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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-110246- -00005-0000

Fecha: 2007-12-21 13:31:35 Dep. 2020 NUECREACIONES
Tra: 11 PATENTE IYII Eve: 378 FASE NACIONAL I
Act. 538 PETICION Folios: 7

Clarke, Modet & C^o

COLOMBIA

SEÑORES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E.S.D

REF EXPEDIENTE No.: 06110246
PATENTE : ANTICUERPOS MONOCLONALES AL
FACTOR DE CRECIMIENTO DE HEPATOCITO
SOLICITANTE : GALAXY BIOTECH , LLC

ASUNTO: SOLICITUD ESTUDIO DE FONDO

DILIA RODRÍGUEZ D'ALEMAN, en mi condición de apoderada de la Sociedad solicitante, por el presente escrito atentamente solicito a Usted que habiéndose surtido la publicación de la presente solicitud, en la Gaceta de la Propiedad Industrial No. 578 de fecha Julio 31 de 2007 y se proceda con el estudio de Fondo.

Para tal fin me permito anexar la tasa correspondiente.

Agradezco continuar con el presente trámite.

Señor Jefe,


DILIA RODRÍGUEZ D'ALEMAN
C.C. 41.654.882 Bogotá
P- 848

167

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RECEPTE DE PAGO DE LA SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-110246-00005-0000

Fecha: 2007-12-21 13:21:35 Dep: 2020 NUECREACION
Dep: 11 PATENTABILIDAD Cve: 378 BASE NACIONAL
Dep: 530 ALIENACION Folio: 7

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 07 - 90,585
FECHA : DICIEMBRE 3 DE 2007

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CLARKE MODET Y CO. COLOMBIA	CONSIGNACION	BANCO POPULAR	050-00110-6	64177535	03/12/2007	22,680,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	114 PTC CAP-II EXAMEN DE PATENTIBILIDAD	202,000.00
		TOTAL :	\$ 202,000.00

MON: DOSCIENTOS DOS MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RECIBO OFICIAL DE CAJA : 07 - 89,827
 FECHA : NOVIEMBRE 1 DE 2007



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***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	Nº. PAGO	FECHA PGO	Vr. PAGO
CLARKE MODET Y CO. COLOMBIA	CONSIGNACION	BANCO POPULAR	050-00110-6	71030245	01/11/2007	32,806,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
17		SUSCRIPCION ANUAL A LA GACETA DE	194,000.00
TOTAL :			\$ 194,000.00

SON: CIENTO NOVENTA Y CUATRO MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900174089-2

CERTIFICACION DE EXCEDENTE : 07 - 96,380
FECHA : NOVIEMBRE 26 DE 2007

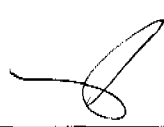
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CLARKE MODET Y CO. COLOMB	CONSIGNACION	BANCO POPULAR	050-00110-6	53267311	26/11/2007	28,480,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		99 OTROS SERVICIOS ADMINISTRATIVOS	5,000.00
		TOTAL :	\$ 5,000.00

SON: CINCO MIL PESOS

RESPONSABLE : 

RECIBO DE CASA APLICADO AL EXPEDIENTE No. _____

170

MIT : 600176089-2

CERTIFICACION DE EXCEDENTE : 07 - 97,300
FECHA : NOVIEMBRE 28 DE 2007

No 06-110245-25048-0000
 1961 JUL 12 11 05 AM EST Dep. 2020 NUCLEACION
 06-110245-25048-0000 Dep. 2020 NUCLEACION
 06-110245-25048-0000 Dep. 2020 NUCLEACION

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林娜	女	34	甘肃	汉族	医生	硕士	无	无	甘肃省人民医院	15145678901	linana@gsrh.com	
刘凯	男	28	青海	汉族	程序员	本科	无	无	青海省软件中心	13956789012	liukaifei@qhsh.com	
宋佳	女	35	四川	汉族	教授	博士	无	无	四川大学	13867890123	songjia@scu.edu.cn	
周明	男	45	陕西	汉族	研究员	本科	无	无	陕西省科学院	13778901234	zhouming@sxky.com	
吴昊	男	39	内蒙古	汉族	律师	硕士	无					

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	Nº. PAGO	FECHA PGO	Vr. PAGO
CLARKE MODET Y CO. COLOMBIA	CONSIGNACION	BANCO POPULAR	050-00110-6	72395998	28/11/2007	8.640.000.00

[illegible]

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-07	OTROS SERVICIOS DE PROPIEDAD I	
		97 OTROS SERVICIOS ADMINISTRATIVOS	1,000.00
		TOTAL :	\$ 1,000.00

CONFIDENTIAL

UNCLASSIFIED

RECIBO DE CAJA APLICADO AL EXPEDIENTE No.

ESTA CERTIFICACION DE EXCEDENTE DE PAGO SOLO PODRA SER UTILIZADA
COMO PARTE DE LA CANCELACION DE UNA TARIFA VIGENTE Y NO PODRA SER
SUSTITUIDA POR NINGUN RECIBO.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

CERTIFICACION DE EXCEDENTE : 07 - 97,298
FECHA : NOVIEMBRE 28 DE 2007

***** CONSIGNACION *****

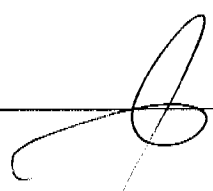
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CLARKE MODET Y CO. COLOMBIA	CONSIGNACION	BANCO POPULAR	050-00110-6	72395998	28/11/2007	8,640,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		99 OTROS SERVICIOS ADMINISTRATIVOS	1,000.00
		TOTAL :	\$ 1,000.00

SON: MIL PESOS

RESPONSABLE :



RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

ESTA CERTIFICACION DE EXCEDENTE DE PAGO SOLO PODRA SER UTILIZADA
COMO PARTE DE LA CANCELACION DE UNA TARIFA VIGENTE Y NO PODRA SER
SUSTITUIDA POR NINGUN RECIBO.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800174089-2

CERTIFICACION DE EXCEDENTE : 07 - 97,299
FECHA : NOVIEMBRE 28 DE 2007

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	Nº. PAGO	FECHA PGO	Vr. PAGO
CLARKE MODET Y CO. COLOMB	CONSIGNACION	BANCO POPULAR	050-00110-6	72395998	28/11/2007	8,640,000.00

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

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		99 OTROS SERVICIOS ADMINISTRATIVOS	1,000.00
		TOTAL :	\$ 1,000.00

SON: MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

ESTA CERTIFICACION DE EXCEDENTE DE PAGO SOLO PODRA SER UTILIZADA
COMO PARTE DE LA CANCELACION DE UNA TARIFA VIGENTE Y NO PODRA SER
SUSTITUIDA POR NINGUN RECIBO.

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DIRECCIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 6-110246

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **578** DE

FECHA **31 DE JULIO DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR LA LEY

EXPIRA EL **26 DE OCTUBRE DE 2007**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X



US006214344B1

(12) **United States Patent**
Schwall et al.

(10) Patent No.: **US 6,214,344 B1**
(45) Date of Patent: ***Apr. 10, 2001**

(54) **HEPATOCTYTE GROWTH FACTOR
RECEPTOR ANTAGONISTS AND USES
THEREOF**

(75) Inventors: **Ralph H. Schwall, Pacifica; Kelly
Helen Tabore, Hillsborough, both of CA
(US)**

(73) Assignee: **Genetech, Inc., South San Francisco,
CA (US)**

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.

This patent is subject to a terminal dis-
claimer.

(21) Appl. No.: **09/006,776**

(22) Filed: **Jan. 14, 1998**

Related U.S. Application Data

(63) Continuation of application No. 08/459,849, filed on Jun. 2,
1995, now abandoned.

(51) Int. Cl.⁷ **C07K 16/28; C07K 16/30;
A61K 39/595**

(52) U.S. Cl. **424/174.1; 424/130.1;
424/138.1; 424/141.1; 424/143.1; 424/152.1;
424/155.1; 424/172.1; 530/387.7; 530/388.22;
530/388.8; 530/388.85; 530/389.7**

(58) Field of Search **424/138.1, 143.1,
424/152.1, 130.1, 141.1, 155.1, 172.1, 174.1;
530/388.8, 388.88, 389.7, 387.7, 388.22**

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5,686,292 * 11/1997 Schwall et al.

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WO 93/23550 11/1993 (WO)
WO 93/04679 3/1994 (WO)
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(List continued on next page.)

Primary Examiner—Nancy A. Johnson
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(57) **ABSTRACT**

Hepatocyte growth factor (HGF) receptor antagonists are
provided. The HGF receptor antagonists include HGF recep-
tor antibodies and fragments thereof. The HGF receptor
antagonists can be employed to block binding of HGF to
HGF receptors or substantially inhibit HGF receptor acti-
vation. The HGF receptor antagonists may be included in
pharmaceutical compositions, articles of manufacture, or
kits. Methods of treating cancer using the HGF receptor
antagonists are also provided.

16 Claims, 3 Drawing Sheets

**1
HEPATOCTYTE GROWTH FACTOR
RECEPTOR ANTAGONISTS AND USES
THEREOF**

This is a continuation of application Ser. No. 08/459,849
filed on Jun. 2, 1995, now abandoned, which application is
incorporated herein by reference and to which application(s)
priority is claimed under 35 USC §120.

FIELD OF THE INVENTION

This application relates to hepatocyte growth factor recep-
tor antagonists. The application also relates to the use of the
antagonists in therapy or diagnosis of particular pathological
conditions in mammals, including cancer.

BACKGROUND OF THE INVENTION

Hepatocyte growth factor ("HGF") functions as a growth
factor for particular tissues and cell types. HGF was iden-
tified initially as a mitogen for hepatocytes [Michalopoulos
et al., *Cancer Res.*, 44:4414-4419 (1984); Russel et al., *J.
Cell. Physiol.*, 119:183-192 (1984); Nakamura et al., *Bio-
chem. Biophys. Res. Comm.*, 122:1450-1459 (1984)]. Naka-
mura et al., supra, reported the purification of HGF from the
serum of partially hepatectomized rats. Subsequently, HGF
was purified from rat platelets, and its subunit structure was
determined [Nakamura et al., *Proc. Natl. Acad. Sci. USA*,
83:6489-6493 (1986); Nakamura et al., *FEBS Letters*,
224:311-316 (1987)]. The purification of human HGF
("huHGF") from human plasma was first described by
Gohda et al., *J. Clin. Invest.*, 81:414-419 (1988).

Both rat HGF and huHGF have been molecularly cloned,
including the cloning and sequencing of a naturally occur-
ring variant lacking 5 amino acids designated "delta5 HGF"
[Miyazawa et al., *Biochem. Biophys. Res. Comm.*,
163:967-973 (1989); Nakamura et al., *Nature*, 342:440-443
(1989); Seki et al., *Biochem. Biophys. Res. Commun.*,
172:321-327 (1990); Tasuro et al., *Proc. Natl. Acad. Sci.
USA*, 87:3200-3204 (1990); Okajima et al., *Eur. J.
Biochem.*, 193:375-381 (1990)].

The mature form of huHGF, corresponding to the major
form purified from human serum, is a disulfide linked
heterodimer derived by proteolytic cleavage of the human
pro-hormone between amino acids R494 and V495. This
cleavage process generates a molecule composed of an
 α -subunit of 440 amino acids (M, 69 kDa) and a β -subunit
of 234 amino acids (M, 34 kDa). The nucleotide sequence
of the huHGF cDNA reveals that both the α - and the
 β -chains are contained in a single open reading frame coding
for a pre-pro precursor protein. In the predicted primary
structure of mature huHGF, an interchain S-S bridge is
formed between Cys 487 of the α -chain and Cys 604 in the
 β -chain [see Nakamura et al., *Nature*, supra]. The
N-terminus of the α -chain is preceded by 54 amino acids,
starting with a methionine group. This segment includes a
characteristic hydrophobic leader (signal) sequence of 31
residues and the prosequence. The α -chain starts at amino
acid (aa) 55, and contains four kringle domains. The kringle
1 domain extends from about aa 128 to about aa 206, the
kringle 2 domain is between about aa 211 and about aa 288,
the kringle 3 domain is defined as extending from about aa
303 to about aa 383, and the kringle 4 domain extends from
about aa 391 to about aa 464 of the α -chain.

