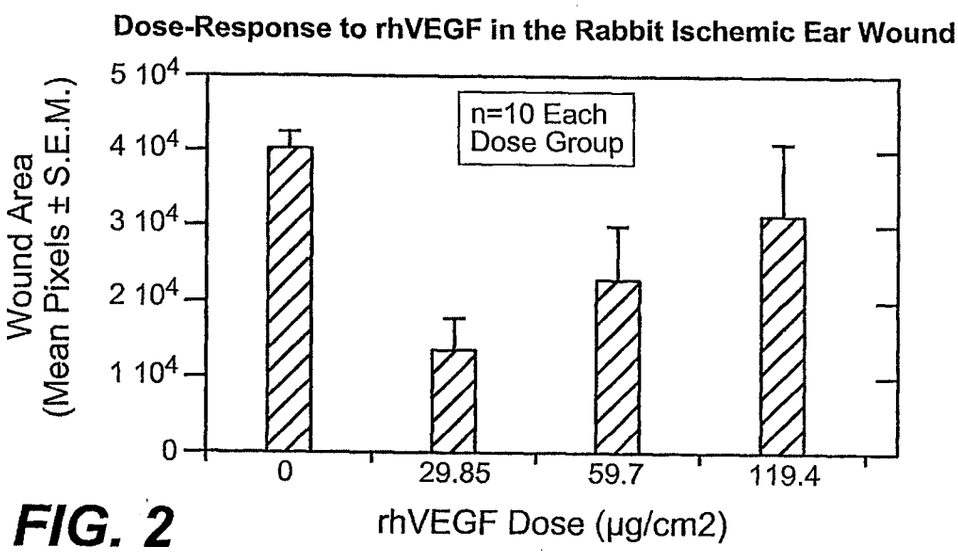

FIG. 2

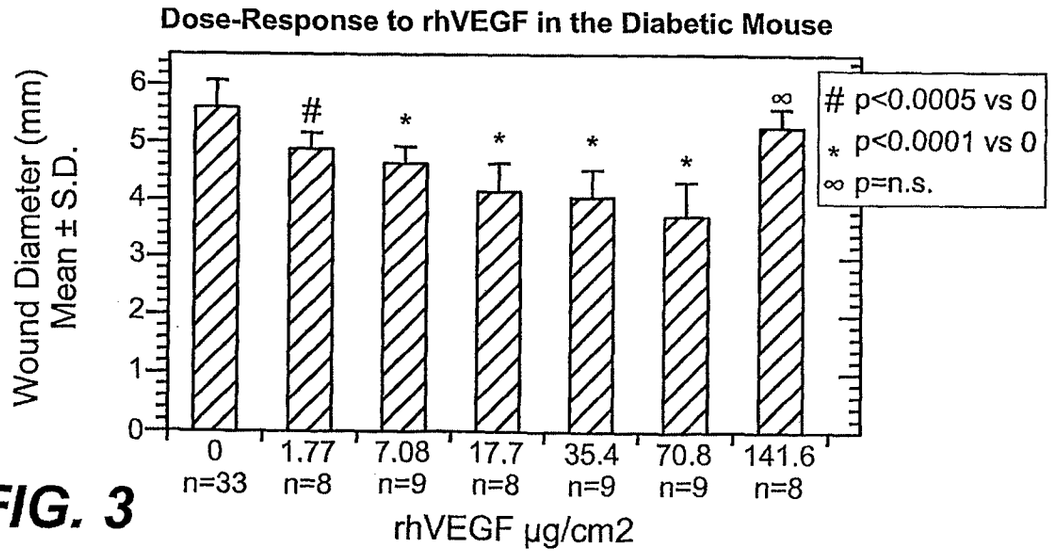

FIG. 3

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(54) Título: USO DE VEGF PARA CICATRIZACIÓN DE HERIDAS

(57) Extracto: Se proveen métodos para acelerar y/o mejorar la cicatrización de heridas en un sujeto, administrándole factor de crecimiento endotelial vascular (VEGF).

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Cicatrización de Heridas

SOLICITUD RELACIONADA

[0001] La presente solicitud reivindica prioridad a la Solicitud Provisional de los Estados Unidos serie No. 60/691,909 y el beneficio de la misma, presentada el 17 de junio de 2005, y a la Solicitud Provisional de los Estados Unidos serie No. 60/794,008, presentada el 21 de abril de 2006, cuyas especificaciones se incorporan en este caso en su integridad.

CAMPO DE LA INVENCIÓN

[0002] La invención se refiere a métodos de acelerar o mejorar la cicatrización de una herida administrando el factor de crecimiento endotelial vascular (VEGF).

ANTECEDENTES

[0003] La cicatrización de una herida es un proceso complejo, que implica una fase de inflamación, una fase de formación de tejido de granulación y una fase de remodelación del tejido. Singer and Clark, Cutaneous Wound Healing, *N. Engl. J. Med.* 341:738-46 (1999). Estos episodios se desencadenan por citocinas y factores de crecimiento que se liberan en el sitio de lesión. Muchos factores pueden complicar o interferir la cicatrización adecuada y normal de una herida. Por ejemplo, tales factores incluyen la edad, la infección, una nutrición deficiente, la inmunosupresión, los medicamentos, la radiación, la diabetes, la vasculopatía periférica, la enfermedad sistémica, el tabaquismo, el estrés, etc.

[0004] El desarrollo de una úlcera del pie diabético (también denominada herida) es una complicación común para los pacientes con diabetes, una enfermedad incapacitante crónica que afectará aproximadamente 20 millones de personas en los Estados Unidos en

2005. Una úlcera crónica se define como una herida que no sigue un proceso de reparación ordenado y oportuno para producir integridad anatómica y funcional (*véase, por ejemplo,* Lazarus et al., Definitions and guidelines for assessment of wounds and evaluation of healing, *Arch. Dermatol.* 130:489-93 (1994)). Por su naturaleza, la úlcera de pie diabético es una herida crónica (American Diabetes Association, Consensus development conference on diabetic foot wound care. *Diabetes Care,* 22(8): 1354-60 (1999)). Debido a que la piel sirve como la barrera primaria contra el ambiente, una herida abierta, resistente al tratamiento, puede ser catastrófica; puede tener como resultado una incapacidad mayor (incluida la pérdida de la extremidad) e, incluso, la muerte. La ulceración del pie es la precursora de alrededor de 85% de las amputaciones de las extremidades inferiores en personas con diabetes. *Veáse, por ejemplo,* Apelqvist, et al., What is the most effective way to reduce incidence of amputation in the diabetic foot? *Diabetes Metab Res. Rev.,* 16(1 Suppl.): S75-S83 (2000).

[0005] Se ha divulgado que hay más de treinta y cinco millones de heridas cutáneas que requieren intervención anualmente en los Estados Unidos. *Véase, por ejemplo,* Tonnesen et al., Angiogenesis in Wound Healing *JID Symposium Proceedings* 5(1):40-46 (2000). Las terapias actuales de cuidado de heridas no han sido suficientemente exitosas debido a su desalentadora eficacia y a su costo. Por consiguiente, subsiste una necesidad de mejorar y optimizar las terapias de cicatrización de heridas para ciertos sujetos. La presente invención aborda estas y otras necesidades, como se hará evidente luego de estudiar la siguiente divulgación.

SUMARIO

[0006] Se proveen métodos para acelerar la cicatrización de heridas, por ejemplo, heridas agudas (por ejemplo, quemaduras, herida

quirúrgica, etc.) o crónicas (por ejemplo, úlcera diabética, úlcera por presión, ulcera de decúbito, úlcera venosa, etc.), o normales. Se ofrecen igualmente métodos para mejorar la cicatrización de una herida, reduciendo al mismo tiempo el número de recurrencias de úlceras, con la administración de factor de crecimiento endotelial vascular (VEGF). Los métodos incluyen, por ejemplo, un método de acelerar la cicatrización de una herida en un sujeto, donde un método comprende administrar una cantidad efectiva de VEGF a una herida, donde la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida en más de 50%, o igual o mayor a 60%, igual o mayor a 70%, igual o mayor a 74%, igual o mayor a 75%, igual o mayor a 80%, igual o mayor a 85%, igual o mayor a 90%, igual o mayor a 95%, igual o mayor a 100%, igual o mayor a 110% o más, cuando se compara con un control. Un control incluye, pero no se limita a, por ejemplo, un sujeto a quien no se administra tratamiento, o un sujeto a quien se administra una cantidad sub-terapéutica de VEGF, o un sujeto a quien se administra otro tratamiento para curación de heridas, o un sujeto a quien se administra un placebo, con o sin buen manejo y cuidado de la herida (GWC), o un sujeto a quien se administra GWC solo. El GWC puede incluir, pero no se limita a, por ejemplo, desbridamiento, limpieza/vendajes, alivio de la presión, control de la infección, y/o sus combinaciones. En una incorporación, un método de acelerar la cicatrización de una herida en un sujeto humano incluye administrar una cantidad efectiva de VEGF a una herida, en donde la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida en más de 60%, cuando se compara con un control, y en donde la herida está presente en el sujeto durante alrededor de 4 semanas o más antes de administrar la cantidad efectiva de VEGF. En una incorporación, un método de acelerar la cicatrización de una herida en un sujeto humano incluye administrar una cantidad efectiva de rhVEGF165 a una herida diabética, donde la administración de la cantidad efectiva de rhVEGF165 acelera la cicatrización de una herida en más de 60%, cuando se compara con un control. En ciertas

incorporaciones, puede emplearse en los métodos un agonista VEGFR en lugar de VEGF, o con él.

[0007] La valoración de la cicatrización de una herida puede determinarse, por ejemplo, mediante el % de reducción en el área de la herida, o el cierre completo de la herida. El área de la herida puede determinarse mediante análisis cuantitativo, por ejemplo, mediciones del área de la herida, registros planimétricos de la herida, etc. El cierre completo de la herida puede determinarse mediante, por ejemplo, el cierre de la piel sin requerimientos de drenaje o vendaje. Igualmente, para valorar la cicatrización de una herida pueden utilizarse fotografías de la herida, exámenes físicos de la herida, etc. La aceleración de la cicatrización de una herida puede expresarse en términos del % de aceleración, o expresarse en términos de la relación de riesgo como tiempo para la cicatrización (por ejemplo, VEGF versus un control, por ejemplo, un placebo), etc. En ciertas incorporaciones de la invención, la relación de riesgo (HR) es mayor que o igual a 1.75, o mayor que o igual a 1.8, o mayor que o igual a 1.85, o mayor que o igual a 1.87, o mayor que o igual a 1.9, o mayor que o igual a 1.95, o mayor que o igual a 1.98, o mayor que o igual a 2.0, o mayor que o igual a 2.1 o más.

[0008] En una incorporación, la herida comprende además una infección. En otra incorporación, la herida es una herida isquémica. En una incorporación, el área de la herida antes del tratamiento es alrededor de 0.4 cm^2 o más, o alrededor de 1.0 cm^2 o más, o entre alrededor de 1.0 cm^2 y alrededor de 10.0 cm^2, o entre alrededor de 1.0 cm^2 y alrededor de 6.5 cm^2, o entre alrededor de 1.0 cm^2 y alrededor de 5.0 cm^2. En una incorporación adicional, el área de la herida se determina antes del tratamiento con VEGF, pero después del desbridamiento. En una incorporación, la herida está presente en el sujeto durante alrededor de 4 semanas o más, o alrededor de 6 semanas o más, antes de administrar el VEGF. En ciertos aspectos de la invención, el sujeto está bajo

tratamiento o se ha sometido a él, en donde el tratamiento retrasa o produce una cicatrización ineficaz de una herida. En otra incorporación, el sujeto tiene una afección secundaria, en donde la afección secundaria retrasa o produce una cicatrización ineficaz de una herida. En una incorporación adicional, la afección secundaria es diabetes.

[0009] En una incorporación, el VEGF administrado es $VEGF_{165}$ (por ejemplo, VEGF humano recombinante (por ejemplo, $VEGF_{165}$ humano)). En una incorporación, el VEGF se administra tópicamente. En ciertas incorporaciones, el VEGF se administra en combinación con otros factores que aceleran la cicatrización de una herida (por ejemplo, factor o agente de angiogénesis, agente o procedimiento de cicatrización de heridas, factor de crecimiento, etc.). El VEGF puede formularse en, por ejemplo, una formulación de liberación lenta, un formulación en gel, un vendaje o apósito, etc. En ciertas incorporaciones, el sujeto es un humano. En una incorporación, la cantidad efectiva de VEGF administrada es alrededor de 20 $\mu g/cm^2$ a alrededor de 250 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 24 $\mu g/cm^2$, o 24 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 72 $\mu g/cm^2$, o 72 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 216 $\mu g/cm^2$, o 216 $\mu g/cm^2$. En una incorporación, la cantidad efectiva de VEGF administrada es 20 $\mu g/cm^2$ a 250 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 24 $\mu g/cm^2$ a alrededor de 216 $\mu g/cm^2$, o 24 $\mu g/cm^2$ a 216 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 24 $\mu g/cm$ a alrededor de 72 $\mu g/cm^2$, o 24 $\mu g/cm^2$ a 72 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 72 $\mu g/cm^2$ a alrededor de 216 $\mu g/cm^2$, o 72 $\mu g/cm^2$ a 216 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 216 $\mu g/cm^2$ a alrededor de 250 $\mu g/cm^2$, o 216 $\mu g/cm^2$ a 250 $\mu g/cm^2$.

[0010] La administración de la cantidad efectiva de VEGF puede ser diaria o, en forma opcional, unas cuantas veces a la semana, por ejemplo, por lo menos dos veces a la semana, o por lo menos tres veces a la semana, o por lo menos cuatro veces a la semana, o por lo menos cinco veces a la semana, o por lo menos seis veces a la semana. En una incorporación, el VEGF se administra durante por lo menos seis semanas, o más de seis semanas, o por lo menos alrededor de doce semanas, o hasta el cierre completo de la herida (por ejemplo, el cual puede determinarse mediante cierre de la piel sin requerimientos de drenaje o apósitos). En una incorporación, el VEGF se administra durante menos de 20 semanas para un ciclo de tratamiento.

[0011] Métodos de la invención incluyen asimismo un método de mejorar la cicatrización de una herida en una población de sujetos. Por ejemplo, un método comprende administrar una cantidad efectiva de VEGF a una herida de un sujeto de la población, donde la administración de la cantidad efectiva de VEGF resulta en un mejora de más de 10% (o más de 12%, o 14%, o 15%, o 17%, o 20%, o 25%, o 30%, o 33%, o 35%, o 40%, o 45%, o 50% o más) en la cicatrización de una herida en la población, comparada con una población de control. Por ejemplo, una población de control incluye, pero no se limita a, por ejemplo, sujetos a los que no se administra tratamiento, o sujetos a quienes se administra una cantidad sub-terapéutica de VEGF, o sujetos a quienes se administra otro tratamiento de la herida, o sujetos a quienes se administra un placebo, con o sin buen manejo y cuidado de la herida (GWC), o sujetos a quienes se administra el GWC solo. En una incorporación, la cicatrización mejorada de una herida se evalúa por la completa cicatrización de una herida. En ciertas incorporaciones de la invención, la población incluye sujetos con cicatrización deficiente de una herida. En una incorporación, la población se compone de pacientes diabéticos con heridas crónicas, por ejemplo, durante alrededor de 4 semanas o más antes del tratamiento.

[0012] La invención provee igualmente métodos para reducir la recurrencia de úlceras. Por ejemplo, un método comprende administrar una cantidad efectiva de VEGF a una úlcera, donde la incidencia de la formación de úlcera se reduce con la administración de VEGF, comparada con un control. Por ejemplo, un control incluye, pero no se limita a, por ejemplo, un sujeto a quien no se administra tratamiento, o un sujeto a quien se administra una cantidad sub-terapéutica de VEGF, o un sujeto a quien se administra otro tratamiento de herida, o un sujeto a quien se administra un placebo, con o sin buen manejo y cuidado de la herida (GWC), o un sujeto a quien se administra GWC solo.

BREVE DESCRIPCIÓN DE LAS FIGURAS

[0013] **Fig. 1** ilustra un diseño de un estudio, por ejemplo, VGF 2763g, para administrar rhVEGF para el tratamiento de heridas diabéticas.

[0014] **Fig. 2** ilustra la curva de respuesta a la dosis de la adición de rhVEGF en un modelo de herida de oreja isquémica en un conejo el día 14.

[0015] **Fig. 3** ilustra una curva de respuesta a la dosis de la adición de rhVEGF en un modelo de ratón diabético el día 8.

DESCRIPCIÓN DETALLADA

[0016] **Definiciones**

[0017] Antes de describir la invención en detalle, hay que entender que esta invención no se limita a composiciones particulares o sistemas biológicos que, lógicamente, pueden variar. Hay que entender también que la terminología empleada en esta solicitud es con el propósito de describir incorporaciones particulares únicamente, y no pretende ser

limitante. Tal como se emplea en esta especificación y las reivindicaciones anexas, las formas del singular "uno", "una" y "el/la" incluyen referentes del plural, a menos que el contenido dicte claramente otra cosa. Por consiguiente, por ejemplo, la referencia a "una molécula" incluye opcionalmente una combinación de dos o más de tales moléculas, y similares.

[0018] El término "VEGF" (también denominado VEGF-A), tal como se emplea en esta solicitud, se refiere a la proteína factor de crecimiento celular endotelial vascular. El término "VEGF humano" (también denominado VEGF-A humano), tal como se emplea en esta solicitud, se refiere al factor de crecimiento celular endotelial humano de 165 aminoácidos, y factores de crecimiento celular endotelial vascular relacionados de 121, 145, 183, 189 y 206 (y demás isoformas) aminoácidos, como se describe por Leung *et al., Science* 246:1306 (1989), y Houck *et al., Mol. Endocrin.* 5:1806 (1991), junto con las formas alélicas, de ocurrencia natural y procesadas, de esos factores de crecimiento.

[0019] Un "polipéptido de secuencia natural" comprende un polipéptido que tiene la misma secuencia de aminoácidos que el polipéptido derivado de la naturaleza. Por lo tanto, un polipéptido de secuencia natural puede tener la secuencia de aminoácidos de cualquier mamífero. Tal polipéptido de secuencia natural puede aislarse de la naturaleza o puede producirse por medios sintéticos o recombinantes. El término polipéptido de "secuencia natural" abarca específicamente formas truncadas o segregadas de ocurrencia natural del polipéptido (por ejemplo, una secuencia de dominio extracelular), formas de variantes de ocurrencia natural (por ejemplo, formas alternativamente empalmadas) y variantes alélicas de ocurrencia natural del polipéptido.

[0020] Una "variante" de polipéptido significa un polipéptido

biológicamente activo que tiene por lo menos alrededor de 80% de identidad de secuencia de aminoácidos con el correspondiente polipéptido de secuencia natural, o su fragmento. Tales variantes incluyen, por ejemplo, polipéptidos en donde se agregan o eliminan uno o más residuos de aminoácidos, en el término N- y/o C del polipéptido. Ordinariamente, una variante tendrá por lo menos alrededor de 80% de identidad de secuencia de aminoácidos, o por lo menos alrededor de 90% de identidad de secuencia de aminoácidos, o por lo menos alrededor de 95% o más de identidad de secuencia de aminoácidos con el polipéptido de secuencia natural, o su fragmento. Análogos o variantes se definen como moléculas en las cuales la secuencia de aminoácidos, la glucosilación, u otra característica del VEGF natural se ha modificado en forma covalente o no covalente.

[0021] El término "variante de VEGF", como se emplea en esta solicitud, se refiere a una variante, como se describe anteriormente, y/o un VEGF que incluye una o más mutaciones de aminoácidos en la secuencia del VEGF natural. Opcionalmente, la una o más mutaciones de aminoácidos incluyen sustituciones de aminoácidos. El VEGF y sus variantes para uso en la invención pueden prepararse por una variedad de métodos bien conocidos en el arte. Variantes de secuencias de aminoácidos de VEGF pueden prepararse mediante mutaciones en el ADN del VEGF. Tales variantes incluyen, por ejemplo, eliminaciones, inserciones o sustituciones de residuos en la secuencia de aminoácidos de VEGF, por ejemplo, una secuencia de aminoácidos humana codificada por el ácido nucleico mostrado en 5,332,671; 5,194,596; o 5,240,848. Puede hacerse cualquier combinación de eliminación, inserción y sustitución para llegar al constructo final, que tiene la actividad deseada, por ejemplo, la actividad de VEGF, por ejemplo, acelerar la cicatrización de una herida. Las mutaciones que se realizarán en el ADN que codifica la variante no deben sacar la secuencia del marco de lectura y, preferiblemente, no crearán regiones

complementarias que puedan producir una estructura ARNm secundaria. EP 75,444A. Variantes de VEGF pueden valorarse en cuanto a la actividad VEGF, por ejemplo, mediante un análisis de proliferación celular. Por ejemplo, un análisis de proliferación celular incluye aumentar la magnitud del crecimiento y/o reproducción de la célula en relación con una célula no tratada, o una célula tratada reducida en *in vitro* o *in vivo*. Un incremento en la proliferación celular en un cultivo de células puede detectarse contando el número de células antes y después de la exposición a una molécula de interés. La magnitud de la proliferación puede cuantificarse mediante examen microscópico del grado de confluencia. De igual modo, la proliferación celular puede cuantificarse usando el análisis de incorporación de timidina.

[0022] Opcionalmente, las variantes de VEGF se preparan por mutagénesis sitio-dirigida de nucleótidos en el ADN, que codifica el VEGF natural, o mediante técnicas de exposición en bacteriófagos, produciendo así ADN que codifica la variante, posteriormente, expresando el ADN en cultivo celular recombinante.

[0023] Aunque el sitio para introducir una variación de una secuencia de aminoácidos está predeterminado, la mutación *per se* no necesita estar predeterminada. Por ejemplo, para optimizar el desempeño de una mutación en un sitio dado, puede conducirse una mutagénesis aleatoria en el codón o región diana, y las variantes de VEGF expresadas pueden cribarse con respecto a la combinación óptima de actividad deseada. Técnicas para hacer mutaciones de sustitución en sitios predeterminados en ADN, que tiene una secuencia conocida, son bien conocidas, tales como, por ejemplo, mutagénesis sitio-específica. La preparación de las variantes de VEGF, descritas en esta solicitud, puede lograrse mediante técnicas de exposición en bacteriófagos, tales como las descritas en la publicación PCT WO 00/63380.

[0024] Una vez seleccionado un clon, la región de la proteína mutada puede retirarse y ponerse en un vector apropiado para la producción de proteínas, generalmente un vector de expresión del tipo que puede emplearse para la transformación de un anfitrión apropiado.

[0025] Las eliminaciones de secuencia de aminoácidos varían generalmente desde alrededor de 1 a 30 residuos, opcionalmente 1 a 10 residuos, opcionalmente 1 a 5 o menos y, típicamente, son contiguos.

[0026] Las inserciones de secuencia de aminoácidos incluyen fusiones amino- y/o carboxilo-terminales de desde un residuo a polipéptidos de longitud esencialmente irrestricta, así como inserciones intrasecuencia de residuos múltiples o individuales de aminoácidos. Las inserciones intrasecuencia (es decir, inserciones en la secuencia del VEGF natural) pueden variar generalmente desde alrededor de 1 a 10 residuos, opcionalmente 1 a 5 o, de manera opcional, 1 a 3. Un ejemplo de una inserción terminal incluye una fusión de una secuencia señal, ya sea heteróloga u homóloga a la célula anfitriona, al término N, para facilitar la secreción de anfitriones recombinantes.

[0027] Variantes adicionales de VEGF son aquellas en las cuales por lo menos un residuo de aminoácidos en el VEGF natural se ha retirado y en su lugar se insertado un residuo diferente. Tales sustituciones pueden hacerse de acuerdo con las ilustradas en la Tabla 1. Variantes de VEGF pueden comprender asimismo aminoácidos no naturales, como se describe en esta solicitud.

[0028] Los aminoácidos pueden agruparse de acuerdo con similitudes en las propiedades de sus cadenas laterales (en A. L. Lehninger, in *Biochemistry,* second ed., pp. 73-75, Worth Publishers, New York (1975)):

1. no polares: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp

(W), Met (M)

2. polares no cargados: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)
3. ácidos: Asp (D), Glu (E)
4. básicos: Lys (K), Arg (R), His(H)

[0029] Alternativamente, residuos de ocurrencia natural pueden dividirse en grupos basados en propiedades comunes de cadena lateral:

(1) hidrófobos: Norleucina, Met, Ala, Val, Leu, Ile;

(2) hidrófilos neutros: Cys, Ser, Thr, Asn, Gln;

(3) ácidos: Asp, Glu;

(4) básicos: His, Lys, Arg;

(5) residuos que influyen en la orientación de la cadena: Gly, Pro;

(6) aromáticos: Tip, Tyr, Phe.

Tabla 1

Residuo Original	**Sustituciones Ejemplares**	**Sustituciones Preferidas**
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucina	Leu
Leu (L)	Norleucina; Ile; Val; Met; Ala; Phe	Ile

Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp(W)	Tyr; Phe	Tyr
Tyr(Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	lie; Leu; Met; Phe; Ala;	Leu

[0030] "Residuos de aminoácidos de ocurrencia natural" (es decir, residuos de aminoácidos codificados por el código genético) pueden seleccionarse del grupo conformado por: alanina (Ala); arginina (Arg); asparagina (Asn); ácido aspártico (Asp); cisteína (Cys); glutamina (Gln); ácido glutámico (Glu); glicina (Gly); histidina (His); isoleucina (Ile): leucina (Leu); lisina (Lys); metionina (Met); fenilalanina (Phe); prolina (Pro); serina (Ser); treonina (Thr); triptófano (Tip); tirosina (Tyr); y valina (Val). Un "residuo de aminoácidos de ocurrencia no natural" se refiere a un residuo, distinto de los residuos de aminoácidos de ocurrencia natural enumerados anteriormente, que es capaz de unir covalentemente residuo(s) de aminoácidos adyacente(s) en una cadena de polipéptido. Ejemplos de residuos de aminoácidos de ocurrencia no natural incluyen, por ejemplo, norleucina, ornitina, norvalina, homoserina y otros análogos de residuos de aminoácidos, tales como los descritos en Ellman et al. *Meth. Enzym.* 202:301-336 (1991) & publicaciones de solicitud de Patente de los Estados Unidos 20030108885 y 20030082575. En síntesis, estos procedimientos implican activar un ARNt supresor con un residuo de aminoácidos de ocurrencia no natural, seguido por trascripción y traducción *in vitro* o *in vivo* del ARN. *Véase, por ejemplo*, las publicaciones de solicitud de

Patentes de los Estados Unidos 20030108885 y 20030082575; Noren et al. *Science* 244:182 (1989); y, Ellman et al., *supra.*

[0031] "Identidad porcentual (%) de secuencia de aminoácidos" se define como el porcentaje de residuos de aminoácidos en una secuencia candidata, que son idénticos con los residuos de aminoácidos en una secuencia seleccionada, luego de alinear las secuencias e introducir hendiduras (***"gaps"***), si es necesario, para lograr la máxima identidad porcentual de secuencia, y sin considerar sustituciones conservadoras de ninguna clase, como parte de la identidad de secuencia. La alineación para efectos de determinar la identidad porcentual de secuencia de aminoácidos puede lograrse en varias formas, que se encuentran al alcance de la habilidad en el arte, por ejemplo, usando software de computador públicamente disponible, tal como software BLAST, BLAST-2, ALIGN, ALIGN-2 o Megalign (DNASTAR). Aquellas personas expertas en el arte pueden determinar parámetros apropiados para medir la alineación, incluidos todos los algoritmos necesarios para lograr la alineación máxima sobre la longitud total de las secuencias que se están comparando. Para los propósitos contemplados en esta solicitud, sin embargo los valores de identidad % de secuencia de aminoácidos se obtienen usando el programa de computador de comparación de secuencia ALIGN-2. El programa de computador de comparación de secuencia ALIGN-2 fue creado por Genentech, Inc., y el código fuente se ha depositado con documentación de usuario ante la Oficina de Derechos de Autor, Washington, D.C, 20559, en donde está registrado bajo el Registro de Derechos de Autor de los Estados Unidos No. TXU510087 y se encuentra a disposición pública a través de Genentech, Inc., South San Francisco, California. El programa ALIGN-2 debe compilarse para uso en un sistema operativo UNIX, preferiblemente UNIX V4.0D digital. Todos los parámetros de comparación de secuencias se fijan por el programa ALIGN-2 y no varían.

[0032] Para los propósitos contemplados en esta solicitud, el % de identidad de secuencia de aminoácidos de una secuencia A de aminoácidos dada con respecto a, con, o frente a una secuencia B de aminoácidos dada (lo cual puede alternativamente expresarse como una secuencia A de aminoácidos dada que tiene o comprende un cierto % de identidad de secuencia de aminoácidos con respecto a, con, o frente una secuencia B de aminoácidos dada) se calcula en la siguiente forma:

100 veces la fracción X/Y

donde X es el número de residuos de aminoácidos calificados como coincidencias idénticas por el programa de alineación de secuencias ALIGN-2 en la alineación de ese programa de A y B, y donde Y es el número total de residuos de aminoácidos en B. Se observará que donde la longitud de la secuencia A de aminoácidos no es igual a la longitud de la secuencia B de aminoácidos, el % de identidad de secuencia de aminoácidos de A a B no equivaldrá al % de identidad de secuencia de aminoácidos de B a A.

[0033] El término "receptor de VEGF" o "VEGFR", tal como se emplea en esta solicitud, se refiere a un receptor celular para VEGF, ordinariamente un receptor de superficie celular hallado en células endoteliales vasculares, así como sus variantes, que conservan la capacidad de unirse a VEGF.

[0034] El término "agonista de VEGFR" se refiere a una molécula que puede activar un receptor de VEGF o aumentar su expresión. Los agonistas de VEGFR incluyen, pero no se limitan a, por ejemplo, agonistas de ligando de un VEGFR, variantes de VEGF, anticuerpos y fragmentos activos. VEGF es un agonista de VEGFR, pero en esta solicitud se enumera y menciona en forma separada. El término "anticuerpo anti-VEGFR" es un anticuerpo que se une a VEGFR con suficiente afinidad y especificidad. En una incorporación, el anticuerpo agonista anti-VEGFR de la invención puede utilizarse como un agente

terapéutico en tratar heridas. En otra incorporación, puede emplearse una variante de VEGF como un agente terapéutico en tratar heridas.

[0035] El término "anticuerpo" se emplea en el sentido más amplio y comprende anticuerpos monoclonales (incluidos anticuerpos monoclonales intactos o de longitud total), anticuerpos policlonales, anticuerpos multivalentes, anticuerpos multiespecíficos (por ejemplo, anticuerpos biespecíficos), y fragmentos de anticuerpo, siempre que exhiban la actividad biológica deseada.

[0036] A menos que se indique lo contrario, la expresión "anticuerpo multivalente" se usa para denotar un anticuerpo que comprende tres o más sitios de unión a antígeno. Típicamente, el anticuerpo multivalente se modifica mediante técnicas de ingeniería genética para tener los tres o más sitios de unión a antígeno y, por lo general, no es un anticuerpo IgA o IgM de secuencia natural.

[0037] "Fragmentos de anticuerpo" comprenden tan sólo una porción de un anticuerpo intacto, incluyendo generalmente un sitio de unión a antígeno del anticuerpo intacto y reteniendo, en consecuencia, la capacidad de unir el antígeno. Ejemplos de fragmentos de anticuerpo abarcados por la presente definición incluyen: (i) el fragmento Fab, que tiene los dominios VL, CL, VH y CH1; (ii) el fragmento Fab', que es un fragmento Fab que tiene uno o más residuos cisteína en el término C del dominio CH1; (iii) el fragmento Fd que tiene los dominios VH y CH1; (iv) el fragmento Fd' que tiene los dominios VH y CH1 y uno o más residuos cisteína en el término C del dominio CH1; (v) el fragmento Fv que tiene los dominios VL y VH de un solo brazo de un anticuerpo; (vi) dAb (Ward et al., *Nature* 341, 544-546 (1989)), que consta de un dominio VH; (vii) regiones CDR aisladas; (viii) los fragmentos F(ab')2, un fragmento bivalente que incluye dos fragmentos Fab' conectados por un puente disulfuro en la región de unión flexible; (ix) moléculas de

anticuerpo de cadena única (por ejemplo Fv de cadena única; scFv) (Bird et al., *Science* 242:423-426 (1988); y Huston et al., *PNAS (USA)* 85:5879-5883 (1988)); (x) "diacuerpos" con dos sitios de unión a antígeno, que comprenden un dominio variable de cadena pesada (VH) conectado a un dominio variable de cadena liviana (VL) en la misma cadena de polipéptido (véase, por ejemplo, EP 404,097; WO 93/11161; y Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993)); (xi) "anticuerpos lineales" que comprenden un par de fragmentos Fd en tándem (VH-CH1-VH-CH1) los cuales, junto con los polipéptidos complementarios de cadena liviana, forman un par de regiones de unión a antígeno (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); y Patente de los Estados Unidos No. 5,641,870).

[0038] El término "anticuerpo monoclonal", como se emplea en esta solicitud, se refiere a un anticuerpo obtenido de una población de anticuerpos sustancialmente homogéneos, es decir, los anticuerpos individuales que componen la población son idénticos, excepto por posibles mutaciones de ocurrencia natural, que puedan estar presentes en cantidades menores. Los anticuerpos monoclonales son altamente específicos, dirigiéndose contra un solo antígeno. Más aún, en contraste con preparaciones convencionales de anticuerpo policlonal, que incluyen diferentes anticuerpos dirigidos contra diferentes determinantes (epítopos), cada anticuerpo monoclonal se dirige contra un solo determinante en el antígeno. El calificativo "monoclonal" no tiene que interpretarse como que requiere la producción del anticuerpo por algún método particular. Por ejemplo, los anticuerpos monoclonales a utilizarse de acuerdo con la presente invención pueden elaborarse por el método del hibridoma, descrito por primera vez por Kohler et al. (1975), *Nature,* 256:495, o pueden elaborarse por métodos de ADN recombinante (véase, por ejemplo, la Patente de los Estados Unidos 4,816,567). Los "anticuerpos monoclonales" pueden aislarse también de bibliotecas de anticuerpos de exposición en bacteriófagos usando las

técnicas descritas en Clackson et al., *Nature*, 352:624-628 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), por ejemplo.

[0039] Los anticuerpos monoclonales en esta solicitud incluyen específicamente anticuerpos "quiméricos", en los cuales una porción de la cadena pesada y/o liviana es idéntica con, u homóloga a, secuencias correspondientes en anticuerpos derivados de una especie particular, o pertenecientes a una clase o subclase de anticuerpos, mientras que el resto de la cadena(s) es idéntico con, u homólogo a, secuencias correspondientes en anticuerpos derivados de otra especie, o pertenecientes a otra clase o subclase de anticuerpos, así como fragmentos de tales anticuerpos, siempre que exhiban la actividad biológica deseada (Patente de los Estados Unidos 4,816,567; y Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

[0040] Formas "humanizadas" de anticuerpos no humanos (por ejemplo, murinos) son anticuerpos quiméricos, que contienen una secuencia mínima derivada de inmunoglobulina no humana. En su mayor parte, los anticuerpos humanizados son inmunoglobulinas humanas (anticuerpo destinatario), en las cuales residuos de una región hipervariable del destinatario se reemplazan por residuos de una región hipervariable de una especie no humana (anticuerpo donante), tal como ratón, rata, conejo o primate no humano, que tiene la especificidad, afinidad y capacidad deseadas. En algunos casos, residuos de la región base (FR) de la inmunoglobulina humana se reemplazan por residuos no humanos correspondientes. Más aún, los anticuerpos humanizados pueden comprender residuos, que no se encuentran en el anticuerpo destinatario o en el anticuerpo donante. Estas modificaciones se hacen para afinar aún más el desempeño del anticuerpo. En general, el anticuerpo humanizado comprenderá sustancialmente la totalidad de por lo menos uno y, típicamente dos, dominios variables, en los cuales la totalidad o sustancialmente todos los bucles hipervariables corresponden

a los de una inmunoglobulina no humana, y todas o sustancialmente todas las FRs son las de una secuencia de inmunoglobulina humana. De manera opcional, el anticuerpo humanizado comprenderá también por lo menos una porción de una región constante de inmunoglobulina (Fc), típicamente la de una inmunoglobulina humana. Para mayores detalles, véase Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); y Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992).

[0041] Un "anticuerpo humano" es uno que posee una secuencia de aminoácidos que corresponde a la de un anticuerpo producido por un humano y/o se ha elaborado usando cualquiera de las técnicas para fabricar anticuerpos humanos, como se divulga en esta solicitud. Esta definición de un anticuerpo humano excluye específicamente un anticuerpo humanizado, que comprende residuos no humanos de unión a antígeno. Anticuerpos humanos pueden producirse usando varias técnicas conocidas en el arte. En una incorporación, el anticuerpo humano se selecciona de una biblioteca de bacteriófagos, donde la biblioteca de bacteriófagos expresa anticuerpos humanos (Vaughan et al. *Nature Biotechnology*, 14:309-314 (1996): Sheets et al. *PNAS, (USA)* 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)). Los anticuerpos humanos pueden elaborarse también introduciendo loci de inmunoglobulina humana en animales transgénicos, por ejemplo, ratones, en los cuales los genes de inmunoglobulina endógena se han inactivado parcial o completamente. Luego de la prueba de provocación, se aprecia la producción de anticuerpos humanos, que se parece mucho a la observada en humanos en todos los aspectos, incluidos rearreglo de genes, ensamble y repertorio de anticuerpos. Este enfoque se describe, por ejemplo, en las Patentes de los Estados Unidos Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, y en las siguientes publicaciones científicas: Marks et al., *Bio/Technology*, 10: 779-783 (1992); Lonberg et al., *Nature*, 368: 856-859 (1994); Morrison,

Nature, 368:812-13 (1994); Fishwild et al., *Nature Biotechnology*, 14: 845-51 (1996); Neuberger, *Nature Biotechnology*, 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.*, 13:65-93 (1995). Alternativamente, el anticuerpo humano puede prepararse mediante inmortalización de linfocitos B humanos, que producen un anticuerpo dirigido contra un antígeno diana (tales linfocitos B pueden recuperarse de un individuo o pueden haberse inmunizado *in vitro*). Véase *por ejemplo*, Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.*, 147 (1):86-95 (1991); y Patente de los Estados Unidos No. 5,750,373.

[0042] La diabetes es un trastorno crónico que afecta el metabolismo de proteínas, grasa y carbohidratos en animales. La diabetes es la causa principal de ceguera, deficiencia renal, y amputaciones de las extremidades inferiores en adultos y un mayor factor de riesgo para enfermedad cardiovascular e infarto.

[0043] La diabetes mellitus tipo I (o diabetes mellitus insulino-dependiente ("IDDM") o diabetes de aparición en jóvenes) comprende aproximadamente 10% de todos los casos de diabetes. La enfermedad se caracteriza por la pérdida progresiva de la función secretora de insulina por las células β del páncreas. Esta característica se comparte además por la diabetes no idiopática, o "secundaria", que tiene sus orígenes en la enfermedad pancreática. La diabetes mellitus tipo I se asocia con los siguientes signos o síntomas clínicos, por ejemplo, concentración persistentemente elevada de glucosa en el plasma o hiperglucemia; poliuria; polidipsia y/o hiperfagia; complicaciones microvasculares crónicas tales como retinopatía, nefropatía y neuropatía; y complicaciones macrovasculares tales como hiperlipidemia e hipertensión, que pueden llevar a ceguera, insuficiencia renal terminal, amputación de extremidades e infarto del miocardio.

[0044] La diabetes mellitus tipo II (diabetes mellitus no insulino-dependiente o NIDDM) es un trastorno metabólico que implica la regulación alterada del metabolismo de glucosa y una sensibilidad deficiente a la insulina. La diabetes mellitus tipo se desarrolla habitualmente en la adultez y se asocia con la incapacidad del cuerpo de utilizar o elaborar suficiente insulina. Aparte de la resistencia a la insulina observada en los tejidos diana, los pacientes que sufren de diabetes mellitus tipo II tienen una insuficiencia de insulina relativa, es decir, los pacientes tienen niveles de insulina más bajos que los predichos para una concentración dada de glucosa en el plasma. La diabetes mellitus tipo II se caracteriza por los siguientes signos o síntomas clínicos, por ejemplo, concentración persistentemente elevada de glucosa en el plasma o hiperglucemia; poliuria; polidipsia y/o hiperfagia; complicaciones microvasculares crónicas tales como retinopatía, nefropatía y neuropatía; y complicaciones macrovasculares tales como hiperlipidemia e hipertensión, que pueden llevar a ceguera, insuficiencia renal terminal, amputación de extremidades e infarto del miocardio.

[0045] "Sujeto" para los propósitos de la invención se refiere a cualquier animal. Generalmente, el animal es un mamífero. "Mamífero", para los propósitos de la invención se refiere a cualquier animal clasificado como un mamífero, incluidos humanos, animales domésticos y de granja, animales de zoológico, deportivos, o mascotas, tales como perros, caballos, gatos, vacas, ovejas, cerdos, etc. Típicamente, el mamífero es un humano.

[0046] El término "acelerar la cicatrización de una herida" o "aceleración de la cicatrización de una herida" se refiere a un aumento en la tasa de cicatrización, por ejemplo, una reducción en el tiempo hasta que ocurra el cierre completo de la herida, o una reducción en el tiempo hasta que ocurra una reducción % en el área de la herida.

[0047] El término "cantidad efectiva" o "cantidad terapéuticamente efectiva" se refiere a una cantidad de un medicamento, efectiva para acelerar o mejorar la cicatrización de una herida en un sujeto, o evitar la recurrencia de una úlcera en un sujeto. Una dosis terapéutica es una dosis que exhibe un efecto terapéutico en el sujeto, y una dosis sub-terapéutica es una dosis que no exhibe un efecto terapéutico en el sujeto tratado.

[0048] Administración "en combinación con" uno o más agentes terapéuticos adicionales incluye la administración simultánea (concurrente) y/o consecutiva en cualquier orden.

[0049] "Tratamiento" se refiere tanto al tratamiento terapéutico como profiláctico o medidas preventivas. Personas necesitadas de tratamiento incluyen aquellas ya con el trastorno, así como aquellas en las cuales el trastorno tiene que prevenirse.

[0050] "Cicatrización de una herida" se refiere a una condición que se beneficiaría del tratamiento con una molécula de la invención.

[0051] Una "herido crónica" se refiere a una herida que no cicatriza. *Véase, por ejemplo,* Lazarus et al., Definitions and guidelines for assessment of wounds and evaluation of healing, *Arch. Dermatol.* 130:489-93 (1994). Las heridas crónicas incluyen, pero no se limitan a, por ejemplo, úlceras arteriales, úlceras diabéticas, úlceras por presión, úlceras venosas, etc. Una herida aguda puede evolucionaren una herida crónica. Las heridas agudas incluyen, pero no se limitan a, heridas causadas, por ejemplo, por lesión térmica, trauma, cirugía, extirpación de cáncer cutáneo extensivo, infecciones fúngicas y bacterianas profundas, vasculitis, escleroderma, pénfigo, necrólisis epidérmica tóxica, etc. *Véase, por ejemplo,* Buford, Wound Healing and Pressure

Sores, *HealingWell.com,* publicada el: 24 de octubre de 2001. Una "herida normal" se refiere a una herida que experimenta reparación normal de la cicatrización de una herida.

[0052] "Buen Manejo y Cuidado de la Herida (GWC)" se refiere a los pasos de cuidar una herida. Por ejemplo, buenas prácticas de cuidado de heridas incluyen, pero no se limitan a, uno o más de los siguientes: desbridamiento (por ejemplo, quirúrgico/extirpación, mecánico, autolítico o químico/enzimático), limpieza (por ejemplo, lavado rutinario de heridas con, por ejemplo, solución salina), apósitos, alivio de la presión (por ejemplo, descargar la presión al pie), mantenimiento de un ambiente húmedo de la herida, y/o control de la infección (por ejemplo, ungüento antibiótico o píldoras). Otros pasos incluyen opcionalmente suministrar al sujeto un calzado cómodo y acolchado, apoyo nutricional, mantener el control de la glucosa sanguínea, manejo de otros factores de riesgo (por ejemplo, peso, tabaquismo), etc. El GWC puede incluir una o más de las prácticas.

[0053] La expresión "trauma que afecta el endotelio vascular" se refiere a un trauma, tal como lesiones, a los vasos sanguíneos o al corazón, incluida al red vascular de órganos, al cual se somete un animal o humano, preferiblemente un mamífero y, muy preferiblemente un humano. Ejemplos de tal trauma incluyen heridas, incisiones, y úlceras, o laceraciones de los vasos sanguíneos o el corazón. El trauma incluye dolencias causadas por episodios internos, así como aquellos que son impuestos por un agente extrínseco, que pueden aliviarse mediante promoción del crecimiento celular endotelial vascular.

[0054] Un "factor o agente angiogénico" es un factor de crecimiento que estimula el desarrollo de los vasos sanguíneos, por ejemplo, promueve la angiogénesis, el crecimiento celular endotelial, la estabilidad de los vasos sanguíneos, y/o la vasculogénesis, etc. Por

ejemplo, factores angiogénicos, incluyen, pero no se limitan a, por ejemplo, VEGF y miembros de la familia VEGF, P1GF, la familia PDGF, la familia de factor de crecimiento de fibroblastos (FGFs), ligandos TIE (angiopoyetinas), efrinas, ANGPTL3, ANGPTL4, etc. Incluye asimismo factores, tales como somatotropina, factor I de crecimiento insulinoide (IGF-I), VIGF, factor de crecimiento epidérmico (EGF), CTGF y miembros de su familia, y TGF-α y TGF-β. *Vease, por ejemplo,* Klagsbrun and D'Amore, *Annu. Rev. Physiol,* 53:217-39 (1991); Streit and Detmar, *Oncogene,* 22:3172-3179 (2003); Ferrara & Alitalo, *Nature Medicine* 5(12): 1359-1364 (1999); Tonini et al., *Oncogene,* 22:6549-6556 (2003) (por ejemplo, Tabla 1 que enumera los factores angiogénicos); y, *Sato Int. J. Clin. Oncol,* 8:200-206 (2003).

[0055] **Cicatrización de una herida**

[0056] La cicatrización de una herida es un proceso complejo que implica tres fases principales: inflamación, tejido de granulación, y remodelamiento del tejido. En el sitio de la herida ocurren muchos procesos, por ejemplo, migración/contracción, producción de metaloproteasas de matriz (MMP), proliferación, y angiogénesis. Existe contracción (reducción de la herida), epitelización (creación de nuevas células epiteliales) y deposición de tejido conectivo, con el fin de cicatrizar la herida. *Véase* Singer & Clark, Cutaneous Wound Healing *N. Engl. J. Med.,* 341:738-46 (1999). Una de las metas de la terapia de curación de heridas es promover la matriz de granulación, donde se necesita un suministro sanguíneo adecuado. Sin embargo, factores de riesgo, asociados a menudo con estados patológicos, (por ejemplo, incluyen, pero no se limitan a, edad, infección, nutrición deficiente, inmunosupresión, medicaciones, radiación, diabetes, vasculopatía periférica, enfermedad sistémica, fumar, estrés, etc.) crean desafíos para la cicatrización de una herida.

[0057] Ejemplos de algunos de los factores de riesgo para úlceras

de pie diabético incluyen neuropatía periférica, que afecta las funciones motoras y sensoriales del pie, movilidad limitada de las articulaciones, deformidades del pie, distribución anormal de la presión sobre el pie, trauma menor repetitivo, y agudez visual deficiente. *Véase, por ejemplo,* Boyko et al., A prospective study of risk factors for diabetic foot ulcer. The Seattle Diabetic Foot Study, *Diabetes Care,* 22:1036-42 (1999); y Apelqvist et al., International consensus and practical guidelines on the management and the prevention of the diabetic foot. International Working Group on the Diabetic Foot., *Diabetes Metab Res. Rev* 16(1 Suppl): S84-S92 (2000). La neuropatía sensorial periférica es un factor primario. Aproximadamente 45%-60% de todas las ulceraciones diabéticas son neuropáticas, mientras que hasta el 45% tienen componentes neuropáticos e isquémicos. Con un pie insensible, el paciente es incapaz de percibir la lesión repetitiva al pie, causada, por ejemplo, por calzado desajustado durante la ambulación y actividades de la vida diaria. La neuropatía, combinada con biomecánica alterada al caminar, lleva a trama contundente repetitivo, y a la distribución de cargas de esfuerzo anormalmente altas a porciones vulnerables del pie, resultando en la formación de callosidades y erosión cutánea. Una vez formada una úlcera, a menudo es lenta en curar, puede continuar ensanchándose, ofrece una oportunidad para una infección local o sistémica, y requiere cuidado médico y quirúrgico integral para promover la cicatrización.

[0058] Existen igualmente retos a la reacción de terapias de proteína para acelerar la cicatrización de una herida, por ejemplo, acelerar la cicatrización de úlceras crónicas, por ejemplo, de pie diabético, y heridas. El área de la herida es un ambiente hostil (enzimas proteolíticas, inhibidores producidos naturalmente de actividad de proteínas junto con infección superimpuesta), donde a menudo los estados patológicos (por ejemplo, en diabetes) los factores del anfitrión se alteran (por ejemplo, en diabetes hay expresión suprimida del VEGF,

respuesta deficiente del VEGF a la hipoxia, metabolismo celular alterado, respuesta inmunitaria/inflamatoria suprimida, etc.). Por ejemplo, basándose en estudios *in vitro*, queratinocitos y fibroblastos de ratones diabéticos (db/db) exhiben un desmejoramiento selectivo de procesos celulares, esenciales para la reparación normal de tejido, y fibroblastos db/db muestran una migración celular significativamente disminuida y alteraciones del factor de crecimiento. *Véase, por ejemplo,* Frank et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing, *J. Biol. Chem.,* 270:12607-13 (1995); y, Lerman et al, Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia, *Am. J. Pathol,* 162:303-12 (2003).

[0059] En esta solicitud se proveen métodos para acelerar y/o mejorar la cicatrización de heridas, administrando una cantidad efectiva de VEGF o un agonista de VEGF. Por ejemplo, un método comprende administrar una cantidad efectiva de VEGF a una herida de un sujeto, donde la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida. Los métodos incluyen igualmente un método de mejorar la cicatrización de una herida en una población de sujetos. Por ejemplo, un método comprende administrar una cantidad efectiva de VEGF a una herida de un sujeto de la población, en donde la administración de la cantidad efectiva de VEGF resulta en una mejora de más de 10% (o más de 12%, o 14%, o 15%, o 17%, o 20%, o 25%, o 30%, o 33%, o 35%, o 40%, o 45%, o 50% o más) en la cicatrización de una herida en la población, comparada con un control. Se proveen asimismo métodos para reducir la recurrencia de úlceras. Por ejemplo, un método comprende administrar una cantidad efectiva de VEGF a una úlcera, en donde la incidencia de recurrencia de úlcera se reduce con la administración de VEGF, comparada con un control.

[0060] Los métodos son igualmente aplicables a sujetos que se están sometiendo o se han sometido a un tratamiento, donde el tratamiento retrasa o permite una cicatrización ineficaz de una herida. Los tratamientos pueden incluir, pero no se limitan a, medicinas, radiación, tratamientos que resultan en sistemas inmunitarios suprimidos, etc. Opcionalmente, un sujeto de la invención tiene una enfermedad secundaria, en donde la enfermedad secundaria retrasa o permite una cicatrización ineficaz de una herida. Enfermedades secundarias incluyen, pero no se limitan a, por ejemplo, diabetes, vasculopatía periférica, infección, trastornos autoinmunitarios vasculares de colágeno, estados patológicos que resultan en sistemas inmunitarios suprimidos, etc.

[0061] La aceleración de la cicatrización de una herida puede describirse por el % de aceleración de la cicatrización de una herida y/o una relación/proporción de riesgo. En ciertas incorporaciones, la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida en más de 50%, o igual o mayor a 60%, igual o mayor a 70%, igual o mayor a 74%, igual o mayor a 75%, igual o mayor a 80%, igual o mayor a 85%, igual o mayor a 90%, igual o mayor a 95%, igual o mayor a 100%, igual o mayor a 110% o más, cuando se compara con un control. En ciertas incorporaciones, la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida entre más de 60% y 110%, cuando se compara con un control. En ciertas incorporaciones, la aceleración de la cicatrización de una herida se describe por una relación/proporción de riesgo, que es igual o mayor a 1.75, o es igual o mayor a 1.80, o es igual o mayor a 1.85, o es igual o mayor a 1.95, o es igual o mayor a 2.0, o es igual o mayor a 2.1, o es igual o mayor a 2.2, o es igual o mayor a 2.3 o más. En ciertas incorporaciones, la aceleración de la cicatrización de una herida se describe por una relación/proporción de riesgo, que está entre 1.75 y 2.3.

[0062] Sujetos de la invención tienen por lo menos una herida. La herida puede ser una herida crónica, aguda o normal. En una incorporación, la herida que se está tratando es una herida estado 1A. Véanse los Estados de Heridas en la Tabla 2. Una herida de la invención puede incluir opcionalmente una infección o isquemia, o incluir tanto una infección como isquemia. En una incorporación, la herida es una úlcera de pie diabético. En una incorporación, la herida está presente en el sujeto durante alrededor de 4 semanas o más, o alrededor de 6 semanas o más, antes de administrar el VEGF.

Tabla 2

	Grado/Profundidad de la Herida			
Estado/Comorbilidades	**0**	**1**	**2**	**3**
A	Lesión pre- o pos-ulcerativa completamente epitelializada	Úlcera superficial que no afecta el tendón, la cápsula, o el hueso	Úlcera que penetra al tendón o la cápsula	Úlcera que penetra al hueso o la articulación
B	Con infección	Con infección	Con infección	Con infección
C	Con isquemia	Con isquemia	Con isquemia	Con isquemia
D	Con infección e isquemia	Con infección e isquemia	Con infección e isquemia	Con infección e isquemia

[0063] Puede emplearse un análisis cuantitativo para evaluar la cicatrización de una herida, por ejemplo, determinando el % de reducción en el área de la herida, o cierre completo de la herida (por ejemplo, medida por cierre de la piel sin requerimientos de drenaje o apósitos). El área de la herida se evalúa antes, durante y después del tratamiento, por métodos conocidos por los expertos en el arte. Por

ejemplo, la valoración puede determinarse mediante, por ejemplo, planimetría cuantitativa (*véase, por ejemplo,* Robson et al., Arch. Surg 135:773-77 (2000)), fotografías, exámenes físicos, etc. El área de la herida puede determinarse antes, durante y después del tratamiento. En una incorporación, el área de la herida puede calcularse midiendo la longitud, L, de la herida, la longitud máxima de un extremo al otro en, por ejemplo, cm, y el ancho, W, el ancho máximo de un extremo a otro, perpendicular a L en, por ejemplo, cm, y multiplicando LxW para obtener el área superficial estimada (cm^2). El tamaño de la herida para tratamiento puede variar. En una incorporación de la invención, el área de la herida antes del tratamiento es alrededor de 0.4 cm^2 o más, o alrededor de 1.0 cm^2 o más, o entre alrededor de 0.4 cm^2 y alrededor de 10 cm^2, o entre alrededor de 1 cm^2 y alrededor de 10 cm^2, o entre alrededor de 1 cm^2 y alrededor de 6.5 cm^2, o entre alrededor de 1 cm^2 y alrededor de 5 cm^2, o más de 4.0 cm^2. El área puede medirse antes o después del desbridamiento.

