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TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 13
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DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado(a)/Señor(a)
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Asunto: Radicación: 10-23987- -19
 Trámite: 11
 Evento: 378
 Actuación: 519
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Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/JP2008/002232				
Fecha de Presentación Internacional	19/08/2008				

1. Documentos estudiados

Descripción:	Radicado N°10-023987-00000-0000	Marzo 1 de 2010
Reivindicaciones	Radicado N°10-023987-00000-0000	Marzo 1 de 2010
	Radicado N°10-023987-00008-0000	Septiembre 29 de 2011
	Radicado N°10-023987-00013-0000	Abril 18 de 2013
Oposiciones	Radicado N°10-023987-00004-0000	Lafrancol S.A. Junio 16 de 2011
	Radicado N°10-023987-00005-0000	Procaps S.A. Junio 16 de 2011
Respuestas del solicitante	Radicado N°10-023987-00007-0000	Septiembre 29 de 2011
	Radicado N°10-023987-00008-0000	Septiembre 29 de 2011
	Radicado N°10-023987-00013-0000	Abril 18 de 2013
Prioridad	US 60/902,949	Agosto 24 de 2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Por lo tanto, las reivindicaciones 1 y 2 contenidas en el radicado N°10-023987-00013-0000 son aceptadas para ser estudiadas.

Título de la solicitud: "Combinación farmacéutica que comprende péptidos derivados de VEGFR2 y Gemcitabina" (Modificado en el radicado N° 10-023987-00008-0000).

El problema planteado en esta solicitud consiste en la necesidad de obtener tratamientos alternativos para el tratamiento del cáncer de páncreas.

Para resolver este problema técnico, la solicitud en estudio plantea la obtención de una combinación que comprende un péptido de VEGFR2 y gemcitabina, para el tratamiento del cáncer de páncreas.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K31/7068; A61K38/00; A61P35/00.

4. De la Solicitud de la Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Para la reivindicación 1, se solicita modificar el término "estuche" por el término "kit de partes", que corresponde al término técnico correcto para reclamar esta modalidad de invenciones que contienen dos composiciones farmacéuticas que conducen a obtener un mismo efecto técnico.

La reivindicación 1 no es concisa porque al mencionar "Un péptido de (a) o (e) en el cual se han sustituido, suprimido o agregado 1 o 2 aminoácidos, donde el péptido induce células T citotóxicas" correspondiente a los literales b) y f), porque a partir de las modificaciones planteadas a los péptidos de a) y e), se pueden obtener muchos péptidos posibles, los cuales no se incluyen dentro del objeto de la solicitud no se encuentran debidamente soportados en la descripción. Por lo tanto, se solicita mencionar de manera específica cuál de todos los aminoácidos de los literales a) y e) se modifican, en que posiciones se dan las modificaciones y cuales aminoácidos son los que se adicionan, se suprimen o se sustituyen.

Para la reivindicación 1 se recomienda modificar la redacción de la misma, de tal forma que se mencione en un solo literal todas las secuencias y en otro literal las modificaciones específicas planteadas.

Se solicita eliminar la reivindicación 2, porque se repite la materia reclamada en la reivindicación 1. A pesar de que el encabezado es diferente, el hecho de mencionar "un agente" no permite diferenciarla del kit de partes que se reclama en la reivindicación anterior.

5. Análisis de las Oposiciones

Argumentaciones de Lafrancol S.A.

La opositora se permite señalar que las composiciones y combinaciones farmacéuticas que comprenden uno o más péptidos y uno o más agentes quimioterapéuticos para el tratamiento del cáncer de páncreas, son prácticas y eventos totalmente conocidos en la industria farmacéutica, por lo que estas composiciones no contienen novedad ni nivel inventivo.

Además, las composiciones farmacéuticas que involucran compuestos tales como gemcitabina, leucina, tirocidina, entre otros, no pueden tener protección debido a que estas moléculas son ampliamente conocidas en el sector químico farmacéutico.

Argumentaciones de Procaps S.A.

La opositora manifiesta que la solicitud se refiere a una terapia en combinación para el tratamiento del cáncer de páncreas, que comprende los péptidos epítopes de VEGFR2 y gemcitabina.

La opositora manifiesta que antes de la fecha de prioridad de la solicitud, los documentos “Brunss, C. J., et al. Effect of the vascular endotelial growth factor receptor – 2 antibody DC101 plus gemcitabine on growth, metástasis and angiogénesis of human pancreatic cáncer growing othotopically in nude mice. *International Journal of Cancer*. 2002, 102; 101 – 108” (de ahora en adelante documento D1) y el documento US2006/216301 (de ahora en adelante documento D2) anticipan el objeto de la solicitud y por lo tanto afectan la patentabilidad de la misma.

El documento D1 menciona que la combinación de anticuerpos anti VEGFR2 y gemcitabina, inhibe el crecimiento del tumor pancreático, por lo que se considera como una estrategia para el tratamiento del cáncer de páncreas. El documento D2 describe un agente anticancerígeno que contiene péptidos epítope de VEGFR2 empleados en vacunas para el tratamiento de tumores. De tal manera que la combinación de los documentos D1 y D2 conducen a que una persona normalmente versada en la materia logre obtener la combinación que contiene péptidos de VEGFR y gemcitabina, tal y como se reclaman en la solicitud.

Respuestas del solicitante

Con respecto a la oposición presentada por la sociedad Lafrancol S.A., la solicitante menciona que para afectar el nivel inventivo de una solicitud, el estado de la técnica debe enseñar o sugerir los elementos que le permitan obtener el objeto de la solicitud. Así que los conceptos mencionados por la opositora no proporcionan la información para obtener el objeto de la solicitud.