The definition of the various kringle domains is based on
their homology with kringle-like domains of other proteins
(such as prothrombin and plasminogen), therefore, the above
limits are only approximate. To date, the function of these

US 6,214,344 B1

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kringles has not been determined. The β -chain of huHGF
shows high homology to the catalytic domain of serine
proteases (38% homology to the plasminogen serine pro-
tease domain). However, two of the three residues which
form the catalytic triad of serine proteases are not conserved
in huHGF. Therefore, despite its serine protease-like
domain, huHGF appears to have no proteolytic activity, and
the precise role of the β -chain remains unknown. HGF
contains four putative glycosylation sites, which are located
at positions 294 and 402 of the α -chain and at positions 566
and 653 of the β -chain.

In a portion of cDNA isolated from human leukocytes,
in-frame deletion of 15 base pairs was observed. Transient
expression of the cDNA sequence in COS-1 cells revealed
that the encoded HGF molecule (delta5 HGF) lacking 5
amino acids in the kringle 1 domain was fully functional
[Seki et al., supra].

A naturally occurring huHGF variant has been identified
which corresponds to an alternative spliced form of the
huHGF transcript containing the coding sequences for the
N-terminal finger and first two kringle domains of mature
huHGF [Chen et al., *Science*, 254:1382-1385 (1991);
Miyazawa et al., *Eur. J. Biochem.*, 197:15-22 (1991)]. This
variant, designated HGF/NK2, has been proposed to be a
competitive antagonist of mature huHGF. Comparisons of
the amino acid sequence of rat HGF with that of huHGF
have revealed that the two sequences are highly conserved
and have the same characteristic structural features. The
length of the four kringle domains in rat HGF is exactly the
same as in huHGF. Furthermore, the cysteine residues are
located in exactly the same positions, an indication of
similar three-dimensional structures [Okajima et al., supra;
Tashiro et al., supra].

HGF and HGF variants are described further in U.S. Pat.
Nos. 5,227,158, 5,316,921, and 5,328,837.

The HGF receptor has been identified as the product of the
c-Met proto-oncogene [Bottaro et al., *Science*, 251:802-804
(1991); Naldini et al., *Oncogene*, 6:501-504 (1991); WO
92/13097 published Aug. 6, 1992; WO 93/15754 published
Aug. 19, 1993]. The receptor is usually referred to as
"c-Met" or "p190^{Met}" and typically comprises, in its native
form, a 190-kDa heterodimeric (a disulfide-linked 50-kDa
 α -chain and a 145-kDa β -chain) membrane-spanning
tyrosine kinase protein [Park et al., *Proc. Natl. Acad. Sci.
USA*, 84:6379-6383 (1987)]. Several truncated forms of the
c-Met receptor have also been described [WO 92/26792;
Prat et al., *Mol. Cell. Biol.*, 11:5954-5962 (1991)].

The binding activity of HGF to its receptor is believed to
be conveyed by a functional domain located in the
N-terminal portion of the HGF molecule, including the first
two kringles [Matsumoto et al., *Biochem. Biophys. Res.
Commun.*, 181:691-699 (1991); Hartmann et al., *Proc. Natl.
Acad. Sci.*, 89:11574-11578 (1992); Lokker et al., *EMBO J.*,
11:2503-2510 (1992); Lokker and Godowski, *J. Biol.
Chem.*, 268:17145-17156 (1994)]. The c-Met protein
becomes phosphorylated on tyrosine residues of the 145-
kDa β -subunit upon HGF binding.

Certain antibodies to HGF receptor have been reported in
the literature. Several such antibodies are described below.

Prat et al., *Mol. Cell. Biol.*, supra, describe several
monoclonal antibodies specific for the extracellular domain
of the β -chain encoded by the c-Met gene [see also, WO
92/20792]. The monoclonal antibodies were selected fol-
lowing immunization of Balb/c mice with whole living
GTL-16 cells (human gastric carcinoma cell line) overex-
pressing the Met protein. The spliced cells obtained from the

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immunized mice were fused with Ag8.653 myeloma cells, and hybrid supernatants were screened for binding to GTL-16 cells. Four monoclonal antibodies, referred to as D1-21, DN-30, DN-31, and DQ-21, were selected.

Prat et al., *Int. J. Cancer*, 49:323-328 (1991) describe using anti-c-Met monoclonal antibody DQ-24 for detecting distribution of the c-Met protein in human normal and neoplastic tissues [see, also, Yamada et al., *Brain Research*, 637:308-312 (1994)]. The murine monoclonal antibody DQ-24 was reported to be an IgG2a isotype antibody.

Crepaldi et al., *J. Cell Biol.*, 125: 313-320 (1994) report using monoclonal antibodies DQ-24 and DN-30 [described in Prat et al., *Mol. Cell Biol.*, supra] and monoclonal antibody DQ-13 to identify subcellular distribution of HGF receptors in epithelial tissues and in MDCK cell monolayers. According to Crepaldi et al., monoclonal antibody DQ-13 was raised against a peptide corresponding to nineteen COOH-terminal amino acids (from Ser¹³⁷² to Ser¹³⁹⁰) of the human c-Met sequence.

A monoclonal antibody specific for the cytoplasmic domain of human c-Met has also been described [Bellaro et al., supra].

Several of the monoclonal antibodies referenced above are commercially available from Upstate Biotechnology Incorporated, Lake Placid, N.Y. Monoclonal antibodies DQ-24 and D1-21, specific for the extracellular epitope of c-Met, are available from Upstate Biotechnology Incorporated. Monoclonal antibody DQ-13, specific for the intracellular epitope of c-Met, is also available from Upstate Biotechnology Incorporated.

Various biological activities have been described for HGF and its receptor [see, generally, Chan et al., *Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c-Met Receptor*, Goldberg and Rosen, eds., Birkhauser Verlag, Basel (1993), pp. 67-79]. It has been observed that levels of HGF increase in the plasma of patients with hepatic failure [Gohda et al., supra] and in the plasma [Lindroos et al., *Hepatology*, 13:734-750 (1991)] or serum [Asami et al., *J. Biochem.*, 109:8-13 (1991)] of animals with experimentally induced liver damage. The kinetics of this response are usually rapid, and precedes the first round of DNA synthesis during liver regeneration. HGF has also been shown to be a mitogen for certain cell types, including melanocytes, renal tubular cells, keratinocytes, certain endothelial cells and cells of epithelial origin [Matsumoto et al., *Biochem. Biophys. Res. Commun.*, 176:45-51 (1991); Igawa et al., *Biochem. Biophys. Res. Commun.*, 174:831-838 (1991); Han et al., *Biochem.*, 30:9768-9780 (1991); Rubin et al., *Proc. Natl. Acad. Sci. USA*, 88:415-419 (1991)]. Both HGF and the c-Met protooncogene have been postulated to play a role in microglial reactions to CNS injuries [DiRenzo et al., *Oncogene*, 8:219-222 (1993)].

HGF can also act as a "scatter factor", an activity that promotes the dissociation of epithelial and vascular endothelial cells in vitro [Sliker et al., *Nature*, 327:239-242 (1987); Weidner et al., *J. Cell Biol.*, 111:2097-2108 (1990); Naldini et al., *EMBO J.*, 10:2867-2878 (1991); Giordano et al., *Proc. Natl. Acad. Sci. USA*, 90:649-653 (1993)]. Moreover, HGF has recently been described as an epithelial morphogen [Montesano et al., *Cell*, 67:901-908 (1991)]. Therefore, HGF has been postulated to be important in tumor invasion [Corioglio, *Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c-Met Receptor*, Goldberg and Rosen, eds., Birkhauser Verlag-Basel (1993), pp. 131-165]. Bellaruci et al., *Oncogene*, 9:1091 (1994) report that HGF can promote motility and invasive properties of NF1-1 bladder carcinoma cells.

c-Met RNA has been detected in several murine myeloid progenitor tumor cell lines [Iyer et al., *Cell Growth and Differentiation*, 1:87-95 (1990)]. Further, c-Met is expressed in various human solid tumors [Prat et al., *Int. J. Cancer*, supra]. Overexpression of the c-Met oncogene has also been suggested to play a role in the pathogenesis and progression of thyroid tumors derived from follicular epithelium [DiRenzo et al., *Oncogene*, 7:2549-2553 (1992)]. Chronic c-Met/HGF receptor activation has also been observed in certain malignancies [Cooper et al., *EMBO J.*, 5:2623 (1986); Giordano et al., *Nature*, 339:155 (1989)].

In view of the role of HGF and/or c-Met in potentiating or promoting such diseases or pathological conditions, it would be useful to have a means of substantially reducing or inhibiting one or more of the biological effects of HGF and its receptor.

SUMMARY OF THE INVENTION

The invention provides HGF receptor antagonists which are capable of specifically binding to a HGF receptor. Preferred HGF receptor antagonists are capable of substantially reducing or inhibiting the mitogenic, motogenic or other biological activity of HGF or HGF receptor activation, and thus are useful in the treatment of various diseases and pathological conditions such as cancer. In one embodiment of the invention, the HGF receptor antagonist is an antibody. Preferably, the antagonist is a monoclonal antibody, and more preferably, is a Fab fragment of a monoclonal antibody.

The invention also provides hybridoma cell lines which produce HGF receptor antagonistic monoclonal antibodies, and isolated nucleic acid encoding HGF receptor antagonistic antibodies.

The invention also provides pharmaceutical compositions comprising one or more HGF receptor antagonists in a pharmaceutically-acceptable carrier. In one embodiment, the pharmaceutical composition may be included in an article of manufacture or kit.

The invention further provides methods for treating cancer comprising administering to a mammal diagnosed as having cancer an effective amount of a HGF receptor antagonist. The HGF receptor antagonist alone may be administered to the mammal, or alternatively, may be administered to the mammal in combination with other therapeutic agents such as anti-cancer agents.

It is believed that the antagonists can be used to block binding of HGF to HGF receptor(s) or substantially prevent receptor activation, thereby treating pathological conditions associated with binding of HGF to HGF receptor(s) or with the activation of HGF receptor(s).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the inhibition of HGF binding to c-Met-IgG fusion protein by monoclonal antibody 1A3.3.13.

FIG. 2 is a bar diagram showing the stimulatory effect of monoclonal antibodies 3D6, 6G1, and 1A3.3.13 on human mammary epithelial cells in a proliferation assay.

FIG. 3 is a bar diagram showing the stimulatory effect of monoclonal antibodies 3D6, Q5-237 and Q5-238 on mink lung cells in a proliferation assay.