[0064] **VEGF**

[0065] Una cantidad efectiva de VEGF se administra en los métodos contemplados en esta solicitud para acelerar o promover la cicatrización de una herida. La familia del gen VEGF, de la cual VEGF es un miembro, es uno de los reguladores claves del desarrollo del sistema vascular. La familia del gen VEGF incluye VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E y factor de crecimiento placentario (P1GF). *Véase, por ejemplo,* Ferrara, Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications, *Sem Oncol,* 29(suppl 16): 10-14 (2002); y, Veikkola and Alitalo, VEGFs receptors and angiogenesis *Semin Cancer Biol.* 9:211-220 (1999). VEGF-A, también conocido como VEGF, es un regulador principal de angiogénesis normal, incluida la cicatrización normal de una herida y curación del hueso, y angiogénesis anormal, tal como proliferación vascular en tumores y trastornos oftalmológicos (por

ejemplo, degeneración relacionada con la edad, retinopatía diabética). *Véase, por ejemplo,* Ferrara, Vascular Endothelial Growth Factor: Basic Science and Clinical Progress, *Endocrine Reviews* 25(4): 581-611 (2004); Ferrara and Henzel, Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells, *Biochem, Biophys Res Comm* 161:851-58 (1989); y Leung et al., Vascular endothelial growth factor is a secreted angiogenic mitogen, *Science* 246:1306-9 (1989). Se encuentra dentro del ámbito de la invención utilizar igualmente variantes de VEGF que tienen actividad VEGF y un agonista de los receptores de VEGF, por ejemplo, los agonistas VEGFR1 y/o VEGFR2, en lugar de VEGF o adicional a él.

[0066] El VEGF humano existe como por lo menos seis isoformas ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, y $VEGF_{206}$) que surgen del empalme alternativo de ARNm de un solo gen organizado en 8 exones, localizados en el cromosoma 6 (*véase, por ejemplo,* Ferrara N, Davis Smyth T. *Endocr Rev* 18:1-22 (1997); y Henry and Abraham, Review of Preclinical and Clinical Results with Vascular Endothelial Growth Factors for Therapeutic Angiogenesis, *Current Interventional Cardiology Reports,* 2:228-241 (2000)). *Véase también* las Patentes de los Estados Unidos No. 5,332,671 y 6,899,882. En una incorporación, $VEGF_{165}$ se administra en los métodos de la invención. Típicamente, se utiliza el $VEGF_{165}$ humano (por ejemplo, el $VEGF_{165}$ humano recombinante). $VEGF_{165}$, la isoforma más abundante, es una glucoproteína dimérica covalente, básica, de unión a heparina, con una masa molecular de ~45,000 daltons *(Id).* El homodímero $VEGF_{165}$ consta de dos cadenas de 165 aminoácidos. La proteína tiene dos dominios distintos: un dominio de unión a receptor (residuos 1-110) y un dominio de unión a heparina (residuos 110-165). Los dominios se estabilizan por siete enlaces disulfuro intramoleculares, y los monómeros se conectan por dos enlaces disulfuro intercadena para formar el homodímero natural. El VEGF121 carece del dominio de unión a heparina (*véase, por ejemplo,* la Patente de los Estados Unidos

No. 5,194,596), mientras que el $VEGF_{189}$ (*véase, por ejemplo,* Las Patentes de los Estados Unidos No. 5,008,196; 5,036,003; y 5,240,848) y el VEGF206 se secuestran en la matriz extracelular. *Véase, por ejemplo,* Ferrara VEGF and the quest for tumor angiogenesis factors, *Nature Rev. Cancer* 2:795-803 (2002).

[0067] Los efectos biológicos del VEGF están mediados a través de receptores tirosina cinasa de alta afinidad. Los agonistas de los receptores de VEGF pueden emplearse asimismo en los métodos de la invención. Las tirosina cinasas de receptor VEGF, VEGFR1 y VEGFR2, han sido identificadas (Shibuya et al. *Oncogene* 5:519-24 (1990); Matthews et al., *Proc Natl Acad Sci USA* 88:9026-30 (1991); Terman et al., *Oncogene* 6:1677-83 (1991); Terman et al. *Biochem Biophys Res Commun* 187:1579-86 (1992); de Vries et al., *Science* 255:989-91 (1992); Millauer et al. *Cell* 72:835-46 (1993); y Quinn et al. *Proc Natl Acad Sci USA* 90:7533-7 (1993)). El VEGFR1 tiene la máxima afinidad para VEGF, con un Kd de ~10-20 pM (de Vries et al., *Science* 255:989-91 (1992)), y el VEGFR2 tiene una afinidad algo menor para el VEGF, con un Kd de ~75-125 pM (Terman et al., *Oncogene* 6:1677-83 (1991); Millauer et al. *Cell* 72:835-46 (1993); y Quinn et al. *Proc Natl Acad Sci USA* 90:7533-7 (1993)). Se ha identificado un tercer receptor tirosina cinasa, el VEGFR3, el cual se encuentra implicado en la regulación de angiogénesis linfática. El VEGFR3 es un receptor para VEGF-C y VEGF-D, los cuales pueden unir también VEGFR2. Estos receptores constan de un dominio extracelular (incluidas siete regiones tipo inmunoglobulina, una región transmembrana) y un dominio intracelular que contiene elementos relacionados con las vías tirosina cinasa. El VEGF-B y el P1GF se unen al VEGFR1 pero no al VEGFR2. De igual modo el $VEGF_{165}$ se une al receptor neuropilina-1 que regula la orientación de las células neuronales. Cuando se co-expresa con VEGFR2, la neuropilina-1 mejora la unión de $VEGF_{165}$ a VEGFR2 y la quimiotaxis mediada por

VEGF. Otros estudios han asociado la neuropilina 2 (NP2) al desarrollo de los vasos linfáticos. *Véase, por ejemplo,* Ferrara, Vascular Endothelial Factor: Basic Science and Clinical Progress. *Endocrine Reviews,* 254(4):581-611. Luego de unirse a VEGF-A, el VEGFR2 experimenta autofosforilación de tirosina que lleva a angiogénesis subsiguiente, permeabilidad vascular incrementada, mitogénesis y quimiotaxis.

[0068] El VEGF tiene varias funciones biológicas, incluidas la regulación de la expresión del gen VEGF bajo condiciones hipóxicas (Ferrara N, Davis Smyth T. *Endocr Rev* 18:1-22 (1997)), actividad mitógena para células endoteliales micro- y macrovasculares (Ferrara N, Henzel WJ. *Biochem Biophys Res Commun* 161:851-8 (1989); Leung et al, *Science* 246:1306-9 (1989); Connolly et al. *J Clin Invest* 84:1470-8 (1989a); Keck et al. *Science* 246:1309-12 (1989); Plouet et al., *EMBO J* 8:3801-6 (1989); Conn et al. *Proc Natl Acad Sci USA* 87:2628-32 (1990); y Pepper et al., *Exp Cell Res* 210:298-305 (1994)), y la inducción de la expresión de activadores de plasminógeno y colagenasa (Pepper et al., *Biochem Biophys Res Commun* 181:902-6 (1991)). Durante la hipoxia, el factor-1 inductor de hipoxia (HIP-1) se regula por aumento y se une a la región promotora del gen VEGF y activa la transcripción (*véase, por ejemplo*, Wang et al., Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular oxygen tension, *PNAS USA* 92:5510 (1995)). Otros agonistas que pueden regular por aumento el VEGF incluyen citocinas (IL-6) y otros factores de crecimiento, incluidos EGF, PDGF, bFGF.

[0069] El VEGF es producido por una amplia variedad de tipos de células normales (por ejemplo, queratinocitos, plaquetas, macrófagos, fibroblastos, células retinianas, células de ovarios) en todo el cuerpo, además de varios tipos de tumores sólidos. El trabajo realizado durante los últimos años ha establecido un papel clave del factor de crecimiento

endotelial vascular (VEGF) en la regulación de la angiogénesis normal y anormal (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). El VEGF es un factor de crecimiento necesario para la vasculogénesis embrionaria normal, desarrollo de miocitos cardiacos (*véase, por ejemplo,* Ferrara et al., Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene, *Nature* 380:439-42 (1996)), formación ósea encondral normal (*véase, por ejemplo,* Gerber et al., VEGF couples hypertrophic cartilage remodeling, ossification, and angiogenesis during enchondral bone formation, *Nat. Med,* 5:623-28 (1999)), reparación de tejido, y en la fisiología del tracto reproductivo femenino, el crecimiento folicular y la función endocrina del cuerpo lúteo son dependientes de la proliferación de nuevos capilares (*véase, por ejemplo*, Phillips et al., Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology,* 127:965-67 (1990)). Más aún, se ha demostrado que el VEGF es un mediador clave de la neovascularización asociada con tumores y trastornos intraoculares (Ferrara *et al.*). El ARNm del VEGF se sobreexpresa por la mayoría de los tumores humanos examinados (Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol..* 26:86-91 (1995); Brown *et al. Cancer Res.* 53:4727-4735 (1993); Mattern *et al. Brit. J. Cancer.* 73:931-934 (1996); y Dvorak *et al. Am J. Pathol.* 146:1029-1039 (1995)).

[0070] El VEGF se conoce también como factor de permeabilidad vascular, basándose en su capacidad de inducir filtración vascular en modelos animales. *Véase, por ejemplo,* Senger et al., Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219: 983-89 (1983). Senger y colegas propusieron que un incremento en la permeabilidad microvascular a las proteínas es un paso crucial en la angiogénesis. Por ejemplo, la filtración inducida de proteínas plasmáticas y la formación de gel de fibrina extracelular pueden constituir una matriz suficiente para el crecimiento de células endoteliales; el papel de factores de crecimiento mitógeno puede ser el

de reforzar este proceso. La angiogénesis se requiere además para permitir la migración de leucocitos, factores de crecimiento y oxígeno durante la formación de tejido de granulación en la cicatrización de una herida.

[0071] En la cicatrización de una herida, el VEGF juega un papel fundamental en la inducción de angiogénesis durante la cicatrización cutánea de una herida. Es un potente mitógeno para las células endoteliales microvasculares dérmicas y se expresa por queratinocitos de heridas en proceso de curación (*véase, por ejemplo,* Nissen et al., Vascular endothelial factor mediates angiogenic activity during the proliferative phase of wound healing. *Am. J. Pathol.,* 152:1445-52 (1998); Corral et al., Vascular endothelial growth factor is more important than basic fibroblast growth factor during ischemic wound healing. *Arch Surg.,* 134:200-5 (1999); Frank et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes, Implications for normal and impaired wound healing. *J. Biol. Chem.,* 270:12607-13 (1995); Ballaun et al., Human keratinocytes express the three major splice forms of vascular endothelial growth factor, *J. Invest Dermatol.,* 104:7-10 (1995). Actúa igualmente en una manera paracrina en los microvasos dérmicos, llevando a una vascularidad cutánea incrementada y formación de la matriz de granulación. *Véase, por ejemplo,* Corral et al., *supra*; y Romano de Peppe, et al., Adenovirus-mediated VEGF165 gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice. *Gen Ther.,* 9:1271-7 (2002). Deficiencias en la reparación de tejido, por ejemplo, cicatrización de una herida, etc., se observan cuando se alteran los niveles de VEGF y otros factores angiogénicos. *Véase, por ejemplo,* Howdieshell, et al., Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation, *J. Surg. Res.* 96:173-82 (2001); Street et al., Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover, *PNAS USA,* 99:9656-61

(2002); Tsou et al., Retroviral delivery of dominant-negative vascular endothelial growth factor receptor type 3 to murine wounds inhibits wound angiogenesis, *Wound Repair Regen.,* 10:222-9 (2002); Frank et al., Regulation of vascular endothelial growth factor expression in cultured keratinocytes, Implications for normal and impaired wound healing. *J. Biol. Chem.,* 270:12607-13 (1995); y Lerman et al., Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia, *Am. J. Pathol.,* 162:303-12 (2003). En algunos modelos animales, el VEGF exógeno promovió la cicatrización de heridas. *Véase, por ejemplo,* Galliano et al., Topical Vascular Endothelial Factor Accelerates Diabetic Wound Healing through increased angiogenesis and by Mobilizing and recruiting bone marrow-derived cells, *American Journal of Pathology,* 164 (6): 1935-1947 (2004); Romano de Peppe S et al, Adenovirus-mediated VEGF165 gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice, *Gen Ther* 9:1271-7 (2002); y Solicitud de Patente de los Estados Unidos No. US20030180259.

[0072] Como se describe anteriormente, existen retos en la creación de terapias a base de proteínas para acelerar la cicatrización de una herida. En esta solicitud describimos un estudio clínico de tratar úlceras con terapia de proteína recombinante VEGF, que resulta en la cicatrización acelerada de una herida. Véase Ejemplo 1, en esta solicitud.

[0073] **Agentes Adicionales**

[0074] Se encuentra dentro del ámbito de esta invención combinar terapia VEGF con uno o más de, por ejemplo, terapia de buen cuidado de heridas (por ejemplo, GWC), otras terapias novedosas o convencionales (por ejemplo, otros miembros de la familia VEGF, factores de crecimiento tales como los enumerados en esta solicitud, factor de crecimiento nervioso (NGF), factores positivos de angiogénesis

o agentes o activadores, esteroides anabólicos, reemplazos de tejido creados por técnicas de bioingeniería (por ejemplo, Apligraph®, Dermagraft™, etc.), terapia en vacío, de oxígeno hiperbárico para mejorar la actividad del VEGF, en acelerar y/o intensificar la cicatrización de una herida. *Véase, por ejemplo,* Meier and Nanney, Emerging New Drugs for Wound Repair, *Expert Opin. Emerging Drugs* (2006) 11(1):23-37.

[0075] Seis principales familias del factor de crecimiento (EGF, FGF, IGF, PDGF, TGF y VEGF) se encuentran asociadas con la cicatrización de una herida. Véase, por ejemplo, Nagai and Embil, Becaplermin: recombinant platelet derived growth factor, a new treatment for healing diabetic foot ulcers, *Exprt Opin Biol. Ther* 2:211-18 (2002). Ejemplos de tales factores de crecimiento incluyen el factor de crecimiento derivado de plaquetas (PDGF-A, PDGF-B, PDGF-C, y PDGF-D), factor I y II de crecimiento insulinoide (IGF-I e IGF-II), factor de crecimiento de fibroblastos ácido y básico (aFGF y bFGF), factor de crecimiento transformante α y β (TGF-α y TGF-β (por ejemplo, TGF-β 1, TGF-β 2, TGF- β 3)), factor de crecimiento epidérmico (EGF) y demás. Véase *Id.* Estos factores de crecimiento estimulan la mitosis de una o más de las células involucradas en la cicatrización de una herida y pueden combinarse con VEGF.

[0076] Otros agentes positivos de angiogénesis que pueden combinarse con VEGF incluyen, pero no se limitan a, por ejemplo, HGF, TNF-α, angiogenina, IL-8, etc. (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991)), activadores de angiogénesis en la Tabla 3, factores de angiogénesis y agentes descritos en esta solicitud, etc.

Tabla 3 Ejemplos de Activadores de Angiogénesis

Angiogénesis
Angiopoyetinas 1 y 2
Tie-2
Alpha-5 integrinas
Metaloproteinasas de matriz
Óxido Nítrico (NO)
COX-2
TGF-β y receptores
VEGF y receptores

[0077] Adicionalmente, los siguientes agentes pueden combinarse con tratamientos de cicatrización de heridas con VEGF, por ejemplo, el factor de crecimiento derivado de plaquetas (PDGF) (por ejemplo, Becaplermin (rhPDGF-BB) tal como Regranex ®; Johnson & Johnson (*véase, por ejemplo*, las Patentes de los Estados Unidos 5,457,093; 5,705,485; y 5,427,778; Perry, BH et al., A meta-analytic approach to an integrated summary of efficacy: a case study of becaplermin gel., *Cont. Clin. Trials* 23:389-408 (2002)), agonistas de receptor A2A-adenosina (por ejemplo, MRE0094 (King Pharmaceuticals)); factor de crecimiento de queratinocitos (KGF-2, repifermina (Human Genome Sciences)); lactoferrina (LF) (Agennix, Inc.,); timosina beta-4 (TJ34 (ReGeneRx Biopharmaceuticals)); péptido de receptor activador derivado de trombina (TP508; Chrysalin® (Chrysalis Biotechnology, Inc.)); vector adenovírico que codifica el factor de crecimiento derivado de plaquetas (PDGF-B) (GAM501) (Selective Genetics); células madre de la médula ósea autóloga (BMSC) (*véase, por ejemplo,* Badiavas & Falanga, Treatment of chronic wounds with bone marrow-derived cells, *Arch Dermatol,* 139:510-16 (2003); e injertos de tejido vivo modificados por técnicas de ingeniería (por ejemplo, Apligraf, etc.). Agentes anti-ulcerantes antibióticos y antisépticos pueden combinarse asimismo con

la administración de VEGF. La administración de VEGF puede aplicarse junto con tratamiento inmunosupresor (por ejemplo, corticosteroides, terapia de radiación, quimioterapia) o tratamiento anti-cáncer.

[0078] No es necesario que tales agentes de co-tratamiento o procedimientos se incluyan *per se* en las composiciones de esta invención, aunque esto será conveniente donde tales agentes son proteináceos. Tales mezclas se administran apropiadamente en la misma manera y para los mismos propósitos que el VEGF empleado solo. La relación útil en moles de VEGF a tales factores terapéuticos secundarios es típicamente 1:0.1-10, utilizándose cantidades casi equimolares en una incorporación de la invención.

[0079] **Dosificación y Administración**

[0080] Dosificaciones y concentraciones de medicamento deseadas de las composiciones farmacéuticas de la invención pueden variar dependiendo del empleo particular vislumbrado. La determinación de la dosificación apropiada o vía de administración está perfectamente dentro de las habilidades de un médico cualquiera. Experimentos animales pueden ofrecer orientación confiable para la determinación de dosis efectivas para terapia humana. El escalonamiento inter-especies de dosis eficaces puede llevarse a cabo siguiendo los principios trazados por Mordenti, J. and Chappell, W. The use of interspecies scaling in toxicokinetics *In Toxicokinetics and New Drug Development,* Yacobi et al., Eds., Pergamon Press, New York 1989, pp. 42-96. Ejemplos de curvas de respuesta a la dosis para modelos de heridas animales medicados con VEGF pueden verse en **Fig. 2**, que es una curva de respuesta a la dosis para VEGF administrado a heridas de oreja isquémica en conejo. **Fig. 3** es una curva de respuesta a la dosis para VEGF administrado a una herida de conejo diabético. Para la prevención o tratamiento de enfermedad o tipo de herida, la dosificación

apropias de VEGF dependerá del tipo de enfermedad por tratar, como se define anteriormente, la gravedad y ciclo de la enfermedad, si el agente se administra para propósitos preventivos o terapéuticos, la terapia previa, la historia clínica del paciente y la respuesta al agente, y la discreción del médico tratante. El VEGF se formulará y dosificará en una forma consistente con la buena práctica médica teniendo en cuenta el trastorno específico por tratar, la afección del paciente individual, el sitio de administración del VEGF, el método de administración y otros factores conocidos por el médico.

[0081] La dosificación a emplear dependen de los factores descritos en esta solicitud. En ciertas incorporaciones de la invención, dependiendo del tipo y la gravedad de la enfermedad del sujeto, alrededor de 1 μg/kg a 50 mg/kg (*por ejemplo* 0.1-20mg/kg) de VEGF y/o un agente adicional, es una dosificación candidata para administración al paciente, ya sea por ejemplo, mediante una o más administraciones separadas, o mediante aplicación continua. Orientación en cuanto a dosificaciones y métodos particulares de administración se provee en la literatura. En una incorporación, la cantidad efectiva de VEGF administrada es alrededor de 20 $\mu g/cm^2$ a alrededor de 250 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 24 $\mu g/cm^2$, o 24 μg/cm. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 72 $\mu g/cm^2$, o 72 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 216 $\mu g/cm^2$, o 216 $\mu g/cm^2$. En una incorporación, la cantidad efectiva de VEGF administrada es 20 $\mu g/cm^2$ a 250 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 24 $\mu g/cm^2$ a alrededor de 216 $\mu g/cm^2$, o 24 $\mu g/cm^2$ a 216 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 24 $\mu g/cm^2$ a alrededor de 72 $\mu g/cm^2$, o 24 $\mu g/cm^2$ a 72 $\mu g/cm^2$. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 72 $\mu g/cm^2$ a alrededor de 216 $\mu g/cm^2$, o 72

μg/cm^2 a 216 μg/cm^2. En ciertas incorporaciones, la cantidad efectiva administrada es alrededor de 216 μg/cm^2 a alrededor de 250 μg/cm^2, o 216 μg/cm^2 a 250 μg/cm^2. El agente se administra convenientemente al sujeto durante una serie de tratamientos o de una vez.

[0082] Para administraciones repetidas durante varios días o más, dependiendo de la afección, el tratamiento se mantiene hasta que ocurra una supresión deseada de los síntomas de la enfermedad, por ejemplo, cierre completo de la herida, o reducción en el área de la herida. No obstante, pueden ser útiles otros regímenes posológicos. Típicamente, el especialista clínico administrará una molécula(s) de la invención hasta que se alcance una dosificación que proporcione el efecto biológico deseado. La administración de la cantidad efectiva de VEGF puede ser diaria o, de manera opcional, una cuantas veces a la semana, por ejemplo, por lo menos dos veces a la semana, o por lo menos tres veces a la semana, o por lo menos cuatro veces a la semana, o por lo menos cinco veces a la semana, o por lo menos seis veces a la semana. En una incorporación, el VEGF se administra por lo menos durante seis semanas, o por lo menos alrededor de doce semanas o hasta el cierre completo de la herida (por ejemplo, el cual puede determinarse mediante cierre de la piel sin requerimientos de drenaje o apósitos). En ciertos aspectos de la invención, el VEGF se administra durante menos de 20 semanas. El progreso de la terapia de la invención se monitorea fácilmente mediante técnicas y exámenes convencionales.

[0083] En general, la composición terapéutica de la invención se administra tópicamente al sujeto. En una incorporación de la invención, el VEGF es una formulación de un gel tópico, por ejemplo, en una jeringa precargada o envase. En ciertas incorporaciones, un agente terapéutico adicional se administra también tópicamente. Opcionalmente pueden emplearse además otras vías de administración del VEGF y/o agentes terapéuticos adicionales, por ejemplo,

administrarse por cualquier medio apropiado, incluida, pero no limitada a, administración parenteral, subcutánea, intraperitoneal, intrapulmonar, intracerobrospinal, intraarticular, intrasinovial, intratecal, oral e intranasal. Las infusiones parenterales incluyen administración intramuscular, intravenosa, intraarterial, intraperitoneal, o subcutánea.

[0084] Como se describe en esta solicitud, el VEGF puede combinarse con uno o más agentes terapéuticos adicionales o procedimientos. La administración combinada incluye coadministración, utilizando formulaciones separadas o una formulación farmacéutica única, y administración consecutiva en cualquier orden. El empleo de múltiples agentes se incluye igualmente en la invención. Por ejemplo, el VEGF puede preceder, seguir, alternar con la administración del agente terapéutico adicional, o puede administrarse con aquel. En una incorporación, hay un período de tiempo mientras ambos (o todos) agentes activos ejercen simultáneamente sus actividades biológicas. En un régimen de terapia de combinación, las composiciones de la invención se administran en una cantidad terapéuticamente efectiva o una cantidad terapéuticamente sinérgica. Tal como se emplea en esta solicitud, una cantidad terapéuticamente efectiva es tal que la co-administración del VEGF y uno o más agentes terapéuticos, o la administración de un procedimiento, resulta en la reducción o inhibición de la enfermedad o afección diana. Una cantidad terapéuticamente sinérgica es aquella cantidad de VEGF y uno o más agentes terapéuticos, por ejemplo, descrita en esta solicitud, necesaria para acelerar sinérgica o significativamente y/o mejorar la cicatrización de una herida.

[0085] **Composiciones Farmacéuticas**

[0086] Formulaciones terapéuticas de moléculas de la invención, por ejemplo, VEGF o agentes terapéuticos adicionales, combinados con VEGF, usados de acuerdo con la invención se preparan para almacenamiento mezclando una molécula, por ejemplo, un polipéptido

que posee el grado deseado de pureza, con sustancias portadoras, excipientes o estabilizadores opcionales farmacéuticamente aceptables (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), en la forma de formulaciones liofilizadas o soluciones acuosas. Sustancias portadoras, diluyentes, excipientes y estabilizadores aceptables son preferiblemente atóxicos a los destinatarios, en las dosis y concentraciones empleadas, e incluyen amortiguadores tales como fosfato, citrato, y otros ácidos orgánicos; antioxidantes incluidos ácido ascórbico y metionina; preservantes (tales como cloruro de octadecildimetilbencil amonio; cloruro de hexametonio; cloruro de benzalconio, cloruro de bencetonio; fenol, alcohol butílico o bencílico; alquil parabenos tales como metil o propil parabeno; catecol; resorcinol; ciclohexanol; 3-pentanol; y m-cresol); anticuerpo de bajo peso molecular (menos de alrededor de 10 residuos); proteínas, tales como albúmina sérica, gelatina, o inmunoglobulinas; polímeros hidrófilos tales como polivinilpirrolidona; aminoácidos tales como glicina, glutamina, asparagina, histidina, arginina, o lisina; monosacáridos, disacáridos, y otros carbohidratos incluidos glucosa, mannosa, o dextrinas; agentes quelantes tales como EDTA; azúcares tales como sacarosa, mannitol, trehalosa o sorbitol; iones contrarios formadores de sales tales como sodio; complejos de metal (por ejemplo, complejos Zn-proteína); y/o surfactantes no iónicos tales como TWEEN™, PLURONICS™ o polietilen glicol (PEG).

[0087] En ciertas incorporaciones, las formulaciones a utilizarse para administración *in vivo* son estériles. Esto se logra fácilmente mediante filtración a través de membranas de filtración estéril. El VEGF puede almacenarse en forma liofilizada o como solución acuosa o forma de gel. El pH de las preparaciones VEGF puede ser alrededor de desde 5 a 9, aunque en ciertos casos pueden ser apropiados valores de pH más altos o más bajos. Se entenderá que el uso de algunos de los excipientes, sustancias portadoras, o estabilizantes pueden resultar en la formación de sales del VEGF.

[0088] Los ingredientes activos pueden atraparse también en microcápsulas preparadas, por ejemplo, por técnicas coacervación o por polimerización interfacial, por ejemplo, hidroximetilcelulosa o microcápsulas de gelatina y microcápsulas de poli-(metilmetacilato), respectivamente, en sistemas coloidales de liberación de medicamento (por ejemplo, liposomas, microesferas de albúmina, microemulsiones, nanopartículas y nanocápsulas) o en macroemulsiones. Tales técnicas se divulgan en *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980). *Véase también* Johnson et al., *Nat. Med.,* 2:795-799 (1996); Yasuda, *Biomed. Ther.,* 27:1221-1223 (1993); Hora et al., *Bio/Technology,* 8:755-758 (1990); Cleland, Design and Production of Single Immunization Vaccines Using Polylactide Polyglycolide Microsphere Systems, in *Vaccine Design: The Subunit and Adjuvant Approach,* Powell and Newman, eds, (Plenum Press: New York, 1995), pp. 439-462; WO 97/03692, WO 96/40072, WO 96/07399; y Patente de los Estados Unidos No. 5,654,010.

[0089] Típicamente, para la cicatrización de una herida, el VEGF se formula en administración específica del sitio. Cuando se aplica tópicamente, el VEGF se combina convenientemente con otros ingredientes, tales como sustancias portadoras y/o coadyuvantes. No existen limitaciones sobre la naturaleza de tales otros ingredientes, excepto que tienen que ser farmacéuticamente aceptables y eficaces para su administración prevista, y no pueden degradar la actividad de los ingredientes activos de la composición. Ejemplos de vehículos apropiados incluyen ungüentos, cremas, geles, atomizadores, o suspensiones, con o sin colágeno purificado. Las composiciones pueden impregnarse igualmente en apósitos estériles, parches transdérmicos, emplastos, y vendajes, opcionalmente en forma líquida o semi-líquida. Pueden usarse también matrices de celulosa regenerada oxidada/colágeno, por ejemplo, Promogran™ Matrix Wound Dressing o

Promogran Prisma Matrix™.

[0090] Pueden elaborarse preparaciones de liberación sostenida. Ejemplos apropiados de preparaciones de liberación sostenida incluyen matrices semi-permeables de polímeros hidrófobos sólidos que contienen polipéptido de la invención, matrices que están en la forma de artículos moldeados, *por ejemplo*, películas, o microcápsulas. Ejemplos de matrices de liberación sostenida incluyen poliésteres, hidrogeles (por ejemplo, poli(2-hidroxietil-metacrilato), o poli(vinilalcohol)), poliláctidos (Patente de los Estados Unidos 3,773,919), copolímeros de ácido L-glutámico y γ etil-L-glutamato, etilen-vinil acetato no degradable, copolímeros degradables ácido láctico-ácido glicólico tales como el LUPRON DEPOT™ (microesferas inyectables compuestas de copolímero ácido láctico-ácido glicólico y acetato de leuprolida), ácido poli-láctico-coglicólico (PGLA) y ácido poli-D-(-)-3-hidroxibutírico. Aunque los polímeros tales como etilen vinil acetato y ácido láctico-ácido glicólico permiten la liberación de moléculas durante más de 100 días, ciertos hidrogeles liberan proteínas durante períodos de tiempo más cortos. Cuando los anticuerpos encapsulados permanecen en el cuerpo por un tiempo prolongado, éstos pueden desnaturalizarse o agregarse como resultado de la exposición a humedad a 37°C, lo cual se traduce en una pérdida de actividad biológica y posibles cambios en la inmunogenicidad. Pueden diseñarse estrategias razonables para la estabilización, dependiendo del mecanismo involucrado. Por ejemplo, si se descubre que el mecanismo de agregación es la formación de enlace S-S intermolecular a través de intercambio tio-disulfuro, la estabilización puede lograrse modificando residuos sulfhidrilo, liofilizando a partir de soluciones ácidas, controlando el contenido de humedad, usando aditivos adecuados, y desarrollando composiciones específicas de matriz polimérica.

[0091] Para obtener una formulación en gel, el VEGF formulado

en una composición líquida puede mezclarse con una cantidad efectiva de de un polisacárido soluble en agua o polímero sintético tal como polietilén glicol para formar un gel de la viscosidad apropiada para aplicarse tópicamente. El polisacárido que puede utilizarse incluye, por ejemplo, derivados de celulosa tales como derivados de celulosa eterificada, incluidas celulosas alquílicas, celulosas hidroxialquílicas, y celulosas alquilhidroxialquílicas, por ejemplo, metilcelulosa, hidroxietilcelulosa, carboximetilcelulosa, hidroxipropilmetilcelulosa, e hidroxipropilcelulosa; almidón y almidón fraccionado; agar; ácido algínico y alginatos; goma arábiga; pululana; agarosa; carragenina; dextranos; dextrinas; fructanas; inulina; mannanas; xilanas; arabinanas; quitosanas; glucógenos; glucanas; y biopolímeros sintéticos; así como gomas tales como goma xantana; goma de guar; goma de algarrobo; goma arábiga; goma tragacanto; y goma karaya; y derivados y sus mezclas. En una incorporación de la invención, el agente gelatinizante en esta solicitud es uno que es, por ejemplo, inerte a sistemas biológicas, no tóxico, sencillo de preparar, y/o no demasiado aguado o viscoso, y no desestabilizará el VEGF contenido dentro de él.

[0092] En ciertas incorporaciones de la invención, el polisacárido es un derivado de celulosa eterificada, en otra incorporación uno que es bien definido, purificado y listado en la Farmacopea de los Estados Unidos (USP), por ejemplo, derivados de metilcelulosa e hidroxialquilcelulosa, tales como hidroxipropilcelulosa, hidroxietilcelulosa, e hidroxipropilmetilcelulosa. En una incorporación, el polisacárido es metilcelulosa.

[0093] El polietilén glicol útil para gelificación es típicamente una mezcla de polietilén glicoles de bajo peso molecular para obtener la viscosidad deseada. Por ejemplo, un mezcla de polietilén glicol de peso molecular 400-600 con uno de peso molecular 1.500 sería efectiva para este propósito cuando se mezcla en la proporción apropiada para obtener

una pasta.

[0094] El término "soluble en agua" como se aplica a los polisacáridos y polietilén glicoles pretende incluir soluciones coloidales y dispersiones. En general, la solubilidad de los derivados de celulosa se determina por el grado de sustitución de grupos éter, y los derivados estabilizantes útiles en esta solicitud deben tener una cantidad suficiente de tales grupos éter por unidad de anhidroglucosa en la cadena de celulosa para hacer los derivados solubles en agua. Un grado de sustitución de éter de por lo menos 0.35 grupos éter por unidad de anhidroglucosa es generalmente suficiente. Además, los derivados de celulosa pueden estar en la forma de sales de metal alcalino, por ejemplo, las sales de Li, Na, K, o Cs.

[0095] Si se emplea metilcelulosa en el gel, por ejemplo, ésta comprende alrededor de 2-5%, o alrededor de 3%, o alrededor de 4% o alrededor de 5%, del gel y el VEGF está presente en una cantidad de alrededor de 100-2.000 μg por ml de gel.

[0096] El VEGF y/o un agente adicional pueden administrarse igualmente a la herida mediante terapia génica. Terapia génica se refiere a una terapia realizada mediante la administración de un ácido nucleico a un sujeto. En aplicaciones de terapia génica, se introducen genes en células con el fin de lograr síntesis *in vivo* de un producto genético terapéuticamente efectivo, por ejemplo para reemplazo de un gen defectuoso. "Terapia génica" incluye tanto la terapia génica convencional, donde se consigue un efecto duradero mediante tratamiento único, como la administración de agentes de terapia génica, la cual implica la administración por una sola vez o repetida de un ADN o ARNm terapéuticamente efectivo. Los oligonucleótidos pueden modificarse para mejorar su absorción, por ejemplo sustituyendo sus grupos fosfodiéster de carga negativa por grupos sin carga. Para reseñas

generales de los métodos de la terapia génica, véase, por ejemplo, Goldspiel et al. *Clinical Pharmacy* 12:488-505 (1993); Wu and Wu *Biotherapy* 3:87-95 (1991); Tolstoshev *Ann. Rev. Pharmacol. Toxicol.* 32:573-596 (1993); Mulligan *Science* 260:926-932 (1993); Morgan and Anderson Ann. *Rev. Biochem.* 62:191-217 (1993); y May *TIBTECH* 11:155-215 (1993). Métodos comúnmente conocidos en el arte de la tecnología de ADN recombinante, que pueden emplearse, se describen en Ausubel et al. eds. (1993) *Current Protocols in Molecular Biology,* John Wiley & Sons, NY; y Kriegler (1990) *Gene Transfer and Expression, A Laboratory Manual,* Stockton Press, NY.

[0097] Existe una variedad de técnicas disponibles para introducir ácidos nucleicos en células viables. Las técnicas varían dependiendo de si el ácido nucleico se transfiere en células cultivadas *in vitro*, o *in vivo* en las células de anfitrión previsto. Técnicas apropiadas para la transferencia de ácido nucleico en células mamíferas *in vitro* incluyen el empleo de liposomas, electroporación, microinyección, fusión de células, DEAE-dextrina, el método de precipitación en fosfato de calcio, etc. Las técnicas de transferencia de genes *in vivo* actualmente preferidas incluyen transfección con vectores víricos (típicamente, retrovíricos) y transfección mediada por liposoma-proteína de cápside vírica (Dzau et al., *Trends in Biotechnology* 11, 205-210 (1993)). Por ejemplo, técnicas de transferencia de ácidos nucleicos *in vivo* incluyen transfección con vectores víricos (tales como adenovirus, Herpes simplex I virus, lentivirus, retrovirus, o virus adeno-asociado) y sistemas a base de lípidos (lípidos útiles para la transgerencia, mediada por lípido, del gen son DOTMA, DOPE y DC-Chol, por ejemplo). Ejemplos de la utilización de vectores víricos en terapia génica pueden hallarse en Clowes et al. *J. Clin. Invest.* 93:644-651 (1994); Kiem et al. *Blood* 83:1467-1473 (1994); Salmons and Gunzberg *Human Gene Therapy* 4:129-141 (1993); Grossman and Wilson *Curr. Opin. in Genetics and Devel.* 3:110-114 (1993); Bout et al. *Human Gene Therapy* 5:3 -10

(1994); Rosenfeld et al. *Science* 252:431-434 (1991); Rosenfeld et al. *Cell* 68:143-155 (1992); Mastrangeli et al. *J. Clin. Invest.* 91:225-234 (1993); and Walsh et al. *Proc. Soc. Exp. Biol. Med.* 204:289-300 (1993).

[0098] En algunas situaciones es deseable para proveer la fuente de ácido nucleico con un agente que reconoce las células de una herida, tal como un anticuerpo específico para una proteína de superficie celular o la célula diana, un ligando para un receptor en la célula diana, etc. Donde se emplean liposomas, proteínas que se unen a una proteína de membrana de superficie celular asociada con endocitosis pueden utilizarse para reconocer y/o facilitar la absorción, por ejemplo proteínas de cápside o sus fragmentos, trópicos para un tipo de célula particular, anticuerpos para proteínas que sufren internalización en su ciclo, proteínas que reconocen localización intracelular y mejoran la semivida intracelular. La técnica de endocitosis mediada por receptor se describe, por ejemplo, por Wu et al., *J. Biol. Chem.* 262, 4429-4432 (1987); y Wagner et al., *Proc. Natl. Acad. Sci. USA* 87, 3410- 3414 (1990). Para una reseña de los protocolos de marcación de genes y terapia génica, véase Anderson et al., *Science* 256, 808- 813 (1992).

[0099] **Modificaciones Covalentes a Polipéptidos de la Invención**

[0100] Modificaciones covalentes de un polipéptido de la invención, por ejemplo, el VEGF u otros agentes polipéptidos terapéuticos adicionales, combinados con VEGF, se incluyen en el ámbito de esta invención. Éstas pueden hacerse mediante síntesis química o escisión enzimática o química del polipéptido, si es aplicable. Otros tipos de modificaciones covalentes de polipéptido se introducen en la molécula haciendo reaccionar residuos de aminoácidos selectivos del polipéptido con un agente derivante orgánico, que es capaz de reaccionar con cadenas laterales seleccionadas o los residuos N- o C-terminales, o incorporando un aminoácido modificado o aminoácido no natural en la cadena polipeptídica en crecimiento, por ejemplo, Ellman

et al. *Meth. Enzym.* 202:301-336 (1991); Noren et al. *Science* 244:182 (1989); y las solicitudes de Patente de los Estados Unidos 20030108885 y 20030082575.

[0101] Muy comúnmente residuos cisteinilo se hacen reaccionar con α-haloacetatos (y aminas correspondientes), tales como ácido cloroacético cloroacetamida, para producir derivados carboximetilo o carboxiamidometilo. Los residuos cisteinilo se derivan igualmente mediante reacción con bromotrifluoroacetona, ácido α-bromo-β-(5-imidozoil)propiónico, fosfato de cloroacetilo, N-alquilmaleimidas, 3-nitro-2-piridildisulfuro, metil 2-piridildisulfuro, p-cloromercuribenzoato, 2-cloromercuri-4-nitrofenol, o cloro-7-nitrobenzo-2-oxa-1,3-diazol.

[0102] Los residuos histidilo se derivan mediante reacción con dietilpirocarbonato a pH 5.5-7.0, porque este agente es relativamente específico para la cadena lateral histidilo. También es útil el bromuro de para-bromofenacilo; la reacción se realiza típicamente en 0.1 M de cacodilato de sodio a pH 6.0.

[0103] Los residuos lisinilo y amino-terminales se hacen reaccionar con anhídridos de ácido succínico u otro carboxílico. La derivación con estos agentes tiene el efecto de invertir la carga de los residuos lisinilo. Otros reactivos apropiados para derivar residuos con contenido α-amino incluyen imidoésteres tales como metil picolinimidato, fosfato de piridoxal, pridoxal, cloroborohidruro, ácido trinitrobencenosulfónico, O-metilisourea, 2,4-pentanodiona, y reacción con glioxilato, caralizada con transaminasa.

[0104] Los residuos arginilo se modifican mediante reacción con uno o varios reactivos convencionales, entre ellos fenilglioxal, 2,3-butanodiona, 1,2-ciclohexanodiona, y ninhidrina. La derivación de residuos de arginina requiere que la reacción se lleve a cabo en

condiciones alcalinas debido al elevado pK_a del grupo funcional guanidina. Más aún, estos reactivos pueden reaccionar con los grupos de lisina, así como el grupo ε-amino arginina.

[0105] La modificación específica de residuos tirosilo puede hacerse, con interés particular en introducir etiquetas espectrales en residuos tirosilo mediante reacción con compuestos diazonio aromáticos o tetranitrometano. Muy comúnmente, N-acetilimidizol y tetranitrometano se utilizan para formar la especie O-acetil tirosilo y derivados 3-nitro, respectivamente. Los residuos tirosilo se yodan usando ^{125}I ó ^{131}I para preparar proteínas marcadas para uso en radioinmunoanálisis.

[0106] Grupos laterales carboxilo (aspartilo o glutamilo) se modifican selectivamente mediante reacción con carbodiimidas (R-N=C=N-R'), donde R y R' son diferentes grupos alquilo, tales como 1-ciclohexil-3-(2-morfolinil-4-etil)carbodiimida o 1-etil-3-(4-azonia-4,4-dimetilpentil)carbodiimida. Más aún, residuos aspartilo y glutamilo se convierten en residuos asparaginilo y glutaminilo mediante reacción con iones de amonio.

[0107] Los residuos glutaminilo y asparaginilo se desamidan frecuentemente en los correspondientes residuos glutamilo y aspartilo, respectivamente. Estos residuos se desamidan bajo condiciones neutras o básicas. La forma desamidada de estos residuos cae dentro del ámbito de esta invención.

[0108] Otras modificaciones incluyen la hidroxilación de prolina y lisina, fosforilación de grupos hidroxilo de residuos serilo o treonilo, metilación de los grupos α-amino de las cadenas laterales de lisina, arginina e histidina (T.E. Creighton, *Proteins: Structure and Molecular Properties,* W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)),

acetilación de la amina N-terminal, y amidación de cualquier grupo carboxilo C-terminal.

[0109] Otro tipo de modificación covalente implica acoplar química o enzimáticamente glucósidos a un polipéptido de la invención. Estos procedimientos son ventajosos en el sentido de que no requieren producción del polipéptido en una célula anfitriona que tiene capacidades de glucosilación para glucosilación N- u O-enlazada. Dependiendo del modo de acoplamiento utilizado, el azúcar(es) puede(n) unirse a (a) arginina e histidina, (b) grupos carboxilo libres, (c) grupos sulfhidrilo libres tales como los de cisteína, (d) grupos hidroxilo libres tales como los de serina, treonina, o hidroxiprolina, (e) residuos aromáticos tales como los de fenilalanina, tirosina, o triptófano, o (f) el grupo amida de glutamina. Estos métodos se describen en WO 87/05330 publicada el 11 de septiembre de 1987, y en Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

[0110] El retiro de cualesquiera fracciones carbohidrato presentes en un polipéptido de la invención puede llevarse a cabo química o enzimáticamente. La desglucosilación química requiere la exposición del polipéptido al compuesto ácido trifluorometanosulfónico, o un compuesto equivalente. Este tratamiento resulta en la escición de la mayoría o la totalidad de los azúcares, con excepción del azúcar enlazante (N-acetilglucosamina o N-acetilgalactosamina), dejando al mismo tiempo el polipéptido intacto. La desglucosilación química se describe por Hakimuddin, *et al. Arch. Biochem. Biophys.* 259:52 (1987) y por Edge *et al. Anal. Biochem.*, 118:131 (1981). La escisión enzimática de fracciones carbohidrato, por ejemplo, en anticuerpos, puede lograrse mediante el uso de una variedad de endo- y exo-glucosidasas como se describe por Thotakura *et al. Meth. Enzymol.* 138:350 (1987).

[0111] Otro tipo de modificación covalente de un polipéptido de la invención comprende conectar el polipéptido a una de una variedad de polímeros no proteináceos, *por ejemplo,* polietilén glicol, polipropilén glicol, o polioxialquilenos, en la manera expuesta en las Patentes de los Estados Unidos No. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 ó 4,179,337.

[0112] **Vectores, Células Anfitrionas y Métodos Recombinantes**

[0113] Los polipéptidos de la invención pueden producirse recombinantemente, usando técnicas y materiales de fácil obtención.

[0114] Para la producción recombinante de un polipéptido de la invención, por ejemplo, el VEGF o agentes polipéptido terapéuticos adicionales combinados con el VEGF, el ácido nucleico que lo codifica se aíslan y se insertan en un vector replicable para clonación posterior (amplificación del ADN) o para expresión. Hay muchos vectores a disposición. Los componentes del vector incluyen generalmente, pero no se limitan a, uno o más de los siguientes: secuencias de control, una secuencia señal, un origen de replicación, uno o más genes marcadores, un elemento potenciador, un promotor, y una secuencia de terminación de transcripción.

[0115] La expresión "secuencias de control" se refieren a secuencias de ADN necesarias para la expresión de una secuencia codificante operacionalmente enlazada en un organismo anfitrión particular. Las secuencias de control que son apropiadas para procariotas, por ejemplo, incluyen un promotor, opcionalmente una secuencia operadora, y un sitio de unión a ribosoma. Las células eucariotas son conocidas por utilizar promotores, señales de poliadenilación y potenciadores.

[0116] El ácido nucleico está "operacionalmente enlazado" cuando

se pone en una relación funcional con otra secuencia de ácidos nucleicos. Por ejemplo, ADN para una pre-secuencia o líder secretor se enlaza operacionalmente a ADN para un polipéptido, si éste se expresa como una pre-proteína que participa en la secreción del polipéptido; un promotor o potenciador se enlaza operacionalmente a una secuencia codificante si afecta la transcripción de la secuencia; o un sitio de unión a ribosoma está operacionalmente enlazado a una secuencia codificante si se posiciona de modo que facilite la traducción. Por lo general, "operacionalmente enlazado" significa que las secuencias de ADN que se están enlazando son contiguas y, en el caso de un líder secretor, contiguas y en el marco de lectura. Sin embargo, los potenciadores no tienen que ser contiguos. El enlazamiento se lleva a cabo por ligamiento en sitios de restricción convenientes. Si tales sitios no existen, los adaptadores oligonucleótido sintéticos o conectores se utilizan de acuerdo con la práctica convencional.

[0117] ADN que codifica el polipéptido de la invención se aísla y/o secuencia fácilmente usando procedimientos convencionales. Por ejemplo, un ADN que codifica VEGF se aísla y se secuencia, *por ejemplo*, utilizando sondas oligonucleótido que son capaces de unirse específicamente al gen que codifica el VEGF. Una molécula de ácido nucleico "aislada" es una molécula de ácido nucleico que se ha identificado y separado de por lo menos una molécula de ácido nucleico contaminante, con la cual se asocia ordinariamente en la fuente natural del ácido nucleico del polipéptido. Una molécula de ácido nucleico aislada es distinta de en la forma o escenario en los cuales se encuentra en la naturaleza. Por consiguiente, moléculas aisladas de ácido nucleico se distinguen de la molécula de ácido nucleico tal como existe en células naturales. Sin embargo, una molécula aislada de ácido nucleico incluye una molécula de ácido nucleico contenida en células que ordinariamente expresan el polipéptido donde, por ejemplo, la molécula de ácido nucleico está en una localización cromosómica diferente de la de las

células naturales.

[0118] *Componente de Secuencia de Señal*

[0119] Polipéptidos de la invención pueden producirse en forma recombinante no sólo directamente, sino también como un polipéptido de fusión con un polipéptido heterólogo, que es típicamente una secuencia señal u otro polipéptido que tiene un sitio de escisión específico en el N-término de la proteína madura o polipéptido. Típicamente, la secuencia señal heteróloga seleccionada es una que es reconocida y procesada (*es decir*, escindida por una polipeptidasa señal) por la célula anfitriona. Para células procariotas anfitrionas que no reconocen y procesan la secuencia señal del polipéptido natural, la secuencia señal se sustituye por una secuencia señal procariota seleccionada, por ejemplo, del grupo de líderes de la fosfatasa alcalina, penicilinasa, Ipp, o enterotoxina II térmicamente estables. Para la secreción de levadura, la secuencia señal natural puede sustituirse, *por ejemplo*, por el líder de invertasa de levadura, un líder del factor (incluidos los líderes factor α *Saccharomyces* y *Kluyveromyces*), o el líder de fosfatasa ácida, el líder de glucoamilasa *C. albicans*, o la señal descrita en WO 90/13646. En la expresión de células mamíferas, se encuentran disponibles secuencias señal mamíferas, así como líderes secretores víricos, por ejemplo, la señal herpes simplex gD.

[0120] El ADN para tal región precursora se liga en el marco de lectura al ADN que codifica el polipéptido de la invención.

[0121] *Componente Origen de Replicación*

[0122] Ambos vectores de expresión y clonación contienen una secuencia de ácidos nucleicos que permite que el vector se replique en una o más células anfitrionas seleccionadas. Generalmente, en los vectores de clonación esta secuencia es una que permite que el vector se replique independientemente del ADN comosómico del anfitrión, e

incluye orígenes de replicación o secuencias autónomamente replicantes. Tales secuencias son bien conocidas para una variedad de bacterias, levaduras y virus. El origen de replicación del plásmido pBR322 es apropiado para la mayoría de las bacterias gram-negativas, el origen del plásmido 2μ es apropiado para levadura, y varios orígenes víricos (SV40, polioma, adenovirus, VSV o BPV) son útiles para clonar vectores en células mamíferas. Generalmente, el componente origen de replicación no se necesita para vectores de expresión mamíferos (el origen SV40 puede usarse típicamente sólo porque contiene el promotor temprano).

[0123] *Componente Gen de Selección*

[0124] Vectores de expresión y clonación pueden contener un gen de selección, denominado también un marcador seleccionable. Genes típicos de selección codifican proteínas que (a) confieren resistencia a antibióticos u otras toxinas, *por ejemplo,* ampicilina, neomicina, metotrexato, o tetraciclina, (b) complementan deficiencias auxotróficas, o (c) aportan nutrientes críticos no disponibles de medios complejos, *por ejemplo*, el gen que codifica la D-alanina racemasa para *Bacilli.*

[0125] Un ejemplo de un esquema de selección utiliza un medicamento para detener el crecimiento de una célula anfitriona. Aquellas células que se transforman exitosamente con un gen heterólogo producen una proteína que confiere resistencia a los medicamentos y, por consiguiente, sobreviven al régimen de selección. Ejemplos de tal selección dominante utilizan los medicamentos neomicina, ácido micofenólico e higromicina.

[0126] Otro ejemplo de marcadores selectivos apropiados para células mamíferas son aquellos que permiten la identificación de células competentes para absorber el ácido nucleico, tales como DHFR, timidina cinasa, metalotioneína-I y -II, típicamente genes de

metalotioneína de primate, adenosina deaminasa, ornitina decarboxilasa, *etc.*

[0127] Por ejemplo, células transformadas con el gen de selección DHFR se identifican primeramente cultivando todos los transformantes en un medio de cultivo que contiene metotrexato (Mtx), un antagonista competitivo de DHFR. Una célula anfitriona apropiada, cuando se emplea DHFR natural es la línea de células de ovario de hámster chino (CHO), deficiente en actividad DHFR.

[0128] Alternativamente, células anfitrionas (particularmente anfitriones naturales que contienen DHFR endógena) transformadas o co-transformadas con secuencias de ADN que codifican un polipéptido de la invención, proteína DHFR natural, y otro marcador seleccionable tal como aminoglucósido 3'-fosfotransferasa (APH) pueden seleccionarse mediante cultivo en medio que contiene un agente se selección para el marcador seleccionable tal como un antibiótico aminoglucosídico, *por ejemplo*, canamicina, neomicina, o G418. Véase la Patente de los Estados Unidos No. 4,965,199.

[0129] Un gen de selección apropiado para uso en levadura es el gen *trp*1 presente en el plásmido de levadura Yrp7 (Stinchcomb *et al., Nature,* 282:39 (1979)). El gen *trp*1 provee un marcador de selección para una cepa mutante de levadura que carece de la capacidad de crecer en triptófano, por ejemplo, ATCC No. 44076 o PEP4-1. Jones, *Genetics,* 85:12 (1977). La presencia de la lesión *trpl* en el genoma de la célula anfitriona de levadura provee entonces un ambiente efectivo para detectar la transformación mediante crecimiento en la ausencia de triptófano. Análogamente, cepas de levadura deficientes en *Leu*2 (ATCC 20,622 ó 38,626) se complementan con plásmidos conocidos que llevan el gen *Leu2*.

[0130] Además, vectores derivados del plásmido circular pKD1 de1.6 μm pueden utilizarse para transformación de levaduras *Kluyveromyces*. Alternativamente, un sistema de expresión para la producción a gran escala de quimosina bovina recombinante se divulgó para *K. lactis*. Van den Berg, *Bio/Technology,* 8:135 (1990). Se han divulgado igualmente vectores de expresión estables multi-copia para secreción de albúmina sérica humana recombinante madura por cepas industriales de *Kluyveromyces*. Fleer *et al, Bio/Technology,* 9:968-975 (1991).

[0131] *Componente Promotor*

[0132] Los vectores de expresión y clonación contienen usualmente un promotor que es reconocido por el organismo anfitrión y está operacionalmente enlazado a un ácido nucleico que codifica un polipéptido de la invención. Promotores apropiados para uso con anfitriones procariotas incluyen el promotor *phoA*, sistemas promotores β-lactamasa y lactosa, fosfatasa alcalina, un sistema promotor triptófano (trp), y promotores híbridos tales como el promotor tac. No obstante, son apropiados otros promotores bacterianos conocidos. Promotores para uso en sistemas bacterianos continuarán asímismo la secuencia Shine-Dalgarno (S.D.) enlazada operacionalmente al ADN que codifica el polipéptido de la invención.

[0133] Se conocen secuencias promotoras para eucariotas. Virtualmente todos los genes eucariotas tienen una región rica en AT localizada aproximadamente 25 a 30 bases corriente arriba del sitio donde se inicia la transcripción. Otra secuencia hallada 70 a 80 bases corriente arriba del inicio de transcripción de muchos genes es una región CNCAAT donde N puede ser cualquier nucleótido. En el extremo 3' de la mayoría de los genes eucariotas es una secuencia AATAAA que puede ser la señal para la adición de la cola poly A al extremo 3' de la secuencia codificante. Todas estas secuencias se

insertan convenientemente en vectores de expresión eucariotas.