En referencia a la oposición presentada por la sociedad Procaps S.A., la solicitante manifiesta que si bien el documento D1 divulga una combinación que comprende un anticuerpo VEGFR2 y gemcitabina, mientras que el documento D2 divulga péptidos epítopes de un anticuerpo VEGFR2, la combinación de los conceptos de los documentos D1 y D2 no conducen al objeto de la solicitud, porque el mecanismo de acción entre un anticuerpo VEGFR2 y los péptidos del epítope VEGFR2 son completamente diferentes; en el caso de los anticuerpos VEGFR2, estos se unen a VEGFR2 inhibiendo el crecimiento celular por falta de vascularización, mientras que los péptidos epítopes VEGFR2, estimulan la producción de células T para matar las células cancerígenas que presentan las secuencias de péptidos. De acuerdo a lo anterior, no existe ninguna motivación

para que una persona normalmente versada en la materia obtenga el objeto de la solicitud.

Respuesta del Examinador Técnico

Con respecto a la oposición de la sociedad Lafrancol S. A., esta no menciona una combinación que contenga un péptido VEGFR2 y gemcitabina, sino que se limita a mencionar que la gemcitabina y otros agentes anticancerígenos son conocidos ampliamente en el campo farmacéutico, así como las composiciones y combinaciones farmacéuticas que los comprenden. Por las razones expuestas anteriormente, este Despacho considera que la oposición presentada a la solicitud por parte de la sociedad Lafrancol S.A., carece de fundamento alguno.

Con respecto a la oposición de la sociedad Procaps S.A., es necesario considerar que los conceptos descritos en los documentos D1 y D2 en conjunto no pueden obtener la combinación propuesta en la solicitud porque no se puede considerar como obvio cambiar un anticuerpo por un péptido. Sin embargo, es necesario resaltar que aunque el efecto técnico y el mecanismo de acción de un compuesto determinado son factores que se tienen en cuenta para la evaluación del nivel inventivo, se considera primordial las características estructurales de dicho compuesto. Este Despacho considera que si bien la combinación documental propuesta por la sociedad Procaps S.A. no permite obtener la combinación de la solicitud, si es necesario establecer que el documento D2 si divulga los péptidos epítotope de VEGFR2 reclamados en la solicitud, por lo que este documento si afecta el nivel inventivo de la solicitud y por lo tanto, la oposición de la sociedad Procaps S.A. se considera como fundada.

En conclusión, el documento US2006/216301 si afecta el nivel inventivo de la solicitud, por lo que se tendrá e4n cuenta dentro del Examen de Patentabilidad.

6. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de prioridad de la solicitud: El 24 de agosto de 2007 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US2006/0216301	"KDR peptides and vaccines containing the same"	28/Septiembre/2006
D3	<i>Oncology</i> . 2005. 69; 81 – 87	"Combination of Low – Dose Gemcitabine and Recombinant Quail Vascular Endothelial Growth Factor Receptor – 2 as a Vaccine Induces Synergistic Antitumor Activities"	02/Agosto/2005

El documento D1¹ divulga polipéptidos de VEGFR2 y vacunas que los contienen, para el tratamiento del cáncer (Pág. 1, párr. [0001], [0002]).

El documento D3² divulga una combinación que comprende proteína de VEGFR2 de codorniz y gemcitabina en bajas dosis con actividad sinérgica par la disminución de tumores (Pág. 81, col. der., abstract).

7. Examen de Patentabilidad.

Nivel Inventivo (Art 18 D 486)

¹[http://worldwide.espacenet.com/publicationDetails/originalDocument?](http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2006216301A1&KC=A1&FT=D&ND=3&date=20060928&DB=worldwide.espacenet.com&locale=es_LP)

[CC=US&NR=2006216301A1&KC=A1&FT=D&ND=3&date=20060928&DB=worldwide.espacenet.com&locale=es_LP](http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2006216301A1&KC=A1&FT=D&ND=3&date=20060928&DB=worldwide.espacenet.com&locale=es_LP)

²<https://www.karger.com/Article/Abstract/87303>

La reivindicación 1 divulga “*Un estuche que comprende dos composiciones farmacéuticas separadas que comprenden los ingredientes activos (i) y (ii) y un vehículo farmacéuticamente aceptable: (i) uno o más péptidos que se seleccionan a partir de grupo que consiste de... (ii) uno o más agentes quimioterapéuticos que se seleccionan a partir de gemcitabina, una sal farmacéuticamente del mismo o un profármaco de la misma*” (Pág. 5 del radicado N° 10–023987–00013–0000).

Comparación de las características técnicas esenciales con el Estado de la Técnica