FIG. 4 is a bar diagram showing the inhibitory effect of monoclonal antibody 1A3.3.13 Fab fragments on Balb-3T3 cells in a proliferation assay.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

As used herein, the terms "hepatocyte growth factor" and "HGF" refer to a growth factor typically having a structure with six domains (finger, Kringle 1, Kringle 2, Kringle 3, Kringle 4 and serine protease domains). Fragments of HGF constitute HGF with fewer domains and variants of HGF may have some of the domains of HGF repeated; both are included if they still retain their respective ability to bind a HGF receptor. The terms "hepatocyte growth factor" and "HGF" include hepatocyte growth factor from humans ("huHGF") and any non-human mammalian species, and in particular rat HGF. The terms as used herein include mature, pre-, pre-pro, and pro forms, purified from a natural source, chemically synthesized or recombinantly produced. Human HGF is encoded by the cDNA sequence published by Miyazawa et al., 1989, supra, or Nakamura et al., 1989, supra. The sequences reported by Miyazawa et al. and Nakamura et al. differ in 14 amino acids. The reason for the differences is not entirely clear; polymorphism or cloning artifacts are among the possibilities. Both sequences are specifically encompassed by the foregoing terms. It will be understood that natural allelic variations exist and can occur among individuals, as demonstrated by one or more amino acid differences in the amino acid sequence of each individual. The terms "hepatocyte growth factor" and "HGF" specifically include the delta5 huHGF as disclosed by Sckl et al., supra.

The terms "HGF receptor" and "c-Met" when used herein refer to a cellular receptor for HGF, which typically includes an extracellular domain, a transmembrane domain and an intracellular domain, as well as variants and fragments thereof which retain the ability to bind HGF. The terms "HGF receptor" and "c-Met" include the polypeptide molecule that comprises the full-length, native amino acid sequence encoded by the gene variously known as p192^{met}. The present definition specifically encompasses soluble forms of HGF receptor, and HGF receptor from natural sources, synthetically produced in vitro or obtained by genetic manipulation including methods of recombinant DNA technology. The HGF receptor variants or fragments preferably share at least about 65% sequence homology, and more preferably at least about 75% sequence homology with any domain of the human c-Met amino acid sequence published in Rodriguez et al., *Mol. Cell Biol.*, 11:2962-2970 (1991); Park et al., *Proc. Natl. Acad. Sci.*, 84:6379-6383 (1987); or Panzotto et al., *Oncogene*, 6:553-559 (1991).

The terms "agonist" and "agonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, substantially inducing, promoting or enhancing HGF biological activity or HGF receptor activation.

The terms "antagonist" and "antagonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, substantially counteracting, reducing or inhibiting HGF biological activity or HGF receptor activation.

The term "HGF biological activity" when used herein refers to any mitogenic, motogenic or morphogenic activities of HGF or any activities occurring as a result of HGF binding to a HGF receptor. The term "HGF receptor activation" refers to HGF receptor dimerization or HGF receptor-induced tyrosine kinase activity. HGF receptor activation may occur as a result of HGF binding to a HGF receptor, but may alternatively occur independent of any HGF binding to a HGF receptor. HGF biological activity may, for example, be determined in an in vitro or in vivo

assay of hepatocyte growth promotion. Adult rat hepatocytes in primary culture have been used to test the effect of HGF on hepatocyte proliferation. Accordingly, the effect of a HGF receptor antagonist can be determined in an assay suitable for testing the ability of HGF to induce DNA synthesis of rat hepatocytes in primary cultures. Human hepatocytes can be cultured similarly to the methods established for preparing primary cultures of normal rat hepatocytes. Alternatively, the effect of a HGF receptor antagonist can be determined in an assay suitable for testing the ability of HGF to induce DNA synthesis in other types of cells expressing HGF receptor(s), such as mink lung cells or human mammary epithelial cells described in Examples 3 and 4. DNA synthesis can, for example, be assayed by measuring incorporation of ³H-thymidine into DNA. The effectiveness of the HGF receptor antagonist can be determined by its ability to block proliferation and incorporation of the ³H-thymidine into DNA. The effect of HGF receptor antagonists can also be tested in vivo in animal models.

The term "antibody" is used herein in a broad sense and includes intact immunoglobulin molecules and immunoglobulin fragments (such as Fab, Fab', or Fv), so long as they exhibit any of the desired antagonistic properties described herein. Antibodies are typically proteins which exhibit binding specificity to a specific antigen. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains [Closa et al., *J. Mol. Biol.*, 186:651-663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA*, 82:4592-4596 (1985)]. The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

The term "variable" is used herein to describe certain portions of the variable domains which differ in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments, called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions,

largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the H/R regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies [see Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md. (1987)]. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a substantially homogeneous population of antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. 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 antagonistic activity [U.S. Pat. No. 4,816,567; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)].

The terms "cancer" and "cancerous" when used herein refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, sarcoma, blastoma and leukemia. More particular examples of such cancers include squamous cell carcinoma, lung cancer, pancreatic cancer, cervical cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer. While the term "cancer" as used herein is not limited to any one specific form of the disease, it is believed that the methods of the invention will be particularly effective for cancers which are found to be accompanied by increased levels of HGF or expression of c-Met in the mammal.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

D. Compositions and Methods of the Invention

In one embodiment of the invention, HGF receptor antagonists are provided. Non-limiting examples of HGF receptor antagonists include antibodies, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, biorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like. The HGF receptor antagonists of the invention are preferably HGF receptor antibodies. For instance, the antagonistic antibodies may be polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intra-

peritoneal injections. Preferably, the immunizing agent includes the c-Met polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL- α -TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for HGF receptor antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

The antagonistic antibodies of the invention may, alternatively, be monoclonal antibodies. Antagonistic monoclonal antibodies of the invention may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro.

Preferably, the immunizing agent includes the c-Met polypeptide or a fusion protein thereof. The immunizing agent may alternatively comprise a fragment or portion of HGF or a HGF receptor having one or more amino acid residues that participate in the binding of HGF to its receptor. In a more preferred embodiment, the immunizing agent comprises an extracellular domain of c-Met fused to an IgG sequence, such as described in Example 1.

Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif., and the American Type Culture Collection, Rockville, Md. An example of such a murine myeloma cell line is P3X63Ag.U1, described in Example 1 below. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the

production of human monoclonal antibodies [Kozbor, J. *Immunol.*, 133:3031 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against a HGF receptor. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Such techniques and assays are known in the art, and are described further in the Examples below. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, *Anal. Biochem.*, 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown in vivo as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Pat. No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a HGF receptor and another antigen-combining site having specificity for a different antigen, such as HER2 or CD3.

It is believed, however, that monovalent antibodies capable of binding to a HGF receptor will be especially useful as HGF receptor antagonists. While not being bound to any particular theory, it is presently believed that activation of c-Met may proceed by a mechanism wherein hHGF binding to c-Met induces aggregation or dimerization of the receptors which in turn activates intracellular receptor kinase activity. Because monovalent antibodies will likely be unable to induce such aggregation or dimerization, the monovalent antibodies should not activate c-Met. Such

monovalent antibodies may be directed against the HGF binding site of the receptor or may otherwise be capable of interfering with HGF, its fragments or its variants binding to the HGF receptor, such as by sterically hindering HGF, its fragments or its variants access to the receptor. Alternatively, the monovalent antibodies may be capable of sterically preventing HGF receptor dimerization.

Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/25348 published Dec. 22, 1994 and U.S. Pat. No. 4,342,566. Papain digestion is also described in Example 6 below. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an F(ab')₂ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH₁) of the heavy chain. Fab fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain (H₁ domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domain bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

In a preferred embodiment of the invention, the antagonists comprise Fab fragments of monoclonal antibodies specific for c-Met. In a more preferred embodiment, the monoclonal antibody Fab fragments have the same biological characteristics as the monoclonal antibody Fab fragments produced by digesting either of the monoclonal antibodies secreted by the hybridoma cell lines deposited under American Type Culture Collection Accession Nos. ATCC HB-11894 or ATCC HB-11895. The term "biological characteristics" is used to refer to the in vitro and/or in vivo activities of the monoclonal antibody, e.g., ability to substantially reduce or inhibit binding of hHGF to c-Met or to substantially reduce or inhibit c-Met activation. Accordingly, the monovalent antibody preferably binds to substantially the same epitope as the 1A3.3.13 antibody or the 5D5.11.6 antibody disclosed herein (this can be determined by conducting assays described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the 1A3.3.13 antibody specifically disclosed (i.e., the antibody having the ATCC deposit No. 11B-11894) or the 5D5.11.6 antibody specifically disclosed (i.e., the antibody having the ATCC deposit No. 11B-11895), one can use a competitive ELISA binding assay. In a preferred embodiment of the invention, the monoclonal antibody or fragment thereof will inhibit the binding of HGF, its fragments or its variants, or the anti-

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genic activity of HGF, its fragments, or its variants at least about 50%, preferably, greater than about 80%, and more preferably, greater than about 90%, as determined by an *in vitro* competitive binding assay or proliferation assay, such as described in the Examples below.

In addition to the antagonistic antibodies described above, it is contemplated that chimeric or hybrid antagonistic antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iodoacetamide and methyl-4-mercaptobutyrimide.

The antagonistic antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂) or other antigen-binding subsequences of antibodies which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found either in the recipient antibody or in the imported CDR or framework sequences. 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 CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "chase-it" method, the sequence of the variable domain of

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a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia and Lesk, *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies. Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/01679 published Mar. 3, 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germline mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germline immunoglobulin gene array in such germline mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551-2555 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immunol.*, 7:33 (1993)]. Human antibodies can also be produced in phage display libraries (Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*, 147(1):86-95 (1991)).

In another embodiment of the invention, methods for treating cancer are provided. In the methods, HGF receptor antagonist is administered to a mammal diagnosed as having cancer. While the term "cancer" as used herein is not limited to any one specific form of the disease, it is believed that the methods will be particularly effective for cancers which are found to be accompanied by increased levels of HGF or expression of c-Met in the mammal. In a preferred method of the invention, the cancer is breast cancer. It is of course contemplated that the methods of the invention can be employed in combination with still other therapeutic techniques such as surgery.

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The antagonist is preferably administered to the mammal in a pharmaceutically-acceptable carrier. Suitable carriers and their formulations are described in *Remington's Pharmaceutical Sciences*, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of antagonist being administered.

The antagonist can be administered to the mammal by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. The antagonist may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Local or intravenous injection is preferred.

Effective dosages and schedules for administering the antagonist may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of antagonist that must be administered will vary depending on, for example, the mammal which will receive the antagonist, the route of administration, the particular type of antagonist used and other drugs being administered to the mammal. Guidance in selection appropriate doses for antibody antagonists is found in the literature on therapeutic uses of antibodies, e.g., *Handbook of Monoclonal Antibodies*, Ferrone et al., eds., Niles Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., *Antibodies in Human Diagnosis and Therapy*, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antagonist used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

The antagonist may also be administered to the mammal in combination with effective amounts of one or more other therapeutic agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and cytokines. Chemotherapeutics contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiopeta, Busulfan, Cytosin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. The antagonist may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of antagonist and therapeutic agent depend, for example, on what type of drugs are used, the cancer being treated, and the scheduling and routes of administration but would generally be less than if each were used individually.

Following administration of antagonist to the mammal, the mammal's cancer and physiological condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques.

In another embodiment of the invention, methods for employing the antagonists *in vivo* in diagnostic assays are

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provided. For instance, the antagonists may be employed in diagnostic assays to detect overexpression of HGF receptor in specific cells and tissues. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases. [Zola, *Monoclonal Antibodies: A Manual of Techniques*, CRC Press, Inc. (1987) pp. 147-158]. The antagonists used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ³H, ¹⁴C, ³²P, ³⁵S, or ¹²⁵I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antagonist to the detectable moiety may be employed, including those methods described by Hunter et al., *Nature*, 144:945 (1962); David et al., *Biochemistry*, 13:1014 (1974); Pain et al., *J. Immunol. Meth.*, 40:219 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30:407 (1982).

Additionally, antagonistic antibodies of the invention can be used to immunopurify HGF receptor(s).

To a further embodiment of the invention, there are provided articles of manufacture and kits containing materials useful for treating cancer or detecting or purifying HGF receptor.