[0134] Ejemplos de secuencias promotoras apropiadas para uso con anfitriones de levadura incluyen los promotores para 3-fosfoglicerato cinasa u otras enzimas glucolíticas, tales como enolasa, gliceraldehído-3-fosfato dehidrogenasa, hexocinasa, piruvato decarboxilasa, fosfo-fructocinasa, glucosa-6-fosfato isomerasa, 3-fosfoglicerato mutasa, piruvato cinasa, triosafosfato isomerasa, fosfoglucosa isomerasa y glucocinasa.

[0135] Otros promotores de levadura, que son promotores inducibles con la ventaja adicional de transcripción controlada por las condiciones de crecimiento, son las regiones promotoras para la alcohol dehidrogenasa 2, isocitocromo C, fosfatasa ácida, enzimas degradantes asociadas con el metabolismo del nitrógeno, metalotioneína, gliceraldehído-3-fosfato dehidrogenasa, y enzimas responsables de la utilización de maltosa y galactosa. Vectores y promotores apropiados para uso en la expresión de levadura se describen adicionalmente en EP 73,657. De igual manera, los potenciadores de levadura se utilizan ventajosamente con promotores de levadura.

[0136] La transcripción de polipéptidos de la invención a partir de vectores en células anfitrionas mamíferas se controla, por ejemplo, por promotores obtenidos de los genomas de virus tales como el virus polioma, el virus de la viruela aviar, adenovirus (tales como Adenovirus 2), virus del papiloma bovino, virus del sarcoma aviar, citomegalovirus, un retrovirus, virus de la hepatitis-B y, típicamente, el virus del mono 40 (SV40), de promotores mamíferos heterólogos, *por ejemplo*, el promotor de actina o un promotor de inmunoglobulina, a partir de promotores de choque térmico, siempre que tales promotores sena compatibles con los sistemas de células anfitrionas.

[0137] Los promotores tempranos y tardíos del virus SV40 se obtienen convenientemente de un fragmento de restricción SV40 que contiene asimismo el origen vírico SV40 de replicación. El promotor temprano inmediato del citomegalovirus humano se obtiene convenientemente como un fragmento de restricción HindIII E. Un sistema para expresar ADN en anfitriones mamíferos usando el virus de papiloma bovino como vector se divulga en la Patente de los Estados Unidos No. 4,419,446. Una modificación de este sistema se describe en la Patente de los Estados Unidos No. 4,601,978. Véase también Reyes *et al., Nature* 297:598-601 (1982) sobre la expresión de ADNc del β-interferón humano en células de ratón bajo el control de un promotor de timidina cinasa del virus herpes simplex. Alternativamente, la repetición terminal larga del virus de sarcoma rous puede utilizarse como el promotor.

[0138] *Componente Elemento Potenciador*

[0139] La transcripción de un ADN que codifica un polipéptido de esta invención por eucariotas superiores se incrementa a menudo insertando una secuencia potenciadora en el vector. Actualmente se conocen muchas secuencias potenciadoras de genes mamíferos (globina, elastasa, albúmina, α-fetoproteína e insulina). Típicamente, se utilizará un potenciador de un virus de célula eurariota. Ejemplos incluyen el potenciador SV40 en el lado tardío del origen de replicación (bp 100-270), el potenciador promotor temprano del citomegalovirus, el potenciador polioma en el lado tardío del origen de replicación, y potenciadores adenovirus. Véase asimismo Yaniv, *Nature* 297:17-18 (1982) sobre elementos potenciadores para activación de promotores eucariotas. El potenciador puede empalmarse en el vector en una posición 5' ó 3' a la secuencia que codifica el polipéptido, pero típicamente se localiza en el sitio 5' del promotor.

[0140] *Componente Terminación de Transcripción*

[0141] Vectores de expresión utilizados en células anfitrionas eucariotas (levaduras, hongos, insectos, plantas, animales, humanos, o

células nucleadas de otros organismos multicelulares) contendrán igualmente secuencias necesarias para la terminación de transcripción y para estabilizar el ARNm. Tales secuencias se obtienen comúnmente de las regiones 5' y, ocasionalmente las regiones no traducidas 3', de los ADN o ADNc eucariotas o víricos. Estas regiones contienen segmentos de nucleótido transcritos como fragmentos poliadenilados en la porción no traducida del ARNm que codifica el polipéptido de la invención. Un componente útil de terminación de transcripción es la región de poliadenilación se la somatotropina bovina. Véase WO94/11026 y el vector de expresión divulgado allí.

[0142] *Selección de Transformación de Células Anfitrionas*

[0143] Células anfitrionas apropiadas para clonar o expresar ADN que codifica los polipéptidos de la invención en los vectores contemplados en esta solicitud son las células procariotas, de lavadura, o células eucariotas superiores descritas anteriormente. Procariotas apropiadas para este propósito incluyen eubacterias, tales como organismos Gram-negativos o Gram-positivos, por ejemplo, Enterobacteriaceae tales como *Escherichia, por ejemplo, E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, por ejemplo, Salmonella typhimurium, Serratia, por ejemplo, Serratia marcescans,* y *Shigella,* así como *Bacilli* tales como *B. subtilis* y *B. licheniformis* (*por ejemplo, B. licheniformis* 41P divulgado en DD 266,710 publicada el 12 de abril de 1989), *Pseudomonas* teles como *P. aeruginosa,* y *Streptomyces.* Típicamente, el anfitrión de clonación *E. coli* es *E. coli* 294 (ATCC 31,446), aunque son apropiadas otras cepas tales como *E. coli* B, *E. coli* X1776 (ATCC 31,537), y *E. coli* W3110 (ATCC 27,325). Estos ejemplos son ilustrativos más que limitantes.

[0144] Aparte de los procariotas, microbios eucariotas tales como hongos filamentosos o levadura son anfitriones apropiados de clonación o expresión para vectores que codifican el polipéptido de la invención.

Saccharomyces cerevisiae, o levadura común de panadería, es el más comúnmente usado entre los microorganismos anfitriones eucariotas inferiores. No obstante, se dispone de un número de otros géneros, especies, y cepas que son útiles en esta solicitud, tales como *Schizosaccharomyces pombe*; anfitriones *Kluyveromyces* tales como, *por ejemplo, K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans,* y *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* tales como *Schwanniomyces occidentalis*; y hongos filamentosos tales como, *por ejemplo, Neurospora, Penicillium, Tolypocladium,* y anfiriones *Aspergillus* tales como *A. nidulans* y *A. niger.*

[0145] Células anfitrionas apropiadas para la expresión de polipéptidos glucosilados de la invención se derivan de organismos multicelulares. Ejemplos de células invertebradas incluyen células vegetales y células de insecto. Se han identificado numerosas cepas baculovíricas y variantes y células anfitrionas de insectos permitidas, correspondientes, de anfitriones tales como *Spodoptera frugiperda* (oruga), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (mosca de la fruta), y *Bombyx mori.* Una variedad de cepas víricas para transfección se encuentran públicamente disponibles, *por ejemplo,* la variante L-1 de *Autographa californica* NPV y la cepa Bm-5 de *Bombyx mori* NPV, y tales virus pueden usarse como el virus contemplado en esta solicitud de acuerdo con la presente invención, particularmente para transfección de células de *Spodoptera frugiperda.* Cultivos de células vegetales de algodón, maíz, papa, fríjol de soya soybean, petunia, tomate y tabaco pueden emplease igualmente como anfitriones.

[0146] No obstante, el interés ha sido máximo en células

vertebradas, y la propagación de células vertebradas en cultivo (cultivo de tejido) se ha vuelto un procedimiento rutinario. Ejemplos de líneas de células anfitrionas mamíferas útiles son la línea de riñón de mono CV1 transformada por SV40 (COS-7, ATCC CRL 1651); la línea de riñón embrionario humano (células 293 ó 293 subclonadas para crecimiento en cultivo de suspensión, Graham *et al, J. Gen Virol* 36:59 (1977)); células de riñón de hámster recién nacido (BHK, ATCC CCL 10); células de ovario de hámster chino ADHFR (CHO, Urlaub *et al, Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); células Sertoli de ratón (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); células de riñón de mono (CV1 ATCC CCL 70); células de riñón de mono African green (VERO-76, ATCC CRL-1587); células de carcinoma cervical humano (HELA, ATCC CCL 2); células de riñón canino (MDCK, ATCC CCL 34); células de hígado de rata buffalo (BRL 3A, ATCC CRL 1442); células de pulmón humano (W138, ATCC CCL 75); células de hígado humano (Hep G2, HB 8065); tumor mamario de ratón (MMT 060562, ATCC CCL51); células TRI (Mather *et al, Annals N.Y. Acad. Sci.* 383:44-68 (1982)); células MRC 5; células FS4; y una línea de hepatoma humano (Hep G2).

[0147] Las células anfitrionas se transforman con los vectores de expresión o clonación ya descritos para la producción del polipéptido de la invención y se cultivan en medios nutrientes convencionales modificados, según lo apropiado, para inducir promotores, seleccionar transformantes, o amplificar los genes que codifican las secuencias deseadas.

[0148] *Cultivo de Células Anfitrionas*

[0149] Las células anfitrionas usadas para producir polipéptidos de la invención pueden cultivarse en una variedad de medios. Medios disponibles en el comercio tales como Ham's F1O (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), y Dulbecco's

Modified Eagle's Medium ((DMEM), Sigma), medios de crecimiento normales para hepatocitos (Cambrex), medios de crecimiento para pre-adipocitos (Cambrex), etc., son apropiados para cultivar las células anfitrionas. Adicionalmente, cualquiera de los medios descritos en Ham *et al., Meth. Enz.* 58:44 (1979), Barnes *et al., Anal. Biochem.* 102:255 (1980), Patentes de los Estados Unidos No. 4,767,704; 4,657,866; 4,927,762; 4,560,655; o 5,122,469; WO 90/03430; WO 87/00195; o Patente de los Estados Unidos Re. 30,985 pueden utilizarse como medios de cultivo para las células anfitrionas. Cualquiera de estos medios puede suplementarse, según sea necesario, con hormonas y/u otros factores de crecimiento (tales como insulina, transferrina, o factor de crecimiento epidérmico), sales (tales como cloruro de sodio, calcio, magnesio, y fosfato), amortiguadores (tales como HEPES), nucleótidos (tales como adenosina y timidina), antibióticos (tales como el fármaco GENTAMYCIN™), elementos trazadores (definidos como compuestos orgánicos usualmente presentes en concentraciones finales en el rango micromolar), y glucosa o una fuente de energía equivalente. Cualesquiera otros suplementos necesarios pueden incluirse asimismo en concentraciones apropiadas que serían conocidas por los expertos en el arte. Las condiciones de cultivo, tales como temperatura, pH, y similares, son las ya previamente utilizadas con la célula anfitriona seleccionada para expresión, y serán evidentes para el profesional de habilidad ordinaria.

[0150] **Purificación del Polipéptido**

[0151] Cuando se emplean técnicas recombinantes, un polipéptido de la invención, por ejemplo, VEGF o agente polipéptido terapéutico adicional que se combina con VEGF, puede producirse intracelularmente, en el espacio periplasmático, o segregarse directamente en el medio. Polipéptidos de la invención pueden recuperarse y/o aislarse de medio de cultivo o de lisados de células anfitrionas. Un polipéptido "aislado" es uno que se ha identificado y

separado y/o recuperado de un componente de su entorno natural. Componentes contaminantes de su entorno natural son materiales que interferirían los usos diagnósticos o terapéuticos del polipéptido, y pueden incluir enzimas, hormonas, y otros solutos proteináceos o no proteináceos. En ciertas incorporaciones, el polipéptido se purificará (1) a más del 95% en peso del polipéptido según se determine por el método de Lowry, o más del 99% en peso, (2) a un grado suficiente para obtener por lo menos 15 residuos de la secuencia de aminoácidos N-terminal o interna mediante el uso de un secuenciador de vaso giratorio, o (3) a homogeneidad por SDS-PAGE bajo condiciones reductoras o no reductoras usando azul Coomassie o, preferiblemente, tintura de plata. Un polipéptido aislado incluye el polipéptido *in situ* en células recombinantes, puesto que por lo menos un componente del entorno natural del polipéptido no estará presente. Ordinariamente, sin embargo, el polipéptido aislado se preparará en por lo menos un paso de purificación.

[0152] Pueden emplearse varios métodos de purificación de proteínas, y tales métodos son conocidos en el arte y se describen por ejemplo en Deutscher, *Methods in Enzymology,* 182 (1990); Scopes, *Protein Purification: Principles and Practice,* Springer-Verlag, New York (1982). El paso(s) de purificación seleccionado(s) dependerá, por ejemplo, de la naturaleza del proceso de producción utilizado y el polipéptido particular de la invención producido. Si se ligan a la membrana, los polipéptidos de la invención pueden liberarse de la membrana utilizando una solución detergente apropiada (por ejemplo Triton-X 100) o mediante escisión enzimática. Las células empleadas en la expresión de un polipéptido de la invención pueden desintegrarse mediante varios medios químicos o físicos, tales como ciclo congelamiento-descongelamiento, sonicación, ruptura mecánica, o agentes de desintegración de células. Los siguientes procedimientos son ejemplares de procedimientos de purificación apropiados: por

fraccionamiento en una columna de intercambio iónico; precipitación en etanol; HPLC de fase reversa; cromatografía en sílice, cromatografía con heparina, cromatografía SEPHAROSE™ en una resina de intercambio iónico o catiónico (tal como una columna de ácido poliaspártico, DEAE, etc.); cromatoenfocado; SDS-PAGE; precipitación en sulfato de amonio; filtración en gel usando, por ejemplo, Sephadex G-75; columnas Sepharose de proteína A para retirar contaminantes tales como IgG; y columnas quelantes de metal para unir formas, marcadas con epítopo, de polipéptidos de la invención.

[0153] Por ejemplo, una composición de VEGF preparada de las células puede purificarse usando, por ejemplo, cromatografía con heparina, electroforesis en gel, y diálisis. Otras técnicas para purificación de proteínas se encuentran también disponibles.

[0154] **Artículos de Fabricación**

[0155] En otra incorporación de la invención, se provee un artículo de fabricación que contiene materiales útiles para los métodos y tratamiento de heridas descritos anteriormente. El artículo de fabricación comprende un envase, una etiqueta y un prospecto de envase. Envases apropiados incluyen, por ejemplo, frascos, frasquitos, jeringas, apósitos, vendajes, etc. Los envases pueden formarse de una variedad de materiales tales como vidrio, plástico, nailon, algodón, poliéster, etc. El envase contiene una composición que es efectiva para tratar la afección y puede tener un puerto de acceso estéril o puede ser un tubo con dosis múltiples o puede ser una jeringa con indicaciones de cantidades medidas de agente activo. Por lo menos un agente activo en la composición se incluye en el envase. La etiqueta sobre el envase, o asociada con él, indica que la composición se utiliza para acelarar o mejorar la cicatrización de una herida. El artículo de fabricación puede comprender además un segundo envase que contiene un amortiguador farmacéuticamente aceptable, tal como solución salina normal, solución

salina amortiguada con fosfato, solución de Ringer y solución de dextrosa, o solución de gel. Puede contener además otros materiales deseables desde el punto de vista del usuario o desde un punto de vista comercial, incluidos otros amortiguadores, diluyentes, filtros, apósitos, vendajes, aplicadores, gasa, barreras, barreras semi-permeables, abate-lenguas, agujas, y jeringas. Opcionalmente, se incluye un conjunto de instrucciones, generalmente instrucciones escritas, que se refieren al uso y dosificación del VEGF para administrarlo a la herida descrita en esta solicitud. Las instrucciones incluidas con el kit contienen generalmente información en cuanto a la dosificación, pauta posológica, y vía de administración para el tratamiento del trastorno. Los envases de VEGF pueden ser dosis unitarias, empaques a granel (por ejemplo, empaques multi-dosis), o dosis sub-unitarias.

[0156] Se considera que la especificación es suficiente para permitir a una persona experta en el arte poner en práctica la invención. Por supuesto, varias modificaciones de la invención, además de las mostradas y descritas en esta solicitud, se harán evidentes a los expertos en el arte a partir de la anterior descripción y caen dentro del ámbito de las reivindicaciones adjuntas.

EJEMPLOS

[0157] Se entiende que los ejemplos e incorporaciones descritos en esta solicitud son para propósitos ilustrativos únicamente y que varias modificaciones o cambios a la luz de los mismos se sugerirán a las personas expertas en el arte y tienen que incluirse en el espíritu y dominio de esta solicitud y el ámbito de las reivindicaciones adjuntas.

[0158] **Ejemplo 1: VEGF tópico en la cicatrización de una herida Estudio clínico con VGF2763g**

[0159] Se realizó un estudio clínico doble ciego (por ejemplo, farmaceuta sin ocultamiento de la asignación, MD con ocultamiento y

paciente con ocultamiento de la asignación) para determinar si la aplicación de VEGF tópico podía promover la cicatrización de una herida en sujetos humanos con ulceraciones diabéticas. Véase Tabla 4 respecto a un diagrama de características de enfermedad de referencia de los pacientes en el estudio, para administrar rhVEGF (denominado en esta solicitud como "telbermina") para el tratamiento de heridas diabéticas. El diseño del estudio se indica en **Fig. 1.**

Tabla 4 Características de la Enfermedad de Referencia

	Placebo (N=26)	Telbermina (N=29)
Edad media, y (rango)	59.3 (38-81)	59.5 (42-74)
Glucosa media, mg/dL (rango)*	225.8 (77-465)	179.1 (29-593)
HbAlC medio, % (rango)†	8.4 (5.5-13.6)	8.3 (5.6-13.6)
Úlcera debridada en el cribado, N (%)		
Sí	21 (80.8)	27 (93.1)
No	5 (19.2)	2 (6.9)
Área de úlcera media, cm^2 (rango)		
Longitud x ancho en el cribado	1.85 (1.08-2.90)	1.92 (0.96-4.08)
Planimetría en el cribado ‡	1.14 (0.50-2.24)	1.35 (0.59-3.51)
Planimetría el día 1	1.05 (0.62-2.34)	1.15 (0.44-2.97)

* Un sujeto tratado con placebo se excluyó del resumen debido a la falta de un valor de glucosa. † Un sujeto tratado con telbermina se excluyó del resumen debido a la falta del valor HbAlC. ‡ Dos sujetos tratados con placebo no tuvieron evaluaciones de planimetría de referencia.

[0160] 24 de los pacientes tratados con placebo completaron la fase de tratamiento y 22 completaron la fase de observación. 27 de los pacientes tratados con telbermina (VEGF recombinante tópico) completaron la fase de tratamiento y 22 completaron la fase de

observación. Pacientes con diabetes mellitus I o II (hemoglobina glucosilada, controlada Alc (HbA1c) ≤ 12%) con área de la úlcera desbridada de ≥0.4 cm^2 y ≤ 4.0 cm^2 el día 1 que eran heridas superficiales (por ejemplo, en la fase UT 1a (no hueso, músculo, tendón), véase Tabla 2) se trataron con VEGF o un placebo 3 veces a la semana durante 6 semanas (18 dosis en total). El tratamiento se realizó cada 48 horas (+/- 24 horas) pero no más de 3 dosis por semana. La cantidad de VEGF por tratamiento fue 72 $\mu g/cm^2$. El VEGF se preparó en el sitio. 0.22 ml de VEGF (5mg/ml) o el placebo (vehículo amortiguador) se retiraron del frasquito se agregaron a alrededor de 5% metilcelulosa (por ejemplo, 4.7%) (por ejemplo, Methocel A4M metilcelulosa premium (The Dow Chemical Company; Midland, MI) formulación en 5mM amortiguador de succinato, pH 5.0. El VEGF o placebo y el gel se mezclaron durante 2 horas, con viscosidad creciente, y redujeron la pérdida de material de dosificación cuando se aplicaron. Un gel VEGF 0.06% final (gel final 3% metilcelulosa) en 5 mM, pH 5.0 amortiguador de succinato (con, por ejemplo, a VEGF 1.8 mg/ml, 0.0036% polisorbato 20 y 100 mM dehidrato de trehalosa) fue el material de dosificación final. El material de dosificación final se aplicó con una jeringa de tuberculina de 1.0 ml, por ejemplo, cargada con 0.6 mL de material de dosificación final.

[0161] Típicamente, la úlcera era un úlcera crónica. La duración de la úlcera fue mayor que o igual a 4 semanas a menos de 6 meses antes del tratamiento. No había infección activa, y el sujeto tenía una extremidad perfundida: índice brazo-tobillo (ABI) ≥ 0.6 y menos de o igual a 1.2 en el pie de estudio. Durante el tratamiento los dos grupos VEGF o placebo tuvieron una buena práctica de cuidado de la herida y evaluaciones semanales, por ejemplo, exámenes físicos, registros planimétricos y/o fotografías de 35 mm (por ejemplo, Food and Drug Administration (FDA) Guidance for Industry 2000, Chronic Cutaneous Ulcer and Burn Wounds-Developing Products for Treatment, June

2000).

[0162] Los criterios de valoración a abordarse fueron la incidencia del cierre completo de la herida, que incluía el cierre de la piel sin requerimientos de drenaje o apósitos, (por ejemplo, evaluarse, 3 meses luego del cierre) y la cicatrización acelerada de una herida, donde la velocidad refleja una disminución clínicamente significativa del tiempo hasta que ocurra, por ejemplo, un cierre completo, y un análisis tiempo a acontecimiento (por ejemplo, tiempo hasta el cierre completo). El criterio de valoración de eficacia primaria fue la reducción porcentual en el área total superficial el día (hasta el día 49) a partir de la línea de referencia se determinó por análisis cuantitativo de registros planimétricos de la úlcera. Los criterios de valoración de eficacia secundarios incluían: reducción porcentual en el área total superficial de la úlcera el día 29 y el día 84, comparada con la línea de referencia (por ejemplo, valor el día 1), incidencia de las cicatrizaciones completas de la úlcera los días 29, 43, y 84, tiempo (por ejemplo, días) en completar la cicatrización de la úlcera, tiempo (por ejemplo, día) hasta la recurrencia de la formación de úlcera para el sujeto con cicatrización completa de la úlcera antes del final del tratamiento, incidencia del área superficial total incrementada de la úlcera (> 15%), comparada con la línea de referencia, incidencia del avance de la etapa de la úlcera (por ejemplo, > UT 1a), y perfusión microcirculatoria del lecho de la úlcera los días 1, 8, 22 y 43.

[0163] Problemas de seguridad que se monitorearon involucraron hipotensión clínicamente significativa (por ejemplo, definida como una caída de ≥ 35 mmHg en la presión sanguínea sistólica relativa a la pre-dosis 60 minutos después de la aplicación de cada dosis del medicamento de estudio durante la primera semana de tratamiento (días 1, 3 y 5), infección de la úlcera clínicamente significativa (por ejemplo, definida por una descarga incrementada y exudados malolientes de la úlcera, fiebre (temperatura de ≥ 38.6°C), y conteo de glóbulos blancos

(WBC) de > 10.000μL), producción de anticuerpos anti-VEGF, etc. Se midió la presión sanguínea antes de cada dosis y 60 minutos después de cada dosis durante la primera semana.

[0164] El volumen total de gel aplicado para cada tratamiento es 0.12-0.48 mL (72 μg-288 μg VEGF). La cantidad de gel aplicado se basó en mediciones de la herida (LxW), donde L es la longitud máxima de un borde al otro en cm y W es el ancho máximo de un borde al otro, perpendicular a L en cm (LxW = área superficial estimada (cm^2)). Por ejemplo, el gel se aplica usando un baja-lenguas esterilizado, donde la cantidad total de gel aplicado sobre la superficie total de la úlcera tuvo un espesor de 1/16". La herida se cubre con una barrera semipermeable estéril (por ejemplo, apósito de película aadptec) y se envolvió con envoltura gasa de algodón (por ejemplo, Kerlix). En el siguiente tratamiento, se retira el apósito y la úlcera se irriga suavemente con solución salina normal estéril. Se mide nuevamente la úlcera, se aplica la dosis apropiada de gel y la herida se vuelve a vendar.

[0165] Resultados: El VEGF tópico parece ser seguro y bien tolerado. La incidencia de episodios adversos fue comparable entre grupos de tratamiento (grupos de telbermina y placebo). Ninguno de los episodios adversos o episodios adversos serios observados se atribuyeron al medicamento del estudio. Dos pacientes no continuaron el estudio debido a episodios adversos serios (1 en el grupo de telbermina → úlcera cutánea infectada; 1 en el grupo de placebo → infección localizada). Hubo un paciente en el grupo de telbermina que falleció 4 días después del último tratamiento, pero la muerte no se atribuyó al medicamento del estudio. No se observaron casos de hipotensión clínicamente significativa en cualquiera de los dos grupos de tratamiento.

[0166] Los datos sugieren la evidencia de actividad biológica. No se observaron señales de seguridad en un estudio con tamaños pequeños

de úlcera que estaban en la fase UT 1a. Véase Tabla 5 para un resumen de los resultados para una reducción % media en el área de la herida, % de sujetos con cicatrización completa y tiempo hasta la cicatrización. En sujetos diabéticos tratados con VEGF durante 6 semanas 3 veces a la semana, la población de sujetos mostró una mejora del 14-25% en la completa cicatrización de una herida después de 6 semanas con VEGF, comparada con el placebo. El estudio demostró que el VEGF tuvo una aceleración de la cicatrización de ~75-100% más rápida que el placebo. Véase Tabla 6, que ilustra el tiempo hasta la primera cicatrización completa de la úlcera en pacientes tratados con telbermina (rhVEGF) o placebo. El tiempo para la completa cicatrización de la úlcera se aceleró en pacientes tratados con VEGF, por ejemplo, el tiempo hasta la completa cicatrización (percentil 25º) fue 32.5 días versus 43.0 días.

Tabla 5

		Reducción % Media en el Área de la Herida (superficie total de la úlcera) (VEGF vs. Placebo)[2]*	**% Sujetos con Cicatrización Completa (VEGF vs. Placebo)[3]***	**Tiempo hasta la Cicatrización (VEGF vs. Placebo)[4]***
Día 43	Seguridad	95% vs. 85%	41% vs. 27%	HR 1.75 (p=0.18)
(Semana 6)	Evaluable	(p=0.67)	(p=0.39)	
	Eficacia[1]	100% vs. 88%	52% vs. 27%	HR 1.98 (p=0.12)
	Evaluable	(p=0.17)	(p=0.13)	
Día 84	Seguridad	100% vs. 92%	52% vs. 35%	HR 1.87 (p=0.13)
(Semana 12)	Evaluable	(p=0.49)	(p=0.28)	
	Eficacia[1]	100% vs. 93%	71% vs. 38%	HR2.10(p=0.08)
	Evaluable	(p=0.05)	(p=0.06)	

[1]Sujetos con eficacia evaluable (especificada antes de revelar el ocultamiento de la asignación - ***"prior to unblinding"***):

Se retiraron los infractores principales del protocolo.

Los sujetos que incumplieron 3 dosis consecutivas – censurados en la última dosificación disponible.

No LOCF (datos faltantes no imputados).

[2] valor p: prueba de suma de rangos Wilcoxon.

[3] valor p: prueba exacta de Fisher.

[4] valor p: prueba Log-Rank.

*método de evaluación – análisis planimétrico cuantitativo

Tabla 6

Tiempo hasta la Cicatrización Completa*	Placebo (N=26)	Telbermina (N=29)
25º percentil, día	43.0	32.5
50º percentil, día	ND	58.0

ND = No detectable

* Estimada usando el método de Kaplan Meier.

[0167] Para los sujetos que lograron una cicatrización completa de la úlcera, se evaluó la recurrencia de la formación de la úlcera entre el tiempo de la primera cicatrización completa de la úlcera y el tiempo de la conclusión o discontinuación del estudio. De los sujetos con seguridad evaluable, que alcanzaron una cicatrización completa de la úlcera, 26.7% de los sujetos tratados con telbermina (4 de 15) y 33.3% de los sujetos tratados con placebo (3 de 9) tuvieron recurrencia de formación de úlcera (valor p log-rank = 0.57). La relación de riesgo por recurrencia de formación de úlcera para los sujetos tratados con telbermina, comparada con los sujetos tratados con placebo fue de 0.63 (95% CI: 0.13, 3.15).

[0168] **Ejemplo 2: VEGF tópico en la cicatrización de una herida**

[0169] Sujetos, por ejemplo, pacientes con diabetes mellitus I o II, con un área estimada de úlcera luego de un exhaustivo desbridamiento de, por ejemplo, ≥ 1.0 cm^2 y ≤ 6.5 cm^2 al comienzo del tratamiento, se tratan con VEGF recombinante tópico (por ejemplo, formulación en gel)

diariamente durante 12 semanas (para un total de hasta 84 dosis) hasta el cierre total o completo de la herida (por ejemplo, cierre de la piel sin requerimientos de drenaje o apósitos), lo primero que ocurra. Los sujetos pueden observarse durante 12 semanas o más, después de la fase de tratamiento. Los sujetos reciben 24 $\mu g/cm^2$, 72 $\mu g/cm^2$, o 216 $\mu g/cm^2$ VEGF en cada tratamiento diario. Se calcula el área superficial de la úlcera (cm^2), por ejemplo, por la longitud (L(cm)), que es la medición máxima de un borde al otro de la úlcera, y el ancho (W(cm)), tomado desde un eje perpendicular a la longitud en la medida máxima de un borde a otro. Entonces, el área superficial calculada es LxW. El tratamiento puede evaluarse midiendo el perímetro del área de la úlcera mediante registros, registros de análisis planimétrico del borde de la úlcera, fotografías, exámenes físicos, etc. El VEGF aplicado será 1.8, 0.6 y 0.2 mg/ml de VEGF, 3% metilcelulosa (por ejemplo, Methocel A4M metilcelulosa premium (The Dow Chemical Company; Midland, MI), en 5 mM, pH 5.0 amortiguador de succinato (con, por ejemplo, con VEGF 1.8 mg/ml, 0.0036% polisorbato 20 y 100 mM dehidrato de trehalosa).

[0170] Se considera que la especificación es suficiente para permitirle a un experto en el arte poner en práctica la invención. Se entiende que los ejemplos e incorporaciones descritos en esta solicitud son para propósitos ilustrativos únicamente. Por supuesto, varias modificaciones de la invención, además de las mostradas y descritas en esta solicitud, se harán evidentes a los expertos en el arte, a partir de la descripción anterior, y caerán dentro del ámbito de las reivindicaciones adjuntas.

REIVINDICACIONES

Reivindicamos:

1. Un método de acelerar la cicatrización de una herida en un sujeto, método que comprende: administrar una cantidad efectiva de VEGF a una herida, en donde la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida en más del 60%, cuando se compara con un control.

2. El método de la reivindicación 1, en donde la aceleración de la cicatrización de una herida es igual o mayor a 74%, cuando se compara con el control.

3. El método de la reivindicación 1, en donde la aceleración de la cicatrización de una herida se evalúa por el % de reducción en el área de la herida.

4. El método de la reivindicación 3, en donde el área de la herida es alrededor de 0.4 cm^2 o más antes del tratamiento.

5. El método de la reivindicación 3, en donde el área de la herida es alrededor de 1.0 cm^2 o más antes del tratamiento.

6. El método de la reivindicación 1, en donde la aceleración de la cicatrización de una herida se evalúa por la tasa de cicatrización completa de una herida

7. El método de la reivindicación 1, en donde la herida es una úlcera de pie diabético.

8. El método de la reivindicación 1, en donde la cantidad efectiva de

VEGF se administra por lo menos tres veces a la semana.

9. El método de la reivindicación 1, en donde la cantidad efectiva de VEGF se administra por lo menos durante seis semanas.

10. El método de la reivindicación 1, en donde la cantidad efectiva de VEGF se administra hasta que hay un cierre completo de la herida.

11. El método de la reivindicación 1, en donde el VEGF es $VEGF_{165}$.

12. El método de la reivindicación 1 u 11, en donde el VEGF es VEGF humano recombinante.

13. El método de la reivindicación 1, en donde la administración es tópica.

14. El método de la reivindicación 1, en donde el VEGF está en una formulación para administración tópica.

15. El método de la reivindicación 1, en donde la herida es una herida crónica.

16. El método de la reivindicación 1, en donde la herida es una úlcera por presión, una úlcera de decúbito, una úlcera venosa, una quemadura, una herida quirúrgica, o una herida normal.

17. El método de la reivindicación 1, en donde el sujeto se está sometiendo o se ha sometido a tratamiento, en donde el tratamiento retrasa o produce una cicatrización ineficaz de una herida.

18. El método de la reivindicación 1, en donde el sujeto tiene una enfermedad secundaria, en donde la enfermedad secundaria retrasa o

produce una cicatrización ineficaz de una herida.

19. El método de la reivindicación 18, en donde la enfermedad secundaria es diabetes.

20. El método de la reivindicación 1, en donde la cantidad efectiva de VEGF es alrededor de 20 $\mu g/cm^2$ a alrededor de 250 $\mu g/cm^2$.

21. El método de la reivindicación 20, en donde la cantidad efectiva de VEGF es alrededor de 24 $\mu g/cm^2$.

22. El método de la reivindicación 20, en donde la cantidad efectiva de VEGF es alrededor de 72 $\mu g/cm^2$.

23. El método de la reivindicación 20, en donde la cantidad efectiva de VEGF es alrededor de 216 $\mu g/cm^2$.

24. El método de la reivindicación 1, en donde el sujeto es humano.

25. Un método de acelerar la cicatrización de una herida en un sujeto humano, método que comprende administrar una cantidad efectiva de VEGF a una herida, en donde la administración de la cantidad efectiva de VEGF acelera la cicatrización de una herida en más del 60%, cuando se compara con un control, y en donde la herida está presente en el sujeto durante alrededor de 4 semanas o más, antes de administrar la cantidad efectiva de VEGF.

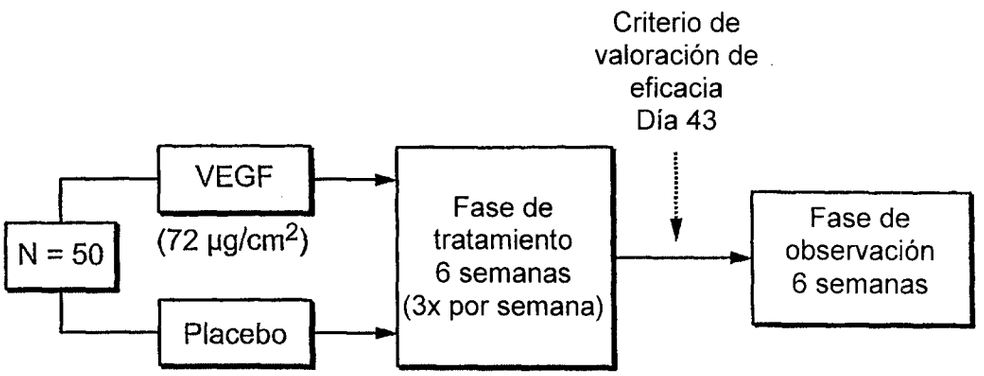

FIG. 1

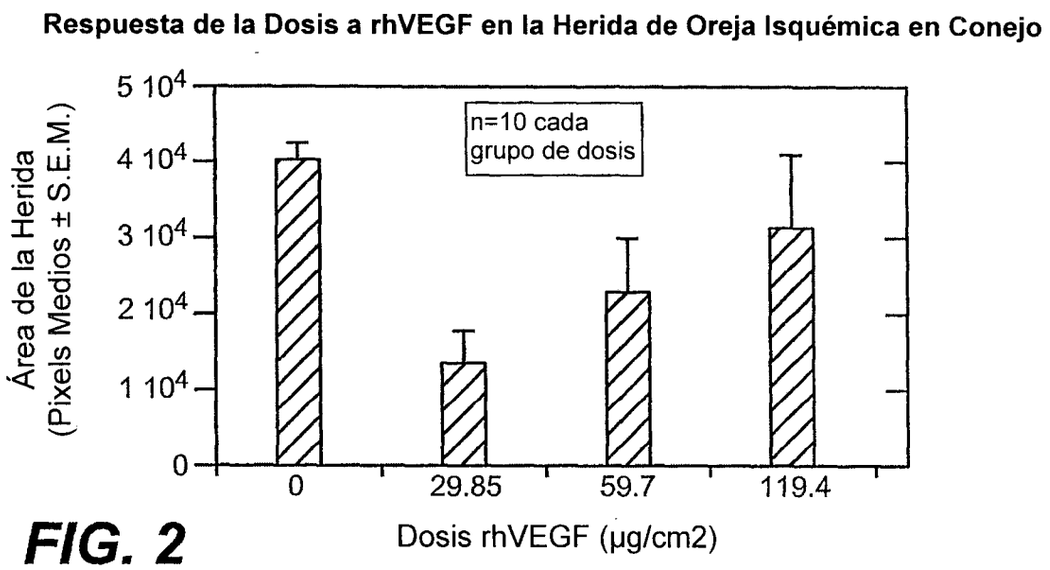

FIG. 2

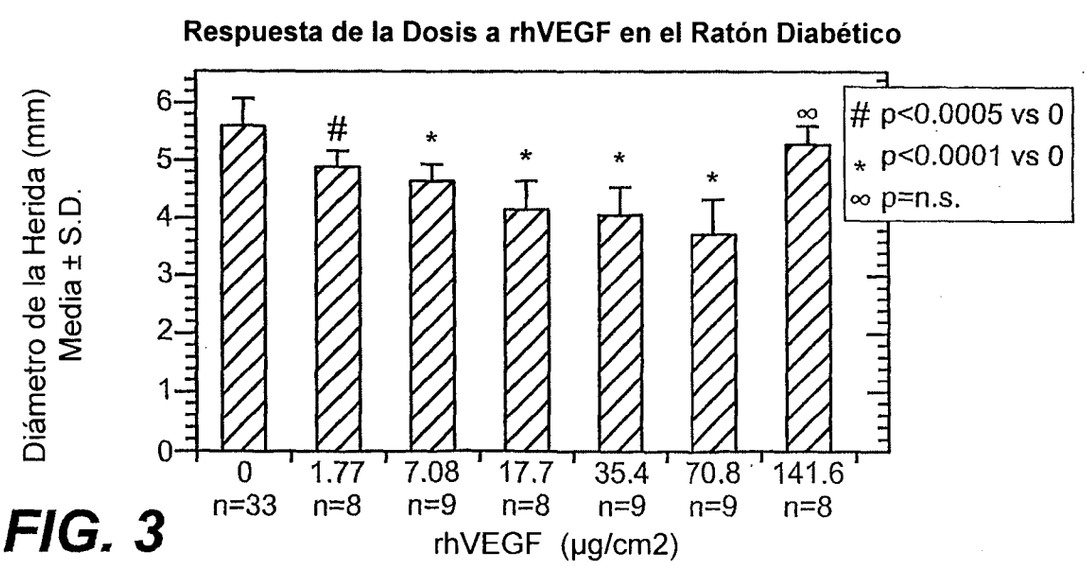

FIG. 3

P23581 - ... COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:

GENENTECH, INC.
Attn. Pleasure, Irene
1 DNA Way MS49
South San Francisco, CA 94080
ETATS-UNIS D'AMERIQUE

RECEIVED
MAY 23 2007
GENENTECH, INC.
LEGAL DEPT.

Calendared/CLK
Resp. to ISR/Consid Ch II - 18JUL07
Ch II Deadline/Resp to WO - 18 Aug07
Due Date

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference P23581R1	Date of mailing (day/month/year) 18/05/2007
International application No. PCT/US2006/023318	FOR FURTHER ACTION See paragraphs 1 and 4 below
Applicant GENENTECH, INC.	International filing date (day/month/year) 16/06/2006

1. [x] The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Fascimile No.: (41-22) 338.82.70

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Gabriele Pfitzner

Form PCT/ISA/220 (October 2005)

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference P23581R1	**FOR FURTHER ACTION** see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2006/023318	International filing date *(day/month/year)* 16/06/2006	(Earliest) Priority Date *(day/month/year)* 17/06/2005
Applicant GENENTECH, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [X] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title**,
   [ ] the text is approved as submitted by the applicant
   [X] the text has been established by this Authority to read as follows:

   USE OF VEGF FOR WOUND HEALING

5. With regard to the **abstract**,
   [X] the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,
   a. the figure of the drawings to be published with the abstract is Figure No. _____
      [ ] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [X] none of the figures is to be published with the abstract

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/19 A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENENTECH WEB PAGE: "PRO-128115 diabetic foot ulcer" 18 May 2004 (2004-05-18), XP002404950 Retrieved from the Internet: URL:www.prous.com> the whole document	1-25
X	KR 2002 020 564 A (GOODGENE INC [KR]) 15 March 2002 (2002-03-15) abstract & DATABASE HCAPLUS abstract	1-7,12, 15,16, 18,19,24

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

'A' document defining the general state of the art which is not considered to be of particular relevance
'E' earlier document but published on or after the international filing date
'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
'O' document referring to an oral disclosure, use, exhibition or other means
'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 2007	18/05/2007
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Authorized officer Fayos, Cécile

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/46249 A (RADIX ORGANIZATION INC [US]; CHEMO PATHOLOGY RESOUR CENTER [US]) 22 October 1998 (1998-10-22) claims 1-15	1-6,8, 10, 12-18, 24,25
Y	the whole document examples 1-3	1-25
X	US 2003/180259 A1 (BREM HAROLD [US]) 25 September 2003 (2003-09-25) claims 1,12,13 page 1, paragraph 9 page 4, paragraph 42-44	1-6, 8-19,24, 25
Y	the whole document	1-25
X	"TOP 10 BIOPHARMA" July 2004 (2004-07), pages 110-112, XP002404951 Retrieved from the Internet: URL:www.contractpharma.com> [retrieved on 2006-10-27]	1-6, 10-14, 24,25
Y	page 110, column 2	1-25
X	US 6 479 654 B1 (BAIRD ANDREW [US] ET AL) 12 November 2002 (2002-11-12) column 5, line 34 - line 46 column 9, line 34 - line 39 figure 1	1-6, 12-16,25
Y	the whole document	1-25

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
KR 2002020564	A		NONE		
WO 9846249	A	22-10-1998	EP	1005355 A1	07-06-2000
US 2003180259	A1	25-09-2003	NONE		
US 6479654	B1	12-11-2002	US	2003144200 A1	31-07-2003

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2006/023318	16.06.2006	17.06.2005

International Patent Classification (IPC) or both national classification and IPC
INV. A61K38/19 A61P17/02

Applicant
GENENTECH, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	see form PCT/ISA/210	Fayos, Cécile Telephone No. +49 89 2399-2180

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed.

   ☐ filed together with the international application in electronic form.

   ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of

☐ the entire international application

☒ claims Nos. 1-25 IA

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international search *(specify)*:

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for the whole application or for said claims Nos. 1-25 IA

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13*ter*.1(a) or (b).

☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See Supplemental Box for further details

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	20-23
	No:	Claims	1-19, 24-25
Inventive step (IS)	Yes:	Claims	-
	No:	Claims	1-25
Industrial applicability (IA)	Yes:	Claims	1-25 see separate sheet
	No:	Claims	-

2. Citations and explanations

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1- Claims 1-25 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

2- Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not defined. Claim 1 attempts to define the subject-matter in terms of the result to be achieved: "accelerates wound healing greater than 60% when compared to control". This feature is therefore not restrictive. The same applies to claims 2-25.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3- Reference is made to the following documents:

D1: GENENTECH WEB PAGE: "PRO-128115 diabetic foot ulcer" 18 May 2004 (2004-05-18), XP002404950 Retrieved from the Internet: URL:www.prous.com>
D2: KR 2002 020 564 A (GOODGENE INC [KR]) 15 March 2002 (2002-03-15)
D3: WO 98/46249 A (RADIX ORGANIZATION INC [US]; CHEMO PATHOLOGY RESOUR CENTER [US]) 22 October 1998 (1998-10-22)
D4: US 2003/180259 A1 (BREM HAROLD [US]) 25 September 2003 (2003-09-25)
D5: "TOP 10 BIOPHARMA" July 2004 (2004-07), pages 110-112, XP002404951 Retrieved from the Internet: URL:www.contractpharma.com> [retrieved on 2006-10-27]
D6: US-B1-6 479 654 (BAIRD ANDREW [US] ET AL) 12 November 2002 (2002-11-12)

3.1- The relevant passages are those indicated in the search report, unless otherwise specified.

NOVELTY - Art. 33 (1) and (2) PCT

4- Claims 1-19, 24-25 are not novel:

4.1- D1 mentions the phase I clinical studies of PRO-128115 (VEGF) as wound healing agent for diabetic ulcer (in particular diabetic foot ulcer). Claims 1-3, 6, 7, 11, 12, 13, 14, 15, 16, 17-19, 24-25 lack novelty.

D2 mentions the use of VEGF for treating diabetic foot ulcer, and is therefore novelty destroying for the subject matte of claims 1-3, 6, 7, 12, 13, 14, 15, 16, 17-19, 24-25 lack novelty.

D3 relates to the use of VEGF for treating ulcers and is novelty destroying for the subject matter of claims 1-6, 8, 10, 12-18, 24, 25.

D4 discloses the use of VEGF for treating diabetic ulcers and is novelty destroying for the subject matter of claims 1-6, 8-19, 24, 25.

D5 mentions the use of PRO128115 for wound healing and is therefore novelty destroying for the subject matter of claims 1-6, 10-14, 24, 25.

D6 discloses the use of VEGF for treating ulcers and anticipates the subject matter of claims 1-6, 12-16, 25.

INVENTIVE STEP - Art. 33 (1) and (3) PCT

5- The features of claims 20-23 are merely some of several straightforward possibilities from which the skilled person would select, in accordance with circumstances, without the exercise of inventive skill, in order to solve the problem posed, in view of the available prior art documents, taken alone or in combination. No inventive step can be acknowledged for the subject matter of claims 1-25.

INDUSTRIAL APPLICABILITY - Art. 33 (1) and (4) PCT

6- For the assessment of the present claims 1-25 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

7- When / if carrying out amendments, and in order to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT, the applicant is requested to clearly identify the amendments carried out, no matter wether they concern amendments by addition, replacement or deletion, and to indicate precisely the passages of the application as filed on which these amendments are based (also rule 66.8 (a) PCT).

Only amendments with a clearly identified basis on the application as originally filed will be taken into account for the international preliminary examination report.

Bitte beachten Sie, dass angeführte Nichtpatentliteratur (wie z. B. wissenschaftliche oder technische Dokumente) je nach geltendem Recht dem Urheberrechtsschutz und/oder anderen Schutzarten für schriftliche Werke unterliegen könnte. Die Vervielfältigung urheberrechtlich geschützter Texte, ihre Verwendung in anderen elektronischen oder gedruckten Publikationen und ihre Weitergabe an Dritte ist ohne ausdrückliche Zustimmung des Rechtsinhabers nicht gestattet.

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XS CPRTENFRDE

XP-002404951

2 GENENTECH

1 DNA Way
South San Francisco, CA 94080
Tel: (650) 225-1000
www.gene.com

HEADCOUNT	6,226	
YEAR ESTABLISHED	1976	
BIOPHARMACEUTICAL REVENUES	$2,621	+21%
TOTAL REVENUES	$3,300	+28%
ROYALTY REVENUES	$500	+37%
NET INCOME	$563	+760%
R&D BUDGET	$722	+16%

DRUGS APPROVED/LAUNCHED

Drug	Indication
RITUXAN	rheumatoid arthritis
RAPTIVA	chronic plaque psoriasis
AVASTIN	metastatic colorectal cancer

DRUGS PENDING

Drug	Indication
NUTROPIN & NUTROPIN AQ	idiopathic short stature

DRUGS IN PHASE IIB AND BEYOND

Drug	Indication
HERCEPTIN	adjuvant breast cancer
LUCENTIS	age-related macular degeneration
NUTROPIN DEPOT	adult growth hormone deficiency
RITUXAN HEMATOLOGY/ ONCOLOGY	aggressive frontline non-Hodgkin's lymphoma
RITUXAN IMMUNOLOGY	ANCA-associated vasculitis, lupus, refractory rheumatoid arthritis
TARCEVA	lung cancer, pancreatic cancer
XOLAIR	pediatric asthma
VELETRI	acute heart failure

EARLY RESEARCH PROJECTS

Drug	Indication
OMNITARG	breast cancer, lung cancer, ovarian cancer, prostate cancer
RAPTIVA	psoriatic arthritis
RITUXAN IMMUNOLOGY	moderate-to-severe rheumatoid arthritis, multiple sclerosis
TARCEVA	glioblastoma multiforme
XOLAIR	peanut allergy
G-024856	basal cell carcinoma
PRO1762 (APO2L/TRAIL)	cancer therapy
PRO70769 (ANTI-CD20)	rheumatoid arthritis
PRO128115 (VEGF)	wound healing

TOP SELLING DRUGS

Drug	Indication	$	(+/- %)
RITUXAN	non-Hodgkin's lymphoma	$1,489	+28%
HERCEPTIN	breast cancer	$425	+10%
NUTROPIN/ PROTROPIN	growth hormone	$322	+8%

Account for 85% of total biopharma sales, same as in 2002

KEY PERSONNEL

Arthur D. Levinson, Ph.D.
chairman and chief executive officer

Susan Desmond-Hellmann, M.D., M.P.H.
executive vice president, development and product operations, chief medical officer

Louis J. Lavigne, Jr.
executive vice president, chief financial officer

Richard H. Scheller, Ph.D.
senior vice president, research

Myrtle S. Potter
executive vice president commercial operations, chief operating officer

If you're looking for a good company to work for, Genentech is it. *Science* magazine named it as "the top employer and most admired company in the biotechnology and pharmaceutical industries" for the second year in a row. In addition, the organization was voted one of *Fortune*'s "100 Best Companies To Work For" for the sixth consecutive year.

The San Francisco Bay Area company also pulled out a solid sales year. For 2003, the company's rheumatoid arthritis drug Rituxan, raked in nearly $1.5 billion in sales, an increase of 28% compared to 2002. Overall, the company's revenues also increased 28% to more than $3.0 billion. Higher revenues were buttressed by the rapid launches of Xolair (asthma) in July 2003 and Raptiva (chronic plaque psoriasis) in November 2003. In early 2004, the company followed the same rapid launch strategy with Avastin (metastatic colorectal cancer), shipping product the same day the FDA approved the company's BLA. The company's total product sales for 2003 totaled $2.6 billion, an increase of 21% compared to 2002. The company's financial results also benefited from increased contract revenues, which totaled $177.9 million compared to $54.5 mil-

lion in 2002. The increase was due to higher revenues from product opt-ins and reimbursement on development efforts from ongoing collaborations.

The company continues its efforts for added indications for therapies, in addition to novel drugs. So far, the FDA has approved Avastin to be used in combination with intravenous 5-Fluorouracil-based chemotherapy as a treatment for patients with first-line (previously untreated) metastatic cancer of the colon or rectum. Avastin is the first FDA-approved therapy designed to inhibit angiogenesis, the process by which new blood vessels develop, which is necessary to support tumor growth and metastasis. Avastin is currently in Phase III trials for adjuvant colorectal cancer, metastatic breast cancer, non-small cell lung cancer, pancreatic cancer and renal cell carcinoma. Rituxan is also in Phase III trials for aggressive frontline non-Hodgkin's lymphoma, indolent frontline non-Hodgkin's lymphoma (for which the company is preparing the filing), indolent maintenance non-Hodgkin's lymphoma, as well as relapsed chronic lymphocytic leukemia. The drug has also shown promise in Phase II trials for rheumatoid arthritis.

However, it's not all wine and roses for the world's number two Biopharma. Genentech and Alkermes recently decided to discontinue commercialization of Nutropin Depot. The decision was based on the significant resources required by both companies to continue manufacturing and commercializing the product. Nutropin Depot, approved in 1999 by the FDA as a treatment for growth hormone deficiency in pediatric patients, is a long-acting form of Genentech's human growth hormone using Alkermes' ProLease injectable extended-release drug delivery system. "We are committed to patients who require growth hormone therapy and will continue to provide options through our current Nutropin line," commented Susan Hellmann, M.D., M.P.H., president, product development at Genentech. "We will collaborate with physicians and the endocrine community over the next several weeks to ensure existing Nutropin Depot patients are informed and transitioned appropriately. Our goal is to work to ensure that patients' therapies are not disrupted during the transition."

The company is also hard at work at preventing generic biologicals from entering the market. Genentech recently filed a petition with the FDA to prevent the agency from publishing a guidance aimed at showing how a new biological drug is similar enough to an existing therapy shown to be safe and effective. Companies such as Genentech and Amgen claim that their biological drugs are quite complicated and very difficult to copy. Therefore, relying on their efficacy and safety testing data would be unsafe. Generic drug makers are clamoring for a piece of the biological drug market pie, which in the U.S. alone, was estimated at $22 billion for 2003. These generic companies are insisting that they can safely produce these biological drugs.