Solicitud	D1	D3
Un estuche	Una combinación (Pág. 4, párr. [0077], [0078])	Una combinación (Pág. 81, col. der., ln. 1 – 6; pág. 82, col. der., 16 – 23; pág. 86, col. der., ln. 3 – 22)
Comprende uno o más péptidos seleccionados de: (a) Uno o más péptidos de RFVPDGNRI (SEQ ID N° 1) VYSSEEAEL (SEQ ID N° 2) GYRIYDVVL (SEQ ID N° 3) SYMISYAGM (SEQ ID N° 4) KWEFPRDRL (SEQ ID N° 5) DFLTLEHLI (SEQ ID N° 6); (b) El péptido de (a) con 1 o 2 aminoácidos suprimidos, agregados o sustituidos; (c) El péptido de (b) donde el segundo aminoácido desde N – terminal es Phe, Tyr, Met, Trp; (d) El péptido de (b) o (c) donde C – terminal es Phe, Leu, Ile, Trp, Met; (e) Uno o más péptidos de AMFFWLLL (SEQ ID N° 7) VIAMFFWLL (SEQ ID N° 8) AVIAMFFWL (SEQ ID N° 9) KLIEIGVQT (SEQ ID N° 10) YMISYAGMV (SEQ ID N° 11) IQSDVWSFGV (SEQ ID N° 12); (f) El péptido de (e) con 1 o 2 aminoácidos suprimidos, agregados o sustituidos; (g) El péptido de (f) donde el segundo aminoácido desde N – terminal es Leu, Met; (h) El péptido de (f) o (g) donde C – terminal es Val, Leu	Comprende los péptidos RFVPDGNRI (SEQ ID N° 8) VYSSEEAEL (SEQ ID N° 2) GYRIYDVVL (SEQ ID N° 3) SYMISYAGM (SEQ ID N° 5) KWEFPRDRL (SEQ ID N° 11) DFLTLEHLI (SEQ ID N° 12) AMFFWLLL (SEQ ID N° 29) VIAMFFWLL (SEQ ID N° 30) AVIAMFFWL (SEQ ID N° 33) KLIEIGVQT (SEQ ID N° 34) YMISYAGMV (SEQ ID N° 40) IQSDVWSFGV (SEQ ID N° 46); Los péptidos tienen uno o dos aminoácidos sustituidos o agregados. Para los péptidos, el segundo aminoácido desde el N – terminal es Phe, Tyr, Met, Trp, Leu; el aminoácido C – terminal es Phe, Leu, Ile, Trp, Met (Pág. 4, párr. [0073] – [0075]; pág. 6, tabla 1; pág. 7, tabla 2)	Comprende una proteína de VEGFR2 de codorniz
Comprende gemcitabina o una sal farmacéuticamente aceptable	Comprende otro agente citotóxico (Pág. 5, párr. [0088])	Comprende gemcitabina

El documento D1 se considera el estado de la técnica más cercano a la combinación de la reivindicación 1, porque se menciona una combinación que contiene péptidos epítope de VEGFR2 seleccionados de las SEQ ID N° 2, 3, 5, 8, 11, 12, 29, 30, 33, 34, 40 y 46 (los cuales se pueden modificar al adicionar o sustituir 1 o 2 aminoácidos) y otro agente citotóxico, para el tratamiento del cáncer (Pág. 4, párr. [0073] – [0075], [0077], [0078]; pág. 5, párr. [0088]; pág. 6, tabla 1; pág. 7, tabla 2).

La diferencia entre la combinación descrita en la solicitud con respecto a la divulgada en el documento D1, consiste en que la combinación del estuche de la solicitud contienen los polipéptidos VEGFR2 y gemcitabina, mientras que la combinación del documento D1 contiene los péptidos VEGFR2 y eventualmente

un agente citotóxico.

No se evidencia un efecto técnico asociado a la administración conjunta de polipéptidos VEGFR2 y gemcitabina, toda vez que los resultados enunciados en la tabla 2 de la descripción no permiten establecer que existe un efecto antitumoral para un número significativo de pacientes a los cuales se les administra dicha combinación.

El problema técnico objetivo que se pretende resolver es el siguiente: Como modificar las combinaciones divulgadas en el documento D1, para obtener combinaciones alternativas que contienen péptidos VEGFR2 para el tratamiento del cáncer.

El documento D3 divulga una combinación que comprende una proteína de VEGFR de codorniz y gemcitabina en bajas dosis como un agente quimioterapéutico para el tratamiento del cáncer (Pág. 81, col. der., ln. 1 – 6; pág. 82, col. der., 16 – 23; pág. 86, col. der., ln. 3 – 22), donde esta combinación genera un efecto sinérgico que inhibe el crecimiento de tumores (Pág. 84, fig. 1) mediante la reducción de angiogénesis (Pág. 85, fig. 3) y mediante incremento de la apoptosis de las células cancerígenas (Pág. 86, fig. 4).

Por lo tanto, una persona normalmente versada en la materia y conocedora de los conceptos divulgados en los documentos D1 y D3, sin ningún esfuerzo, modificaría la combinación divulgada en el documento D1, incluyendo la gemcitabina en la combinación con el fin de obtener un efecto sinérgico para disminuir la angiogénesis e incrementar la apoptosis en células cancerígenas, tal como lo sugiere la combinación del documento D3, para obtener la combinación propuesta en la solicitud de manera obvia o evidente.

En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en los documentos D1 y D3, carece de Nivel Inventivo.

Las reivindicaciones 2, dependientes de la reivindicación 1, no menciona características inventivas adicionales a las comprendidas dentro del objeto de la reivindicación 1. Por lo tanto, esta reivindicación también carece de Nivel Inventivo.

8. Conclusión

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US2006/0216301	1 y 2	Nivel Inventivo
D2	<i>Oncology</i> . 2005. 69; 81 – 87	1 y 2	Nivel Inventivo

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 del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-05-26

Elaboró: Dayron Alexander Ramirez Reyes
Revisó: Sandra Carolina Crespo

Combination of Low-Dose Gemcitabine and Recombinant Quail Vascular Endothelial Growth Factor Receptor-2 as a Vaccine Induces Synergistic Antitumor Activities