The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating cancer or for detecting or purifying HGF receptor. The active agent in the composition is a HGF receptor antagonist and preferably, comprises Fab fragments of monoclonal antibodies specific for c-Met. The label on the container indicates that the composition is used for treating cancer or detecting or purifying HGF receptor, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention comprises the container described above and a second container comprising a buffer. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All reference citations herein are incorporated by reference.

EXAMPLE I

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 2.5 µg/50 µl c-Met-IgG fusion protein (cloned in MPI-TPM adjuvant purchased from R&B Immunochemical Research, Inc., Hampton, Mont.) five times into each hind foot pad. Injections were administered on Day 0 and Days 56, 63, 66 and 73. The c-Met-IgG fusion protein (including the extracellular domain of c-Met fused to a human IgG1 heavy chain) was constructed essentially as described by Mark et al., *J. Biol. Chem.*, 267:26166-26171 (1992) and produced in Chinese hamster ovary (CHO) cells. The c-Met-IgG was subsequently purified in a single step using affinity chromatography on immobilized Protein A (BioProcessing, Inc., Princeton, N.J.), using an elution scheme modified from Charnow et al., *J. Immunol.*,

153,4268-4280 (1994). Culture supernatant was loaded onto a Protein A column equilibrated in 20 mM Tris, pH 7.4, 0.15 M NaCl. The column was washed, first with equilibration buffer, then with equilibration buffer containing 0.5 M tetramethylammonium chloride, to remove non-specifically bound protein. e-Met-IgG was eluted with 20 mM Tris, pH 7.4, 3.5 M MgCl₂. This e-Met-IgG eluate was concentrated and exchanged into 20 mM Tris, pH 7.4, 0.15 M NaCl by gel filtration on Sephadex G25 to a final concentration of about 2-4 mg/ml.

On Day 77, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowittaker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the e-Met-IgG fusion protein.

In the ELISA, 96-well microtiter plates were coated by adding 50 μ l of 2 μ g/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) to each well and incubating at 4° C. overnight. The plates were then washed three times with distilled water. The wells in the microtiter plates were blocked with 200 μ l of 2% bovine serum albumin and incubated at room temperature for 1 hour. The plates were then washed again three times with distilled water.

After the washing step, 100 μ l of 0.4 μ g/ml e-Met-IgG fusion protein (as described above) was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with distilled water.

Then, 100 μ l of the hybridoma supernatants was added to designated wells. 100 μ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with distilled water.

Next, 50 μ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimerosal in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with distilled water, followed by addition of 50 μ l of substrate (5 mg OPD, 12.5 ml PBS, 5 μ l H₂O₂) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50 μ l of 2 N H₂SO₄ to each well, and absorbance at 490 nm was read in an automated microtiter plate reader.

Of 912 hybridoma supernatants screened in the ELISA, 24 supernatants tested positive (calculated as approximately 2 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using A549 cells (human epidermoid cell line expressing e-Met; ATCC CCL 185) and fluorescein-conjugated mouse anti-IgG. The FACS analysis showed 19/24 supernatants were positive for anti-e-Met antibodies.

EXAMPLE 2

Balb/c mice were immunized as described in Example 1 except that the e-Met-IgG fusion protein injections were administered on Day 0 and Days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105, 112, 119, 126, 133, 140, 147, 154, 161, 168, 175, 182, 189, 196, 203, 210, 217, 224, 231, 238, 245, 252, 259. On Day 282, lymph nodes were removed and a fusion was conducted as described in Example 1. Hybridoma

supernatants were tested according to the ELISA described in Example 1. One of the positive anti-e-Met monoclonal antibodies was called 5D5.11.6. Further antibody analysis showed that the 5D5.11.6 monoclonal antibody is an IgG1 isotype antibody comprising a kappa light chain.

EXAMPLE 3

An inhibition assay was conducted to examine the ability of the antibodies (described in Example 1) to block binding of HGF to e-Met-IgG fusion protein. Prior to conducting the inhibition assay, the 24 hybridoma supernatants determined positive in the ELISA in Example 1 were purified on Protein A-Sepharose columns to yield antibody preparations of about 1 μ g/ml.

For the assay, 96-well microtiter plates were coated by adding 100 μ l of 2 μ g/ml goat anti-human Fc (purchased from Cappel Laboratories) to each well and incubating overnight at 4° C. The plates were then washed with a washing buffer (0.05% Tween-20, 0.01% Thimerosal in PBS). Nonspecific binding in the wells was blocked by adding 150 μ l blocking buffer (0.5% BSA, 0.01% Thimerosal in PBS, pH 7.4) to each well and incubating at room temperature for 2 hours with rapid agitation on an orbital shaker.

Next, continuing at room temperature and agitation on the orbital shaker, the blocking buffer was removed from the wells, and the plates were washed with washing buffer. Next, 100 μ l of 10 ng/ml e-Met-IgG fusion protein (described in Example 1) was added to the wells. The plates were incubated for 2 hours and then washed 3 times with washing buffer.

Recombinant human HGF (rhHGF) was produced in CHO cells using a procedure modified from Naka et al., *J. Biol. Chem.*, 267:20114-20119 (1992). rhHGF-transfected cells were grown in a 400 L bioreactor in medium containing 20% fetal bovine serum for 8 days. Culture supernatant containing rhHGF was concentrated and clarified, then conditioned by the addition of solid NaCl to 0.3 M. rhHGF was then purified in a single step using cation exchange chromatography. Conditioned, concentrated culture supernatant was loaded onto a column of S-Sepharose Fast Flow equilibrated in 20 mM Tris, pH 7.5, 0.3 M NaCl. After washing out unbound protein, rhHGF was eluted in a linear gradient from 20 mM Tris, pH 7.5, 0.3 M NaCl to 20 mM Tris, pH 7.5, 1.2 M NaCl. rhHGF-containing fractions were pooled based on SDS-PAGE analysis. The S-Sepharose Fast Flow pool was concentrated and exchanged into 20 mM Tris, pH 7.5, 0.5 M NaCl by gel filtration on Sephadex G25 to a final concentration of about 3-5 mg/ml. A rhHGF stock solution was then prepared by diluting the rhHGF in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimerosal in PBS) to a concentration of 10 μ g/ml. A stock solution of control gp120 antibody (Genentech, Inc.) was also prepared by diluting the antibody in assay buffer to a concentration of 10 μ g/ml.

Then, 50 μ l of either rhHGF, gp120 antibody or one of the 24 monoclonal antibodies was added to designated wells to yield a final concentration of 1000, 100, 10, or 1 ng/ml/well. Immediately, 50 μ l of 200 ng/ml biotinylated rhHGF (rhHGF biotinylated using Biotin-X-NHS obtained from Research Organics, Inc.) was also added to each well. After 2 hours incubation, the wells were washed 3 times with washing buffer. 100 μ l HRP-streptavidin (1:2000 dilution in assay buffer) (purchased from Zymed Laboratories), was added to the wells and the plates were incubated for 30 minutes. The plates were washed again 3 times with washing

buffer. 50 μ l of substrate (5 mg OPD, 12.5 ml PBS, 5 μ l H₂O₂) was added to the wells and color was allowed to develop for 20 minutes. The reaction was stopped by adding 100 μ l 4.5 N sulfuric acid to each well. Absorbance at 492 nm was quantitated in an automated microtiter plate reader.

One of the monoclonal antibodies, referred to as 1A3.3.13, significantly blocked binding of HGF (FIG. 1). Further antibody analysis showed that the 1A3.3.13 monoclonal antibody is an IgG1 isotype antibody comprising a kappa light chain.

EXAMPLE 4

Several different antibodies produced in the fusion described in Example 1, referred to as 3D6, 6G1, and 1A3.3.13, and purified as described in Example 3, were tested and compared for their ability to induce DNA synthesis in a human mammary epithelial cell bioassay.

Human mammary epithelial cells (obtained from Clonetics Corp., No. CC-2551) were passaged in Mammary Epithelial Cell Basal Medium (Clonetics Corp., No. CC-3151). Prior to conducting the bioassay, the cells were trypsinized, washed, and resuspended in assay medium (Basal Medium supplemented with 1 mg/ml BSA, penicillin, streptomycin and L-glutamine) to a concentration of 1x10⁵ cells/ml. Next, 100 μ l of the cells were added to the wells of 96-well culture plates. rhHGF (described in Example 3) was diluted in assay medium at concentrations of 20 ng/ml and 200 ng/ml. The 3D6, 6G1, 1A3.3.13, and control gp 120 antibodies were diluted in assay medium at concentrations of 200 ng/ml and 2 μ g/ml. 100 μ l of the rhHGF and antibody preparations was then added to designated wells. The plates were incubated at 37° C. in 5% CO₂ for 16 hours.

Next, 1 μ Ci [³H]-thymidine was added to each well, and the plates were incubated for 24 hours at 37° and 5% CO₂. The human mammary epithelial cells were harvested and the amount of radioactivity incorporated into the DNA was then quantitated in a microplate scintillation counter.

The results showed that, at a concentration of 10 ng/ml, the 3D6, 6G1 and 1A3.3.12 antibodies have some HGF-agonistic effect. (See FIG. 2)

EXAMPLE 5

Anti-e-Met antibodies 05-237 and 05-238 (purchased from Upstate Biotechnology Inc., Lake Placid, N.Y.; see also, Prat et al., *Mol. Cell. Biol.*, supra; Prat et al., *Int. J. Cancer*, supra) and antibody 3D6 (described in Example 4) were tested and compared for their ability to induce DNA synthesis in a mink lung bioassay.

Mink lung cells (Mv 1 Lu, ATCC CCL 64) were passaged in DME/F12 (50:50) supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine. Prior to conducting the bioassay, the mink lung cells were trypsinized, washed, and resuspended in assay medium (DME/F12 medium supplemented with 1 mg/ml BSA, penicillin, streptomycin and L-glutamine) to a concentration of 1x10⁵ cells/ml. The bioassay was then conducted as described in Example 4.

The results showed that antibodies 05-237 and 05-238 have HGF agonistic effect. (See FIG. 3)

EXAMPLE 6

Antagonistic activity of 1A3.3.13 monoclonal antibody Fab fragments was determined using a thymidine incorporation assay. Monoclonal antibody 1A3.3.13 (described in Example 3) was digested with papain to obtain the Fab

fragments. The papain digestion was performed by initially dialyzing the antibody against a 20 mM phosphate/10 mM EDTA, pH 7.0, buffer overnight. The antibody was then concentrated to approximately 10 mg/ml. Next, 0.5 ml immobilized papain (crosslinked 6% beaded agarose, obtained from Pierce Chemicals) was added to a 16x100 mm tube. The papain beads were washed 3 times with 4 ml of digestion buffer (42 mg cysteine-HCl in 12 ml phosphate buffer, pH 10). Each wash was removed using a separator. About 0.5 to 1 ml of the 1A3.3.13 antibody was added to the papain beads and then incubated in a heated shaker bath (37° C., 200 rpm) overnight. 1.5 ml of binding buffer (Immunopure IgG Binding Buffer obtained from Pierce Chemicals) was added to the tube, and the supernatant was separated from the beads with a separator. The supernatant was then passed over a Protein A column equilibrated with the binding buffer. Additional binding buffer was passed over the column and the eluate containing the Fab fragments was collected in 1 ml fractions. The fractions were analyzed by absorbance at 280 nm and the Fab containing fractions dialyzed against PBS overnight. Absorbance at 280 nm was read again to determine the concentration of Fab (about 1.53). The fractions were also run on a 7.5% SDS gel to determine the purity of the Fab in the fraction. The 1A3.3.13 Fab fragments were further tested in an inhibition assay (as described in Example 3). The Fab fragments did inhibit HGF binding but showed a weaker inhibitory effect as compared to intact 1A3.3.13 antibody (data not shown).