If the FDA publishes this guidance (and insiders are saying it's a done deal) then the issue will be taken to court, as generic biologicals are not covered under the Hatch-Waxman Act that allowed small molecule generics to enter the market in 1984. And you can bet Senator Orrin Hatch (R-UT) won't be far behind with his support for cheaper, generic biologicals. Genentech will fiercely defend its ground, but it looks like an uphill battle. ■—SWM

3 SERONO

Chemin des Mines 5bis
PO Box 54
1211 Geneva 20
Switzerland
Tel: (41) 22 739 3000
www.serono.com

HEADCOUNT	4,597	
YEAR ESTABLISHED	1987	–
BIOPHARMACEUTICAL REVENUES	$1,858	+31%
TOTAL REVENUES	$2,019	+31%
ROYALTY REVENUES	$161	+31%
NET INCOME	$390	+21%
R&D BUDGET	$468	+31%

DRUGS APPROVED/LAUNCHED

Drug	Indication
ZORBTIVE	short bowel syndrome

DRUGS PENDING

Drug	Indication
LUVERIS	female infertility
GONAL-F PREFILLED PEN	dosage form
RAPTIVA	psoriasis
SAIZEN	small gestational age babies

DRUGS IN PHASE IIB AND BEYOND

Drug	Indication
MICROENCAPSULATED R-FSH	female infertility
ANASTROZOLE	ovulation induction, improved follicular development
EMFILERMIN (R-LIF)	embryo implantation enhancement
ATEXAKIN-A (R-IL-6)	peripheral neuropathy
SEROSTIM	HARS/lipodystrophy
R-INTERFERON BETA	chronic hepatitis C in Asian patients

XP-002404950

Records Retrieved 1 in Drugs & Biologics

Options

Drugs & Biologics Search Results

Entry Number	369180
Highest Phase	Phase I
Under Active Development	
Chemical Name/Description	

Recombinant Vascular Endothelial Growth (VEGF)

Code Name	Generic Name	Brand Name
PRO-128115	-	
Therapeutic Group	**Cellular / Molecular Mechanism**	**Biological / Chemical Group**
Wound-Healing Agents		

Organization

Genentech (Originator)

Product Summary REPORT

Development Status Summary DETAILS

Phase	Organization	Condition
Phase I	Genentech	Ulcer, diabetic

Related Information

Literature | Companies & Research Institutions 1

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http://integrity.prous.com

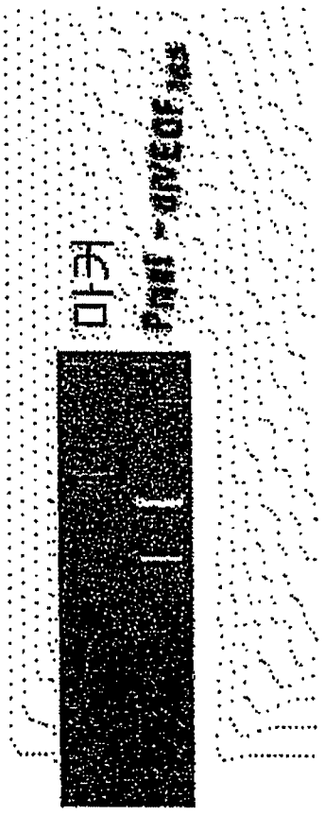
Entry Number: 369180
PRO-128115

Development Status - Launched, Registered or Under Active Development

Country/Area	Phase	Organization	Brand Name	Condition	Notes
	Phase I	Genentech		Ulcer, diabetic	Topical (In patients with diabetic foot ulcers)

도면 2

도면 3



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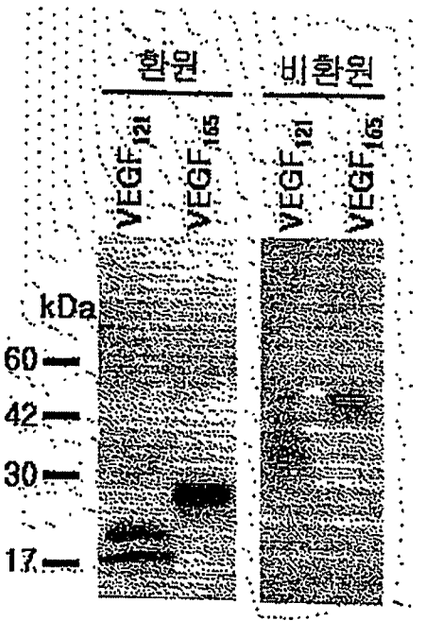
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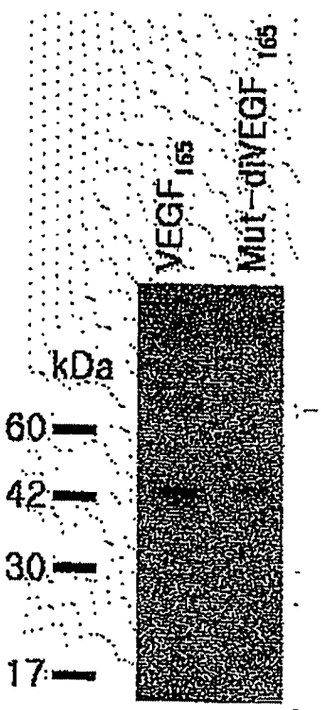
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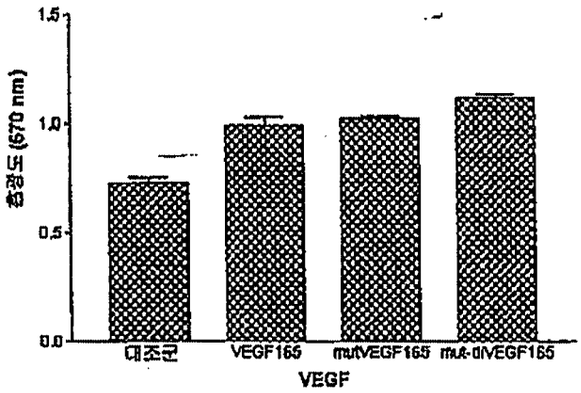

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(54) Title: METHOD OF TREATING GASTROINTESTINAL ULCERS AND COMPOSITIONS THEREFOR

(57) Abstract

The invention provides methods and compositions for providing chronic gastrointestinal ulcer healing or acute gastroprotection. In particular, administration of a pharmaceutically effective amount of VEGF, such as from about 30 ng/kg to about 1000μg/kg per day for about a week to two months results in healing of GI ulcers in a mammal, such as a human. Examples of gastrointestinal ulcers include duodenal ulcers, peptic ulcers, drug-induced ulcers, or intestinal ulcers, including but not limited to damage due to non-steroidal (NSAID) and anti-inflammatory drugs, gastro-esphogheal reflux disease or inflammatory bowel disease such as ulcerative colitis and Crohn's disease. Similarly, administration of a pharmaceutically effective amount of VEGF, such as from about 3μg/kg to about 1000μg/kg VEGF, from 0 minutes to about 60 minutes prior to potential injury to the GI tract, such as ingestion of an NSAID or ethanol, results in acute gastroprotection.

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Method of Treating Gastrointestinal Ulcers and Compositions Therefor

Field of the Invention

The present invention relates to methods and compositions for treating gastrointestinal (GI) ulcers as well as for providing acute gastroprotection. Examples of GI ulcers include duodenal ulcers, peptic ulcers, drug-induced ulcers, or intestinal ulcers, including but not limited to damage due to non-steroidal (NSAID) and anti-inflammatory drugs, gastro-esphogheal reflux disease or inflammatory bowel disease such as ulcerative colitis and Crohn's disease. In particular, the present invention relates to treatment of a mammal, including a human, with vascular endothelial cell growth factor (VEGF), which is also a potent vascular permeability factor (VPF), to provide healing of GI ulcers or to provide acute gastroprotection.

Background of the Invention

The vascular system of organs and tissues develops by either of two processes, vasculogenesis or angiogenesis. Vasculogenesis is the *de novo* formation of blood vessels from differentiating endothelial cell precursors or angioblasts. Angiogenesis, the sprouting of new capillaries from pre-existing blood vessels, further refines the vascular network and is the principal process by which certain organs, such as the stomach, intestine or brain, become vascularized. Vasculogenesis and angiogenesis are complex multistep processes that involve remodelling of the extracellular matrix, migration and proliferation of endothelial cells, lumen formation and functional maturation of blood vessels. Multiple cell-cell or cell-extracellular matrix interactions occur, which require signalling via soluble or cell-bound molecules. A variety of

molecules have been implicated in these processes, in particular angiogenic growth factors and their endothelial receptors and cell adhesion molecules. Soluble secreted molecules are likely to function as paracrine signalling molecules in these processes. Recent evidence suggests that the angiogenic factor vascular endothelial cell growth factor (VEGF) and its cellular receptors lie at the heart of the regulatory network controlling blood vessel growth and differentiation in both embryonic development and several angiogenesis-dependent diseases. While proliferation of the vascular endothelial cells is vital to angiogenesis, uncontrolled proliferation can lead to cancer and metastasis, diabetic retinopathy, atherosclerosis, rheumatoid arthritis, synovitis, psoriasis, dermatitis, endometriosis, encephalitis, and tonsilitis.

VEGF is a disulfide-linked 46-kDa dimeric glycoprotein first isolated from media conditioned by bovine folliculo stellate cells and its partial amino acid sequence determined (Gospodarowicz et al., *PNAS* 86(19):7311-7315; Ferrara and Henzel, *BBRC* 161(2):851-858 (1989)). It has several properties expected of a candidate regulator of angiogenesis: it is secreted by normal and tumor cell lines; its is an endothelial-cell-specific mitogen; it is angiogenic in *in vivo* test systems such as the chorioallantoic membrane and the rabbit cornea; it is chemotactic for endothelial cells and monocytes; and it induces in endothelial cells the production of plasminogen activators, which are involved in the proteolytic degradation of the extra-cellular matrix during capillary sprouting. Several VEGF isoforms are known, which have similar biological activities but differ in their secretion from producer cells and in heparin binding (Georg Breier and Werner Risau, *Trends in Cell Biology*, 6:454-456(December 1996)).

US Pat. Nos. 5,194,596 and 5,219,739, which are hereby incorporated by reference, disclose methods and means for producing vascular endothelial cell growth factor by means of recombinant DNA technology, such as bovine VEGF sub 120, bovine VEGF sub 164, human VEGF sub 121 and human VEGF sub

165, for use as a wound healing agent following vascular graft surgery, balloon angioplasty or to promote collateral circulation in post-myocardial infarction patients or in the treatment of wounds, such as dermal ulcers, including pressure sores, venous ulcers and diabetic ulcers, in the treatment of burns and injuries where angiogenesis is required to prepare the burn or injured site for a skin graft or flap, in cases where plastic surgery reconstruction is required and administration to the site of a bone fracture. An additional use relates in keeping wounds clean and non-infected in association with general surgery and following the repair of cuts and lacerations. Parenteral and topical compositions are disclosed.

US Pat. No. 5,332,671, which is hereby incorporated by reference, discloses the production of a vascular endothelial cell growth factor and/or derivatives thereof by recombinant techniques for use in treating conditions in which a selective action on the vascular endothelial cells, in the absence of excessive tissue growth, is important, e.g., surface ulcers such as diabetic, hemophiliac and varicose ulcers and vascular injuries resulting from trauma such as subcutaneous wounds, incisions and injuries to the blood vessels or heart. Compositions for continuous infusion, sustained release, topical application or injection at empirically determined frequencies are disclosed.

PCT Publication No. 9639515 discloses DNA encoding human VEGF-2 used to develop products to promote angiogenesis (e.g., stimulate growth of transplanted tissue following coronary bypass surgery) or endothelialisation in vascular graft surgery. PCT Publication No. 9639421 discloses DNA encoding human VEGF-3 used to develop products to promote angiogenesis and wound healing and to promote vascular tissue repair.

Our previous studies demonstrated that basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) which stimulate angiogenesis and granulation tissue production accelerated experimental duodenal ulcer

healing that was more than two million times more potent on a molar basis that the similar effect of cimetidine (S. Szabo et al., *Gastroenterology*, 106:1106-1111 (1994)).

Summary of the Invention

Surprisingly, we have discovered that administration of VEGF (VPF), which is highly specific for endothelial cells, is sufficient for both acute gastroprotection and chronic GI ulcer healing in mammals. In particular, administration of a pharmaceutically effective amount of VEGF, such as from about 30 ng/kg to about 1000μg/kg, preferably about 80 ng/kg to about 800 μg/kg, more preferably about 400 ng/kg to about 700 μg/kg per day for about a week to two months, preferably two to three weeks, results in healing of GI ulcers in a mammal, such as a human. In particular, improved or enhanced mucosal restoration or prominent stimulation of angiogenesis and granulation tissue production at the ulcer edge results from this treatment.

Similarly, administration of from about 3 μg/kg to about 1000μg/kg, preferably about 8μg/kg to about 800 μg/kg, more preferably about 400 μg/kg to about 700 μg/kg VEGF, which is administered from 0 minutes to about 60 minutes prior to potential injury to the GI tract, such as ingestion of an NSAID or ethanol, results in acute gastroprotection.

Preferably, the administration is targeted to the GI tract, such as by oral administration, for example liquid solutions, immediate release dosage forms, controlled or sustained release dosage forms, or gastro-retentive dosage forms or by rectal administration, for example enemas or suppositories.

Detailed Description of the Invention

As used herein, the term "VEGF" or "vascular endothelial cell growth factor" refers to the disulfide-linked 46-kDa dimeric glycoprotein (a mammalian

growth factor) first isolated from media conditioned by bovine folliculo stellate cells and its partial amino acid sequence determined (Gospodarowicz et al., *PNAS* 86(19):7311-7315; Ferrara and Henzel, *BBRC* 161(2):851-858 (1989))(hereinafter native VEGF) together with analogs and variants thereof having the biological activity of the corresponding native VEGF, including human VEGF. Analogs or variants are defined as molecules in which the amino acid sequence, glycosylation, or other features of native VEGF have been modified covalently or noncovalently, such as the analogs or variants disclosed above, and including salts thereof. An example of VEGF is the human VEGF obtainable from PeproTech, Canton, MA.

The present invention encompasses administration of a pharmacologically effective amount of VEGF, such as from about 30 ng/kg to about 1000 μg/kg, preferably about 80 ng/kg to about 800 μg/kg, more preferably about 400 ng/kg to about 700 μg/kg per day for about a week to two months, preferably two to four weeks, to treat duodenal ulcers or other GI ulcers in a mammal, such as a human. Examples of GI ulcers include but are not limited to duodenal ulcers, peptic ulcers, drug-induced ulcers, or intestinal ulcers, including but not limited to damage due to non-steroidal (NSAID) and anti-inflammatory drugs, gastro-esphogheal reflux disease or inflammatory bowel disease such as ulcerative colitis and Crohn's disease. The treatment regimen may consist of once daily administration or dividing the daily dose into multiple administrations, such as administration from 2 to 4 times per day.

The present invention also encompasses administration of from about 3 μg/kg to about 1000 μg/kg, preferably about 8 μg/kg to about 800 μg/kg, most preferably 400 μg/kg to about 700 μg/kg VEGF to provide for acute gastroprotection. Typically, the administration of VEGF occurs from about 0 minutes to about 60 minutes, preferably from about 0 minutes to about 30 minutes prior to insult to the GI tract, such as ingestion of NSAIDS or ethanol.

Preferably, the administration is targeted to the GI tract. For instance, oral administration is a preferred administration route. In particular, liquid solutions, immediate release dosage forms, controlled or sustained release dosage forms or gastro-retentive dosage forms are preferred. Additionally, oral buccal dosage forms are useful for the delivery of VEGF.

Oral immediate release, sustained release, controlled release, oral buccal and/or gastro-retentive dosage forms can be prepared from nano- or microparticulates including but not limited to biodegradable matrix nano- or microparticles, nanocapsules, microcapsules or spray-dried vehicles. Emulsions, capsules or tablets incorporating these nano- or microparticulate forms can be prepared. Additionally, solid dosage forms can be prepared as tablets, mini-tablets, beads, capsules, etc., including forms with a controlled/sustained release coating. In these forms, the VEGF is typically released by erosion, diffusion, osmotic pressure, or by a combination of these mechanisms. Oral solutions, including elixirs, emulsions and effervescent solutions, can also be employed in this invention.

Typical controlled, sustained, or gastro-retentive excipients may include, but are not limited to, biodegradable polymers or biopolymers, such as poly-lactide, poly-glycolide, poly(lactide-co-glycolide), other polymers or copolymers of poly(α-hydroxycarboxylic acids), poly(lactones), poly(acetals), poly(orthoesters), poly(alkylcyanoacrylates) or poly(orthcarbonates). Additional excipients useful for oral administration forms include, but are not limited to, polysaccharides, for example cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; agarose; carrageenan; dextrans; destins; fructans; chitosans; glycogens; and glucans.

Similarly, rectal administration is also a preferred administration route. In particular, enemas or rectal suppositories are preferred.

While the compositions for administering VEGF are preferably those which target the VEGF to the GI tract, such as oral or rectal administration, less preferred but still useful are compositions adapted for other routes of administration, such as vaginal or parenteral administration.

Example 1

Chronic duodenal ulcers were induced in Sprague-Dawley female rats (160-200g) by administering cysteamine-HCl (25 mg/100g) 3 times at 4-hour intervals by gavage and cortisone (5 mg/100g) s.c. 4 hours after the last dose of cystemine. To randomize rats with equally severe penetrating or perforated duodenal ulcers, the animals were anesthetized and laparatomized on the third day following treatment with cysteamine-HCl. After randomization, treatment was started with either vehicle saline (controls) or human VEGF (PeproTech, Canton, MA) at a dose of 1μg/100g once daily by gavage for 21 days.

At autopsy, the size of duodenal ulcers was measured, evaluated by stereomicroscopic planimetry and histologic sections taken. The results revealed that the size of duodenal ulcers in controls was 7.4 ± 1.6 mm^2. In contrast, the size of the ulcers in the VEGF-treated group were 1.9 ± 0.9 mm^2 ($p<0.05$), histologically accompanied by complete healing or prominent angiogenesis and granulation tissue production. The density of blood vessels per x400 magnification field in the granulation tissue at the ulcer edge was 8.0 ± 0.5 in the controls as opposed to 16.9 ± 1.9 ($p<0.001$) in the VEGF-treated rats. Thus, oral treatment of rats with VEGF (VPF) accelerated the healing of cysteamine-induced chronic duodenal ulcers. In addition, the healing was accompanied by complete mucosal restoration and stimulation of angiogenesis and granulation tissue production.

Example 2

To investigate whether VEGF provides acute gastroprotection, groups of fasted rats were given by gavage human VEGF (PeproTech, Canton, MA) at 0.1μg/100g [VEGF group 1] or 1μg/100g [VEGF group 2] followed by 1 ml of 75% ethanol 30 minutes later. The control group was treated with 1 ml of 75% ethanol only.

Autopsy was performed 1 hour after treatment with the alcohol and the area of hemorrhagic mucosal lesions (HML) was measured by computerized stereomicroscopic planimetry. In the controls, HML involved 7.1 ± 1.4% of glandular stomach. VEGF group 1 showed HML which involved 7.7 ± 2.7% while VEGF group 2 showed HML which involved 4.0 ± 1.6% ($p<0.05$) of the grandular stomach. Thus, VEGF (VPF) exerted gastroprotection which might be associated with the known acute vascular effects of the peptide.

Example 3

Normally fed female Sprague-Dawley rats (170-200 g) were given 0.1 ml of 6% iodoacetamide (Sigma) in 1% methyl-cellulose intracolonically (i.c.) with Nelston catheter 7 cm from the anus to induce ulcerative colitis-like lesions. From the second day, the rats were treated with vehicle saline or VEGF (rhVEGF, 38kDa, 165 AA, PeproTech) 10, 100 or 500 ng/100g i.c. once daily while the clinical signs of the illness, such as diarrhea, lethargy and weight loss were monitored. On the 10th day, the animals were killed and the area and severity of colonic lesions were evaluated macroscopically, with stereomicroscopic planimetry ,and histologically. The results of this evaluation are tablulated in Table I.

TABLE 1				
Treatment (*p<0.05; **p<0.01)	**Diarrhea** (Scale:0-3)	**Lethargy** (Scale:0-3)	**Lesion area** (nm^2)	**Severity of colitis** (Scale: 0-3)
Vehicle	0.92±0.08	0.56±0.06	55.9±8.1	2.3±0.2
VEGF 10ng/100g	1.0±0.02	0.71±0.18	32.5±14.5	1.5±0.5**
VEGF 100ng/100g	0.67±0.1**	0.47±0.1**	26.1±6.2**	1.3±0.2**
VEGF 500ng/100g	0.60±0.1**	0.58±0.1	32.7±9.1*	1.8±0.3

Histologic examination revealed extensive granulation tissue production with partial or complete colonic re-epithelialization. The VEGF (10-500ng/100g) dose-dependently reduced the severity of diarrhea in the iodoacetamide-induced colitis. The VEGF also decreased the area and severity of the colonic lesions. On a molar basis, these small doses of VEGF indicate that this peptide is very potent in the healing of not only upper but also lower GI tract lesions as well.

The skilled artisan in the field would expect that the examples given above as practiced in the rat are predictive of healing in other mammals, particularly humans.

What is Claimed is:

1. A method of treating a GI ulcer in a mammal comprising administering a pharmacologically effective amount of VEGF to the mammal.

2. A method of providing acute gastroprotection in a mammal comprising administering a pharmacologically effective amount of VEGF to the mammal.

3. The method of claims 1 or 2, wherein the mammal is a human.

4. The method of any of the previous claims, wherein the administration of the pharmacologically effective amount of VEGF is by oral administration.

5. The method of any of the previous claims, wherein the administration of the pharmacologically effective amount of VEGF is by rectal administration.

6. The method of any of the previous claims, wherein the pharmacologically effective amount of VEGF ranges from about 30 ng/kg to about 1000 μg/kg per day.

7. The method of any of the previous claims, wherein the administration of the pharmacologically effective amount of VEGF is once daily.

8. The method of any of the previous claims, wherein the GI ulcer is selected from the group consisting of duodenal ulcers, peptic ulcers, drug-induced ulcers, intestinal ulcers, GI damage due to NSAID ingestion, GI damage due to anti-inflammatory drugs, GI damage due to gastro-esphogheal reflux disease or GI damage due to inflammatory bowel disease.

9. The method of claim 1 or claims 3 through 8, wherein the VEGF is administered daily from about 1 week to about a month to provide for healing of the GI ulcer.

10. The method of any of claims 2 through 8, wherein the VEGF is administered from about 0 minutes to about 60 minutes prior to insult to the GI tract to provide acute gastroprotection.

11. A composition for treating a GI ulcer or for providing acute gastroprotection in a mammal comprising a pharmacologically effective amount of VEGF in combination with a pharmaceutically acceptable carrier.

12. The composition of Claim 11, wherein the composition is adapted to target release to the GI tract.

13. The composition of either claim 11 or 12, wherein the pharmacologically effective amount of VEGF ranges from about 30 ng/kg to about 1000 μg/kg.

14. The composition of any of claims 11 through 13, wherein the pharmacologically effective amount of VEGF ranges from about 80 ng/kg to about 800 μg/kg.

15. The composition of any of claims 11 through 14, wherein the composition is adapted for oral or rectal administration.

16. The composition of any of claims 11 through 15, wherein the pharmaceutically acceptable carrier comprises a liquid solution selected from the group consisting of elixirs, emulsions and effervescent solutions.

17. The composition of any of claims 11 through 15, wherein the pharmaceutically acceptable carrier is selected from the group consisting of an immediate release dosage form, a controlled release dosage form, a sustained release dosage form, a gastro-retentive dosage form, an oral buccal dosage form, an enema and a rectal suppository dosage form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07686

A. CLASSIFICATION OF SUBJECT MATTER

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US CL : 514/21

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,219,998 A (LEVIN et al.) 15 June 1993, see the entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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(54) METHOD FOR TREATING DIABETIC ULCERS

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(57) ABSTRACT

A method and means have been developed to deliver a therapeutic dose or dosages of the angiogenic molecule, Vascular Endothelial Growth Factor (VEGF) that results in a statically significant decrease in the time to achieve 100% wound closure and accelerates the rate of healing in experimental diabetic ulcers. Toxicity is evaluated by measuring any local inflammatory response at the wound site, the systemic absorption of VEGF, and the effect on distant organs that may be particularly susceptible to VEGF therapy (e.g., retinopathy and hepatitis) The angiogenic response is quantified by measuring the change in collagen deposition, epithelialization, and the closure rates of diabetic ulcers after therapeutic dosing with ADV-VEGF. Sustained administration of VEGF stimulates and accelerates the healing process as evidenced by a reduced time to complete healing (defined by 100% epithelialization and no drainage) in experimental diabetic ulcers, with minimal to no toxicity. Important features of the method and reagents for use therein are that the VEGF is released into the ulcer in a sufficient quantity over a period of time for at least two to six weeks, or to closure of the wound.

Proliferation Assay of HUVECS Stimulated With SUP of Infected JC cells

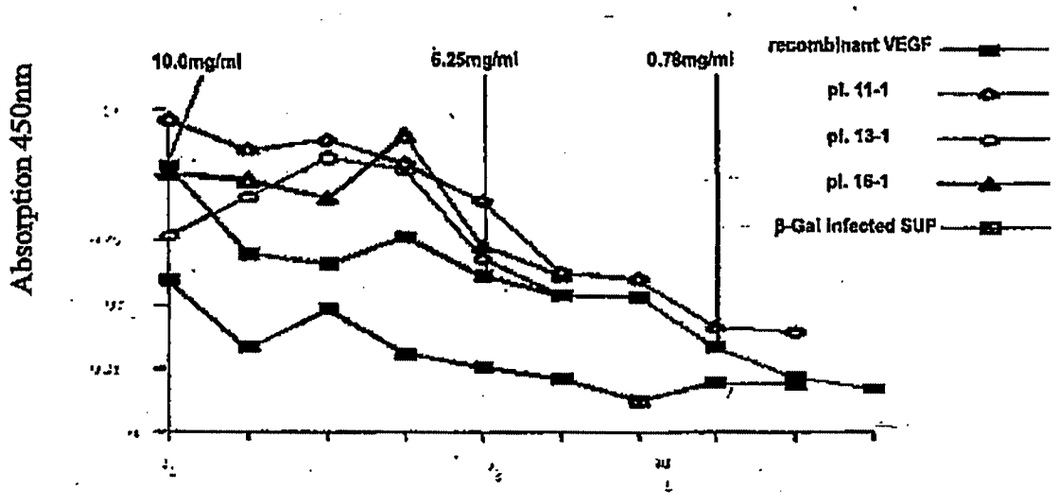

FIGURE 1

METHOD FOR TREATING DIABETIC ULCERS

[0001] This application claims priority to U.S. Ser. No. 60/363,584 filed Mar. 12, 2002.

[0002] The United States government has certain rights in this invention by virtue of a NIH, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grant R21DK060214-01 and NIH, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grant K08DK059424-01 to Dr. Harold Brem.

BACKGROUND OF THE INVENTION

[0003] The present invention is a method of tissue engineering, and specifically relates to administration of angiogenic factors directly to diabetic wound ulcers, in an effective amount for a sustained period of time effective to promote closure.

[0004] Among the 16 million diabetic patients (diagnosed and undiagnosed) in the United States, an estimated 1200 amputations are performed each week (Pecoraro, et al., *Diabetes Care.* 1990; 13:213-521); 84% of which are preceded by a foot ulcer. Limb amputation in diabetics is associated with an increased risk for further amputation, with a five-year mortality rate of 39 to 68% (Reiber et al. *Diabetes in America.* Washington, D.C.; U.S. Government Printing Office, 1995:409-428). The direct costs of a lower extremity amputation range from $20,000 to $60,000. When failed vascular reconstruction, rehabilitation, and lost productivity within society are considered, these costs greatly exceed financial analysis. The grave consequences, pain, and suffering endured by patients with diabetic foot ulcers mandate determination of the best combination of therapies to prevent progression and, consequent occurrence of these complications.

[0005] In addition to amputation, the need to have accelerated healing in diabetic patients with foot ulcers is accentuated because these patients have impaired immunity (Geerlings et al. *FEMS Immunol Med Microbiol.* 1999;3-4:259-265; Feige et al., *EXS.* 1996;77:359-373; Bessman et al., *J Diabetes Complications.* 1992;4:258-262; Abraham, et al., *J Dermatol.* 1990;7440-447; Loots et al., *J Invest Dermatol.* 1998;5:850-857; Brown et al., *J Surg Research* 1994;56:562-570; Greenhalgh et al., *Am J Pathol* 1990;1361235-1246). Since unhealed open wounds are portals for systemic infection, they can have particularly devastating effects for the diabetic patient.

[0006] It is well known that diabetic patients are predisposed to ulceration. This predisposition has multiple etiologies, including endothelial cell dysfunction, accelerated atherosclerosis, and peripheral neuropathy, which relate to the endothelium and contribute to deficits in healing. One of the most central etiologies for this predisposition is the reduced angiogenic response in the diabetic patient.

[0007] Recently, local use of growth factors has been shown to be promising for the treatment of diabetic ulcers. Two new agents have been FDA approved for the treatment of diabetic foot ulcers. The first, platelet derived growth factor (PDGF-BB), has shown efficacy. The second, human skin equivalent, has also shown efficacy in the treatment of diabetic foot ulcers. However, despite their successes, there remain several significant problems with these therapies: neither has demonstrated efficacy in ischemic diabetic foot ulcers, and both have a minimum failure rate of 45% in well vascularized limbs. Although this is better than the failure rate of standard therapies (i.e., off-loading and saline dressing), the number of amputations and non-healed diabetic foot ulcers remains excessive. Although both of these therapies have also demonstrated that local therapy is clinically effective in the treatment of diabetic foot ulcers, more therapies are clearly needed.

[0008] Therefore, it is an object of the present invention to provide a method and means to provide increased blood flow to diabetic ulcers, and thereby promote wound closure.

SUMMARY OF THE INVENTION

[0009] A method and means have been developed to deliver a therapeutic dose or dosages of the angiogenic molecule, Vascular Endothelial Growth Factor (VEGF) that results in a statistically significant decrease in the time to achieve substantially 100% wound closure and accelerates the rate of healing in experimental diabetic ulcers. Toxicity is evaluated by measuring any local inflammatory response at the wound site, the systemic absorption of VEGF, and the effect on distant organs that may be particularly susceptible to VEGF therapy (e.g., retinopathy and hepatitis) The angiogenic response is quantified by measuring the change in collagen deposition, epithelialization, and the closure rates of diabetic ulcers after therapeutic dosing with adenoviral vector (ADV)-VEGF or VEGF. Sustained administration of VEGF stimulates and accelerates the healing process as evidenced by a reduced time to complete healing (defined by 100% epithelialization and no drainage) in experimental diabetic ulcers, with minimal to no toxicity. Important features of the method and reagents for use therein are that the VEGF is released into the ulcer in a sufficient quantity over a period of time for at least two to six weeks, or to closure of the wound. The VEGF can be administered directly or as the gene which is expressed at the site in need of treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIG. 1 is a graph of the proliferation of human vascular endothelial cells (HUVECs) stimulated with the supernatant of JC cells infected with adenoviral vectors expressing VEGF, as a function of number of cells (absorption) versus dilution of conditioned supernatant, compared to control (beta-galactosidase).

DETAILED DESCRIPTION OF THE INVENTION

[0011] VEGF and Angiogenesis

[0012] Local angiogenic therapy is used to treat one of the primary etiologies of complications in patients with diabetic foot ulcers, decreased angiogenesis in the wound. It has been well established that distal bypass alone is not enough to sufficiently accelerate the healing of most diabetic foot ulcers.

[0013] There are multiple physiological processes that result in decreased angiogenesis in a diabetic ulcer; including:

[0014] 1. Decreased VEGF expression in experimental diabetic wounds. VEGF normally stimulates endothelial cell proliferation and experimental dia-

betic wounds show a marked decrease in angiogenesis (Frank et al., *J Biol Chem* 1995;270:12607-12613; Tsuboi, et al., *J Explorer Med* 1990;172:245-251).

[0015] 2. Endothelial dysfunction results in several metabolic anomalies in diabetes, including oxidative stress, hyperglycemic pseudohypoxia, nonenzymatic glycosylation, and activation of the coagulation cascade (Stehouwer et al., *Cardiovasc Res* 1997;34:55-68)

[0016] 3. Accelerated atherosclerosis may impair function by damaging the transduction cascades that synthesize nitrous oxide or by decreasing expression of nitrous oxide synthase, consistent with the finding of decreased nitrous oxide synthase expression in skin endothelium from diabetic patients (Liao, et al., *J Biol Chem* 1995;270:319-324; Veves et al., *Diabetes* 1998;47:457-463).

[0017] 4. Neuropathy is associated with endothelium dependent and independent dysfunction in diabetic patients, predisposing them to foot ulceration (Pasini et al., *Angiology* 1996;47:569-577).

[0018] 5. Arterial occlusive disease is associated with peripheral neuropathy, manifested as slower conduction velocity of motor and sensory nerves and depression of automatic responses.

[0019] Other factors are thought to be significant variables in the treatment of diabetic wound ulcers, especially the high pressure within the wound, which contributes to the ischemia and neuropathy, although it has not been apparent what single factor, if any, could be effective in promoting wound closure.

[0020] VEGF has been shown to be efficacious in the treatment of cardiac ischemia and leg ischemia, and it has demonstrated effectiveness in the treatment of diabetic limbs. However, it has not previously been tested in diabetic ulcers. VEGF possesses certain key advantages for use in diabetic foot ulcers over other angiogenic agents, for example, basic fibroblast growth factor (bFGF). Although bFGF has been one of the most widely studied angiogenic growth factors for the treatment of experimental diabetic ulcers (Albertson et al. *Surgery* 1993;1 14:368-373), other chronic wounds (Quirinia, et al., *Ann Surg* 1998;227:446-454), and accelerating acute wound healing (Hebda et al., *J Invest Dermatol* 1990;95:626-631), it has not been shown to be effective in treating human diabetic ulcers (Richard, et al., *Diabetes Care* 1995;18:64-69). Unlike VEGF, bFGF is not acid stable, which is critical in the low pH environment of a diabetic wound (Ferrara, et al., *Biochem Biophys Res Comm* 1989; 161:851 -858). The impaired wound healing in diabetics may be due to fibroblast dysfunction (Lerman et al. Am. J. Pathol. 2003; 162:303-312). Fibroblasts from diabetic db/db mice maintain selective impairments in multiple cellular processes which are accentuated by hypoxic environments such as those in a healing wound. These impairments include a severe reduction in VEGF expression, and release in response to injury. These changes were only observed after the development of the diabetic phenotype in these animals. Additionally, VEGF has been shown to be a more potent angiogenic molecule than bFGF in experimental diabetic wound (Corral, et al., *Arch Surg.* 199; 134:200-5). Also, VEGF levels are apparently decreased by endogenous proteases (Lauer, et al., *J Invest Derm* 2000;115:12-18) in chronic wounds. This provides a basis from which to develop gene therapy with VEGF for use in diabetic wounds. The intense and sustained local synthesis of VEGF by local angiogenic therapy should obviate the proteolytic degradation of endogenous VEGF in the diabetic wound.

[0021] The role of VEGF in angiogenesis reflects its function as an endothelial cell mitogen, (Ferrara N., *J Mol Med* 1999;77:524-543; Gospodarowicz D, et al., *Proc Natl Acad Sci* 1989;86:7311-7315) chemotactic agent, (Leung D W, et al., *Science* 1989;246: 1306-1309; Keck P J, et al., *Science* 1989;246: 1309-1312) and inducer of vascular permeability (Connolly D T, et al., *J Clin Invest* 1989;84: 1470-1478; Yoshida A, et al., *Growth Factors* 1996;13:57-64; Senger D R, et al., *Cancer Res* 1990;50:1774-1778). It exists in many isoforms with a common amino terminus that contains a signal sequence that allows the protein to be secreted. VEGF 165 is the most common isoform and is preferred, although it is understood that other equivalent forms of VEGF are known and could be used alone or in combination with each other as described herein.

[0022] Many of the cells (Banks, et al., *Br J Cancer* 1998;77:956-964, Nathan, *J Clin Invest* 1987;79:319-326; Berse, et al., *Mol Biol Cell* 1992;3:211 -220; Leibovich, et al., *Am J Pathol* 1975;78:71-100; Koch, et al., *Science* 1993;258:1798-1801; Uchida, et al., *Am J Physiol* 1994;266:F81-F88; Namiki, et al., *J Biol Chem* 1995;270:31189-31195; Nissen, et al., *Am J Path* 1998;152:1445-1452; Brogi, et al., *Circulation* 1994;90:649-652; Stavri, et al. *Circulation* 1995;92:11-14) recruited into a wound synthesize VEGF. VEGF serves distinct paracrine and autocrine roles upon endothelial cells. By stimulating the endothelial cells, multiple phases of the angiogenic cascade are enhanced by VEGF. VEGF has been shown in experimental studies to enhance vascularization of both autologous bone grafts and skin flaps in rats (Padubidri, et al., *Ann Plast Surg* 1996;37:604-611; Li, et al. *Surg Forum.* 1999;50:586-587). One of the mediators of VEGF activity, nitric oxide, enhances collagen deposition in diabetic wounds, (Witte, et al., *Surg Forum* 1997:48:665-667) and may restore endothelial function to improve both nerve conduction and tissue oxygenation. This supports the concept that VEGF enhances wound healing primarily by stimulating angiogenesis and possible secondary stimulation of collagen production.

[0023]– Local Sustained Release of VEGF in a Diabetic Wound

[0024] In the twelve years that VEGF has been available, local therapy with recombinant forms has not yet been shown to be effective for wound closure. One limitation to this technique is that it fails to sustain sufficient levels of VEGF in the wound for a significant period of time. Gene therapy is an effective means for delivering VEGF in a sustained release fashion in vivo. ADV-VEGF has already been tested in patients with cardiac ischemia and leg ischemia to stimulate angiogenesis. Additionally, it has also been tested for diabetic ischemia and neuropathy. Other means of obtaining sustained release of an effective amount of compound include providing sustained release formulations such as polymeric delivery systems, mini-pumps, and hydrogels. These can be loaded with VEGF, injected or

implanted into the ulcers, where the VEGF is released over a therapeutically effective time period.

[0025] The principle of gene therapy is that a therapeutic gene must first be efficiently delivered to the specific target cell (Nabel, et al., *Science*. 1990;249:1285-1288). Second, it must be expressed and sustained at a certain level to achieve its therapeutic purpose (Sauter, et al., *Proc Natl Acad Sci USA*. 2000;9:4802-4807).

[0026] The principle components of gene therapy are a vector or other means of delivering a nucleic acid of interest, and the nucleic acid. Many appropriate viral vectors are known, most of which are adenoviral vectors, adeno-associated viral vectors or retroviral vectors. Other means of delivery include liposomes, direct delivery of naked DNA, and hydrogels. The vectors will typically include a promoter that can contain enhancers, inverted terminal repeats (ITRs), inducible promoters, and polyA sequences, followed by a termination sequence. All of these are known to those skilled in the art, and commercially available or described in the literature.

[0027] The discovery that naked DNA is taken up by muscle cells and transiently expressed in vivo, was reported twelve years ago, by Wolff, al, et, *Science*, 1990;247,1465-1468,; and Wolff, *Nature*, 1991;352, 815-818.

[0028] Plasmid DNA, which can function episomally, has been used with liposome encapsulation, $CaPO_4$ precipitation, and electroporation as an alternative to viral transfections. Clinical trials with liposome encapsulated DNA in treating melanoma illustrates this approach to gene therapy, as reported by Nabel, et al., *Proc. Nat. Acad. Sci. USA.*, 1993;90, 11307-11311 and Wolff, *Experimental Neurology*, 1992;115, 400-413, also reported expression of plasmid DNA. There have been many confirmatory reports since the initial studies.

[0029] Viral vectors are preferred for gene therapy. Human adenoviruses have a 36-kilobase double-stranded DNA genome that undergoes a highly regulated program of gene expression during the normal life cycle of the virus. The advantages of adenoviruses over other chemical, physical, or biological gene transfer techniques include several unique features of this system (Molnar-Kimber, et al., *Hum Gene Ther* Sep. 20, 1998;9(14):2121-33). First, adenoviruses infect human skin cells at more than 95% efficiency and do not require that cells are dividing, making a lengthy selection process unnecessary (Kozarsky, et al., *Curr Opin Genet Dev* June 1993;3(3):499-503; Mulligan, *Science* May 14, 1993;260(5110):926-32; Kremer, *Gene Ther* 1995;2:564-5; Yang, et al., *Immunity August* 1994;1(5):433-42; Mitani, et al., *Proc Natl Acad Sci USA* Apr 25, 1995;92(9):3854-8). Second, adenoviruses remain episomal and thus do not normally integrate into the human genome (Bett, et al., *J Virol* October 1993;67(10):5911-21 Losordo, et al., *Am Heart J.* 1999;138:132-141). Third, adenovirus-mediated gene expression in keratinocytes, melanocytes, and fibroblasts remains stable in vitro for at least 2-6 weeks, depending on the proliferation rate of cells (Feng, et al., *Cancer Res* May 15, 1995;55(10):2024-8). Adenoviral vectors are commonly constructed by deletion of the essential ELAM-1 gene to prevent viral replication.

[0030] For the purpose of wound healing, ADV-VEGF gene therapy may offer substantial advantages, one of which is its ability to transduce both resting and dividing cells at very high efficiency without integration into the host cell's genome. The inflammatory response that is often associated with adenoviral mediated gene transfer for the induction of endogenous growth factor overexpression may actually enhance wound healing, since wound healing is itself fundamentally an inflammatory response. Furthermore, the limited duration of high level transgene expression of a growth factor known to have significant vulnerary effects (like VEGF) may be all that is necessary to affect wound healing. Recombinant ADV can be generated in high titers, is known to infect both resting and dividing cells with high efficiency, and its transgene expression is limited by the host's cellular immunity. The high prevalence of anti-adenovirus antibodies in the general population was thought to preclude adenovirus-mediated gene therapy. However, it was shown that local injection of rADV in pre-sensitized hosts results in levels of transgene expression at the injection site comparable to that of native animals (Bramson, et al., *Gene Ther* 1997;4:1069-76). These findings stress both the applicability of this local gene and the potential for repeated treatments, if required. Although, for effective gene therapy, the transgene may need to be expressed in a higher percentage of cells in the target population, lower rates of expression may be sufficient if the gene product exerts a paracrine effect on neighboring cells. Toxicity to tissues other than the target, due to the broad tropism of the adenovirus, is usually seen only with systemic administration.

[0031] Adenoviruses can transduce both dividing and non-dividing cells and the viral genome, remains episomal and does not integrate into host chromosomes. They can infect a broad range of human cells such as those in skin, lung, liver, brain, and blood vessels. It has recently been shown that adenoviral vectors are very effective for in vivo gene transfer to porcine and other experimental skin wounds and nearly microgram quantities of therapeutic protein can be expressed in the wound microenvironment (Liechty, et al., *Wound Rep Reg* 1999;7:148-53; Liechty, et al., *J Invest Dermatol* 1999;113:375-83).

[0032] The usefulness of administration of ADV-VEGF in reducing the time needed for wound healing, as described in U.S. Ser. No. 60/363,584 filed Mar. 12, 2002, was recently confirmed in experimentally-induced excision wounds in diabetic mice (Romano Di Peppe, et al *Gene Therapy*, 9: 1271-1277 (October 2002)). A statistically significant reduction in wound healing time as compared to untreated mice was noted as early as 3 days after treatment, in mice treated with 10^8 p.f.u. of AdCMV.$VEGF_{165}$ directly on the wound. There was also an increase in VEGF expression in the wounded skin and significant angiogenic response. The concentration of AD-VEGF can be titrated accordingly to result in expression of an effective amount of VEGF.

[0033] Polymeric Matrices

[0034] Alternatively, the VEGF is delivered using a sustained release device. Both non-biodegradable and biodegradable matrices can be used for delivery of genes, although biodegradable matrices are preferred. These may be natural or synthetic polymers, although synthetic polymers are preferred due to the better characterization of degradation and release profiles. The polymer is selected based on the period over which release is desired, generally in the range of at least two to six weeks, although longer

periods may be desirable. In some cases linear release may be most useful, although in others a pulse release or "bulk release" may provided more effective results. The polymer may be in the form of a hydrogel (typically in absorbing up to about 90% by weight of water), and can optionally be crosslinked with multivalent ions or polymers.

[0035] High molecular weight genes can be delivered partially by diffusion but mainly by degradation of the polymeric system. In this case, biodegradable polymers, bioerodible hydrogels, and protein delivery systems are particularly preferred. Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof. Examples of biodegradable polymers include synthetic polymers such as hydroxyacid polymers, for example, polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-co-caprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water in vivo, by surface or bulk erosion.

[0036] In the preferred embodiment, the polymeric matrix is a microparticle between nanometers and one millimeter in diameter, more preferably between 0.5 and 100 microns for administration via injection. The microparticles can be microspheres, where the gene is dispersed within a solid polymeric matrix, or microcapsules, where the core is of a different material than the polymeric shell, and the gene is dispersed or suspended in the core, which may be liquid or solid in nature. Unless specifically defined herein, microparticles, microspheres, and microcapsules are used interchangeably.

[0037] Alternatively, the polymer may be cast as a thin slab or film, ranging from nanometers to four centimeters, a powder produced by grinding or other standard techniques, or even a gel such as a hydrogel. The polymer can also be in the form of a coating or part of a stent or catheter, vascular graft, or other prosthetic device.

[0038] The matrices can be formed by solvent evaporation, spray drying, solvent extraction and other methods known to those skilled in the art.

[0039] The release of VEGF from fibrin-based biomaterials was demonstrated by Wong et al., *Thromb Haemost* 89(3):573-82 March 2003). Fibrin-based biomaterial preparations can be used as provisional growth matrices for cells important in tissue repair during wound healing in vivo. VEGF was incorporated into the fibrin biomaterials prior to formation of the Fibrin Sealant clots. Clotting resulted in sustained release of VEGF causing angiogenic activity.

[0040] Prolonged controlled release has been achieved using several different devices. Examples include mini-implantable pumps for a variety of drugs especially chemotherapeutics and highly potent neuroactive drugs, silicon tubing with release controlling pores in the ends for birth control agents, and co-axial implants. Currently approved infusion procedures generally use an externally-worn or implanted pump. DUROS® sufentanil, an osmotic pump designed for 100-day delivery of sufentanil, is currently undergoing clinical testing. This implant is much smaller and easier to administer, and is described in WO 00/54745.

[0041] Effective Dosages of VEGF

[0042] Unlike in the case of an ischemic ulcer, for a diabetic ulcer, the challenge remains to keep a steady rate of angiogenic stimulation with minimal toxicity. At this stage in the field of wound healing, it appears that diabetic wounds have impaired angiogenesis. VEGF administered locally into the wound is the safest and most effective method to stimulate angiogenesis and to facilitate healing the diabetic wound.

[0043] The use of $VEGF_{165}$ has been established in multiple models of angiogenesis (Kaner, et al., *Am J Respir Cell Mol Biol.* 2000;6:640-641; Muhlhauser, et al., *Circ Res.* 1995;6:1077-1086) Human $VEGF_{165}$ molecule (rather than the murine molecule) has already been established in the preclinical studies for cardiac ischemia (using a porcine model, Esakol, et al., *Hum Gene Ther.* 1999;10:2307-2314; Lopez, et al., *Cardiovasc Res.* 1998;40:272-281; Baumgartner, et al., *Annu Rev Physiol.* 2001;63:427-450; Losordo, et al., *Am Heart J.* 1999;138:132-141; Pearlman, et al., *Nat Med.* 1995;1:1085-1089; Hariawala, et al., *J Surg Res.* 1996;63:77-82), and a dog model (Banai, et al., *Circulation.* 1994;89:2183-2189; Lazarous, et al., *Cardiovasc Res.* 1999:44;294-302) which led to its eventual use and demonstrated efficacy in humans (Esakof, et al., *Hum Gene Ther.* 1999;10:2307-2314; Sumes, et al., *Ann Thorac Surg* 1999;68:830-7; Losordo, et al., *Circulation.* 1998;98:2800-2804; Baumgartner, et al., *Am Heart J.* 1999;138:132-41; Hendel, et al., *Circulation.* 2000;101:118-121; Hammond, et al., *Cardiovasc Res.* 2001;49:561-567; Sylven, et al., *Coron Artery Dis.* 2001;12:239-243; Tio, et al., *Human Gene Therapy.* 1999;10:2953-2960; Laitinen, et al., *Hum Gene Ther.* 2000;11:263-270; Vale, et al., *Circulation.* 2000;102:965-974).

[0044] Similarly, $VEGF_{165}$ was used in preclinical studies for limb ischemia and atherosclerosis in rabbits (Gennaro et al., *Circulation* Jan. 21, 2003;107(2):230-3; Takeshita, et al., *Biochem Biophys Res Commun.* 1996;227:628-635; Tsurumi, et al., *Circulation.* 1996;94:3281-3290; Takeshita, et al., *J Clin Invest.* 1994;93:662-670; Banters, et al., *Circulation.* 1995;91:2802-2809; Bauters, et al., *J Vase Surg.* 1995;21:314-325; Takeshita, et al., *Circulation.* 1994;90(suppl1II):II-228-234; Vincent, *Circulation.* 2000;102:2255-2261) and diabetic mice, which led to its eventual clinical trial testing and demonstrated efficacy for patients (Simovic, et al., *Arch Neurol.* 2001;58:761-768; Isner, et al., *J Vasc Surg.* 1998;28:964-75). Recently, human VEGF has been used in diabetes (e.g., for diabetic neuropathy in rats, (Samii, et al., *Neurosci Lett.* 1999;262:159-162; Schratzberger, et al., *J Clin Invest.* 2001;107:1083-1092) for diabetes-related impairment of angiogenesis in mice, (Rivard, et al., *Am J Pathol.* 1999;154:355-363) for ischemic neuropathy in rabbits, (Schratzberger, et al., *Nat Med.* 2000;4:405-413) and in patients (Baumgartner, et al., *Circulation.* 1998;97:1114-1123; Isner, et al., *Hum Gene Ther.* 1996;7:959-988; Isner, et al., *Lancet.* 1996;10:370-374; Schratzberger, et al., *J Clin Invest.* 2001;107:1083-1092). Furthermore, successful treatment of diabetic neuropathy in

rodents led to human trials for efficacy of chronic ischemic neuropathy in patients (Simovic, et al., *Arch Neurol.* 2001;58:761-768). A vast amount of literature has demonstrated that the use of human VEGF in a mouse shows functionality due to a high degree of homology between the two species-specific proteins (90%, data from BLAST® protein homology searching). Finally, according to the European Molecular Biology Laboratory (EMBL) database, the amino acid of human VEGF protein and murine VEGF proteins demonstrate only one amino acid difference (215 vs. 214).

[0045] An effective dosage can be determined by extrapolation based on animal studies, for example, using a mouse model. In previous local therapy studies, the use of full thickness excisional wounds in diabetic mice has been shown to be useful for developing therapies for eventual use in patients. The mouse model has been successful in bringing growth factor therapy from the lab to the bedside. Both FDA approved drugs that are currently efficacious for diabetic ulcers (PDGF-BB and human skin equivalent) utilized the murine model in the vast majority of their preclinical testing.

[0046] The C57BL/KsJ db/db mouse is a particularly useful model since it has been shown to have decreased angiogenesis (Allavilla, et al., *Diabetes.* 2001;50:667-673; Coleman, *Diabetes.* 1982;31 (Suppl: 1 Pt 2):1-6). Tissue repair in C57BL/KsJ db/db mice has proven to be a clinically relevant model of impaired wound healing. The animals exhibit several characteristics of adult onset diabetes, including obesity, insulin-resistant hyperglycemia and markedly delayed wound closure.

[0047] C57BL/KsJ-db/db mice, homozygous for the diabetes spontaneous mutation, become identifiably obese around 3 to 4 weeks of age. Elevations of plasma insulin begin at 10 to 14 days and of blood sugar at 4 to 8 weeks. Homozygous mutant mice are polyphagic, polydipsic, and polyuric. The course of the disease is markedly influenced by genetic background. A number of features are observed on the C57BL/KsJ db/db background, including an uncontrolled rise in blood sugar, severe depletion of the insulin-producing beta-cells of the pancreatic islets, and death by 10 months of age. Exogenous insulin fails to control blood glucose levels and gluconeogenic enzyme activity increases. The diabetic mutation is a result of a point mutation in the leptin receptor gene, lepr. This point mutation promotes abnormal splicing creating a stop codon that shortens the intracellular domain of the receptor, so that its signaling capacity is curtailed. The ligand, Leptin, has been shown to be a key weight control hormone that takes a mutant form in the mouse obesity mutation, Lepob (JAX Mice database: http://jaxmice.jax.org/jaxmice-cgi/jaxmicedb.cgi).

[0048] C57BL/KsJ-db/db mice exhibit characteristics similar to those of human adult onset diabetes (NIDDM Type II) as a result of a single autosomal recessive mutation on chromosome 4. Only the homozygous animals develop diabetes. This strain also expresses lower levels of several growth factors and receptors, accounting, at least in part, for the reduced rate of healing (Werner, et al., *J Invest Dermatol* 1994;103:469-473).

[0049] The streptozotocin diabetic mouse is another model for studying the pathology of diabetes. Mice are rendered diabetic by intraperitoneal injection of streptozotocin administered for five consecutive days. Streptozotocin-treated mice become hyperglycemic and also show impaired wound healing when compared to healthy animals (Matsuda et al. *J Exp Med* 1998; 187:297-306; Brown et al *Am J Pathol* 1997; 151:715-724). The streptozotocin-induced diabetic mouse has been widely studied and is known to those of skill in the art.

[0050] The diabetic mouse model (Geerlings et al., *FEMS Immunol Med Microbial.* 1999;3-4:259-265; Feige, et al., *EXS.* 1996;77:359-373; Bessman, *J Diabetes Complications.* 1992;4:258-262; Abraham, et al., *J Dermatol.* 1990;7440-447; Loots, et al., *J Invest Dermatol.* 1998;5:850-857; Brown, et al., *J Surg Research* 1994;56:562-570; Greenhalgh, et al., *Am J Pathol* 1990;136:1235-1246; Tsuboi, et al., *J Explorer Med* 1990;172:245-251; Matuszewska, et al., *Pharm Res* 1994;11:65-71; Darby, et al., *Int J Biochem Cell Biol* 1997;29:191-200; Livant, et al., *J Clin Invest.* 2000;105:1537-1545; Yamamota, et al., *Europ J Pharm* 1996;302:53-60; Wetzler, et al., *J Invest Dermatol.* 2000;115:245-253; Sun, et al., *J Invest Dermatol* 1997; 108:313-318; Sun, et al., *J Invest Dermatol.* 1996;106:232-237; Zykova, et al., *Diabetes.* 2000;49:1461-1458; Beer, et al., *J Invest Dermatol.* 1997;109:132-138) has been widely accepted in the study of therapeutic agents that may be effective in the treatment of chronic wounds, it has been successfully used in preclinical testing for other growth factor therapies, and it offers a good model for patients with diabetic foot ulcers.

[0051] The present invention will be further understood by reference to the following non-limiting examples.

EXAMPLE 1

Non Diabetic Wounds are Angiogenic Dependent and have Delayed Contraction with Angiogenic Inhibitors

[0052] There are multiple stimuli for angiogenesis in the first few hours after a wound is created, but it is usually 3 days before new vessel formation can be visualized histologically in the wound. Throughout the wound healing process, angiogenesis maintains a critical role in the healing response (Brem, et al., *Bone Formation and Repair American Academy of Orthopedic Surgeons*/Rosemont, Ill., 1994. pp 213-222; Arbiser. *J Am Acad Dermatol* 1996;34:486-497; Pettet G, et al., *Proc R. Soc Lond* 1996;263: 1487-1493; Arnold, et al., *Pharmacol Ther* 1991;52:407-422; Seifert, et al., *Explorer Mol Pathol* 1997;64:31-40; Folkman, *Growth Factors: From Genes to Clinical Application.* Karolinska Institute Nobel Conference Series. New York, N.Y., Raven Press, 1990, pp 201-216; Folkman, et al., *Inflammation: Basic Principles and Clinical Correlates,* ed 2. New York, N.Y., Raven Press, 1992, pp 821-839; Hunt, et al., *Surgery* 1984;96:48-54).

[0053] Angiogenesis occurs in a time-dependent manner in relation to the wound healing process. For example, when a potent angiogenic inhibitor (e.g., TNP-470, also known as AGM-1470) is given systemically, wound closure and breaking strength are significantly decreased in a time-dependent manner. If the angiogenesis inhibitor is given before or 5 days after wound creation, there is no delay in wound closure, but if it is given in the first 5 days after wounding a sharp delay in closure is noted.