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Key Words

Gemcitabine · Vascular endothelial growth factor · Vaccine · Tumor

Abstract

Vascular endothelial growth factor receptor-2 (VEGFR-2) has been shown to play a major role in inducing the full spectrum of VEGF biological response which is essential for tumor angiogenesis. We have demonstrated that immunotherapy of tumors with a vaccine based on quail homologous VEGFR-2 (qVEGFR) was effective in providing both protective and therapeutic antitumor immunity in several tumor models in mice. The purpose of this study was to determine whether the combination therapy of low-dose gemcitabine with qVEGFR as a vaccine could inhibit tumor growth to a greater extent. To test this concept, H22 hepatoma and Lewis lung carcinoma models were established in BALB/c mice and C57BL/6

mice, respectively. Mice were treated with either qVEGFR as a protein vaccine, gemcitabine, or both agents together. qVEGFR or low-dose chemotherapy treatment individually resulted in tumor inhibition to a certain extent. Remarkably, the combination therapy resulted in synergistic antitumor activity. Histological examination revealed that there was endothelial deposition of immunoglobulins within tumor tissues from mice treated with vaccine or combination therapy, especially intratumor angiogenesis was suppressed more significantly for the combination group. Also, ELISPOT analysis showed that mice treated with either qVEGFR alone or in combination with low-dose chemotherapy produced similar amount of anti-VEGFR antibody-producing B cells, which suggested that low-dose gemcitabine did not suppress the host's immune response, but potentiated the antitumor activity of the qVEGFR vaccine. Furthermore, TUNEL staining demonstrated a significant increase in the number of TUNEL-positive cells in the combination group compared with those of other groups. The observations may provide a new bio-chemotherapeutic approach for cancer.

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Introduction

New blood vessel formation, or angiogenesis, plays an important role in both tumor growth and metastasis [1]. As a strategy of cancer therapy, antiangiogenic therapy attempts to stop new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass [2, 3]. Vascular endothelial growth factor receptor-2 (VEGFR-2) is the main receptor responsible for the angiogenic activity of VEGF [4]. Recent results from our laboratory have shown that immunotherapy of tumors with a vaccine based on quail homologous VEGFR-2 (qVEGFR) resulted in both protective and therapeutic antitumor effect through the induction of autoimmunity against VEGFR-2 in mice [5].

It is well known that tumors and metastases originate as small avascular masses [6]. In the absence of local neovascular formation, tumors may not grow beyond 2–3 mm in diameter [7]. Although antiangiogenic and -vascular therapy have shown both protective and therapeutic antitumor effect to varying extents [8, 9], cancer therapy targeting only tumor-existing vessels or tumor angiogenesis may not eradicate the tumor completely. Chemotherapy is one of the mainstays for cancer therapy. However, acquired drug resistance is one of the major reasons which account for the limited advances in cancer chemotherapy [10]. Also, toxicity, sometimes even unbearable, is another major problem in the chemotherapy of cancer. Thus, it is conceivable that antiangiogenic or -vascular therapy combined with chemotherapy may exert greater effect on tumor growth, even eliminate tumor. Recently, many researches based on this strategy did show better effect than either therapy alone [11–13].

Gemcitabine is a new deoxycytidine analog that inhibits DNA synthesis. After cellular uptake, gemcitabine is phosphorylated to its active metabolites, which inhibits DNA chain elongation, leading to DNA fragmentation and cell death. Gemcitabine has shown cytotoxicity against a wide range of cancer cell lines in vitro and a number of murine and human tumors in vivo [14, 15].

In this study, we decided to test the effect of combination treatment of tumors with low-dose gemcitabine and qVEGFR as a vaccine in mice.

Materials and Methods

Vaccine Preparation

The qVEGFR was prepared as described by us previously [5]. Briefly, A PCR-amplified cDNA clone encoding the N-terminal 134–417 amino acid residues of extracellular domains of qVEGFR-

2 (Quek-1) was inserted into pQE30 expression vector (Qiagen Inc., Chatsworth, Calif., USA). The ligated DNA was used to transform *Escherichia coli* JM109 strains. The correct transformant was cultured for recombinant protein expression by adding isopropyl- β -D-thiogalactoside. Recombinant proteins were purified from the bacterial lysates by affinity chromatography on a nickel-nitrilotriacetic acid gel matrix (Qiagen Inc.). Thereafter, the purified proteins were extensively dialyzed to allow refolding. The refolded proteins were further purified by ion exchange and gel filtration chromatography, then lyophilized and stored in 4°C.

Design of Animal Experiment

Female mice at 6–8 weeks of age were transplanted with 1×10^6 live tumor cells. The Lewis lung carcinoma (LL/2) model was established in syngeneic C57BL/6 mice, and H22 hepatoma model in syngeneic BALB/c mice. After the tumors had grown for 7 days, the mice were randomly divided into the following 4 groups of 10 animals each. Group 1, combination of low-dose gemcitabine and qVEGFR vaccine, mice received qVEGFR vaccine plus low-dose gemcitabine as follows: day 0 (7 days after tumor cell injection), qVEGFR vaccine was injected subcutaneously (s.c.) once a week for 4 weeks with a dose of 10 μ g per mouse. At day 7 (14 days after tumor cell injection), 20 mg/kg of gemcitabine was injected by intraperitoneal (i.p.) route at an interval of every 3 days for a total of 4 doses. Group 2, qVEGFR vaccine alone, mice received qVEGFR vaccine in a scheme as that in group 1, but not gemcitabine. Group 3, low-dose gemcitabine alone, mice received the same dose of gemcitabine in a scheme as that in group 1, but not qVEGFR vaccine. Group 4, untreated group, mice received sterile normal saline s.c. as the scheme of vaccination and i.p. as the scheme of gemcitabine in the group 1, respectively. Tumor growth was evaluated every 4 days, and tumor volumes were calculated according to the formula: Tumor volume (mm^3) = $\pi/6 \times \text{length (mm)} \times \text{width (mm)} \times \text{width (mm)}$. All studies involving mice were approved by the institute's Animal Care and Use Committee.

B-Cell ELISPOT Assay

B-cell ELISPOT assay was used to detect and enumerate anti-VEGFR antibody-producing B cells (APBCs) [16]. Briefly, polyvinylidene-difluoride-bottomed 96-well filtration plates (Millipore Cor., Bedford, Mass., USA) were coated with 30 μ g/ml of recombinant mouse VEGFR protein. Mononuclear cells prepared from spleen were incubated on the plates at 37°C for 4 h. IgG bound to the membrane was revealed as spots using alkaline-phosphatase-conjugated anti-mouse IgG antibodies.