An expression plasmid was prepared by inserting a full-length cDNA for human e-Met (described as pOK met cDNA in Rodrigues et al., supra) into a pRK5.1k.neo vector [de Sauvage et al., *Nature*, 369:533-538 (1994); Gorman, *DNA Cloning: A New Approach*, 2:143-190 (IRI, Washington 1985)]. The resulting plasmid was linearized and transfected into the IL-3 dependent cell line, BaF3 [Palacios et al., *Cell*, 41:727-734 (1985)] by electroporation (800 microfarad, 250 V, 300 pF electroporator). Selection of transfectants was performed by culturing the cells for 2-3 weeks in the presence of 2 ng/ml G418. One of the selected transfectant cell lines, referred to as BaF3-hmet.8, was confirmed by Western blotting to express e-Met. BaF3-hmet.8 also tested positive for response to HGF in a proliferation assay measuring incorporation of [³H]-thymidine. Neither the parental BaF3 cells nor any cells derived by transfection with the pRK5.1k.neo vector alone were found to express e-Met or respond to HGF in the proliferation assay.

The BaF3-hmet.8 cells were passaged in RPMI medium supplemented with 10% fetal bovine serum, 5% WEHI-conditioned medium (as a source of IL-3) and 2 mM glutamine. Prior to conducting the assay, the cells were washed twice with assay medium (RPMI medium supplemented with 10% fetal bovine serum) and resuspended in assay medium to a concentration of 5x10⁵ cells/ml. Next, 100 μ l of the cells was added to each well in the 96-well culture plates. Various concentrations (0.2 μ g/ml, 2 μ g/ml, 20 μ g/ml) of control gp 120 Fab fragments (gp 120 monoclonal antibody digested with papain as described above) and the 1A3.3.13 Fab fragments were prepared in assay medium and 100 μ l was added to designated wells. The plates were incubated at 37° C. in 5% CO₂ for 15 hours.

One μ Ci [³H]-thymidine was added in each well of the culture plates. The cells were harvested 7 hours later and the amount of radioactivity incorporated into the DNA was quantitated (CPM) in a microplate scintillation counter.

The results are shown in FIG. 4. At a concentration of 10 μ g/ml, the 1A3.3.13 Fab fragments significantly blocked BaF3-hmet.8 cell proliferation in the presence of HGF.

Deposit of Materials

The following cultures have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC):

Hybrid no.	ATCC No.	Deposit Date
IA33.13	HB-11894	May 23, 1995
SDS.11.6	HB-11895	May 23, 1995

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strains are not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the cultures deposited, since the deposited embodiments are intended as an illustration of an aspect of the invention and any cultures that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents.

What is claimed is:

1. A method of treating cancer in a mammal, comprising administering an effective amount of hepatocyte growth factor (HGF) receptor antagonist to a mammal diagnosed as having cancer, wherein said hepatocyte growth factor (HGF) receptor antagonist is an antibody.

2. The method of claim 1 wherein said cancer is breast cancer.

3. The method of claim 1 wherein said cancer is pancreatic cancer.

4. The method of claim 1 wherein said cancer is colon cancer.

5. The method of claim 1 wherein said cancer is lung cancer.

6. The method of claim 1 wherein said antibody is a monoclonal antibody.

7. The method of claim 1 wherein said antibody binds to the c-Met receptor.

8. The method of claim 7 wherein said antibody inhibits binding of human HGF to the c-met receptor.

9. The method of claim 1 wherein said antagonist is a Fab fragment of a monoclonal antibody.

10. The method of claim 6 wherein said monoclonal antibody is produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-11894.

11. The method of claim 6 wherein said monoclonal antibody is produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-11895.

12. An article of manufacture, comprising:

a container;

a label on said container; and

a composition contained within said container;

wherein the composition is effective for treating cancer, the label on said container indicates that the composition can be used for treating cancer, and the active agent in said composition comprises HGF receptor antagonist; and

wherein said HGF receptor antagonist is an antibody.

13. The article of manufacture of claim 12 further comprising instructions for administering the HGF receptor antagonist to a mammal.

14. A kit, comprising:

a first container, a label on said first container, and a composition contained within said first container;

wherein said composition is effective for treating cancer, the label on said first container indicates that said composition can be used for treating cancer, and the active agent in said composition comprises HGF receptor antagonist; and

wherein said HGF receptor antagonist is an antibody;

a second container comprising a pharmaceutically-acceptable buffer; and

instructions for using said HGF receptor antagonist to treat cancer.

15. The method of claim 1, wherein said antibody is an antagonist for HGF that has the antigen binding ability of the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-11894.

16. The method of claim 1, wherein said antibody is an antagonist for HGF that has the antigen binding ability of the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-11895.

ANTI-HEPATOCYTE GROWTH FACTOR ANTIBODY INHIBITS HEPATOCYTE PROLIFERATION DURING - LIVER REGENERATION

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SUMMARY

In vitro studies have shown hepatocyte growth factor (HGF) to be a potent mitogen for hepatocytes. Direct evidence of a mitogenic role *in vivo* was sought by inhibiting HGF activity, using continuous administration of neutralizing antibody to rats which had a stimulus for liver regeneration. Alzet osmotic mini-pumps, administering a constant supply of anti-HGF monoclonal antibody (clone D9), were inserted intraperitoneally into male Wistar rats; an irrelevant isotypical antibody was administered to controls. Forty-five animals received an intragastric bolus of 40 per cent carbon tetrachloride (CCl₄) and groups of three test and control animals were killed at 24 h intervals for 7 days. Treatment with anti-HGF monoclonal antibody significantly inhibited the levels of immunodetectable HGF in the sera of rats following CCl₄ administration. In comparison with controls, hepatocyte proliferation as assessed by bromodeoxyuridine labelling in anti-HGF-treated animals was significantly inhibited at 24 h ($P < 0.001$), 48 h ($P < 0.001$), and 96 h ($P < 0.05$) post-CCl₄ administration. In contrast, sinusoidal cell proliferation was not significantly different from controls at any time point. Inhibition of the parenchymal proliferative response to acute CCl₄-induced liver injury by the *in vivo* neutralization of HGF provides direct evidence that this growth factor plays an important role in liver regeneration following necrosis. © 1998 John Wiley & Sons, Ltd.

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KEY WORDS—hepatocyte growth factor

INTRODUCTION

Liver regeneration involves a complex array of tightly regulated responses to loss of functional hepatic mass.¹ Hepatocyte growth factor (HGF), a pleiotropic factor with the ability to regulate cell growth, development, motility, and morphogenesis,² is thought to play a central role in this repair process. More recently, HGF has also been assigned a cytoprotective role in response to various acute liver injuries.³⁻⁵ HGF was originally identified in the sera of partially hepatectomized rats and is now recognized to be identical to scatter factor;² it is the most potent complete mitogen for rat and human hepatocytes in primary culture.² Under non-reducing conditions, purified rat HGF is an 82 kD disulphide-linked heterodimeric protein, while under reducing conditions it dissociates into a 59 kD α -subunit and a 34 kD β -subunit;² there is 90 per cent homology with human HGF. The α -subunit contains four kringle domains and the β -subunit structure resembles that of serine proteases, although there is no evidence of protease activity.

HGF is thought to be synthesized and released only by cells of mesenchymal origin. In the liver, HGF messenger ribonucleic acid (mRNA) has been detected in non-parenchymal cells following injury⁶ and in normal liver it has been localized in hepatic stellate

cells.⁷ The protein has been localized to basement membrane structures.^{8,9} Extracellular matrix may serve as an HGF reservoir with mobilization of the protein occurring during hepatic repair; HGF acts predominantly on epithelial cells and thus satisfies the requirements for a paracrine factor. Its expression in extra-hepatic organs following liver injury also suggests that it may function as an endocrine factor¹⁰ and its supply from extra-hepatic sources may contribute to early elevation of plasma levels following injury. Its receptor, the *c-met* proto-oncogene product, is expressed in the liver and a large number of extra-hepatic tissues.¹¹

Much of the evidence that HGF is involved in liver regeneration is based on *in vitro* data. In addition, however, liver HGF mRNA expression has been shown to be upregulated after acute liver injury in rats, increasing prior to the onset of DNA synthesis; this is associated with elevated serum levels in experimental animals and humans following liver injury.^{6,12-14} More direct evidence for a role for HGF in controlling liver cell proliferation is the capacity for recombinant HGF to elevate hepatocyte deoxyribonucleic acid (DNA) synthesis *in vivo* in normal adult rat livers.¹⁵ Furthermore, intravenous injection of recombinant HGF has also been shown to enhance replication of hepatocytes following liver injuries in experimental animals.^{16,17} These approaches, however, do not establish whether HGF activity is an absolute requirement for liver regeneration following injury. We have used an alternative approach to address this issue and have obtained more direct evidence by inhibiting its activity *in vivo*, using continuous administration of a neutralizing antibody to rats

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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DETALLE DE DEVOLUCION CORREO

PERIODO 11/08/2008 AL 11/08/2008

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which had a stimulus for liver regeneration: acute CCl₄-induced liver necrosis.

MATERIALS AND METHODS

Anti-hepatocyte growth factor monoclonal antibody

This antibody, designated as clone D9, is an IgG₁ mouse monoclonal which was raised against a synthetic peptide immunogen with the amino acid sequence DSQYPHKHDIT. This sequence corresponds to the rat sequence of amino acids 330–340¹⁸ and to the human sequence of amino acids 6–16.⁶ In the amino acid sequence of human HGF, Ile is replaced by Met and a possible further substitution of Lys for Glu has also been reported.¹⁹ D9 was purified either using a two-step precipitation method involving 4 per cent caprylic acid²⁰ followed by 50 per cent ammonium sulphate or on a Prosep-A column (Bioprocessing U.K. Ltd.). Purified antibody was dialysed against phosphate buffered saline (PBS) (44 mM NaH₂PO₄, 84 mM Na₂HPO₄, 137 mM NaCl in dH₂O) and stored at –70°C, with or without 0.2 per cent sodium azide.

Under non-reducing conditions, D9 identified a band of 82 kD from immunoblotted rat liver tissue total protein extracts. Under reducing conditions, it detected a band at 69 kD which corresponded to the molecular weight of the HGF α -subunit. Although D9 could detect rhHGF (pET61) in enzyme-linked immunosorbent assays (ELISAs) (see below), it did not recognize rhHGF in immunoblots. The D9 anti-HGF monoclonal antibody did not demonstrate any cross-reactivity with several proteins possessing structural similarity to HGF (human: plasminogen, plasmin, thrombin, prothrombin, apolipoprotein A-1, kallikrein, and Hageman factor; rabbit plasma plasminogen and porcine pancreas elastase). All chemicals were obtained from Sigma Chemical Co. U.K. Ltd.

Osmotic mini-pump preparation

Under sterile conditions, each osmotic mini-pump was loaded with sterile filtered D9 or sterile filtered irrelevant IgG₁ antibody in the same diluent. Osmotic mini-pumps were allowed to equilibrate in sterile 0.9 per cent saline at 37°C for 4 h prior to insertion into rats. These pumps delivered monoclonal antibody at a rate of 1 μ g/h directly into the peritoneal cavity; this approach is associated with uptake of released proteins into the splanchnic circulation.

Animal model

Forty-eight male Wistar rats (mean weight 250 g) were housed in 12/12 h light/dark cycle enclosures and permitted *ad libitum* consumption of a standard laboratory pellet diet. All experiments were carried out under appropriate licence from the U.K. Home Office. Twenty-four animals were anaesthetized and underwent laparotomy with peritoneal insertion of Alzet 2001 mini-pumps containing anti-HGF D9 monoclonal

antibody. Prior to recovery from anaesthesia, 21 rats were given a 1 ml intragastric bolus of 40 per cent CCl₄ in liquid paraffin. Upon recovery from anaesthesia, the remaining three rats that did not receive CCl₄ were given a 1 ml injection of bromodeoxyuridine (BrdU; 50 mg/kg body weight) intraperitoneally (i.p.). One hour after BrdU administration, these animals were killed by administration of terminal anaesthetic (pentobarbitone sodium BP Vet. 200 mg/ml saline; 160 μ l i.p.). One group of three animals was killed daily thereafter, up to and including 7 days. One hour prior to death, all animals received i.p. BrdU (50 mg/kg body weight) injections. A further 24 animals were used as controls. These animals were submitted to identical procedures as described above, except that D9 monoclonal antibody was replaced by irrelevant IgG1 monoclonal antibody.

Tissue collection and processing

Blood was collected by cardiac puncture and serum used for HGF and anti-HGF monoclonal antibody ELISA determinations. Blocks of liver tissue were fixed in formal sublimate (4 h; 4 per cent neutral phosphate-buffered formaldehyde in saturated aqueous mercuric chloride) and processed to paraffin embedding for immunohistochemistry. Further liver tissue samples were snap-frozen in liquid nitrogen.

Sandwich ELISA for detection of serum HGF

Wells of 96-well Immulon IV (Dynatech Ltd.) ELISA plates were incubated with 100 μ l of the D9 monoclonal antibody (1:100 in 0.1 M NaHCO₃) overnight at 20°C. Non-specific protein binding was blocked by incubation of the plates with 3 per cent bovine serum albumin (BSA; 100 μ l/well) for 90 min at 20°C. Rat sera were added to the first row of wells at a dilution of 1:5 in PBS. Subsequent rows received sera at increasing doubling dilutions. Recombinant human HGF (pET61) was similarly assayed starting with 100 ng/ml and doubling the dilutions thereafter to give a final dilution of 0.049 ng/ml. All incubations took place for 150 min at 37°C. A rabbit anti-mouse polyclonal antibody directed against rhHGF and designated 201 (1:4000 dilution; kind gift of Professor T. Nakamura, Kyushu University, Japan) was then added (100 μ l/well) and incubated for 1 h at 20°C. Plates were then incubated with biotin-conjugated swine anti-rabbit immunoglobulins (Dako U.K. Ltd.) for 1 h at 20°C. Binding was detected using a streptavidin biotin peroxidase conjugate developed with *ortho*-phenylenediamine (OPD). Absorbance was read at 490 nm with a reference wavelength of 630 nm. Using rhHGF at known concentrations, a standard curve was constructed and from this, serum HGF levels were estimated.

Quantitative analysis of liver cell proliferation

Cells in S-phase were identified by nuclear localization of incorporated BrdU using an anti-BrdU monoclonal antibody (clone B44; Becton Dickinson U.K. Ltd.) with

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Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models

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Contributed by George F. Vande Woude, April 24, 2001

The hepatocyte growth factor (HGF/SF) receptor, Met, regulates mitogenesis, motility, and morphogenesis in a cell type-dependent fashion. Activation of Met via autocrine, paracrine, or nutritional mechanisms can lead to tumorigenesis and metastasis and numerous studies have linked inappropriate expression of this ligand-receptor pair to most types of human solid tumors. To prepare mAbs to human HGF/SF, mice were immunized with native and denatured preparations of the ligand. Recloned mAbs were tested *in vitro* for blocking activity against scattering and branching morphogenesis. Our results show that no single mAb was capable of neutralizing the *in vitro* activity of HGF/SF, and that the ligand possesses a minimum of three epitopes that must be blocked to prevent Met tyrosine kinase activation. *In vivo*, the neutralizing mAb combination inhibited s.c. growth in athymic *nu/nu* mice of tumors dependent on an autocrine Met-HGF/SF loop. Importantly, growth of human glioblastoma multiforme xenografts expressing Met and HGF/SF were markedly reduced in the presence of HGF/SF-neutralizing mAbs. These results suggest interrupting autocrine and/or paracrine Met-HGF/SF signaling in tumors dependent on this pathway is a possible intervention strategy.

Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional heterodimeric polypeptide produced by mesenchymal cells, and an effector of cells expressing the Met tyrosine kinase receptor (1, 2). HGF/SF has been shown to mediate the growth and scattering of various cell types, to mediate epithelial-mesenchymal transition (3) and the formation of umbilical and lumens (4, 5), and to promote angiogenesis (6, 7). *In vivo*, this ligand-receptor pair is believed to play a role in neural induction (8), liver regeneration, wound healing (9, 10), and normal embryological development. Both Met and HGF/SF knockout mice are embryonic lethal and show developmental defects in the placenta, fetal liver, limb/muscle formation, etc. (11, 12).

In addition to the role of HGF/SF-Met signaling in development and homeostasis, this ligand-receptor has also been shown to be uniquely involved in most human solid tumors (13) and to participate in tumor development, invasion, and metastasis (14). Met was originally isolated as the product of the human oncogene, *lmi-met*, which encodes an altered Met protein possessing constitutive, ligand-independent tyrosine kinase activity and transforming ability (15). Acting through Met, HGF/SF plays multifunctional roles on different tumor cells by autocrine or paracrine mechanisms, and HGF/SF transgenic mice develop a remarkably broad array of histologically distinct tumors of both mesenchymal and epithelial origin (16). Met activation has also been shown to markedly enhance the metastatic spread of cancer stemming from its stimulatory influence of processes such as angiogenesis (6, 7), cell motility (17), and cell surface protease regulation (18, 19). HGF/SF has been shown to induce invasiveness *in vitro* in various types of tumor cells (18, 20, 21). Neovascularization is also essential for the growth of solid tumors, and tumor cells are thought to secrete angiogenic factors

that promote neovascularization. HGF/SF is angiogenic and along with basic fibroblast growth factor has been shown to stimulate blood vessel formation directly or mediate angiogenesis through paracrine induction of vascular endothelial growth factor (VEGF) (22). HGF/SF also displays antiapoptotic activity (23–25) and is reported to be involved in other diseases such as cardiovascular disease and Alzheimer's disease (26, 27).

Malignant gliomas are extremely aggressive solid tumors, and their poor prognosis is linked to their ability to induce vascular proliferation and to invade surrounding brain. Both Met and HGF/SF are expressed in glioblastoma multiforme (GBM) (28), and ectopic expression of HGF/SF in GBM enhances both tumor growth and angiogenesis *in vivo* (29). Moreover, HGF/SF biological activity is inhibited by a chimeric U1 small nuclear RNA/ribozyme (30) or by the NK2 antagonist of HGF/SF interferes with GBM growth (31).

A rabbit polyclonal Ab can neutralize HGF/SF (28, 32), but there is no single mAb able to significantly inhibit all of the biological activities of HGF/SF. Here, we describe the generation of neutralizing mAbs to HGF/SF. We have determined that at low IgG concentration, a minimum of three mAbs used in combination are required to inhibit the Met-HGF/SF signaling pathway *in vitro*. Importantly, growth in athymic *nu/nu* mice of a human GBM tumor xenograft autocrine for Met and HGF/SF is markedly inhibited with the neutralizing combination of mAbs.

Materials and Methods

Cell Lines. Madin-Darby canine kidney (MDCK) cells were cultured in DMEM supplemented with 5% FBS. S-114 cells (NIH 3T3 cells transformed with human HGF/SF and Met; ref. 33) were grown in DMEM containing 8% of calf serum. SK-LMS-1 human leiomyosarcoma cell line (19) was maintained in DMEM containing 10% FBS. C-127 cells transformed with human HGF/SF and mouse Met (20), and U-118 cells (a human glioma cell line autocrine for endogenous HGF/SF and Met; ref. 28), were maintained in DMEM supplemented with 10% FBS. All cell lines were cultured at 37°C with 5% CO₂.

Neutralizing mAb Production. HGF/SF was prepared from S-114 cells and mouse mAbs against the ligand were produced by injecting BALB/c mice i.p. with purified native and denatured (boiling in SDS sample buffer) HGF/SF protein in complete Freund's adjuvant, followed by four additional injections in incomplete Freund's adjuvant. After 1 mo, a final HGF/SF injection was given i.p. and i.v. without adjuvant. Polyclonal

Abbreviations: HGF/SF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; GBM, glioblastoma multiforme; MDCK, Madin-Darby canine kidney; RT, room temperature.

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antisera from immunized mice were tested for neutralizing activity of HGF/SF in the MDCK cell scatter assay. Spleen cells were fused with P3X63A8/653 myeloma cells using standard techniques 3 days after the final injection. Rabbit polyclonal Ab to HGF/SF was used as positive control (28).

ELISA Screening. Hybridoma cells were screened for reactivity to HGF/SF by ELISA using 96-well plates coated with 2.5 µg/ml HGF/SF in coating buffer (0.2 M Na₂CO₃/NaHCO₃, pH 9.6, 50 µl per well) overnight at 4°C. The plates were then blocked with PBS containing 1% BSA (200 µl/well) overnight at 4°C. Fifty microliters of hybridoma supernatant were added to wells for 1.5 h at room temperature (RT). Plates were washed twice in washing buffer (PBS with 0.05% Tween 20), and alkaline phosphatase-coupled goat anti-mouse IgG (Sigma) was added (50 µl/well) at 1:3,000 dilution for 1.5 h at RT. After washing four times in washing buffer, phosphatase substrate CPN-triophenyl phosphate (Kirkegaard & Perry Laboratories) was added for 30 min, and absorbance was measured at 405 nm. Hybridomas with strong reactivity with HGF/SF (OD value >0.5, negative controls <0.02) were recloned twice, and reactivity was again confirmed by ELISA.

HGF/SF Neutralization in the MDCK Scatter Assay. Recloned hybridomas supernatants, either individually or in pools, were screened for neutralizing activity to HGF/SF using the MDCK cell scatter assay (34). Briefly, MDCK cells were plated at 7.5×10^4 cells per 100 µl per well with or without HGF (5 ng/well) in DMEM with 5% FBS. Three hundred microliters of supernatants (either individually or as pools), at 2-fold serial dilutions, were then added to 96-well plates. A rabbit polyclonal neutralizing antiserum (1 µl/well; ref. 28) was included as control. Following overnight incubation at 37°C, cells were then stained with 0.5% crystal violet in 30% ethanol (vol/vol) for 10 min at RT, and scattering was viewed using a light microscope. The IgGs were purified from the hybridoma cell lines showing the strongest neutralizing activity by using protein G columns and were adjusted to a final concentration of 2 mg/ml. The purified mAbs were assayed for neutralizing activity in the MDCK scatter assay.

Branching Morphogenesis Assay. Branching morphogenesis assay using SK-LMS-1 cells was conducted as described (19). Briefly, cell suspensions were mixed with an equal volume of GFR-Matrigel (Becton Dickinson), plated at 5×10^4 cells per 125 µl per well in a 96-well culture plate, and incubated for 30 min at 37°C. HGF/SF, with or without neutralizing mAbs, was added along with DMEM containing 10% FBS on top of the gel. After 72–96 h of incubation at 37°C, representative wells were photographed at ×400 magnification.

HGF/SF Immunoprecipitation and Western Blotting. S-114 cells were grown in serum-free medium for 48 h. The supernatant containing HGF/SF was collected and precleared with normal rabbit serum and protein G-Sepharose (Sigma). One milliliter of precleared supernatant was then immunoprecipitated with 1 µg of HGF/SF mAb and 20 µl of protein G-Sepharose, followed by immunoblotting as described (28). Briefly, the immune complexes were washed, and the proteins were separated by 10% SDS/PAGE gel and transferred onto polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with PBS containing 1% BSA (Sigma) at 4°C overnight, incubated with rabbit polyclonal anti-HGF/SF at 1:4,000 dilution for 1.5 h at RT, washed in PBS with 0.05% Tween 20, and reacted with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at 1:10,000 dilution for an additional 1.5 h at RT. Following the same washing, the proteins were detected with chemiluminescent substrate as suggested by the manufacturer (Bio-Rad).

mAb Inhibition of Tumor Growth in Athymic Nude Mice. Animal experiments were performed using female athymic nude (*nu/nu*) mice at 6 weeks of age. mAb combinations (e.g., mAbs A-1, -5, and -7) prepared against native HGF/SF were compared with nonneutralizing mAbs prepared against denatured HGF/SF (e.g., mAbs 7-2 and -3).

C-127 cells expressing human HGF/SF and mouse Met (35) were trypsinized, washed twice, and resuspended in PBS prior to injection. Mice were divided into five groups of five mice per group, and each mouse was injected s.c. with the cell suspension (2×10^5 cells per 0.1 ml per mouse). The first day after cell injection, the animals were administered Abs (0.2 mg per 100 µl per animal). Group one animals were injected s.c. with mAbs A-1, -5, and -7. Group 2 animals were injected i.p. with the same mAb pool. Groups 3 and 4 were injected with a combination of mAbs 7-2, -3, and -4, either s.c. or i.p., respectively. Group 5 animals received C-127 tumor cells without Abs. The Ab injections were repeated every day for 30 days, and tumor size was measured twice a week. The final data were collected when the control group was killed due to tumor size.

Xenografts of the U-118 human GBM tumor cell line, expressing HGF/SF and Met (28), were produced by injecting 5×10^5 cells s.c. into seven mice per group. As in the C-127 studies above, neutralizing mAb combination A-1, -5, and -7 and nonneutralizing mAb combination 7-2 and -3 were injected twice a week (0.2 mg per 100 µl/animal) for 10 weeks after cell injection. An additional experiment was performed using the U-118 cell line to test the ability of the mAbs to interfere with the growth of established tumors. At 30 days after s.c. injection (5×10^5 cells per mouse), mice were divided into five groups (10 mice per group) with an average tumor size about 100 mm³. Neutralizing (A-1, -5, and -7) and nonneutralizing (7-2 and -3) mAb combinations were injected either s.c. (intratumor) or i.p. every 2 days (0.2 mg per 100 µl per mouse) for 10 weeks. Tumor size was measured twice a week, and animals were killed when the health of the animal was severely affected by the size of the tumor.

Results

Production of mAbs to HGF/SF. We raised mAbs to both native and denatured forms of human HGF/SF. Sera from immunized mice were tested for neutralizing activity in the MDCK scatter assay. We found that only the sera from mice immunized with native HGF/SF inhibited scattering. After fusion, single clones of hybridoma cells positive by ELISA reactivity with HGF/SF were selected. mAbs prepared against native and denatured HGF/SF were tested individually for neutralizing activity in the MDCK scatter assay. No single mAb displayed neutralizing activity (data not shown). Because the sera from the mice immunized with native HGF/SF routinely displayed neutralizing activity (data not shown), we pooled mAb culture supernatants to test for neutralization activity against HGF/SF. One group of 10 pooled mAbs (A-1 to -10) showed strong neutralizing activity (Table 1).

To further characterize the mAb A-1 to -10 pool, culture supernatants were produced individually, and mAbs were purified on a protein G column. Various combinations of mAbs were tested to determine which members of the pool contributed to neutralizing activity. We found that combinations of any of two mAbs of A-1 to -10 did not neutralize MDCK scattering, even when mAbs were used at molar ratios 1,000 times greater than HGF/SF (data not shown). However, when three or more mAbs were combined, seven different combinations were identified with neutralizing activity at molar ratios of <30-fold (Table 1). Combinations of four mAbs, which include A-1, and any three of mAbs A-4, -5, -7, or -10, had the highest neutralizing activity. However, combinations of mAb A-1, plus either A-4 or -5 and -7 or -10, also efficiently neutralized HGF/SF-mediated MDCK scatter activity with mAbs A-1, -5, and -7 and/or A-1, -5, and -10

Table 1. *In vitro* neutralizing activity by mAbs to H5N1

proton transfer HSG-SF transfer ratio	MDCK cell	scattering	protonation ^a
Blanking	Blanking		
morphogen	morphogen		
1.0	30.1	ProA, A, 10 proA, 6	
1.0	Neg	ProA, B, 10 mAbB	
1.0	Neg	ProA, C, 11 mAbB	
1.0	7.41	A, 1, 4, 5, 7 and 10	
1.0	76.1	A, 1, 4, 5, 7 and 10	
1.0	20.1	A, 1, 4, 5, 7 and 10	
1.0	20.1	A, 1, 4, 5, 7 and 10	
1.0	20.1	A, 1, 4, 5, 7 and 10	
1.0	Neg	A, 1, 4, 5, 7 and 10	
1.0	69.1	A, 1, 4, 5, 7 and 10	
1.0	69.1	A, 1, 4, 5, 7 and 10	
1.0	33.1	A, 1, 4, 5, 7 and 10	
1.0	40.1	A, 1, 4, 5, 7 and 10	
1.0	20.1	A, 1, 4, 5, 7 and 10	
1.0	40.1	A, 1, 4, 5, 7 and 10	
1.0	40.1	A, 1, 4, 5, 7 and 10	
1.0	40.1	A, 1, 4, 5, 7 and 10	

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showing the general activity of side 1 and the 11. The magnetic field around the detector is 10 T/ST. A and ω are also indicated around the detector. The induced scattering of ω and ω are shown.

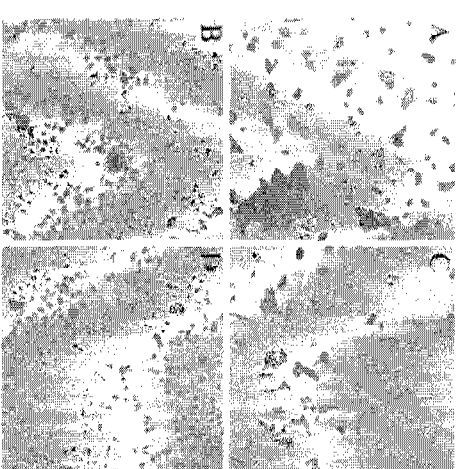


Fig. 1. Neutralization of Hsp 70 produced in MCF-7 cells with varying levels of glucose during treatment with or without human HGF. For each glucose concentration, 100% neutralization was achieved with 100 μ g/ml of human HGF. The neutralization of Hsp 70 produced in MCF-7 cells with 100 μ g/ml of human HGF was 100% for 0, 2, and 4 mM glucose.

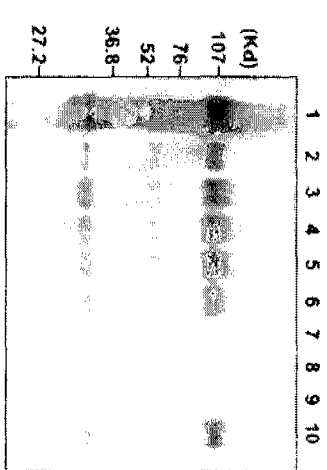


Fig. 2. Immunoprecipitation of HGF/SF from 5-11A cell culture supernatant by mAb₈. Lane 1, positive control, purified human HGF/SF; lanes 2-6, mAb₈ 1, 4, 5, 7, and 10; lane 7, IgG; lanes 7-9, mAb₈ 7, 2, 3, and lane 10, positive control from enzyme immunoprecipitation with native HGF/SF. The molecular weight standards are shown on the left.

Branching Morphogenesis. We tested for neutralizing activity of the mAb in the HGF/SF-mediated branching morphogenesis assay using SK-LA5-1 cells. The mAb combinations A-1, -5, and -7 and A-1, -5, and -10 displayed the greatest inhibitory activity (Table 1). However, A-1 is not required for blocking HGF/SF-induced branching morphogenesis and combinations of mAbs 2, 5, and 10 showed the highest neutralizing activity. These results suggest that a component(s) generated by the basement membrane, in addition to HGF/SF, is required for branching morphogenesis. Although the SK-LA5-1 cells show the 100% neutralization for mAb A-1, the mAb's activity decreases 110% to 75% for mAb A-1. The mAb's activity decreases 110% to 75% for mAb A-1. The mAb's activity decreases 110% to 75% for mAb A-1. The mAb's activity decreases 110% to 75% for mAb A-1.

immunoprecipitation, and Western blot analysis of mAb to HGF/SF. The starting tissue mAb's are also very effective for immunoprecipitating native HGF/SF from cell supernatants, whereas the mAb raised against recombinant HGF/SF are not (Tables 2, 3, 4, Fig. 2). The protein migrating at ~66 kDa is molecular weight of HGF/SF, and the smaller protein represents the α -2-macroglobulin complex product. By contrast, the α -macroglobulin does not precipitate recombinant HGF/SF on Western blot, while mAb raised against recombinant HGF/SF does (data not shown).

Antitumor Activity of HGF-SF-Normalizing mAbs. We have previously demonstrated that cells with reductive Met-1/4/18 signaling are tumorigenic and metastatic in nude mice [4,18,20]. We have characterized several human tumor types that display authentic HGF-SF-Met signaling, such as human osteosarcoma (33) and GBM (29). To determine whether the neutralizing mAb to human HGF-SF has any effect on tumor growth, we performed antitumor experiments with C-127 mouse fibroblasts that express mouse Met and human HGF-SF to generate an autocrine loop [29]. These cells (2×10^4) when injected subcutaneously in syngeneic nude mice in 2-3 weeks formed tumors in 17 syngeneic nude mice. In 2-3 weeks, animals injected with C-127 tumor cells were also injected with pools of either mAb A-1, A-2, or F, or F-2, -3, and -4 either intratumorally or i.p. every 2 days for 20 days. The experiment was terminated at 37 days after C-127 cell injection. Dramatically, the animals treated with the combination of mAbs A-1, -3, and F showed 90% inhibition of tumor growth while either A-1 or F alone had no impact with the control mice. The control mice in which injected mice compared with the control (Fig. 2a).

Using the same procedure described above, we tested the effect of A-1, -2, and -7 combination in two different concentrations with the U-7418 (GBA) to see cell senescence. Human GBA cell lines (couple: H3h-SF and Met, which is isolated from a fibro-

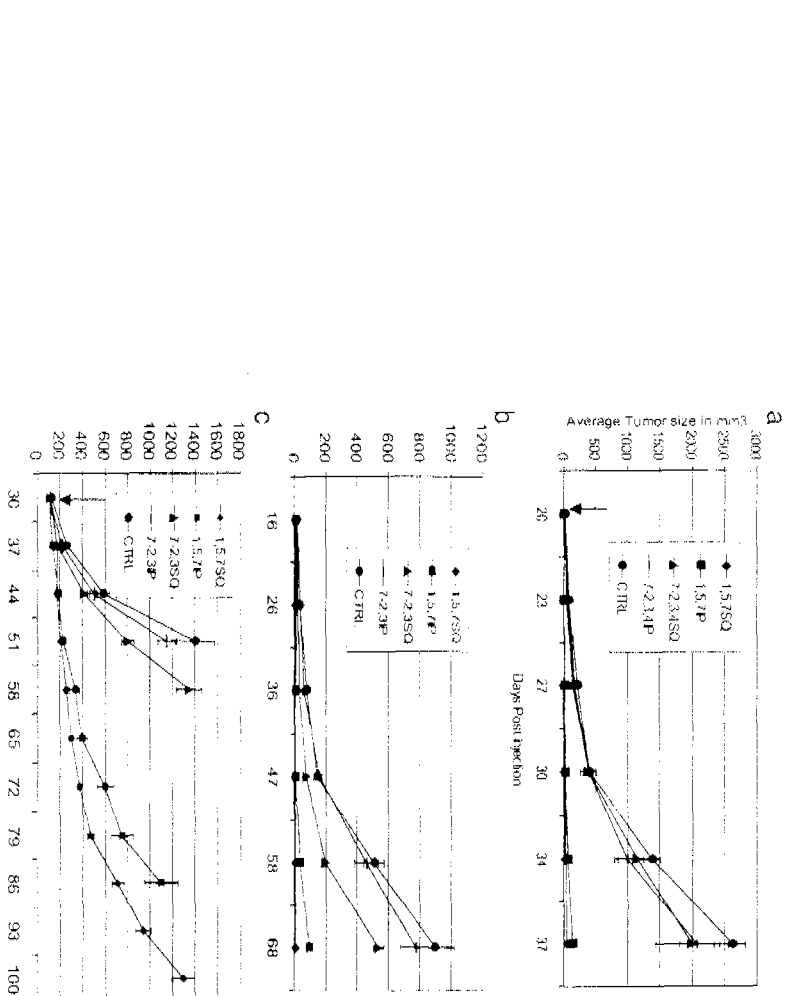


Fig. 3. (a) Titration of C122 (mean $\pm$ S.E.) in 400 μ l H₂O, 5% (v/v) DMSO, 5, and 7.5 mM β -glucuronidase. The values are an average of the two or three independent experiments. (b) Titration of C122 in 400 μ l H₂O, 5% (v/v) DMSO, 5, and 7.5 mM β -glucuronidase. The values are an average of the two or three independent experiments.

to tumor themselves. In this experiment, tumor growth in the combination of irradiation and growth factor injection was the same as the average tumor size in the AP group with the controls (Fig. 3b). We also found that 30 days after the D₂O/CMV treatment, there was no significant delay in tumor growth (Fig. 3c). The regression map A₁, A₂, and A₃ began reflecting the delay for up to 70 days. A total of eight tumor growths were detected.

Discussion

Multiple biological activities in a unique structure. The ligand is containing the N-teriminal domain and covalently insoluble linked to a

Altogether, these results suggest that the β -subunit of the HGF/SF receptor is a transcriptionally regulated gene. The expression of the β -subunit is down-regulated in the rat hepatoma cell line HCC-T2, which is a well established model for the study of hepatocarcinogenesis [10]. In this cell line, the expression of the β -subunit is down-regulated in a dose-dependent manner by the addition of the carcinogen aflatoxin B₁ [11].

It has been shown that HGF/SF-induced biological activities are mediated by the heterotetrameric complex formed by the heterodimeric ligand and the heterodimeric receptor [12]. The heterodimeric receptor is composed of an α -chain consisting of two extracellular domains (E1 and E2), a transmembrane domain (TM), and an intracellular domain (IC). The E1 domain is responsible for the high-affinity binding of the ligand, while the E2 domain is involved in the heterodimerization of the receptor [13]. The TM domain is responsible for the anchoring of the receptor in the cell membrane, and the IC domain is responsible for the transduction of the signal [14].

It has been shown that HGF/SF-induced biological activities are mediated by the heterotetrameric complex formed by the heterodimeric ligand and the heterodimeric receptor [12]. The heterodimeric receptor is composed of an α -chain consisting of two extracellular domains (E1 and E2), a transmembrane domain (TM), and an intracellular domain (IC). The E1 domain is responsible for the high-affinity binding of the ligand, while the E2 domain is involved in the heterodimerization of the receptor [13]. The TM domain is responsible for the anchoring of the receptor in the cell membrane, and the IC domain is responsible for the transduction of the signal [14].

chains of the α -chain and the β -chain are all required for the full spectrum of biological activity of hSCF/SF (49).

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DIRECCIÓN DE NUEVAS CREACIONES

2020
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	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	012

06-1007246

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☒	Cap. I	☒
Vía PCT (fase nacional)	☒	Cap. II	☐

No. Solicitud Internacional PCT/US2004/026565
Fecha de Presentación Internacional 13 Agosto 2004

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 46 a 96	30 Octubre 2006
Reivindicaciones:	Folios 97 a 99	30 Octubre 2006
	Folios 127 a 129	08 Noviembre 2006
Dibujos:	Folios 101 a 112	30 Octubre 2006
Prioridad:	US 10.825060	15 Abril 2004

2. Objeto de la invención

Título de la solicitud: "Anticuerpos monoclonales al factor de crecimiento de hepatocito"

El problema planteado en esta solicitud consiste en la necesidad de proporcionar anticuerpos que se enlacen y neutralicen el factor de crecimiento de hepatocito para el tratamiento del cáncer.

Para resolver este problema técnico la solicitud en estudio proporciona un anticuerpo monoclonal neutralizante al factor de crecimiento de hepatocito y una composición farmacéutica que comprende el mismo.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K39/395; C07K16/22; C07K16/30; C12N5/20; G01N33/53; G01N33/563; G01N33/574; G01N33/577.

3. No es una invención (Art 15 D 486 literal (b))

El artículo 15 en su literal b) señala que:

No se consideran invenciones el todo o parte de los seres vivos tal como se encuentran en la naturaleza, los procesos biológicos naturales, el material biológico existente en la naturaleza o aquel que pueda ser aislado inclusive genoma o germoplasma de cualquier ser vivo natural.

El anticuerpo humano reclamado en la reivindicación 4 obedece a material biológico que puede existir en la naturaleza, por lo que a menos que se especifique el carácter artificial de dicho anticuerpo, no se puede considerar como una invención.

Por tal razón la reivindicación 4 no puede ser considerada invención.

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- Las reivindicaciones 1 y 12 hacen referencia a un anticuerpo monoclonal de manera general, dichos anticuerpos no se encuentran caracterizados adecuadamente puesto que no se dan a conocer las características técnicas esenciales, es decir un anticuerpo debe definirse por su estructura, las secuencias que corresponden a la región variable del anticuerpo (cadenas pesada y liviana) ó los CDRs que los conforman. Asimismo, no se pueden caracterizar los anticuerpos por su función ya que esto corresponde a un resultado a alcanzar como se indica más adelante. En el caso que se identifique el anticuerpo por su hibridoma debe definirse por su número de depósito y el epítotope al cual va ligado.
- Las Reivindicaciones 1, 5 a 11, 13 y 15 no son claras puesto que no caracterizan adecuadamente el anticuerpo monoclonal sino por el contrario se refieren a una serie de actividades farmacológicas o mecanismos de acción que realiza dicho anticuerpo. Dichas actividades farmacológicas o mecanismos de acción son un resultado a alcanzar, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, estructurales del anticuerpo, que contribuyen a la obtención de ese efecto.
- Las reivindicaciones 2, 3 y 14 no se encuentran soportadas en la descripción debido a que en la descripción no hay indicación que los anticuerpos de las realizaciones preferidas sean de características quiméricas o humanizadas, de la misma forma, dichas reivindicaciones no son claras puesto que no definen de que manera correcta las características de los anticuerpos quimérico y humanizado. Se sugiere al solicitante definir su estructura, las secuencias que corresponden a la región variable del anticuerpo (cadenas pesada y liviana) ó los CDRs que los conforman. Asimismo, las modificaciones realizadas para dichos anticuerpos.
- Se sugiere al solicitante que para la reivindicación 16 se adjunte el número de depósito de tal manera que se defina el tipo de línea celular reclamada en la presente solicitud.

- Las composiciones reclamadas en las reivindicaciones 17 a 19 no definen los componentes y proporciones que la caracterizan, se sugiere al solicitante definir dichas características técnicas esenciales.

Se sugiere al solicitante realizar la modificación al capítulo reivindicatorio de tal manera que se caracterice adecuadamente el objeto de la invención.

Descripción (Art 28 D 486)

La Descripción no divulga la invención de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla, ya que no se establecen las secuencias ni de nucleótidos ni de aminoácidos que caracterizan y llevan a la generación de los anticuerpos reclamados.

5. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad (15 abril 2004) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D2	US 6214344	Hepatocyte grown factor receptor antagonists and uses thereof ,	10 Abril 2001
D1	JOURNAL OF PATHOLOGY. (1998) Vol. 185, pag 298-302	Anti-hepatocyte growth factor antibody inhibits hepatocyte proliferation during liver regeneration	Julio 1998

D1 (folios 174 a 176) divulga antagonistas que inhiben la activación del factor de crecimiento de hepatocito (HGF) que comprenden anticuerpos monoclonales quiméricos o humanizados y fragmentos de estos de tipo FAb para el tratamiento del cáncer.
URL:http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&adjacent=true&locale=en_gb&FT=D&date=20010410&CC=US&NR=6214344B1&KC=B1

D2 (folios 177 a 178) divulga anticuerpos monoclonales que neutralizan el factor de crecimiento de hepatocito (HGF) y disminuyen los niveles de dicho factor en ratas tratadas.
URL:[http://dx.doi.org/10.1002/\(SICI\)1096-9896\(199807\)185:3<298::AID-PATH88>3.3.CO;2-2](http://dx.doi.org/10.1002/(SICI)1096-9896(199807)185:3<298::AID-PATH88>3.3.CO;2-2)

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La **reivindicación 1** consiste en un anticuerpo monoclonal que se enlaza y neutraliza al factor de crecimiento de hepatocito humano.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Anticuerpo monoclonal	Anticuerpo monoclonal	Anticuerpo monoclonal
Se enlaza y neutraliza al factor de crecimiento de hepatocito humano.	Se enlaza y neutraliza al factor de crecimiento de hepatocito humano.	Se enlaza y neutraliza al factor de crecimiento de hepatocito humano.

Frente a D1

Las características esenciales del anticuerpo de la reivindicación 1 habían sido divulgadas previamente en **D1**, el cual enseña antagonistas del receptor del factor de crecimiento de hepatocito (HGF) (folio 174, abstract) y composiciones farmacéuticas que los contienen (folio 174, columna 4, línea 35 a 39). Dichos antagonistas corresponden a anticuerpos monoclonales quiméricos (folio 175, columna 11, línea 16 a 20) o humanizados (folio 175, columna 11, línea 22 a 27) o fragmentos FAb de un anticuerpo monoclonal (folio 174, columna 4, línea 26 a 30; folio 175, columna 10, línea 16 a 29) los cuales neutralizan la actividad de este factor e inhiben su actividad mitogénica. Por otro lado D1 hace referencia a líneas celulares que producen dichos anticuerpos monoclonales como son células inmortalizadas de mieloma murino (folio 175, columna 8, línea 57 a 65).

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 a 13 y 16 a 19 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Frente a D2

Las características esenciales del anticuerpo de la reivindicación 1 habían sido divulgadas previamente en **D2**, el cual revela anticuerpos monoclonales de tipo quimérico y humanizado anti-HGF (folio 178, pagina 299, columna 1, párrafo 1) que se enlazan y neutralizan la acción del factor de crecimiento de hepatocito (HGF) (folio 177, pagina 298, columna 2) y son útiles para inhibir la proliferación de hepatocitos (folio 177, abstract). De igual manera revela composiciones para administración por infusión de dichos anticuerpos folio 178, pagina 299, columna 1, párrafo 3).

En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2 a 11, 13, 17 a 19 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

7. Conclusión

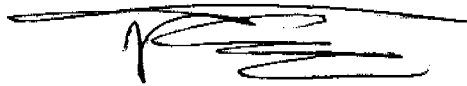
Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 6214344	1 a 13 y 16 a 19	Novedad
D2	JOURNAL OF PATHOLOGY. (1998) Vol. 185, pag 298-302	1 a 11, 13, 17 a 19	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días

contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o sí a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

13 DIC 2011

El anterior requerimiento fue notificado por fijación en lista, de fecha _____

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