[0054] These experiments demonstrate the importance of the initiation of angiogenesis in contributing to the multiple processes in normal wound healing, such as wound closure and breaking strength. In the healing of acute wounds, these results show that the angiogenic response is time-dependent. This experiment establishes that decreased angiogenesis delays wound closure. In chronic wounds, such as diabetic ulcers, the angiogenic response is impaired as long as the wound is present, secondary to multiple etiologies.

EXAMPLE 2

The Viral Vector

[0055] The human cDNA for VEGF was cloned into a recombinant adenovirus vector. Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical cords using a 0.2% collagenase solution in Hanks Balanced Salt Solution (HBSS) for 20 minutes at room temperature. The cells were washed with HBSS and plated on collagen-coated (1% in PBS) tissue culture dishes in M13 medium supplemented with 20% FBS and 1 mg/ml of bFGF. HUVEC from a confluent 10-cm plate were homogenized and total RNA was extracted with the RNEASY™ kit from QIAGEN® Inc. First strand of cDNA was amplified from the RNA by RT-PCR with oligo-dT primers using the SUPERSCRIPT™ II RT PCR kit from LIFE TECHNOLOGIES®. The full-length human VEGF cDNA was then amplified by PCR with appropriate primers (sense 5'-CCCAAGCTTGCCGCCGCCATGAACTTTC TGCTGTCT-3' (SEQ ID NO:1); Hind III linker antisense, 5'-GCTCTAGAATCTGGTTCCCGAAACCCTGA-3' (SEQ ID NO:2), Xba I linker) using pfu (plaque forming units), DNA polymerase (STRATAGENE®). The product corresponding to the size of $VEGF_{165}$ was gel-purified and cloned into pBluescript (STRATAGENE®), to be spliced between the Hind III and Xba I sites. After confirmation by automatic sequencing, the $hVEGF_{165}$ cDNA was cloned into the ADV shuttle vector pADV RSV-bPA downstream of the RSV LTR. The ADV shuttle vector was then co-transfected with the adenovirus type 5 genome containing backbone vector pBHG10 into 293 cells (E1 positive packaging cell line) by standard calcium phosphate precipitation. The ensuing viral plaques were isolated and in vitro screened for transgene expression and functional activity of the transgene. A positive plaque (#11-1) was then selected and expanded. The virus ($ADV.hVEGF_{165}$) was released from the producer cells by three freeze/thaw cycles and purified over a CsCl gradient. The viral particles were measured by spectrophotometric analysis (OD_{260}) and the plaque-forming units (pfu) were determined by standard agarose overlay plaque assay on 293 cells.

[0056] The vector, as constructed, resulted in gene transfer, ascertaining transgene expression in target cells in culture. JC cells, a VEGF-negative murine breast cancer cell line which is easily transducible by adenovirus, were infected with virus of different plaques of $ADV.hVEGF_{165}$ or ADV.beta Gal (negative control) with an MOI of 100. VEGF was measured after 48 hours in the conditioned supernatant by hVEGF ELISA (R&D Systems), according to the manufacturer's instructions. The ELISA results for hVEGF of conditioned supernatant of JC cells transduced are:

[0057] 1) Virus of different plaques of $ADV.hVEGF_{165}$: (plaques 11-1; 11-2; 11-3; 11-9; 13-1; 16-1);

[0058] 2) Control vector (beta-Gal); and supernatant of uninfected JC cells. (VEGF standard curve with recombinant VEGF protein supplied by the manufacturer).

[0059] The transgene for VEGF stimulates endothelial cell proliferation in vitro. The function of the transgene was assessed through induction of HUVEC proliferation by conditioned supernatant of $ADV.hVEGF_{165}$ or ADV.beta Gal transduced JC cells, respectively. HUVECs were seeded in flat-bottom well plates pre-coated with 1% gelatin (in PBS) at 2×10^3 cells/well and serum-starved overnight (M13 medium without FBS). Conditioned supernatant was then added in several dilutions and cells were cultured for an additional 48 hours in M13 supplemented with 20% FBS, but no growth factors. HUVEC proliferation was measured by a tetrazolium-based assay at an absorption of 450 nm according to the manufacturer's instructions, EZ4U™ kit by Biomedica. Measurements of HUVEC proliferation by a tetrazolium-based assay after a 48-hour stimulation with log 2 dilution of conditioned supernatant of JC cells were transduced with:

[0060] 1) virus of different plaques of $ADV.hVEGF_{165}$: (plaques 11-1, 13-1, 16-1); and

[0061] 2) control vector (b-Gal).

[0062] Addition of recombinant VEGF protein (Pepro Tech Inc.) to the culture medium in a log 2 dilution series from 100 ng/ml to <1 ng/ml was used as a positive control.

[0063] The results are shown in FIG. 1.

EXAMPLE 3

ADV-VEGF Leads to Angiogenesis

[0064] Mice received subcutaneous injections of ADV-VEGF in one thigh and ADV.LacZ in the other. At various time intervals, these mice were sacrificed and perfusion-fixed. The thighs were harvested and processed for histology and immunohistochemistry. Histology revealed no significant changes in the control (ADV.LacZ)P group and marked presence of new blood vessels in the ADV-VEGF group.

[0065] Thus, it was established that ADV-VEGF infected cells secreted human $VEGF_{165}$ and that this protein product stimulated endothelial cell proliferation in vitro and angiogenesis in vivo, without local necrosis.

[0066] Examples 4-7 use a mouse model. For all mouse wound experiments, mice were purchased from Jackson Laboratories, and housed one per cage. Animals were anesthetized in a chamber in preparation for all experiments and shaved the day before experimentation. Full thickness wounds were made with a template of 0.8 or 1.4 cm in diameter. All wounds were created in exactly the same location, 15 mm from the last cervical vertebra on the mouse dorsum. The marked area was excised with scissors to include the epidermis, dermis, and panniculus carnosus. The wounds were then photographed on the day of wounding. The photographs and digital images were taken with a NIKON® 35 mm camera and a NIKON® digital camera, respectively, at a fixed distance from the wound. A photo ruler was placed in the field of the photograph and labeled with the mouse's identification number and date. Digital photos were used to assess length and width measurements

and the area was calculated from planimetry. Length and width were also recorded in the lab notebook at the time of photography. The data included in these studies are maintained in a database, created with MICROSOFT ACCESS®, on a DELL™ Dimension XPS R450 Computer, and backed up on an IOMEGA® Jaz Drive. Both film and digital photographs of each mouse were printed within 7 days of being taken and kept in a secure area. The digital images were measured using Med-Data Systems' Wound Imager program. An identical data system is utilized to follow clinical patients treated for diabetic foot ulcers. In all experiments, the ADV-VEGF was injected into 4 quadrants of the dermis of the newly created wound edge utilizing a Hamilton™ microliter syringe in 4 equal volumes.

[0067] This model is analogous to clinical situations where patients are routinely treated for diabetic foot ulcers, where the wound is debrided prior to initiation of growth factor therapy (PDGF-BAL) or cell therapy (human skin equivalent comprising cultured keratinocytes and fibroblasts on type I collagen). The emphasis is on converting a chronic wound or ulcer into an acute wound, in part, by debridement of tissue, leaving fresh wound edges. This approach is a standard of care and is the basis of the experimental design, allowing for the maximally effective injection of VEGF into the sharply excised wound edges.

EXAMPLE 4

Effect of ADV-VEGF

[0068] The dosage of 5×10^{11} viral particles (VPs) of ADV-VEGF was used because this was the maximal amount of viral particles that could be placed in a limited volume (up to 400 μl, which is the maximum that can be administered without the volume affecting the wound). This dosage was also based on multiple other preclinical and clinical trials performed at our institution, which determined this to be safe for ADV particles. It has become clear that the total load of ADV particles contributes to toxicity. Therefore, the FDA has requested that experiments use viral particles rather than pfus. Pfu is a biological assay measuring activity of the virus, which can vary by up to one log using the same batch of virus in different assays. If different batches of virus are used, there may be a significant variation in particle measurements. To prevent this potential problem from occurring and to keep the loads constant, the same batch of virus was used for all experiments.

[0069] Six groups, comprising 5 mice each, were used. Wounds of 0.8 cm were made with a template, as described above. All, except group F, used the C57BL/KsJ-db/db mouse model.

[0070] Group A: (ADV-VEGF) received 5×10^{11} ADV-VEGF VPs at the time of wounding. The wounds closed on average in 13.6±days.

[0071] Group B: (ADV-DL312, control null vector) received 5×10^{11} VPs at the time of wounding. The wounds closed in 26.9±0.4 days.

[0072] Group C: (saline control) received PBS and their wounds closed in 26 days, on average.

[0073] Group D: (2nd injection of ADV-VEGF) received a second injection of ADV-VEGF 7 days after wounding. The results were not statistically different from Group A.

[0074] Group E: (human recombinant VEGF) received 2.5 μg recombinant VEGF at the time of wounding. The wounds closed at a rate similar to Groups B and C.

[0075] Group F: (wild type, non-diabetic mice) received saline. The wounds closed in 12 days, on average.

[0076] The results show that treatment with ADV-VEGF resulted in a (highly) significant reduction in time to closure of wounds in diabetic mice ($p<0.01$, Student's t-test and nonparametric Mann-Whitney/Wilcoxon's test). Although these results suggested that a single treatment with ADV-VEGF was maximally effective in accelerating wound closure, the study was limited.

EXAMPLE 5

Dose-Response Study

[0077] Despite the limitations of the above experiment and the small number of mice, the promising results motivated determining if a lower dose may be effective. In order to determine if gene therapy is effective, it is first necessary to perform a dose-response experiment. This allows one to establish that there is a causal relationship between the therapy and its therapeutic effects. It was found that in these small 0.8 cm wounds 5×10^{10} and 5×10^{11} VPs of ADV-VEGF both significantly accelerated the closure of diabetic ulcers in db/db male mice, as compared to 1.6×10^{10} ADV-VEGF VPs and PBS.

[0078] There was a significantly ($p<0.05$) greater improvement in the rate of healing for the high dose group, compared to the control group.

[0079] A limitation in this experimental design was that the occlusive dressing placed on the wound after each photograph was taken resulted in broad contamination with culture-proven gross purulence with *Pseudomonas aeruginosa*. The technique was modified to utilize only a clear occlusive dressing during the first 5 days after the wound was made. No infection has been seen in subsequent experiments. However, this provided a serendipitous and important finding. This experiment suggests that ADV-VEGF may reverse bacterial contamination of a wound. This is particularly important, because almost all patients with diabetic foot ulcers have some degree of infection or, more specifically, bacterial contamination, which ~~is the single~~ greatest contributing factor to the morbidity and mortality related to these wounds.

EXAMPLE 6

Effect of VEGF on Larger Wounds

[0080] Example 5 established that even a lower dose of gene therapy might be effective. However, these experiments were limited by the occurrence of infection and the small number of groups. The next experiment determined whether a single high dose of ADV-VEGF would be effective in larger wounds.

[0081] This experiment tested the hypothesis that ADV-VEGF will be effective in accelerating wound closure, even when the size of the wound is increased, with metabolic control ascertained (therefore female mice were utilized).

Since patients usually present with larger wounds, the size of the wound was doubled to 1.4 cm which represents 35% of the total diameter of the mouse dorsum. Four treatment groups at 0.5 log differences and two control groups were tested. One set of controls was injected with saline at the time of injection and the other with ADV-DL312 5×10^{10} particles/wound.

[0082] The only group showing a statistically significant acceleration of closure was the group treated with 5×10^{11} VPs ADV-VEGF (which was the maximum dose tested). Therefore, it is necessary to determine if a greater acceleration of healing can be obtained. Another limitation of this experiment was that these mice were 12-14 weeks old, older mice exhibit poor metabolic control. The glucose levels in all groups were between 300-310. This is too high and may have affected the results, since this places the mice in a particularly catabolic state, which is not analogous to the clinical situation.

EXAMPLE 7

Determination of Toxicity (10 Mice per Group)

[0083] A comprehensive examination was initiated to look for indicators of toxicity. The liver was examined for any signs of hepatitis. The lungs were examined because angiogenic therapy can potentially cause petechia in lungs and bleeding. The kidneys were examined at the histological level because nephropathy is very common in diabetics and could be precipitated by angiogenic therapy. The cecum was analyzed because it is the most rapidly proliferating organ in the body and any therapy that may effect a wound could also effect the rapidly dividing epithelium. The retina was examined closely because it is of great concern whether systemic therapy could cause retinopathy. In addition, the heart was analyzed because of concerns that angiogenic therapy could adversely effect this muscle. The brain was examined because it is known that angiogenic therapy can cause petechia and bleeds in the brain, which is particularly susceptible in the diabetic CNS vasculature.

[0084] There were no pathologic findings in any organs 90 days after 5×10^{11} VPs of ADV-VEGF was administered into the diabetic ulcer. In the liver, there was neither acute hepatitis from the ADV nor proliferation of the sinusoidal lining of the endothelial cells from the VEGF. The kidneys, lungs, retina, or cecum had neither hemorrhage nor any other pathologic findings. Similarly, neither the heart or brain (not shown) had any abnormal pathologic findings. This is significant, because in past experience, angiogenic therapy was reported to have a significant effect on the immune system, however no gross difference in weight, size, or histological examination of the organs were manifested.

[0085] At this point, the experiment was limited, because only 3 time points were assayed and, at most, only 5 mice were tested at any time point. The consistency of the results suggests that they can be duplicated in significantly higher numbers. After 3 months a toxic effect may have been realized, but it was transient.

[0086] It was then determined whether VEGF levels in the serum of diabetic mice were increased after ADV-VEGF local administration. VEGF serum levels were determined by a commercially available ELISA kit in accordance with the manufacturer's protocol. It is emphasized that human VEGF was assayed in the serum. A linear VEGF standard curve was generated for each assay (r=0.99 to 1 between calculated and measured VEGF levels) with the supplied recombinant VEGF protein in the range from 10000 pg/ml to 15.6 pb/ml and serum levels were calculated accordingly. Only 3-5 mice per group were studied, enough to determine that the assay was accurate and consistent. VEGF levels demonstrated a peak of activity in day 10 after the wound was made. However, there were no differences in the control group treated with saline versus the ADV-VEGF group.

[0087] Utilizing the same series of mice, larger quantifies were studied (5×10^{11} vp of ADV-VEGF and 5×10^{11} vp of ADV-DL312 vs. PBS, respectively) and the effects on the organ weights of the lungs, kidneys, and spleen measured. No differences were found for tested time points. However, the sampling size is too small to draw any definitive conclusions. Preliminary data from days 5, 10, 15 and 90 5×10^{11} ADV-VEGF, 5×10^{11} ADV-DL^{312} vs. PBS, respectively) of serum chemistries: ALT, AST, ALP, Bilirubin, Creatinine, Glucose, BUN, Platelets, WBC, and Hemoglobin show no differences detected to date.

[0088] In summary, the preliminary data suggest that 5×10^{11} vp of ADV-VEGF accelerates ulcer healing in C57BL/KsJ-db/db mice. VEGF stimulates angiogenesis and accelerates the healing process as evidenced by:

[0089] 1) a reduced time to closure (a healed wound is defined as 100% epithelialization and no drainage) in experimental diabetic ulcers, and

[0090] 2) accelerated epithelialization and increased cellular density.

[0091] The preliminary data suggest that a high dose of 5×10^{11} ADV-DL312 VPs/wound is effective. However, such a high dose of ADV particles unlikely to be utilized in patients in years to come because of concerns of potential toxicity.

[0092] Modifications and variations of the present invention will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the following claims.

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I claim:

1. A method for tissue engineering a diabetic ulcer comprising administering to the wound an effective amount of VEGF for a sustained period of time of at least two weeks to enhance the rate of closure of the wound.

2. The method of claim 1 wherein the VEGF is administered in the form of a nucleic acid molecule encoding the VEGF.

3. The method of claim 1 wherein the VEGF reverses bacterial contamination of the wound in a patient with a diabetic ulcer.

4. The method of claim 2 wherein the nucleic acid molecule is a viral vector.

5. The method of claim 4 further comprising administering the adenoviral vector at a concentration of at least 1.6×10^{10} adenoviral-VEGF particles.

6. The method of claim 4 further comprising administering the adenoviral vector at a concentration of at least 5×10^{10} adenoviral-VEGF particles.

7. The method of claim 4 further comprising administering the adenoviral vector at a concentration of at least 5×10^{11} adenoviral-VEGF particles.

8. The method of claim 2 wherein the nucleic acid molecule is in the form of genetically engineered cells secreting VEGF.

9. The method of claim 2 wherein the nucleic acid molecule is in the form of naked DNA encoding VEGF.

10. The method of claim 1 wherein the VEGF is administered by a sustained delivery pump.

11. The method of claim 1 wherein the VEGF is administered in a sustained delivery polymeric device.

12. The method of claim 8 wherein the device is formed of a biodegradable polymer in the form of microspheres, slabs, disks, or gels.

13. The method of claim 1 wherein the VEGF is released over a period of between two and six weeks.

14. A composition for tissue engineering a diabetic ulcer comprising an effective amount of VEGF administered for a sustained period of time of at least two weeks to enhance the rate of closure of the wound.

15. The composition of claim 14 wherein the VEGF is in the form of a nucleic acid molecule encoding the VEGF.

16. The composition of claim 14 wherein the VEGF is in the form of a viral vector encoding the VEGF.

17. The composition of claim 15 wherein the nucleic acid molecule is in the form of genetically engineered cells secreting VEGF.

18. The composition of claim 15 wherein the nucleic acid molecule is in the form of naked DNA encoding VEGF.

19. The composition of claim 14 wherein the VEGF is formulated in a sustained delivery pump.

20. The composition of claim 14 wherein the VEGF is formulated in a sustained delivery polymeric device.

21. The composition of claim 20 wherein the device is formed of a biodegradable polymer in the form of microspheres, slabs, disks, or gels.

* * * * *

US006479654B1

(12) **United States Patent**
Baird et al.

(10) **Patent No.: US 6,479,654 B1**
(45) **Date of Patent: Nov. 12, 2002**

(54) **FORMS OF THE ANGIOGENIC FACTOR VASCULAR ENDOTHELIAL CELL GROWTH FACTOR: VEGF**

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(52) **U.S. Cl.** 536/23.5; 536/23.1; 435/320.1

(58) **Field of Search** 424/93.1, 93.2; 435/69.1, 320.1, 172.3; 514/44; 536/23.1, 23.5

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(57) **ABSTRACT**

Novel forms of vascular endothelial growth factor genes and the novel proteins encoded by these genes are disclosed. More particularly, novel forms of human VEGF-A which contain exon 6*b* and do not contain exon 6*a* are disclosed. Other novel forms of human VEGF-A contain exon 6*b* in addition to exon 6*a*. These novel forms of VEGF-A include VEGF-A_{138}, VEGF-A_{162}, and VEGF-A_{182}. Such novel VEGF proteins may be used in treatment of the cardiovascular system and its diseases through effects on anatomy, conduit function, and permeability, and more particularly in the treatment of cardiovascular disease by stimulating vascular cell proliferation using a growth factor, thereby stimulating endothelial cell growth and vascular permeability. The invention also relates to nucleic acids encoding such novel VEGF proteins, cells, tissues and animals containing such nucleic acids; methods of treatment using such nucleic acids; and methods relating to all of the foregoing.

6 Claims, 6 Drawing Sheets

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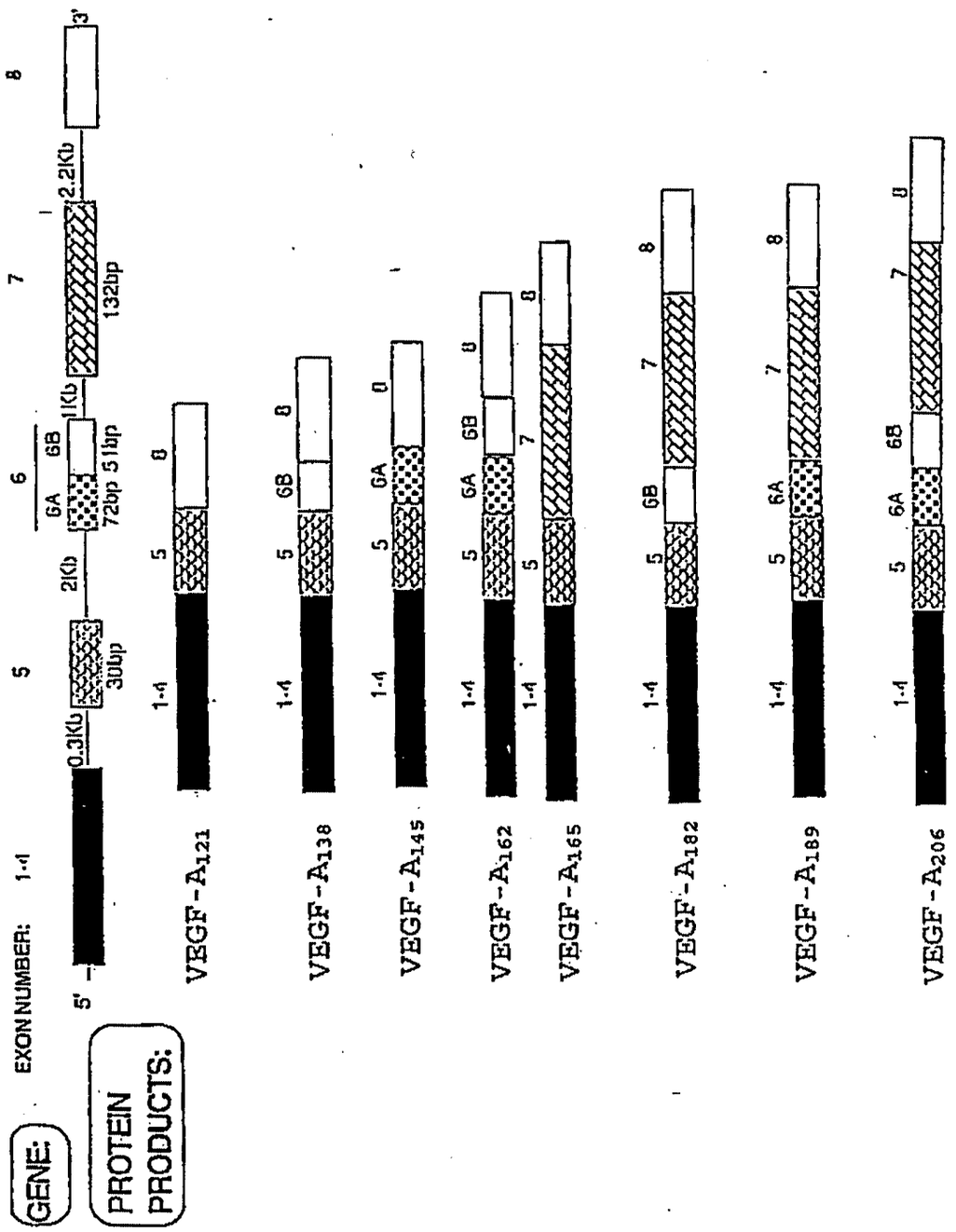
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EXON NUMBER:
1-4
5
6
6A
6B
7
8
5'
3'
0.3Kb
300bp
2Kb
72bp
51bp
1Kb
132bp
2.2Kb
PROTEIN PRODUCTS:
VEGF-A121
VEGF-A138
VEGF-A145
VEGF-A162
VEGF-A165
VEGF-A182
VEGF-A189
VEGF-A206

FIG 1

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- 100 AAGAGTAGCTCGCCGAGGCGCCGAGGAGAGCGGGCCGCCCCACAGCCCGAGCCGGAGAGGGAGCGCGAGCCGCGCCGGCCCCGGTCGGGCCTCCG

      Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys
AAACC ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG gtaag
                                        Exon 1

cggtcgtgccct..........

                                  *
                    Trp Ser Gln Ala Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu V
..tctctttctgtcctcag TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA G gtgagt
                                                Exon 2

                                   al Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro
cccccctggctg........catcgcctctcatgcag TG GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA

Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro
ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC

Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr
CTG ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC
                                                Exon 3

Met Gln                                                 Ile Met Arg Ile Lys Pro His Gln Gly Gln His
ATG CAG gtgggcatctttgggaa...........gcttccttcctttccag ATT ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC

Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys Glu Cys Ar
ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AG gtgaggatgtagtcacg
                Exon 4

                                   g Pro Lys Lys Asp Arg Ala Arg Gln Glu Ly
............ctccctacccattgcag A CCA AAG AAA GAT AGA GCA AGA CAA GAA AA  gtaagtggccct
                                            Exon 5
```

FIG 2A

196

```
                                        s Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser
gactt...........gtttttttattttccag A AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC
                                                                    Exon 6a

Arg Tyr Lys Ser Trp Ser Va  l Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro Hi
CGG TAT AAG TCC TGG AGC GT  G TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG AGC CTC CCT GGC CCC CA....
                                                    Exon 6b

                                        s Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln
............cttttgcctttttgcag T CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG

Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Ar
ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC AG
                                                    Exon 7

                                                g Cys Asp Lys Pro Arg Arg
gttggttcccagaggca............ttttccatttccctcag A TGT GAC AAG CCG AGG CGG TGA
                                                    Exon 8