Immunohistochemistry

To identify the endothelial deposition of autoantibody, immunofluorescent staining was performed as described previously [5]. Briefly, frozen sections were fixed in acetone, washed with PBS, and incubated with goat FITC-conjugated antibody against mouse IgG, IgA, or IgM (Sigma, St. Louis, Mo., USA). Slides were examined by fluorescence microscopy. For the determination of vessel density in tumor tissue, the sections were processed and stained with rat anti-mouse CD31 antibody by an avidin-biotin-peroxidase method using VECTASTAIN Elite ABC KIT (Vector Laboratories Burlingame, Calif., USA), following the manufacturer's instructions. Microvessel density (MVD) was determined by counting the number of microvessels per high-power field in the sections as described [17].

Apoptosis Detection

TUNEL staining was performed using an In Situ Cell Death Detection Kit AP (Roche Molecular Biochemicals, Indianapolis, Ind., USA) following the manufacturer's protocol. It is based on the enzymatic addition of digoxigenin-nucleotide to the nicked DNA by terminal deoxynucleotidyl transferase. The results were imaged in a Nikon (ECLIPSE E600) fluorescence microscope and the apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each field.

Calculation of Synergistic Indexes

Mean values of tumor volume, MVD, and cell apoptosis were used for calculation of correspondent synergistic indexes using the methods described before [18]. Briefly, the observed relative ratio of tumor volume, MVD, and cell apoptosis in each treatment group, was obtained by dividing its mean value by that in the untreated control group. The expected relative ratio of the combination treatment group was obtained by timing the observed relative ratio of the low-dose gemcitabine treatment group to that of qVEGFR vaccine treatment group. Then the correspondent synergistic index of tumor volume and MVD was obtained by dividing the expected relative ratio by the observed relative ratio, whereas the synergistic index of apoptosis was obtained by dividing the observed relative ratio by the expected relative ratio. The example for calculation of synergistic index of tumor volume was more detailed in table 1. An index of greater than 1 indicates a synergistic effect; otherwise an index of less than 1 indicates a less than additive effect.

Statistic Analysis

For comparison of individual time points, ANOVA and an unpaired Student's *t* test were used. Survival curves were constructed according to the Kaplan-Meier method. Statistical significance was determined by the log-rank test. *p* values <0.05 were considered significant.

Results and Discussion

Combination of Low-Dose Gemcitabine and qVEGFR Inhibited Tumor Growth Synergistically and Led to Prolonged Survival

The treatment began on the 7th day after the mice were transplanted with tumor cells. QVEGFR or low-dose gemcitabine treatment resulted in tumor inhibition to a certain extent. Remarkably, the combination therapy resulted in significant antitumor activity (fig 1). Mice treated with either low-dose gemcitabine or qVEGFR showed an appreciable decrease in mean tumor volume compared with those of control group during treatment period, but without complete tumor regression. After treatment, the tumor residues of mice of low-dose chemotherapy group grew progressively again and tumor-bearing mice died successively due to large tumor burden. In comparison with the low-dose chemotherapy group, the mice immunized with qVEGFR had a relatively smaller mean tumor volume and longer survival. However, in combina-

Table 1. Synergistic indexes of combination treatment calculated by relative ratio of tumor volume (RRTV)^a

Day ^b	Gem- citabine RRTV	qVEGFR RRTV	Combination treatment		Index ^d
			expected ^c RRTV	observed RRTV	
<i>Lewis lung carcinoma model</i>					
30	0.17	0.33	0.05	0.13	0.38
34	0.35	0.40	0.14	0.16	0.87
38	0.85	0.44	0.37	0.17	2.18
42	1.26	0.52	0.65	0.18	3.61
46	1.37	0.56	0.77	0.19	4.05
<i>H22 hepatoma model</i>					
30	0.18	0.32	0.05	0.13	0.38
34	0.35	0.39	0.14	0.17	0.82
38	0.80	0.45	0.36	0.21	1.71
42	1.51	0.58	0.88	0.25	3.52
46	1.58	0.69	1.09	0.29	3.89