GCCGGGCAGGAGGAAGGAGCCTCCCTCAGGGTTTCGGGAACCAGATCTCTCACCAGGAAAGACTGATACAGAACGATCGATACAGAAACCACGCTGCCG
```

FIG. 2B

197

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys
ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG
Exon 1

Trp Ser Gln Ala Ala Pro Met Ala
TGG TCC CAG GCT GCA CCC ATG GCA

Glu Gly Gly Gly Gln Asn His His Glu V
GAA GGA GGA GGG CAG AAT CAT CAC GAA G
Exon 2

al Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu
TG GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG
Exon 3

Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg Cys Gly
GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA TGC GGG

Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys
GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA

Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys Glu Cys Ar g Pro Lys Lys Asp Arg
CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AG A CCA AAG AAA GAT AGA
Exon 4 Exon 5

Ala Arg Gln Glu Ly s Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro Gl
GCA AGA CAA GAA AA G TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG AGC CTC CCT GGC CCC CA
Exon 6b

n Cys Asp Lys Pro Arg Arg
A TGT GAC AAG CCG AGG CGG TGA
Exon 8

FIG 3

198

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys
ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG
Exon 1

*

Trp Ser Gln Ala Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu V
TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA G
Exon 2

al Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu
TG GTG AAG TTC ATG GAT GTC TAT CAG CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG

Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly
GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCC CTG ATG CGA TGC GGG GGC

Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His Gln
TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC CAA
Exon 3

Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys Glu Cys Ar g Pro Lys Lys Asp Arg Ala Arg Gln Glu Ly
GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT GAA TGC AG A CCA AAG AAA GAT AGA GCA AGA CAA GAA AA
Exon 4 Exon 5

s Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro Hi s Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys
G TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG AGC CTC CCT GGC CCC CA T CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG
Exon 6b

His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu
CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA

Asn Glu Arg Thr Cys Ar g Cys Asp Lys Pro Arg Arg
AAC GAA CGT ACT TGC AG A TGT GAC AAG CCG AGG CGG TGA
Exon 7 Exon 8

FIG 4

192

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys
ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTT GCC TTG CTG CTC TAC CTC CAC CAT GCC AAG
Exon 1

*
Trp Ser Gln Ala Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His His Glu V al Val Lys Phe Met Asp Val Tyr Gln Arg
TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA GGA GGG CAG AAT CAT CAC GAA G TG GTG AAG TTC ATG GAT GTC TAT CAG CGC
Exon 2

Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser
AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC

Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr
TGT GTG CCC CTG ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC ACT GAG GAG TCC AAC ATC ACC
Exon 3

Met Gln Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys
ATG CAG ATT ATG CGG ATC AAA CCT CAC CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Exon 4

Glu Cys Ar g Pro Lys Lys Asp Arg Ala Arg Gln Glu Ly
GAA TGC AG A CCA AAG AAA GAT AGA GCA AGA CAA GAA AA
Exon 5

s Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr Lys Ser Trp Ser Va
A AAA TCA GTT CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT AAG TCC TGG AGC GT
Exon 6a

l Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro Gl n Cys Asp Lys Pro Arg Arg
G TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG AGC CTC CCT GGC CCC CA A TGT GAC AAG CCG AGG CGG TGA
Exon 6b Exon 8

GCCGGGCAGGAGGAAGGAGCCTCCCTCAGGGTTTCGGGAACCAGATCTCTCACCAGGAAAGACTGATACAGAACGATCGATACAGAAACCACGCTGCCG

FIG 5

200

FORMS OF THE ANGIOGENIC FACTOR VASCULAR ENDOTHELIAL CELL GROWTH FACTOR: VEGF

This application claims priority to Provisional Application Baird, et al., U.S. Ser. No. 60/073,979, filed Feb. 6, 1998, entitled NOVEL FORMS OF THE ANGIOGENIC FACTOR VASCULAR ENDOTHELIAL CELL GROWTH FACTOR: VEGF.

FIELD OF THE INVENTION

The present invention relates to novel forms of vascular endothelial growth factor genes and the novel proteins encoded by these genes. More particularly, the invention relates to novel forms of human VEGF-A. These novel forms of VEGF-A include VEGF-A_{138}, VEGF-A_{162}, and VEGF-A_{182}. Such novel VEGF proteins may be used in the treatment of the cardiovascular system and its diseases through effects on anatomy, conduit function, and permeability, and more particularly in the treatment of cardiovascular disease by stimulating vascular cell proliferation using a growth factor, thereby stimulating endothelial cell growth and vascular permeability.

The invention also relates to nucleic acids encoding such novel VEGF proteins, cells, tissues and animals containing such nucleic acids; methods of treatment using such nucleic acids; and methods relating to all of the foregoing.

BACKGROUND OF THE INVENTION

Cardiovascular-diseases are generally characterized by an impaired supply of blood to the heart or other target organs. Myocardial infarction (MI), commonly referred to as heart attacks, is a leading cause of mortality as 30% are fatal in the first months following the heart attack. Heart attacks result from narrowed or blocked coronary arteries in the heart which starves the heart of needed nutrients and oxygen. When the supply of blood to the heart is compromised, cells respond by generating compounds that induce the growth of new blood vessels so as to increase the supply of blood to the heart. These new blood vessels are called collateral blood vessels. The process by which new blood vessels are induced to grow out of the existing vasculature is termed angiogenesis, and the substances that are produced by cells to induce angiogenesis are the angiogenic factors.

Unfortunately, the body's natural angiogenic response is limited and often inadequate. For this reason, the discovery of angiogenic growth factors has lead to the emergence of an alternative therapeutic strategy which seeks to supplement the natural angiogenic response by supplying exogenous angiogenic substances.

Attempts have been made to stimulate angiogenesis by administering various growth factors. U.S. Pat. No. 5,318,957 to Cid et al. discloses a method of stimulating angiogenesis by administering haptoglobins (glyco-protein with two polypeptide chains linked by disulfide bonds). Intracoronary injection of a recombinant vector expressing human fibroblast growth factor-5 (FGF-5) (i.e., in vivo gene transfer) in an animal model resulted in successful amelioration of abnormalities in myocardial blood flow and function. (Giordano, F. J., et. al. *Nature Med* 2, 534–539, 1996). Recombinant adenoviruses have also been used to express angiogenic growth factors in-vivo. These included acidic fibroblast growth factor (Muhlhauser, J., et. al. *Hum. Gene Ther.* 6:1457–1465, 1995), and one of the VEGF forms, VEGF-A_{165} (Muhlhauser, J., et. al. *Circ. Res.* 77:1077–1086, 1995).

One of the responses of heart muscle cells to impaired blood supply involves activation of the gene encoding Vascular Endothelial Growth Factor ("VEGF"), also known as VEGF-A, (Banai, S., et. al. *Cardiovasc. Res.* 28:1176–1179, 1994). VEGF-A is actually a family of angiogenic factors that induce the growth of new collateral blood vessels. These growth factors are specific angiogenic growth factors that have vaso-permeability activity and target endothelial (blood vessel-lining) cells almost exclusively. (Reviewed in Ferrara et al., *Endocr. Rev.* 13:18–32 (1992); Dvorak et al., *Am. J. Pathol.* 146:1029–39 (1995); Thomas, *J. Biol. Chem.* 271:603–06 (1996)). Expression of the VEGF-A gene is linked in space and time to events of physiological angiogenesis, and deletion of the VEGF-A gene by way of targeted gene disruption in mice leads to embryonic death because the blood vessels do not develop. It is therefore the only known angiogenic growth factor that appears to function as a specific physiological regulator of angiogenesis.

When tested in cell culture, VEGF-A, and, because of its structure, likely VEGF-B, (VEGF's) are potently mitogenic (Gospodarowicz et al., *Proc. Natl. Acad. Sci. USA* 86:7311–15, 1989) and chemotactic (Favard et al., *Biol. Cell* 73:1–6, 1991). Additionally, VEGFs induce plasminogen activator, plasminogen activator inhibitor, and plasminogen activator receptor (Mandriota et al., *J. Biol. Chem.* 270:9709–16, 1995; Pepper et al., 181: 902–06, 1991), as well as collagenases (Unemori et al., *J. Cell. Physiol.* 153:557–62, 1992), enzyme systems that regulate invasion of growing capillaries into tissues. VEGFs also stimulate the formation of tube-like structures by endothelial cells, an in vitro example of angiogenesis (Nicosia et al., *Am. J. Pathol.*, 145:1023–29, 1994).

In vivo, VEGFs induce angiogenesis (Leung et al., *Science* 246:1306–09, 1989) and increase vascular permeability (Senger et al., *Science* 219:983–85, 1983). VEGFs are now known as important physiological regulators of capillary blood vessel formation. They are involved in the normal formation of new capillaries during organ growth, including fetal growth (Peters et al., *Proc. Natl. Acad. Sci. USA* 90:8915–19, 1991), tissue repair (Brown et al., *J. Exp. Med.* 176:1375–79, 1992), the menstrual cycle, and pregnancy (Jackson et al., *Placenta* 15:341–53, 1994; Cullinan & Koos, *Endocrinology* 133:829–37, 1993; Kamat et al., *Am. J. Pathol.* 146:157–65, 1995). During fetal development, VEGFs appear to play an essential role in the de novo formation of blood vessels from blood islands (Risau & Flamme, *Ann. Rev. Cell. Dev. Biol.* 11:73–92, 1995), as evidenced by abnormal blood vessel development and lethality in embryos lacking a single VEGF allele (Carmeliet et al., *Nature* 380:435–38, 1996). Moreover, VEGFs are implicated in the pathological blood vessel growth characteristic of many diseases, including solid tumors (Potgens et al., *Biol. Chem. Hoppe-Seyler* 376:57–70, 1995), retinopathies (Miller et al., *Am. J. Pathol.* 145:574–84, 1994; Aiello et al., *N. Engl. J. Med.* 331:1480–87, 1994; Adamis et al., *Am. J. Ophthalmol.* 118:445–50, 1994), psoriasis (Detmar et al., *J. Exp. Med.* 180:1141–46, 1994), and rheumatoid arthritis (Fava et al., *J. Exp. Med.* 180:141–46, 1994).

VEGF expression is regulated by hormones (Schweiki et al., *J. Clin. Invest.* 91:2235–43, 1993) growth factors (Thomas, *J. Biol. Chem.* 271:603–06, 1996), and by hypoxia (Schweiki et al., *Nature* 359:843–45, 1992, Levy et al., *J. Biol. Chem.* 271:2746–53, 1996). Upregulation of VEGFs by hypoxic conditions is of particular importance as a compensatory mechanism by which tissues increase oxygenation through induction of additional capillary vessel

formation and resulting increased blood flow. This mechanism is thought to contribute to pathological angiogenesis in tumors and in retinopathies. However, upregulation of VEGF expression after hypoxia is also essential in tissue repair, e.g., in dermal wound healing (Frank et al., *J. Biol. Chem.* 270:12607–613, 1995), and in coronary ischemia (Banai et al., *Cardiovasc. Res.* 28:1176–79, 1994; Hashimoto et al., *Am. J. Physiol.* 267:H1948-H1954, 1994).

Using the rabbit chronic limb ischemia model, it has been shown that repeated intramuscular injection or a single intra-arterial bolus of VEGF-A can augment collateral blood vessel formation as evidenced by blood flow measurement in the ischemic hindlimb (Pu, et al., *Circulation* 88:208–15, 1993; Bauters et al., *Am. J. Physiol.* 267:HI263-71, 1994; Takeshita et al., *Circulation* 90 [part 2], II-228–34, 1994; Bauters et al., *J. Vasc. Surg.* 21:314–25, 1995; Bauters et al., *Circulation* 91:2802–09, 1995; Takeshita et al., *J. Clin. Invest.* 93:662–70, 1994). In this model, VEGF has also been shown to act synergistically with basic FGF to ameliorate ischemia (Asahara et al., *Circulation* 92:[suppl 2], II-365–71, 1995). VEGF was also reported to accelerate the repair of balloon-injured rat carotid artery endothelium while at the same time inhibiting pathological thickening of the underlying smooth muscle layers, thereby maintaining lumen diameter and blood flow (Asahara et al., *Circulation* 91:2793–2801, 1995). VEGF has also been shown to induce EDRF (Endothelin-Derived Relaxin Factor (nitric oxide))-dependent relaxation in canine coronary arteries, thus potentially contributing to increased blood flow to ischemic areas via a secondary mechanism not related to angiogenesis (Ku et al., *Am. J. Physiol* 265:H586-H592, 1993).

Activation of the gene encoding VEGF-A results in the production of several different VEGF-A variants, or isoforms, produced by alternative splicing wherein the same chromosomal DNA yields different mRNA transcripts containing different exons thereby producing different proteins. Such variants have been disclosed, for example, in U.S. Pat. No. 5,194,596 to Tischer et al. which identifies human vascular endothelial cell growth factors having peptide sequence lengths of 121, and 165 amino acids (i.e., VEGF-A_{121} and VEGF-A_{165}). Additionally, VEGF-A_{189} and VEGF-A_{206} have also been characterized and reported (Neufeld, G., et. al. *Cancer Metastasis Rev.* 15:153–158, 1996).

The mitogenic activity of the various VEGF-A isoforms varies depending on each isoform. For example, VEGF-A_{121} and VEGF-A_{165} have very similar mitogenic activity for endothelial cells. However, VEGF-A_{189} and VEGF-A_{206} are only weakly mitogenic (Ferrara et al., *Endocr. Rev.* 13:18–32, 1992). The reduced activity of these isoforms is attributed to their strong association with cells and matrix, as evidenced by the normal mitogenic activity of a mutant of VEGF-A_{206} which lacks the 24-residue "matrix targeting" sequence common to VEGF-A_{189} and VEGF-A_{206} (residues 115–139 in FIG. 2) (Ferrara et al., *Endocr. Rev.* 13:18–32, 1992).

Four known forms of VEGF-A arise from alternative splicing of up to eight exons of the VEGF-A gene (VEGF-A_{121}, exons 1–5, 8; VEGF-A_{165}, exons 1–5,7,8; VEGF-A_{189}, exons 1–5, 6a, 7, 8; VEGF-A_{206}, exons 1–5, 6a, 6b, 7, 8 (exon 6a and 6b refer to 2 alternatively spliced forms of the same exon)) (Houck et al., *Mol. Endocr.*, 5:1806–14 (1991)). All VEGF-A genes encode signal peptides that direct the protein into the secretary pathway. For example, VEGF-A_{165} cDNA encodes a 191-residue amino acid sequence consisting, of a 26-residue secretary signal peptide sequence, which is cleaved upon secretion of the protein from cells, and the 165-residue mature protein subunit. However, only VEGF-A_{121} and VEGF-A_{165} are found to be readily secreted by cultured cells whereas VEGF-A_{189} and VEGF-A_{206} remain associated with the producing cells. These VEGF-A forms possess an additional highly basic sequence encoded by exon 6 corresponding to residues 115–139 in VEGF-A_{189} and residues 115–156 in VEGF-A_{206}. These additions confer a high affinity to heparin and an ability to associate with the extracellular matrix (matrix-targeting sequence) (Houck, K. A. et al., *J. Biol. Chem.* 267:26031–37 (1992) and Thomas, *J. Biol. Chem,* 271:603–06 (1996)). The mitogenic activities of VEGF-A_{121} and VEGF-A_{165} are similar according to the results of several groups (Neufeld, G., et al., *Cancer Metastasis Rev.* 15:153–158 (1996) although one research group has shown evidence indicating that VEGF-A_{121} is significantly less active (Keyt, B. A., et al. *J. Biol. Chem.* 271:7798–7795 (1996). It is unclear whether the two longer VEGF-A forms, VEGF-A_{189} and VEGF-A_{206}, are as active or less active than the two shorter forms since it has not been possible to obtain them in pure form suitable for quantitative measurements. This failure is due in part to their strong association with producing cells and extracellular matrices which is impaired by the presence of exon-6 derived sequences apparently acting in synergism with exon-7 derived sequences groups (Neufeld, G., et al., *Cancer Metastasis Rev.* 15:153–158 (1996).

As depicted in FIG. 1, the domain encoded by exons 1–5 contains information required for the recognition of the VEGF receptors flt-1 (R_1 and R_2) and KDR/flk-1 (Keyt, B. A., et. al. *J. Biol Chem* 271:5638–5646, 1996), and is present in all known VEGF isoforms. The amino-acids encoded by exon 8 are also present in all known isoforms. The isoforms may be distinguished however by the presence or absence of the peptides encoded by exons 6 and 7 of the VEGF-A gene, and the presence or absence of the peptides encoded by these exons results in structural differences which are translated into functional differences between the VEGF-A forms (reviewed in: Neufeld, G., et. al. *Cancer Metastasis Rev.* 15, 153–158. 1996).

Exon 6 can terminate after 72 bp at a donor splice site wherein it contributes 24 amino acids to VEGF forms that contain it such as VEGF-A_{189}. This exon 6 form is referred to herein as exon 6a. However, the VEGF-A RNA can be spliced at the 3' end of exon 6 using an alternative splice site located 51 bp downstream to the first resulting in a larger exon 6 product containing 41 amino-acids. The additional 17 amino-acids added to the exon 6 product as a result of this alternative splicing are referred to herein as exon 6b. VEGF-A_{206} contains the elongated exon 6 composed of 6a and 6b, but this VEGF form is much rarer than VEGF-A_{189}. (Tischer, E., et al., *J. Biol. Chem.* 266, 11947–11954; Houck, K. A., et al., *Mol. Endocrinol.*, 1806–1814, 1991).

A putative fifth form of VEGF-A, VEGF-A_{145}, has been noted in the human endometrium, using PCR. The authors state that the sequence of the cDNA of the VEGF-A_{145} splice variant indicated that it contained exons 1–5, 6 and 8. However, it is uncertain whether the authors found that the splice variant contained exons 6a and 6b as in VEGF-A_{206}, exon 6a as in VEGF-A_{189}, or exon 6b. The authors state that since the splice variant retains exon 6 it is probable that it will be retained by the cell as are the other members of the family that contain this exon. (Charnock-Jones et al., *Biology of Reproduction* 48, 1120–1128 (1993); see also, Bacic M., et al. *Growth Factors* 12, 11–15, 1995). The biologic activity of this form was not established in that report (Cheung, C. Y., et al., *Am. J. Obstet. Gynecol.*, 173,

751–759, 1995); Anthony, F. W. et al., *Placenta*, 15, 557–561, 1994). The various isoforms, and the exons that encode the isoforms, are depicted in FIG. 1.

More recently, a VEGF-A protein of 145 amino acids, and nucleic acid encoding this protein, have been identified, and its use for treating the cardiovascular system and its diseases has been identified (U.S. Ser. No. 08/784,551 now U.S. Pat. No. 6,013,780, filed Jan. 21, 1997, published as WO 98/10071 on Mar. 12, 1998).

VEGF-A is known to bind to two of three different endothelial cell receptors, each of which is a single transmembrane protein with a large extracellular portion comprised of 7 immunoglobulin-type domains and a cytoplasmic portion that functions as a tyrosine kinase. These receptors are R_1 (flt-1) (De Vries et al., *Science* 255:989–91, 1992), R_2 (KDR/flk-1) (Terman et al., *Biochem. Biophys. Res. Commun.* 187:1579–86, 1992), and R_3 (flk-4) (Pajusola et al., *Cancer Res.* 52:5738–43, 1992). There are distinct selectivities between these receptors and the various VEGF-A and VEGF-related protein ligands that have not been completely elucidated as yet. However, it is known that VEGF-A binds to R_1 and R2 (Terman et al., *Growth Factors* 11:187–95, 1994) but not R_3 (Joukov et al., *EMBO J.* 15:290–98, 1996). R_2 is thought to be primarily responsible for the angiogenic response of endothelial cells to VEGF-like growth factors (Gitay-Goren et al., *J. Biol. Chem.* 271:5519–23 (1996)).

Accordingly, there is a need for new forms of VEGF-A proteins that have modified affinities for matrix and low affinity receptor. This modified affinity will alter its bioavailability when administered.

SUMMARY OF THE INVENTION

The present invention is directed to novel forms of VEGF proteins, preferably human VEGF-A proteins. The preferred use of the VEGF proteins and nucleic acid molecule compositions of the invention is to use such compositions to treat the cardiovascular system and its diseases through effects on anatomy, conduit function, and permeability. Such proteins and compositions may be used to aid in the treatment of patients with heart disease, wounds, or other ischemic conditions by stimulating angiogenesis in such patients. These novel forms of VEGF proteins will have a modified affinity for matrix and low affinity receptors. This modification alters the bioavailability of the proteins when administered directly to cells or when cells are transduced with DNA encoding these proteins.

These novel VEGF proteins will possess a unique combination of biological properties that will distinguish them from other VEGF forms. The unique combination of properties of these VEGF proteins will render them preferred therapeutic agents in certain circumstances for the treatment of the cardiovascular system and its diseases as well as other diseases characterized by vascular cell proliferation. In particular, the cDNA coding for these VEGF proteins may be employed in gene therapy for treating the cardiovascular system and its diseases.

The novel VEGF-A proteins comprise amino acid sequences coded for, for example, by VEGF-A exons 1–5, 6b, and 8, or 1–5, 6b, 7, and 8 or a derivative thereof. These proteins preferably do not comprise the full amino acid sequence of exon 6a and, preferably, do not have the same properties, activity, and function of the corresponding VEGF-A proteins that comprise the full amino acid sequence of exon 6a. Other novel VEGF-A proteins comprise amino acid sequences coded for, for example, by VEGF-A exons 1–5, 6a, 6b, and 8. These proteins preferably do not have the same properties, activity, and function of the corresponding VEGF-A proteins that do not comprise exon 6b.

Thus, in preferred embodiments of the invention, a purified polypeptide is provided, comprising an amino acid sequence coded for by VEGF-A exons 1–5, 6b, and 8, or a derivative thereof. In other preferred embodiments of the invention, a purified polypeptide is provided comprising an amino acid sequence coded for by VEGF-A exons 1–5, 6b, 7, and 8, or a derivative thereof.

In other preferred embodiments of the invention, a purified polypeptide is provided comprising an amino acid sequence coded for by VEGF-A exons 1–5, 6a, 6b, and 8, or a derivative thereof. Preferably, the VEGF-A polypeptide of the invention is human VEGF-A. In preferred aspects, the purified polypeptide comprises the amino acid sequence of FIG. 3, FIG. 4, or of FIG. 5.

Also provided in the invention are purified and isolated nucleic acid molecules coding for the purified polypeptides of the invention. Preferably, the nucleic acid molecules have the nucleotide sequence of FIG. 3 or FIG. 4, or of FIG. 5.

Also provided in the present invention are purified and isolated nucleic acid molecules coding for a biologically active fragment of a 6b-modified VEGF protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, or a derivative thereof. Such fragments maintain most preferably up to 100% of the activity of the full length 6b-modified and have at least 10%, more preferably at least 40%, more preferably at least 80% of the activity of the full length VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182} polypeptides.

Also provided are expression vectors comprising the nucleic acid molecules of the invention. Preferably, these vectors comprise adenovirus sequences; preferably the expression vector is an adenovirus vector. More preferably, the nucleic acid is operably linked to a promoter sequence that is active in vascular endothelial cells. The expression vector preferably further comprises a partial adenoviral sequence from which the E1A/E1B genes have been deleted.

In another embodiment of the invention, a kit is provided for intracoronary injection of a recombinant vector expressing a 6b-modified protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, comprising: a nucleic acid molecule encoding a 6b-modified protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, cloned into a vector suitable for expression of said polynucleotide in vivo, a suitable container for said vector, and instructions for injecting said vector into a patient. Preferably, in the kit, the polynucleotide is cloned into an adenovirus expression vector.

In yet another aspect of the invention, a method is provided for treating vascular disease in a mammal comprising the step of administering to said mammal a 6b-modified VEGF-A protein, preferably, VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, or other 6b-modified VEGF-A protein in a therapeutically effective amount to stimulate vascular cell proliferation.

In another aspect of the invention, a method is provided for enhancing endothelialization of diseased vessels comprising the step of administering to a mammal a therapeutically effective amount of a 6b-modified protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}. Preferably, the endothelialization is reendothelialization after angioplasty. More preferably, the reendothelialization reduces or prevents restenosis.

In further aspects of these methods of the invention, the patient is treated with or without a stent. Preferably, the

mammals used in the methods of the invention are human, however, it is contemplated that all mammals would be candidates for these methods. In the methods of the invention, the administration may comprise gene therapy. In preferred methods, the gene for gene therapy is administered using an inflatable balloon catheter coated with a polynucleotide encoding 6b-modified VEGF protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}.

In yet other preferred embodiments of the present invention, the methods, compositions, and vectors of the invention may be used to enhance drug permeation by tumors comprising administering to a patient a 6 b-modified VEGF-A protein or a nucleic acid molecule coding for a 6b-modified VEGF-A protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}. The 6b-modified VEGF-A protein may be delivered directly to a tumor cell, or it may be delivered into the vascular system, preferably at a site located close to the site of the tumor. Thus, delivery of the 6b-modified VEGF-A protein in conjunction with chemotherapy to remove or reduce the size of a tumor, will help to enhance the effectiveness of the chemotherapy by increasing drug uptake by the tumor. The 6b-modified VEGF-A protein delivered in this method may either be through direct delivery of the polypeptide or protein, or through gene therapy.

Thus, provided in the present invention is a method of enhancing drug permeation by tumors comprising administering to a patient a nucleic acid molecule coding for a 6b-modified VEGF-A protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF$_{182}$. Preferably, the 6b-modified VEGF-A protein is delivered directly into a tumor cell.

In another aspect of the invention, a therapeutic composition is provided comprising a pharmaceutically acceptable carrier and a 6b-modified VEGF protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF$_{182}$, in a therapeutically effective amount to stimulate vascular cell proliferation. Also provided as an aspect of the invention is a filtered injectable adenovirus vector preparation, comprising: a recombinant adenoviral vector, said vector containing no wild-type virus and comprising: a partial adenoviral sequence from which the E1A/E1B genes have been deleted, and a transgene coding for a 6b-modified VEGF protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, driven by a promoter flanked by the partial adenoviral sequence; and a pharmaceutically acceptable carrier.

In further embodiments of the invention are provided methods for treating cardiovascular disease in a mammal comprising the step of transfecting cells of said mammal with a nucleic acid molecule which encodes an amino acid sequence coded for by VEGF-A exons 1–5, 6b and 8. Also provided are methods for treating cardiovascular disease in a mammal comprising the step of transfecting cells of said mammal with a nucleic acid molecule which encodes an amino acid sequence coded for by VEGF-A exons 1–5, 6b, 7, and 8. Also provided are methods for treating cardiovascular disease in a mammal comprising the step of transfecting cells of said mammal with a nucleic acid molecule which encodes an amino acid sequence coded for by VEGF-A exons 1–5, 6a, 6b, and 8.

Preferably, the nucleic acid molecule employed in the methods of the invention codes for VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}. In preferred embodiments, the nucleic acid molecule is cloned into a vector. Preferably, the vector comprises adenovirus particles. Preferably, the adenovirus vector particles are delivered to said mammal by injection. Preferably, the number of said adenovirus particles is between about 10^8 to about 10^{14}, more preferably between about 10^{10} to about 10^{14}.

In further preferred aspects, the polynucleotide which encodes a 6b-modified VEGF protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, is delivered to the heart of a mammal. Intracoronary delivery of the polynucleotide is preferred, preferably according to the methods set forth in PCT/US96/02631, published Sep. 6, 1996 as WO96/26742, hereby incorporated by reference herein. Preferably, an intracoronary injection is conducted about 1 cm into the lumens of the left and right coronary arteries.

Thus, in preferred embodiments, the transfected cells are muscle cells, including but not limited to myoblasts, myocytes, cardiomyocytes, cardioblasts, and smooth muscle cells. Preferably, the cells are cardiomyocytes. More preferably, the transfected cells are coronary artery cells and said injection is intracoronary injection. More preferably, the adenovirus particles are injected at about 1 cm into the lumens of the left and right coronary arteries.

Preferably, these methods further comprise administering a potentiating agent that potentiates the angiogenic effect of the 6b-modified VEGF-A polypeptide. Preferably, the potentiating agent is an angiogenic FGF. More preferably, the potentiating agent is selected from the group consisting of FGF-1, FGF-2, FGF-4, FGF-5, and FGF-6.

In one preferred aspect of the methods of the invention, the cells are transfected in vivo. In other preferred aspects, the said cells are transfected ex vivo.

Endothelial cell proliferation, such as that which occurs in angiogenesis, is also useful in preventing restenosis following balloon angioplasty. The balloon angioplasty procedure often injuries the endothelial cells lining the inner walls of blood vessels. Smooth muscle cells often infiltrate into the opened blood vessels causing a secondary obstruction in a process known as restenosis. The proliferation of the endothelial cells located at the periphery of the balloon-induced damaged area in order to cover the luminal surface of the vessel with a new monolayer of endothelial cells would potentially restore the original structure of the blood vessel.

Thus, the present invention provides a method of treating cardiovascular disease in a mammal comprising the step of transfecting cells of said mammal with a polynucleotide which encodes a 6b-modified VEGF-A protein, preferably VEGF$_{138}$, VEGF-A_{162}, or VEGF-A_{182}. In preferred aspects, the polynucleotide is cloned into a vector. In further preferred aspects, the vector is an adenovirus vector. The adenovirus vector is preferably delivered to the mammal by injection; preferably, about 10^8 to about 10^{14} adenovirus vector particles are delivered in the injection. More preferably, about 10^{11} to about 10^{13} adenovirus vector particles are delivered in the injection. Most preferably, about 10^{12} adenovirus vector particles are delivered in the injection.

Thus in further preferred embodiments, the nucleic acid molecule is introduced into the coronary artery by a catheter inserted into said artery. Preferably, the catheter comprises an inflatable balloon having an outer surface adapted to engage the inner wall of said artery, and wherein said nucleic acid molecule is disposed upon said balloon outer surface.

In preferred aspects of the methods of the invention, the nucleic acid molecule comprises the nucleotide sequence of FIG. 3, FIG. 4, or FIG. 5.

In other aspects of the invention, transformed or transfected host cells are provided which comprise the expression vectors of the invention. Such host cells may be used in, for example, methods of producing a VEGF-A polypeptide

comprising growing, under suitable conditions, a host cell transformed or transfected with the recombinant DNA expression vector of the invention in a manner allowing expression of said polypeptide, and isolating said polypeptide from the host cell.

Also provided in the present invention is a method of treating a patient suffering from an ischemic condition comprising administering a therapeutic amount of a pharmaceutical composition comprising a 6b-modified VEGF protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182}, in a suitable carrier. Preferably, this method further comprises administering an agent that potentiates the therapeutic effect of said 6b-modified VEGF-A polypeptide. Preferably, the potentiating agent is selected from the group consisting of FGF-1, FGF-2, FGF-4, FGF-5, and FGF-6. Further, said ischemic condition is preferably selected from the group consisting of: cardiac infarction, chronic coronary ischemia, chronic lower limb ischemia, stroke, and peripheral vascular disease.

For the treatment of peripheral conditions, such as peripheral vascular disease, administration of the pharmaceutical compositions of the present invention is preferably by delivery of a modified VEGF-A polypeptide or polynucleotide (or a vector comprising such a polynucleotide) to a peripheral tissue in vivo. Preferably, this is achieved by direct injection into the peripheral tissue, or by introduction into a blood vessel that supplies the peripheral tissue.

Also provided in the present invention are methods of increasing vascular permeability comprising administering a therapeutic amount of a pharmaceutical composition comprising a 6b-modified VEGF-A protein, preferably VEGF-A_{138}, VEGF-A_{162}, or VEGF-A_{182} in a suitable carrier.

Also provided in the present invention are methods of treating a patient suffering from a wound comprising administering a therapeutic amount of a pharmaceutical composition comprising a 6b-modified VEGF protein preferably VEGF-A_{138} VEGF-A_{162}, or VEGF-A_{182} in a suitable carrier.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the invention, the figures, and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic depiction of the exons that encode various VEGF isoforms.

FIG. 2 depicts the nucleotide [SEQ ID NOS. 9, 11, 13, 15, 17, 19, 21 and 25] and amino acid sequences [SEQ ID NOS. 8, 10, 12, 14, 16, 18, 20 and 22] of VEGF-$_{206}$, with the exons indicated by underline. Nucleotides present in mature VEGF-A_{206} transcripts are shown in uppercase letters. Nucleotides present in the 5' flanking regions and introns are in lower case letters. The alanine residue corresponding to the amino terminus of the mature protein, after the amino terminal secretion signal sequence is cleaved off, is indicated with an asterisk.

FIG. 3 depicts the nucleotide [SEQ ID NO. 25] and amino acid sequences [SEQ ID NO. 24] of the VEGF-A_{138} protein coding region. The alanine residue corresponding to the amino terminus of the mature protein, after the amino terminal secretion signal sequence is cleaved off, is indicated with an asterisk.

FIG. 4 depicts the nucleotide [SEQ ID NO. 27] and amino acid sequences [SEQ ID NO. 26] of the VEGF-A_{182} protein coding region. The alanine residue corresponding to the amino terminus of the mature protein, after the amino terminal secretion signal sequence is cleaved off, is indicated with an asterisk.

FIG. 5 depicts the nucleotide [SEQ ID NO. 29] and amino acid sequences [SEQ ID NO. 28] of the VEGF-A_{162} protein coding region. The alanine residue corresponding to the amino terminus of the mature protein, after the amino terminal secretion signal sequence is cleaved off, is indicated with an asterisk.

DETAILED DESCRIPTION OF THE INVENTION

BCE	Bovine corneal endothelial cells
BFGF	Basic fibroblast growth factor
ECM	Extracellular matrix
HUVEC	Human umbilical vein derived endothelial cells
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VEGF-A_{xxx}	Vascular endothelial growth factor-A form containing a designated number (xxx) of amino-acids.
6b-modified VEGF-A	Vascular endothelial growth factor protein comprising exon 6b, not including VEGF-A_{206}.

The present invention relates to novel VEGF-A protein products, and nucleic acids encoding the novel protein products comprising, for example, exons 1–5, 6b and 8, 1–5, 6b, 7 and 8, or 1–5, 6a, 6b, and 8, of VEGF-A proteins, and their uses thereof in treating cardiovascular disease. As used herein "cardiovascular disease" means disease which results from a cardiovascular insufficiency, including, but not limited to, coronary artery disease, congestive heart failure, and peripheral vascular disease. The methods of the present invention relate to the treatment of mammalian patients, preferably humans.

"VEGF-A_{138}" refers to a form of VEGF-A containing about 138 amino-acids and containing the peptides encoded by exons 1–5, 6b and 8 of the VEGF-A gene. The term "VEGF-A_{138}" also refers to derivatives and functional equivalents of the native VEGF-A_{138} nucleic acid or amino acid sequence. Mature VEGF-A_{138} monomers comprise the amino acid sequence shown in FIG. 3. However, as used herein, the term VEGF-A_{138} refers to both the mature form and the pro-form of VEGF-A_{138}, including a signal sequence, or derivatives or functional equivalents thereof.

"VEGF-A_{182}" refers to a form of VEGF-A containing about 182 amino-acids and containing the peptides encoded by exons 1–5, 6b, 7 and 8 of the VEGF-A gene. The term "VEGF-A_{182}" also refers to derivatives and functional equivalents of the native VEGF-A_{182} nucleic acid or amino acid sequence. Mature VEGF-A_{182} monomers comprise the amino acid sequence shown in FIG. 4. However, as used herein, the term VEGF-A_{182} refers to both the mature form and the pro-form of VEGF-A_{182}, including a signal sequence, or derivatives or functional equivalents thereof.

"VEGF-A_{162}" refers to a form of VEGF-A containing about 162 amino-acids and containing the peptides encoded by exons 1–5, 6a, 6b and 8 of the VEGF-A gene. The term "VEGF-A_{162}" also refers to derivatives and functional equivalents of the native VEGF-A_{162} nucleic acid or amino acid sequence. Mature VEGF-A_{162} monomers comprise the amino acid sequence shown in FIG. 5. However, as used herein, the term VEGF-A_{162} refers to both the mature form

and the pro-form of VEGF-A_{162}, including a signal sequence, or derivatives or functional equivalents thereof.

"Derivatives" of a VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182} or other 6b-modified VEGF protein are functional equivalents having similar amino acid sequences and retaining, to some extent, the activities of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein. By "functional equivalent" is meant the derivative has an activity that can be substituted for the activity of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein. Preferred functional equivalents retain the full level of activity of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182} or other 6b-modified VEGF protein as measured by assays known to these skilled in the art, and/or in the assays described herein. Preferred functional equivalents have activities that are within 1% to 10,000% of the activity of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182} or other 6b-modified VEGF protein, more preferably between 10% to 1000%, and more preferably within 50% to 200%. Derivatives have at least 50% sequence similarity, preferably 70%, more preferably 90%, and even more preferably 95% sequence similarity to VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182} or similarly 6b-modified VEGF protein. "Sequence similarity" refers to "homology" observed between amino acid sequences in two different polypeptides, irrespective of polypeptide origin.

The ability of the derivative to retain some activity can be measured using techniques described herein and/or using techniques known to those skilled in the art for measuring the activity of other VEGF-A isoforms. Derivatives include modification occurring during or after translation, for example, by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand (see Ferguson et al., 1988, *Annu. Rev. Biochem.* 57:285–320).

Specific types of derivatives also include amino acid alterations such as deletions, substitutions, additions, and amino acid modifications. A "deletion" refers to the absence of one or more amino acid residue(s) in the related polypeptide. An "addition" refers to the presence of one or more amino acid residue(s) in the related polypeptide. Additions and deletions to a polypeptide may be at the amino terminus, the carboxy terminus, and/or internal. Amino acid "modification" refers to the alteration of a naturally occurring amino acid to produce a non-naturally occurring amino acid. A "substitution" refers to the replacement of one or more amino acid residue(s) by another amino acid residue(s) in the polypeptide. Derivatives can contain different combinations of alterations including more than one alteration and different types of alterations.

Although the effect of an amino acid change varies depending upon factors such as phosphorylation, glycosylation, intra-chain linkages, tertiary structure, and the role of the amino acid in the active site or a possible allosteric site, it is generally preferred that the substituted amino acid is from the same group as the amino acid being replaced. To some extent the following groups contain amino acids which are interchangeable: the basic amino acids lysine arginine, and histidine; the acidic amino acids aspartic and glutamic acids; the neutral polar amino acids serine, threonine, cysteine, glutamine, asparagine and, to a lesser extent, methionine; the nonpolar aliphatic amino acids glycine, alanine, valine, isoleucine, and leucine (however, because of size, glycine and alanine are more closely related and valine, isoleucine and leucine are more closely related); and the aromatic amino acids phenylalanine, tryptophan, and tyrosine. In addition, although classified in different categories, alanine, glycine, and serine seem to be interchangeable to some extent, and cysteine additionally fits into this group, or may be classified with the polar neutral amino acids.

Although proline is a nonpolar neutral amino acid, its replacement represents difficulties because of its effects on conformation. Thus, substitutions by or for proline are not preferred, except when the same or similar conformational results can be obtained. The conformation conferring properties of proline residues may be obtained if one or more of these is substituted by hydroxyproline (Hyp).

Examples of modified amino acids include the following: altered neutral nonpolar amino acids such as amino acids of the formula $H_2N(CH_2)_nCOOH$ where n is 2–6, sarcosine (Sar), t-butylalanine (t-BuAla), t-butylglycine (t-BuGly), N-methyl isoleucine (N-MeIle), and norleucine (Nleu); altered neutral aromatic amino acids such as phenylglycine; altered polar, but neutral amino acids such as citrulline (Cit) and methionine sulfoxide (MSO); altered neutral and nonpolar amino acids such as cyclohexyl alanine (Cha), altered acidic amino acids such as cysteic acid (Cya); and altered basic amino acids such as ornithine (Orn).

Preferred derivatives have one or more amino acid alteration(s) that do not significantly affect the receptor-binding or other activity of the 6b-modified VEGF protein. In regions of the 6b-modified VEGF polypeptide sequence not necessary for the 6b-modified VEGF protein activity, amino acids may be deleted, added or substituted with less risk of affecting activity. In regions required for the 6b-modified VEGF protein activity, amino acid alterations are less preferred as there is a greater risk of affecting activity. Such alterations should be conservative alterations. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent.

Conserved regions tend to be more important for protein activity than non-conserved regions. Standard procedures can be used to determine the conserved and non-conserved regions important for receptor activity using in vitro mutagenesis techniques or deletion analyses and measuring receptor activity as described by the present disclosure.

Derivatives can be produced using standard chemical techniques and recombinant nucleic acid molecule techniques. Modifications to a specific polypeptide may be deliberate, as through site-directed mutagenesis and amino acid substitution during solid-phase synthesis, or may be accidental such as through mutations in hosts which produce the polypeptide. Polypeptides including derivatives can be obtained using standard techniques such as those described in Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Laboratory Press (1989). For example, Chapter 15 of Sambrook describes procedures for site-directed mutagenesis of cloned DNA.

In one aspect the invention features a nucleic acid molecule, or polynucleotide encoding a 6b-modified VEGF protein. In some situations it is desirable for such nucleic acid molecule to be isolated or enriched, or purified. By the use of the term "enriched" in reference to nucleic acid molecule, polypeptide, or protein is meant that the specific DNA or RNA sequence, polypeptide, or protein constitutes a significantly higher fraction (2–5 fold) of the total DNA, RNA, polypeptide or protein present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused, for example, by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA

sequence, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term significant here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other nucleic acids of about at least 2 fold, more preferably at least 5 to 10 fold or even more. The term also does not imply that there is no DNA or RNA from other sources. The other source DNA may, for example, comprise DNA from a yeast or bacterial genome, or a cloning vector such as pUC19. This term distinguishes from naturally occurring events, such as viral infection, or tumor type growths, in which the level of one mRNA may be naturally increased relative to other species of mRNA. That is, the term is meant to cover only those situations in which a person has intervened to elevate the proportion of the desired nucleic acid.

Use of the term "isolated" indicates that a DNA, RNA or protein has been removed from its naturally occurring environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide chain or polypeptide present, but that it is essentially free (about 90–95% pure at least) of non-nucleotide or non-peptide material naturally associated with it.

It is also advantageous for some purposes that a nucleotide sequence or polypeptide be in purified form, e.g., cloned or recombinant. The term "purified" does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level this level should be at least 2–5 fold greater, e.g., in terms of mg/ml).

The nucleic acid molecule may be constructed, for example, from an existing VEGF-A nucleotide sequence by modification using, for example, oligonucleotide site-directed mutagenesis, by deleting sequences using restriction enzymes, by adding sequences obtained by subcloning or PCR, or as described herein. Standard recombinant techniques for mutagenesis such as in vitro site-directed mutagenesis (Hutchinson et al., *J. Biol. Chem.* 253:6551, (1978), Sambrook et al., Chapter 15, supra), use of TAB® linkers (Pharmacia), and PCR-directed mutagenesis can be used to create such mutations. The nucleic acid molecule may also be synthesized by the triester method or by using an automated DNA synthesizer.

The invention also features recombinant DNA vectors, preferably in a cell or an organism. The recombinant DNA vectors may contain a sequence coding for a VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF-A protein or a functional derivative thereof in a vector containing a promoter effective to initiate transcription in a host cell. The recombinant DNA vector may contain a transcriptional initiation region functional in a cell and a transcriptional termination region functional in a cell. Where the DNA vector contains sufficient control sequences, such as initiation and/or termination regions, such that the inserted nucleic acid molecule may be expressed in a host cell, the vector may also be called an "expression vector."

The present invention also relates to a cell or organism that contains the above-described nucleic acid molecule or recombinant DNA vector and thereby is capable of expressing a VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF-A peptide. The peptide may be purified from cells which have been altered to express the polypeptide. A cell is said to be "altered to express a desired polypeptide" when the cell, through genetic manipulation, is made to produce a protein which it normally does not produce or which the cell normally produces at lower levels. One skilled in the art can readily adapt procedures for introducing and expressing either genomic, cDNA, or synthetic sequences into either eukaryotic or prokaryotic cells.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. The precise nature of the regulatory regions needed for gene sequence expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal synthesis initiation. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

For example, the entire coding sequence of VEGF-A_{138} may be combined with one or more of the following in an appropriate expression vector to allow for such expression: (1) an exogenous promoter sequence (2) a ribosome binding site (3) a polyadenylation signal (4) a secretion signal (5) a promiscuous or tissue specific enhancer. Modifications can be made in the 5'-untranslated and 3'-untranslated sequences to improve expression in a prokaryotic or eukaryotic cell, or codons may be modified such that while they encode an identical amino acid, that codon may be a preferred codon in the chosen expression system. The use of such preferred codons is described in, for example, Grantham et al., *Nuc. Acids Res.*, 9:43–74 (1981), and Lathe, *J. Mol. Biol.* 183:1–12 (1985) hereby incorporated by reference herein in their entirety. These publications, and all other publications referenced herein, are hereby incorporated by reference in their entirety.

If desired, the non-coding region 3' to the genomic VEGF-A protein sequence may be operably linked to the nucleic acid molecule encoding, for example, VEGF-A_{138}. This region may be used in the recombinant DNA vector for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3' region naturally contiguous to the DNA sequence encoding VEGF-A transcriptional termination signals, or sequences involved in message stability may be provided. Alternatively, a 3' region functional in the host cell may be substituted.

An operable linkage is a linkage in which the regulation, DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene sequence expression. Two DNA sequences (such as a promoter region sequence and a VEGF-A_{138} protein sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation in the coding sequence, (2) interfere with the ability of the promoter region sequence to direct the transcription of VEGF-A_{138} protein gene sequence, or (3) interfere with the ability of the VEGF-A_{138} protein gene sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express a VEGF-A_{138}, transcriptional and translational signals recognized by an appropriate host are necessary.

Those skilled in the art will recognize that the novel VEGF proteins of the present invention may also be expressed in various cell systems, both prokaryotic and eukaryotic, all of which are within the scope of the present invention.

Although the novel VEGF proteins of the present invention may be expressed in prokaryotic cells, which are generally very efficient and convenient for the production of recombinant proteins, the VEGF proteins produced by such cells will not be glycosylated and therefore may have a shorter half-life in vivo. Prokaryotes most frequently are represented by various strains of *E. coli*. However, other microbial strains may also be used, including other bacterial strains. Recognized prokaryotic hosts include bacteria such as *E. coli*, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, and the like. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

In prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host may be used. Examples of suitable plasmid vectors may include pBR322, PUC118, pUC119 and the like; suitable phage or bacteriophage vectors may include λgt10, λgt11 and the like; and suitable virus vectors may include pMAM-neo, pKRC and the like. Preferably, the selected vector of the present invention has the capacity to replicate in the selected host cell.

To express VEGF pplypeptides or subunits (or a functional derivative thereof) in a prokaryotic cell, it is necessary to operably link the 6b-modified VEGF nucleic acid sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or repressible). Examples of constitutive promoters include the int promoter of bacteriophage λ., the bla promoter of the β-lactamase gene sequence of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), the trp, recA, lacZ, lacI, and gal promoters of *E. coli*, the α-amylase (Ulmanen et al., *J. Bacteriol.* 162:176–182 (1985)) and the _-28-specific promoters of *B. subtilis* (Gilman et al., *Gene sequence* 32:11–20(1984)), the promoters of the bacteriophages of Bacillus (Gryczan, In: *The Molecular Biology of the Bacilli*, Academic Press, Inc., NY (I 982)), and Streptomyces promoters (Ward et al., *Mol. Gen. Genet.* 203:468–478(1986)). Prokaryotic promoters are reviewed by Glick (*J. Ind. Microbiot.* 1:277–282(1987)); Cenatiempo (*Biochimie* 68:505–516(1986)); and Gottesman (*Ann. Rev. Genet.* 18:415–442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene sequence-encoding sequence. Such ribosome binding. sites are disclosed, for example, by Gold et al. (*Ann. Rev. Microbiol.* 35:365–404(1981)). The tibosome binding site and other sequences required for translation initiation are operably linked to the nucleic acid molecule coding for VEGF-A_{145} by, for example, in frame ligation of synthetic oligonucleotides that contain such control sequences. For expression in prokaryotic cells, no signal peptide sequence is required. The selection of control sequences, expression vectors, transformation methods, and the like, are dependent on the type of host cell used to express the gene.

As used herein, "cell", "cell line", and "cell culture" may be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" include the primary subject cell and cultures derived therefrom, without regard to the number of transfers. For example, VEGF-A_{138} expressed in prokaryotic cells is expected to comprise a mixture of properly initiated VEGF-A_{138} protein peptides with the N-terminal sequence predicted from the sequence of the expression vector, and VEGF-A_{138} protein peptides that have an N-terminal methionine resulting from inefficient cleaving of the initiation methionine during bacterial expression. Both types of VEGF-A_{138} peptides are considered to be within the scope of the present invention as the presence of an N-terminal methionine is not expected to affect biological activity. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. However, as defined, mutant progeny have the same functionality as that of the originally transformed cell.

Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColEl, pSC101, pACYC 184, πVX. Such plasmids are, for example, disclosed by Sambrook (cf. "*Molecular Cloning: A Laboratory Manual*", second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)). Bacillus plasmids include pC194, pC221, pT127, and the like. Such plasmids are disclosed by Gryczan (In: *The Molecular Biology of the Bacilli*, Academic Press, NY (1982), pp. 307–329). Suitable Streptomyces plasmids include pIJ101 (Kendall et al., *J. Bacteriol.* 169:4177–4183 (1987)), and streptomyces bacteriophages such as φC31 (Chater et al., In: *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kaido, Budapest, Hungary (1986), pp. 4554). Pseudomonas plasmids are reviewed by John et al. (*Rev. Infect. Dis.* 8:693–704 (1986)), and Izaki (*Jpn. J. Bacteriol.* 33:729–742(1978)).

Eukaryotic host cells that may be used in the expression systems of the present invention are not strictly limited, provided that they are suitable for use in the expression of the 6b-modified VEGF proteins of the invention. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, mammalian cells either in vivo, or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as Vero or CHO-K1, or cells of lymphoid origin and their derivatives.

The 6b-modified VEGF proteins of the present invention may also be expressed in human cells such as human embryo kidney 293EBNA cells, which express Epstein-Barr virus nuclear antigen 1, as described, for example, in Olofsson, B. et al., *Proc. Natl. Acad. Sci. USA* 93:2576–2581 (1996). The cells are transfected with the expression vectors by using, for example, a calcium phosphate precipitation or lipofection method, and the cells are then incubated-for at least 48 hours. The VEGF peptides may then be purified from the supernatant as described in Example II.

In addition, plant cells are also available as hosts, and control sequences compatible with plant cells are available, such as the cauliflower mosaic virus 35S and 19S, and nopaline synthase promoter and polyadenylation signal sequences. Another preferred host is an insect cell, for example the Drosophila larvae. Using insect cells as hosts, the Drosophila alcohol dehydrogenase promoter can be used. Rubin, *Science* 240:1453–1459(1988).

Any of a series of yeast gene sequence expression systems can be utilized which incorporate promoter and termination elements from the actively expressed gene sequences coding for glycolytic enzymes are produced in large quantities when yeast are grown in mediums rich in glucose. Known glycolytic gene sequences can also provide very efficient

transcriptional control signals. Yeast provides substantial advantages in that it can also carry out post-translational peptide modifications. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene sequence products and secretes peptides bearing leader sequences (i.e., prepeptides). For a mammalian host, several possible vector systems are available for the expression of the 6b-modified VEGF peptides and of the invention.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

Expression of the 6b-modified VEGF protein of the invention in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, for example, the promoter of the mouse metallothionein I gene sequence (Hamer et al., *J. Mol. Appl. Gen.* 1:273–288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell* 31:355–365 (1982)); the SV40 early promoter (Benoist et al., *Nature* (London) 290:304–310(1981)); the yeast gal4 gene sequence promoter (Johnston et al., *Proc. Natl. Acad. Sci.* (USA) 79:6971–6975(1982); Silver et al., *Proc. Natl. Acad. Sci.* (USA) 81:5951–5955 (1984)).

Translation of eukaryotic MRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence that encodes a VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein, a functional derivative thereof, does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as, for example, the VEGF-A_{138} protein coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as, for example, the VEGF-A_{138} protein coding sequence).

A 6b-modified VEGF nucleic acid molecule and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a nonreplicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Because such molecules are incapable of autonomous replication, the expression of the gene may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced DNA sequence into the host chromosome.

A vector may be employed that is capable of integrating the desired gene sequences into the host cell chromosome. Cells that have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers that allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, *Molec. Cell. Biol.* 3:280(1983).

The introduced nucleic acid molecule can be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred eukaryotic plasmids include, for example, BPV, vaccinia, SV40, 2-micron circle, and the like, or their derivatives. Such plasmids are well known in the art (Botstein et al., *Miami Wntr. Symp.* 19:265–274(1982); Broach, In: *"The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance"*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 445–470 (1981); Broach, *Cell* 28:203–204 (1982); Bollon et al., *J. Clin. Hematol. Oncol.* 10:39–48 (1980); Maniatis, In: *Cell Biology: A Comprehensive Treatise*, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563–608(1980).

A "vector" (sometimes referred to as gene delivery or gene transfer "vehicle") refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either in vitro or in vivo. The polynucleotide to be delivered may comprise a coding sequence of interest in gene therapy. Vectors include, for example, viral vectors (such as adenoviruses ('Ad'), adeno-associated viruses (AAV), and retroviruses), liposomes and other lipid-containing complexes, and other macromolecular complexes capable of mediating delivery of a polynucleotide to a host cell. Vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. As described and illustrated in more detail below, such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such as agents mediating nuclear localization); and components that influence expression of the polynucleotide. Such components also might include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities. A large variety

of such vectors are known in the art and are generally available (see, e.g., the various references cited herein).

In preferred methods of enhancing cardiac function according to one of the preceding embodiments, the vector is a viral vector or a lipid-based vector, preferably a viral vector. The vector can be a targeted vector, especially a targeted vector that preferentially binds to ventricular myocytes. Presently preferred viral vectors are derived from adenovirus (Ad) or adeno-associated virus (AAV). Both human and non-human viral vectors can be used but preferably the recombinant viral vector is replication-defective in humans. Where the vector is an adenovirus, it preferably comprises a polynucleotide having a promoter operably linked to a gene encoding an angiogenic protein or peptide, and is replication-defective in humans.

Presently preferred replication-defective adenoviral vectors have deletions that remove the E1A and E1B genes, or have deletions that remove the E1A, E1B and E4 genes. Preferably about 10^{10} to 10^{14} adenovirus vector particles, more preferably about 10^{11} to 10^{13} vector particles, most preferably about 10^{12} vector particles, are introduced into a blood vessel, preferably a blood vessel supplying the myocardium.

For AAV vectors, the vector preferably comprises a polynucleotide having a promoter operably linked to a gene encoding an angiogenic protein or peptide, and preferably, the gene encoding the angiogenic protein or peptide is flanked by AAV inverted terminal repeats (ITRs). Preferably, the AAV vector is replication-defective in humans. Presently preferred replication-defective AAV vectors have deletions affecting one or more AAV replication or encapsidation sequences. Alternatively, the vector can be a lipid-based vector comprising a gene encoding an angiogenic protein or peptide as described herein.

Once the vector or nucleic acid molecule containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, i.e., transformation, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, lipofection, calcium phosphate precipitation, direct microinjection, DEAE-dextran transfection, and the like. The most effective method for transfection of eukaryotic cell lines with plasmid DNA varies with the given cell type. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene molecule(s) results in the production of a 6b-modified VEGF. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like). A variety of incubation conditions can be used to form the peptide of the present invention. The most preferred conditions are those which mimic physiological conditions.

Production of the stable transfectants, may be accomplished by, for example, by transfection of an appropriate cell line with a eukaryotic expression vector, such as pCEP4, in which the coding sequence for the 6b-modified VEGF protein has been cloned into the multiple cloning site. These expression vectors contain a promoter region, such as the human cytomegalovirus promoter (CMV), that drive high-level transcription of desired DNA molecules in a variety of mammalian cells. In addition, these vectors contain genes for the selection of cells that stably express the DNA molecule of interest. The selectable marker in the pCEP4 vector encodes an enzyme that confers resistance to hygromycin, a metabolic inhibitor that is added to the culture to kill the nontransfected cells.

Cells that have stably incorporated the transfected DNA may be identified by their resistance to selection media, as described above, and clonal cell lines will be produced by expansion of resistant colonies. The expression of a 6b-modified VEGF protein by these cell lines may be assessed by methods known in the art, for example, by RNAse protection assay, Northern blot, or Western blot analysis.

Pharmaceutical Compositions and Therapeutic Uses

One object of this invention is to provide VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein in a pharmaceutical composition suitable for therapeutic use. Thus, in one aspect the invention provides a method for stimulating vascular cell proliferation in a patient by administering a therapeutically effective amount of pharmaceutical composition comprising VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein.

By "therapeutically effective amount" is meant an amount of a compound that produces the desired therapeutic effect in a patient. For example, in reference to a disease or disorder, it is the amount which reduces to some extent one or more symptoms of the disease or disorder, and returns to normal, either partially or completely, physiological or biochemical parameters associated or causative of the disease or disorder. When used to therapeutically treat a patient it is an amount expected to be between 0.1 mg/kg of protein to 100 mg/kg, preferably less than 50 mg/kg, more preferably less than 10 mg/kg, more preferably less than 1 mg/kg. The amount of compound depends on the age, size, and disease associated with the patient and can be determined by standard procedure. Thus, for gene therapy, a therapeutically effective amount would be an amount sufficient to direct the expression and secretion of such an amount of protein in a patient.

The optimal formulation and mode of administration of compounds of the present application to a patient depend on factors known in the art such as the particular disease or disorder, the desired effect, and the type of patient. While the compounds will typically be used to treat human patients, they may also be used to treat similar or identical diseases in other mammals such as other primates, farm animals such as swine, cattle and poultry, and sports animals and pets such as horses, dogs and cats.

Preferably, the therapeutically effective amount is provided as a pharmaceutical composition. A pharmacological agent or composition refers to an agent or composition in a form suitable for administration into a multicellular organism such as a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should allow the agent or composition to reach a target cell whether the target cell is present in a multicellular host or in culture. For example, pharmacological agents or compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the agent or composition from exerting its effect.

The claimed compositions can also be formulated as pharmaceutically acceptable salts (e.g., acid addition salts) and/or complexes thereof. Pharmaceutically acceptable salts are non-toxic salts at the concentration at which they are administered. The preparation of such salts can facilitate the pharmacological use by altering the physical-chemical characteristics of the composition without preventing the composition from exerting its physiological effect. Examples of

useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing the solubility to facilitate the administration of higher concentrations of the drug.

Pharmaceutically acceptable salts include acid addition salts such as those containing sulfate, hydrochloride, phosphate, sulfonate, sulfamate, sulfate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclohexylsulfonate, cyclohexylsulfamate and quinate. Pharmaceutically acceptable salts can be obtained from acids such as hydrochloric acid, sulfuric acid, phosphoric acid, sulfonic acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, cyclohexylsulfonic acid, cyclohexylsulfamic acid, and quinic acid. Such salts may be prepared by, for example, reacting the free acid or base forms of the product with one or more equivalents of the appropriate base or acid in a solvent or medium in which the salt is insoluble, or in a solvent Such as water which is then removed in vacuo or by freeze-drying or by exchanging the ions of an existing salt for another ion on a suitable ion exchange resin.

Carriers or excipients can also be used to facilitate administration of the compound. Examples of carriers and excipients include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. The compositions or pharmaceutical composition can be administered by different routes including intravenously, intraperitoneal, subcutaneous, and intramuscular, orally, topically, or transmucosally.

The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or the inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

The compounds of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co., Easton, Pa., 1990. See, also, Wang, Y. J. and Hanson, M. A. "Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers ", *Journal of Parenteral Science and Technology*, Technical Report No. 10, Supp. 42:2S (1 988). A suitable administration format may best be determined by a medical practitioner for each patient individually.

For systemic administration, injection is preferred, e.g., intraperitbneal, subcutaneous, intrathecal, or intracerebroventricular. For injection, the compounds of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. Alternatively, the compounds of the invention are formulated in one or more excipients (e.g., propylene glycol) that are generally accepted as safe as defined by USP standards. They can, for example, be suspended in an inert oil, suitably a vegetable oil such as sesame, peanut, olive oil, or other acceptable carrier. Preferably, they are suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 5.6 to 7.4. These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH buffering agents. Useful buffers include for example, Goodman acetate/acetic acid buffers. A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the bloodstream over many hours or days following transdermal injection or delivery. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. An inflatable balloon catheter with VEGF-A_{145} protein coating the balloon may also be employed to deliver the substance to a targeted artery.

Alternatively, the compounds may be administered orally. For oral administration, the compounds are formulated into conventional oral dosage forms such as capsules, tablets and tonics.

Systemic administration can also be by transmucosal or transdermal means, or the molecules can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be, for example, through nasal sprays or using suppositories. For oral administration, the molecules are formulated into conventional oral administration dosage forms such as capsules, tablets, and liquid preparations.

For topical administration, the compounds of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

If desired, solutions of the above compositions may be thickened with a thickening agent such as methyl cellulose. They may be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acacia powder, a non-ionic surfactant (such as a Tween), or an ionic surfactant (such as alkali polyether alcohol sulfates or sulfonates, e.g., a Triton).

Compositions useful in the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be simply mixed in a blender or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

The amounts of various compounds of this invention to be administered can be determined by standard procedures.

For use by the physician, the compositions will be provided in dosage unit form containing an amount of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein.

Gene Therapy

Nucleic acids coding for VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF proteins will also be useful in gene therapy (reviewed in Miller, *Nature* 357:455–460 (1992)). Miller states that advances have resulted in practical approaches to human gene therapy that have demonstrated positive initial results. The basic science of gene therapy is described in Mulligan, *Science* 260:926–931 (1993). One example of gene therapy is presented in Example IV, which describes the use of adenovirus-mediated gene therapy.

As another example, an expression vector containing the VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified

VEGF protein coding sequence may be inserted into cells, the cells are grown in vitro and then infused in large numbers into patients. In another example, a DNA segment containing a promoter of choice (for example a strong promoter) is transferred into cells containing an endogenous VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein, should such endogenous forms exist, in such a manner that the promoter segment enhances expression of the endogenous VEGF gene (for example, the promoter segment is transferred to the cell such that it becomes directly linked to the endogenous VEGF gene.

The gene therapy may involve the use of an adenovirus or other vector including a nucleotide sequence coding for VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein, or a naked nucleic acid molecule coding for these proteins. Alternatively, engineered cells containing a nucleic acid molecule coding for these modified proteins may be injected. Example IV illustrates the method of gene therapy using an adenovirus vector to provide angiogenesis therapy.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA viruses, or bovine papilloma virus, may be used for delivery of nucleotide sequences (e.g., cDNA) encoding recombinant VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences. See, for example, Nabel, E. G., *Circulation*, 91, 541–548 (1995), the techniques described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked DNA or in reconstituted system e.g., liposomes or other lipid systems for delivery to target cells (See e.g., Felgner et al., *Nature* 337:397–8, 1989). Several other methods for the direct transfer of plasmid DNA into cells exist for use in human gene therapy and involve targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins. See, Miller, *Nature* 357:455–60, 1992.

In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the nucleus of a cell, through a process of microinjection. Capecchi, M. R., *Cell* 22:479–88 (1980). Once recombinant genes are introduced into a cell, they can be recognized by the cells' normal mechanisms for transcription and translation, and a gene product will be expressed. Other methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with $CaPO_4$ and taken into cells by pinocytosis (Chen, C. and Okayama, H., *Mol. Cell Biol.* 7:2745–52 (1987)); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane (Chu, G. et al., *Nucleic Acids Res.*, 15:1311–26 (1987)); lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell (Felgner, P. L., et al., *Proc. Natl. Acad. Sci.* USA. 84:7413–7 (1987)); and particle bombardment using DNA bound to small projectiles (Yang, N. S., et al., *Proc. Natl. Acid Sci.*. 87:9568–72 (1990)). Another method for introducing DNA into cells is to couple the DNA to chemically modified proteins.

It has also been shown that adenovirus proteins are capable of destabilizing endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the recombinant gene. Curiel, D. T., et al., *Am. J. Respir. Cell. Mol. Biol.* 6:247–52 (1992).

A balloon catheter, such as those used in angioplasty may be employed wherein the balloon is coated with VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified protein, DNA coding for such protein, or vectors as described in Riessen, R., *Human Gene Therapy*, 4, 749–758 (1993) incorporated herein by reference.

As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, antisense DNA or RNA, or enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid molecule contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid molecule into the cell through the membrane or by endocytosis, and release of nucleic acid molecule into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid molecule into the nucleus of the cell and binding to appropriate nuclear factors for transcription.

As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell in vivo or in vitro. Gene transfer can be performed ex vivo on cells which are then transplanted into a patient, or can be performed by direct administration of the nucleic acid molecule or nucleic acid-protein complex into the patient.

In another preferred embodiment, a vector having nucleic acid molecule sequences encoding VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein is provided in which the nucleic acid molecule sequence is expressed only in a specific tissue. Methods of achieving tissue-specific gene expression as set forth in International Publication No. WO 93/09236, filed Nov. 3, 1992 and published May 13, 1993.

In all of the preceding vectors set forth above, a further aspect of the invention is that the nucleic acid sequence contained in the vector may include additions, deletions or modifications to some or all of the sequence of the nucleic acid, as defined above.

In another preferred embodiment, ~~a method of~~ gene replacement is set forth. "Gene replacement" as used herein means supplying a nucleic acid molecule sequence which is capable of being expressed in vivo in an animal and thereby providing or augmenting the function of an endogenous gene which is missing or defective in the animal.

Vectors for Gene Delivery In Vivo

In general, the gene of interest is transferred to the target organ, preferably the heart, including cardiac myocytes, or skeletal muscle, including skeletal myocytes in vivo and directs production of the encoded protein. Such production may be relatively constitutive. A variety of different gene transfer vectors, including viral as well as non-viral systems, can be employed to deliver transgenes for use in the present invention.

Preferred vectors for use in the present invention include viral vectors, lipid-based vectors and other vectors that are capable of delivering DNA to non-dividing cells in vivo.

Presently preferred are viral vectors, particularly replication-defective viral vectors (including, for example replication-defective adenovirus vectors and adeno-associated virus (AAV) vectors. For ease of production and use in the present invention, replication-defective adenovirus vectors are presently most preferred.

References describing a variety of other gene delivery vectors are known in the art, some of which are cited herein. Such other vectors include, for example, other viral vectors (such as adeno-associated viruses (AAV), liposomes and other lipid-containing complexes, and other macromolecular complexes capable of mediating delivery of a polynucleotide to a host cell. As described above and in the cited references, vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. Such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such as agents mediating nuclear localization); and components that influence expression of the polynucleotide. Such components also might include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow selection for cells carrying the marker, whereas negative selectable markers allow cells carrying the marker to be selectively eliminated. A variety of such marker genes have been described, including bifunctional (i.e. positive/negative) markers (see, e.g., Lupton, S., WO 92/08796, published May 29, 1992; and Lupton, S., WO 94/28143, published Dec. 8, 1994). Such marker genes can provide an added measure of control that can be advantageous in gene therapy contexts. A large variety of such vectors are known in the art and are generally available (see, e.g., the various references cited above).

Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M. S., Adenoviridae and Their Replication, in Fields, B., et al.. (eds.) Virology, Vol. 2, Raven Press New York, pp. 1679–1721, 1990); Graham, F., et al., pp. 109128 in Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al., FASEB Journal 9: 190–199, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68: 145–159, 1994; Schneider and French, Circulation 88:1937–1942, 1993; Curiel D. T., et al., Human Gene Therapy 3: 147–154, 1992; Graham, F. L., et al., WO 95/00655 (Jan. 5, 1995); Falck-Pedersen, E. S., WO 95/16772 (Jun. 22, 1995); Denefle, P. et al., WO 95/23867 (Sep. 8, 1995); Haddada, H. et al., WO 94/26914 (Nov. 24, 1994); Perricaudet, M. et al., WO 95/02697 (Jan. 26, 1995); Zhang, W., et al., WO 95/25071 (Oct. 12, 1995). A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996).

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Carter, B., Handbook of Parvoviruses, vol. 1, pp. 169–228, 1990; Berns, Virology, pp. 1743–1764 (Raven Press 1990); Carter, B., Curr. Opin. Biotechnol., 3: 533–539, 1992; Muzyczka, N., Current Topics in Microbiology and Immunology, 158: 92–129, 1992; Flotte, T. R., et al., *Am. J. Respir. Cell Mol. Biol.* 7:349–356, 1992; Chatterjee et al., Ann. NY Acad. Sci., 770: 79–90, 1995; Flotte, T. R., et al., WO 95/13365 (May 18, 1995); Trempe, J. P., et al., WO 95/13392 (May 18, 1995); Kotin, R., Human Gene Therapy, 5: 793–801, 1994; Flotte, T. R., et al., Gene Therapy 2:357–362, 1995; Allen, J. M., WO 96/17947 (Jun. 13, 1996); and Du et al., Gene Therapy 3: 254261, 1996.

Additional references describing non-viral vectors which could be used in the methods of the present invention include the following: Ledley, F D, Human Gene Therapy 6: 11 29–1144, 1995; Miller, N., et al., FASEB Journal 9: 190–199, 1995; Chonn, A., et al., Curr. Opin. in Biotech. 6: 698–708, 1995; Schofield, J P, et al., British Med. Bull. 51: 56–71, –1995; Brigham, K. L., et al., J. Liposome Res. 3: 31 49, 1993; Brigham, K. L., WO 91/06309 (May 16, 1991); Felgner, P. L., et al., WO 91/17424 (Nov. 14, 1991); Solodin et al., Biochemistry 34: 13537–13544, 1995; WO 93/19768 (Oct. 14, 1993); Debs et al., WO 93125673; Felgner, P. L., et al., U.S. Pat. No. 5,264,618 (Nov. 23, 1993); Epand, R. M., et al., U.S. Pat. No. 5,283,185 (Feb. 1, 1994); Gebeyehu et al., U.S. Pat. No. 5,334,761 (Aug. 2, 1994); Felgner, P. L., et al., U.S. Pat. No. 5,459,127 (Oct. 17, 1995); Overell, R. W., et al., WO 95/28494 (Oct. 26, 1995); Jessee, WO 95/02698 (Jan. 26, 1995); Haces and Ciccarone, WO 95/17373 (Jun. 29, 1995); Lin et al., WO 96/01840 (Jan. 25, 1996).

Helper Independent Replication Deficient Human Adenovirus 5 System

In general, the gene of interest is transferred to the heart (or skeletal muscle), including cardiac myocytes (and skeletal myocytes), in vivo and directs constitutive production of the encoded protein. Several different gene transfer approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus 5 system. Using this system, Giordano and Hammond have demonstrated transfection of greater than 60% of myocardial cells in vivo by a single intracoronary injection (Giordano and Hammond, *Clin. Res.*, 42:123A, 1994). Non-replicative recombinant adenoviral vectors are particularly useful in transfecting coronary endothelium and cardiac myocytes resulting in highly efficient transfection after intracoronary injection. The same will be true for transfecting desired cells of the peripheral vascular system.

The helper-independent replication-defective human adenovirus 5 system can be used effectively to transfect a large percentage of myocardial cells in vivo by a single intracoronary injection. Hammond, et al. have also shown that such a delivery technique can be used to effectively target vectors to the myocardium of a large mammal heart. Additional means of targeting vectors to particular cells or tissue types are described below and in the art.

In various illustrations described below, recombinant adenovirus vectors are based on the human adenovirus 5 (as described by McGrory W J, et al., Virology 163:614–617, 1988) which are missing essential early genes from the adenovirus genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products in trans. In place of the missing adenovirus genomic sequences, a transgene of interest can be cloned and expressed in tissue/cells infected with the replication-defective adenovirus. Although adenovirus-

based gene transfer does not generally result in stable integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), adenovirus vectors can be propagated in high titer and transfect non-replicating cells; and, although the transgene is not passed to daughter cells, this is suitable for gene transfer to adult cardiac myocytes, which do not actively divide. Retrovirus vectors provide stable gene transfer, and high titers are now obtainable via retrovirus pseudotyping (Burns, et al., Proc Nat'l Acad Sci (USA) 90: 8033–8037, 1993), but current retrovirus vectors are generally unable to efficiently transduce nonreplicating cells.

An advantage associated with nondividing cells such as myocytes is that the viral vector is not readily "diluted out" by host cell division. To further enhance the duration of transgene expression in the heart, however, it is also possible to employ various second generation adenovirus vectors that have both E1 and E4 deletions, which can be used in conjunction with cyclophosphamide administration (See, e.g., Dai et al., Proc. Nat'l Acad Sci. (USA) 92: 1401–1405, 1995). To further increase the extent of initial gene transfer, multiple infusions, or infusion in an isolated coronary circuit can also be employed.

Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines for the production of such replication-defective vectors. However, other cell lines which allow replication-defective adenovirus vectors to propagate therein can also be used, such as HeLa cells.

The recombinant adenoviral vectors based on the human adenovirus 5 (*Virology*, 163:614–617, 1988) are missing essential early genes from the adenoviral genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products in trans. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in tissue/cells infected with the replication deficient adenovirus. Although adenovirus-based gene transfer does not result in integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and transfect non-replicating cells. Although the transgene is not passed to daughter cells, this is acceptable for gene transfer to adult skeletal muscle and cardiac myocytes, which do not divide. Retroviral vectors provide stable gene transfer, and high titers are now obtainable via retrovirus pseudotyping (Burns, et al., *Proc. Natl. Acad. Sci. (USA)*, 90:8033–8037, 1993), but current retroviral vectors are unable to transduce nonreplicating cells (adult skeletal muscle and cardiac myocytes) efficiently. In addition, the potential hazards of transgene incorporation into host DNA are not warranted if short-term gene transfer is sufficient. Indeed, Hammond, et al. have discovered that a limited duration expression of an angiogenic protein is sufficient for substantial angiogenesis, and transient gene transfer for cardiovascular disease and peripheral disease processes is therapeutically adequate.

Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines. However, other cell lines which allow replication-deficient adenoviral vectors to propagate therein can be used.

Construction of Recombinant Adenoviral Vectors

All adenoviral vectors used in the present invention can be constructed by the rescue recombination technique described in Graham, *Virology*, 163:614–617, 1988. Briefly, the transgene of interest is cloned into a shuttle vector that contains a promoter, polylinker and partial flanking adenoviral sequences from which E1A/E1B genes have been deleted. As the shuttle vector, plasmid pAC1 (*Virology*, 163:614–617, 1988) (or an analog) which encodes portions of the left end of the human adenovirus 5 genome (*Virology*, 163:614–617, 1988) minus the early protein encoding E1A and E1B sequences that are essential for viral replication, and plasmid ACCMVPLPA (*J. Biol. Chem.*, 267:25129–25134, 1992) which contains polylinker, the CMV promoter and SV40 polyadenylation signal flanked by partial adenoviral sequences from which the E1A/E1B genes have been deleted can be exemplified. The use of plasmid pAC1 or ACCMVPLA facilitates the cloning process. The shuttle vector is then co-transfected with a plasmid which contains the entire human adenoviral 5 genome with a length too large to be encapsidated, into 293 cells. Co-transfection can be conducted by calcium phosphate precipitation or lipofection (*Biotechniqtes*, 15:868–872, 1993). Plasmid JM17 encodes the entire human adenovirus 5 genome plus portions of the vector pBR322 including the gene for ampicillin resistance (4.3 kb). Although JM17 encodes all of the adenoviral proteins necessary to make mature viral particles, it is too large to be encapsidated (40 kb versus 36 kb for wild type). In a small subset of co-transfected cells, rescue recombination between the transgene containing the shuttle vector such as plasmid pAC1 and the plasmid having the entire adenoviral 5 genome such as plasmid pJM17 provides a recombinant genome that is deficient in the E1A/E1B sequences, and that contains the transgene of interest but secondarily loses the additional sequence such as the pBR322 sequences during recombination, thereby being small enough to be encapsidated. With respect to the above method, successful results have been reported (Giordano, et al., *Circulation*, 88:I-139, 1993, and Giordano and Hammond, *Clin. Res.*, 42:123A, 1994). The CMV driven β-galactosidase encoding adenovirus HCMVSP11acZ (*Clin. Res.*, 42:123A, 1994) can be used to evaluate efficiency of gene transfer using X-gal treatment.

The initial mode of gene transfer uses adenoviral vectors as delineated above. The advantages of these vectors include the ability to effect high efficiency gene transfer (more than 60% of target organ cells transfected in vivo), the ease of obtaining high titer viral stocks and the ability of these vectors to effect gene transfer into cells such as cardiac myocytes which do not divide.

Tissue-Specific Promoters

The present invention also contemplates the use of cell targeting not only by delivery of the transgene into the coronary artery, or femoral artery, or other localized site for example, but also the use of tissue-specific promoters. By fusing, for example, tissue-specific transcriptional control sequences of left ventricular myosin light chain-2 (MLC_{2v}) or myosin heavy chain (MHC) to a transgene such as the VEGF-A_{138} gene within the adenoviral construct, transgene expression is limited to ventricular cardiac myocytes. The efficacy of gene expression and degree of specificity provided by MDC_{2v} and MHC promoters with lacZ have been determined, using the recombinant adenoviral system of the present invention. Cardiac-specific expression has been reported previously by Lee, et al.. (*J. Biol. Chem.*, 267:15875–15885, 1992). The MLC_{2v} promoter is comprised of 250 bp, and fits easily within the adenoviral-5 packaging constraints. The myosin heavy chain promoter, known to be a vigorous promoter of transcription, provides a reasonable alternative cardiac-specific promoter and is

comprised of less than 300 bp. Other promoters, such as the troponin-C promoter, while highly efficacious and sufficiently small, does less than lack adequate tissue specificity. By using the MLC_{2v} or MHC promoters and delivering the transgene in vivo, it is believed that the cardiac myocyte alone (that is without concomitant expression in endothelial cells, smooth muscle cells, and fibroblasts within the heart) will provide adequate expression of an angiogenic protein such as VEGF-A_{138} to promote angiogenesis. Limiting expression to the cardiac myocyte also has advantages regarding the utility of gene transfer for the treatment of CHF. By limiting expression to the heart, one avoids the potentially harmful effect of angiogenesis in non-cardiac tissues such as the retina. In addition, of the cells in the heart, the myocyte would likely provide the longest transgene expression since the cells do not undergo rapid turnover; expression would not therefore be decreased by cell division and death as would occur with endothelial cells. Endothelial-specific promoters are already available for this purpose (Lee, et al., *J. Biol. Chem.*, 265:10446–10450, 1990).

In the present invention, with regard to the treatment of heart disease, targeting the heart by intracoronary injection with a high titer of the vector and transfecting all cell types is presently preferred.

Propagation and Purification of Adenovirus Vectors

Successful recombinant vectors can be plaque purified according to standard methods. The resulting viral vectors are propagated on 293 cells which provide E1A and E1B functions in trans, to titers in the preferred 10^{10}–10^{12} viral particles/ml range. Cells can be infected at 80% confluence and harvested 48 hours later. After 3 freeze-thaw cycles the cellular debris is pelleted by centrifugation and the virus purified by CsCl gradient ultracentrifugation (double CsCl gradient ultracentrifugation is preferred). Prior to in vivo injection, the viral stocks are desalted by gel filtration through Sepharose columns such as G25 Sephadex. The product is then filtered through a 0.3 micron filter, thereby reducing deleterious effects of intracoronary injection of unfiltered virus (life threatening cardiac arrhythmias) and promoting efficient gene transfer. The resulting viral stock has a final viral titer in the range of 10^{10}–10^{12} viral particles/ml. The recombinant adenovirus must be highly purified, with no wild-type (potentially replicative) virus. Impure constructs can cause an intense immune response in the host animal. From this point of view, propagation and purification may be conducted to exclude contaminants and wild-type virus by, for example, identifying successful recombinants with PCR using appropriate primers, conducting two rounds of plaque purification, and double CsCl gradient ultracentrifugation. The recombinant adenovirus can also be passed through an appropriately-sized filter prior to intracoronary injection

Delivery of Recombinant Adenovirus Vectors

The viral stock can be in the form of an injectable preparation containing pharmaceutically acceptable carrier such as saline, for example, as necessary. The final titer of the vector in the injectable preparation is preferably in the range of 10^{7}–10^{13} viral particles which allows for effective gene transfer. Other pharmaceutical carriers, formulations and dosages are described below. For delivery to the myocardium, the adenovirus transgene constructs are preferably delivered by direct intracoronary (or graft vessel) injection using standard percutaneous catheter based methods under fluoroscopic guidance, at an amount sufficient for the transgene to be expressed to a degree which allows for highly effective therapy. The injection should be made deeply into the lumen (about 1 cm within the arterial lumen) of the coronary arteries (or graft vessel), and preferably be made in both coronary arteries.

By injecting the material directly into the lumen of the coronary artery by coronary catheters, it is possible to target the gene rather effectively, and to minimize loss of the recombinant vectors to the proximal aorta during injection. The vector stock, preferably containing no wild-type virus, may be injected deeply into the lumen of one or both coronary arteries (or grafts), preferably into both the right and left coronary arteries (or grafts), and preferably in an amount of 10^{10}–10^{14} viral particles as determined by optical densitometry (more preferably 10^{11}–10^{13} viral particles, most preferably 10^{12} viral particles). This type of injection enables local transfection of a desired number of cells, especially cardiac myocytes, in the affected myocardium with angiogenic protein- or peptide-encoding genes, thereby maximizing therapeutic efficacy of gene transfer, and minimizing undesirable angiogenesis at extracardiac sites and the possibility of an inflammatory response to viral proteins. A ventricular myocyte-specific promoter may be used, for example, to securely enable expression limited to the cardiac myocytes so as to avoid the potentially harmful effects of angiogenesis in non-cardiac tissues such as the retina. Thus delivery of the transgenes in this matter may result in targeted gene expression in, for example, the cells of the left ventricle.

It has been found that gene expression when delivered in this manner does not occur in hepatocytes and viral RNA cannot be found in the urine at any time after intracoronary injection. Any variety of coronary catheter, or a Stack perfusion catheter, for example, can be used in the present invention. In addition, other techniques known to those having ordinary skill in the art can be used for transfer of genes to the arterial wall.

Clinical Applications

Stimulating angiogenesis in mammals by transfecting the cells with a polynucleotide coding for VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may employ the procedure described by Giordano et al. in "Intracoronary Gene Transfer of Fibroblast Growth Factor-5 Increases Blood Flow and Contractile Function in an Ischemic Region of the Heart", Nature Medicine, Vol. 2 No. 5, pp. 534–539, May 1996 which is incorporated herein by reference. VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein should be released from cells infected by adenovirus vectors directing expression of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein in cells of the heart. This releasability is also found in VEGF-A_{121} and VEGF-A_{165} but not in VEGF-A_{189} or VEGF-A_{206}. However, VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein in contrast to VEGF-A_{121}, or VEGF-A_{165}, may be partially retained by ECM molecules as it diffuses towards target endothelial cells in adjacent blood vessels. The bound VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may be slowly released later thus prolonging the angiogenic effect as compared to VEGF-A_{121} or VEGF-A_{165}. Furthermore, the ECM bound VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein will be active, and will support the newly synthesized blood vessels during the critical period of blood vessel maturation, until the existence of blood-vessels is no longer dependent upon the presence of angiogenic growth factors. Thus, VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein will be more effective than some other VEGF forms as a therapeutic agent to be used for induction of collateral

blood vessels. These advantages may be critical when usage of adenovirus based expression vectors for gene therapy delivery of angiogenic agents is considered. An advantage of using adenovirus based vectors is that they are generally safe. Because the VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may have binding characteristics that allow it to clear at a slower rate compared to some other secreted VEGF forms, they may be more effective therapeutic agents compared to the other VEGF forms.

Balloon angioplasty is a major treatment of ischemic heart disease which involves the inflation of a balloon in a clogged blood vessel in order to open the blocked blood vessel. Unfortunately, this method of treatment frequently results in injury to the endothelial cells lining the inner walls of blood vessels. Smooth muscle cells often infiltrate into the opened blood vessels causing a secondary obstruction in a process called restenosis. VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may be employed to induce proliferation of the endothelial cells located at the periphery of the balloon induced damaged area in order to cover the luminal surface of the vessel with a new monolayer of endothelial cells, hoping to restore the original structure of the blood vessel. Adenovirus mediated gene therapy may also be applicable in this case as a method aimed at the delivery of inducers of endothelial cell proliferation to the lesion created by the balloon angioplasty procedure. The ability to bind to the ECM may offer several advantages for this application.

To prevent restenosis following balloon angioplasty, two types of approaches may be considered. It is possible to deliver a protein, or deliver an expression vector which will direct the expression of such a protein, to the site of occlusion using the balloon that is used to open the clogged vessel. Such a protein will also inhibit the proliferation of the non-endothelial cells which invade the reopened blood vessel until the endothelial cells on both sides of the wounded endothelial cells monolayer have a chance to re-grow. This can be combined with the delivery of a protein or a vector such as a recombinant adenovirus which will speed the re-growth of the endothelial cell layer. However, growth factors such as FGF-5, bFGF or HGF are also mitogenic to smooth muscle cells, and will induce their proliferation, which is the opposite of the desired effect. VEGFs on the other hand are generally specific for endothelial cells. VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may be especially useful in this context, because of its ECM binding properties. Following application, for example, by infection of adjacent cells with adenovirus encoding the protein, direct transfection with plasmid DNA encoding the protein, or the direct delivery of the protein, VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein have the potential to stick to the exposed extracellular matrix in the balloon treated vessel, and thus promote proliferation and re-growth of endothelial cells specifically at the site of the lesion. Thus, VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein can localize and concentrate in the very region where its activity is required, making it a particularly attractive candidate for the treatment of restenosis.

Coronary angioplasty is frequently accompanied by deployment of an intravascular stent to help maintain vessel function and avoid restenosis. Stents have been coated with heparin to prevent thrombosis until the new channel formed by the stent can endothelialize. The 6b-modified VEGF protein can be applied directly to the stent, or nucleic acids encoding the 6b-modified protein, such as plasmids, cDNA, or adenovirus vectors, may be applied to the stent for direct transfection of neighboring cells, using methods known to those of skill in the art. VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein that is locally applied, or produced through transfection, will enhance endothelialization of the stent and thus reduce thrombosis and restenosis.

Other applications for use of the growth factor of the present invention are contemplated. One example is for the treatment of ulcers. An ulcer is in effect a wound residing in the stomach. It was shown that angiogenic growth factors may be effective for the treatment of duodenal ulcers, and that stabilization of angiogenic growth factors may be a mechanism by which some therapeutic agents such as sucralfate produce their beneficial effects (Szabo, S., et. al. *Gastroenterology* 106, 1106–1111, 1994). Since VEGF is an angiogenic growth factor that is very stable under acidic conditions, its employment for the treatment of stomach and duodenal ulcers is contemplated. The heparin binding ability of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein which acts to preserve it in an active state, and its expected ability to bind to exposed ECM at the wound site, indicate that VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may be more suitable than other VEGF forms for treating stomach and duodenal ulcers.

To assist in understanding the present invention, the following Examples are included that describe the results of a series of experiments. The experiments relating to this invention should not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the scope of the invention as described herein and hereinafter claimed.

EXAMPLE I

Preparation of a Nucleic Acid Sequence Coding for VEGF-A_{138}

At least 5 differentially-spliced VEGF-A isoforms exist. These include VEGF-A 121, 145, 165, 189 and 206. (See FIG. 1, and Houck et. al. (1991) *Molecular Endocrinology* 5(12): 1806–1814 & Poltorack et. al. (1997) Journal of Biological Chemistry 272(11): 7151–7158). Only one of these, VEGF-A_{206} (Houck et. al. (1991) *Molecular Endocrinology* 5(12): 1806–1814, includes an exon denoted as 6b. The proposed name, VEGF-A_{138}, reflects the composition of the processed isoform which would be 138 amino acids in length. Two strategies are employed for the construction of VEGF-A_{138}. In one strategy, VEGF-A_{206} is cloned, and exon 6B is generated by polymerase chain reaction (PCR). In a second strategy, the replacement exon is generated by synthesis of two complementary oligonucleotides which are annealed to each other before sub-cloning. In both strategies, Exon 8 is excised in the first step and cloned back in at the last step. Exon 8 can be generated by PCR or by synthesis of complementary oligonucleotides. Synthesis of Exon 8 is described. Those of ordinary skill in the art will understand how to apply the teachings of the invention to the construction of VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182} or other 6b-modified protein, using these or analogous strategies.

A. Background Information

1. Sequence of VEGF-A_{145} (the sequence is shown with flanking BamHI sites; the insert is cloned into the Invitrogen vector, pCRII) [SEQ ID NO.1]. Exon 6a is shown in bold type. GGATCCGAAACCAT-GAACTTTCTGCTGTCTTGGGTGCATTG-

GAGCCTTGCCTTGCTGCTCTACCTCCAC-CATGCCAAGTGGTCCCAGGCTGCACCCATG GCAGAAGGAGGAGGGCAGAATCATCAC-GAAGTGGT

GAAGTTCATGGATGTCTATCAGCGCAGC-TACTGCCATCCAATCGAGACCCTGGTG-GACATCTTCCAGGAGTACCCTGAT-GAGATCGAGTACATCTTCAAGCCATCCTGTG TGCCCCTGATGCGATGCGGGGGCTGCTG-CAATGACGAGGGCCTGGAGTGTGTGC-CCACTGAGGAGTCCAACATCACCATGCA-GATTATGCGGATCAAACCTCACCAAGGCC AGCACATAGGAGAGATGAGCTTCCTA-CAGCACAACAAATGTGAATGCAGAC-CAAAGAAAGATAGAGCAAGACAA-GAAAAAAAATCAGTTCGAGGAAAGGGAAAG GGGCAAAAACGAAAGCGCAAGAAATC-CCGGTATAAGTCCTGGAGCGTATGTGA-CAAGCCGAGGCGGTGATGAATGAATGAG-GATCC

2. Sequence of the Replacement cassette (Exon 6b from VEGF-A_{206}) [SEQ ID NO.2]. GTACGTTGGTGC-CCGCTGCTGTCTAATGCCCTGGAGCCTC-CCTGGCCCCCA

B. Strategy for Subcloning

VEGF-A_{138} can be generated by replacement of the exon, 6a, of VEGF-A_{145}, with an alternative exon, 6b, which has been reported only in VEGF-A_{206}. The requisite steps are:

1. Generate a site-specific mutation in VEGF-A_{145} that will allow restriction immediately 5' of Exon 6a (i.e., between nucleotides 434 and 435). This mutation will create Apo I site.

Nucleotides 437 and 438 will be converted, by site-directed mutagenesis from Adenines to Thymidine. This will change the existing sequence from "AAAAAT" to "AAATTT". (Apo I cleaves the site "Pu\AATTPy").

2. Excise Exon 6a and Exon 8 by restriction endonuclease cleavage of the site-specifically mutated VEGF-A_{145} with Apo I and EcoR5. EcoR5 creates a blunt end in the vector, 3' of the VEGF insert.

3. Remove the Apo I 5' overhang by digestion with Mung Bean nuclease.

4. This will create a truncated, linearized, VEGF-A_{145} vector. Gel-purify this vector.

5. Generate the replacement exon (see "A.2.", above) by PCR from VEGF-A_{206} or by synthesis of complementary oligonucleotides.

a. For PCR, the 5' oligo would be: $(n)_6$tacGTACGTTGGTGCCCGCTGCT [SEQ ID NO.3]. The 3' oligo would be:

ACCTCGGAGGGACCGGGGGcccatag$(n)_2$ [SEQ ID NO.4].

Uppercase letters represent nucleotides which comprise VEGF-A sequence.

Lowercase letters represent nucleotides added to create appropriate restriction sites for cloning.

The PCR product will be endonuclease restricted with SnaB1 (cleaves TAC/GTA) and EcoR5 (cleaves GAT/ATC).

Note that the 3' oligo does not include the most 5' nucleotide from Exon 6b. This nucleotide will be added to the Exon 8 thereby completing the requisite sequence and preserving the proper reading frame.

b. If the replacement exon is directly synthesized, the oligos will be:

$(n)_6$tacGTACGTTGGTGCCCGCTGCTGTCTAATGC CCTGGAGCCTCCCTGGCCCCCgggatatc$(n)_2$ [SEQ ID NO:5] and its reverse complement.

After complementary oligonucleotides are annealed, they will be endonuclease restricted with SnaB1 (cleaves TAC/GTA) and EcoR5 (cleaves GAT/ATC).

6. After gel purification, ligate the replacement into the truncated, linearized VEGF-A_{145}. The product, pE-1–5/6b, contains Exons 1–5 and all but the most 3' nucleotide of Exon 6b.

7. Screen for proper orientation by restriction mapping.

8. Linearize pE1–5/6b. Endonuclease restriction with SmaI (cleaves at CCC/GGG) and NotI (cleaves at GC/GGCCGC). NotI will cleave 3' of the VEGF-A sequence in the pCRII-vector.

9. Generate Exon 8 by synthesis of the oligonucleotide: AATGTGACAAGCCGAGGCGGTGATGAATGAAT GAGGATGCGGCCGCAAAAGGAA[SEQ ID NO. 6] and its reverse complement. Those of ordinary skill in the art will recognize that this exon may also be synthesized using preferred codons of the cell type in which the transgene will be expressed.

10. After annealing, add 5' phosphates with T4 polynucleotide kinase.

11. Ligate the synthesized Exon 8 into the linearized vector p1–5/6b.

12. Screen for proper orientation by restriction mapping and confirm insert integrity by sequencing.

EXAMPLE II

Expression of VEGF-A_{138}

This recombinant VEGF-A_{138} cDNA is used to construct fa recombinant baculovirus containing the VEGF-A_{138} cDNA. The virus is used to infect Sf9 cells as described for VEGF-A_{165} by Cohen, T., et al. *Growth Factors*. 7:131–138, 1992, incorporated herein by reference. Most of the VEGF-A_{138} produced by the infected Sf9 cells should be found in the conditioned medium. Dithiotreitol may be used to dissociate VEGF-A_{138} dimers for purification. VEGF-A_{138} is partially purified using heparin-sepharose. The protein is eluted from the column using a stepwise salt gradient. The recombinant VEGF-A_{138} is biologically active and induces the proliferation of human umbilical vein derived endothelial cells (HUVEC cells). Other 6b modified VEGF-A proteins can be expressed using analogous methods.

EXAMPLE III

Proliferation of Endothelial Cells and Angiogenesis

To confirm that VEGF-A_{138} can induce angiogenesis in vivo, the VEGF-A_{138} DNA is subcloned into the BamHI site of the mammalian expression vector MIRB using the technique described by Macarthur, C. A., et al. *Cell Growth Differ.* 6, 817–825, 1995, which is incorporated herein by reference. The MIRB/VEGF-A_{138} plasmid is transfected into BHK-21 hamster kidney derived cells (a cell line that does not produce VEGF-A_{145}), and stable cell lines producing VEGF-A_{138} are isolated. The VEGF-A_{138} produced by the mammalian cells is biologically active and is secreted into the growth medium. The VEGF-A_{138} expressing cells are embedded in alginate beads, and the beads are implanted under the skin of Balb/c mice using the method described by Plunkett, M. L., et. al. *Lab. Invest.* 62, 510–517, 1990, which is incorporated herein by reference. Alginate pellets containing the entrapped cells are removed after four days and photographed. Clusters of alginate beads containing VEGF-A_{138} expressing cells are dark red with blood, while beads containing cells transfected with vector alone have a much lower content of blood. When examined under higher magnification, pellets containing VEGF-A_{138} producing cells appear much more vascularized than pellets containing control cells. These results are consistent with the expected

behavior of a vascular cell proliferation or angiogenesis-promoting factor.

EXAMPLE IV

Gene-Transfer-Mediated Angiogenesis Therapy Using VEGF-A_{138}

DNA encoding VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein is used for gene-transfer-mediated angiogenesis therapy as described, for example, in International Patent Application No. PCT/US96/02631, published Sep. 6, 1996, as WO 96/26742, hereby incorporated by, reference herein in its entirety.

Adenoviral Constructs

A helper independent replication deficient human adenovirus 5 system may be used for gene-transfer. A nucleic acid molecule coding for VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein may be cloned into the polylinker of plasmid ACCMVPLPA which contains the CMV promoter and SV40 polyadenylation signal flanked by partial adenoviral sequences from which the E1A and E1B genes (essential for viral replication) have been deleted. This plasmid is co-transferred (lipofection) into 293 cells with plasmid JM17 which contains the entire human adenoviral 5 genome with an additional 4.3 kb insert making pJM17 too large to be encapsulated. Homologous rescue recombination results in adenoviral vectors containing the transgene in the absence of E1A/E1B sequences. Although these recombinants are nonreplicative in mammalian cells, they can propagate in 293 cells which have been transformed with E1A/E1B and provided these essential gene products in trans. Transfected cells are monitored for evidence of cytopathic effect which usually occurs 10–14 days after transfection. To identify successful recombinants, cell supernatant from plates showing a cytopathic effect is treated with proteinase K (50 mg/ml with 0.5% sodium dodecyl sulfate and 20 mM EDTA) at 56° C. for 60 minutes, phenol/chloroform extracted and ethanol precipitated. Successful recombinants are then identified with PCR using primers (*Biotechniques*, 15:868–72, 1993) complementary to the CMV promoter and SV40 polyadenylation sequences to amplify the VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein nucleic acid insert and primers (*Biotechniques*, 15:868–72, 1993) designed to concomitantly amplify adenoviral sequences. Successful recombinants then are plaque purified twice. Viral stocks are propagated in 293 cells to titers ranging between 10^{10} and 10^{12} viral particles, and are purified by double CsCl gradient centrifugation prior to use. The system used to generate recombinant adenoviruses imposed a packing limit of 5 kb for transgene inserts. The VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein genes, driven by the CMV promoter and with the SV40 polyadenylation sequences are well within the packaging constraints. Recombinant vectors are plaque purified by standard procedures. The resulting viral vectors are propagated on 293 cells to titers in the 10^{10}–10^{12} viral particles range. Cells are infected at 80% confluence and harvested at 36–48 hours. After freeze-thaw cycles the cellular debris is pelleted by standard centrifugation and the virus further purified by double CsCl gradient ultracentrifugation (discontinuous 1.33/1.45 CsCl gradient: cesium prepared in 5 mM Tris, 1 mM EDTA (pH 7.8); 90,000×g (2 hr), 105,000×g (18 hr)). Prior to in vivo injection, the viral stocks are desalted by gel filtration through Sepharose columns such as G25 Sephadex. The resulting viral stock has a final viral titer approximately in the 10^{10}–10^{12} viral particles range. The adenoviral construct should thus be highly purified, with no wild-type (potentially replicative) virus.

Porcine Ischemia Model for Angiogenesis

A left thoracotomy is performed on domestic pigs (30–40 kg) under sterile conditions for instrumentation. (Hammond, et al., *J. Clin Invest* 92:2644–52, and Roth, et al., *J. Clin. Invest.* 91:939–49, 1993). Catheters are placed in the left atrium and aorta, providing a means to measure regional blood flow, and to monitor pressures. Wires are sutured on the left atrium to permit ECG recording and atrial pacing. Finally, an ameroid is placed around the proximal LCx. After a stable degree of ischemia develops, the treatment group receives an adenoviral construct that includes a VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene driven by a CMV promoter. Control animals receive gene transfer with an adenoviral construct that includes a reporter gene, lacZ, driven by a CMV promoter.

Studies are initiated 35±3 days after ameroid placement, at a time when collateral vessel development and pacing-induced dysfunction are stable (Roth, et al., *Am. J. Physiol* 253: 1-11279–1288, 1987, and Roth, et al., *Circulation* 82:1778–89). Conscious animals are suspended in a sling and pressures from the LV, LA and aorta, and electrocardiogram are recorded in digital format on-line (at rest and during atrial pacing at 200 bpm). Two-dimensional and M-mode images are obtained using a Hewlett Packard ultrasound imaging system. Images are obtained from a right parasternal approach at the mid-papillary muscle level and recorded on VHS tape. Images are recorded with animals in a basal state and again during right atrial pacing (HR=200 bpm). These studies are performed one day prior to gene transfer and repeated 14±1 days later. Rate-pressure products and left atrial pressures should be similar in both groups before and after gene transfer, indicating similar myocardial oxygen demands and loading conditions. Echocardiographic measurements are made using standardized criteria (Sahn, et al., *Circulation* 58:1072, 1978). End-diastolic wall thickness (EDWTh) and end-systolic wall thickness (ESWTh) are measured from 5 continuous beats and averaged. Percent wall thickening (% WTh) is calculated [(EDWTh-ESWTh)/EDWTh]×100. Data should be analyzed without knowledge of which gene the animals had received. To demonstrate reproducibility of echocardiographic measurements, animals should be imaged on two consecutive days, showing high correlation (r^2=0.90; p=0.005).

35±3 days after ameroid placement, well after ameroid closure, but before gene transfer, contrast echocardiographic studies are performed using the contrast material (Levovist) which is injected into the left atrium during atrial pacing (200 bpm). Studies are repeated 14±1 days after gene transfer. Peak contrast intensity is measured from the video images using a computer-based video analysis program (Color_Vue II, Nova Microsonics, Indianapolis, Ind.), that provides an objective measure of video intensity. The contrast studies are analyzed without knowledge of which gene the animals have received.

At completion of the study, animals are anesthetized and midline thoracotomy performed. The brachycephalic artery is isolated, a canula inserted, and other great vessels ligated. The animals receive intravenous heparin (10,000 IU) and papaverine (60 mg). Potassium chloride is given to induce diastolic cardiac arrest, and the aorta cross-clamped. Saline is delivered through the brachycephalic artery cannula (120 mm Hg pressure), thereby perfuming the coronary arteries. Glutaraldehyde solution (6.25%, 0.1 M cacodylate buffer) was perfused (120 mm Hg pressure) until the heart is well fixed (10–15 min). The heart is then removed, the beds identified using color-coded dyes injected anterograde through the left anterior descending (LAD), left circumflex

(LCx), and right coronary arteries. The ameroid is examined to confirm closure. Samples taken from the normally perfused and ischemic regions are divided into thirds and the endocardial and epicardial thirds are plastic-imbedded. Microscopic analysis to quantitate capillary number is conducted as previously described (Mathieu-Costello, et al., *Am. J. Physiol* 359:H204, 1990). Four 1 μm thick transverse sections are taken from each subsample (endocardium and epicardium of each region) and point-counting is used to determine capillary number per fiber number ratio at 400x magnification. Twenty to twenty-five high power fields are counted per subsample. Within each region, capillary number to fiber number rations should be similar in endocardium and epicardium so the 40–50 field per region should be averaged to provide the transmural capillary to fiber number ratio.

To establish that improved regional function and blood flow result from transgene expression, PCR and RT-PCR may be used to detect transgenic VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein DNA and mRNA in myocardium from animals that have received $VEGF_{38}$, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene transfer. Using a sense primer to the CMV promoter [GCAGAGCTCGTTTAGTGAAC] and antisense [SEQ ID NO. 7] primer to the internal VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene sequence PCR is used to amplify the expected 500 bp fragment. Using a sense primer to the beginning of the VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein sequence and an antisense primer to the internal VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene sequence RT-PCR is used to amplify the expected 400 bp fragment.

Finally, using an antibody directed against $VEGF_{145}$ VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein, protein expression maybe demonstrated 48 hours as well as 14±1 days after gene transfer in cells and myocardium from animals that have received gene transfer with a VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene.

The helper independent replication deficient human adenovirus 5 system can be employed to prepare transgene containing vectors. The material injected in vivo should be highly purified and should preferably contain no wild-type (replication competent) adenovirus. Thus adenoviral infection and inflammatory infiltration in the heart are minimized. By injecting the material directly into the lumen of the coronary artery by coronary catheters, it is possible to target the gene effectively. When delivered in this manner there should be no transgene expression in hepatocytes, and viral RNA should not be found in the urine at any time after intracoronary injection.

Injection of the construct (4.0 ml containing about 10^{11} viral particles of adenovirus) can be performed by injecting 2.0 ml into both the left and right coronary arteries (collateral flow to the LCx bed appeared to come from both vessels). Animals are anesthetized, and arterial access acquired via the right carotid by cut-down; a 5F Cordis sheath is then placed. A 5F Multipurpose (A2) coronary catheter is used to engage the coronary arteries. Closure of the LCx ameroid is confirmed by contrast injection into the left main coronary artery. The catheter tip is then placed 1 cm within the arterial lumen so that minimal material is lost to the proximal aorta during injection. This procedure is carried out for each of the pigs.

Once gene transfer is performed, three strategies are used to establish successful incorporation and expression of the gene: (1) Some constructs may include a reporter gene (lacZ); (2) myocardium from the relevant beds is sampled, and immunoblotting is performed to quantitate the presence of $VEGF_{145}$ protein; and (3) PCR is used to detect VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein mRNA and DNA.

The regional contractile function data obtained should show that control pigs show a similar degree of pacing-induced dysfunction in the ischemic region before and 14±1 days after gene transfer. In contrast, pigs receiving VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene transfer should show an increase in wall thickening in the ischemic region during pacing, demonstrating that VEGF-A_{138}, VEGF-A_{162}, VEGF-A_{182}, or other 6b-modified VEGF protein gene transfer in accordance with the invention is associated with improved contraction in the ischemic region during pacing. Wall thickening in the normally perfused region (the interventricular septum) should be normal during pacing and unaffected by gene transfer. The percent decrease in function measured by transthoracic echocardiography should be very similar to the percentage decrease measured by sonomicrometry during atrial pacing in the same model (Hammond, et al. *J. Clin. Invest.* 92:2644, 1993), documenting the accuracy of echocardiography for the evaluation of ischemic dysfunction.

Although preferred embodiments are specifically described herein, it will be appreciated that many modifications and variations of the present invention are possible in light of the above teachings and within the purview of the appended claims without departing from the spirit and intended scope of the invention.

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Glu

<210> SEQ ID NO 11
<211> LENGTH: 86
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 11

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atcacgaagg tgagtccccc tggctg 86

<210> SEQ ID NO 12
<211> LENGTH: 65
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 12

Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu
1 5 10 15

-continued

Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile
20 25 30

Phe Lys Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn
35 40 45

Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met
50 55 60

Gln
65

<210> SEQ ID NO 13
<211> LENGTH: 231
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 13

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aatcgagacc ctggtggaca tcttccagga gtaccctgat gagatcgagt acatcttcaa 120

gccatcctgt gtgcccctga tgcgatgcgg gggctgctgc aatgacgagg gcctggagtg 180

tgtgcccact gaggagtcca acatcaccat gcaggtgggc atctttggga a 231

<210> SEQ ID NO 14
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 14

Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met Ser
1 5 10 15

Phe Leu Gln His Asn Lys Cys Glu Cys
20 25

<210> SEQ ID NO 15
<211> LENGTH: 111
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 15

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tgagcttcct acagcacaac aaatgtgaat gcaggtgagg atgtagtcac g 111

<210> SEQ ID NO 16
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 16

Pro Lys Lys Asp Arg Ala Arg Gln Glu
1 5

<210> SEQ ID NO 17
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 17

ctccctaccc attgcagacc aaagaaagat agagcaagac aagaaaagta agtggccctg 60

actt 64

<210> SEQ ID NO 18

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<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 18

Lys Ser Val Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys
1 5 10 15

Ser Arg Tyr Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu
20 25 30

Met Pro Trp Ser Leu Pro Gly Pro
35 40

<210> SEQ ID NO 19
<211> LENGTH: 140
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 19

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aagaaatccc ggtataagtc ctggagcgtg tacgttggtg cccgctgctg tctaatgccc 120

tggagcctcc ctggccccca 140

<210> SEQ ID NO 20
<211> LENGTH: 43
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 20

Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp
1 5 10 15

Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys
20 25 30

Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys
35 40

<210> SEQ ID NO 21
<211> LENGTH: 166
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<213> ORGANISM: Homo sapien

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caagatccgc agacgtgtaa atgttcctgc aaaaacacag actcgcgttg caaggcgagg 120

cagcttgagt taaacgaacg tacttgcagg ttggttccca gaggca 166

<210> SEQ ID NO 22
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 22

Cys Asp Lys Pro Arg Arg
1 5

<210> SEQ ID NO 23
<211> LENGTH: 138
<212> TYPE: DNA
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<400> SEQUENCE: 23

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tccctcaggg tttcgggaac cagatctctc accaggaaag actgatacag aacgatcgat 120

acagaaacca cgctgccg 138

<210> SEQ ID NO 24
<211> LENGTH: 164
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 24

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu
1 5 10 15

Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly
20 25 30

Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln
35 40 45

Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu
50 55 60

Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu
65 70 75 80

Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro
85 90 95

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His
100 105 110

Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys
115 120 125

Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Tyr Val Gly
130 135 140

Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro Gln Cys Asp
145 150 155 160

Lys Pro Arg Arg

<210> SEQ ID NO 25
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<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 25

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gccaagtggt cccaggctgc acccatggca gaaggaggag ggcagaatca tcacgaagtg 120

gtgaagttca tggatgtcta tcagcgcagc tactgccatc caatcgagac cctggtggac 180

atcttccagg agtaccctga tgagatcgag tacatcttca agccatcctg tgtgcccctg 240

atgcgatgcg ggggctgctg caatgacgag ggcctggagt gtgtgcccac tgaggagtcc 300

aacatcacca tgcagattat gcggatcaaa cctcaccaag gccagcacat aggagagatg 360

agcttcctac agcacaacaa atgtgaatgc agaccaaaga aagatagagc aagacaagaa 420

aagtacgttg gtgcccgctg ctgtctaatg ccctggagcc tccctggccc ccaatgtgac 480

aagccgaggc ggtga 495

<210> SEQ ID NO 26
<211> LENGTH: 208
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 26

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Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu
1 5 10 15

Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly
20 25 30

Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln
35 40 45

Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu
50 55 60

Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu
65 70 75 80

Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro
85 90 95

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His
100 105 110

Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys
115 120 125

Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Tyr Val Gly
130 135 140

Ala Arg Cys Cys Leu Met Pro Trp Ser Leu Pro Gly Pro His Pro Cys
145 150 155 160

Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln
165 170 175

Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg
180 185 190

Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg
195 200 205

<210> SEQ ID NO 27
<211> LENGTH: 627
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 27

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gccaagtggt cccaggctgc acccatggca gaaggaggag ggcagaatca tcacgaagtg 120

gtgaagttca tggatgtcta tcagcgcagc tactgccatc caatcgagac cctggtggac 180

atcttccagg agtaccctga tgagatcgag tacatcttca agccatcctg tgtgcccctg 240

atgcgatgcg ggggctgctg caatgacgag ggcctggagt gtgtgcccac tgaggagtcc 300

aacatcacca tgcagattat gcggatcaaa cctcaccaag gccagcacat aggagagatg 360

agcttcctac agcacaacaa atgtgaatgc agaccaaaga aagatagagc aagacaagaa 420

aagtacgttg gtgcccgctg ctgtctaatg ccctggagcc tccctggccc ccatcccctgt 480

gggccttgct cagagcggag aaagcatttg tttgtacaag atccgcagac gtgtaaatgt 540

tcctgcaaaa acacagactc gcgttgcaag gcgaggcagc ttgagttaaa cgaacgtact 600

tgcagatgtg acaagccgag gcggtga 627

<210> SEQ ID NO 28
<211> LENGTH: 188
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 28

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

-continued

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1               5                   10                  15

Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly
            20                  25                  30

Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln
        35                  40                  45

Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu
    50                  55                  60

Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu
65                  70                  75                  80

Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro
                85                  90                  95

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His
            100                 105                 110

Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys
        115                 120                 125

Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val
    130                 135                 140

Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr
145                 150                 155                 160

Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp
                165                 170                 175

Ser Leu Pro Gly Pro Gln Cys Asp Lys Pro Arg Arg
            180                 185


<210> SEQ ID NO 29
<211> LENGTH: 665
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

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gtgaagttca tggatgtcta tcagcgcagc tactgccatc caatcgagac cctggtggac     180

atcttccagg agtaccctga tgagatcgag tacatcttca agccatcctg tgtgccccctg    240

atgcgatgcg ggggctgctg caatgacgag ggcctggagt gtgtgcccac tgaggagtcc     300

aacatcacca tgcagattat gcggatcaaa cctcaccaag gccagcacat aggagagatg     360

agcttcctac agcacaacaa atgtgaatgc agaccaaaga aagatagagc aagacaagaa     420

aaaaaatcag ttcgaggaaa gggaaagggg caaaaacgaa agcgcaagaa atcccggtat     480

aagtcctgga gcgtgtacgt tggtgcccgc tgctgtctaa tgccctggag cctccctggc     540

ccccaatgtg acaagccgag gcggtgaccg ggcaggagga aggagcctcc ctcagggttt     600

cgggaaccag atctctcacc aggaaagact gatacagaac gatcgataca gaaaccacgc     660

tgccg                                                                 665
```

We claim:

1. A nucleic acid sequence, encoding a 6b- modified human VEGF-A polypeptide, comprising exons 1–5, 6b and 8 of VEGF-A and lacking exon 7 of VEGF-A.

2. The nucleic acid sequence of claim 1, encoding the amino acid sequence of SEQ ID NO 24.

3. The nucleic acid sequence of claim 1, encoding the amino acid sequence of SEQ ID NO 28.

4. An expression vector comprising a nucleic acid according to claim 1.

5. The expression vector according to claim 4, wherein said nucleic acid encodes the amino acid sequence of SEQ ID NO 24.

6. The expression vector according to claim 4, wherein said nucleic acid encodes the amino acid sequence of SEQ ID NO 28.

* * * * *

PATENT COOPERATION TREATY

RECEIVED

OCT 16 2006

GENENTECH, INC.
LEGAL DEPT.

CCC/TTP/EC

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

PLEASURE, Irene
c/o GENENTECH, INC.
1 Dna Way
Ms 49
South San Francisco, California 94080
ETATS-UNIS D'AMERIQUE

Date of mailing *(day month year)* 29 September 2006 (29.09.2006)	
Applicant's or agent's file reference P2358R1 (WO)	IMPORTANT NOTIFICATION
International application No. PCT/US2006/023318	International filing date *(day month year)* 16 June 2006 (16.06.2006)
International publication date *(day month year)* Not yet published	Priority date *(day month year)* 17 June 2005 (17.06.2005)
Applicant GENENTECH, INC. et al	

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. *(If applicable)* The letters "NR" appearing in the right-hand column denote a **priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau** under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

3. *(If applicable)* **An asterisk (*)** appearing next to a date of receipt, in the right-hand column, denotes a **priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b)** (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
17 June 2005 (17.06.2005)	60/691,909	US	11 August 2006 (11.08.2006)
21 April 2006 (21.04.2006)	60/794,008	US	11 August 2006 (11.08.2006)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Simin Baharlou Facsimile No. +41 22 338 71 30 Telephone No. +41 22 338 99 32

22

UNITED STATES PATENT AND TRADEMARK OFFICE

UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

JULY 13, 2006

PTAS

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IRENE PLEASURE
1 DNA WAY
SOUTH SAN FRANCISCO, CA 94080

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RECORDATION DATE: 07/13/2006

REEL/FRAME: 017926/0621
NUMBER OF PAGES: 6

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).
DOCKET NUMBER: PR2358

ASSIGNOR:
SEMBA, CHARLES

DOC DATE: 06/08/2006

ASSIGNOR:
BUNTING, STUART

DOC DATE: 06/12/2006

ASSIGNOR:
BREEN, TIMOTHY J.

DOC DATE: 06/20/2006

ASSIGNEE:
GENENTECH INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CALIFORNIA

94080

SERIAL NUMBER: 60794008
PATENT NUMBER:
TITLE: WOUND HEALING

FILING DATE: 04/21/2006
ISSUE DATE:

ASSIGNMENT SERVICES BRANCH
PUBLIC RECORDS DIVISION

PATENT ASSIGNMENT

Electronic Version v1.1
Stylesheet Version v1.1

07/13/2006
500125252

SUBMISSION TYPE:	NEW ASSIGNMENT
NATURE OF CONVEYANCE:	ASSIGNMENT

CONVEYING PARTY DATA

Name	Execution Date
Charles Semba	06/08/2006
Stuart Bunting	06/12/2006
Timothy J. Breen	06/20/2006

RECEIVING PARTY DATA

Name:	Genentech Inc.
Street Address:	1 DNA Way
City:	South San Francisco
State/Country:	CALIFORNIA
Postal Code:	94080

PROPERTY NUMBERS Total: 1

Property Type	Number
Application Number:	60794008

CORRESPONDENCE DATA

Fax Number: (650)952-9881
Correspondence will be sent via US Mail when the fax attempt is unsuccessful.
Phone: 650/467-6829
Email: patty@gene.com
Correspondent Name: Irene Pleasure
Address Line 1: 1 DNA Way
Address Line 4: South San Francisco, CALIFORNIA 94080

ATTORNEY DOCKET NUMBER:	PR2358
NAME OF SUBMITTER:	Irene Pleasure

Total Attachments: 4
source=Assignment60794008#page1.tif
source=Assignment60794008#page2.tif

CH $40.00 60794008

IN WITNESS WHEREOF we undersign as follows:

South San Francisco

Dated: _____

_____
Charles Semba

South San Francisco

Dated: 6/12/06

_____
Stuart Bunting

Half Moon Bay

Dated: _____

_____
Timothy J. Breen

IN WITNESS WHEREOF we undersign as follows:

South San Francisco

Dated: _____

_____
Charles Semba

South San Francisco

Dated: _____

_____
Stuart Bunting

Half Moon Bay

Dated: 6-20-06

Timothy J. Breen
Timothy J. Breen

UNITED STATES PATENT AND TRADEMARK OFFICE
UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
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DECEMBER 07, 2005

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GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CALIFORNIA 94080

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RECORDATION DATE: 10/07/2005

REEL/FRAME: 016859/0809
NUMBER OF PAGES: 3

BRIEF: ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

ASSIGNOR:
SEMBA, CHARLES

DOC DATE: 10/04/2005

ASSIGNOR:
BUNTING, STUART

DOC DATE: 10/04/2004

ASSIGNOR:
BREEN, TIMOTHY J.

DOC DATE: 10/04/2005

ASSIGNEE:
GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CALIFORNIA

94080-4990

SERIAL NUMBER: 60691909
PATENT NUMBER:
TITLE: WOUND HEALING

FILING DATE: 06/17/2005
ISSUE DATE:

ALLYSON PURNELL, EXAMINER
ASSIGNMENT DIVISION
OFFICE OF PUBLIC RECORDS

10/07/2005
700216298

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Patent and Trademark Office

RECORDATION FORM COVER SHEET
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Atty Dkt No: FR2258

1. Name of conveying party(ies):

Charles Semba
Stuart Bunting
Timothy J. Breen

Additional name(s) of conveying party(ies) attached? _ Yes X No

2. Name and address of receiving party(ies):

Name: GENENTECH, INC.
Internal Address:
Street Address: 1 DNA Way
City: South San Francisco State: California
ZIP: 94080-4990

Additional name(s) & address(es) attached? _ Yes X No

3. Nature of conveyance:

X Assignment Merger
Security Agreement
Change of Name
Other:

Execution Date: October 4, 2005, October 4, 2005, October 4, 2005

4. Application number(s) or patent number(s):

If this document is being filed together with a new application, the execution date of the application is

A. Patent Application No.(s)
60/691,909

B. Patent No.(s)

Additional numbers attached? _ Yes X No

5. Name and address of party to whom correspondence concerning document should be mailed:

Irene Pleasure
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080

6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 3.41): $ 40.00
_ Enclosed
X Authorized to be charged to deposit account

8. Deposit account number:
07-0630

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9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct and any attached copy is a true copy of the original document.

Irene Pleasure
Name of Person Signing
Registration No. 45,506

Signature

October 7, 2005
Date

Total number of pages comprising cover sheet, attachments and document: 3

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CH $40.00 070690 60691909

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Patent and Trademark Office

RECORDATION FORM COVER SHEET
PATENTS ONLY

Atty Dkt No: **PR2258**

Commissioner for Patents: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies):

Charles Semba
Stuart Bunting
Timothy J. Breen

Additional name(s) of conveying party(ies) attached? _ Yes X No

2. Name and address of receiving party(ies):

Name: GENENTECH, INC.

Internal Address:

Street Address: 1 DNA Way

City: South San Francisco State: California

ZIP: 94080-4990

Additional name(s) & address(es) attached? _ Yes X No

3. Nature of conveyance:

X Assignment Merger

Security Agreement

Change of Name

Other:

Execution Date: October 4, 2005, October 4, 2005, October 4, 2005

4. Application number(s) or patent number(s):

If this document is being filed together with a new application, the execution date of the application is:

A. Patent Application No.(s)

60/691,909

B. Patent No.(s)

Additional numbers attached? _ Yes X No

5. Name and address of party to whom correspondence concerning document should be mailed:

Irene Pleasure
GENENTECH, INC.
1 DNA Way
South San Francisco, CA 94080

6. Total number of applications and patents involved: 1

7. Total fee (37 CFR 3.41): $ 40.00

_ Enclosed

X Authorized to be charged to deposit account

8. Deposit account number:
07-0630

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Name of Person Signing
Registration No. 45,506

Irene Pleasure
Signature

October 7, 2005
Date

Total number of pages comprising cover sheet, attachments and document: 3

Revised (10/11/95)

238

ASSIGNMENT

WHEREAS, Charles Semba, a citizen of USA, residing at 1925 Byron Street, Palo Alto, CA 94301, and Stuart Bunting, a citizen of GB, residing at 220 Miramontes Ave., Half Moon Bay, CA 94019, and Timothy J. Breen, a citizen of USA, residing at 432 Coronado Ave., Half Moon Bay, CA 94019, (hereinafter "ASSIGNORS"), have invented a new and useful invention in

Wound Healing

for which a provisional application Serial No. 60/691,909 (Docket No. PR2258) has been filed by them on June 17, 2005; and

WHEREAS, GENENTECH, INC., a corporation organized and existing under and by virtue of the laws of the State of Delaware, having a place of business at 1 DNA Way, South San Francisco, California 94080-4490, is desirous of acquiring an interest in and to said invention, and in and to the Letters Patents to be obtained therefor;

NOW, THEREFORE, be it known by all whom it may concern;

That for good and valuable consideration the receipt of which is hereby acknowledged, the said ASSIGNORS have and do hereby sell, assign, transfer and set over unto the said GENENTECH, INC., its successors and assigns, the full and exclusive right, title and interest including all rights under the Paris Convention for the Protection of Industrial Property, in and to said invention, and in and to any and all Letters Patents to be granted and issued therefor or any continuation, division, renewal, or substitute thereof, and as to Letters Patents any reissue or re-examination thereof, not only for, to, and in the United States of America, its territories and possessions, but for, to and in all other countries; and it has been and is hereby authorized and requested that the appropriate government agencies issue said Letters Patents to said GENENTECH, INC., in accordance with this Assignment.

Said ASSIGNORS covenant and agree to cooperate with GENENTECH, INC., to enable said GENENTECH, INC. to enjoy to the fullest extent the right, title and interest herein conveyed in the United States and foreign countries. Such cooperation by said ASSIGNORS includes prompt production of pertinent facts and documents, giving of testimony, execution of petitions, oaths, specifications, declarations or other papers, and other assistance all to the extent deemed necessary or desirable by said GENENTECH, INC., (a) for perfecting the right, title and interest herein conveyed; (b) for prosecuting any of said applications; (c) for filing and prosecuting applications for reissuance of any of said patents; (d) for interference or other priority proceedings involving said invention; and (e) for legal proceedings involving said invention and any applications therefor and any patents granted thereon, including without limitation opposition proceedings, cancellation proceedings, priority contests, public use proceedings, infringement actions and court actions; provided, however, that the expense incurred by said ASSIGNORS in providing such cooperation shall be paid for by said GENENTECH, INC.

The terms and covenants of this assignment shall inure to the benefit of said GENENTECH, INC., its successors, assigns and other legal representatives, and shall be binding upon said ASSIGNORS, their respective heirs, legal representatives and assigns.

Said ASSIGNORS hereby warrant and represent that they have not entered and will not enter into any assignment, contract, or understanding in conflict herewith.

IN WITNESS WHEREOF we undersign as follows;

South San Francisco

Dated: October 4, 2005

Charles Semba

South San Francisco

Dated: 10/4/2005

Stuart Bunting

South San Francisco

Dated: 10/4/2005

Timothy J. Breen

#190441

PCT REQUEST

Original (for SUBMISSION)

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.005.180 MT/FOP 20060401/0.20.4rc.2.7
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	Applicant's or agent's file reference	P2358R1
I	Title of Invention	WOUND HEALING
II	Applicant	
II-1	This person is	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	GENENTECH, INC.
II-5	Address	1 DNA Way MS 49 South San Francisco, California 94080 United States of America
II-6	State of nationality	US
II-7	State of residence	US
II-8	Telephone No.	650-225-1000
II-9	Facsimile No.	650-952-9881
III-1	Applicant and/or inventor	
III-1-1	This person is	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	BREEN, Timothy J.
III-1-5	Address	432 Coronado Avenue Half Moon Bay, California 94019 United States of America
III-1-6	State of nationality	US
III-1-7	State of residence	US

PCT REQUEST

Original (for SUBMISSION)

III-2	Applicant and/or inventor	
III-2-1	This person is	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	BUNTING, Stuart
III-2-5	Address	220 Miramontes Avenue Half Moon Bay, California 94019 United States of America
III-2-6	State of nationality	GB
III-2-7	State of residence	US
III-3	Applicant and/or inventor	
III-3-1	This person is	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	SEMBA, Charles P.
III-3-5	Address	1925 Byron Street Palo Alto, California 94301 United States of America
III-3-6	State of nationality	US
III-3-7	State of residence	US
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	PLEASURE, Irene
IV-1-2	Address	c/o GENENTECH, INC. 1 DNA Way MS 49 South San Francisco, California 94080 United States of America
IV-1-3	Telephone No.	650-467-6829
IV-1-4	Facsimile No.	650-952-9881
IV-1-6	Agent's registration No.	45,506
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	ATULYA,Agarwal R.; ANDRUS,Alex; BARNES,Elizabeth M.; CARPENTER,David A.; COBURN,Cara M.; CONLEY,Deirdre L.; CUI,Steven X.; ELLIOTT,Jennifer; EVANS,David; HASAK,Janet E.; JOHNSTON,Sean A.; KALINOWSKI,Grant; KRESNAK,Mark T.; KUBINEC,Jeffrey S.; LEE,Wendy M.; MARSCHANG,Diane L.;

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		NAIK,Paul; PASQUALONE,Danielle; SCHWARTZ,Timothy R.; SHERMAN,Daniel; SHIN,Elinor K.; SVOBODA,Craig G.; TAN,Lee K.; YEUNG,Bonnie
V	DESIGNATIONS	
V-1	The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.	
VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	17 June 2005 (17.06.2005)
VI-1-2	Number	60/691,909
VI-1-3	Country	US
VI-2	Priority claim of earlier national application	
VI-2-1	Filing date	21 April 2006 (21.04.2006)
VI-2-2	Number	60/794,008
VI-2-3	Country	US
VI-3	Priority document request The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1, VI-2
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)

VIII	Declarations	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	

PCT REQUEST

Original (for SUBMISSION)

IX	Check list	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	4	✓
IX-2	Description	48	-
IX-3	Claims	2	-
IX-4	Abstract	1	✓
IX-5	Drawings	1	-
IX-7	TOTAL	56	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-11	Copy of general power of attorney	✓	-
IX-17	PCT-SAFE physical media	-	✓
IX-19	Figure of the drawings which should accompany the abstract	NONE	
IX-20	Language of filing of the international application	English	
X-1	Signature of applicant, agent or common representative	Irene Pleasure	
X-1-1	Name (LAST, First)	PLEASURE, Irene	
X-1-2	Name of signatory		
X-1-3	Capacity		

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

State of California

SECRETARY OF STATE

APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. *Country: United States of America*
   This public document
2. *has been signed by AIDA MICLAT GUIAM*
3. *acting in the capacity of Notary Public, State of California*
4. *bears the seal/stamp of AIDA MICLAT GUIAM, Notary Public, State of California*

CERTIFIED

5. *At Sacramento, California*
6. *the 15th day of December 2005*
7. *by Deputy Secretary of State, State of California*
8. *No. 267083*
9. *Seal/Stamp:*

10. *Signature*

Secretary of State

BY

NP-24 A (REV. 4-05)

OSP 05 90772

20 NOV. 2005

LUZ DEYANIRA AVELLA ROMERO
NOTARIO CUARENTA Y OCHO ENCARGADO

CALIFORNIA ALL-PURPOSE ACKNOWLEDGMENT

State of California
County of San Mateo } ss.

On December 6, 2005 (Date) before me, Aida Miclat Guiam (Name and Title of Officer (e.g., "Jane Doe, Notary Public")),
personally appeared Timothy R. Schwartz (Name(s) of Signer(s)),

☐ personally known to me
☒ proved to me on the basis of satisfactory evidence

to be the person~~(s)~~ whose name~~(s)~~ is/~~are~~ subscribed to the within instrument and acknowledged to me that he/~~she/they~~ executed the same in his/~~her/their~~ authorized capacity~~(ies)~~, and that by his/~~her/their~~ signature~~(s)~~ on the instrument the person~~(s)~~, or the entity upon behalf of which the person~~(s)~~ acted, executed the instrument.

AIDA MICLAT GUIAM
Commission # 1455662
Notary Public - California
San Mateo County
My Comm. Expires Dec 9, 2007

WITNESS my hand and official seal.

Aida Miclat Guiam
Signature of Notary Public

OPTIONAL

Though the information below is not required by law, it may prove valuable to persons relying on the document and could prevent fraudulent removal and reattachment of this form to another document.

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Document Date: _____ Number of Pages: _____

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Capacity(ies) Claimed by Signer

Signer's Name: _____

☐ Individual
☐ Corporate Officer — Title(s): _____
☐ Partner — ☐ Limited ☐ General
☐ Attorney-in-Fact
☐ Trustee
☐ Guardian or Conservator
☐ Other: _____

Signer Is Representing: _____

RIGHT THUMBPRINT OF SIGNER
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20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

RECONOCIMIENTO TODO-PROPOSITO DE CALIFORNIA

Estado de California)
} ss.
Condado de San Mateo)

El 6 de diciembre de 2005, ante mi, AIDA MICLAT GUIAM, apareció personalmente Thimoty R. Schwartz

☐ a quien conozco personalmente

☒ quien demostró ante mi en base a evidencia satisfactoria ser la persona cuyo nombre esta suscrito en el instrumento y quien reconoció ante mi que ejecutó el mismo en su capacidad autorizada, y que por su firma en el instrumento la persona o la entidad a nombre de la cual la persona actuó, ejecutó el instrumento.

Hay un sello que dice:
AIDA MICLAT GUIAM
COMISIÓN # 14556612
Notario Público-California
Condado de San Mateo
Mi comisión expira Dic. 9 de 2007

Atestigua mi firma y sello oficial,

AIDA MICLAT GUIAM

----------**OPCIONAL**----------

Aunque la información en esta sección no es requerida por la ley, puede probar ser valiosa para las personas que se basan en el documento y puede prevenir la remoción y readhesión fraudulenta de esta forma a otro documento

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Nombre del firmante: _____

☐ Individuo
☐ Funcionario Corporativo – Titulo(s): _____
☐ Socio - ☐ Limitado ☐ General
☐ Abogado de hecho
☐ Fideicomisario
☐ Guardián o conservador
☐ Otro: _____

El firmante representa: _____

Huella Índice derecho del firmante
Parte alta del dedo aquí

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20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

TRADUCCION AL ESPAÑOL del Documento de Poder otorgado por **Genentech, Inc.** domiciliada en **1 DNA Way, South San Francisco, California 94080-4990, Estados Unidos de América**.

ESTADO DE CALIFORNIA
SECRETARIA DE ESTADO

APOSTILLA
(Convención de La Haya del 5 de Octubre de 1961)

1 País: Estados Unidos de América
Esta documento público
2. ha sido firmado por AIDA MICLAT GUIAM
3. actuando en capacidad de Notario Público, Estado de California
4. lleva el sello/timbre de AIDA MICLAT GUIAM, Notario Público Estado de California

CERTIFICADO

5. en Sacramento, California
6. el 15 de diciembre de 2005
7. por el Secretario de Estado Delegado, Estado de California
8. No. 267083
9. Sello/Timbre

10. Firma

(Firma)
Secretario de Estado

NOTARIO CUARENTA Y SEIS ENCARGADO
DEL CIRCULO DE BOGOTA, HAGO CONSTAR
QUE ESTA COPIA FOTOSTATICA COINCIDE CON
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20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

AUTORIZACION

Genentech, Inc. por la presente autoriza a cualquiera de Janet E. Hasak, Mark T. Kresnak, Jeffrey S. Kubinec y Thomity R. Schwartz, mientras permanezcan como empleados de Genentech, Inc., a ejecutar, a su nombre, cualquier solicitud asociada con presentación, procesamiento, consecución, y extensión y mantenimiento de concesión de solicitudes de patente y marca registrada en los Estados Unidos y países extranjeros, contratos relacionados con depósitos biológicos con la Colección Americana de Tipo de Cultivo, y cesiones a publicistas de derechos de autor que cubran los trabajos de autoría de los empleados de Genentech.

27 de Agosto de 2002
Fechado

(Firma)
Sean A. Johnston
Vicepresidente, Propiedad Intelectual
Genentech, Inc.

NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE PLENAMENTE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
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LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

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AUTHORIZATION

Genentech, Inc. hereby authorizes any of Janet E. Hasak, Mark T. Kresnak, Jeffrey S. Kubinec and Timothy R. Schwartz, for so long as they remain employees of Genentech, Inc., to execute, on its behalf, any submissions associated with the filing, prosecution, granting, and extension and maintenance of grant of patent and trademark applications in the U.S. and foreign countries, contracts relating to biological deposits with the American Type Culture Collection, and assignments to publishers of copyrights covering works authored by Genentech employees.

August 27, 2002
Dated

Sean Johnston

Sean A. Johnston
Vice President,Intellectual Property
Genentech, Inc.

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20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

Brigard & Castro

BOGOTA - COLOMBIA

PODER

Yo (nosotros)

domiciliado(s) en

Nombro(amos) a: **JUAN PABLO CADENA SARMIENTO** y/o **BEATRIZ JARAMILLO** y/o **CARLOS H. PALACIO EASTMAN** y/o **HECTOR GOMEZ MEJIA**, de Bogotá, Colombia, apoderados verdaderos y legales con poder especial, amplio y suficiente, para recabar de las oficinas y autoridades colombianas administrativas, judiciales y de policía, en cualquier instancia o recurso, la obtención de patentes de invención, registros de marcas, diseños industriales, modelos de utilidad, depósitos de nombres comerciales y enseñas, registros de derechos de autor y contratos sobre los mismos, obtención de certificados de obtentor de variedades vegetales, así como sus prórrogas, renovaciones, anotaciones de traspaso, cambios de nombre y/o domicilio; elaborar, tramitar y registrar contratos de licencia y licencias de cualesquiera clases; intervenir como demandante o como demandado en cualquier instancia y recurso ante cualquier juez, corporación, funcionario público o autoridad en acciones judiciales, administrativas y de policía tales como: oposiciones al registro de marcas; acciones de caducidad, cancelación y nulidad; acciones reivindicatorias, acciones derivadas de la obtención o explotación de licencias; acciones de competencia desleal; demandas penales; acciones de indemnización de perjuicios, reclamos y observaciones a solicitudes de patentes, diseños industriales, modelos de utilidad, en el ejercicio de medidas cautelares y en otras acciones o medidas similares.

A este efecto, lo(s) faculto(amos) para elevar solicitudes, formular descripciones, enmiendas, protestas, declaraciones, oposiciones, cancelaciones, nulidades, relaciones y reclamos; pagar impuestos, cuotas y gravámenes prescritos por la ley; renunciar o desistir a derechos concedidos o en curso de concesión; recibir toda clase de documentos y valores, dando el descargo respectivo, y, en general, para dar ante dichas autoridades todos los pasos necesarios al efecto indicado y tomar todas las medidas que creyeren conducentes al resguardo de mis (nuestros intereses), con todas las demás facultades legales necesarias.

Este poder comprende la facultad de ratificar o confirmar en mi (nuestro) nombre lo actuado así como la facultad de desistir, transigir, comprometer, y la de sustituirlo en todo o en parte y revocar sustituciones. Asimismo nombro(amos) a: **JUAN PABLO CADENA SARMIENTO** y/o **BEATRIZ JARAMILLO** y/o **CARLOS H. PALACIO EASTMAN** y/o **HECTOR GOMEZ MEJIA**,, domiciliado(s) en la Carrera 7 No. 16-56 Piso 9, mis (nuestros) apoderados en Colombia, con facultades para recibir notificaciones y designar apoderados judiciales o extrajudiciales.

Otorgado y firmado en

hoy de de

Firma

Nombre
Cargo

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BOGOTA, D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

POWER OF ATTORNEY

I (We) **Genentech, Inc.**
1 DNA Way

domiciled in **South San Francisco, California 94080-4990 USA**

Hereby appoints **JUAN PABLO CADENA SARMIENTO** and/or **BEATRIZ JARAMILLO** and/or **CARLOS H. PALACIO EASTMAN** and/or **HECTOR GOMEZ MEJIA**, of Bogotá, Colombia, my (our) true and lawful attorneys with special, ample and sufficient power to obtain from the Colombian administrative, judicial and police offices and authorities, in any instance, privileges of patents of invention, registration of trademarks, industrial designs and utility models; registration of commercial names and emblems, registration of copyright and contracts related thereto, certificates of obtainers of vegetables varieties, as well as extensions, renewals and recordal of assignments and changes of name and domicile related to the above; to prepare, prosecute and register licenses of any kind; to act as plaintiff or defendant in any instance or recourse, and before any judge, corporation, public official or authority in judicial, administrative and police actions, such as: oppositions to the registration of trademarks; caducity, cancellation and nullity actions; claim actions derived from the obtaining or explotation of licenses, action of unfair competition; criminal proceedings, actions for payment of damages: claims or observations on patent applications, models, industrial designs and utility models; in the exercise of preventive measures and in other actions or similar proceedings.

To that effect they hereby empowered to file applications, formulate descriptions, amendments, protests, declarations, oppositions, cancellations, nullities, appeals and claims; to pay taxes, quotas and assessments prescribed by law, renounce or waive the rights granted or in the process of granting; to receive all kinds of documents and valuables, giving the respective release of receipt, and, in general, to take before said authorities the necessary steps to such effect, and any measures that they may deem pertinent to the safeguard of my (our) interest(s) with all the other necessary legal powers.

This power includes the faculty to ratify or confirm desist, settle and compromise on my (our) behalf, and to substitute this power in whole or in part and to revoke substitutions. At the same time I (We) hereby appoints **JUAN PABLO CADENA SARMIENTO** and/or **BEATRIZ JARAMILLO** and/or **CARLOS H. PALACIO EASTMAN** and/or **HECTOR GOMEZ MEJIA**, domiciled in Carrera 7 No. 16-56, Piso 9, my (our) attorney(s) in fact, with powers to receive notifications and to designate judicial and extrajudicial attorneys.

Granted and signed in **South San Francisco, California USA**

this 6th day of December 2005

Signature

Name Timothy R. Schwartz, Ph.D.
Title Section Patent Counsel
Authorized Corporate Signatory
Genentech, Inc.

Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

This public document:

2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254030

9. Seal/Stamp:

10. Signature:

Harriet Smith Windsor

Secretary of State

DOMO NOTARIO CUARENTA Y SEIS (46) DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE PE... TENIDO A LA VISTA

BOGOTA D.C. ...