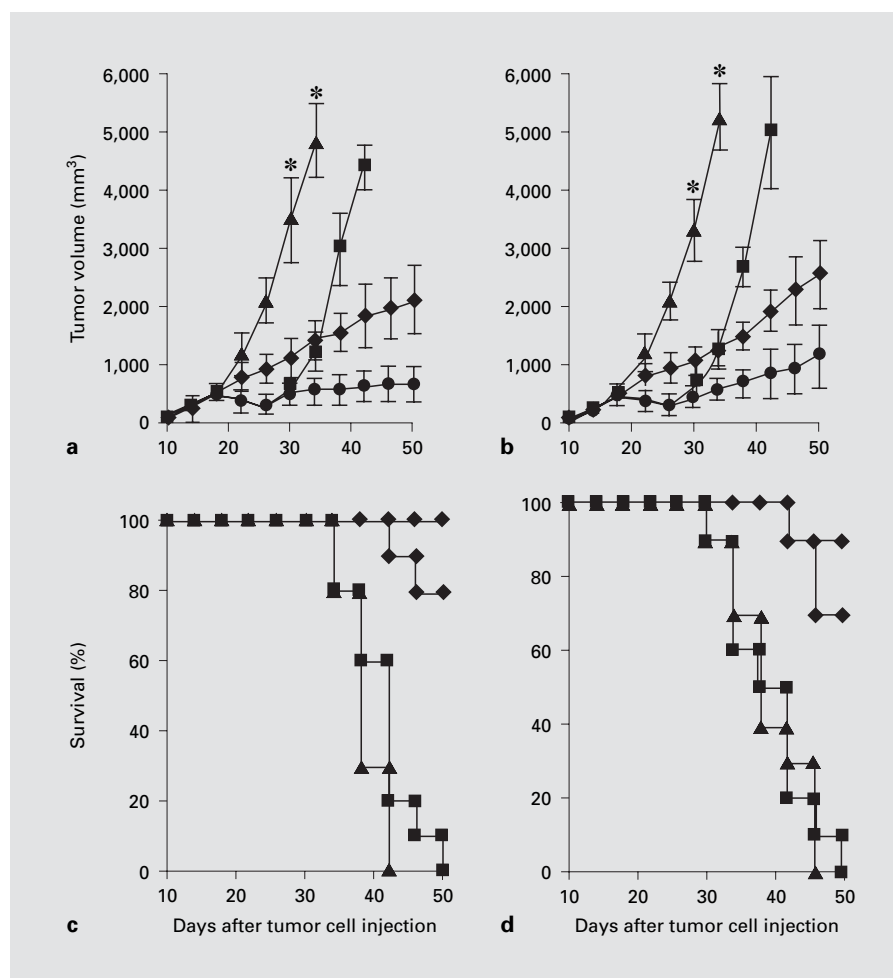
^a RRTV = (mean tumor volume experimental)/(mean tumor volume untreated on day 30). ^b Day after tumor cell transplantation. ^c (RRTV of gemcitabine) × (RRTV of qVEGFR vaccine). ^d Obtained by dividing the expected RRTV by the observed RRTV. An index of greater than 1 indicates a synergistic effect; an index of less than 1 indicates a less than additive effect.

For example, the observed mean tumor volumes (mean value of 10 mice in the Lewis lung carcinoma model; shown in fig. 1a) on day 30 after tumor cell injection were: 466.1 (combination treatment group); 1,152.3 (qVEGFR vaccine group); 619.3 (gemcitabine treatment group) and 3,529.5 (untreated control group).

The following numbers shown in row 1 of table 1 (under Lewis lung carcinoma model) were calculated based on the above value: 0.17 = 619.3/3,529.5 (the observed relative ratio of the gemcitabine treatment group); 0.33 = 1,152.3/3,529.5 (the observed relative ratio of the qVEGFR vaccine treatment group); 0.05 = 0.17 × 0.33 (the expected relative ratio of the combination treatment group); 0.13 = 466.1/3,529.5 (the observed relative ratio of the combination treatment group); 0.38 = 0.05/0.13 (the synergistic index of the combination treatment group).

tion treatment group, complete tumor eradication was observed in 3 of 10 mice bearing Lewis lung carcinoma and 1 of 10 mice bearing H22 hepatoma (fig. 1 a, b). The tumor growth inhibition in mice which received combination therapy was remarkable and was maintained even at day 100 after tumor cell injection. The relative ratio of tumor volume of the combination group showed a synergistic relationship about 38–40 days later after tumor cell transplantation (table 1) in both tumor models. In addition, the survival of the tumor-bearing mice treated in the experiment showed similar results to that of the tumor growth (fig. 1 c, d).

Fig. 1. Antitumor effect of qVEGFR in combination with gemcitabine in 2 murine tumor models. Mice (10 mice in each group) treated with qVEGFR in combination with gemcitabine (●), qVEGFR (◆), gemcitabine alone (■), or saline (▲) at day 7 after s.c. inoculation of 1×10^6 LL/2 (a and c) and hepatoma cells (b and d). The results are expressed as mean $\pm$ SEM. * Indicated significant difference when compared with the untreated group ($p < 0.05$). Also, combined therapy with qVEGFR and gemcitabine resulted in the prolongation of the survival compared with the control groups in both tumor models ($p < 0.01$, by log-rank test).



The mice treated with low-dose chemotherapy plus qVEGFR have been investigated for potential toxicity. There was no more significant body weight loss between combination and single-agent group. The only side effect observed in mice treated with gemcitabine was less feeding, which was both minor and transient, compared with mice which received no chemotherapy. No adverse consequences were indicated in other gross measures such as ruffling of fur, lifespan, and behavior. Furthermore, no pathological changes in liver, lung, kidney, spleen, brain and heart, etc. were found by microscopic examination.

Combination Therapy Had No Inhibitory Effect on Anti-VEGFR Antibody Formation

Anti-VEGFR APBCs were detected by ELISPOT. The number of APBCs was elevated in the spleens of mice immunized with qVEGFR, compared with those in non-

immunized groups. At the same time, the number of APBCs of mice belonging to the combination group was comparable with that of mice belonging to qVEGFR immunized group, which suggested that low-dose chemotherapy had no inhibitory effect on mice immune response to qVEGFR immunization (fig. 2a). To identify the possible deposition of autoantibodies within tumor tissues, we investigated the tissues by immunofluorescence staining. There was the endothelial deposition of IgG within tumor tissues from qVEGFR-immunized mice (fig. 2b, c), but not from nonimmunized mice (fig. 2d, e). Also, no IgM- or IgA-specific fluorescence was found. In addition, no detectable deposition of autoantibodies was found within the major organs, such as kidney, liver, spleen, brain, etc., in the immunized or nonimmunized mice.

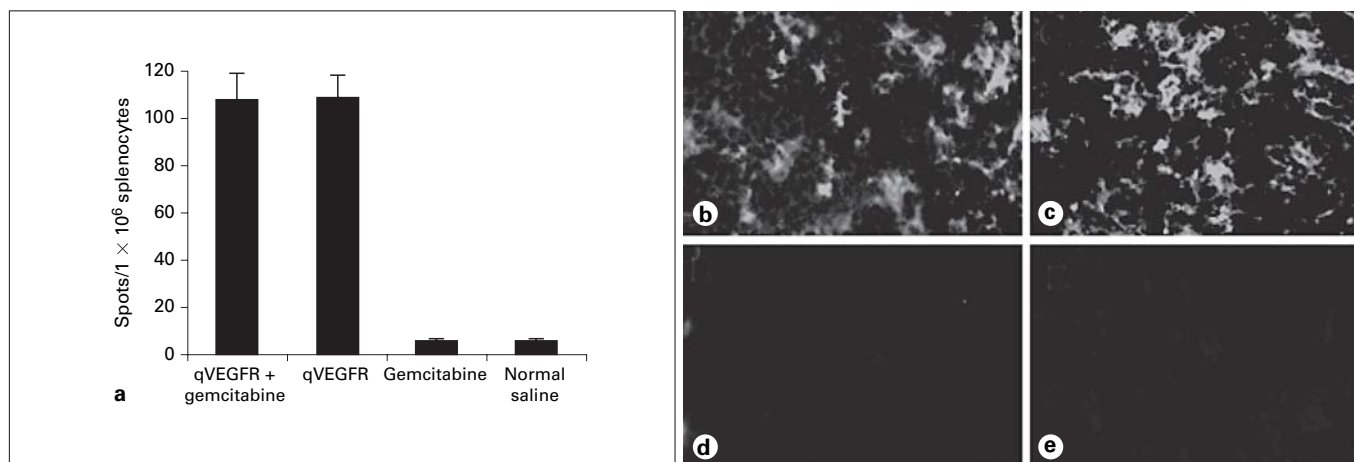


Fig. 2. The identification of autoantibodies on the endothelial cells by fluorescence microscopy and the analysis of anti-qVEGFR antibody-producing B cells by ELISPOT assay. Mice burdened with LL/2 tumor were killed on day 35 after tumor cell injection. Quantification of the number of B cells secreting antibodies specific for qVEGFR were analyzed by ELISPOT, as described in Materials and Methods. Data was represented as the mean $\pm$ SD values for each group (a). The deposition of autoantibodies on the endothelial cells from mice immunized with qVEGFR was observed by immunofluorescent staining without using primary antibody (b and c). No fluorescent signal was detected within the tumor tissues from control groups (d and e).

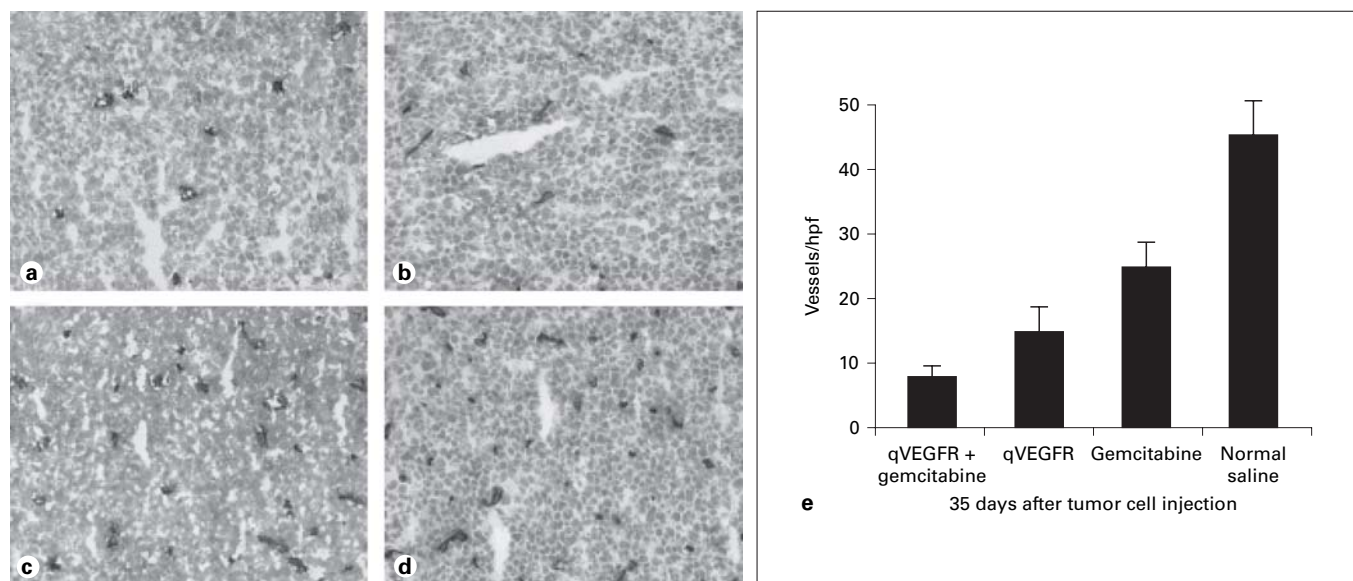


Fig. 3. Inhibition of angiogenesis within tumors from combination-treated mice was estimated by immunohistochemical analysis. The frozen sections of LL/2 tumor tissues were obtained from mice treated with qVEGFR and gemcitabine (a), qVEGFR (b), gemcitabine alone (c), and saline (d). Vessel density (e) was determined by counting the number of the microvessels per high-power field (hpf) in the section with an antibody reactive to CD31, as described in Materials and Methods.

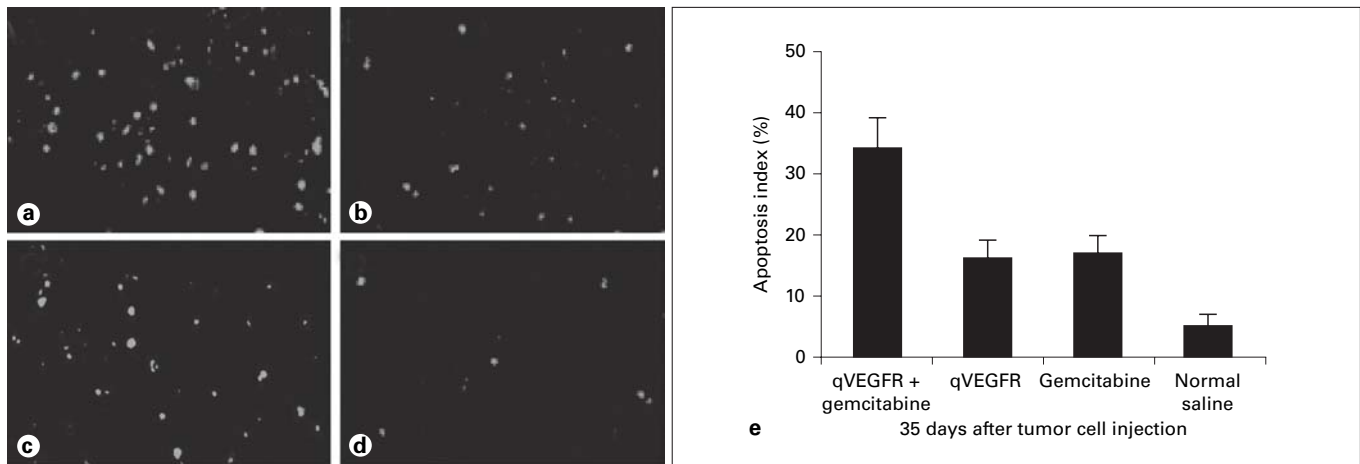


Fig. 4. Induction of tumor cell apoptosis by the combined therapy with qVEGFR and gemcitabine. Apoptosis was estimated by TUNEL assay, as described in Materials and Methods. The frozen sections of LL/2 tumor tissues were obtained from mice at day 35 after tumor cell injection. Apoptotic tumor cells were elevated within tumor tissues obtained from mice treated with qVEGFR in combination with gemcitabine (a), compared with control groups (b–d). The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each field (e).

Combination Therapy Resulted in Synergistic Inhibition of Tumor Angiogenesis

Angiogenesis within tumor tissue was estimated by counting the number of microvessels on the section stained with an antibody reactive to CD31. The combination treatment with low-dose chemotherapy and qVEGFR (fig. 3a) resulted in synergistic inhibition of angiogenesis in tumors (synergistic index = 1.07), compared with either agent alone or normal saline (fig. 3b–d). Similar findings were confirmed in 2 tumor models.

Combination Therapy with Low-Dose Gemcitabine and qVEGFR Increased Tumor and Endothelial Cell Apoptosis

VEGF is a very specific mitogen for vascular endothelial cells. It also functions as a potent antiapoptotic factor for endothelial cells in newly formed vessels [19]. Inhibition of VEGF/VEGFR signal transduction by anti-VEGFR antibody induced by qVEGFR immunization resulted in increased endothelial cell apoptosis. Also, gemcitabine has been identified as an apoptosis-inducing agent [20, 21]. Thus, in comparison with single agent alone or normal saline (fig. 4b–d), the combination therapy (fig. 4a) resulted in more apoptosis of both endothelial (data not shown) and tumor cells which could be detected by TUNEL staining under fluorescence microscope. The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each

field (fig. 4e). The apoptotic index showed a significant synergistic effect (the synergistic index = 1.54). These results suggest that the increased apoptosis of tumor and endothelial cell may play an important role in the enhanced antitumor activity of combination therapy.

The mechanism responsible for the interaction between qVEGFR and low-dose chemotherapy may involve a synergistic antiangiogenic effect and synergistic apoptosis of both tumor and endothelial cells. On one hand, previous study from our laboratory has proved that the protein vaccine qVEGFR could induce both protective and therapeutic antitumor immunity. Autoimmune response against mouse VEGFR-2 (Flk-1) may be provoked in a cross-reaction by the immunization with qVEGFR vaccine, and the induction of the autoantibody response to Flk-1, which is responsible for qVEGFR-induced antitumor activity, may involve CD4⁺ T lymphocytes. On the other hand, being cytotoxic agents, low-dose chemotherapy with gemcitabine could interfere with DNA synthesis and induce DNA breakage, thus cause tumor cell apoptosis [13–15, 20].

Our data indicated that low-dose gemcitabine and xenogeneic qVEGFR vaccine could potentiate each other's antitumor activities, which were demonstrated in the synergistic indexes of MVD and cell apoptosis. It has been reported that gemcitabine could result in downregulation of tumor-cell-produced VEGF by way of inducing tumor cell apoptosis [21]. Also, qVEGFR immunization in-

duced the response of autoantibodies to Flk-1. The above two effects may contribute to the synergistic inhibition of tumor angiogenesis. On the other hand, autoantibodies against Flk-1 induced by qVEGFR immunization could block the VEGF/VEGFR signal transduction and might lead to the apoptosis of endothelial cells [19]. Thus, tumor cells could not acquire necessary oxygen and nutrients, which would result in more tumor cell apoptosis. Therefore, the interaction between qVEGFR and low-dose gemcitabine showed a significant synergistic effect in the increased amount of cell apoptosis.

Collectively, the present study showed that the treatment with qVEGFR and low-dose gemcitabine induced synergistic effect on apoptosis induction and angiogenesis inhibition. Although the mechanism for the enhanced antitumor activity is not known exactly and needs further investigation, the combined treatment with qVEGFR plus low-dose gemcitabine may present a potential approach for cancer therapy without significant undesired side effects.

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