20 NOV. 2006

LUZ ESPER...

NOTARIO CUARENTA Y SEIS (46) ...

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE RESTATED CERTIFICATE OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE TWENTY-SECOND DAY OF JULY, A.D. 1999, AT 9 O'CLOCK A.M.

2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3900384

DATE: 05-24-05

COMO NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
BOGOTA D.C.
20 NOV. 2006
LUZ ESPERANZA AVELLA NUMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

AMENDED AND RESTATED

CERTIFICATE OF INCORPORATION

GENENTECH, INC.

Genentech, Inc. (the "**Corporation**"), a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware, does hereby amend the Certificate of Incorporation of the Corporation, which was originally filed on December 23, 1986 under the name "Genentech Delaware, Inc.", and subsequently amended.

The Certificate of Incorporation of the Corporation is hereby restated and further amended to read in its entirety as follows:

ARTICLE 1

The name of the Corporation is: Genentech, Inc.

ARTICLE 2

The address of the registered office of the Corporation in the State of Delaware is Corporation Service Company, 1013 Centre Road, City of Wilmington, County of New Castle, Delaware 19805. The name of its registered agent at such address is Corporation Service Company.

ARTICLE 3

The purpose of the Corporation is to engage in any lawful act or activity for which corporations may be organized under the General Corporation Law of the State of Delaware as the same exists or may hereafter be amended ("**Delaware Law**").

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 09:00 AM 07/22/1999
991302331 - 2112549

20 NOV. 2006
AVELLA ROMERO

ARTICLE 4

SECTION 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is four hundred million (400,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value $0.02 per share (**"Preferred Stock"**). Three hundred million (300,000,000) shares shall be designated common stock, par value $0.02 per share (**"Common Stock"**) The Common Stock of the Corporation shall be all of one class.

(b) The number of authorized shares of any class or classes of stock may be increased or decreased (but not below the number of shares thereof then outstanding) by the affirmative vote of a majority in voting power of the outstanding stock of the Corporation entitled to vote thereon irrespective of the provisions of Section 242(b)(2) of Delaware Law.

SECTION 4.02. *Common Stock.* (a) *Issuance and Consideration.* Any unissued or treasury shares of the Common Stock may be issued for such consideration as may be fixed in accordance with applicable law from time to time by the Board of Directors.

(b) *Dividends.* Subject to the rights of holders of the Preferred Stock, the holders of the Common Stock shall be entitled to receive, when and as declared by the Board of Directors, out of the assets of the Corporation which are by law available therefor, dividends payable either in cash, in property, or in shares of stock and the holders of the Preferred Stock shall not be entitled to participate in any such dividends (unless otherwise provided by the Board of Directors in any resolution providing for the issue of a series of Preferred Stock).

SECTION 4.03. *Preferred Stock.*

(a) *Series and Limits of Variations between Series.* Any unissued or treasury shares of the Preferred Stock may be issued from time to time in one or more series for such consideration as may be fixed from time to time by the Board of Directors. Before any shares of Preferred Stock of any particular series shall be issued, a certificate shall be filed with the Secretary of State of Delaware setting forth the designation, rights, privileges, restrictions, and conditions to be attached to the Preferred Stock of such series and such other matters as may be required, and the Board of Directors shall fix and determine, and is hereby expressly empowered to fix and determine, in the manner provided by law, the particulars of

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the shares of such series (so far as not inconsistent with the provisions of this Article 4 applicable to all series of Preferred Stock), including, but not limited to, the following:

(i) the distinctive designation of such series and the number of shares which shall constitute such series, which number may be increased (except where otherwise provided by the Board of Directors in creating such series) or decreased (but not below the number of shares thereof then outstanding) from time to time by like action of the Board of Directors;

(ii) the annual rate of dividends payable on shares of such series, the conditions upon which such dividends shall be payable and the date from which dividends shall be cumulative in the event the Board of Directors determines that dividends shall be cumulative;

(iii) whether such series shall have voting rights, in addition to the voting rights provided by law and, if so, the terms of such voting rights;

(iv) whether such series shall have conversion privileges and, if so, the terms and conditions of such conversion, including, but not limited to, provision for adjustment of the conversion rate upon such events and in such manner as the Board of Directors shall determine;

(v) whether or not the shares of such series shall be redeemable and, if so, the terms and conditions of such redemption, including the date or dates upon or after which they shall be redeemable, and the amount per share payable in case of redemption, which amount may vary under different conditions and at different redemption dates;

(vi) whether such series shall have a sinking fund for the redemption or purchase of shares of that series and, if so, the terms and amount of such sinking fund;

(vii) the rights of the shares of such series in the event of voluntary or involuntary liquidation, dissolution or winding up of the Corporation, and the relative rights of priority, if any, of payment of shares of that series; and

(viii) any other relative rights, preferences and limitations of such series.

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ARTICLE 5

SECTION 5.01. *Amendment of Bylaws by Directors.* (a) In furtherance and not in limitation of the powers conferred by statute, the Board of Directors is expressly authorized to make, alter, amend, repeal and rescind the bylaws of the Corporation, subject to Sections 5.01(b) and 5.01(c) hereof.

(b) Sections 2.02, 3.02, 3.03(a), 3.03(b), 3.04, 3.05, 3.14, and 5.06 of the bylaws may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 60% of the shares entitled to be voted thereon.

(c) Sections 3.03(c) and 3.15 of the bylaws may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 90% of the shares entitled to be voted thereon.

SECTION 5.02. *Amendment of Certificate of Incorporation.* Section 5.01(b) of this Certificate of Incorporation may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 60% of the shares entitled to be voted thereon. Section 5.01(c) and Articles 8 and 9 of this Certificate of Incorporation may only be altered, amended, repealed, or rescinded by the affirmative vote of holders of capital stock entitled to vote thereon representing more than 90% of the shares entitled to be voted thereon.

ARTICLE 6

SECTION 6.01. *Election of Directors by Holders of Preferred Stock.* During any period when the holders of any Preferred Stock or any one or more series thereof, voting as a class, shall be entitled to elect a specified number of directors, by reason of dividend arrearages or other provisions giving them the right to do so, then and during such time as such right continues (i) the then otherwise authorized number of directors shall be increased by such specified number of directors, and the holders of such Preferred Stock or such series thereof, voting as a class, shall be entitled to elect the additional directors so provided for, pursuant to the provisions of such Preferred Stock or series; (ii) each such additional director shall serve for such term, and have such voting powers, as shall be stated in the provisions pertaining to such Preferred Stock or series; and (iii) whenever the holders of any such Preferred Stock or series thereof are

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divested of such rights to elect a specified number of directors, voting as a class, pursuant to the provisions of such Preferred Stock or series, the terms of office of all directors elected by the holders of such Preferred Stock or series, voting as a class pursuant to such provisions or elected to fill any vacancies resulting from the death, resignation or removal of directors so elected by the holders of such Preferred Stock or series, shall forthwith terminate and the authorized number of directors shall be reduced accordingly.

SECTION 6.02. *Ballots*. Elections of directors at an annual or special meeting of stockholders need not be by written ballot unless the bylaws of the Corporation shall provide otherwise.

SECTION 6.03. *Elimination of Certain Personal Liability of Directors*. (a) A director of the Corporation shall not be liable to the Corporation or its stockholders for monetary damages for breach of fiduciary duty as a director to the fullest extent permitted by Delaware Law.

(b) Each person (and the heirs, executors or administrators of such person) who was or is a party or is threatened to be made a party to, or is involved in any threatened, pending or completed action, suit or proceeding, whether civil, criminal, administrative or investigative, by reason of the fact that such person is or was a director of the Corporation or is or was serving at the request of the Corporation as a director of another corporation, partnership, joint venture, trust or other enterprise, shall be indemnified and held harmless by the Corporation to the fullest extent permitted by Delaware Law. The right to indemnification conferred in this Section 6.03 shall also include the right to be paid by the Corporation the expenses incurred in connection with any such proceeding in advance of its final disposition to the fullest extent authorized by Delaware Law. The right to indemnification conferred in this Section 6.03 shall be a contract right.

(c) The Corporation may, by action of its Board of Directors, provide indemnification to such of the officers, employees and agents of the Corporation to such extent and to such effect as the Board of Directors shall determine to be appropriate and authorized by Delaware Law.

(d) The Corporation shall have power to purchase and maintain insurance on behalf of any person who is or was a director, officer, employee or agent of the Corporation, or is or was serving at the request of the Corporation as a director, officer, employee or agent of another corporation, partnership, joint venture, trust or other enterprise against any expense, liability or loss incurred by such person in any such capacity or arising out of his status as such, whether or

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not the Corporation would have the power to indemnify him against such liability under Delaware Law.

(e) The rights and authority conferred in this Section 6.03 shall not be exclusive of any other right which any person may otherwise have or hereafter acquire.

(f) Neither the alteration, amendment, repeal or recission of this Section 6.03, nor the adoption of any provision of this Certificate of Incorporation or the bylaws of the Corporation, nor, to the fullest extent permitted by Delaware Law, any modification of law, shall eliminate or reduce the effect of this Section 6.03 in respect of any acts or omissions occurring prior to such alteration, amendment, repeal, recission, adoption or modification.

ARTICLE 7

SECTION 7.01. *Section 203 of Delaware Law.* The Corporation hereby elects not to be governed by Section 203 of Delaware Law.

ARTICLE 8

SECTION 8.01. *Relationship With Roche.* In anticipation that the Corporation will cease to be a wholly owned subsidiary of Roche Holdings, Inc., but that Roche (as defined in Section 8.05) will remain a stockholder of the Corporation, and in anticipation that the Corporation (as defined in Section 8.05) and Roche may engage in the same or similar activities or lines of business and have an interest in the same areas of corporate opportunities, and in recognition of (i) the benefits to be derived by the Corporation through its continued contractual, corporate and business relations with Roche (including service of officers and directors of Roche as directors of the Corporation) and (ii) the difficulties attendant to any director, who desires and endeavors fully to satisfy such director's fiduciary duties, in determining the full scope of such duties in any particular situation, the provisions of this Article 8 are set forth to regulate, define and guide the conduct of certain affairs of the Corporation as they may involve Roche and its officers and directors, and the powers, rights, duties and liabilities

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of the Corporation and its officers, directors and stockholders in connection therewith.

SECTION 8.02. *Similar Business Activities.* Except as Roche Holdings, Inc. may otherwise agree in writing,

(a) Roche shall not have a duty to refrain from engaging directly or indirectly in the same or similar business activities or lines of business as the Corporation, and

(b) neither Roche nor any officer or director thereof shall be liable to the Corporation or its stockholders for breach of any fiduciary duty by reason of any such activities of Roche or of such person's participation therein.

In the event that Roche acquires knowledge of a potential transaction or matter that may be a corporate opportunity for both Roche and the Corporation, Roche shall have no duty to communicate or offer such corporate opportunity to the Corporation and shall not be liable to the Corporation or its stockholders for breach of any fiduciary duty as a stockholder of the Corporation or controlling person of a stockholder by reason of the fact that Roche pursues or acquires such corporate opportunity for itself, directs such corporate opportunity to another person or entity, or does not communicate information regarding, or offer, such corporate opportunity to the Corporation.

SECTION 8.03. *Corporate Opportunities.* In the event that a director, officer or employee of the Corporation who is also a director, officer or employee of Roche acquires knowledge of a potential transaction or matter that may be a corporate opportunity for the Corporation and Roche (whether such potential transaction or matter is proposed by a third-party or is conceived of by such director, officer or employee of the Corporation), such director, officer or employee shall be entitled to offer such corporate opportunity to the Corporation or Roche as such director, officer or employee deems appropriate under the circumstances in his sole discretion, and no such director, officer or employee shall be liable to the Corporation or its stockholders for breach of any fiduciary duty or duty of loyalty or failure to act in (or not opposed to) the best interests of the Corporation or the derivation of any improper personal benefit by reason of the fact that (i) such director, officer or employee offered such corporate opportunity to Roche (rather than the Corporation) or did not communicate information regarding such corporate opportunity to the Corporation or (ii) Roche pursues or acquires such corporate opportunity for itself or directs such corporate opportunity to another person or does not communicate information regarding such corporate opportunity to the Corporation.

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SECTION 8.04. *Deemed Consent of Stockholders.* Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 8.

SECTION 8.05. *Definitions.* For purposes of this Article 8 and Article 9 only, (i) the term **"Corporation"** shall mean the Corporation and all corporations, partnerships, joint ventures, associations and other entities in which the Corporation beneficially owns (directly or indirectly) fifty percent or more of the outstanding voting stock, voting power or similar voting interests, and (ii) the term **"Roche"** shall mean Roche Holdings, Inc. and all corporations, partnerships, joint ventures, associations and other entities (other than the Corporation, defined in accordance with clause (i) of this Section 8.05) that are **"affiliates"** (as defined in Rule 12b-2 under the Securities Exchange Act of 1934, as amended) of Roche Holdings, Inc.

SECTION 8.06. *Termination: Binding Effect.* Notwithstanding anything in this Certificate of Incorporation to the contrary, the foregoing provisions of this Article 8 shall expire on the date that Roche ceases to own beneficially Common Stock representing at least 5% of the number of outstanding shares of Common Stock of the Corporation and no person who is a director or officer of the Corporation is also a director or officer of Roche. Neither such expiration, nor the alteration, amendment, change or repeal of any provision of this Article 8 nor the adoption of any provision of this Amended and Restated Certificate of Incorporation inconsistent with any provision of this Article 8 shall eliminate or reduce the effect of this Article 8 in respect of any matter occurring, or any cause of action, suit or claim that, but for this Article 8, would accrue or arise, prior to such expiration, alteration, amendment, repeal or adoption.

SECTION 8.07. *Article 9.* The provisions of this Article 8 are in addition to the provisions of Article 9.

ARTICLE 9

SECTION 9.01. *Contracts Not Void.* No contract, agreement, arrangement or transaction (or any amendment, modification or termination thereof) between the Corporation and Roche shall be void or voidable solely for the reason that Roche is a party thereto, or solely because any directors or officers of the Corporation who are affiliated with Roche are present at or participate in the

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meeting of the Board of Directors or committee thereof which authorizes the contract, agreement, arrangement, transaction, amendment, modification or termination or solely because his or their votes are counted for such purpose, but any such contract, agreement, arrangement or transaction (or any amendment, modification or termination thereof) shall be governed by the provisions of this Amended and Restated Certificate of Incorporation, the Corporation's Bylaws, Delaware Law and other applicable law. For purposes of this Article 9, the terms the "**Corporation**" and "**Roche**" have the meanings set forth in Section 8.05.

SECTION 9.02. *Quorum.* Directors of the Corporation who are also directors or officers of Roche may be counted in determining the presence of a quorum at a meeting of the Board of Directors or of a committee that authorizes or approves any such contract, agreement, arrangement or transaction (or amendment, modification or termination thereof). Outstanding shares of Common Stock owned by Roche may be counted in determining the presence of a quorum at a meeting of stockholders that authorizes or approves any such contract, agreement, arrangement or transaction (or amendment, modification or termination thereof).

SECTION 9.03. *No Liability For Good Faith Actions.* Neither Roche nor any officer or director thereof shall be liable to the Corporation or its stockholders for breach of any fiduciary duty or duty of loyalty or failure to act in (or not opposed to) the best interests of the Corporation or the derivation of any improper personal benefit by reason of the fact that Roche or an officer of director thereof in good faith takes any action or exercises any rights or gives or withholds any consent in connection with any agreement or contract between Roche and the Corporation. No vote cast or other action taken by any person who is an officer, director or other representative of Roche, which vote is cast or action is taken by such person in his capacity as a director of this Corporation, shall constitute an action of or the exercise of a right by or a consent of Roche for the purpose of any such agreement or contract.

SECTION 9.04. *Deemed Consent by Stockholders.* Any person or entity purchasing or otherwise acquiring any interest in any shares of capital stock of the Corporation shall be deemed to have notice of and to have consented to the provisions of this Article 9.

SECTION 9.05. *Contracts Covered.* For purposes of this Article 9, any contract, agreement, arrangement or transaction with the Corporation or any corporation, partnership, joint venture, association or other entity in which the Corporation beneficially owns (directly or indirectly) fifty percent or more of the outstanding voting stock, voting power or similar voting interests shall be deemed to be a contract, agreement, arrangement or transaction with the Corporation.

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SECTION 9.06. *Binding Effect.* Neither the alteration, amendment, change or repeal of any provision of this Article 9 nor the adoption of any provision inconsistent with any provision of this Article 9 shall eliminate or reduce the effect of this Article 9 in respect of any matter occurring, or any cause of action, suit or claim that, but for this Article 9, would accrue or arise, prior to such alteration, amendment, change, repeal or adoption.

SECTION 9.07. *Article 8.* The provisions of this Article 9 are in addition to the provisions of Article 8.

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TRADUCCION AL ESPAÑOL del Certificado de Incorporación de Genentech, Inc.

APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
   Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254030
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado

20 NOV. 2006
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NOTARIO CUARENTA Y SEIS ENCARGADO

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DELAWARE

EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO VUELTO A REDACTAR DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL VIGÉSIMO SEGUNDO DÍA DE JULIO, A. D. 1999, A LAS 9 EN PUNTO DE LA MAÑANA.

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Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN: 3900384

FECHA: 05 – 24 – 05

preferenciales, por un valor de $0.02 por acción ("**Acción Preferencial**"). Trecientos millones (300,000,000) de acciones serán designadas acciones comunes, por un valor de $0.02 por acción ("**Acción Común**"). La acción común de la Corporación será toda de una misma clase.

(b) El número de acciones autorizadas de cualquier clase o clases de acciones puede incrementarse o disminuirse (pero no por debajo del número del número de acciones de las mismas que en su momento están vigentes) por el voto afirmativo de una mayoría con poder de votación de las acciones vigentes de la Corporación con derecho a votar por la misma independiente de las consideraciones de la Sección 242(b)(2) de la Ley de Delaware.

SECCION 4.02. *Acción Común.* (a) *Emisión y Consideración.* Cualquier acción sin emitir o del tesoro del las Acciones Comunes puede emitirse para tal consideración como pueda fijarse de acuerdo con la ley aplicable cada cierto tiempo por la Junta Directiva.

(b) *Dividendos.* Sujeto a los derechos de los accionistas de las Acciones Preferenciales, los accionistas de Acciones Comunes deberán tener el derecho a recibir, cuando y como se declare por la Junta Directiva, de los activos de la Corporación los cuales están disponibles para ello por ley, dividendos pagables ya sea en dinero, propiedad, o acciones y los accionistas de la Acción Preferencial no tendrán derecho a participar en tales dividendos (a menos que la Junta Directiva determine lo contrario en cualquier resolución que condiciona la emisión de una serie de Acciones Preferenciales).

SECCIÓN 4.03. *Acción Preferencial*

(a) *Series y Límites de Variaciones entre Series.* Cualquier acción no emitida o del tesoro de las Acciones Preferenciales puede emitirse cada cierto tiempo en una o más series para tal consideración como pueda ser fijada cada cierto tiempo por la Junta Directiva. Antes de que se emita cualquier acción de las Acciones Preferenciales de cualquier serie particular, se deberá presentar un certificado ante el Secretario de Estado de Delaware estableciendo las designaciones, derechos, privilegios, restricciones, y condiciones que irán anexas a las Acciones Preferenciales de tal serie y tales otras cuestiones como puedan requerirse, y la Junta Directiva deberá fijar y determinar, y por la presente está expresamente autorizada para fijar y determinar, en la forma provista por la ley, los

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CORREGIDO Y VUELTO A REDACTAR
CERTIFICADO DE INCORPORACIÓN
GENENTECH, INC.

Genentech, Inc (la "**Corporación**"), una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware, por la presente corrige el Certificado de Incorporación, el fue presentado originalmente el 23 de diciembre de 1986, bajo el nombre "Genentech Delaware, Inc.", y corregido subsiguientemente.

Por la presente se vuelve a redactar el certificado de incorporación de la Corporación y se corrige aun más para que diga en su totalidad lo que sigue:

ARTICULO 1

El nombre de la Corporación es: Genentech, Inc.

ARTICULO 2

La dirección de la oficina registrada de la Corporación en el Estado de Delaware es Corporation Service Company, 1013 Centre Road, Ciudad de Wilmington, Condado de New castle, Delaware 19805. El nombre de su agente registrado en tal dirección es Corporation Service Company.

ARTICULO 3

El propósito de a Corporación es el de participar en cualquier acto o actividad legal para las cuales las corporaciones pueden ser organizadas bajo la Ley General de Corporación del Estado de Delaware como existe o en adelante se modificada la misma en adelante ("**Delaware Law**").

ARTICULO 4

SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de cuatrocientos millones (400,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones

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particulares de las acciones de tal serie (siempre y cuando no sean inconsistentes con las condiciones de este Artículo 4 aplicables a todas las series de Acciones Preferenciales), incluyendo, pero sin limitarse a, las siguientes:

(i) la designación distintiva de tal serie y el número de las acciones que han de constituir tal serie, numero que puede incrementarse (excepto cuando lo determine de otro modo la Junta Directiva al crear tal serie) o reducida (pero no por debajo del número de acciones de las mismas que están vigentes) cada cierto tiempo por una acción semejante de la Junta Directiva;

(ii) la tasa anual de dividendos a pagar sobre las acciones de tal serie, las condiciones sobre las cuales se pagarán tales dividendos y le fecha a partir de la cual los dividendos serán acumulativos en el evento que la Junta Directiva determine que los dividendos serán acumulativos;

(iii) si la serie tendrá o no derechos de voto, además de los derechos de voto provistos por la ley, y de ser así, los términos de tales derechos de voto;

(iv) si tal serie tendrá o no privilegios de conversión y, de ser así, los términos y condiciones de tal conversión, incluyendo, pero sin estar limitado a, la condición para ajuste de la tasa de conversión en tales eventos y de tal forma como la Junta Directiva lo determine;

(v) si las acciones de tal serie serán o no redimibles y, de ser así, los términos y condiciones de tal redención, incluyendo la fecha o fechas en las cuales o después de las cuales pueden ser redimibles, y la cantidad a pagar por acción en caso de redención, cantidad que variará bajo diferentes condiciones y en diferentes fechas de redención;

(vi) si tal serie tendrá o no un fondo de inversión para la redención o compra de acciones de esa serie y, de ser así, los términos y la cantidad de tal fondo de inversión;

(vii) los derechos de las acciones de tal serien caso de una liquidación, disolución o cierre voluntaria o involuntaria de la Corporación, y

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los derechos relativos de prioridad, de haber, de pago de acciones de esa serie; y

(viii) cualquier otro derecho relativo, preferencia y limitación de tal serie.

ARTICULO 5

SECCION 5.01. *Enmiendas a los estatutos por los Directores.* (a) En adición y no limitando los poderes conferidos por estatutos, la Junta Directiva esta expresamente autorizada para hacer, alterar, enmendar, rechazar y rescindir los estatutos de la Corporación, sujetos a las secciones 5.01(b) y 5.01(c) a continuación.

(b) Las secciones 2.02, 3.02, 3.03(a), 3.04, 3.05, 3.14 y 5.06 de los estatutos solo pueden alterarse, enmendarse, repelerse, o rescindirse por el voto afirmativo de los accionistas de capital con derecho a votar las mismas que representen más del 60% de las acciones con derecho a votar las mismas.

(c) Las secciones 3.03(c) y 3.15 de los estatutos solo pueden ser alteradas, enmendadas, repelidas, o rescindidas por le voto afirmativo de los accionistas de capital con derecho a votar las mismas que representen más del 90% de las acciones con derecho a votar las mismas.

SECCION 5.02. *Enmienda del Certificado de Incorporación.* La sección 5.01 (b) de este Certificado de Incorporación solo puede ser alterada, enmendada, repelida, o rescindida por el voto afirmativo de los accionistas de capital con derecho a voto sobre la misma que representen a más del 60% de las acciones con derecho a votar la misma. La sección 5.01(c) y los Artículos 8 y 9 de este Certificado de Incorporación solo pueden alterarse, enmendarse, repelerse, o rescindirse por el voto afirmativo de los accionistas de capital con derecho a votar la misma que representen más del 90% de las acciones con derecho a votar la misma.

ARTICULO 6

SECCION 6.01. *Elección del Directorio por los Accionistas de Acción Preferencial.* Durante cualquier periodo cuando los accionistas de cualquier

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NOTARIO CUARENTA Y SEIS (46) DEL CIRCULO

acción preferencial cualquiera de una o más series de las mismas, que voten como una clase, tengan derecho a elegir un número específico de directores, por razón a atrasos en los dividendos u otras condiciones que les dan el derecho a hacerlo, entonces y durante el tiempo que continúe el derecho (i) el número de directores autorizado de otro modo será incrementado por tal número de directores especificado, y los accionistas de tales Acciones Preferenciales o tales series de las mismas, que votan como una clase, tendrán derecho a elegir los directores adicionales para lo que se provee, de acuerdo con las condiciones de tales Acciones Preferenciales o series; (ii) cada uno de tales directores adicionales servirá durante tal termino, y tendrá tales poderes de voto, como se han de otorgar en las provisiones pertinentes a tales Acciones Preferenciales o series; y (iii) siempre que los accionistas de cualquiera de tales Acciones Preferenciales o series de las mismas están desprovistos de tales derechos a elegir un número específico de directores, votando como clase, de acuerdo con las condiciones de tales Acciones preferenciales o series, los términos de oficio de todos los directores elegidos por los accionistas de tales Acciones preferenciales o series, que votan como clase, de acuerdo con las condiciones o elegidos para llenar cualquier vacante resultante de la muerte, renuncia o remoción de directores así elegidos por los accionistas de tales Acciones Preferenciales o series, terminarán en adelante y el número autorizado de directores será reducido en concordancia.

SECCION 6.02. *Votaciones.* Las elecciones de directores en una reunión anual o especial de accionistas necesaria no serán por votos escritos a menos que los estatutos de la Corporación digan lo contrario.

SECCION 6.03. *Eliminación de cierta responsabilidad personal de los directores.* (a) Un director de la Corporación no será responsable ante la Corporación o sus accionistas por daños monetarios por violación de deber fiduciario como director hasta donde lo permita la Ley de Delaware.

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(b) Cada persona (y sus herederos, ejecutores o administradores de tal persona) quien fue o es una parte o esta amenazado de ser parte de esta involucrado en cualquier acción, demanda o proceso amenazado, pendiente, o completado, ya sea civil, criminal, administrativo o investigativo, por razón del hecho de que esa persona es o fue un director de la Corporación o es o estaba sirviendo por solicitud de la Corporación como un director de otra corporación, sociedad, sociedad de riesgo compartido, fideicomiso u otra empresa, será indemnizado y completamente protegido por la Corporación hasta donde lo

permita la Ley de Delaware. El derecho a la indemnización concedido en esta sección 6.03 también debe incluir el derecho a que la Corporación le pague los gastos incurridos en conexión cuna cualquiera de tales procesos por adelantado de su decisión final hasta donde los autoriza la Ley de Delaware. El derecho a indemnización conferido en esta sección 6.03 será un derecho contractual.

(c) La Corporación puede, por acción de su Junta Directiva, proporcionar indemnización a tales funcionarios, empleados y agentes de la Corporación hasta tal punto y a tal efecto como lo determine apropiado la Junta Directiva y lo autorice la Ley de Delaware.

(d) La Corporación tendrá el poder de comprar y mantener un seguro a nombre de cualquier persona quien es o fue director, funcionario, empleado o agente de la corporación, o esta o estuvo en servicio por pedido de la Corporación como director, funcionario, empleado o agente de otra corporación, sociedad, sociedad de riesgo compartido, fideicomiso u otra empresa contra cualquier gasto, responsabilidad o pérdida incurrida por tal persona en cualquier capacidad o que surge de su estado como tal, ya sea si la Corporación tiene o no el poder de indemnizarlo contra tal responsabilidad bajo la ley de Delaware.

(e) Los derechos y autoridad conferidos en esta Sección 6.03 no serán exclusivos de ningún otro derecho el cual cualquier otra persona pueda tener o adquirir de aquí en adelante.

(f) Ni la alteración, enmienda, rechazo o rescisión de esta Sección 6.03, ni la adopción de ninguna condición de este Certificado de Incorporación o de los Estatutos de la Corporación, ni, hasta donde lo permite la Ley de Delaware, ninguna modificación de la ley, debe eliminar o reducir el efecto de esta Sección 6.03 respecto a ninguno de los actos u omisiones que ocurran antes de tal alteración, enmienda, rechazo, rescisión, adopción o modificación.

ARTICULO 7

SECCION 7.01, *Sección 203 de la Ley de Delaware.* Por la presente la Corporación elige no ser gobernada por la Sección 203 de la Ley de Delaware.

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ARTICULO 8

SECCION 8.01. *Relación con Roche.* Anticipándose a que la Corporación cese de ser un subsidiaria completamente de propiedad de Roche Holdings, Inc., pero que Roche (como se define en la sección 8.05) permanezca siendo un accionista de la Corporación, y anticipándose a que la Corporación (como se define en la Sección 8.05) y Roche puede entrar en actividades o líneas de negocios semejantes o similares y tener un interés en las mismas áreas de oportunidades corporativas, y en reconocimiento de (i) los beneficios a ser derivados por la Corporación a través de sus relaciones contractuales, corporativas y de negocios con Roche (incluyendo el servicio de funcionarios y directores de Roche como directores de la Corporación) y (ii) las dificultades concomitantes a cualquier director, quien desea y se propone satisfacer completamente tales deberes fiduciarios del director, al determinar el alcance completo de tales deberes en cualquier situación particular, las condiciones de este artículos 8 se establecen para regular, definir y guiar la conducta de ciertas cuestiones de la Corporación ya que pueden involucrar a Roche y sus funcionaros y directores, y los poderes, derechos, deberes y responsabilidades de la Corporación y sus funcionarios, directores y accionistas en conexión con éste.

SECCION 8.02. *Actividades en Negocios Similares.* Excepto que Roche Holdings, Inc. pueda de otra forma acordarlo por escrito,

(a) Roche no tendrá el deber de abstenerse de entrar directamente o indirectamente en actividades de negocios o líneas de negocios semejantes o similares a las de la Corporación, y

(b) ni Roche ni ninguno de sus funcionarios o director de la misma será responsable a la Corporación o sus accionistas por violar cualquier deber fiduciario por razón de alguna de tales actividades de Roche o de la participación de tal persona en las mismas.

En el evento que Roche adquiera conocimientos de una transacción o materia potencial que pueda ser una oportunidad corporativa tanto para Roche como para la Corporación, Roche no tendrá el deber de comunicar u ofrecer tal oportunidad corporativa a la Corporación y no será responsable ante la Corporación o sus accionistas por violar ningún deber fiduciario como accionista de la Corporación o la persona que controla a un accionista por razón de dicha

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NOTARIO CUARENTA Y SEIS (E.)

que Roche prosigue o adquiere tal oportunidad corporativa para si misma, dirige tal oportunidad corporativa a otra persona o entidad, o no comunica información respecto, u ofrece, tal oportunidad corporativa a la Corporación.

SECCION 8.03. *Oportunidades Corporativas.* En el evento que un director, funcionario o empleado de la Corporación quien también es director, funcionario o empleado de Roche adquiera conocimiento de una transacción o materia potencial que pueda ser una oportunidad corporativa para la Corporación y Roche (ya se si esta transacción o materia potencial es propuesta por un tercero o es concebida por tal director, funcionario o empleado de la Corporación o no), tal director, funcionario o empleado debe tener el derecho a ofrecer tal oportunidad corporativa a la Corporación o Roche como tal director, funcionario o empleado los considere apropiado bajo las circunstancias de su discreción, y tal director, funcionario o empleado no será responsable a la Corporación o sus accionistas por violar ningún deber fiduciario o deber de lealtad o faltar en actuar en (o no oponerse a) los mejores intereses de la Corporación o la derivación de ningún beneficio personal inapropiado por razón del hecho que (i) tal director, funcionario o empleado ofreció tal oportunidad corporativa a Roche (en lugar de a la Corporación) o no comunicó información respecto a tal oportunidad corporativa a la Corporación o (ii) Roche busca o adquiere tal oportunidad corporativa para si misma o dirige tal oportunidad corporativa a otra persona o no comunica información respecto a tal oportunidad corporativa a la Corporación.

SECCION 8.04. *Consentimiento estimado de los accionistas.* Se considerará que cualquier persona o entidad que compre o de otro modo adquiera algún interés en cualquiera de las acciones de capital de la Corporación ha tenido noticia o ha estado de acuerdo con las condiciones de este Artículo 8.

SECCION 8.05, *Definiciones.* Para propósitos de este Artículo 8 y el Artículo 9 solamente, (i) el término ***"Corporación"*** debe significar la Corporación y todas las corporaciones, sociedades, sociedades de riesgo compartido y otras entidades en las cuales la Corporación posee con beneficio (directa o indirectamente) cincuenta por ciento o más de las acciones con voto, poder de voto o intereses de voto similares, y (ii) el término ***"Roche"*** debe significar Roche Holdings, Inc y todas las corporaciones, sociedades, sociedades de riesgo compartido, asociaciones y otras entidades (diferentes a la Corporación, definida de acuerdo con la cláusula (i) de esta sección 8.05) que son ***"afiliados"*** (como se

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define en la Regla 12b-2 bajo el Acta de Intercambio de Valores de 1934, como se enmendó) de Roche Holdings, Inc.

SECCION 8.06, *Finalización: Efecto Obligante.* Son consideración con nada en este Certificado de Incorporación que lo contradiga, las condiciones anteriores de este Artículo 8 deberán expirar en la fecha que Roche cese de poseer con beneficios Acciones Comunes que representen al menos 5% del número de acciones vigentes de las Acciones Comunes de la Corporación y nadie que se director o funcionario de la Corporación sea también director o funcionario de Roche. Ni tal vencimiento, ni la alteración, enmienda, cambio o rechazo de alguna de las condiciones de este Artículo 8 así como tampoco ninguna condición de este Certificado de Incorporación Enmendado y Vuelto a Redactar inconsistente con alguna de las condiciones de este Artículo 8 eliminará o reducirá el efecto de este Artículo 8 respecto a cualquier materia, o cualquier causa de acción, demanda o reclamo que, excepto por este Artículo 8, pueda acaecer o surgir, antes de tal vencimiento, alteración, enmienda o adopción.

SECCION 8.07. *Artículo 9.* Las condiciones de este Artículo son adicionales a las condiciones del Artículo 9.

ARTICULO 9

SECCION 9.01. *Contratos no nulos.* Ningún contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización de los mismos) entre la Corporación y Roche será nulo u anulable solamente por razón de que Roche es parte del mismo, o solamente porque cualquiera de los directores o funcionarios de la Corporación quienes están afiliados a Roche están presentes en o participan en la reunión de la Junta Directiva o comité de la misma que autoriza el contrato, acuerdo, arreglo, transacción, enmienda, modificación o finalización o solamente porque su o sus votos se cuentan para tal propósito, pero cualquiera de tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo) estará gobernado por las condiciones de este Cerificado de Incorporación Enmendado y vuelto a Redactar, los Estatutos de la Corporación, la Ley de Delaware y otras leyes aplicables. Para propósitos de este Artículo 9, los términos "***Corporación***" y "***Roche***" tienen los significados establecidos en la Sección 8.05.

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SECCION 9.02. *Quórum.* Los Directores de la Corporación que son también directores o funcionarios de Roche pueden contarse al determinar la presencia de un quórum en una reunión de la Junta Directiva o de un comité que autoriza o aprueba cualquiera de tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo). Las acciones vigentes de las Acciones Comunes de propiedad de Roche pueden contarse al determinar la presencia de un quórum en una reunión de accionistas que autorice o apruebe tal contrato, acuerdo, arreglo o transacción (o cualquier enmienda, modificación o finalización del mismo).

SECCION 9.03. *No responsabilidad por acciones de buena fe.* Ni Roche ni ningún funcionario o director de la misma será responsable ante la Corporación o sus accionistas por violar cualquiera de los deberes fiduciarios o deberes de lealtad o por fallar en actuar en (o no oponerse a) los mejores intereses de la Corporación o por derivación de cualquier beneficio personal por razón del hecho que Roche o un funcionario o director de la misma de buena fe tome cualquier acción o ejerza cualquier derecho o de o retenga cualquier consentimiento en conexión con cualquier acuerdo o contrato entre Roche y la Corporación. Ningún voto dado u otra acción tomada por cualquier persona que es un funcionario, director o representante de Roche, donde el voto o acción es tomado por tal persona en su capacidad como director de esta Corporación, constituirá una acción de o el ejercicio de un derecho o un consentimiento de Roche para los propósitos de cualquiera de tal acuerdo o contrato.

SECCION 9.04. *Consentimiento estimado de los accionistas.* Se considerará que cualquier persona o entidad que compre o de otro modo adquiera algún interés en cualquiera de las acciones de capital de la Corporación ha tenido noticia o ha estado de acuerdo con las condiciones de este Artículo 9.

SECCION 9.05. *Contratos cubiertos.* Para propósitos de este Artículo 9, cualquier contrato, acuerdo, arreglo o transacción con la Corporación o cualquier corporación, sociedad, sociedad de riesgo conjunto, asociación u otra entidad en la cual la Corporación posee con beneficio (directa o indirectamente) el cincuenta por ciento o más de las acciones con voto, poderes de voto o intereses de voto similares vigentes se considerará un contrato, acuerdo o transacción con la Corporación.

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SECCION 9.06. *Efecto obligante.* Ni la alteración, enmienda, cambio o rechazo de alguna de las condiciones de este Artículo 9 ni la adopción de alguna condición inconsistente con cualquier condición de este Artículo 9 eliminará o reducirá el efecto de este Artículo 9 respecto a cualquier materia que ocurra, o cualquier causa de acción, demanda o reclamo que, a no ser por este Artículo 9, pueda acaecer o surgir, antes de tal alteración, enmienda, cambio, rechazo o adopción.

SECCION 9.07. *Artículo 8.* Las condiciones de este Artículo 9 son adicionales a las condiciones del Artículo 8.

EN TESTIMONIO DE LO CUAL, este Certificado de Incorporación Enmendado y vuelto a Redactar, el cual ha sido adoptado de acuerdo con las secciones 242 y 245 de la Ley General de Corporación del Estado de Delaware y por consentimiento escrito de el único accionista de la Corporación de acuerdo con la sección 228 de Ley General de Corporación del Estado de Delaware, ha sido ejecutado este 22 de Julio de 1999.

GENENTECH, INC.
Por: /s/ Stephen G. Juelsgaard
Nombre: Stephen G. Juelsgaard
Título: Vicepresidente
Consejo General y Secretaría

NOTARIO CUARENTA Y SEIS ENCARGADO
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Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

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2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

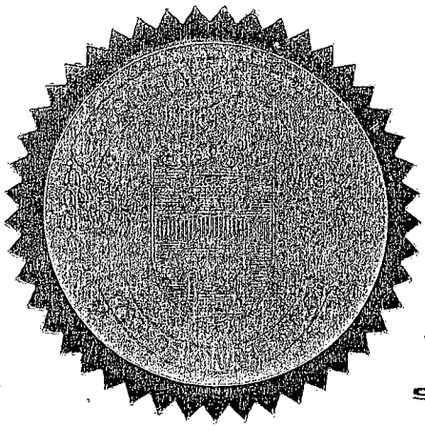
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254031

9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor

Secretary of State

Delaware

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The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE FIFTEENTH DAY OF MAY, A.D. 2000, AT 5 O'CLOCK P.M.

2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3900385

DATE: 05-24-05

20 NOV. 2006

LUZ LOPEZANZA AVELLA ROMERO

NOTARIO CUARENTA Y SEIS ENCARGADO

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 05:00 PM 05/15/2000
001245251 - 2112549

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CERTIFICATE OF AMENDMENT OF AMENDED AND RESTATED CERTIFICATE OF INCORPORATION

Genentech, Inc. a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware.

DOES HEREBY CERTIFY:

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows:

> "Section 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is seven hundred million (700,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value $0.02 per share ("Preferred Stock"). Six hundred million (600,000,000) shares shall be designated common stock, par value $0.02 per share ("Common Stock"). The Common Stock of the Corporation shall be all of one class."

SECOND: That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 15th day of May, 2000.

GENENTECH, INC.

By: Stephen Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President, General Counsel and Secretary

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20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

TRADUCCION AL ESPAÑOL del Certificado de Incorporación de Genentech, Inc.

APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
   Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254031
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado

EL DECANO DE BOGOTA, D.C. PARA CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
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NOTARIO CUARENTA Y SEIS ENCARGADO

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DELAWARE

EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO VUELTO A REDACTAR DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMOQUINTO DIA DE MAYO, A. D. 2000, A LAS 5 EN PUNTO DE LA TARDE.

NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA BOGOTA D.C. REPUBLICA DE COLOMBIA

20 NOV. 2005

LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN: 3900385

FECHA: 05 – 24 – 05

CERTIFICADO DE ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmiende cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

> SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de setecientos millones (700,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de $0.02 por acción ("**Acción Preferencial**"). Seiscientos millones (600,000,000) de acciones serán designadas acciones comunes, por un valor de $0.02 por acción ("**Acción Común**"). La acción común de la Corporación será toda de una misma clase.

SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva la reunión ordinaria de accionistas de dicha corporación se convocó debidamente y se sostuvo al ser anunciada de acuerdo con la sección 222 de la Ley General de Corporación del Estado de Delaware, en donde el número necesario de acciones requerido por los estatutos votaron a favor de la enmienda.

20 NOV 2003
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NOTARIO CUARENTA Y SIETE ENCARGADO

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TERCERO: Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO: Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 15 de Mayo de 2000.

GENENTECH, INC.

Por: (Firma)
Nombre: Stephen G. Juelsgaard
Título: Vicepresidente, Consejo
y Secretaría General.

20 NOV. 2000
LUZ ESPERANZA AVELLA ROMERO
NOTARIO ENCARGADO

Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

This public document:

2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254033

9. Seal/Stamp:

10. Signature:

Harriet Smith Windsor

Secretary of State

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE SIXTEENTH DAY OF APRIL, A.D. 2004, AT 7:39 O'CLOCK P.M.

2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3900387

DATE: 05-24-05

COMO NOTARIO GUARDO LOS ENCARGADO DEL CONSULADO DE REPUBLICA DOMINICANA COMPARAR QUE HA TENIDO A LA VISTA EL ORIGINAL DE REPUBLICA DE COLOMBIA

03 ABR. 2007

ROSA PAULA LABOZCA

NOTARIO CONSULADO DE REPUBLICA DOMINICANA

State of Delaware
Secretary of State
Division of Corporations
Delivered 07:44 PM 04/16/2004
FILED 07:39 PM 04/16/2004
SRV 040281669 - 2112549 FILE

CERTIFICATE OF THIRD AMENDMENT OF AMENDED AND RESTATED CERTIFICATE OF INCORPORATION

Genentech, Inc., a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware,

DOES HEREBY CERTIFY:

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows:

> Section 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is three billion one hundred million (3,100,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value $0.02 per share ("Preferred Stock"). Three billion (3,000,000,000) shares shall be designated common stock, par value $0.02 per share ("Common Stock"). The common stock of the Corporation shall be all of one class.

SECOND: That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Third Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 16th day of April, 2004.

GENENTECH, INC.

By: Stephen Juelsgaard
Name: Stephen G. Juelsgaard
Title: Executive Vice President, General Counsel and Secretary

#153087

COMO NOTARIO DEL CIRCULO DE ... QUE ESTA COPIA ... EL ORIGINAL QUE HE TENIDO A LA VISTA ... 03 ABR. 2007
ROSA PAULA LABOCA
NOTARIO CUMPLIDA Y SOBRE ENCARGADA

TRÁDUCCION AL ESPAÑOL del Certificado de Enmienda de Genentech, Inc.

APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
   Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254033
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado

COMO NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
03 ABR. 2007
ROSA TALLA LABRADA
NOTARIO CUARENTA Y SEIS (E)

287

DELAWARE

EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO DE ENMIENDA DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMOSEXTO DIA DE ABRIL, A. D. 2004, A LAS 7:39 EN PUNTO DE LA TARDE.

Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado

COMO NOTARIO CERTIFICO Y SELLO CON MI FIRMA QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
03 ABR. 2007
ROSA PALLA LABBECA
NOTARIO CERTIFICADO

CERTIFICADO DE TERCERA ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmiende cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

> SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de tres mil cien millones (3,100,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de $0.02 por acción ("**Acción Preferencial**"). Tres mil millones (3,000,000,000) de acciones serán designadas acciones comunes, por un valor de $0.02 por acción ("**Acción Común**"). La acción común de la Corporación será toda de una misma clase.

SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente

03 ABR 2007

TERCERO: Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO: Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 16 de Abril de 2004.

GENENTECH, INC.

Por: (Firma)
Nombre: Stephen G. Juelsgaard
Título: Vicepresidente, Consejo
y Secretaría General.

COMO NOTARIO CERTIFICO QUE LA PRESENTE ES COPIA FIEL DEL ORIGINAL QUE HE TENIDO A LA VISTA
03 MAR. 2007
ROSA PALLA LANSCA
NOTARIO CERTIFICADO

Apostille

(Convention de La Haye du 5 Octobre 1961)

1. Country: United States of America

This public document:

2. has been signed by Harriet Smith Windsor

3. acting in the capacity of Secretary of State of Delaware

4. bears the seal/stamp of Office of Secretary of State

Certified

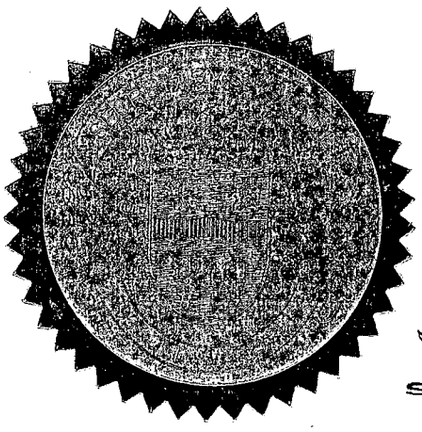
5. at Dover, Delaware

6. the twenty-fourth day of May, A.D. 2005

7. by Secretary of State, Delaware Department of State

8. No. 0254032

9. Seal/Stamp:



10. Signature:

Harriet Smith Windsor

Secretary of State

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "GENENTECH, INC.", FILED IN THIS OFFICE ON THE TENTH DAY OF MAY, A.D. 2001, AT 2:05 O'CLOCK P.M.

2112549

050426506

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3900386

DATE: 05-24-05

20 NOV. 2006

LUZ ESPERANZA AVELLA ROMERO

NOTARIO CUARENTA Y SEIS ENCARGADO

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 02:05 PM 05/10/2001
010226224 - 2112549

S ////STATE/OF/DELAWARE///// V
T ///SECRETARY/OF/STATE///// O
A DIVISION/OF/CORPORATIONS// I
M FILED/09/00/AM/05/10/2001/ D
P ///0102262 24/+/2112549////
BY Sandy Miller

292

CERTIFICATE OF AMENDMENT OF AMENDED AND RESTATED CERTIFICATE OF INCORPORATION

Genentech, Inc. a corporation organized and existing under and by virtue of the General Corporation Law of the State of Delaware.

DOES HEREBY CERTIFY:

FIRST: That at a meeting of the Board of Directors of Genentech, Inc. resolutions were duly adopted setting forth a proposed amendment of the Amended and Restated Certificate of Incorporation of said corporation, declaring said amendment to be advisable and calling a meeting of the stockholders of said corporation for consideration thereof. The resolution setting forth the proposed amendment is as follows:

RESOLVED, that the Amended and Restated Certificate of Incorporation of this corporation be amended by changing "Section 4.01(a)" of the Article thereof numbered "4" so that, as amended, said Section of said Article shall be and read as follows:

> "Section 4.01. *Capital Stock.* (a) The Corporation is authorized to issue two classes of stock to be designated, respectively, preferred stock and common stock. The total number of shares which the Corporation is authorized to issue is one billion three hundred million (1,300,000,000) shares. One hundred million (100,000,000) shares shall be designated preferred stock, par value $0.02 per share ("Preferred Stock"). One billion two hundred million (1,200,000,000) shares shall be designated common stock, par value $0.02 per share ("Common Stock"). The Common Stock of the Corporation shall be all of one class."

SECOND: That thereafter, pursuant to resolution of its Board of Directors, the regular meeting of the stockholders of said corporation was duly called and held upon notice in accordance with Section 222 of the General Corporation Law of the State of Delaware at which meeting the necessary number of shares as required by statute were voted in favor of the amendment.

THIRD: That said amendment was duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware.

FOURTH: That the capital of said corporation shall not be reduced under or by reason of said amendment.

IN WITNESS WHEREOF, said Genentech, Inc. has caused this Certificate of Amendment to be signed by Stephen G. Juelsgaard, an Authorized Officer, this 10th day of May, 2001.

GENENTECH, INC.

By: Stephen Juelsgaard
Name: Stephen G. Juelsgaard
Title: Senior Vice President, General Counsel and Secretary

86747

EL NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
BOGOTA D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

TRADUCCION AL ESPAÑOL del Certificado de Enmienda de Genentech, Inc.

APOSTILLA

(Convención de La Haya del 5 de Octubre de 1961)

1. País: Estados Unidos de América
   Este documento público
2. ha sido firmado por Harriet Smith Windsor
3. actuando en capacidad de Secretaria de Estado de Delaware
4. lleva el sello/timbre de la Oficina del Secretario de Estado

Certificado

5. en Dover, Delaware
6. el veinticuatro de mayo, A. D. 2005
7. por el Secretario de Estado, Departamento de Estado de Delaware
8. No. 0254032
9. Sello/timbre
10. Firma

Harriet Smith Windsor (Firma)
Secretaria de Estado

COMO NOTARIO CUARENTA Y SEIS ENCARGADO DEL CIRCULO DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
BOGOTA D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUZ ESPERANZA AVELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

294

DELAWARE

EL PRIMER ESTADO

YO, HARRIET SMITH WINDSOR, SECRETARIA DE ESTADO DEL ESTADO DE DELAWARE, CERTIFICO POR LA PRESENTE QUE EL ANEXO ES UNA COPIA AUTÉNTICA Y CORRECTA DEL CERTIFICADO DE ENMIENDA DE "GENENTECH, INC", PRESENTADO EN ESTA OFICINA EL DECIMO DIA DE MAYO, A. D. 2001, A LAS 2:05 EN PUNTO DE LA TARDE.

20 NOV. 2006

LUZ ESPERANZA AVELLA RONDON
NOTARIO CUARENTA Y SEIS DEL CIRCULO

Harriet Smith Windsor (Firma)
Harriet Smith Windsor, Secretaria de Estado
AUTENTICACIÓN: 3900386

FECHA: 05 – 24 – 05

CERTIFICADO DE TERCERA ENMIENDA DEL
CERTIFICADO DE INCORPORACIÓN
CORREGIDO Y VUELTO A REDACTAR

Genentech, Inc. una corporación organizada y existente bajo y por virtud de la Ley General de Corporación del Estado de Delaware.

CERTIFICA POR LA PRESENTE:

PRIMERO: Que en una reunión de la Junta Directiva de Genentech, Inc. se adoptaron debidamente resoluciones que establecen una enmienda propuesta del certificado de Incorporación Corregido y vuelto a Redactar de dicha corporación, declarando dicha enmienda como aconsejable y llamando a una reunión de accionistas de dicha corporación para consideración de la misma. La resolución establece la enmienda propuesta de la siguiente manera:

RESUELVE, que el Certificado de Incorporación Corregido y vuelto a Redactar de esta corporación se enmiende cambiando "la Sección 4.01(a)" del Artículo allí numerado "4" tal que, como se enmienda, dicha Sección de dicho Artículo se lea de la siguiente manera:

> SECCION 4.01. *Acciones de Capital.* (a) la Corporación está autorizada a emitir dos clases de acciones que deben designarse, respectivamente, acciones preferenciales y acciones comunes. El número total de acciones que la Corporación esta autorizada a emitir es de mil trescientos millones (1,300,000,000) de acciones. Cien millones (100,000,000) de acciones serán designadas acciones preferenciales, por un valor de $0.02 por acción ("**Acción Preferencial**"). Mil doscientos millones (1,200,000,000) de acciones serán designadas acciones comunes, por un valor de $0.02 por acción ("**Acción Común**"). La acción común de la Corporación será toda de una misma clase.

SEGUNDO: Que por lo tanto, de acuerdo con la resolución de su Junta Directiva, la reunión ordinaria de accionistas de dicha corporación se convocó debidamente y se sostuvo al ser anunciada de acuerdo con la sección 222 de la Ley General de Corporación del Estado de Delaware, en donde el número necesario de acciones requerido por los estatutos votaron a favor de la enmienda.

COMO NOTARIO QUARENTA DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCUERDA CON EL ORIGINAL QUE HE TENIDO A LA VISTA DEL CIRCULO DE BOGOTA, D.C. REPUBLICA DE COLOMBIA
20 NOV. 2006
LUIS ESPERANZA DELLA ROMERO
NOTARIO CUARENTA Y SEIS ENCARGADO

TERCERO: Que dicha enmienda se adoptó debidamente de acuerdo con lo previsto en la Sección 242 de la Ley General de Corporación del Estado de Delaware.

CUARTO: Que el capital de dicha corporación no será reducido bajo o por razón de dicha enmienda

EN TESTIMONIO DE LO CUAL, dicha Genentech, Inc. ha causado este Certificado de Enmienda para que sea firmado por Stephen G. Juelsgaard, un Funcionario Autorizado, este 10 de Mayo de 2001.

GENENTECH, INC.

Por: (Firma)
Nombre: Stephen G. Juelsgaard
Título: Vicepresidente, Consejo
y Secretaría General.

20 NOV. 2006

297

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 07-133662- -00000-0000

Fecha: 2007-12-18 16:45:49 Dep. 2020 NUECREACIONI
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 296

Industria y Comercio
SUPERINTENDENCIA

REQUISITOS MINIMOS PARA ADMISION A TRAMITE PCT

PATENTE DE INVENCION [✓] MODELO DE UTILIDAD [ ]

- Indicación de que se solicita la concesión de una patente [✓]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [✓]
- Descripción de la invención [✓]
- Dibujos de ser estos pertinentes [ ]
- Comprobante de pago de las tasas establecidas [✓]

COMPLETA [ ] INCOMPLETA [ ]

DISEÑO INDUSTRIAL [ ]

- Indicación de que se solicita el registro de Diseño Industrial [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

ESQUEMA DE TRAZADO [ ]

- Indicación de que se solicita el registro de un Esquema de Trazado [ ]
- Datos de identificación del solicitante o de la persona que presenta la solicitud [ ]
- Representación gráfica del esquema trazado [ ]
- Comprobante de pago de las tasas establecidas [ ]

COMPLETA [ ] INCOMPLETA [ ]

Claudia M Neira Cortes
Firma Responsable

Fecha_____

Carrera 13 No. 27-00 Piso 5, 7 y 10
Conmutador: 3 82 08 40
Fax: 350 52 20
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia