FACULTAD DE MEDICINA

DEPARTAMENTO DE ANATOMÍA Y EMBRIOLOGÍA HUMANA

TESIS DOCTORAL:
"DESARROLLO DE UNA NUEVA ESTRATEGIA DE TERAPIA GÉNICA PARA EL CÁNCER: EFECTO ANTITUMORAL DE LOS GENES "KILLER" GEF Y E EN CÁNCER DE PULMÓN Y MELANOMA"

Memoria presentada por D. Raúl Ortiz Quesada

Para la obtención del grado de Doctor Europeo

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CERTIFICA:

Que el trabajo de investigación que se expone en la presente Tesis, **"DESARROLLO DE UNA NUEVA ESTRATEGIA DE TERAPIA GÉNICA PARA EL CÁNCER: EFECTO ANTITUMORAL DE LOS GENES "KILLER" GEF Y E EN CÁNCER DE PULMÓN Y MELANOMA"** ha sido realizado bajo mi dirección por el licenciado **D. Raúl Ortiz Quesada** corresponde fielmente a los resultados obtenidos.

Una vez redactada la presente memoria ha sido revisada por mí y la encuentro conforme para ser presentada y aspirar al grado de Doctor Europeo ante el tribunal que en su día se designe.

Y para que conste, en cumplimiento de las disposiciones vigentes, expido el presente en Granada a 22 de Junio de 2009.

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ÍNDICE

I.- SUMMARY

Currently, the cancer is a major cause of mortality in the world, mainly due to late diagnosis, metastatic ability, and that many current treatments do not have the desired effect on the disease. Among the various types of tumors there are lung cancer and melanoma are quite interesting from the standpoint of the development of new therapies.

Lung cancer is the leading cause of cancer-related mortality in both men and women. Non-small cell lung cancer (NSCLC) represents about 75-80% of all lung cancers, and most of these patients are in advanced stage at diagnosis. Although chemotherapy has recently shown promising results in adjuvant strategies for early-stage patients and some progress has been made in the treatment of locally progressive and advanced disease, latest studies suggest that a therapeutic plateau has been reached and that novel, more specific and less toxic therapeutic strategies are needed.

In the case of melanoma, that represents only 4% of all skin cancers but nearly 80% of total skin cancer deaths, predominantly because of metastatic spread. Apart from surgery, the treatment options for melanoma, particularly metastatic melanoma, are relatively limited and emphasize the need for the development of novel efficacious therapies. As melanoma is a highly therapy-refractory tumor, it demands effective therapeutic combinations.

Suicide gene therapy has been proposed as a strategy for the treatment of intractable cancers and has been assayed in some clinical trials by itself or in combination with other therapies (tumor irradiation or chemotherapy). Recently, novel advances in the combined use of suicide gene therapy and antitumour drugs have been reported in bladder cancer, pancreatic cancer and breast or colorectal cancer. However, few studies of this type have been performed in lung cancer and melanoma. In fact, classical strategies using a suicide gene e.g., herpes simplex virus thymidine kinase (HSV-tk), have shown beneficial effects but with some limitations. They are able to convert a non-toxic prodrug

into a toxic metabolite, but the release of toxic metabolites and their bioavailability are two important shortcomings of the use of these systems. Therefore, increasing attention is being paid to the transfer of genes that are not dependent on the use of a prodrug.

Therapeutic genes which encode cytotoxic proteins directly could be an attractive alternative to this strategy. In contrast to classical suicide genes, which act by disrupting DNA synthesis and therefore target only rapidly dividing cells, these new toxins may act by killing both quiescent and rapidly dividing tumor cells and may be effective for aggressively growing tumors as well as for those that grow more slowly. The most recent experiences with genes expressing toxins from bacteria such as diphtheria toxin or streptolysin O, plants such as saporin (SAP), viruses such as the matrix protein of vesicular stomatitis virus, and bacteriophages such as alpha-holin, have shown a high cytotoxicity for tumoral cells derived from different tissues.

In this context, the gef and E genes are another potentially interesting prokaryotic lysis genes for cancer therapy. The E gene, in contrast to most double-stranded DNA phages, which generally encode two genes that elicit host-cell lysis (endolysin and holing protein), the small single-stranded DNA phage φX174 has only one lysis gene. The 91-aa E protein encoded by this causes cell lysis at concentrations of 100–300 molecules per cell, although its mechanism of action is controversial. Gene fusion analysis has revealed that only the 29 amino-terminal amino acids of the E polypeptide encompassing the putative transmembrane domain are required for lytic activity. However, this polypeptide has no detectable cell-wall-degrading activity, and given its simple primary structure it is unlikely to have any enzymatic activity at all. Scanning electron microscopy images of cells undergoing E-mediated lysis have shown discrete 50- to 200-nm holes in the cell membrane. This observation has led to the proposal of a model in which the E protein oligomerizes to form a

"transmembrane tunnel" spanning the entire cell envelope, thereby releasing the cytoplasmic content.

The gef gene is a member of a gene family with homologous cell-killing functions, encodes a membrane protein of 50 amino acids which is anchored in the cytoplasmic membrane by the N-terminal portion, whereas the C-terminal part is located in the periplasm. Although activation of this protein induces arrest of respiration and death in bacterial cells the mechanism of action in tumoral cells is unclear.

Here we report the antitumor effect of E and gef genes in studies in vivo and in vitro, as well as in combination with drugs. We evaluated the:

-**Combined therapy using suicide *gef* gene and paclitaxel in multicellular tumour spheroids of A-549 human lung cancer cells:** To improve the antitumoral effect of the paclitaxel in lung cancer cells, we investigated a combined suicide gene therapy using this drug and *gef* gene *in vitro*, using A-549 lung cancer cells in culture and forming multicellular tumour spheroids (MTS). Our results showed that *gef* expression in A-549 cells led to an ultrastructural changes, including dilated mitochondria with clear matrices and disrupted cristae and cell surface alterations such as reduction in length and number of microvilli and cytoplasmic membrane evaginations. The use of paclitaxel in A-549 lung cancer cells transfected with *gef* gene enhanced the chemotherapeutic effect of this drug. Volume analyses showed an 87.4% decrease in the A-549 MTS growth after 96 h in comparison with control MTS. This inhibition was greater than that obtained using the gene therapy or chemotherapy alone.

-**The cytotoxic activity of the E and gef genes on the murine B16 melanomas in vitro and in vivo**: Firstly, we used a non-viral gene delivery approach (pcDNA3.1/gef or E) to study the inhibition of melanoma cells (B16-

F10) proliferation in vitro. Secondly, we used direct intratumoral injection of pcDNA3.1/gef or E complexed with jetPEI to deliver E and gef cDNA to rapidly growing murine melanomas. In the case of E gene we used also a GFP vector with the objective of localize the the GFP-E fusion protein (that was located in the mitochondria). We demonstrated that gef and E genes not only has an antiproliferative effect on B16-F10 cells in vitro, but also induces an important decrease in melanoma tumor volume (around 70% in both genes on 8 days) in vivo. Interestingly, after E and gef gene treatment, melanoma showed apoptosis activation associated with the mitochondrial pathway, suggesting that the induction of this death mechanism may be an effective strategy for its treatment.

In conclusion, These results show *that gef* gene has a cytotoxic effect in lung cancer cells and enhances cell growth inhibition when used with paclitaxel (these results indicate that this combined therapy may be of potential therapeutic value in lung cancer) and E and gef gene expression in melanoma cells has an extraordinary antitumor effect, which means it may be a new candidate for an effective strategy for melanoma treatment.

II.- INTRODUCCIÓN

1.- MELANOMA

1.1.- CONCEPTO

El melanoma cutáneo (MC) es una neoplasia maligna derivada de los melanocitos de la piel o de tumores melanocíticos inicialmente benignos (nevus) (Clark y cols., 1975) (Figura 1). Aunque, a nivel mundial, no es un tumor muy frecuente, en los últimos tiempos su relevancia en los países occidentales, habitados en gran medida por población de raza blanca, ha aumentado de manera importante debido al llamativo incremento de sus tasas de incidencia.

Su importancia, por tanto, radica fundamentalmente en esta marcada tendencia ascendente, una de las más agudas en los países occidentales, y en su capacidad letal, ya que, aunque en general la supervivencia es alta gracias a la detección temprana de las lesiones (Berrino y cols., 2003), la posibilidad de controlar la enfermedad una vez que ya ha metastatizado es muy baja.

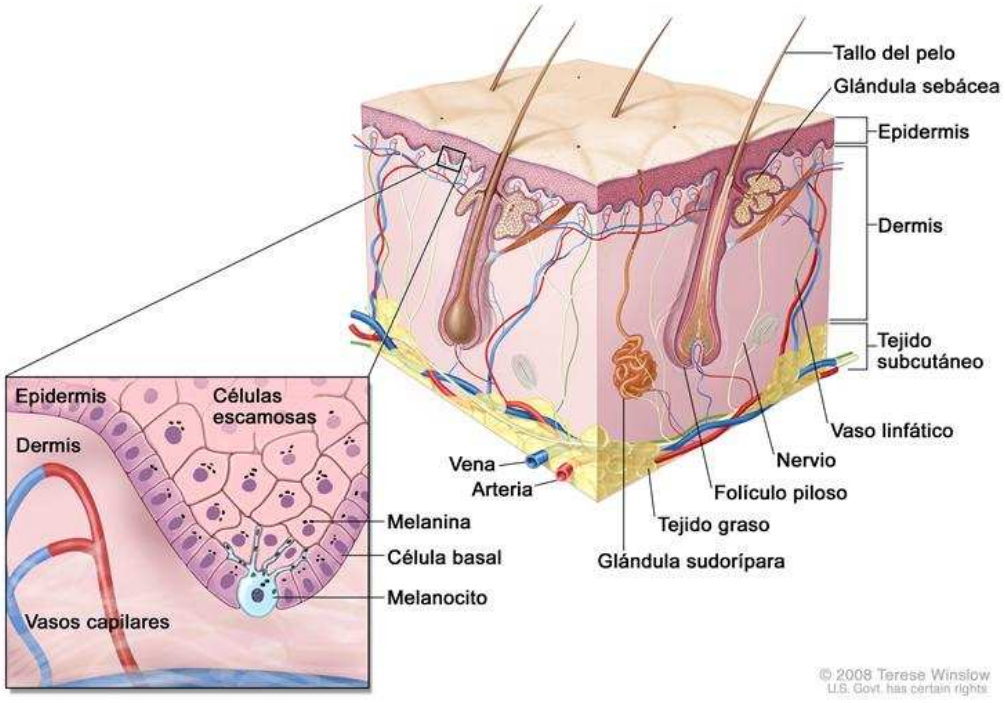

Figura 1. Estructura y composición de la piel: ubicación de los melanocitos

La localización cutánea de este tumor, el conocimiento de la clínica y los estudios histológicos han permitido conocer con detalle su evolución. Sin embargo, han sido los avances en biología molecular los que han permitido determinar marcadores específicos de melanocitos mediante los cuales podemos analizar el comportamiento de este tumor, avanzar en su diagnóstico precoz y desarrollar nuevos tratamientos dirigidos a modificar los factores que contribuyen a su diseminación metastásica

1. 2.-EPIDEMIOLOGÍA

El melanoma es un tumor que presenta índices epidemiológicos diferentes a cualquier otro tumor, el aumento anual de las tasas de incidencia varía entre el 3-7% en la población caucásica. Es uno de los tumores en los que más ha aumentado la incidencia en la población blanca. Es uno de los diez tumores malignos más frecuentes en el mundo, sobre todo en Australia, Nueva Zelanda, América y Europa del Norte; siendo por el contrario poco frecuente en África, Asia y Sudamérica. Australia y Nueva Zelanda presentan tasas que duplican a las más altas de EEUU o Europa.

En España la incidencia es ligeramente mayor en las mujeres que en las hombres. Presentan mayores tasas sobre todo en Girona y Granada, seguidas de Navarra, Mallorca, Murcia y Tarragona. Si analizamos la incidencia específica por edad se duplican a partir de los 55 años tanto en hombres como en mujeres, pero es a partir de los 75 cuando se observa mayor incremento.

Un reciente análisis de la mortalidad por melanoma en España (Tardon y cols., 2002) desde 1975 a 2001 confirma que la mortalidad por este tumor aumentó dramáticamente desde mediados de los setenta hasta los noventa, en que la tendencia se estabiliza de forma similar a otros países de Europa. Sin embargo, esta tendencia al aumento no ha sido tan llamativa como el de la incidencia suponiendo el 2% de todas las muertes (Lopez-Abente y cols.,

2002). En razón de género en España el riesgo acumulado de mortalidad por melanoma en los últimos 50 años ha sido siempre superior en hombres que en mujeres al igual que su incremento a lo largo de los años (INE).

1.3.- ETIOLOGÍA Y FACTORES DE RIESGO

Los factores asociados a un mayor riesgo de desarrollo de melanoma son diversos aunque los estudios más amplios corroboran que entre los significativamente asociados a este tipo de tumor se encuentran la exposición al sol, sobretodo intensa e intermitente con quemaduras solares, la historia familiar, el hecho de haber padecido previamente melanoma y la existencia de lesiones precursoras.

1.3.1.- Melanoma familiar

Se estima que la prevalencia del melanoma se sitúa entre un 5-10%. Es una variable compleja ya que la existencia de varios casos en una familia puede representar susceptibilidad genética o la exposición común a un agente externo procancerígeno. El pronóstico y la histología son similares a los casos esporádicos de melanoma, sin embargo en los casos familiares parece existir una tendencia a la aparición temprana, escaso grosor y desarrollo de múltiples melanomas primarios.

Varios grupos han estudiado las bases genéticas de la predisposición hereditaria a padecer melanoma, desgraciadamente el número de enfermos no ha sido suficiente para contestar a cuestiones fundamentales. Los locus principales identificados en melanoma familiar son CDKN2a, p16 y CDK4 (Nelson y cols., 2009)

1.3.2.- Factores de riesgo

Los factores de riesgo a destacar y en los que coinciden la mayoría de los estudios son:

- Lesiones precursoras: Xeroderma pigmentoso, Léntigo Maligno (Melanosis precancerosa de Dubreuilh o peca melanocítica de Hutchinson) Nevus Congénito Gigante

- Alteraciones en nevus preexistentes

- Raza y fenotipo: raza caucásica, piel clara, cabello rubio o pelirrojo, incapacidad para el bronceado.

- Exposición a Rayos Ultravioleta: en especial RUV-B. El riesgo se ve incrementado con intensas exposiciones intermitentes y con el número de quemaduras solares.

- Historia familiar y personal de melanoma.

- Presencia de nevus displásicos, “síndrome de nevus displásico”. Son nevus con alteraciones arquitecturales, con displasia melanocítica lentiginosas y atípica citológica en los nevomelanocitos, preferentemente localizados en la unión dermoepidérmica. Los sujetos con nevus displásicos albergan un mayor riesgo de desarrollar melanoma, ya sea sobre lesión displásica o sobre piel normal, siendo la incidencia de la neoplasia sobre estos nevus de 1:3.000 al año. Por tanto la existencia de nevus displásicos es un marcador que identifica a personas a riesgo de desarrollar melanoma (INE).

- Presencia de multitud de nevus no displásicos. La cantidad, y no la arquitectura, de nevus normales sobre piel sana también incrementan el riesgo de padecer melanoma.

- Inmunosupresión.

1.4.- LESIONES PRECURSORAS DE MELANOMA

Una proporción sustancial de melanomas se origina en nevus preexistentes (Figura 2). El número de nevus que tiene una persona es un fuerte factor predictivo del riesgo de melanoma.

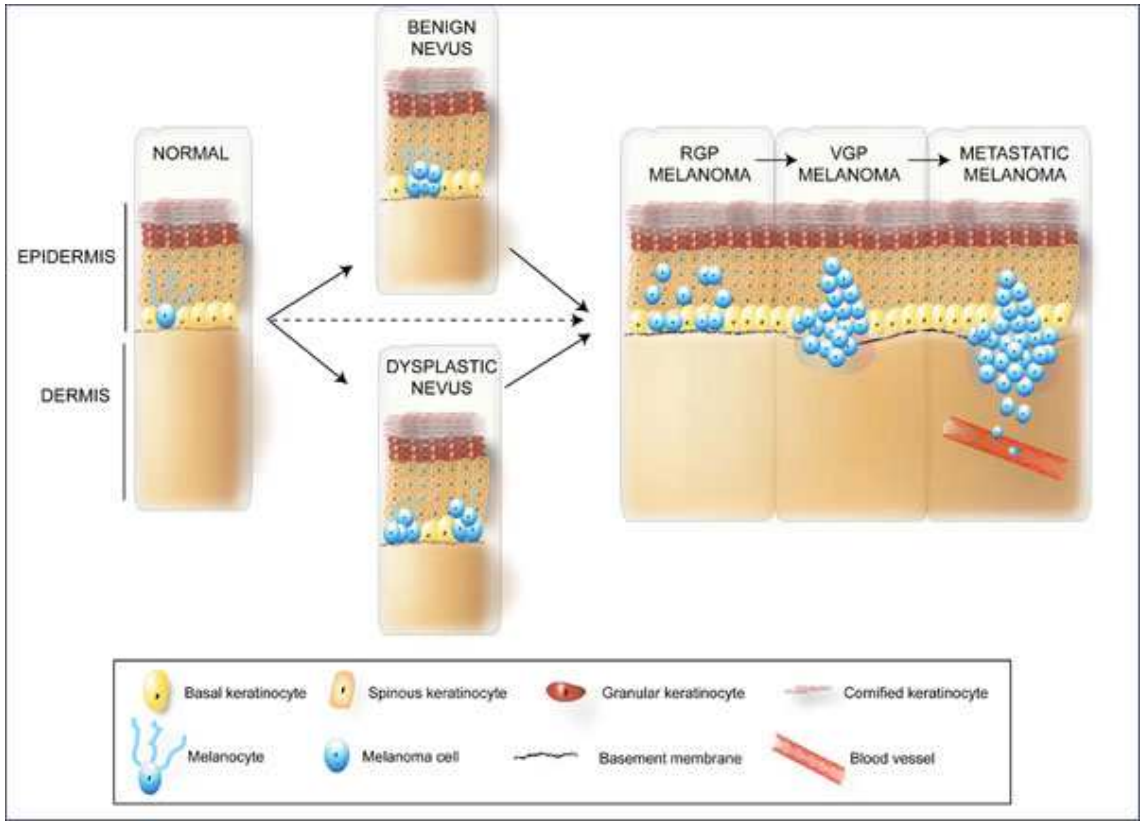

Figura 2. Desarrollo del melanoma maligno a partir de un nevus preexistente

Dentro de este tipo de lesiones cabe distinguir entre:

-El nevus congénito, presente en el momento del nacimiento y de tamaño muy variable (pequeño, mediano y gigante) (Gruber y Armstrong., 2006). Cuanto mayor sea su tamaño, mayor es el riesgo de que aparezca un MC. El riesgo de que aparezca un melanoma en pacientes con nevus congénitos grandes (mayores de 20 cm de diámetro) oscila entre un 5-20%.

-Los nevus pigmentocelulares, pecas o lunares que todos poseemos, aparecen en un promedio de 20 por persona y su número es un fuerte predictor de riesgo de melanoma (Green y cols., 1999; Rodenas y cols., 1996). El desarrollo de estos nevus está relacionado con la raza y el color de la piel; los caucasianos presentan un mayor número de nevus que los no caucasianos (Nelemans y cols., 1995).

-Los nevus clínicamente atípicos (nevus displásicos) son nevus de diámetro mayor de 5 mm con bordes irregulares y superficie poco elevada. Pueden diferenciarse dos subgrupos: de tipo familiar, enfermos con antecedentes familiares de nevus displásicos y melanomas; y de tipo esporádico, persona con más de dos nevus displásicos sin antecedentes familiares de nevus displásicos ni melanomas. Se ha observado una asociación histológica entre estos nevus benignos y los melanomas en el 20% de los casos (Miller y Mihm-MC, 2006)

1.5.- FORMAS CLÍNICO-HISTOLÓGICAS DE MELANOMA

Desde el punto de vista clínico-histológico podemos diferenciar entre cuatro tipos de melanoma: melanoma lentigo maligno, melanoma de diseminación superficial, melanoma nodular, melanoma lentiginoso acral (Armstrong., 1988; DeVita., 2000) (Figura 3).

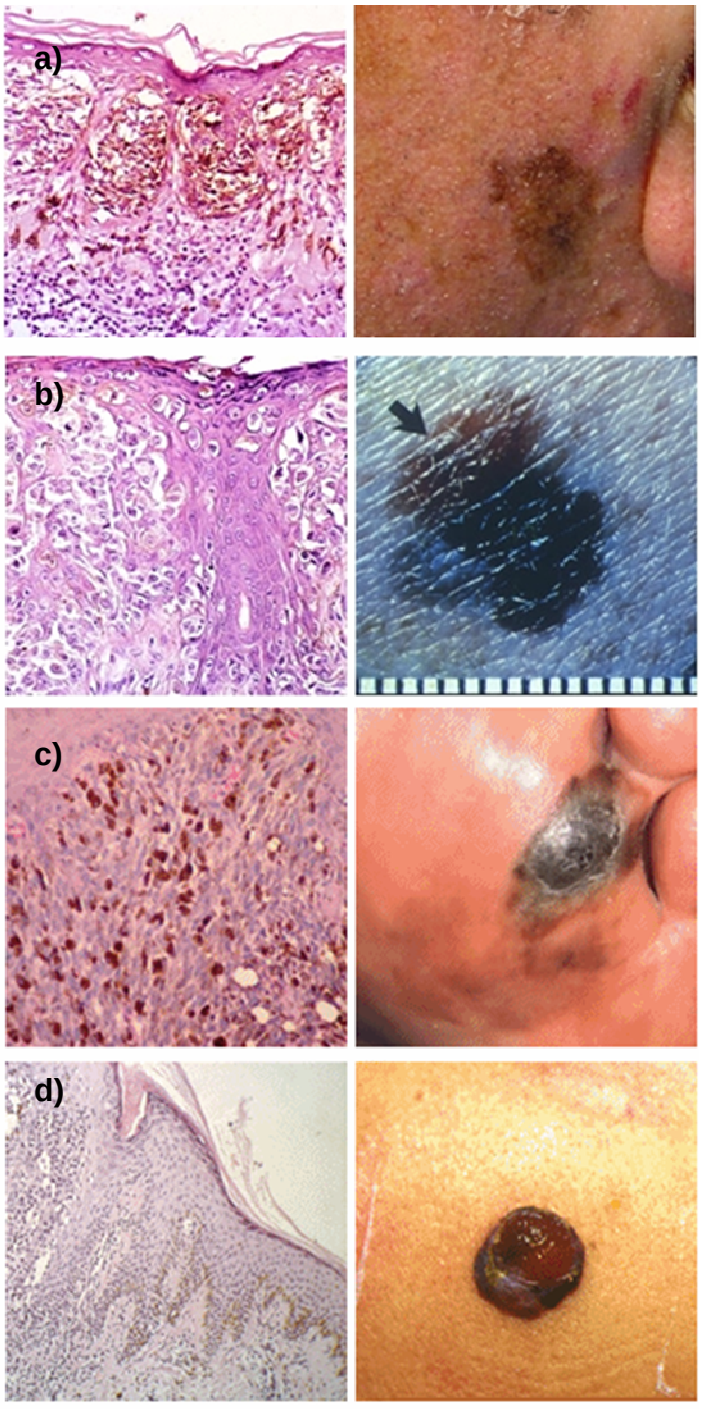

Figura 3. Tipos de melanoma, a) LMM; b) MLA; c) MSS; d) MN

El diagnóstico de melanoma debe sospecharse al observar lesiones pigmentadas con asimetría, bordes irregulares, color no uniforme y diámetro mayor de 6mm. Estos datos se resumen en la denominada regla ABCD.

Las características más importantes de los 4 tipos de melanoma son las siguientes:

- El melanoma lentigo maligno (LMM) es un melanoma intraepidérmico que todavía no ha rebasado la membrana basal. Aparece como una mácula pigmentada en zonas fotoexpuestas (principalmente en cara y dorso de manos), en personas de más de cincuenta años y sobre una piel con síntomas evidentes de degeneración solar. Su característica es la falta de uniformidad en el color que varía de tostado suave a marrón oscuro con contornos muy irregulares. Algunas zonas son hiperqueratósicas pero en conjunto existe una atrofia epidérmica.

- En el 30% de los casos, tras un período de latencia que suele ser largo (cinco a treinta años), evoluciona hacia un melanoma invasor con capacidad de dar metástasis (Farshad y cols., 2002). Clínicamente se detecta por la aparición de un nódulo y una erosión o la extensión rápida en alguna zona de la mácula. Histológicamente se observa la invasión de la dermis. Su pronóstico es más favorable que el de otros tipos de MC debido a que el crecimiento vertical se desarrolla relativamente tarde. Representa el 4-10% de los MC y en él la exposición solar es un factor etiológico demostrado.

- El melanoma de extensión superficial (SSM) generalmente aparece sobre un nevus previo y es la forma clínico-histológica más frecuente (65% al 80% de todos los MC) (Porras y Cockerell., 1997). La lesión suele afectar a personas de mediana edad y se presenta como una mácula que progresivamente crece (en un periodo de 1 a 5 años) con contornos irregulares, límites poco precisos, desbordamiento de pigmento y grandes variaciones en la

hiperpigmentación (pardas, azules y negras). Cuando las lesiones son más avanzadas muestran un característico aspecto rojizo, blanquecino y azulado (Fitzpatrick., 1993).

- El SSM presenta dos fases de crecimiento. La primera ó fase de crecimiento radial se caracteriza por la presencia de células de melanoma en la epidermis. En este periodo el pronóstico es bueno con un desarrollo durante meses o años (Breuninger y cols., 1994). En la segunda fase ó fase de crecimiento vertical se desarrolla con mayor rapidez (semanas o meses) apreciándose la aparición de nódulos. Las lesiones en esta fase dan metástasis en un 35-85 % de casos (Castel y cols., 1991). Su pronóstico, entre el relativamente benigno del LMM y el muy maligno del MN, viene determinado por el grosor del tumor en la parte nodular.

- El melanoma nodular (MN) es el más maligno de las cuatro variedades y representa del 10 al 30% de todos los MC. La edad media de su diagnóstico es de 50 años, presentándose sobre la piel sana y preferentemente en hombres, en los que es dos veces más frecuente que en las mujeres. Esto puede explicar, al menos en parte, el peor pronóstico del melanoma en hombres (Wick y cols., 1980). Su crecimiento es vertical invadiendo la dermis profunda. La epidermis suprayacente suele estar ulcerada, destruida o reemplazada por el tumor y desde el punto de vista clínico evoluciona con rapidez (varios meses a dos años) siendo las lesiones iniciales sobreelevadas y normalmente de un color gris con tintes rosados. La ausencia del crecimiento radial hace difícil el diagnóstico precoz (Fitzpatrick., 1993.)

- El melanoma lentiginoso acral (MLA) constituye el 2-8% de los MC en la raza caucasiana aunque es mucho más frecuente en negros y orientales en los que llega a representar el 35-60%. Suele aparecer en zonas donde no hay normalmente melanocitos: palmas de las manos, plantas de los pies, lechos ungueales y membranas de mucosas (Saida y cols., 1999).

- Morfológicamente es una mácula hiperpigmentada de contornos irregulares y límites poco netos que suele estar muy modificada por la gruesa capa córnea que la cubre. No suele producir molestias subjetivas y el diagnóstico se realiza en fases muy avanzadas de la enfermedad cuando se erosiona. Este diagnóstico tardío hace que tenga el peor pronóstico de los MC (Schmuth y cols., 1999). Su desarrollo biológico se caracteriza por fases de crecimiento radial y vertical. En el periodo de crecimiento radial la lesión es extensa, aumenta de tamaño, el borde no se palpa y el color es un mosaico de ricos bronceados marrones y negros. Clínicamente produce metástasis ganglionar y hemática con una alta mortalidad (Blessing y cols., 1993). El pronóstico va a depender del grado de desarrollo, de su grosor y de su actividad mitótica.

1.6.- TRATAMIENTO DEL MELANOMA CUTÁNEO

La cirugía, la radioterapia y la inmunoterapia han sido utilizadas para el tratamiento de los MC. No obstante, sólo el diagnóstico precoz y la extirpación quirúrgica de la lesión ha conseguido la curación total de este tumor. El resto de los tratamientos deben ser considerados como coadyuvantes.

1.6.1.-Tratamiento Quirúrgico:

La cirugía del tumor primario debe realizarse con escisión quirúrgica amplia de acuerdo al grado de infiltración en profundidad determinado por el índice de Breslow, dejando desde 0.5cm en los melanomas in situ hasta 2cm en lesiones con espesor superior a 2mm.

La linfadenectomía electiva es la disección de la estación ganglionar metastásica. Actualmente no se recomienda de rutina por la alta tasa de complicaciones que conlleva y escasa evidencia de beneficio. Si se lleva a cabo la biopsia selectiva del ganglio centinela guiada por sonda y previa

realización de linfoescintigrafía con coloides marcado con tecnecio 99. La indicación de esta es para los MM mayores de 1mm y para los menores que presentan ulceración. Si la técnica es positiva se realizará vaciamiento ganglionar.

1.6.2.- Tratamiento Radioterápico

La radioterapia adyuvante puede utilizarse cuando la enfermedad residual micro o macroscópica permanece in situ y una nueva intervención no está aconsejada (Elsmann y cols., 1991). Se emplea en lesiones de alto riesgo de recurrencias con alto nivel de invasión: por ejemplo, pacientes tratados quirúrgicamente en áreas de cabeza y cuello (Storper y cols., 1993).

1.6.3.-Tratamiento Adyuvante

Es tratamiento sistémico en melanoma es considerado, la quimioterapia convencional no ha demostrado ningún beneficio en el contexto adyuvante. La inmunoterapia con interferón-α-2b (IFN) es la única opción que ha demostrado cierta eficacia en el tratamiento adyuvante del melanoma. Una reciente revisión del año 2001 de los estudios randomizados publicados con IFN a dosis altas, intermedias y bajas, concluye que de acuerdo a los datos maduros en supervivencia libre de enfermedad (SLE) y supervivencia global (SG) no existe beneficio alguno al emplear IFN a dosis bajas, mientras que parece existir una ventaja en SLE cuando se emplea a dosis altas a costa de una elevada toxicidad. (Tanner., 1999). Por tanto la recomendación como tratamiento adyuvante de IFN a altas dosis se apoya en una evidencia tipo 2 que no puede considerarse definitiva.

1.6.4.- Tratamiento del melanoma metastásico (MM)

La cirugía es el tratamiento de elección en el caso de metástasis (M) en tránsito, satelitosis y recidivas locorregionales. También esta indicada en el tratamiento de metástasis únicas, ya que en algunos casos seleccionados la metastasectomía puede lograr supervivencias prolongadas (Chin y cols., 1997). Cuando el tratamiento quirúrgico en metástasis en tránsito, satelitosis y recidivas locorregionales no es posible, se puede emplear la quimioterapia (QT) de miembro afecto que ha demostrado un elevado índice de respuestas (80-90%) y control local. Consiste en el aislamiento de la circulación del miembro afecto y perfundiendo altas dosis de QT con hipertermia de la extremidad, evitando de este modo la exposición sistémica y, por ende, los efectos secundarios de la misma (Arndt y Rank., 1997).

La QT sistémica en el MM tiene una modesta actividad. Las drogas que presentan mayor eficacia son la dacarbacina (DTIC), nitrosureas, cisplatino y alcaloides de la vinca (Niu y cols., 1999). Las respuestas del DTIC en monoterapia son en torno al 12-20%, ocurriendo estas con mayor frecuencia en localizaciones de partes blandas ya que en metástasis viscerales y, especialmente en las cerebrales, son anecdóticas. Una droga que presenta cierta eficacia en metástasis cerebrales (un 20%) es la fotemustina. Recientemente ha cobrado importancia la Temozolamida, profármaco del DTIC de administración oral y que parece tener una mayor distribución en el líquido cefalorraquideo.

Existen una gran cantidad de esquemas de combinación. En los últimos años se ha suscitado un gran interés por los esquemas combinados de quimio-inmunoterapia tales como interleuquina 2 (IL-2), cisplatino o IFN-α-2b y DTIC. Los resultados de momento son contradictorios (Huang y cols., 1994; Gutzmer y Guerry., 1998). Si bien en algunos se demuestra cierto incremento en tasas de respuesta, esto no se corresponde con un aumento en SG. Dos

metaanálisis recientemente publicados de estudios randomizados en los que se comparaba DTIC en monoterapia con QT de combinación con o sin inmunoterapia, concluían que la bioquimioterapia con DTIC e IFN-α-2b obtenía mayores tasas de respuesta pero a expensas de gran toxicidad y sin repercusión en la SG, que es el objetivo fundamental (Boulaiz y cols., 2003).

Otra opción terapéutica explorada es la inmunoterapia sin combinación con QT. El IFN-α-2b administrado con dosis variables entre 3-18 millones de unidades, 3 veces por semana, alcanza respuestas del 12-18%. La IL-2 a dosis entre 9 y 18 millones de unidades por m^2 logra respuestas del 15-25% (Carlow y cols., 1989). Cabe decir que pese a la multitud de tratamientos ensayados en el melanoma en fase metastásica, incluyendo vacunas, terapia génica con oligonucleótidos antisentido, inhibidores de RAF Kinasa, etc, no existe en el momento actual un tratamiento estándar que se pueda considerar absolutamente eficaz, por tanto el esfuerzo debe ir encaminado a trabajar en conjunto y a desarrollar líneas de investigación enraizadas en la investigación transnacional, alumbre una importante interrelación entre el laboratorio de investigación básica y la práctica clínica, para beneficio de nuestros pacientes.

1.7. BIOLOGÍA MOLECULAR EN MELANOMA MALIGNO

La biología molecular del melanoma maligno involucra dos situaciones clínicas distintas, el estudio de las alteraciones genéticas somáticas en los melanomas malignos esporádicos y el estudio del comportamiento genético del melanoma en las familias con predisposición genética a esta enfermedad.

1.7.1. Alteraciones genéticas en melanoma maligno

Como prácticamente en todos los tumores malignos, en el melanoma hay una acumulación progresiva de anormalidades del ADN que genera una mayor inestabilidad genética que, finalmente, conduce a transformación

maligna. Entre estas anormalidades figuran pérdidas cromosómicas, duplicaciones, translocaciones y delecciones. Además hay otros cambios más sutiles, como mutaciones puntuales y variabilidad microsatélite. Determinar cuáles de estos cambios son causales de la tumorogénesis o son producto de la inestabilidad genética inicial es difícil en muchos casos, reforzando el concepto de múltiples etapas en la transformación maligna (Pons y Quintanilla., 2006).

Uno de los cambios somáticos más frecuentes en melanoma maligno es la pérdida de material genético del brazo corto del cromosoma 9, observado en casi el 50% de los casos. Aparentemente la pérdida de heterocigocidad del cromosoma 9 es un evento temprano en la transformación maligna del melanoma. Otras lesiones genéticas asociadas a melanoma involucran pérdida de heterocigocidad de los cromosomas 3p, 6p, 10q 11q y l7p especialmente en lesiones menores de l,5mm sugiriendo que el compromiso de genes supresores de tumor es un evento genético relativamente importante en los estadios tempranos del melanoma. Cerca del 90% de los melanomas malignos tienen un origen genético no hereditario o esporádico, situación similar a la de la mayoría de tumores sólidos. Así el melanoma esporádico puede resultar simplemente de la exposición aleatoria a sustancias carcinógenas como la radiación solar o puede ser el producto de la alteración de múltiples genes o alelos de débil penetrancia que modifican el riesgo individual moderadamente pero que en conjunción, afectan fuertemente la incidencia total de esta enfermedad en la población.

1.7.2. Genes de predisposición a melanoma

Cuando se analiza los casos de melanomas familiares, análisis de linajes entre las familias afectadas permitieron identificar un locus de susceptibilidad a melanoma (MLM). Este MLM se trasmite en forma mendeliana dominante, así una copia simple defectuosa del gen es capaz de predisponer a

melanoma. La penetrancia de este gen ha sido estimada en 53% y en algunas familias la penetrancia llega a 100%. Como en otros genes de predisposición a cáncer, la herencia de un gen MLM defectuoso incrementa las posibilidades de desarrollar melanoma (Lynch y cols., 2007). Así se calcula que el riesgo de desarrollar melanoma se incrementa 50 veces cuando uno hereda un alelo MLM de predisposición, siendo este riesgo dependiente de la exposición a la luz solar. Este gen de susceptibilidad para melanoma se comporta como un clásico gen supresor de tumores, esto quiere decir que siguen el comportamiento de "doble golpe" descrito por Knudson para el gen del retinoblastoma, donde el "primer golpe" ocurre cuando el paciente hereda el alelo defectuoso y el "segundo golpe" ocurre con una mutación somática del alelo restante (Fecher y cols., 2007).

Entre los genes considerados candidatos a MLM e involucrados en la susceptibilidad genética a melanoma han sido descritos p16, p15, cdk4 y otros. Las mutaciones de células germinales del gen p16 incrementan el riesgo de melanoma; así numerosas mutaciones puntuales se presentan en varios casos de melanomas y en otros tipos de neoplasias. La sobreexpresión de p16 causa parada del ciclo celular en G1/S, siendo un inhibidor in vitro de cdk4, que se encarga de la migración de la célula en el ciclo celular a fase S. Sin embargo, la naturaleza precisa de su rol en la tumorogénesis no está clara. Interesantemente p16 no se limita a intervenir en la predisposición a tumorogénesis del melanoma sino además interviene en la génesis de otros tumores sólidos esporádicos. Otros inhibidores de ciclinas, como p15 y p21, son capaces de inducir detención del ciclo celular en diferentes fases del mismo en forma fisiológica y se postula su rol en la predisposición genética a melanoma (Lynch y cols., 2007, Fecher y cols., 2007).

1.7.3. Progresión molecular en melanoma maligno

Existe una correlación entre los estadios clínicos de melanoma y las alteraciones moleculares y genéticas involucradas en la progresión del melanoma (Hoek y cols., 2006). Así, la evolución de inestabilidad genética, proliferación celular desrregulada y desarrollo de capacidad invasiva y metastásica son eventos complejos desde el punto de vista molecular, producto de la inexorable acumulación de alteraciones moleculares que involucran a oncogenes activados inapropiadamente o a genes supresores inadecuadamente bloqueados o inhibidos. Este proceso además se da a través del tiempo lo cual demuestra no sólo la gran estabilidad genómica inicial del queratinocito sino además la acumulación temporal de defectos específicos dentro de la célula pigmentaria. El proceso puede agruparse en cuatro fases de progresión del melanoma: Inestabilidad genómica, desregulación de la proliferación del queratinocito, desarrollo de potencial invasivo y desarrollo de potencial metastásico.

La inestabildad genómica es un evento crítico en la progresión del melanoma que induce inestabilidad genética dentro del melanocito. Se ha descrito múltiples alteraciones cromosómicas en el melanoma, las más frecuentes en los cromosomas 9, 1, 6, 7, 10 y 11, siendo el gen p16 probablemente crítico en la transformación maligna del melanoma (Cretnik y cols., 2009). Este gen se ubica en el cromosoma 9 y como se ha señalado, su función sería la de un gen supresor de tumor

Como resultado del daño de genes críticos en el control del ciclo de división celular y el continuo acúmulo de lesiones genéticas secundarias se produce proliferación desrregulada de los melanocitos. Como se señaló, es interesante observar que si bien normalmente el melanocito no tiende a dividirse en el adulto normal, el daño sobre los genes reguladores del ciclo

celular genera que, al menos una subpoblación de melanocitos, prolifere inadecuadamente perpetuando la inestabilidad genómica originada por la exposición a luz solar. Ante la exposición solar el melanocito epidérmico está influenciado por dos señales conflictivas, por un lado a la inhibición de la replicación del ADN dañado y reparación de dicha molécula, y por otro, la replicación de ADN y proliferación transitoria de un grupo de melanocitos. La regulación de estas dos señales celulares es crítica en la prevención de la transformación maligna.

Por otro lado, el estatus del gen supresor p53 en el melanoma maligno es complejo (Box y Terzian., 2008). Uno esperaría que este gen supresor estuviese mutado como en la mayoría de tumores sólidos y de esta manera favoreciera la transformación maligna. Sin embargo mutaciones de p53 sólo se presentan entre el 5 y 30% de los casos, especialmente en los casos de melanoma metastásico. El comportamiento tan contradictorio de p53 en el melanoma es aún motivo de controversia. La heterogeneidad de la expresión de p53 en melanoma y si la sobreexpresión del p53 mutado en realidad favorece al estado de inestabilidad genómica o la capacidad metastásica.

Posteriormente, en la fase de desarrollo del potencial invasivo, las células de melanoma son capaces de activar tres mecanismos de invasión de tejidos bien a través de la desrregulaclón directa del tejido normal que rodea a las células de melanoma, bien al atenuar las señales bloqueadoras de motilidad y crecimiento celular de las células vecinas, o a través de la producción de sustancias paracrinas y autocrinas como factores de crecimiento hematopoyético (GCSF, PDGF-A) o factores de crecimiento epitelial (TGF alfa y beta , TNF , interleuquinas). Así se establece una interacción entre las células de melanoma y la matriz extracelular de soporte provocando finalmente que las células neoplásicas invadan los tejidos sanos (Zigler y cols., 2008).

Por último, el desarrollo de potencial metastásico está asociado a la producción de neoangiogénesis inducida por las células neoplásicas a través de la producción de sustancias o factores como los factores de crecimiento endotelial (VEGF), TGF beta l y TNF (Rinderknecht y Detmar., 2008). El resultado final de esta inducción de neovasos es el desarrollo de la capacidad metastásica de las células tumorales. Sin embargo una vez que las células de melanoma tienen esta capacidad, aún se necesita una serie de eventos moleculares y locales para que pueda completarse el proceso de metástasis desde el punto de vista clínico, ya que es claro que sólo un número reducido de células neoplásicas lograrán completar todos los eventos biológicos requeridos para la metástasis clínica.

Podemos finalmente señalar que a pesar de los estudios cada vez más profundos y esclarecedores sobre el comportamiento biológico del melanoma, ésta es una neoplasia que continúa incrementándose en incidencia a pesar de diagnósticos más precoces, tratamientos agresivos y estrategias terapéuticas que incluyen algunos de los conceptos nuevos de la biología del melanoma (Kasper., 2007). La descripción de oncogenes, genes supresores, factores de crecimiento, factores angiogénicos, citoquinas y otras moléculas y su rol en la progresión del melanoma maligno ha permitido proveernos de nuevas perspectivas para el desarrollo de estrategias terapéuticas más racionales y biológicas en el control del melanoma que están destinadas a delinear los tipos precisos de perturbaciones moleculares que caracterizan el proceso maligno en sus diferentes etapas de progresión y definir el impacto biológico y bioquímico de estos defectos moleculares en la interacción de mecanismos que regulan y gobiernan la proliferación, diferenciación y relaciones intercelulares de los melanocitos normales. Al igual que en el caso de cáncer de piel no melanoma, estos conocimientos podrán ser usados para mejorar el nivel diagnóstico, desde un punto de vista molecular y genético, mejorando el pronóstico de un caso individual y, finalmente, permitirá un tratamiento más adecuado y efectivo contra esta neoplasía.

El mejor conocimiento de las bases biológicas, moleculares y genéticas de las neoplasias de la piel nos permitirá mejorar nuestra conducta terapéutica lo cual aumentará las posibilidades de supervivencia y curación de nuestros pacientes (Kasper, 2007). En este contexto, y como ya hemos mencionado la terapía génica, desarrollada como una nueva vía terapéutica dirigida hacia dianas específicas de este tipo de tumor abre una nueva posibilidad para mejorar el pronóstico de estos pacientes.

2.- CÁNCER DE PULMÓN

2.1.- CONCEPTO

El cáncer de pulmón (CP) o carcinoma broncogénico, fue considerado una enfermedad rara hasta la segunda mitad del siglo XX (González Barón., 2006). A pesar de esa concepción inicial hoy en día se ha convertido en uno de los mayores problemas de salud en el mundo, suponiendo el 12.4% de los nuevos casos de enfermedades tumorales y el 17.5% de las muertes por cáncer, además de tener una incidencia anual de 1,2 millones de casos y una mortalidad global anual de 1,1 millones (Parkin y cols., 2002), siendo la media de supervivencia inferior al año y la supervivencia relativa a los 5 años menor del 15%. Los avances para tratar de paliar esta situación hoy en día son lentos y poco efectivos.

2.2.- EPIDEMIOLOGÍA

En España se diagnostican unos 18500 casos nuevos de CP al año. En cuanto a la incidencia en varones o mujeres, en España, y en el resto del mundo, se inclina más hacia los varones (11 casos de varones por cada caso de mujer en España), aunque el incremento del consumo de tabaco por parte de las mujeres ha hecho incrementar la mortalidad por CP, empezando a

superar la mortalidad por cáncer de mama en algunos países, hecho que se ha constatado ya en EEUU (Peto y cols., 1992).

2.3.- ETIOLOGÍA Y FACTORES DE RIESGO

Tabaco

El tabaco es el agente etiológico más importante en el desarrollo del CP, estando estrechamente relacionado (López Vivanco, 2005), como demuestran bastantes estudios. Se estima que el tabaco es el responsable del 90% del CP. El riesgo de padecer un CP a lo largo de la vida es entre 20 y 30 veces mayor en un fumador que en un no fumador. Así, aproximadamente el 18% de los fumadores desarrollará esta enfermedad.

Exposición ambiental

Existen agentes documentados como carcinógenos relacionados con la actividad laboral, perteneciendo a este grupo sustancias como radón (Darby y cols., 2005) y asbesto (Coggon y cols., 2003), y otros como arsénico, berilio, cromo, hidrocarburos, níquel y radiación ionizante (Sekido y cols., 2005). El riesgo de CP que supone la exposición a éstas sustancias es difícil de cuantificar porque generalmente se trata de exposiciones prolongadas a bajos niveles de estos agentes, y el intervalo de tiempo entre la exposición y el desarrollo de la enfermedad es variable y a menudo largo. Además existen factores de confusión como la exposición a diferentes sustancias al mismo tiempo, como el tabaco. En general, fumar potencia los efectos carcinógenos de estas sustancias.

Predisposición genética

La mayor parte del CP se atribuye al hábito de fumar, aunque sólo el 20% de los fumadores desarrolla un CP, lo que sugiere que es necesaria una susceptibilidad genética. La familia de genes implicada en la carcinogénesis pulmonar incluye oncogenes, principalmente familia ras, erb1 y 2, TGF alfa; y, genes supresores, fundamentalmente p53, p16 y bcl2 (Sekido y cols., 2005).

Dieta

Hoy en día los estudios entre dieta y CP no dejan clara una relación, aunque parece que concentraciones bajas de vitaminas A y E se asocian al desarrollo de CP, pero los datos son contradictorios. No está claro que el consumo de frutas y verduras disminuya el riesgo de CP (Sekido y cols., 2005).

2.4.- FORMAS CLÍNICO-HISTOLÓGICAS

De entre las distintas clasificaciones nosológicas que existen, la más usada es la de la Organización Mundial de la Salud (OMS) (Travis y cols., 2004). En líneas generales, el CP se divide en dos grandes grupos por sus características histológicas, curso clínico y respuesta al tratamiento: el cáncer de pulmón células pequeñas (CPCP) o microcítico, que representa el 20% del total de cánceres de pulmón; y el cáncer de pulmón células no pequeñas (CPCNP) o no microcítico, que correspondería al 80% restante.

Aunque su origen celular es el mismo, debido a distintos factores estimuladores y diferenciadores celulares autocrinos y paracrinos, constituyen dos entidades con distinto comportamiento biológico y manejo terapéutico. El CP derivaría de una célula pluripotencial, capaz de expresar una variedad de fenotipos (Sekido y cols., 2005; Mariño y cols., 2006).

El CPCNP esta compuesto por diferentes clases de células cancerosas, cada una de las cuales crecen y se diseminan de diferentes maneras. Los tipos de cáncer de pulmón de células no pequeñas se denominan según las clases de células que se encuentran en el cáncer y la apariencia de las células bajo un microscopio (Figura 4):

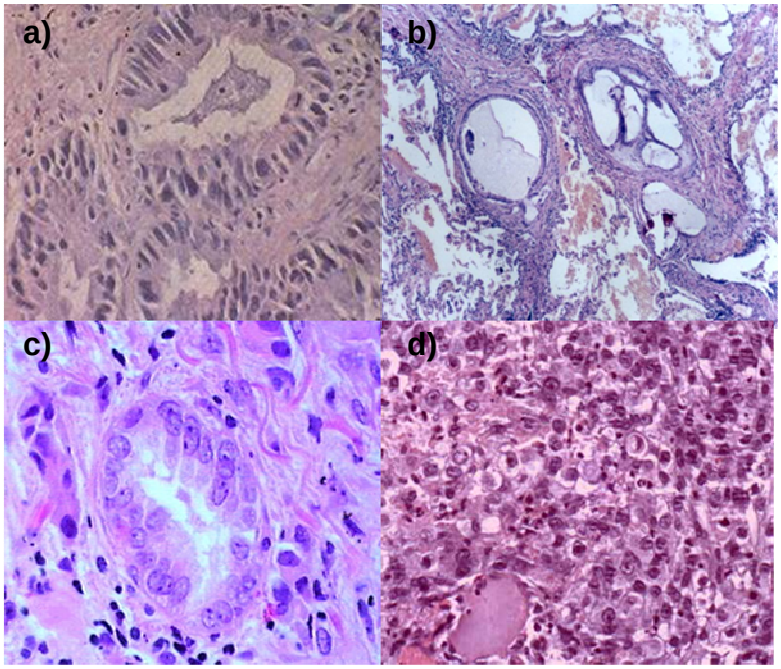

Figura 4. Subtipos histológicos de CPCNP: a) Epidermoide; b) Adenocarcinoma; c) Adenocarcinoma bronquioloalveolar; d) Carcinoma de células grandes.

El carcinoma epidermoide representa aproximadamente el 30% del CP. Es un tumor epitelial maligno con diferenciación escamosa que se caracteriza histológicamente por presencia de puentes intercelulares, perlas córneas y queratinización celular. Es un tumor de crecimiento lento, pudiendo pasar 3-4 años desde carcinoma in situ a tumor que produzca síntomas. Tiende a ser agresivo localmente, y afectar estructuras adyacentes por contigüidad, con menos frecuencia de metástasis a distancia que el adenocarcinoma.

El carcinoma de células grandes es el de menor incidencia, aproximadamente un 15% de los CP. Se caracteriza por ser un tumor muy indiferenciado, con células grandes, con abundante citoplasma y nucleolo

pigmentado. Se puede presentar como un tumor central o periférico. En numerosas ocasiones, se identifican características neuroendocrinas. El carcinoma de células grandes tiene un pronóstico similar al adenocarcinoma.

El adenocarcinoma supone el 30-40% de los CP. Actualmente es la entidad más frecuente. Es un tumor epitelial maligno que se caracteriza histológicamente por formación de glándulas y producción de mucina. Se origina en zonas periféricas, en el epitelio alveolar o glándulas de la submucosa, incluso sobre áreas de fibrosis como cicatrices antiguas. Presenta varios patrones de crecimiento que suelen combinarse, sobre todo en tumores de gran tamaño, lo que se denomina adenocarcinoma mixto.

El CPCNP a pesar de tener un crecimiento más lento y menos agresivo que el CPCP, supone la causa de muerte por cáncer más importante, a través principalmente de diseminación metastásica. La posibilidad de curación está por debajo del 15%, aunque el riesgo de muerte ha disminuido en los últimos años.

El CP permanece silente durante mucho tiempo. En el momento del diagnóstico el 90% de los enfermos presentan algún síntoma y cuando se detecta radiológicamente, se estima que ha completado las tres cuartas partes de su historia natural (Dómine y cols., 2006).

El CP es más frecuente en los lóbulos superiores que en los inferiores, y en el hemitórax derecho que en el izquierdo. Los tumores superiores son más difíciles de localizar en una radiografía de tórax por la superposición de otras estructuras, como arcos costales y clavícula. Las manifestaciones clínicas del CP dependen de muchas variables, entre ellas, el tipo histológico; su localización y afectación regional; las metástasis a distancia; y, los síndromes paraneoplásicos, como la presencia del síndrome constitucional.

Manifestaciones relacionadas con el tumor primario.

Los síntomas locales van a estar relacionados con el crecimiento y la posición del tumor, que puede ser central o periférico

-Tumores centrales (que suelen ser los epidermoides y microcíticos) van a dar síntomas relacionados con la afectación de bronquios principales, lobares o segmentarios proximales consistiendo esta clínica en: tos irritativa, expectoración, disnea obstructiva, neumonitis obstructiva, recidivante o atelectasia, sibilancias y estridor y dolor torácico

-Tumores de crecimiento periférico (adenocarcinomas y carcinomas de células grandes). Su sintomatología es tardía y suele ser secundaria a invasión de estructuras vecinas. Consiste en: dolor pleurítico y dolor costal, disnea restrictiva, derrame pleural y pleuritis, absceso pulmonar y tos.

Manifestaciones relacionadas con la existencia de metástasis

El 40-50% de los pacientes con CP presentan metástasis en el momento del diagnóstico ocurriendo la diseminación por vía linfática, hematógena o interalveolar, siendo las localizaciones más frecuentes son pulmón, glándulas suprarrenales, hígado, cerebro y hueso.

Síndromes paraneoplásicos.

Los síndromes paraneoplásicos son el conjunto de síntomas o signos no atribuidos a la invasión local del tumor ni a sus metástasis, sino que están relacionados con la liberación de sustancias biológicamente activas, con acción hormonal o humoral, por las células tumorales.

El CP es uno de los tumores en los que con más frecuencia se dan síndromes paraneoplásicos (10-20% de los pacientes). Pueden constituir el primer síntoma de esta enfermedad, e incluso pueden ser más graves que las consecuencias del propio tumor. Entre estos síndromes, destaca el síndrome de anorexia-caquexia o síndrome constitucional.

2.5.- CLASIFICIACIÓN POR ESTADIOS

En el CPCNP se puede hablar de cuatro estadios según la clasificación TNM de 1997, establecida según el tamaño y afectación a estructuras vecinas (T); afectación ganglionar (N); y afectación de órganos a distancia (M), aunque durante el 2009 será publicada una nueva. Los estadios serían: I, II, III y IV, dividiéndose los 3 primeros tres primeros a su vez en A y B. (Tabla 1) (figura 5).

TAMAÑO	**T1**	Tumor ≤ 3 cm sin afectación pleural ni del bronquio principal
	T2	Tumor > 3 cm o que afecta al bronquio principal ≥ 2 cm de la carina, a vísceras, a la pleura, o que produce atelectasia lobar
	T3	Tumor con afectación de la pared torácica (incluido los tumores de la cisura superior), del diafragma, pleura mediastínica, pericardio, bronquio principal a < 2 cm de la carina, o que produce atelectasia completa de un pulmón
	T4	Tumor con invasión del mediastino, corazón grandes vasos, tráquea, cuerpos vertebrales, o a la carina o con derrame pleural con células malignas
AFECTACIÓN GANGLIONAR	**N0**	Sin metástasis demostrables en los ganglios linfáticos
	N1	Afectación ganglionar ipsolateral hiliar o peribronquial
	N2	Metástasis en ganglios linfáticos ipsolaterales, mediastínicos o subcarinales
	N3	Metástasis en ganglios linfáticos contralaterales, mediastínicos o subcarinales, en el escaleno ipsolateral o contralateral, o ganglios supra claviculares
METASTASIS	**M0**	Sin metástasis a distancia conocidas
	M1	Con metástasis a distancia

			T	N	M
ESTADIOS	E.I	IA	1	0	0
		IB	2	0	0
	E.II	IIA	1	1	0
		IIB	2	1	0
			3	0	0
	E.III	IIIA	1-2	2	0
			3	0-2	0
		IIIB	1-4	3	0
			4	0-3	0
	E.IV	IV	0-4	0-4	1

Tabla 1. Estadificación del cancer de pulmon en base a la clasificación TNM

2.6.- TRATAMIENTO DEL CÁNCER DE PULMÓN.

El CP es un grave problema sanitario causado en la mayoría de los casos por un hábito, el tabaco. Por tanto sería evitable y posible de erradicar. En la actualidad el tratamiento cura a menos del 15% de los pacientes. Sin embargo, los resultados de la investigación básica y clínica han producido un aumento en la supervivencia y calidad de vida de estos pacientes. Se trata de una enfermedad compleja y heterogénea.

2.6.1.- Estadios IA y IB

En estos estadios en los que la enfermedad aún se considera regional localizada, el tratamiento de elección, previa cuidadosa valoración preoperatoria, es la cirugía. El objetivo de la cirugía curativa es la exéresis de la masa tumoral, dejando bordes de resección negativos y procurando respetar al máximo el parénquima sano circundante. En caso de que la enfermedad sea inoperable por las circunstancias médicas del paciente, o por su rechazo a la cirugía, se debe administrar radioterapia radical (RT) con intención curativa. En

estos estadios tras la cirugía la supervivencia oscila entre el 67% del estadio IA y el 57% del IB.

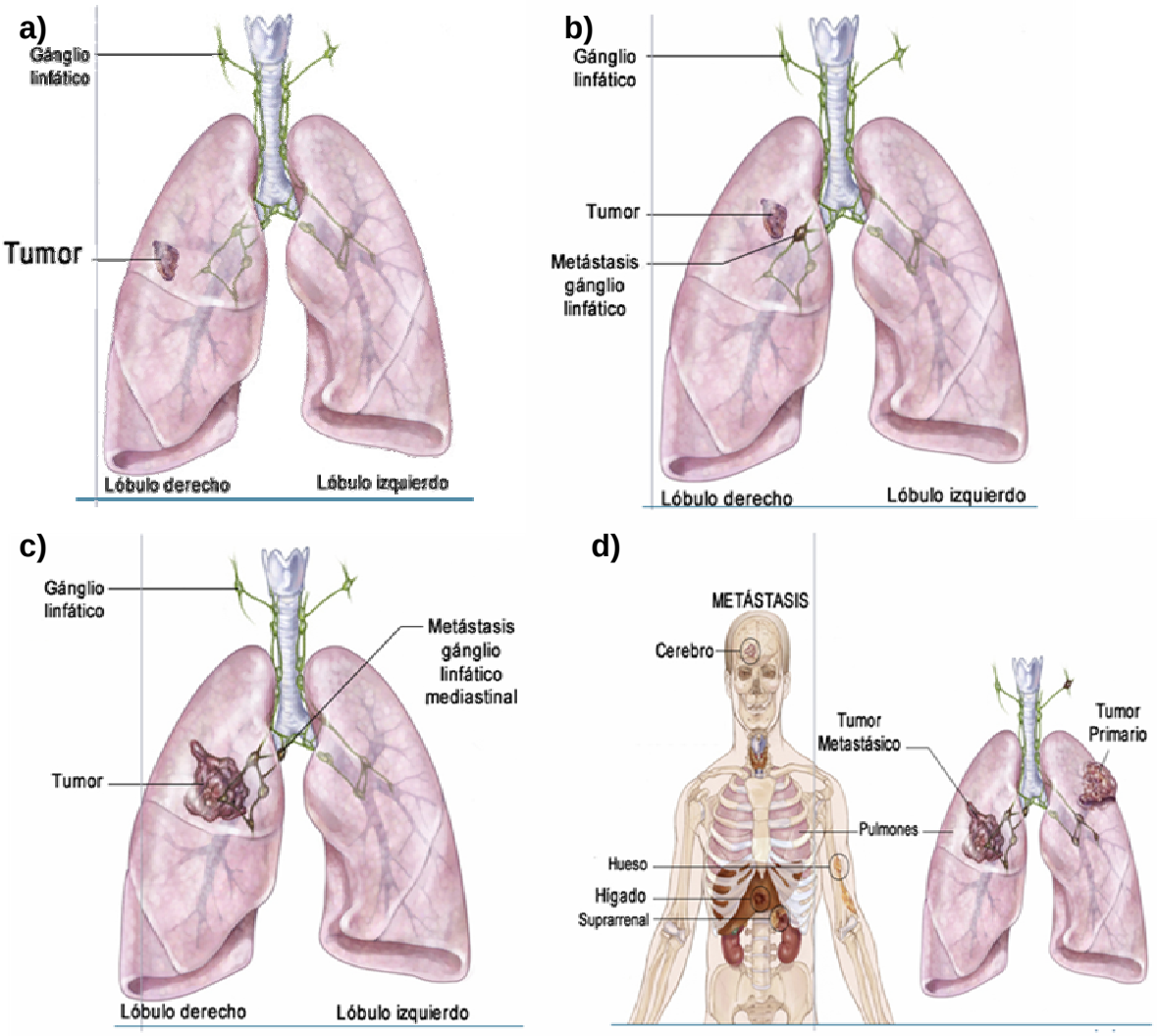

Figura 5. Estadios de cancer de pulmon en base a la localización y metastasis. a)I, b)II,c)III y d) IV

2.6.2.- Estadios IIA y IIB

Al igual que en el caso anterior, el tratamiento ideal es la resección quirúrgica. La radioterapia postoperatoria estaría indicada en pacientes con resección incompleta, porque reduce el riesgo de recidiva local, que suele aparecer durante los 2 primeros años en la mayoría de los pacientes, lo que

hace pensar en un proceso metastásico oculto en el momento del diagnostico (Dômont y cols., 2005).

2.6.3.- Estadio IIIA y IIIB

Dentro de este estadio pueden establecerse varios subgrupos pronósticos para valorar las posibilidades de resección quirúrgica. En los pacientes de pronóstico incierto, médicamente operables, podría plantearse la posibilidad de terapia neoadyuvante para intentar conseguir una reducción local del tumor y metástasis ganglionares, lo que aumentaría las posibilidades de resección quirúrgica y erradicar micrometástasis. Dicho tratamiento puede ser llevado a cabo con radioterapia o quimioterapia concomitante, aunque es esta última la que ofrece unos mejores resultados.

El grupo del estadio III-B representa la enfermedad locorregional avanzada, considerada como irresecable. La recidiva local constituye el principal problema, pudiendo ser del 90 % a los dos años tras radioterapia con intenciones presuntamente curativas siendo el tratamiento combinado quimio-radioterapia más beneficioso que la RT sola. Los regímenes de QT con cisplatino, asociados a RT concomitante, parecen ser más eficaces que otras pautas en el control de las metástasis y la recidiva regional.

2.6.4.- Estadio IV

El objetivo del tratamiento es fundamentalmente paliativo, y serían subsidiarios de quimioterapia.

2.7.- BILOGÍA MOLECULAR EN EL CPCNP

La tumorogénesis es el proceso por el cual los eventos genéticos se acumulan en el tiempo provocando la transformación maligna de la célula. Se

estima que son necesarias 10-20 alteraciones de oncogenes y/o genes supresores para la tumorogénesis del CP. Entre los genes implicados están RAS, p53, gen RB, familia EGFR, MYC, bcl-2, fenotipo mdr.

RAS

La familia de genes ras (H-ras, N-ras y K-ras) es uno de los grupos de oncogenes más frecuentemente alterados en las neoplasias humanas (Aunoble y cols., 2000). Las proteínas codificadas por estos genes se ensamblan entre sí, conformando una estructura proteica con un peso de 21Kd que le otorga el nombre (p21). Poseen actividad GTP-asa, participando en la vía de transducción de señales de crecimiento y diferenciación celular (Ellis y cols., 2000). Las proteínas p21 mutadas se activan constitutivamente y estimulan el crecimiento y la diferenciación de manera autónoma. Aproximadamente del 25 al 48% del CPCNP presenta mutación en RAS, principalmente adenocarcinomas. El 90% de las mutaciones ocurren en K-RAS y en el codón 12. Además conocemos que generalmente los cambios son de Guanina a Timina. Estudios in vitro relacionan la exposición a los carcinógenos del humo del tabaco, como benzo-alfa- pireno y N- nitrosamina, con las mutaciones RAS.

MYC

La familia de oncogenes Myc son oncogenes los cuales son expresados de manera anormal en muchos tipos de cánceres, incluyendo el cáncer del pulmón. La proteína myc actúa como un factor de transcripción del tipo hélice-bucle-hélice-cremallera de leucina (bHLZHZ) para regular la expresión de varios genes. La familia MYC incluye c-MYC, N-MYC y L-MYC. El que con mayor frecuencia está alterado en el CPCNP es el c-MYC, mientras que N-MYC y L-MYC se encuentran alterados en el cáncer microcítico. (Fong y cols., 2003)

Gen p53

El gen P53 es uno de los genes supresores de tumores más importantes y codifica una proteína imprescindible para mantener la integridad genómica. La inactivación de p53 se debe a una delección de una copia, existiendo una mutación en la otra. Alteraciones en este gen probablemente las más citadas en el Cáncer de Pulmón, están presentes en más del 50% de todos los tipos morfológicos. Inactivación de P53 correlaciona con el tabaquismo y confiere mal pronóstico (Rom y cols., 2000)

Gen p16

El gen p16 regula la función del gen RB e inhibe la actividad cinasa CDK4 y CDK6. p16 puede sufrir la pérdida de un alelo o de ambos, pero con frecuencia se inactiva por mutilación aberrante del promotor del gen, de manera que se inactiva su función como gen supresor. En un 30-50% de los CPCNP se produce la pérdida de función de RB (Chen y cols., 2001), por delecciones o mutaciones, que se traducen en una proteína aberrante. Tiene un gran interés teórico como posible marcador biológico (Field y cols., 2002)

Bcl-2

El protooncogen bcl-2 codifica una proteína mitocondrial que aumenta la supervivencia celular inhibiendo la apoptosis. Esta sobrexpresión impide la muerte celular y aparece sobre todo en el CPCP (kim y cols., 2004).

Familia EGFR

EGFR es un miembro de una familia de receptores, incluyendo EGFR (ErbB1), HER-2/neu (ErbB2), HER-3 (erbB3) y HER-4 (ErbB4). La unión del ligando del EGFR va de la mano de la activación de la tirosina kinasa y una

serie de eventos como incremento en la proliferación celular, invasión, bloqueo de la apoptosis y resistencia a la quimioterapia (Janne y cols., 2005). El EGFR está sobreexpresado en un 40-80% de los CP, conllevando que se desarrollasen inhibidores de tirosina kinasa (TKIs), como el gefitinib y luego con erlotinib.

3.- APOPTOSIS

3.1.- CONCEPTO

La apoptosis o "muerte celular programada" es una forma de suicidio celular genéticamente definida, que ocurre de manera fisiológica durante la morfogénesis, la renovación tisular, en la regulación del sistema inmunitario y en el mantenimiento de la homeostasis del organismo (ya que es necesario mantener un equilibrio entre las células que se generan y las que mueren). Fue descrita por primera vez por en 1972 (Kerr y cols., 1972). En este caso las células mueren de una manera morfológicamente distinta a la muerte por necrosis, ya que se producen una serie de cambios secuenciales que no tienen como resultado la pérdida de la integridad de la membrana celular y que además no desencadenan una respuesta inflamatoria, tal y como ocurre en la muerte por necrosis.

Debido al papel esencial que juega en la fisiología del organismo es normal que defectos en la regulación de la apoptosis den lugar a enfermedades como el SIDA, patologías neurodegenerativas, que se dan por un exceso de apoptosis u otras en las que se da por el supuesto contrario, falta de apoptosis, como son malformaciones embrionarias, enfermedades autoinmunes o el cáncer (Cory y Adams., 2002; Cory y cols., 2003). En el caso del cáncer estas alteraciones en la apoptosis posibilitan a las células tumorales el sobrevivir más tiempo, haciéndolas independientes de factores de supervivencia exógenos, de la hipóxia y del estrés oxidativo conforme la masa

tumoral se expande, con lo que ganan tiempo para la acumulación de alteraciones genéticas que desregulan la proliferación celular, interfieren en la diferenciación, promueven angiogénesis, y aumentan la movilidad e invasividad durante la progresión tumoral (Green y Evan., 2002). También defectos en la apoptosis se asocian a fenómenos de quimioresistencia del tumor ya que se incrementa el umbral de supervivencia y por tanto la dosis terapéuticas para eliminar el tumor son más altas (Makin y Hickman., 2000).

3.2.- CAMBIOS MORFOLÓGICOS DURANTE LA APOPTOSIS

Durante el proceso apoptótico se producen una serie de cambios morfológicos y estructurales en la célula (Leist y Jaattela., 2001) (Figura 6), que se caracterizan por:

– Incremento brusco de la densidad citoplasmática. Se produce una dilatación del retículo endoplásmico que forma vesículas que se unen con la membrana citoplásmica liberando su contenido al exterior. También se inhibe el sistema de cotransporte Na+-K+-Cl-, impidiéndose así la pérdida de agua y sodio por las células afectadas.

– Incremento moderado, pero mantenido en el tiempo, de la concentración de calcio libre citoplasmático ($[Ca^{+2}]i$), diferenciándose con la necrosis en que en ésta el incremento es brusco.

– Cambios en la composición de la membrana celular. Translocación de la fosfatidilserina a la cara externa de la membrana celular, permitiendo el reconocimiento y la unión de la célula por parte de los macrófagos, y de ésta manera se evita la liberación del contenido celular y la posible reacción inflamatoria.

– Alteración en la conformación de elementos del citoesqueleto. Aparecen deformaciones celulares, pareciendo que la célula hierve, como resultado de la actividad de las proteasas sobre el citoesqueleto.

– Aumento y activación de la síntesis de determinadas proteínas necesarias en las rutas metabólicas de los procesos de muerte celular.

– Compactación de la cromatina dando lugar a la formación de densos agregados que se deslocalizan para situarse junto a la membrana nuclear.

– Fragmentación de la cromatina por acción de endonucleasas que cortan el ADN en una serie de fragmentos oligonucleosomales de 180pb o múltiplos de éstos, que dan un patrón característico de la apoptosis en los geles de electroforesis.

– Por último, aparecen los cuerpos apoptóticos. Estos son fragmentos de membrana intacta formados por las invaginaciones citoplásmicas y que mantienen sus mitocondrias intactas, y contienen cuerpos densos granulares que corresponden a fragmentos nucleares, los cuales, también se producen por las grandes invaginaciones de la membrana nuclear. Estos cuerpos apoptóticos pueden ser reconocidos por macrófagos y ser fagocitados.

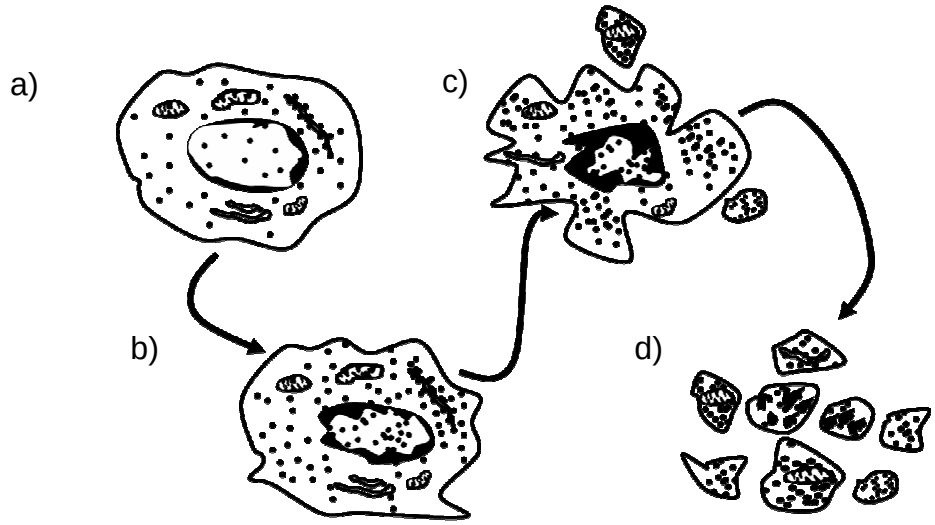

Figura 6. Cambios morfológicos producidos durante la apoptosis: a) Célula normal; b) Compactación celular y condensación de la cromatina; c) Los cambios en el citoesqueleto provocan las marcadas invaginaciones a los cuales se dirigen los organulos, el núcleo comienza a fracturarse; d) Aparecen los cuerpos citoplasmicos portanto en su interior mitocondrias intactas, contenido citoplásmico y nuclear.

3.3.- CASPASAS

Los cambios morfológicos observados en las células apoptóticas descritos anteriormente, son iniciados por la activación de una serie de proteasas denominadas caspasas, (cysteine aspartyl-specific proteases (Thornberry y Lazebnik., 1998).

Las caspasas son una familia de cisteín-proteasas que se encuentran como moléculas precursoras inactivas (procaspasas) y que al recibir la señal apoptótica sufren una ruptura proteolítica dando lugar a dos subunidades que constituyen la enzima activa o caspasa. Las procaspasas constan de un predominio N-terminal y dos subunidades, una grande p20 y otra pequeña p10. La activación supone un corte entre la subunidad larga y la pequeña, y además la eliminación del pre-dominio. Basándose en su función dentro de la cascada apoptótica (Degterev y cols., 2003) las caspasas se pueden clasificar en dos grupos: Las caspasas iniciadoras y las caspasas efectoras o ejecutoras.

- Caspasas iniciadoras: Son las que primero se activan tras un estímulo apoptótico. Poseen prodominios largos que contienen unos motivos de interacción proteína-proteína característicos: dominios efectores de muerte (DED) y dominios de activación y reclutamiento de caspasas (CARD). Por medio de ellos, pueden interaccionar con proteínas adaptadoras que presenten dominios homólogos. Dentro de este grupo se encuentran la caspasa-8, -9, -10, -2, -1, -4 y -5. Las caspasas -1, -4 y -5 forman una subclase dentro de este grupo que están implicadas en apoptosis y además en el control de ciertas respuestas inflamatorias, en concreto, de la maduración de citoquinas.

- Caspasas efectoras: En este grupo se incluyen las caspasas encargadas de cortar múltiples sustratos celulares necesarios para la supervivencia de la célula como proteínas del citoesqueleto (ej. actina, fodrina y plectina), proteínas reguladoras de la reparación del ADN (como la PARP), proteínas del ciclo celular (ej. MDM2), etcétera. A diferencia de las iniciadoras, estas caspasas poseen un prodominio corto y no contienen dominios DED o CARD. Caspasas efectoras son la caspasa-3, -6 y -7. Son normalmente procesadas y activadas por las caspasas iniciadoras.

3.4.- VÍAS DE LA APOPTOSIS

Existen 2 vías clásicas de activación de la apoptosis: la ruta de los receptores de muerte o ruta extrínseca (figura 7), disparada por la activación de los miembros de la superfamilia de receptores del factor de necrosis tumoral (TNF) en la superficie celular, y por otro lado la mitocondrial o ruta intrínseca, inducida por diferentes formas de estrés.

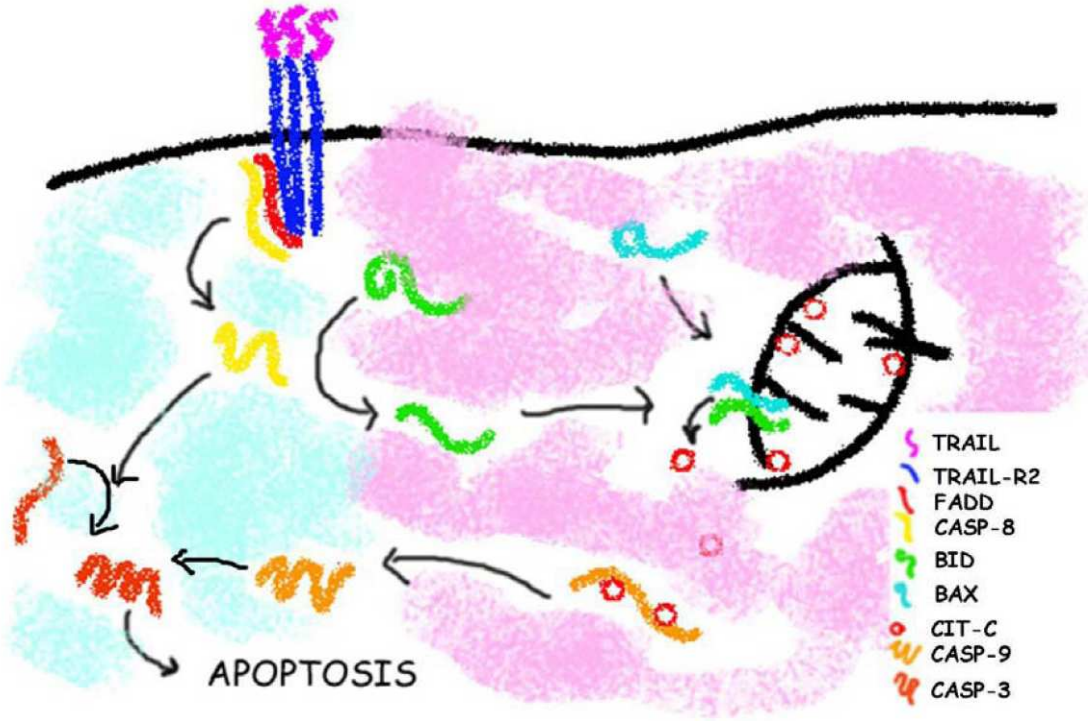

Figura 7. Vías intrínseca y extrínseca de apoptosis: La parte morada definiría la ruta intrínseca que implica a la mitocondria. La parte de citoplasma azul define la ruta exrínseca que implica a los receptores de muerte. (G. Ortiz-Ferron 2005)

3.4.1.- Vía extrínseca

Se inicia a través de la membrana citoplásmica con la unión de una serie de ligandos específicos a lo que se conoce como "Death Receptors" (DR) de la superficie celular. A esta vía se la denomina también vía extrínseca. Los receptores de muerte son proteínas transmembrana tipo I que conectan señales extracelulares inductoras de muerte con la apoptosis intracelular. Pertenecen a la superfamila de receptores del factor de necrosis tumoral (TNF). Se caracterizan porque en la parte intracelular contienen un dominio de interacción con otras proteínas denominado dominio de muerte (DD). Incluye a Fas (CD95), al TNFR1, a DR-3 y a los receptores TRAIL (DR-4 y DR-5). Los ligandos activadores de estos DR están estructuralmente relacionados entre sí,

perteneciendo todos ellos a la superfamilia de genes TNF. Cuando Fas une su ligando (FasL) ocurren fundamentalmente tres eventos: trimerización, reclutamiento y activación.

La unión de FasL a Fas induce la trimerización de Fas (un solo FasL une tres Fas). Fas a través de su región citoplasmática DD, recluta una serie de moléculas adaptadora que también poseen DD, como son FADD y RIP (Schmitz y cols., 2000). La FADD ("Fas Asociating protein with Death Domain") contiene dos dominios importantes, que son el dominio DD, con el cual interacciona Fas, y el dominio DED (dominio efector de muerte), que es el encargado de transmitir la señal apoptótica a los efectores, es decir a la procaspasa 8 y/o la procaspasa 10. El reclutamiento se realiza gracias a que la procaspasa 8 tiene dos DED en su N-ter a través de los cuales se une al DED de FADD. Justo después de su reclutamiento se produce el procesamiento proteolítico de la procaspasa 8 generándose los dos fragmentos catalíticos que forman la caspasa 8 activa. La caspasa 8 activa actuaría también sobre la mitocondria permitiendo la liberación del citocromo c, interaccionando así con la vía intrínseca.

3.4.2.- Vía intrínseca

En esta vía la mitocondria juega un papel fundamental, ya que numerosas moléculas señalizadoras de la apoptosis, así como estímulos patológicos, convergen en ella provocando la permeabilización de la membrana mitocondrial externa (PMME), que conlleva la liberación de una serie de moléculas que se encuentran en el espacio intermembrana y que juegan un papel importante en la apoptosis, como por ejemplo el citocromo c. Antes, durante o después de esta permeabilización se produce la disipación del potencial de membrana mitocondrial interno. El proceso que lleva a la PMME es aún controvertido, postulándose 2 mecanismos: uno en el que participa la membrana mitocondrial interna, a través de la formación de un poro

de membrana (poro de permeabilidad transitoria) permitiendo el paso de agua y moléculas de hasta aproximadamente 1.5kD. Este poro conduce a la pérdida del potencial de membrana ya que a través de esta membrana se produce el equilibrio iónico, y al hinchamiento de la matriz mitocondrial debido a la entrada de agua, llevando, en caso de un hinchamiento suficiente, a la ruptura de la membrana mitocondrial externa y por tanto dando lugar a la PMME (Kokoszka y cols., 2004).

En el otro mecanismo no intervendría directamente ni el poro PT ni la membrana mitocondrial interna. Dicho mecanismo parece estar mediado por proteínas reguladoras de la apoptosis pertenecientes a la familia de Bcl-2 y que actúan directamente en la membrana mitocondrial externa formando poros o canales proteicos que darían lugar a PMME (Green y Kroemer., 2004). Una vez producido el PMME, se produciría la liberación de citocromo c. Una vez liberado al citosol, el citocromo c se une a la proteína Apaf-1, permitiendo la unión del nucleótido dATP o ATP. La unión de este nucleótido al complejo Apaf-1 – citocromo c provoca su oligomerización para formar el llamado apoptosoma, el cual recluta a la procaspasa-9. La unión de la procaspasa-9 al apoptosoma forma la holoenzima caspasa- 9 que es capaz de cortar y activar caspasas efectoras, como la caspasa-3. Por tanto, la liberación del citocromo c de la mitocondria lleva a la célula a la muerte bien rápidamente por apoptosis mediante un mecanismo que implica la activación de caspasas por medio de Apaf-1, como acabamos de describir, o bien lentamente por un proceso de necrosis debido al colapso de la cadena de transporte de electrones, lo cual ocurre cuando el citocromo c es eliminado de la mitocondria, dando como resultado una serie de secuelas incluyendo la generación de radicales libres de oxígeno y un descenso en la producción de ATP (Van Loo y cols., 2002).

Resumiendo lo anterior, existen 2 vías de activación de la apoptosis: 1. cuando los receptores de muerte son reconocidos por su ligando específico, o en su defecto se presenta una supresión de los factores de crecimiento u

hormonales y 2. activación de la vía mitocondrial por daño al citosol o núcleo, los ejemplos más estudiados son la radiación y compuestos químicos que dañan al ADN, dando lugar a que el gen supresor p53 active la transcripción de varios genes, entre ellos los proapoptóticos de la familia Bcl-2: Bax, Bak, Bid, Bad.

Tanto la vía extrínseca como la intrínseca dan lugar a la activación de las caspasas efectoras por parte de las activadoras. Entre sus acciones hay que destacar:

• Inactivación de proteínas del citoesqueleto: laminina, actina, gelsolina, vicentina etc.

• Sobre las proteínas asociadas a DNA como PARP (Poli ADP-ribosa polimerasa), ICAD y DFF etc.

• Activación por proteolisis de Bid, que activado por caspasa-8, se transloca a la mitocondria para cooperar con Bax y Bak provocando la liberación de moléculas apoptóticas mitocondriales.

• La caspasa-3 es capaz de cortar a Bcl-2, eliminando su dominio intermembrana y dejando siempre expuesto su dominio BH3, lo que la convierte en una molécula proapoptótica al igual que Bax .

• Inactivación de NF-KB, lo que bloquea la transcripción de genes de supervivencia, mediante el corte en la zona de regulación de su inhibidor IkB y también por corte directo de P65.

• Estabilización de p53 por corte de la región inactivadora de MDM2 (inhibidor de p53) lo que facilita la acción de P53.

• Inactivación de Rb con lo que E2F está activo.

• El corte de MEKK-1 (quinasa activada por mitogenos/ERKquinasa) provoca un cambio en su acción (principalmente es de supervivencia) ante respuestas de estrés, al ser cortada promueve la apoptosis.

Todas estas actuaciones van a desencadenar el fenómeno de apoptosis celular.

4.-TERAPIA GÉNICA

4.1.-CONCEPTO DE TERAPIA GÉNICA

Uno de los posibles conceptos de terapia génica es el siguiente: “Estrategia terapéutica basada en la modificación del repertorio genético de células somáticas mediante la administración de ácidos nucleicos y destinada a curar tanto enfermedades de origen hereditario como adquirido”.

La terapia génica engloba un amplio rango de posibilidades que no pueden ser incluidas en una descripción tan general. Actualmente el término terapia génica se ha visto "aumentado" hasta englobar las transferencias génicas de naturaleza preventiva y aquellas que contribuyen al avance de la investigación médica.

4.2.-APLICACIONES DE LA TERAPIA GÉNICA

La terapia génica al involucrar la manipulación genética del organismo humano podría ser utilizada, en principio, en cualquier enfermedad que haya surgido por la modificación de un factor genético, ya sea de tipo heredado, como las enfermedades monogénicas con patrón de herencia mendeliano tales

como la hipercolesterolemia familiar, en la que el fallo en el receptor del LDL es corregido mediante terapia génica ex vivo mediante la transducción con retrovirus que contienen la copia correcta para el receptor del LDL de los hepatocitos del paciente (Huy y cols., 2009); el caso de la fibrosis quística se debe a la mutación en el gen CFTR (cystic fibrosis transmembrane conductance regulador), por tanto las terapias van orientadas a corrección de este gen. Actualmente se está usando aerosoles que contienen liposomas o vectores virales con el gen CFTR y que llegan al epitelio respiratorio (Figueredo y cols., 2007); otra enfermedad es la hemofilia A, que se debe a una deficiencia en el factor VIII, muy importante para los procesos de coagulación, y que se está tratando en ratones mediante la reimplantación de fibroblastos modificados genéticamente para sobreexpresar este factor VIII (Viiala y cols., 2009).

También es útil para enfermedades con herencia multifactorial, en las que hay una influencia de los genes y el ambiente, como la diabetes tipo I en la que la terapia génica trata de modificar la respuesta anómala del sistema inmunitario y de incrementar el número de células que secreten insulina (D'Anneo y cols., 2009); enfermedades coronarias, en las cuales se busca una mejora de los tejidos tras el infarto (White y cols., 2007), etc. También puede ser importante en enfermedades genéticas de tipo adquirido como es el cáncer en el que la terapia génica tiene como objetivo la destrucción de las células tumorales, o como el SIDA, en el que se extraen células del paciente y, por inyección directa o por vectores virales, se inserta el gen terapéutico (Lanao y cols., 2007) (como genes inhibidores de la replicación viral en monocitos y linfocitos T con el objetivo de inhibir la producción del VIH; oligonucleótidos antisentido complementarios en secuencia génica al VIH, Uso de ribozimas para que uniéndose a secuencias complementarias del RNA diana puedan romperlo con su actividad ribonucleasa, etc.). Las células que han incorporado y expresado el transgén son reintroducidas al paciente por vía intravenosa. También podría utilizarse en el mejoramiento de los procesos de curación y

regeneración tisular, y en el tratamiento de enfermedades neurológicas degenerativas como la enfermedad de Parkinson y de Alzheimer, existiendo en esta última un ensayo en fase I (Tuszynski y cols., 2005) donde se emplean fibroblastos autólogos genéticamente modificados de ocho individuos con la enfermedad de Alzheimer en un grado leve, para que expresen NGF (nerve growth factor) humano. Estos fibroblastos manipulados genéticamente son inyectados en la zona del telencéfalo y el diencéfalo para actuar como fuentes del factor de crecimiento. Durante el proceso se observa una mejoría del declive cognitivo y no se aprecian reacciones adversas.

A pesar de lo excesivo de esta lista, que haría sospechar de la terapia génica como una panacea, en cada uno de los grupos mencionados hay numerosos experimentos en animales (fase preclínica) y estudios clínicos que arrojan resultados prometedores, anunciando así una verdadera era de la terapéutica.

4.3.- ESTRATEGIAS EN TERAPIA GÉNICA

La terapia génica presenta tres componentes indisociables y necesarios: el primero, un gen de interés, del cual se espera que la expresión en una célula normal se acompañe de un efecto terapéutico, el segundo lo constituye la célula diana, sobre la cual hay que realizar la modificación y el tercero es el vector, vehículo que transporta el material genético y permite su introducción en la célula diana. Los diferentes modelos experimentales sobre los que se lleva a cabo la manipulación genética se clasifican en transferencia génica ex vivo (Figura 8), in vivo (Figura 9) dependiendo del tipo de muestra y del método utilizado.

-Transferencia génica ex vivo: se realiza la corrección del defecto genético en células extraídas del propio paciente que son cultivadas y modificadas genéticamente en el laboratorio y que, al dividirse, transmiten el

transgén a sus células hijas, devolviéndose al paciente sólo aquellas poblaciones celulares en las que se ha comprobado la integración y funcionamiento correcto del transgén. Este procedimiento se utiliza cuando las células diana son células o tejidos que presentan la capacidad de renovarse a partir de células precursoras, como es el caso de la piel, los endotelios, el hígado o los mioblastos musculares. Ejemplos de esta estrategia están representados por la modificación de linfocitos T en el tratamiento de la deficiencia en adenosina desaminasa de hepatocitos en la hipercolesterolemia familiar y de linfocitos infiltrados de tumores en algunas enfermedades neoplásicas.

-Transferencia génica in vivo: se inocula al paciente directamente con el vector y los genes a transfectar alcanzan las células diana a través del torrente circulatorio, utilizándose este método para el tratamiento de enfermedades tales como la fibrosis quística y algunas neoplasias.

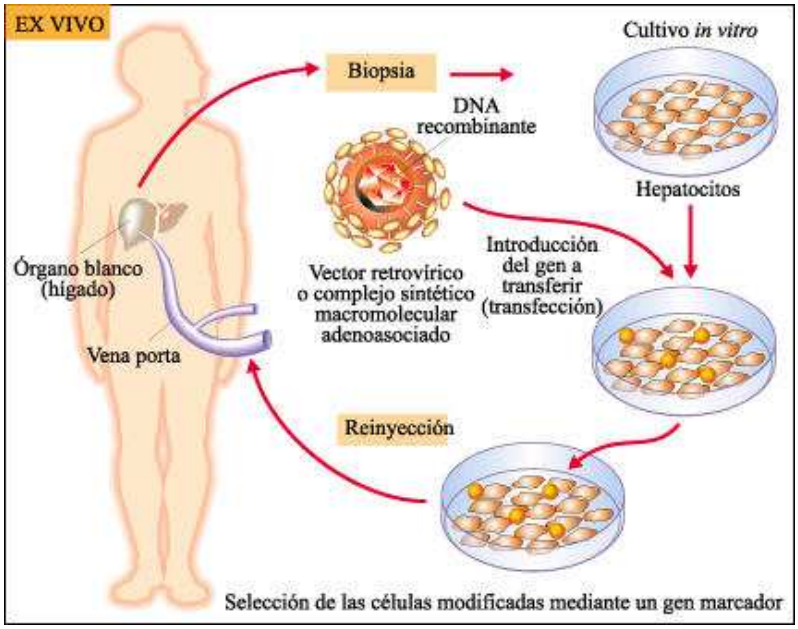

Figura 8. Representación de la terapia génica ex vivo

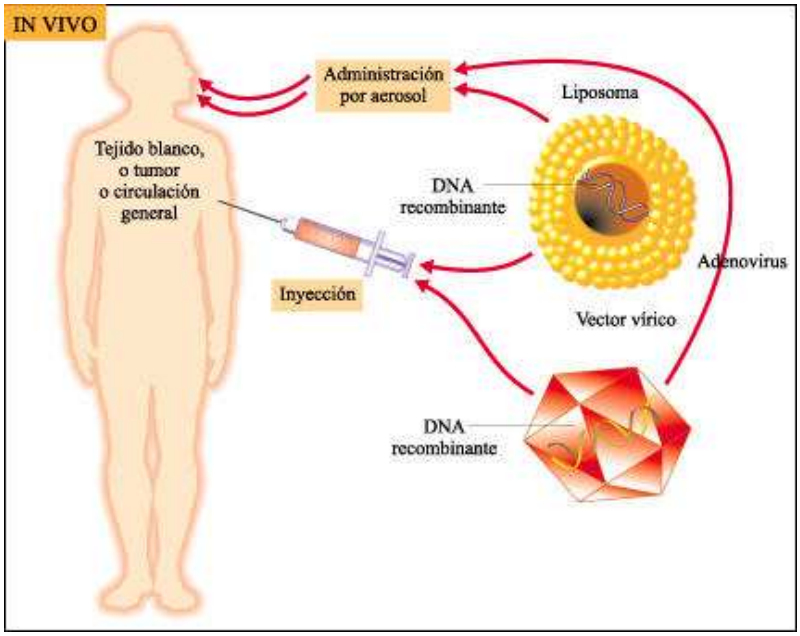

Figura 9. Representación de la terapia génica in vivo

4.4.- SISTEMAS DE TRANSFERENCIA

Los genes usados en la terapia génica pueden ser llevados a la célula por medio de los llamados vehículos o "vectores", denominados así por su similitud con los agentes biológicos que transmiten enfermedades. El término vector anteriormente se utilizaba sólo para designar a los plásmidos, a los virus modificados que se utilizaban como vehículos que transportaban el gen deseado a una célula. El mismo ha pasado a ser un término genérico que involucra los diversos medios, ya sean biológicos, químicos o físicos, por medio de los cuales se puede hacer llegar el gen a la célula. Es por eso que hablamos de métodos de transferencia o vectores no virales y virales.

4.4.1.-Vectores no virales

Los vectores no virales engloban aquellas técnicas de transducción donde el material genético es introducido utilizando tanto métodos químicos (fosfato cálcico, liposomas) como físicos (biobalística, electroporación, microinyección). Los vectores no virales sintéticos se han desarrollado como una alternativa para superar muchos de los problemas de seguridad asociados a los vectores virales.

4.4.1.1. Químicos:

-La utilización del fosfato de calcio se basa en la capacidad que presentan los iones calcio para precipitar el ADN provocando que la célula, mediante endocitosis, introduzca el ADN en su interior.

-Los liposomas constituyen bolsas rodeadas de una membrana lipídica, a semejanza de una célula eucariota animal, capaces de introducir ADN en la célula diana (Figura 10). Los liposomas catiónicos interaccionan tanto con el material genético a transferir como con las membranas celulares que deben atravesar, debido a la presencia de cargas netas negativas originadas por los grupos fosfato en el ADN y por residuos del ácido siálico de la superficie celular. Así, los lípidos catiónicos condensan el ADN y, ya en forma de complejos transfectantes, se unen a las proteínas azucaradas de la membrana plasmática mediante enlaces electrostáticos. Los complejos transfectantes con carga neta positiva utilizan las proteínas azucaradas de la membrana plasmática para fijarse en la célula.

Los lípidos catiónicos interaccionan con las cargas negativas del ADN condensándolo, mientras que los transportadores catiónicos interaccionan con las cargas negativas que presenta la membrana celular gracias al exceso de cargas positivas, mediante enlaces electrostáticos. Una condensación

controlada del ADN permite la formación de partículas, con un diámetro de 50-150 nanómetros, que contienen una sola molécula de ADN, hecho que facilita notablemente la penetración en las células y posteriormente en el núcleo. Se produce la captura del 100% de los complejos estables de polinucleótidos por atracción de cargas y los lípidos se absorben a la membrana celular debido a propiedades de fusión, liberando el ácido nucleico directamente dentro del citoplasma. De esta manera se evita la degradación lisosomal (Merdan y cols., 2002).

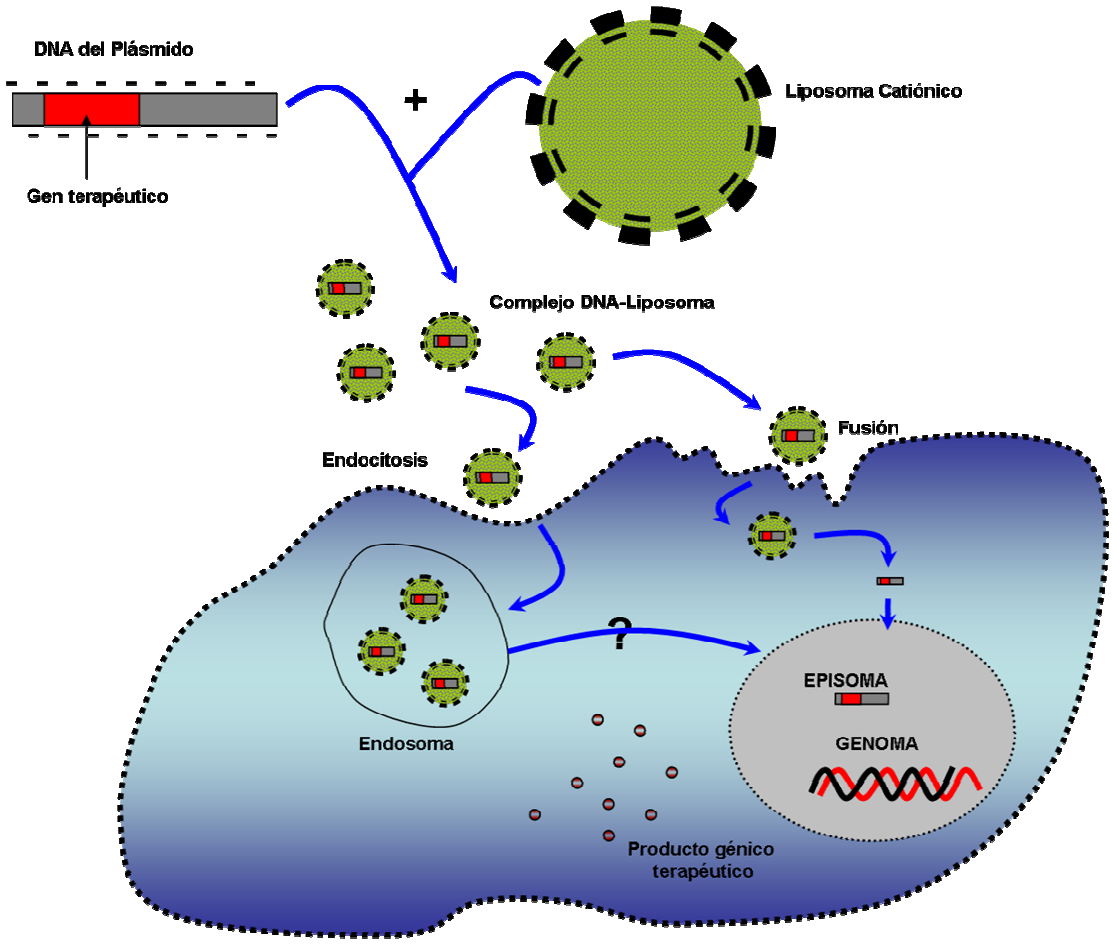

Figura 10. Representación de transferencia génica mediante liposomas

Entre los liposomas destacan el DMRIE (dimiristiloxi-propil-3- dimetil-hidroxietilamonio/DOPE), o las combinaciones DMRIE/DOPE y DC-chol/DOPE.

En cultivos de células eucariotas se utiliza con frecuencia la lipofectamina, un liposoma de formulación 3:1 (w/w) del lípido policatiónico 2,3-dioleiloxi- N-[2(sperminecarboxamido)etil]- N,N-dimetil-1-propanaminio trifluoroacetato (DOSPA) y el lípido neutro dioleoil fosfatidil etanolamina (DOPE) en agua (Tros de Ilarduya y cols., 2002) Sin embargo, los resultados de la utilización in vivo de los lipoplejos no son muy alentadores hasta el momento, ya que su efectividad in vivo es muy baja, sobre todo cuando se compara con la de los vectores virales.

Parece ser que la escasa eficacia de los lipoplejos in vivo es debida a la interacción de éstos con las proteínas del suero (Li y Tseng., 1999), que induce su agregación y la activación del sistema del complemento, que conduce ala eliminación de los lipocomplejos antes de que puedan alcanzar las células diana. Los liposomas suelen ser administrados por vía intravenosa, con la ayuda de catéteres, presentando muy buenos resultados en las células de pulmón e hígado, quizás debido a que los complejos transfectantes se unen a partículas de gran tamaño, lo que impide que puedan abandonar eficazmente el sistema vascular, siendo retenidas mecánicamente por filtros naturales, como los pulmones y el hígado.

4.4.1.2.- Físicos

- Disparo de partículas, biolística o bombardeo de microproyectiles. En él, el plásmido de ADN a transferir es situado sobre la superficie de pequeñas gotas de 1 a 3 micras de diámetro de oro o tungsteno que posteriormente son aceleradas, «disparadas» bien mediante una descarga eléctrica o por un pulso de gas, hacia la célula diana.

- Microinyección, en el que el ADN es introducido por inyección directamente en el núcleo de las células gracias a la ayuda de un

micromanipulador, evitando de este modo la degradación citoplasmática y lisosomal.

- Electroporación: La aplicación de una corriente eléctrica a células es capaz de abrir poros en la membrana celular que permiten la entrada del gen en su interior.

4.4.2.-Vectores virales

Hoy en día los virus empiezan a ser considerados como herramientas de trabajo, vectores, que sirven para introducir material genético con fines terapéuticos en las células diana. Los virus no pueden ser utilizados directamente como se encuentran en la naturaleza, necesitan ser modificados para poder ser utilizados como vectores. Es necesario convertirlos en entes deficientes en replicación en el interior de la célula diana. Se trata, por tanto, de producir una anulación parcial del genoma viral. Frecuentemente se retiran regiones codificadoras de algunas proteínas estructurales como gag, pol y env en los vectores retrovirales, o de los elementos E1 en adenovirus, que son remplazadas por el gen o genes de interés. Estas partículas son incapaces de replicarse, pero mantienen la capacidad de infectar. Así, el vector viral sólo podrá multiplicarse o crecer en cultivos de líneas celulares modificadas genéticamente que sobreexpresan la parte genoma viral que ha sido delecionado; a estas células se las conoce como «células de empaquetamiento». La cantidad de ADN que puede ser insertado en un vector viral varía dependiendo del tipo. Con todo, los vectores virales presentan el inconveniente de que, junto a la información genética que pretendemos transducir, se encuentra el material genético propio del virus y que, en algunos casos, al igual que los adenovirus, pueden resultar inmunogenéticas.

a) Retrovirus: fueron la estrategia pionera en la terapia génica, en las técnicas ex vivo. Sin embargo, los niveles de expresión en una variedad de

tipos celulares eran considerablemente mayores en cultivo que después de ser trasplantados, presentando además una expresión transitoria en las células transducidas in vivo. Los retrovirus comprenden una gran clase de virus desarrollados que contienen ARN de cadena sencilla como genoma viral.

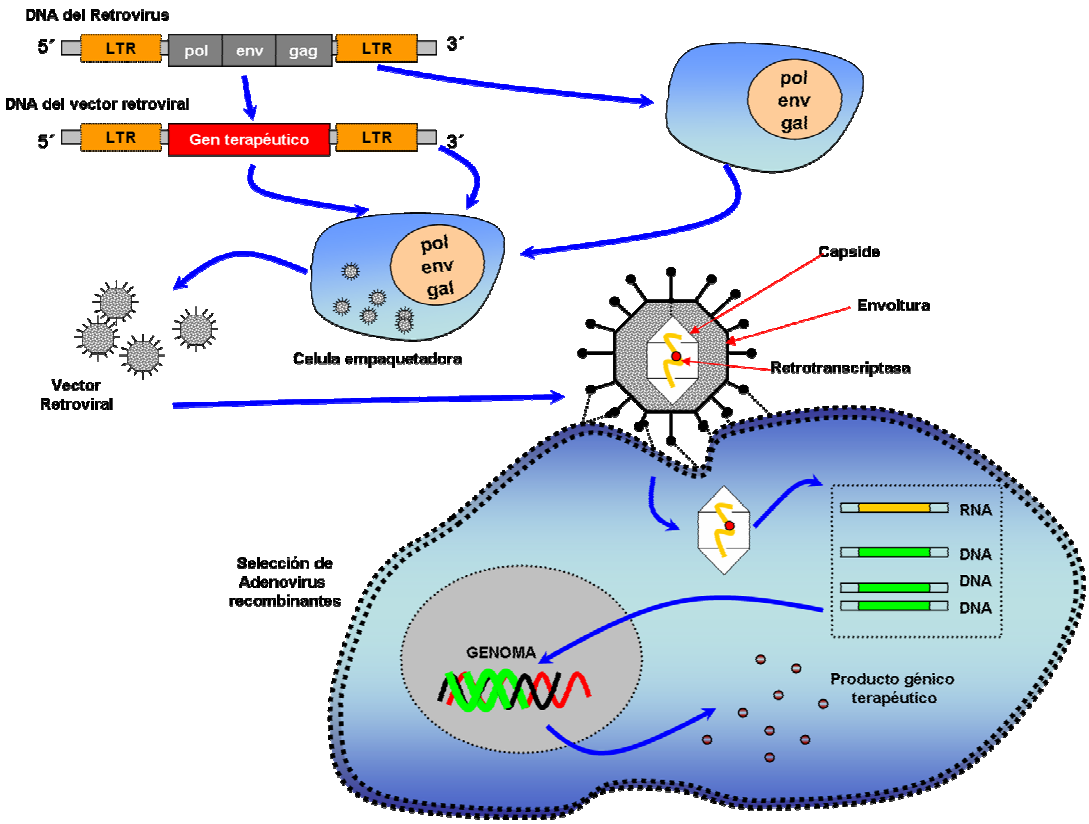

Figura 11. Representación de transferencia génica mediante retrovirus

Durante el ciclo de vida vírico normal, el ARN vírico se transcribe a la inversa para producir ADN de cadena doble (gracias a la acción del enzima reversotranscriptasa) que se integra en el genoma de la célula hospedadora y se expresa en períodos prolongados (Figura 11). Como resultado, las células infectadas vierten virus de forma constante sin daño aparente en la célula hospedadora. El genoma viral es pequeño (aproximadamente 10 kilobases).

Las formas deficientes en replicación de estos vectores se obtienen remplazando las regiones codificadoras para las proteínas estructurales gag, pol y env por el gen o genes de interés, lo que permite la incorporación de cADN de hasta 8 kb.

b) Adenovirus: son virus no envueltos de doble cadena de ADN. Son deficientes en replicación y requieren de un sistema de complementación que es la línea celular HEK293 (Human Embryonic Kidney 293) modificada para que produzca constitutivamente los elementos E1 virales, que son suprimidos en el vector adenoviral (Figura 12).

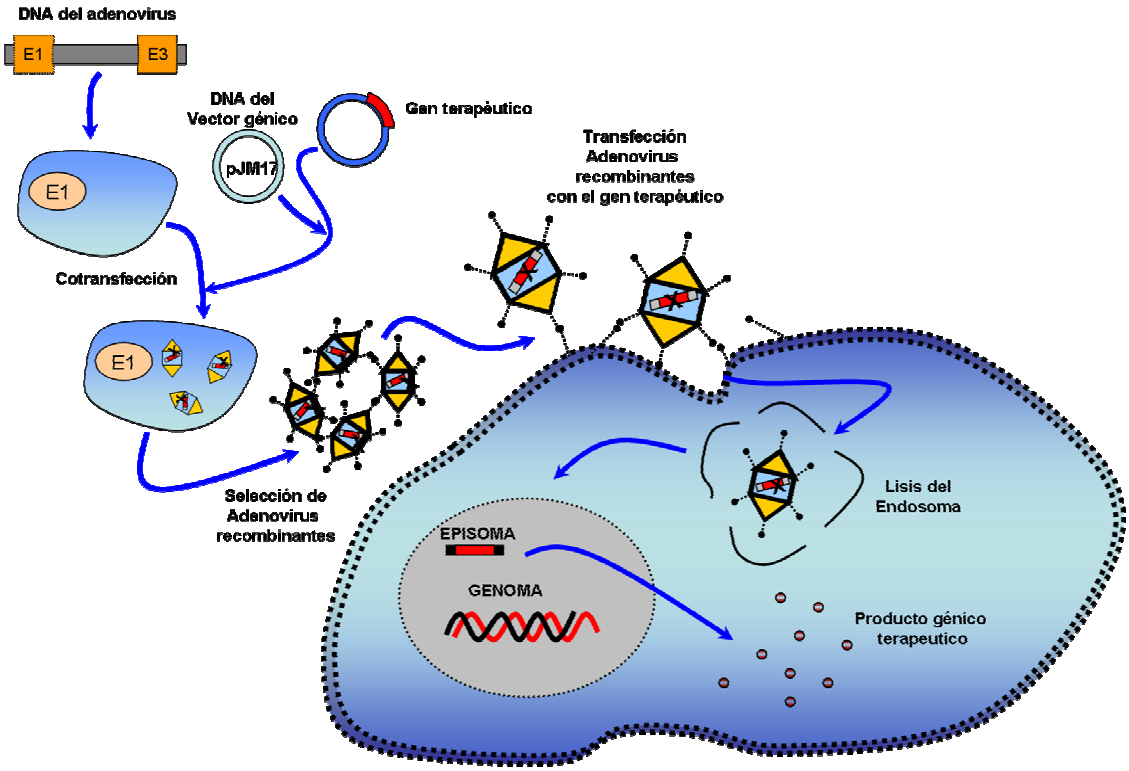

Figura 12. Representación de transferencia génica mediante adenovirus

Entre las ventajas de este vector viral destacamos que pueden insertar hasta 20 kb de gen, además de transducir células con gran número de partículas virales y de infectar células tanto en reposo como en división. Sin

embargo, presentan el inconveniente de expresar varias proteínas virales que resultan inmunogenéticas, lo que conduce a una separación más rápida del vector y las células transducidas.

c) Virus asociados a los adenovirus: son capaces de integrar de forma específica en el cromosoma 19 humano. Sin embargo, éstos se han visto relegados a un plano más discreto cuando se observó que esta integración específica no ocurría con los vectores derivados de estos virus, el tamaño del gen a transducir es bastante limitado (menos de 4 kb), y sólo transducirían células en presencia de un adenovirus.

d) Herpesvirus: virus que presenta un material genético compuesto por ADN bicatenario lineal de 100 a 250 Kb. En el virus del herpes simplex ronda las 50 Kb. mientras que en citomegalovirus las 220 Kb. El potencial de estos virus como vectores génicos recae en la habilidad de llevar grandes secuencias de ADN extraño insertadas y su habilidad para establecer infecciones latentes de larga duración en las cuales el genoma del virus existe como un episoma con efectos no aparentes en la célula hospedadora.

4.5.-TERAPIA GÉNICA CONTRA EL CÁNCER: ESTRATEGIAS

En la terapia génica contra el cáncer se requiere de una población celular blanco, la cual puede estar constituida por las células tumorales mismas, o bien, por células efectoras específicas del huésped con actividad antitumoral. La terapia génica ofrece el potencial de inducir la regresión tumoral con un posible resultado clínico curativo y menos efectos colaterales que otros tratamientos como la quimioterapia.

La terapia génica antitumoral puede ofrecer el potencial para llevar a cabo un nivel mucho más alto de especificidad de acción que la terapéutica de

los medicamentos convencionales, gracias al control fino de los mecanismos reguladores y la especificidad de la expresión génica.

La elección de la estrategia de terapia génica antineoplásica depende del método que se desea emplear para destruir a las células tumorales. Según el método seleccionado, las células malignas pueden ser reconvertidas a un fenotipo no maligno, o pueden ser eliminadas por el sistema inmune, o por inhibición de la neovascularización tumoral, o por efectos tóxicos directos. Estos métodos se explicarán a continuación.

Inmunoterapia. Esta estrategia involucra la activación de la respuesta inmune contra las células tumorales in vivo. En esta modalidad, las células que se han modificado ex vivo con genes que codifican para citocinas, antígenos o moléculas alogénicas del complejo mayor de histocompatibilidad, potencian la inmunogenicidad del tumor (Figura 13). Otra opción ha sido inmunizar activamente con antígenos asociados a tumor que han sido clonados (Chaudhuri y cols., 2009).

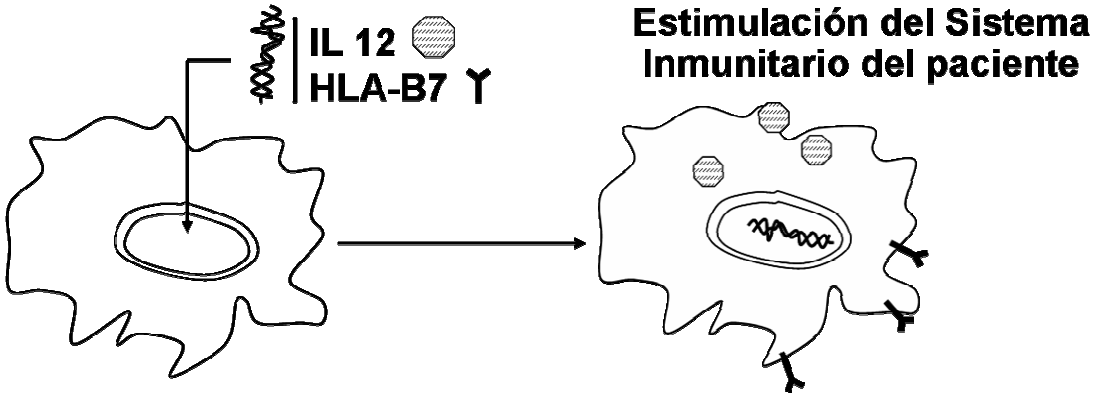

Figura 13. Inmunoterapia frente al cancer

Citoxicidad condicionada. Esta modalidad también se conoce como terapia génica suicida y consiste en la administración de genes tóxicos para

eliminar células tumorales (figura 14). Estos genes codifican para enzimas que convierten a una prodroga inocua en un agente tóxico. Los genes representantes de este grupo son los de timidina quinasa del virus herpes simplex I (HSV-tk) (Cascante y cols., 2005), citosina desaminasa (CD) (Izumi y cols., 2005), que convierte a la 5-fluorocitosina (F-FC) en 5-fluorouracilo (5-FU), desoxicitidina quinasa que convierte la citarabina en citarabina 5' fosfato, nitrorreductasa que activa la 5-(aziridin-1 yl)-2,4-dinitrobenzamida (CB1954), y carboxipeptidasa G2 que activa el 4-[(2cloroetil) (2mesiloxietil) amino] benzoil-L-ácido gutámico (CDMA) (Altaner C, 2008).

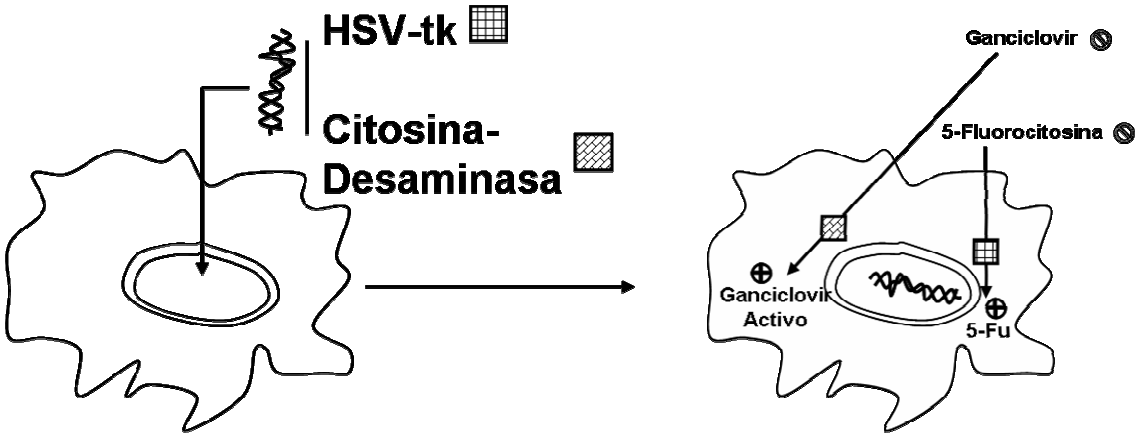

Figura 14. Ejemplo de terapia genica con genes suicidas

En el primer caso, el gen codifica para la timidina quinasa (TK), que convierte a la prodroga ganciclovir (GCV) en la forma activa de GCV-monofosfato, el cual posteriormente es convertido a la forma trifosfato por las nucleótido-quinasas celulares, transformando a la prodroga en una análogo anormal de los nucleótidos de tipo purina. El compuesto trisfosfatado se incorpora en las cadenas crecientes generadas durante la síntesis del DNA en las células cancerosas y bloquea su reproducción. Adicionalmente, el compuesto trifosfatado se puede explotar a las células tumorales adyacentes que no recibieron el gen tk, eliminándolas por el mismo mecanismo.

Este efecto se conoce como "bystander" (Freeman y cols., 1993). Finalmente, también se ha descrito que este método es capaz de generar una respuesta celular inmune antitumoral, la cual, incluso, ha demostrado ser eficiente en contra de metástasis a distancia en modelos murinos. Actualmente este sistema se está empleando bastante, tratando de mejorar el sistema de la TK mejorando su transducción (Matono y cols., 2003) o combinando con otros sistemas citotóxicos como es la lisis adenoviral (Zheng y cols., 2009).

Corrección fenotípica o compensación de mutaciones. Consiste en la adición de genes y en el uso de moléculas antisentido, para anular el fenotipo maligno. Esto se logra mediante la sobrexpresión de genes supresores de tumor o la inactivación de oncogenes involucrados en la progresión tumoral, como es la regulación de expresión de p53 (Halaby y Yang., 2007). Esta corrección difícilmente es curativa, pues con los métodos actuales es imposible introducir el gen en todas las células malignas que constituyen una masa tumoral (Figura 15).

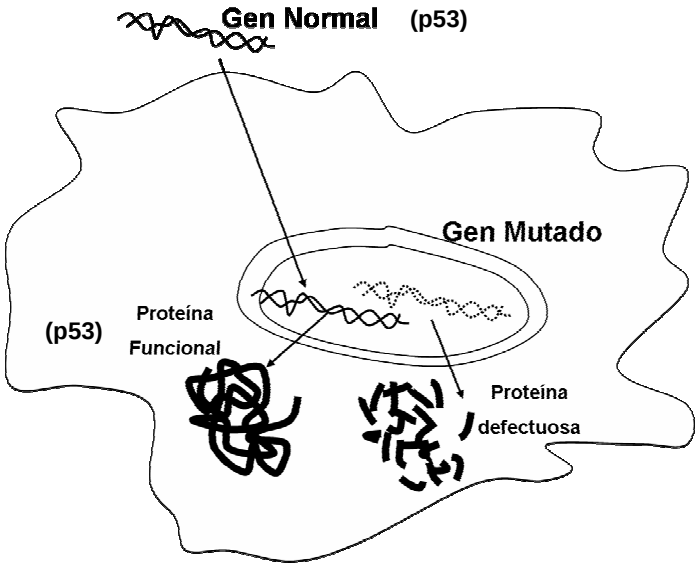

Figura 15. Corrección fenotipica de p53 en tumores

Inhibición de la neoangiogénesis tumoral. Se están estudiando moléculas y mecanismos que bloquearían la perfusión sanguínea de la neoplasia y provocarían una inhibición persistente del crecimiento tumoral. La tarea es facilitada por el hecho que la neovascularización tumoral es muy particular y completamente diferente de la vascularización de los tejidos sanos, lo cual ha sido observado en diferentes estudios microscópicos y moleculares. Por estos motivos, los tratamientos para alterar la neovascularización tumoral tendrían un efecto localizado y probablemente no afectarían a los tejidos normales (Figura 16). Se suele usar para este cometido la tecnología del RNAi o del antisenso, usando como diana principal el VEGF (Kunze y cols., 2008. Zhou y cols., 2009).

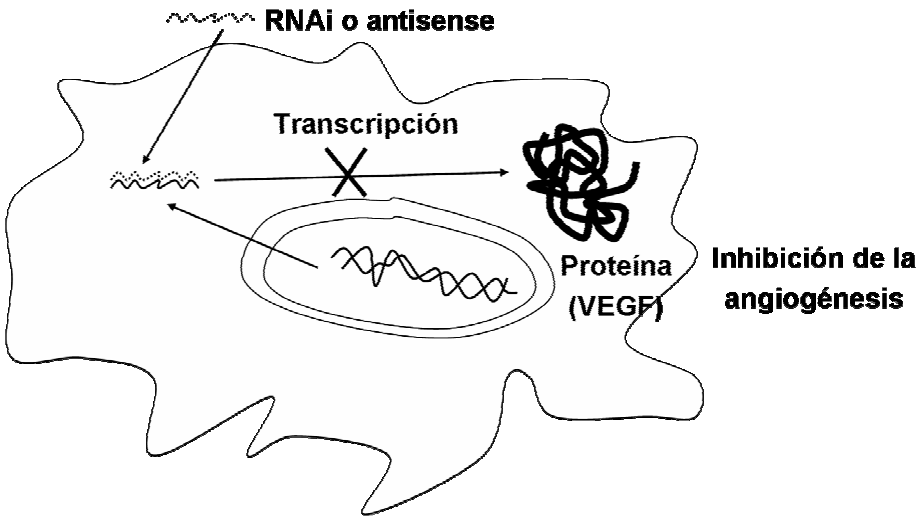

Figura 16. Representación del uso de RNAi o antisense para silenciamiento de genes proangiogénicos

Quimioprotección: Consiste en la administración de genes de resistencia a drogas para proteger la médula ósea de la mielosupresión inducida por quimioterapia. En la estrategia de quimioprotección se introducen genes que incrementan la resistencia a medicamentos. Para este fin se pueden utilizar genes como el de resistencia múltiple a medicamentos (MDR-1), el cual se usaría como una terapia adyuvante que permitiría la administración de altas dosis de quimioterapia en pacientes con cáncer avanzado de mama y ovario

(Wang y cols., 2006). La terapia génica en este caso se administra ex-vivo, en células extraídas de la médula ósea de la paciente, que se reintroducen después de la transducción y previamente al tratamiento por quimioterapia. Este tratamiento permitiría disminuir la magnitud de los efectos tóxicos en las células de la línea mieloide.

4.5.1.- Terapia génica en melanoma

Introducción de genes suicidas, como es el gen de la timidin kinasa del Herpes virus. Este gen fosforila análogos de acíclicos de nucleósidos (Ganciclovir) dando lugar a formas fosforiladas que interrumpen la elongación del DNA durante la fase S de las células transducidas del melanoma. Esta técnica se basa en que la timidin kinasa humana tiene muy baja afinidad por el ganciclovir, mientras que la del herpes virus si, no teniendo apenas efecto la prodroga en células no transducidas (Vile y cols., 1994). Además de matar a la célula transducida se ha visto un efecto bystander en las células vecinas (Blaese y cols., 1994). Con este gen se hicieron estudios clínicos en fase I/II con melanoma metastático (Klatzmann y cols., 1998), pero los resultados no fueron muy buenos, no apareciendo una regresión tumoral clara.

Transferir genes supresores de tumores, como por ejemplo el p53 (Benjamin y cols., 2007) se ha demostrado que induce apoptosis en las celulas tumorales de melanoma. Otro gen supresor de tumores más implicado en la patogénesis tumoral del melanoma es el p16INK4a, que codifica para un inhibidor de ciclinas dependientes de quinasas que actúa bloqueando la CDK4 y la CDK6, provocando el bloqueo de la célula entre la fase G1 y S. De tal manera que mutaciones en este gen provocan la progresión tumoral. Se han hecho estudios clínicos con construcciones para p16INK4a (Gallagher y cols., 2008), y se ha visto que mejora el pronóstico e induce apoptosis.

Inactivación de rutas de señalización oncogénicas sobre-expresados en el melanoma, como ese es el ras o el c-myc, mediante el uso de RNA de interferencia con capacidad catalítica o mediante el uso de RNA antisenso, que se traducen en una disminución de la expresión del oncogen y una reducción en la agresividad tumoral (Li y cols., 2008). Por esta vía también tenemos la actuación sobre los elementos de activación de transducción y transcripción Stat-3. Los STATs son factores de transcripción que se encuentran en el citoplasma y juegan un papel central en la proliferación, difierenciación y apoptosis, demostrándose que su sobreexpresión contribuye al proceso oncogénico en el melanoma. Se ha usado inyecciones intratumorales en ratones de plásmidos que codificaban para una forma negativa de Stat-3 (Beta) que actuaba bloqueando a la Stat-3 tumoral. Una significante regresión tumoral es debida a la apoptosis de las células del tumor (Niu y cols., 2001).

Introducción de genes que codifiquen moléculas inmunológicamente relevantes, como son genes de MHC de clase I (Chang., 2006), genes de citoquinas (GM-CSF, IL-2, INFs, etc) (Perales y cols., 2008) y moléculas co-estimuladoras (B7.1) (Dietrich y cols., 2006). Esto se ha conseguido a través de la modificación genética de las células tumorales, una vez extraídas del tumor, mediante procesos de transfección para que sobre expresen estas moléculas, su irradiación para disminuir su capacidad proliferativa, y reimplantación en el tumor a modo de vacuna. Estas experiencias han dado lugar a resultados interesantes en el tratamiento del melanoma, pero todavía hay que mejorar las técnicas ya que son muchos los efectos secundarios observados en los pacientes.

Otra técnica es ***modificar las células T del paciente*** y reinsertárselas una vez modificadas genéticamente. Esto se ha realizado modificando las células T para que reconozcan el antígeno MELOE-1, que se sobreexpresa

sólo en células melánicas malignas, de tal manera que las células T actúan específicamente sobre células del tumor (Gode y cols., 2008)

También se puede ***actuar sobre las Células dendríticas*** (DCs) del paciente para que expresen antígenos melánicos como el MART-1 o el Melan A (Ribas y cols., 2000). Con esta expresión se consigue potenciar la respuesta inmune del paciente sobre el tumor.

4.5.2.-Terapia génica en cáncer de pulmón

Terapia génica basada en células dendríticas: El SI juega un papel importante en la vigilancia tumoral (inmunovigilancia), dándose fenómenos de activación de células presentadoras de antígenos (APCs) cuando se produce el desarrollo de alguna célula normal, así como se ha demostrado que terapias inmunosupresoras pueden desencadenar el desarrollo tumoral. También se ha demostrado que a veces no se desarrolla respuesta al tumor por el desarrollo de una serie de protecciones frente al SI, como por ejemplo la pérdida de moléculas coestimuladoras, producción de antagonistas de la respuesta inmune, inducción de tolerancia por parte de las células T, etc. (Dubinett y cols., 2004).

En base a ello, se ha demostrado que las APCs del paciente pueden ser recuperadas de pacientes, activadas in vitro mediante una sobrexposición a antígenos tumorales y reinsertadas in vivo a través de numerosas rutas sistémicas o locales, manteniendo estas APCs su potencial inmunogénico durante largo tiempo (numerosos días e incluso semanas). (Sharma y cols., 2003). Las ventajas de este sistema son notables, como por ejemplo que todos los pacientes pueden ser tratados, no son necesarias manipulaciones en vivo muy invasivas para el paciente, etc.

Células dendríticas e interleukina 7: La interleukina 7 se ha demostrado que tiene una actividad in vivo de carácter proinmunogénico, inhibiendo la producción y señalización del TGF-Beta e incrementando la expresión de moléculas de adhesión y de antígenos de histocompatibilidad. Existen numerosos estudios utilizando esta molécula para potenciar la respuesta inmune frente al cáncer de pulmón con resultados prometedores (Andersson y cols., 2009).

Células dendríticas y el CCL21: Resultados similares, e incluso mejores se han encontrado con otra citoquina, la CCL21, que es una potente quimioquina que atrae la movilización de células dendríticas hacia los lugares donde se encuentra. Existen estudios en los que mediante inyecciones intratumorales de CCL21 han potenciado la respuesta antitumoral por parte de las células T CD4 y CD8 (Yang y cols., 2004).

Vacunas para el cáncer de pulmón. Otra opción en términos de terapia adyuvante es el uso de vacunas, para incrementar la capacidad inmunogénica de los antígenos tumorales y su reconocimiento por parte de las células T (Xiang, 2008). Esto se ha realizado en pacientes con estadio I-IIIA reseccionables mediante biopsias del tejido tumoral que se someten a una digestión enzimática para obtener células tumorales. Luego estas células tumorales son transfectadas con una construcción adenoviral que contenga el GM-CSF, luego son irradiadas y ya estarían listas para ser inyectadas como vacunas (Rüttinger y cols., 2007).

RNA antisenso frente al TGF-beta: Se ha demostrado que en los pacientes de cáncer de pulmón de células no pequeñas aparecen elevados niveles de TGF-beta. Esta citoquina es un potente supresor de la estimulación de las células T, con lo que utilizando RNA antisenso se podrían bajar sus niveles y reprimir la inmunosupresión que se da en el tumor. Se han realizado estudios en pacientes con estadios II, IIIA, IIIB y IV, extrayendo células

tumorales, transfectándoles con una construcción que produjera el antisenso, irradiándolas y usándolas como vacuna (Nemunaitis y cols., 2006).

Genes supresores de tumores: Uno de los genes más comúnmente mutados (50-70 % en pacientes) es el p53, el cual controla la progresión tumoral llevando la célula a apoptosis. Se han hecho numerosos estudios en CPNSC estimulando a p53 a través de una sobreexpresión de MdM-2, mediante la introducción de vectores que expresan el MdM-2 (Wang y cols., 2009).

Otro gen supresor de tumores, el FUS1, que se localiza en el cromosoma región 3p21.3, y cuya pérdida de expresión o deficiencia por modificación postrascripcional se ha encontrado en la mayoría de cáncer de pulmón humano. La restauración de la actividad de FUS1 en los tumores de pulmón deficientes se ha demostrado, tanto in vitro como in vivo que tiene una potente actividad supresora del tumor (Ji y cols., 2002). Un estudio reciente (Deng y cols., 2007) ha combinado el p53 y el FUS1 transfectando líneas tumorales así como ratones, mediante el uso de nanopartículas, demostrando la inducción de apoptosis por parte de estos genes supresores de tumores.
Otros genes utilizados para inhibir el crecimiento tumoral en modelos animales son p16 y retinoblastoma (Rb).

Inhibición de oncogénesis: Este tipo de terapia se basa en la identificación e inhibición de aquellos genes críticos para el desarrollo de una carcinogénesis. Los oncogenes de la familia ras son algunos de los más comunes oncogenes activados en cáncer de pulmón y por lo tanto son blanco para este tipo de terapia. En estudios preclínicos, mediante un plásmido con la secuencia antisentido para k-ras, se logró bloquear selectivamente el RNAm de k-ras mutante y reducir el crecimiento de tumores de cáncer de pulmón in vitro e in vivo en modelos murinos (Zhang y cols., 2006).

Transferencia de genes pro-apoptóticos: Una gran proporción de la regresión tumoral que se observa con la radio y quimioterapia es debida a la inducción de apoptosis. Entre los genes candidatos para este tipo de terapia se encuentran los genes de la familia de Bcl-2 los cuales son importantes en la regulación de apoptosis, éstos son homólogos celulares de los genes pro-apoptóticos (Bax, Bak) o antiapoptóticos (Bcl-2, Bcl-XL). Se ha demostrado que la transferencia de los genes Bak y Bax inducen altos niveles de apoptosis en células cancerosas de pulmón, tanto in vivo como in vitro independientemente del estatus de p53 (Sun y cols., 2007). Otro gen que induce apoptosis es el gen fas, el cual en pacientes con cáncer de pulmón de células no pequeñas está relacionado con una mayor supervivencia, ya que su sobreexpresión mediada por adenovirus induce apoptosis en las células tumorales (Zhang y cols., 2009).

5.- GENES "KILLERS"

5.1.-EL GEN KILLER "E"

El gen E es un gen que pertenece a una familia de genes de lisis y se encuentra en el fago ΦX174, el cual se diferencia de otros fagos más grandes por presentar unos requerimientos genéticos mínimos para inducir la lisis. (Blasi y cols., 1989). Tradicionalmente el gen E es uno de los más usados para la generación de "fantasmas bacterianos" (usados como vacunas inactivas genéticamente de gram negativas): se introduce en un vector de expresión controlada en procariotas y se induce su expresión (generalmente por temperatura), provocando en poco tiempo estos "fantasmas" constituidos únicamente por la envuelta celular, sin contenido citoplásmico ni DNA.

5.1.1.- Estructura

La proteína E presenta una serie de características que podemos resumir de la siguiente forma:

-Un total de 91 aminoácidos (aa), con carácter muy hidrofóbico

-Comparte algunas características llamativas con otras proteínas de lisis (como la λ-protein-S), como son la región N-terminal (con residuos aminoacídicos cargados positivamente), que parece estar implicada en la inserción en la membrana. Esta región parece estar muy conservada en las proteínas de lisis, al contrario que la región C-terminal que presenta gran variabilidad. Parece ser que, para su función requiere una oligomerización en el interior de la membrana (de 3 a 4 monómeros), ya que se ha observado que al reducir la fluidez de la membrana se reduce la actuación, y con esta reducción lo que se consigue es disminuir la oligomerización (Blasi y cols., 1989).

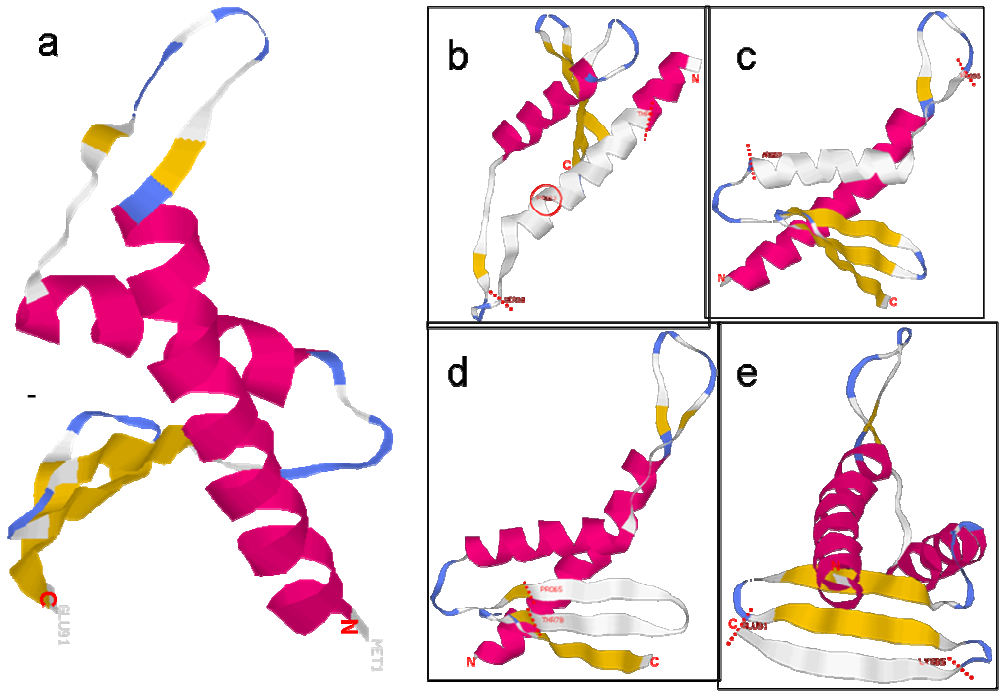

Figura 17. Imagen de la estructura terciaria de la proteína E: a) imagen general; b) dominio 1; c) dominio 2; d) dominio 3; e) dominio 4.

La proteína E se localiza predominantemente en la membrana interna de Coli, y en menos cantidad en la externa. Se ha demostrado que no presenta actividad de lisozima (parece que no tiene actividad enzimática). La predicción de estructura secundaria divide a la proteína en 4 dominios funcionales:

-Dominio 1: (del aa 9-30), con estructura en alfa hélice muy hidrofóbica.

-Dominio 2: (del aa 35-55), con estructura en lámina beta plegada.

-Dominio 3: (del aa 65-78), con estructura muy similar a la del segundo dominio 2 y en ambos casos muy hidrofóbicas

-Dominio 4: (del aa 85-91), es hidrofílico y muy desestructurado

El dominio 1 (Figura 17) parece ser el más importante en la lisis, ya que se ha visto que la pérdida o alteración de alguno de sus aa provoca la pérdida de función. Se ha demostrado que a través de este dominio se ancla a la membrana, recorriendo la membrana interna y situando el extremo N-terminal en la región periplasmática (Lubitz y col, 1984). El extremo C-terminal se encuentra en la región citoplásmica. En mitad de este dominio se encuentra la Prolina 21, que ocupa una posición similar a otras Pro de dominios de membrana de proteínas transportadoras. Se ha propuesto que el cambio conformacional que se produce en la proteína E puede ser iniciado por Peptidil-Prolil cis-trans isomerasas.

5.1.2.- Función

La función de la proteína E es la lisis bacteriana mediante interacción con la membrana bacteriana mediante una serie de pasos, que aun no están totalmente definidos:

- Paso 1: N-terminal en la región periplásmica y el C-terminal en el citoplasma.

- Paso 2: 2 segmentos de la proteína atraviesan la membrana (el dominio 1 y 2). El tránsito de paso 1 a 2 es catalizado por una Peptidil-Prolil cis-trans isomerasa, que generaría un cambio conformacional en la Pro 21. Aquí también se formaría un puente disulfuro importante para la actividad lítica (Schon y cols., 1995).

- Paso 3: fusión de la membrana interna a la externa y la conformación de un túnel transmembrana. No se sabe si en la formación del túnel interviene una sola o más proteínas, por lo tanto no tendrían un diámetro parecido ya que puede haber una o varias proteínas. (Witte y cols., 1997).

En estudios posteriores se postulo con la posibilidad que la actuación lítica del gen E fuese a traves de la inhibición de la síntesis lípidica del peptido-glicano de la pared celular (Bernhardt y cols., 2001)

5.2.-EL GEN KILLER GEF

Es un gen de Escherichia coli que sintetiza una proteína conocida como proteína Gef o también como proteína Hok. Esta proteína pertenece a la familia de proteínas asesinas Hok, cuya función y estructura está muy conservada en todas las bacterias Gram Negativas.

5.2.1.- Estructura

La proteína codificada por el gen Gef, tiene un tamaño de 50 aminoácidos y un peso molecular de 5502 Da (Figura 18). Es un homodímero cuyas subunidades interaccionan a través de puentes disulfuro, presentando una estructura común a la de las proteínas de la familia hok/gef:

Su extremo amino N-terminal, le confiere posibilidad de interaccionar con la membrana, mientras que en el extremo carboxilo C-terminal, situado en la región periplasmática, se encuentra la actividad citotóxica. Este extremo C-terminal presenta una cisteína (posición 30) muy conservada que es la que permite la homodimerización, aunque a veces ésta no es necesaria para el efecto citotóxico. En el resto de la estructura proteica cabe destacar:

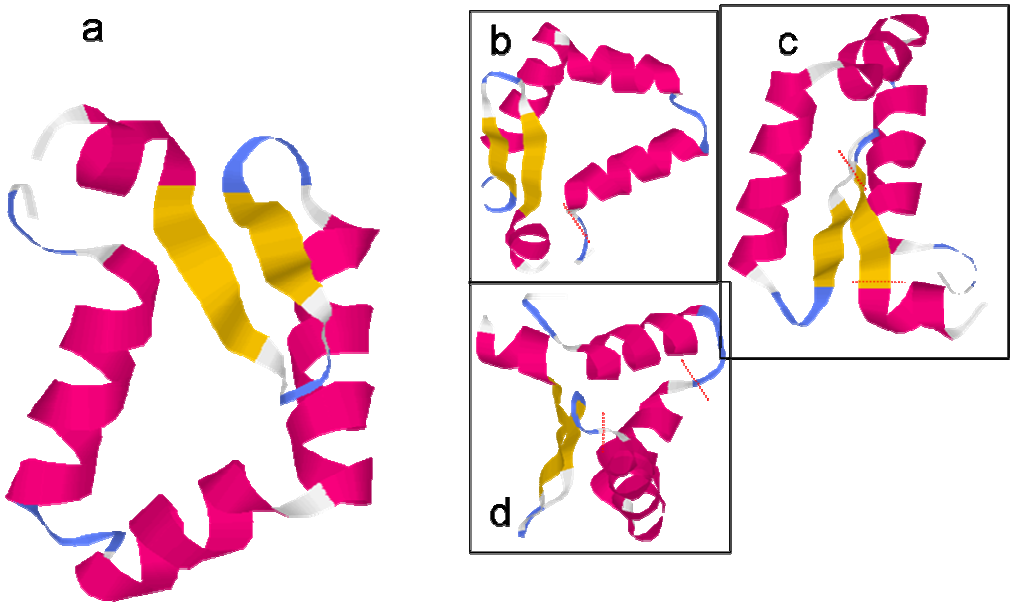

Figura 18. Imagen de la estructura terciaria de la proteína Gef: a) imagen general; b) dominio 1; c) dominio 2; d) dominio 3;

-La región que va del aa 1-5 es citoplasmática.

-La región que va del aa 6-24 contiene una señal que permite el anclaje a proteínas de membrana tipo II

-La región que va del aa 25-50 es periplasmática.

5.2.2.- Función

La expresión de la proteína codificada por el gen Gef provoca la muerte celular desde el interior por interferir en funciones vitales de la membrana celular. Aunque no se encuentra completamente dilucidado este gen es capaz de inducir lesiones celulares generando poros que provocan la aparición de las denominadas “células fantasma”. Experiencias previas con este gen han demostrado su acción sobre la membrana de células eucarióticas pero no se conoce su efecto in vivo (Boulaiz y cols. 2003).

III.- OBJETIVOS

- Determinar la eficacia del gen killer gef en el tratamiento mediante terapia génica del cáncer de pulmón utilizando sistemas experimentales in vitro que incluirán sistemas multicelulares (MTS) como nuevo modelo experimental para reproducir el comportamiento de crecimiento tumoral in vivo.

- Determinar el efecto potenciador que la terapia génica con el gen gef ejerce sobre agentes antitumorales clásicamente usados en el tratamiento del cáncer de pulmón, determinado la eficacia del tratamiento combinado gef-citotóxicos en este tipo de tumor.

- Determinar la eficacia del gen killer en el tratamiento mediante terapia génica del melanoma , utilizando sistemas experimentales in vitro (cultivos celulares) e in vivo (inducción de tumores en ratones).

- Determinar la utilidad en un nuevo gen killer, el gen E, para el desarrollo de un sistema de terapia génica para el tratamiento del melanoma en sistemas experimentales in vitro (cultivos celulares) e in vivo (inducción de tumores en ratones). .

- Analizar los mecanismos de acción por los que los genes E y gef provocan un efecto antitumoral. Este objetivo será llevado a cabo en modelos experimentales in vivo e in Vitro de melanoma

IV.- RESULTADOS

"COMBINED THERAPY USING SUICIDE GEF GENE AND PACLITAXEL ENHANCES GROWTH INHIBITION OF MULTICELLULAR TUMOUR SPHEROIDS OF A-549 HUMAN LUNG CANCER CELLS"

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Key words: lung cancer, gene therapy, gef gene, combined therapy, cytotoxic therapy
Short running title: Experimental lung cancer treatment with *gef* gene and paclitaxel

1.1.- ABSTRACT

The low efficiency of conventional therapies in achieving long-term survival of lung cancer patients calls for development of novel options. The potential use of combined gene therapy is under intensive study. One approach uses the expression of genes encoding cytotoxic proteins that affect cellular viability. The *gef* gene from *E. coli*, identified as a member of a gene family encoding homologous cell-killing functions, codes for a membrane protein with a toxic domain which leads to a decrease in the rate of tumor cell growth. To improve the antitumoral effect of the paclitaxel in lung cancer cells, we investigated a combined suicide gene therapy using this drug and *gef* gene *in vitro*, using A-549 lung cancer cells in culture and forming multicellular tumour spheroids (MTS). Our results showed that *gef* expression in A-549 cells led to an ultrastructural changes, including dilated mitochondrias with clear matrices and disrupted cristae and cell surface alterations such as reduction in length and number of microvilli and cytoplasmic membrane evaginations. The use of paclitaxel in A-549 lung cancer cells transfected with *gef* gene enhanced the chemotherapeutic effect of this drug. Volume analyses showed an 87.4% decrease in the A-549 MTS growth after 96 h in comparison with control MTS. This inhibition was greater than that obtained using the gene therapy or chemotherapy alone. In conclusion, *gef* gene has a cytotoxic effect in lung cancer cells and enhances cell growth inhibition when used with paclitaxel. These results indicate that this combined therapy may be of potential therapeutic value in lung cancer.

1.2.- INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in both men and women. Non-small cell lung cancer (NSCLC) represents about 75–80% of all lung cancers, and most of these patients are in advanced stage at diagnosis (1). Although chemotherapy has recently shown promising results in adjuvant

strategies for early-stage patients (2) and some progress has been made in the treatment of locally progressive and advanced disease (3), latest studies suggest that a therapeutic plateau has been reached and that novel, more specific, and less toxic therapeutic strategies are needed (4). A number of gene therapy techniques have been developed, but their safety and efficiency remain unsatisfactory. However, interest is growing in the development of combined approaches using gene therapy and local tumour irradiation or chemotherapy (5). The combination of gene therapy with various drugs has been shown to enhance tumour cell killing. Recently, novel advances in the combined use of suicide gene therapy and antitumour drugs have been reported in bladder cancer (6), pancreatic cancer (7) and breast or colorectal cancer (8). However, few studies of this type have been performed in lung cancer. In fact, classical strategies using a suicide gene e.g., herpes simplex virus thymidine kinase (HSV-tk), have shown beneficial effects but with some limitations (9). They are able to convert a nontoxic prodrug into a toxic metabolite, but the release of toxic metabolites and their bioavailability are two important shortcomings of the use of these systems (10). Therefore, increasing attention is being paid to the transfer of genes that are not dependent on the use of a prodrug. Our group recently developed a new cancer gene therapy strategy using a toxic gene from the chromosome of E. coli (*gef*) which does not need a prodrug to be effective in tumour cells (11,12). The *gef* gene, a member of a gene family with homologous cell-killing functions, encodes a membrane protein of 50 amino acids that is anchored in the cytoplasmic membrane by the N-terminal portion. The C-terminal part is located in the periplasm (13). Mutagenesis studies have shown that this periplasmic portion encodes the toxic domain and that its dimerization is not essential for the toxic effect. Activation of this protein induces arrest of cellular respiration and cell death (14). Studies of suicide cassettes consisting of members of the gene family plus inducible promoters have documented their efficacy (15).

Based on the knowledge that the *gef* gene encodes a cytotoxic protein that binds to cell membranes, we investigated whether this gene can be used in a combined therapy with the antitumour drug paclitaxel in an experimental protocol to the treatment of lung cancer cells. Results obtained suggest that the combination of these treatments enhanced the anticancer effect and could be potentially used for cancer gene therapy approaches.

1.3.- MATERIAL AND METHODS

Cell culture and MTS formation*.* The lung carcinoma cell line A549 (ATCC-CCL185) was grown with Ham's F12K (Sigma Chemical co., St. Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 40 mg/l gentamicin and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37°C in an atmo sphere containing 5% CO2. To generate multicellular tumour spheroids (MTS), exponentially growing monolayer A-549 cells were harvested by trypsinization and counted using a haemocytometer. Dead cells were excluded using trypan blue stain, then 10 × 103 cells/well were grown in a 24-well microplate (BD Biosciences) previously coated with 400 µl 1.33 % agarose type II in FCS-free medium and allowed to dry for 30 min. Plates were incubated at 37º C in a 5% CO2 atmosphere to promote aggregation and transferred onto a rocker designed for three-dimensional agitation (70 cycles/min) as described previously (16). Growth of the spheroids was monitored and measured to obtain a median relative volume (volume at day x/volume at day 0), as previously described by Boyd *et al.* (17).
Vector construction. The *gef* gene was kindly provided by Dr. J. L. Ramos from the Zaidín Experimental Station, CSIC, Granada, Spain. After its amplification using specific primers (sense 5´ATGAAGCAGCATAAGGCGATG3´ and antisense 5´TTACTCGGATTCGTAAGCCGTC3) gef gene was subcloned into the pcDNA3.1 vector following manufacturer's instructions (Invitrogen). The resulting plasmid pcDNA3.1/*gef* was confirmed by sequence analysis using the T7 primer 5′TAATACGACTCACTATAGGG3′. Plasmid DNA was amplified in *E*.

coli DH5α and purified by large-scale plasmid preparation using columns (Qiagen, Barcelona, Spain). DNA was dissolved in free TE buffer for storage. To optimize transfection conditions, the pcDNA3.1/*lac*Z encoding β-galactosidase under the CMV promoter was used as a positive control vector for transfection and expression. A control pcDNA 3.1 plasmid in which the *gef* gene was absent was used as a negative control.

gef transfection in A-549*.* One day before transfection, confluent cells were seeded into 6-well plates (0.8 x 10^5 cells per well). Briefly, a transfection mixture was prepared by adding 94 µl of the serum-free medium and 6 µl FuGENE-6 reagent (Roche Diagnostic, Barcelona, Spain). After 5 min of incubation at room temperature, 2 µg of plasmid DNA (pcDNA3.1/*gef*) was added (ratio 2:6). The transfection mixture was incubated for 45 min at room temperature. A-549 cells, yielding approximately 70% confluence, were transfected with empty (control) or *gef* gene-containing pcDNA vector. Cells were cultivated for 8 h at 37º C, and the medium containing transfection mixture was then replaced with the growth medium. The β-galactosidase-positive cells were counted microscopically to determine the transfection efficiency which was between 40 and 50%.

In Vitro Expression of gef gene*.* Upregulation of mRNA expression of *gef* cDNA was determined by RT- PCR. Total RNA was extracted from transfected (24, 48, 72 and 96 h) and parental cells with the Rneasy Mini kit **(**Qiagen), and cDNA was generated by means of the Promega reverse transcription system using total cellular RNA (1µg). PCR amplification of *gef* gene took place under the above-described conditions and was run on a 2% agarose gel and visualized by ethidium bromide staining. RNA integrity was assessed by amplification of β-*actin* mRNA (sense 5'ATCATGTTTGAGACCTTCAA3' and antisense 5'CATCTCTTGCTCGAAGTCCA3'). Images were scanned and analysed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative

gef mRNA expression was calculated as the ratio of *gef* to *β-actin*.

Proliferation assays. Haemocytometer analysis and sulphorhodamine B proliferation assay were performed to evaluate the effects of *gef* gene on cell growth. Parental and transfected cells (including cells transfected with empty vector) growing in well plates were trypsinized after 24, 48, 72 and 96 h and collected. Then, cells were counted with a haemocytometer. Trypan blue dye exclusion was used to determine cell viability. The same experiment was repeated using sulphorhodamine-B (SRB). Cells were fixed with 10% trichloroacetic acid for 60 min at 4º C and stained with 0.4% sulphorhodamine B/1% acetic acid by incubating for 10 min with constant shaking. Cells previously washed with 0.1% acetic acid were left in 10 mM Trizma for 15 min at room temperature with constant shaking. Optical density was then determined using a Titertek multiscan (Flow, Irvine, California) colorimeter at 492 nm. Linearity of the SRB assay with cell number was tested for each A-549 cell stock before each cell growth experiment. A-549 cells transfected with empty vector were used in the proliferation assay as controls.

Measurement of Annexin V and PI Staining*.* Annexin V and PI staining was used to assess apoptosis (Pharmingen, San Diego, CA, USA). Briefly, medium was removed, then cells were washed twice with PBS and incubated in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2 1.8 mM CaCl2, pH 7.4) containing annexin V-FITC (25 µg/ml) and PI (25 µg/ml) in the dark for 15 min at room temperature. Then, 500 µl binding buffer was added and cells were immediately processed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analysis. Parental and transfected A-549 cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. Pellet and monolayer were post-fixed with 1% osmium

tetroxide in 0.1 cacodylate buffer for 1 h at room temperature and dehydrated in ethanol. Cells were detached from culture vessel by rapid treatment with propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. Sections were contrasted with uranyl acetate-lead citrate and examined in a Hitachi H7000 transmission electron microscope. For SEM, adherent transfected and parental tumour cells on coverslips were fixed with 2% glutaraldehyde, dehydrated in graded concentrations of ethanol and died using the critical point method. These preparations were coated with platinum and observed under a Hitachi S-800 scanning electron microscope (Hitachi, Tokyo).

Combined therapy in MTS. MTS from A-549 cells were transferred, using a Pasteur pipette, from the 24-well microplate to a96-well plate (one MTS per well) coated with agarose and containing 200 µl of medium. MTS were transfected with pcDNA3.1/*gef* as reported above. Four groups of MTS were analysed: control MTS, transfected MTS, paclitaxel-treated transfected MTS and paclitaxel-treated non-transfected MTS. Paclitaxel was used at 10 nM, 100 nM and 1 µM according to Monazzam *et al* (18). The experiment was carried out four times with six replicates in each group. The response to each anticancer treatment was evaluated by measuring MTS volume during treatment, as reported above.

Statistical Analysis. SPSS 7.5 software (SPSS, Chicago, IL, USA) was used for all statistical analyses. Results were compared by using the Student´s *t* test. All data are expressed as means ± SD. Differences were considered statistically significant at a *P* value of <0.05.

1.4.- RESULTS

***In Vitro Evaluation of gef expression*.** *In vitro* evaluation of *gef* gene expression was performed by RT-PCR. As shown in Fig. 1, an amplification fragment of 153 pb was found in A549 cells transfected with pcDNA3.1/*gef* for different times, indicating the effectiveness and ability of the construction to be used in the subsequent *in vitro* experiment. To demonstrate the integrity of the RNA preparations, PCR was performed using β-*actin* primers (Fig. 1). Studies of the bands, normalized by comparison with the β-*actin* signal, showed that the highest *gef* expression occurred at 72 and 96 h after transfection (6.8- and 9-fold higher, respectively, versus A-549 cells at 24 h).

Inhibition of the A-549 growth rate by gef gene. After establishing that transfected A549 cells expressed *gef* transcripts, we analyzed the potential of *gef* gene to decrease the growth of lung cancer cells. Cell growth was measured in A-549 cells transfected by either pcDNA3.1/*gef* (experimental group) or empty pcDNA3.1 (control group) at 24, 48, 72 and 96 h. As shown in Fig. 2, the growth of A549 cells transfected with the empty vector was similar to that of the parental cells. In contrast, A-549 cells transfected with pcDNA3.1/*gef* showed a significant and time-dependent decrease in growth. No significant differences were found at 24 h after transfection, then transfected cells showed a significant decrease in growth rate (39%) versus empty vector transfected cultures at 48 h, with the largest decrease in the proliferation rate observed at 72 and 92 h (55 and 42.2%, respectively).

Morphologic characteristics of transfected A-549 cells. Light microscopy observations typically showed A549 lung cancer cells with polygonal shape and sheet-like pattern in normal monolayer culture, compatible with their epithelial origin. Cells were attached to the bottom of the flasks with an irregular arrangement in confluent cultures, although some cells showed short cytoplasmic projections. Cultures of transfected cells with empty vector

showed no morphological changes with respect to the parental cell line (data not shown). However, microscopic comparisons between control group cultures and cultures of cells transfected with pcDNA3.1/*gef* over four days showed a progressive loss of monolayer culture uniformity, with the presence of irregular zones without cells (Fig. 3). Conventional electron microscopy and confocal microscopy were used for ultrastructural analyses of transfected A-549 cells. Control cells showed the characteristic features of undifferentiated cells, i.e., polygonal shape, large nucleus and scant cytoplasm (Fig. 4a). In transfected A-549 cells, the most relevant ultrastructural features were the presence of dilated mitochondrias with clear matrices and disrupted cristae and of cell surface alterations, i.e., reduction in length and number of microvilli and appearance of cytoplasmic membrane evaginations (Fig. 4b and c). No compaction or segregation of chromatin was observed, indicating absence of apoptosis (data not shown). Similar morphological alterations were observed in SEM images. Cells with both morphological changes (microvilli reduction and membrane evaginations) were observed (Fig. 4e and f). In contrast, A-549 parental cells were characterized by numerous microvilli on their surface (Fig. 4d)

Apoptosis analysis. A-549 cells were studied by means of an annexin V-FITC apoptosis detection kit to determine possible apoptotic cell death resulting from *gef* gene transfection. Treatment with empty vector had no significant apoptotic effect on these cells (data not shown), and no significant differences in apoptosis level were observed between transfected (24- 96 h in culture) and control A-549 cells (Fig. 5). No typical apoptotic changes were observed under microscopy, as reported above.

Combined therapy with gef gene and paclitaxel in A-549 cells. The therapeutic potential of combined *gef* gene and paclitaxel therapy was evaluated in A-549 lung cancer cells. The response to each anticancer treatment was evaluated by measuring A-549 MTS volumes, as described above. The largest decrease in growth rate after treatment with pcDNA3.1/*gef*

or paclitaxel was observed at 96 h (Fig. 6). At this time, a 35.2% volume decrease was observed in *gef* gene transfected A-549 MTS. With paclitaxel treatment, a dose-dependent reduction in A-549 MTS volume was detected, with a decrease of 20.3, 35 and 54.6% versus control MTS after administration of 10nM, 100nM and 1µM of paclitaxel, respectively (Fig. 6). However, a more effective inhibition of MTS growth was obtained by the combined therapy (pcDNA3.1/*gef* and paclitaxel treatments) than by each treatment alone. The effect of paclitaxel at different concentration was enhanced by gef gene expression but the largest reduction in A-549 MTS volume was obtained with 1µM paclitaxel. This treatment produced an 87.4% decrease in the MTS growth rate at 96 h versus control MTS (Fig. 6).

1.5.-DISCUSSION

Despite therapeutic advances, conventional lung cancer therapy has failed to improve survival rates in NSCLC patients. The habitually late diagnosis and high mortality of lung cancer, alongside the ineffective and harmful effects of chemotherapy and radiotherapy, mandate the adoption of novel treatment approaches. Thus, research is in progress into combined modality treatment strategies for cancer, including gene therapy with anti-tumour drugs (19). The present study explored a combined therapy with *gef* suicide gene and paclitaxel as a new approach to the treatment of lung cancer.

To date, isolated gene therapy has not guaranteed the successful treatment of lung cancer. Gene therapy with antiangiogenic factors, proapoptotic genes or autologous tumour cells modified with an adenovirus vector have induced a partial response (5). Thus, HSV-tk/ganciclovir (GCV) treatment was shown to selectively kill lung cancer cells (20). However, despite the use of new specific promoters (e.g., INSM1) (21), this approach is limited by the release of toxic metabolites and their bioavailability (10). Moreover, the development of chemoresistance in lung cancer cells (such as GLC4)

significantly changes GCV sensitivity, reducing the efficacy of HSV-tk/GCV (22). Therefore, therapeutic systems are required that are not dependent on the use of a prodrug. In fact, bacterial genes that encode toxins, viral genes, and even plant genes have been shown to be able to induce tumour cell death (23-25).

With this background, we assayed the *gef* gene in A-549 lung cancer cells. The *gef* protein is known to form pores in bacterial cell membranes, promoting host cell lysis, and it has demonstrated a cytotoxic effect in melanoma and breast cancer cells (11,12). When we transfected *gef* gene in A-549 lung cancer cells, the number of surviving cells was significantly lower versus control cells at 48-96 h, with the largest decrease in the proliferation rate observed at 72 h (55%). A similar growth inhibition effect was found in A-549 cells by Narumi *et al.* (26) using a cytolytic pore-forming protein (perforin) that also binds to the tumour cell membrane. Interestingly, the decrease in tumour cell number observed in our experiments was not associated with apoptosis, despite a progressive loss of monolayer culture uniformity. Other non-mammalian genes used in cancer gene therapy induce cell death by a non-apoptotic mechanism (27). It was recently demonstrated that breast cancer cell growth is inhibited by bacteriophage λ-holin, a protein that can permeabilise the bacterial membrane (24). Forty-eight hours after induction cells became multinucleated, in some cases extensively vacuolated, and finally detached from the culture dish surface. These findings indicate that the cytotoxic effects of the *λ*-holin protein include alterations in cellular morphology preceding cell death. The *gef* gene diminishes the membrane potential, leads to membrane leakiness and also induces morphological changes (28). However, its specific mechanism of action in eukaryotic cells has not been elucidated. Eukaryotic cells fundamentally differ from prokaryotic cells in terms of their cellular structure, organisation, metabolism and membrane composition. Nevertheless, because the eukaryotic endomembrane system arose in an ancestral prokaryotic lineage (29), *gef* gene may act in cell organelle membranes. In fact,

bacterial toxins such as *Vibrio cholerae* cytolysin or *Helicobacter pylori* VacA protein directly interact with the eukaryotic cytoplasm membrane (30,31). This hypothesis is strongly supported by our ultrastructural findings in the transfected A-549 cells of dilated mitochondrias with disrupted cristae, cytoplasmic membrane evaginations and smaller and fewer microvilli.

After establishing the efficacy of the *gef gene to* affect the growth of human lung cancer cells, we investigated its use in a combined therapy with paclitaxel, a drug of choice for treating lung cancer. Addition of gene therapy strategies to conventional therapies appears to improve their effectiveness. Thus, the anti-tumour response was enhanced by combining chemotherapeutic drugs with HSV-tk in bladder cancer (6), with p53 in breast cancer (32) and with E2F-1 in melanoma (33). Combined therapy with docetaxel or paclitaxel and p53 or interleukin 12 genes improved outcomes in lung cancer (34). Paclitaxel, which has a known activity against a broad range of tumour types, also showed higher efficacy when combined with gene therapy in ovarian and metastatic breast cancers (35,36). We tested the combination of paclitaxel and gene therapy in lung cancer cells by using A-549 MTS. This model mimics the real biological environment and gives a more relevant picture of the drug effects by including limitations in penetration, distribution and feedback mechanisms in cell signalling (37). Volume analyses of the A-549 MTS showed that the combined therapy induced significant MTS growth inhibition after 96 h in comparison with control MTS. The largest reduction was obtained with the use of gef gene and paclitaxel 1µM. This inhibition was greater than that obtained using the gene therapy or chemotherapy alone. These results showed that the combination of *gef* gene and paclitaxel enhanced cell growth inhibition in A-549 MTS, suggesting its therapeutic potential in lung cancer. However, as in most gene therapy systems, gene delivery and selectivity for cancer cells remain a challenge. In this respect, retroviral vectors have the advantage of selectively transducing dividing cells and of integrating into the genome of the infected target cell. Recent developments in vector design, such as the reconstituting

retroviral vector system (39), allow the generation of high-titre vector viruses expressing genes that encode cytotoxic products. By replacing the constitutively active viral promoters with tissue- or tumour-specific promoters (40), a targeted delivery of cytoxically acting gene products appears to be feasible. In summary, our data demonstrate the potential clinical relevance of a new combined therapy which could be usedor lung cancer gene therapy.

1.6.- FIGURES

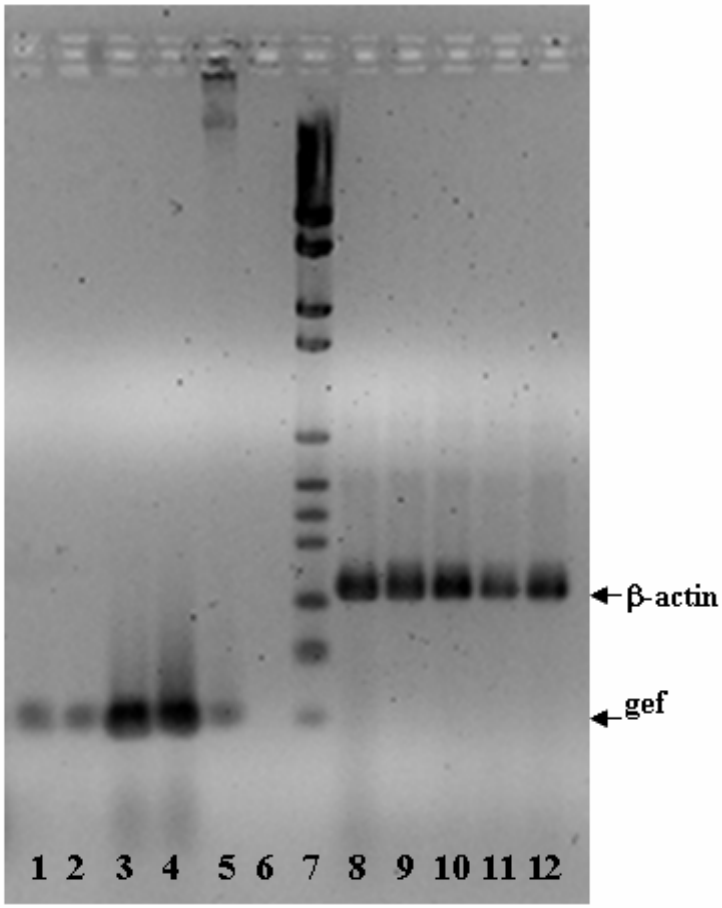


Fig. 1. Determination of *gef* gene expression by RT-PCR. Total RNA isolated from transfected and parental A-549 lung cancer cells was transcribed to cDNA using reverse transcriptase PCR amplification as described in Material and Methods. Amplified PCR products of gef mRNA and β-actin mRNA were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. PCR of gef gene: Lane 1-4; transfected A-549 (24, 48, 72 and 96h respectively); Lane 5, pcDNA3.1/Gef (positive control); Lane 6, Parental A-549 cells (negative control); Lane 7, Molecular weight. PCR of β-actin: Lanes 8-11, transfected A-549 cells (24, 48, 72 and 96 h respectively); Lane 12, parental A-549 cells.

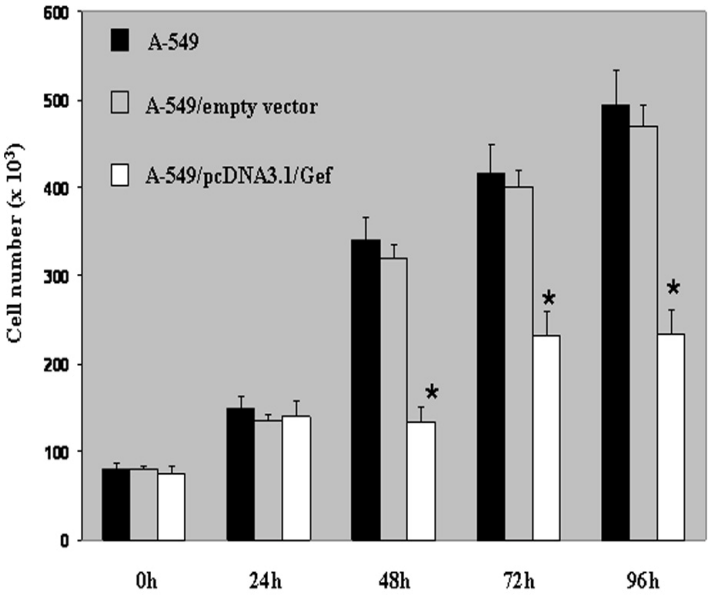


Fig. 2. Effects of *gef* transfection on growth of A-549 cells. Parental A-549 cells and A-549 cells transfected with empty vector or pcDNA3.1/Gef were seeded at a density of 8 × 104 in plastic dishes and cultured for four days. Cell numbers were measured daily (24, 48, 72 and 96 h) by sulphorhodamine B assay. Values represent means ± SD of quadruplicate cultures (*p< 0.05 compared with empty vector transfected cells)

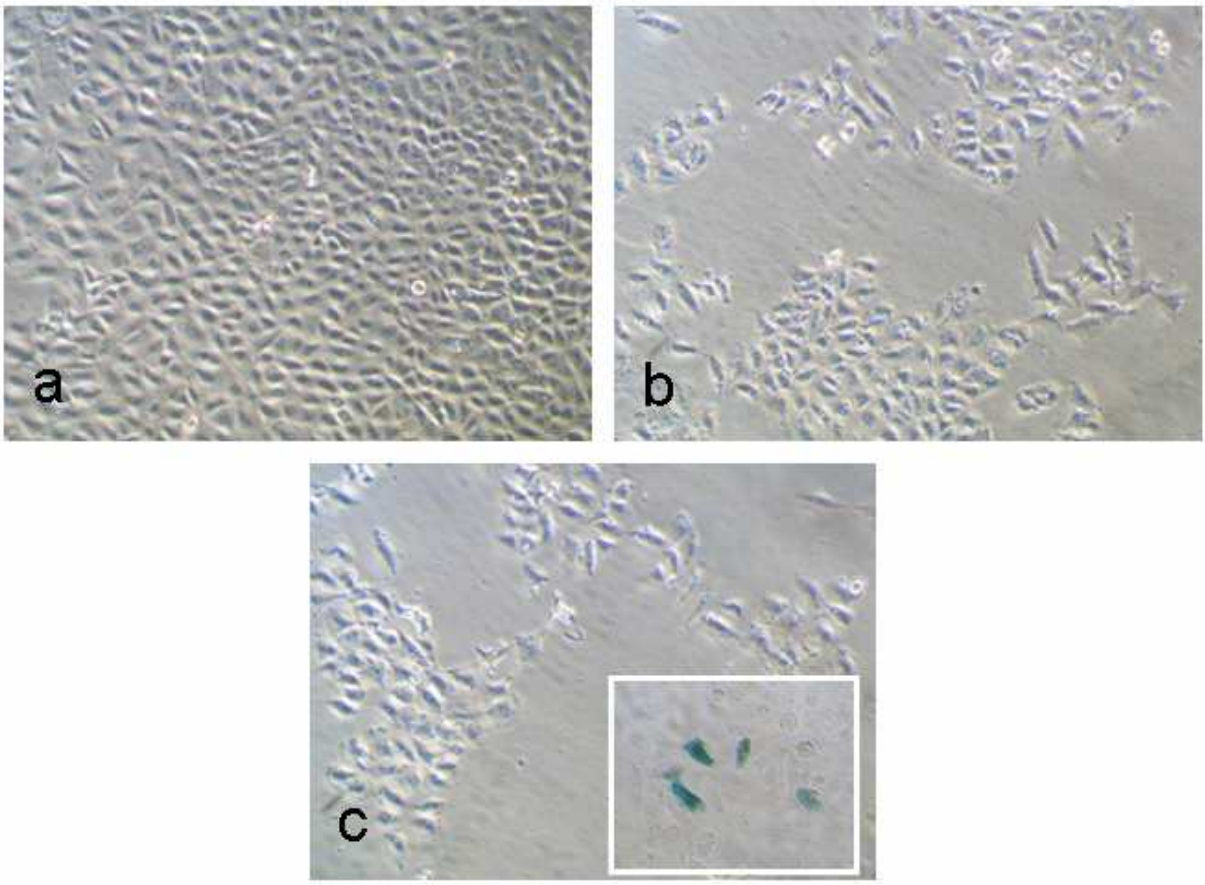


Fig. 3. Phase-contrast photomicrographs showing morphology of parental and transfected A-549 cells. Parental A-549 cells (a) grew in clumps, were typically polygonal and formed a monolayer culture on the entire flask surface at 96 h. In contrast, A549 pcDNA3.1/V5/His-Gef transfected cells at 48 h (data not shown), 72 h (b) (cells stained with X-gal in corner), and 96 h (c) (x 40) formed an irregular monolayer culture with the progressive presence of zones without cells.

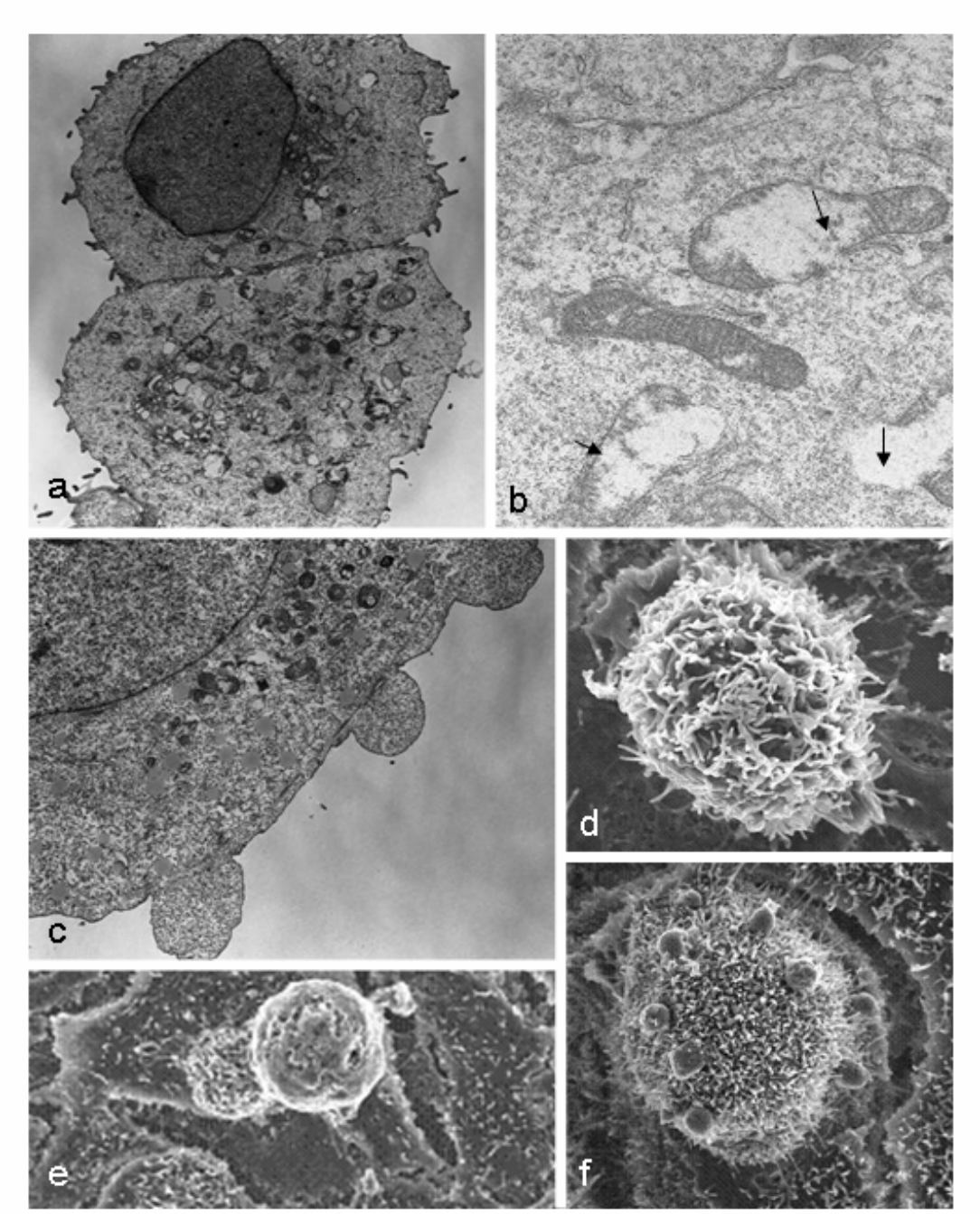


Fig. 4. TEM and SEM analyses of A-549 cells. Conventional electron microscopy of parental A-549 cells (a) showed typical tumour cells with polygonal shape, large nucleus and light cytoplasmic complexion (x1100). Transfected A-549 cells showed dilated mitochondrias with disrupted cristae (arrows) (b) (x12000) and cytoplasmic membrane evaginations (c) (x4400). Confocal microscopy of parental A-549 cells (d) showed numerous microvilli on cell surface. In contrast, transfected A-549 cel(e and f) and membrane evaginations (f) ls were characterized by progressive disappearance of microvilli

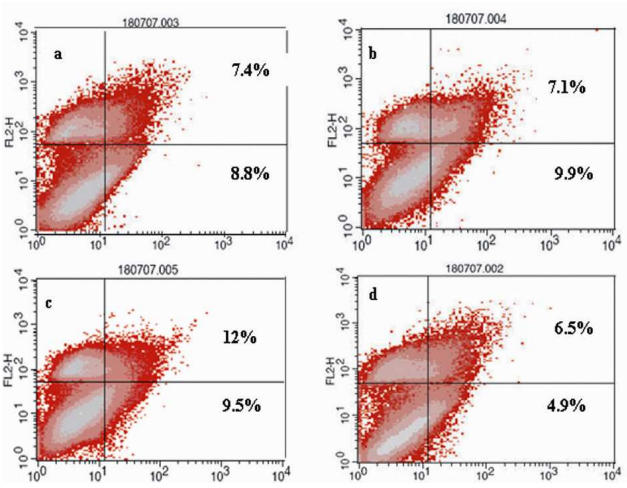
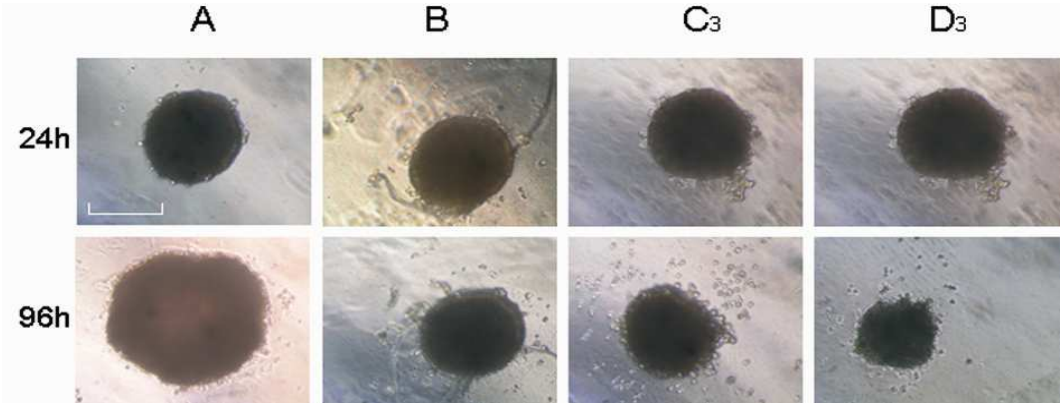


Fig. 5. Fluorescence-activated cell sorting analysis of apoptosis induction by *gef* gene in A-549 lung cancer cells. Cells were stained with annexin V and propidium iodide to evaluate apoptotic cell death, as described in Material and Methods. Representative images for comparisons between parental A-549 cells (a) and transfected A-549 cells at 48 h (b), 72 h (d) and 96 h (d). These data are mean results of four separate experiments.



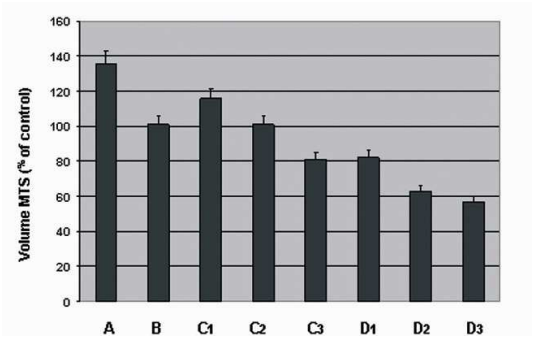


Fig. 6. Analysis of the combined therapy (pcDNA3.1/Gef/paclitaxel) in A-549 cells, using multicellular tumour spheroids (MTS). Growth of MTS was monitored by measurement of their cross-sectional area, calculating the median relative volume (volume at day x/volume at day 0). The graph depicts percentage volume changes in MTS after 96 h treatment in each experimental group. Group A, control A-549 MTS; Group B, A-549 MTS treated with pcDNA3.1/Gef; Group C, A-549 MTS treated with 10nM (C_1), 100nM (C_2) and 1µM (C_3) paclitaxel; Group D, A-549 MTS treated with combined therapy pcDNA3.1/Gef and 10nM (D_1), 100nM (D_2) and 1µM (D_3) paclitaxel; Bar = 300 µm. These data are mean results of four separate experiments. Light microscopic image represents A-549 MTS of the experimental group A, B, C3 y D3.

Acknowledgments

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"REGRESSION OF ESTABLISHED SUBCUTANEOUS B16-F10 MURINE MELANOMA TUMORS AFTER *GEF* GENE THERAPY ASSOCIATED WITH THE MITOCHONDRIAL APOPTOTIC PATHWAY"

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Short title: *In vivo* melanoma gene therapy with the suicide *gef* gene

2.1.-ABSTRACT

Novel treatment modalities, including gene therapy, are needed for patients with advanced melanoma. We evaluated whether the *gef* gene, a suicide gene from *E. coli,* had a significant cytotoxic impact on melanoma *in vivo*. Firstly, we used a non-viral gene delivery approach (pcDNA3.1/*gef*) to study the inhibition of melanoma cells (B16-F10) proliferation *in vitro*. Secondly, we used direct intratumoral injection of pcDNA3.1/*gef* complexed with jetPEI to deliver *gef* cDNA to rapidly growing murine melanomas. We demonstrated that *gef* gene not only has an antiproliferative effect on B16-F10 cells *in vitro,* but also induces an important decrease in melanoma tumor volume (77.7% in 8 days) *in vivo* . Interestingly, after *gef* gene treatment, melanoma showed apoptosis activation associated with the mitochondrial pathway, suggesting that the induction of this death mechanism may be an effective strategy for its treatment. Our in vivo results indicate that *gef* gene might become a suitable therapeutic strategy for patients with advanced melanoma.

2.2.- INTRODUCTION

Melanoma represents only 4% of all skin cancers, but nearly 80% of skin cancer deaths, predominantly because of metastatic spread (1). Apart from surgery, treatment options for melanoma, particularly metastatic melanoma, are relatively limited**.** As melanoma is a highly therapy-refractory tumor, it demands effective therapies combinations (2). Suicide gene therapy has been proposed as a strategy for the treatment of intractable cancers and has been assayed in some clinical trials by itself or in combination with other therapies (tumor irradiation or chemotherapy). In melanoma, strategies to facilitate apoptosis by gene therapy may be an alternative or complementary strategy for its treatment (3) since it has been demonstrated that apoptosis deficiency is a critical factor for therapy resistance in this tumor (4).

Classical cancer suicide gene therapy employs genes encoding enzymes that convert non-toxic prodrugs into cytotoxic compounds (5). However, these prodrug systems have been assayed in melanoma both *in vitro* and *in vivo* with limited results (6,7). As an attractive alternative to this strategy, therapeutic genes that directly encode cytotoxic proteins could be used. In contrast to classical suicide genes that act by disrupting DNA synthesis (targeting only rapidly dividing cells) these new toxins may act killing both quiescent and rapidly dividing tumor cells and may be effective for aggressively growing tumors as well as for those that grow more slowly. Many genes encoding cytotoxic products have been evaluated as gene therapy approaches (8,9). The most recent experiences with genes expressing toxins from bacteria (10), from plants (11) or from bacteriophage (12) showed a high cytotoxic impact on tumoral cells derived from different tissues.

In this context, our group has developed last year a direct cancer gene therapy system based in the suicide gene named *gef*. The *gef* gene, member of a gene family with homologous cell-killing functions, encodes a membrane protein of 50 amino acids which is anchored in the cytoplasmic membrane by the N-terminal portion, whereas the C-terminal part is located in the periplasm (13). Although activation of this protein induces arrest of respiration and death in bacterial cells the mechanism of action in tumoral cells is unclear. We have previously demonstrated that *gef* protein is able to induce changes in proliferation rate and differentiation degree of tumoral cells without having to use prodrugs (14,15). However, its possible *in vivo* application could not be demonstrated yet.

In this study, we have evaluated for the first time the potential use of the *gef* gene for the treatment of melanoma tumors *in vivo*. Moreover, based on the knowledge that the *gef* gene encodes a cytotoxic protein that binds to cell membranes, we analyzed *gef´*s mechanism of action. We used the B16-F10 murine melanoma model due to its highly invasive and metastatic nature and

the cationic lipids jetPEI to deliver *gef* cDNA to rapidly growing murine melanomas. Results obtained suggest that treatment with the *gef* gene significantly decreases tumor growth, inducing apoptosis in melanoma tumor cells by means of the mitochondrial pathway.

2.3.- MATERIAL AND METHODS

***Cells and reagents*:** The B16-F10 murine melanoma cell line (CRL6475) was obtained from American Type Culture Collection (ATCC) and was grown in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2mM L-glutammine, 40 mg/l gentamicin and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37° C in an atmosphere containing 5% CO_2.

***gef transfection in B16-F10 cells*:** The *gef* gene was amplified using specific primers (sense 5´ATGAAGCAGCATAAGGCGATG3´ and antisense 5´TTACTCGGATTCGTAAGCCGTC3´) under the following conditions: 94 ℃ for 1min, 35 cycles at 94℃ for 1 min, 53℃ for 30 seg, and 72℃ for 30 seg and 72℃ for 10 min and was subcloned into the pcDNA3.1 -TOPO vector (Invitrogen, Barcelona, Spain) following the manufacturers' instructions. The resulting plasmid pcDNA3.1/*gef* was transformed into the subcloning efficiency DH5 alpha chemically competent *E. coli* (Invitrogene). The correct DNA sequence was confirmed by sequence analysis using the T7 primer. One day before transfection, confluent cells were seeded into 6-well plates (2 x 10^5 cells per well). Briefly, a transfection mixture was prepared by adding 94 µl of the serum-free medium and 6 µl FuGENE-6 reagent (Roche Diagnostic, Barcelona, Spain). After 5 min of incubation at room temperature, 2 µg of plasmid DNA (pcDNA3.1/*gef*) were added (ratio 1:3). B16-F10 cells, yielding approximately 70% confluence, were transfected with *gef* gene-containing pcDNA3.1 vector. Cells were cultivated for 8 h at 37º C, and the medium containing transfection

mixture was then replaced with the growth medium. A pcDNA3.1 plasmid in which the *gef* gene was absent was used as a negative control. The pcDNA3.1/green fluorescent protein (GFP) (provided by Dr. G. Ortiz) was used to optimize transfections conditions.

***Proliferation assays*:** Parental and transfected cells growing in well plates were trypsinized after 24, 48, 72 and 96 h and collected. Cells were fixed and stained with 0.4% sulphorhodamine B/1% acetic acid. Cells previously washed with 0.1% acetic acid were left in 10 mM Trizma for 15 min at room temperature. Optical density was then determined using a Titertek multiscan (Flow, Irvine, California) colorimeter at 492 nm. Linearity of the SRB assay with cell number was tested for each B16-F10 cell stock before each cell growth experiment. B16-F10 cells transfected with empty vector were used in the proliferation assay as controls.

***In Vitro and In vivo Expression of gef gene*:** Upregulation of mRNA expression of *gef* cDNA was determined by RT-PCR. RNA was extracted from transfected and parental cells with the RNeasy Mini kit **(**Qiagen). RNA from tumor was obtained with the RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was generated by means of the Promega reverse transcription system using total cellular RNA (1µg). PCR amplification of *gef* gene took place under the above-described conditions. RNA integrity was assessed by amplification of β-actin mRNA. Images were scanned and analyzed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative *gef* mRNA expression was calculated as the ratio of *gef* to β-actin.

***Annexin V and PI Staining*:** Parental and transfected cells were washed twice with PBS and incubated in binding buffer containing annexin V-FITC (25 µg/ml) and PI (25 µg/ml) in the dark for 15 min at room temperature (Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA, USA). Then, binding buffer (500 µl) was added and cells were immediately

processed with a FACScan flow cytometer. Microscopy analysis was carried out by Technical Services from the Granada University in a Leica DMI6000 (Heidelberg, Germany) confocal microscope with laser Argon/Krypton.

Assay for cytoplasmic mono- and oligonucleosomes*:*The Cell Death Detection ELISA Kit (Boehringer Mannheim) was used for assessing apoptosis in transfected cells following the manufacturer's protocol. Parental and transfected cells (2×10^4) were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Samples were then incubated with anti-DNA peroxidase followed by color development with ABTS substrate. Samples absorbance was determined with Titertek multiscan at 405 nm.

Measurement of mitochondrial membrane potential: Parental and transfected cells were washed twice with cold PBS and incubated with 40 nM DiOC6(3) for 15 min at 37ºC. Then, cells were washed with ice-cold PBS and resuspended in 500 ml of PBS. Fluorescence intensities of DiOC6(3) were analyzed on a FACScan flow cytometer with excitation and emission settings of 484 and 500 nm, respectively.

Caspase activity assay:Caspase-9 and -8 activities were measured using caspase colorimetric assay kits (R&D Systems, Minneapolis, MN). Briefly, parental and transfected cells were washed twice with cold PBS and resuspended in 50 µl of cold lysis buffer, incubated for 10 min, and centrifuged for 1 min at 10,000 x *g* to precipitate cellular debris. Assay was performed in triplicate on a 96-well plate following the manufacturers' protocol. Results are expressed as the fold increase in pcDNA3.1/*gef* treated cells over that of control cells. Etoposide (Sigma) (50 µmol/L) was used as positive control of caspase activities in B16-F10 cells.

Tumor induction and measurement: For *in vivo* study, female C57BL/6 mice (Scientific Instrumentation Centre, Granada University) were used. All mice (weighing 25-30 g) were maintained in a laminar air-flow cabinet at a room kept at 37ºC temperature and 40% to 70% humidity with a 12-hour light/dark cycle under specific pathogen-free conditions. All studies on animal models were approved by the Ethical Committee of the Medical School of Granada University and performed according to its guidelines. Tumors were induced by subcutaneous injection of $5x10^5$ B16-F10 cells into the left flanks of C57BL/6 mice. Tumors were allowed to grow to the appropriate size (75 mm^3) before treatment (ideal minimal size for intratumoral injection). After reaching this volume (treatment day 0), tumors were measured at periodic intervals following treatment using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a). Using these measurements the tumor volume was calculated by the formula $V = ab^2x\pi/6$.

Intratumoral plasmid treatment: *In vivo* JetPEI (Polyplus-transfection Inc.) was used as a transfection enhancer reagent. PEI/DNA complexes with a ratio of 1:6 were prepared in a solution of 10% w/v glucose. This was carried out in a two-step procedure for the preparation of a standard quantity of 20 µg of PEI/DNA complex, according to the manufacturer's instructions. Tumors were then treated intratumorally with pcDNA3.1/*gef* plasmid. The pcDNA3.1 LacZ plasmid was used to normalize transfection efficiency. Moreover, control groups (without treatment and treated with empty vector) were included. Treatments were administered during 14 days. Comparative study between treated and non treated groups was realized during the first 8 days due to the high rate of mortality in control group.

Histologic analysis*:*Tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and cut into 3–5 µm sections. Cells were immunofluorescently labeled with primary anti-tubulin mouse monoclonal antibody (1:500) (Sigma) followed by Texas Red dye-conjugated affinitPure

Goat Anti-Mouse IgG+IgM (1:500) (Jackson ImmnoResearch Laboratories, West Grove, PA). The presence of apoptotic cells within the tumor sections was evaluated by the TUNEL technique using the In Situ Cell Death Detection Kit Fluorescein (Roche, Mannheim, Germany) according to manufacturers´ recommendations. Cell nuclei were counterstained with DAPI. Percent apoptosis (apoptotic index) was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high power fields/slide). Immunohistochemical analyses of caspases were realized using anti-active forms of caspase-9 (1:50) (Cell Signaling Technology, Inc) and caspase-8 (1:100) (Imgenex, San Diego, CA). FITC-conjugated anti-rabbit secondary antibody at room temperature for 1 h was used for the detection. Cell nuclei of cultures were counterstained with DAPI. Fluorescence images were captured using an Olympus DP11 microscope with a Nikon Eclipse Ti digital imaging system.

Transmission electron microscopy (TEM): Melanoma tumors grown in mice were collected, cut up into small pieces and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 1 h. After postfixation with 1% OsO_4 in cacodylate buffer (room temperature, 2 h), sections were dehydrated through graded ethanol concentrations with a final propylene oxide dehydration. Samples were then embedded in Epon 812 resin. Ultrathin sections, were stained with uranyl acetate and lead citrate and examined in a Hitachi H7000 transmission electron microscope.

Statistical analysis: SPSS 14 software (SPSS, Chicago, IL, USA) was used for all statistical analyses. Results were compared by using the Student´s *t* test. All data are expressed as means ± SD. Differences were considered statistically significant at a *P* value of <0.05.

2.4.- RESULTS

Expression of gef gene and inhibition of cell growth in B16-F10 cells in culture *In vitro* evaluation of *gef* gene expression was performed by RT-PCR As shown in Fig. 1A, an amplification fragment of 153 bp was found in B16-F10 cells transfected with pcDNA3.1/*gef* for different time periods, indicating the effectiveness and ability of the construction in order to be used in the subsequent experiment. To demonstrate the integrity of the RNA preparations, PCR was performed using β-actin primers. Analysis of the bands, normalized by comparison with the β-actin signal, showed a progressive increase of *gef* expression; this was 3.9- and 4.5-fold higher at 48 and 72 h versus B16-F10 cells at 24 h and was maximal at 96 h after transfection (6 fold higher versus B16-F10 cells at 24 h). Previously, cell transfection was optimized by pcDNA3.1/GFP (Fig 1B). As shown in Fig. 1C the B16-F10 cells transfected with pcDNA3.1/*gef* showed a significant and time-dependent decrease in growth. Twenty four hours after transfection a 28% decrease in growth rate versus control cultures was observed. The decrease in proliferation was 45.5% at 48 h. The main decrease in proliferation rate occurred at 72 h and 96 h, when similar ratios of growth decrease were observed (64.6% and 69.7%, respectively). In contrast, the growth of B16-F10 cells transfected with the empty vector (control group) was similar to that of the parental cells.

Expression of gef gene induced apoptosis in B16-F10 cells**.** Apoptotic rates of B16-F10 cells untreated or transfected with empty vector revealed no significant difference. Only 8.7 % of the pcDNA3.1/*gef* transfected cells showed apoptosis after 24 h. However, at 48 and 72 h a significant increase was found (19 % and 34%, respectively) (Fig. 2A). At 96 h apoptosis was similar to that found at 72 h (data not shown). These results indicated the ability of *gef* gene to stimulate apoptosis in B16-F10 melanoma cells after *in vitro* transfection. The induction of apoptosis by *gef* gene was also evident by confocal laser-scanning microscopy (Fig. 2B). Furthermore, to confirm whether

the growth inhibitory effects of *gef* gene are related to the induction of apoptosis, we used an ELISA-based assay. Amounts of cytoplasmic oligonucleosomes (an indicator of apoptosis) increased between 24-96 hours after *gef* transfection as compared to untreated cells. As shown in Fig. 2C, the strongest enrichment factor (EF) was obtained at 72 and 96 h (3.6 and 5.4, respectively). These results provide convincing data that up-regulation of *gef* induces apoptosis in B16-F10 cancer cells.

Gef gene therapy-induced modulation of mitochondrial membrane potential and caspase-9 activation. To determine if induced apoptosis by *gef* gene in B16-F10 cells is mediated via the mitochondrial pathway, mitochondrial membrane integrity was measured by DiOC6(3) dye staining. As shown Figure 3A, a significant decrease in membrane potential was detected in transfected cells at 24, 48 and 72h compared to parental cells (control) indicating a mitochondrial membrane permeability increase after *gef* gene treatment. At 96 h membrane potential modulation was similar to that found at 72 h (data not shown). Caspase-9 activity was induced after *gef* gene treatment in B16-F10 cells. Although its activity was modulated at different times, the largest increase was observed at 72 and 96 h (3- and 4.1-fold; respectively). The caspase-8 activity remained unchanged (Fig. 3B). These data support the hypothesis that *gef* gene induced apoptosis through the mitochondrial-mediated pathway.

Gef gene effects on melanoma growth in vivo. The potential of *gef* gene to promote tumor cell killing *in vivo* was evaluated by direct injection of the plasmid complexed with jetPEI in B16-F10 subcutaneous mice tumors. Fig. 4 shows that *gef* gene was able to inhibit tumor growth. During the first two days following treatment, tumor volume decreased by 40.4% in the *gef* gene treated group, as compared to the control group. On post-treatment days 4 and 6 the observed reduction was 45.2 and 54.3% respectively. Following a similar trend, a 77.7% volume reduction was observed on day 8. After this time, the control group showed a high mortality rate (Fig. 4). Mice treated with *gef* gene showed

no evidence of systemic toxicity (i.e. animal death, loss of body weight, other tissue damage or changes in behavior or aspect). ***After empty vector injection*** with jetPEI, no tumor growth modifications were observed (Fig. 4A and B). RT-PCR was performed to ensure *gef* gene expression in all treated tumors. Fig. 4C shows a comparison of *gef* gene expression in melanoma tissue between days 2 and 8 after the treatment.

gef gene induced apoptosis in melanoma cells in vivo. To determine the *gef* expression efficiency to induce apoptosis *in vivo* we analyzed established subcutaneous B16-F10 tumors with a TUNEL reaction mixture. As showed Fig. 5A the number of apoptotic cells (green) was significantly higher in tumors treated with pcDNA3.1/*gef* in comparison with the tissue control. Analysis of the melanoma sections showed a progressive increase of percent apoptosis which was maximal at 8 days after treatment (Fig. 5B). To examine possible caspase-9 or -8-activation by *gef* gene treatment we used immunohistochemistry. Weak or absent expression of caspase-8 protein was detected in tissue samples (data not shown). However, all samples of melanoma tissue treated with pcDNA3.1/*gef* at different times showed a clear caspase-9 activation, with the strongest staining observed from the 4th day of treatment until the end of the experiment. Caspase-9 expression was not seen in any of the controls or in melanoma tissue treated with the empty vector (Fig. 5C).

Transmission electron microscopy. To further investigate the nature of *gef* gene-mediated cytotoxicity, B16-F10 mouse melanoma-induced tumors treated with *gef* gene *in vivo* were analyzed by transmission electron microscopy. The control tissue showed giant malignant cells with an intact cell membrane and single- or multiple-nuclei cells. The first identifiable morphological change after treatment was the pronounced swelling in the mitochondria seen within 2 days of treatment. Mitochondria in control cells and cells treated with vehicle alone remained unaffected. Forty eight hours later,

apoptosis ultrastructural characteristics such as chromatin condensation, crescent formation and margination were seen by electron microscopy in the treated melanoma, but not in the control group. Similar pictures were observed 6 and 8 days after treatment (Fig. 6).

2.5.- DISCUSSION

New treatment strategies for malignant melanoma are urgently needed because conventional approaches like chemotherapy and radiation have little impact on patient survival in the advanced stages of the disease. As a promising alternative, gene therapeutic strategies based on suicide gene expression in tumor cells have been developed. In this study, we have demonstrated the *in vivo* potential use of the *gef* cDNA as a suicide gene in a new melanoma gene therapy approach.

To date, classical suicide gene therapy systems have not guaranteed the successful treatment of melanoma and have induced only a partially positive response (16, 17). One of the main limitations of these indirect action systems is the need to use prodrugs (18).The use of genes encoding toxins avoids the administration of a prodrug, eliminating its side effects, its bioavailability limitations and the consecutive applications of vector and prodrug. Moreover, these genes can be directly expressed in the cytosol of the target cells, thus overcoming the problems (cytotoxicity, internalization efficiency and resistance acquired by cancer cells) originated by their use as components of immunotoxins or recombinant chimeras (19). In melanomas, viral genes encoding toxins such as viral protein R and some plant genes such as saporin (SAP) have been applied with a significant result to induce tumoral cell death (20,21). Our previous results *in vitro* showed that *gef* is effective in melanoma MS-36TG cells, modulating their proliferation capacity, differentiation degree and tumor malignancy (22). In the present work we have demonstrated that the transfection of the pcDNA 3.1/*gef* not only inhibits *in vitro* melanoma

proliferation but also that it is highly toxic for tumors *in vivo*. The *gef* gene treatment induced a significant decrease in tumor growth (77.7 % relative volume reduction after 8 days of treatment), an effect that was clearly improved upon repeated administrations. Experimental treatment with HSV-tk/GCV (23) or more recently with the SAP gene (21) in the same tumor type, induced a 40-50% and 67% relative volume reduction, respectively. McCray *et al.* (20), who used the Vpr gene integrated in the pcDNA3.1 (100 µg) vector in melanoma tumor from B16-F10 culture cells, described an 86% of tumor volume reduction which required 25 days of treatment. Therefore, the main advantage of the *gef* gene is not only its efficacy in melanoma cells but also the shorter latency for effective antitumoral action

Although we have demonstrated the cytotoxic effect of *gef* gene in cancer cells the specific mechanism of action has remained unclear so far. In prokaryotic cells, the *gef* gene diminishes the membrane potential, leads to membrane leakiness and also induces morphological changes (24). Eukaryotic cells fundamentally differ from prokaryotic cells in terms of their cellular structure, organization, metabolism and membrane composition. Nevertheless, since the eukaryotic endomembrane system arose in an ancestral prokaryotic lineage (25) *gef* gene might act in cell organelle membranes. Recently, it was demonstrated that breast cancer cells growth was inhibited by bacteriophage λ-holin, a protein that can permeabilize the bacterial membrane (12). Our results showed that forty eight hours after induction B16-F10 cells become multinucleated, in some cases extensively vacuolated, and finally detached from the culture dish surface. Experiments with annexin, confocal laser-scanning microscopy and nucleosomes clearly showed that the *gef* gene is able to induce apoptosis in a time dependent manner. These results are similar to those obtained with the SAP gene which also induces programmed cell death and direct DNA fragmentation in B16-F10 cells (21). Interestingly, the pronounced clinical chemoresistance of melanoma is strongly suggestive of an inactivation of apoptotic programmes. Defects in proapoptotic signaling

pathways and enhancement of antiapoptotic pathways may synergistically contribute to this apoptosis deficiency (26). Immunohistochemical analysis by TUNEL assay revealed that pcDNA3.1/*gef* treatment significantly increased apoptosis in established subcutaneous B16-F10 tumors in vivo. The incidence of apoptosis in the tumor almost corresponded to the effect of tumor growth inhibition, suggesting that our experimental treatment resulted in tumor regression by significant augmentation of apoptosis.

Apoptosis may occur via death-receptor dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is triggered by the activation of death receptors, such as Fas and TRAIL receptors (DR4, DR5) activating initiator caspase-8, which then cleaves executioner caspase-3. The mitochondrial pathway of cell death is mediated by Bcl-2 family proteins, which disrupt the mitochondria membrane potential and result in release of apoptogenic factors, such as cytochrome c, from the mitochondria into cytosol; in turn, these factors would form an apoptosome with apoptosis activating factor 1 and caspase-9 (27). Treatments modulating apoptosis phenomenon, for example with bcl-2-targeted antisense, are a promising new strategy in melanoma (28). Assays with drugs such as hydroquinone or thiobenzanilides in this tumor type have demonstrated an action mechanism related to caspase-9 activation (29,30). This tumoral cellular injury mediated by caspases may also be induced by suicide genes and it may be relevant in relation to their application in tumors. In fact, CD/5FC system induces apoptosis in human malignant glioma cells by the activation of caspases-3 and -9 but not caspase-8 (31) while a certain modification, the bifunctional *E. coli* cytosine deaminase and uracil phosphoribosyltransferasefusion, is able to induce caspase-3 activation only (32). HSV*tk*/GCV activates caspase-3, -8 and -9 in rat bladder carcinomas (33) and a variant, the thymidylate kinase, induces apoptosis in Jurkat cells by activation of caspase-3 only (34). Our studies in B16-F10 cells expressing *gef* showed alteration of the mitochondrial membrane integrity suggesting that apoptosis is mediated by the mitochondrial pathway. This

hypothesis is supported by the caspase-9 activity increase in B16-F10 transfected cells. Moreover, the mitochondrial transmembrane potential is altered in most of the cellular population, supporting the hypothesis on the possible effect of *gef* once it is released from the apoptotic cells. The mitochondrial-mediated apoptotic pathway is strongly supported by our ultrastructural findings in the induced B16-F10 tumors in mice which showed dilated mitochondrias with disrupted cristae. Moreover, the *in vivo* assay shows that caspase-9 activity increases significantly after *gef* gene treatment, supporting the participation of a mitochondrial-mediated apoptotic pathway in our gene therapy system. However, we can not exclude the possible participation of other apoptosis-mediated molecule in treated B16-F10 induced tumors such as endonuclease G, Smac/DIABLO and HtrA2 (35). Further studies are required to elucidate the exact mechanisms involved.

We have reported the successful use of the *gef* gene as an anticancer gene therapy system, not only in melanoma cells in culture but in melanoma tumors *in vivo*. Our *in vivo* experiments show that *gef* gene has a rapid and efficient activity in relation to tumor volume decrease. However, *gef* gene binds to the mitochondrial membrane and its activity is not tumor-specific. Therefore, it will be necessary to create this specificity as in most of the toxic genes used in gene therapy (8-12). We have injected intratumorally the pcDNA3.1/gef plasmid to observe its activity in melanoma cells. Adenoviral vectors modified by attaching tumor-specific promoters should be used to assay metastatic melanoma treatment. Currently, we are using specific enhancer/promoter genes (such as tyrosinase) (36), new vectors (such as ReCon) (37) and combined therapy with citotoxic drugs (38) to improve the tumoral response against *gef* gene. Moreover, it will be necessary to demonstrate the apoptosis induction in human melanoma by the extopic *gef* gene expression. In summary, our results suggest that *gef* is a suicide gene candidate for oncologic *in vivo* applications and that it may contribute to eradicate tumor mass in combination with surgery or classic radio- or chemotherapy.

2.6.- FIGURES

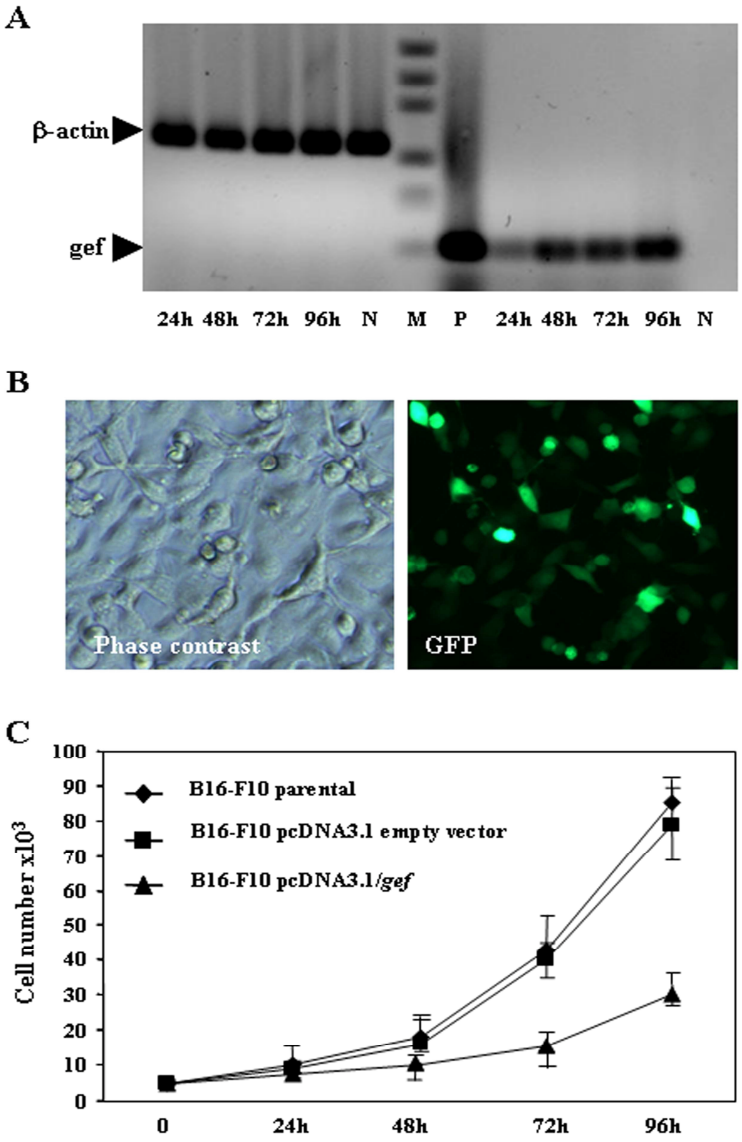

Fig. 1. *gef* gene expression and growth rate inhibition in B16-F10 cells. A. RT-PCR showing *gef* gene expression in B16-F10 transfected cells at different time periods. The integrity of the RNA was demonstrated using β-actin primers. N, negative control (B16-F10 non-transfected cells). M, molecular weight. P, positive control (pcDNA3.1/*gef)*. B. Representative photomicrograph (phase contrast and fluorescent images) of B16-F10 cells transfected with pcDNA3.1/GFP to optimize transfections conditions. C. Growth of B16-F10 cells expressing *gef* was detected by sulphorhodamine B assay. Cells transfected with pcDNA3.1/*gef* showed a clearly decreased growth rate compared to the control cells and cells transfected with pcDNA3.1 empty vector ($P<0.05$). Data represent the mean ± SD of four independent experiments.

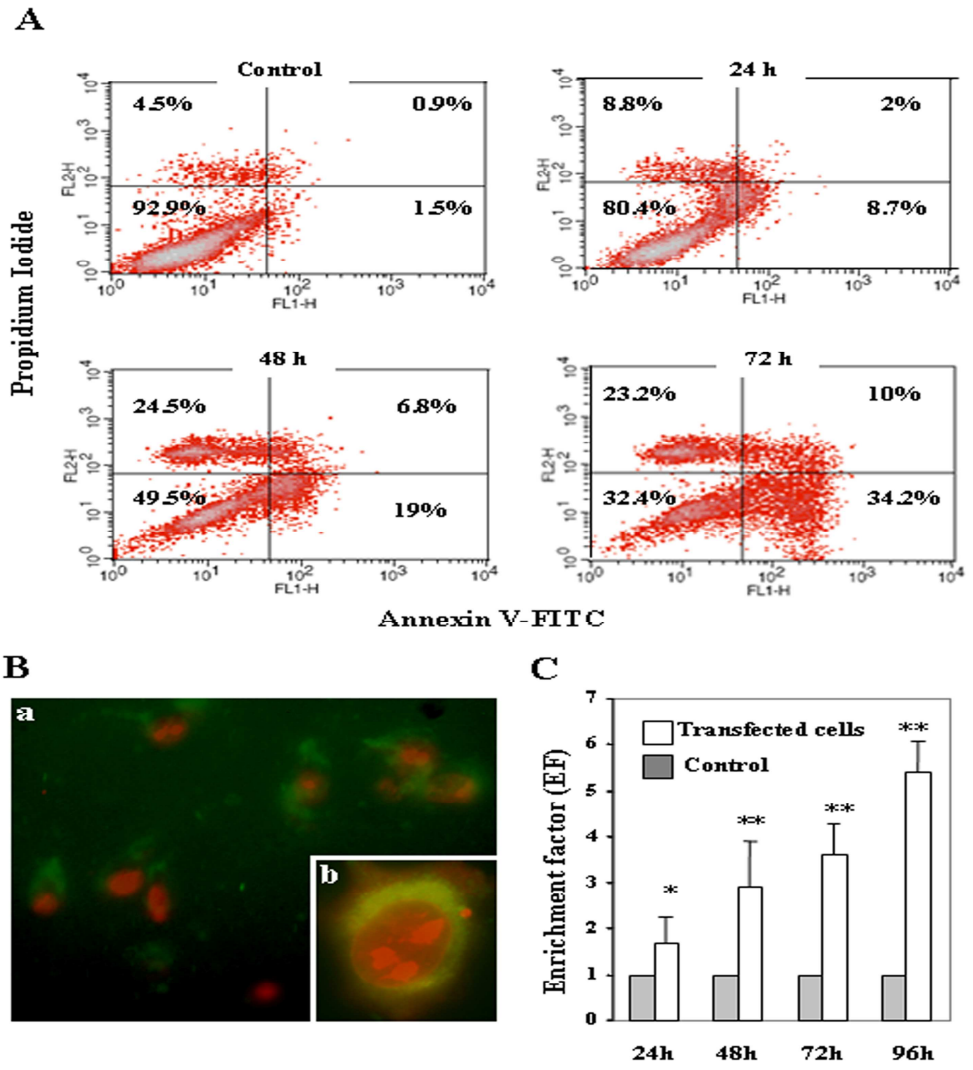

Figure 2. Analysis of apoptosis induction by *gef* gene in B16-F10 cells. A, Fluorescence-activated cell sorting. These data are representative results from four separate experiments. B, Annexin V-FITC staining and confocal microscopy. The annexin V-FITC fluorescence localized at the periphery of the cells consequent to the translocation of phosphatidylserine (PS) residues from the inner leaflet of the plasma membrane to the outer leaflet. B16-F10 cells 48 h after transfection (a). Cell nuclei were counterstained with PI before examination under a confocal laser-scanning microscope. A stronger binding was observed when the study was carried out 96 h after *gef* transfection (b). The experiment was performed three times with identical results. Magnification: a, 20x; b, 40x. C, ELISA apoptosis assay of cytoplasmic nucleosomes. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the formula: mean of absorbance of transfected cells/ mean of absorbance of control cells **=** enrichment factor (EF). The enrichment factor was calculated relative to the control value (equal to 1) of untreated cells. *P<0.05 and ** P<0.01 compared to control cells.

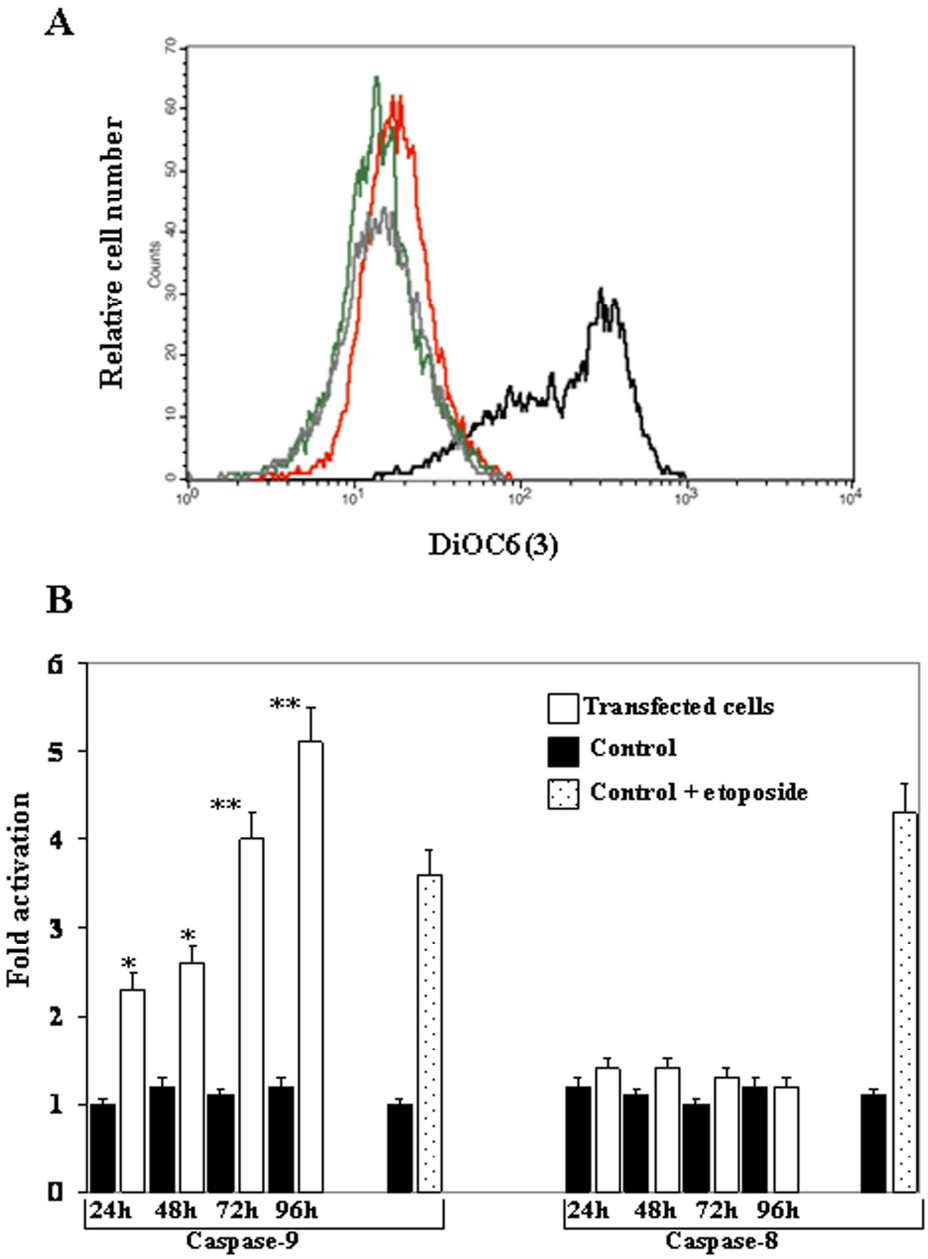

Figure 3. Mitochondrial membrane potential and caspase activity. A. Reduction of mitochondrial transmembrane potential ($\Delta\Psi_m$) in B16-F10 cells (black) after 24 h (red), 48 h (gray) and 72 h (green) of *gef* transfection. The fluorescence intensity of DiOC6(3) was analyzed by flow cytometry. Data shown are representative of three independent experiments. B. Caspase-9 and -8 activities were determined (as described in Methods) in pcDNA 3.1/*gef* transfected B16-F10 cells at indicated time points in comparison with parental cells (percentage values). Etoposide treatment (6 h) was used to demonstrate caspase 8 and caspase 9 activities in B16-F10 cells. Experiments were performed four times with identical results. *P<0.05 and ** P<0.01 compared to control cells.

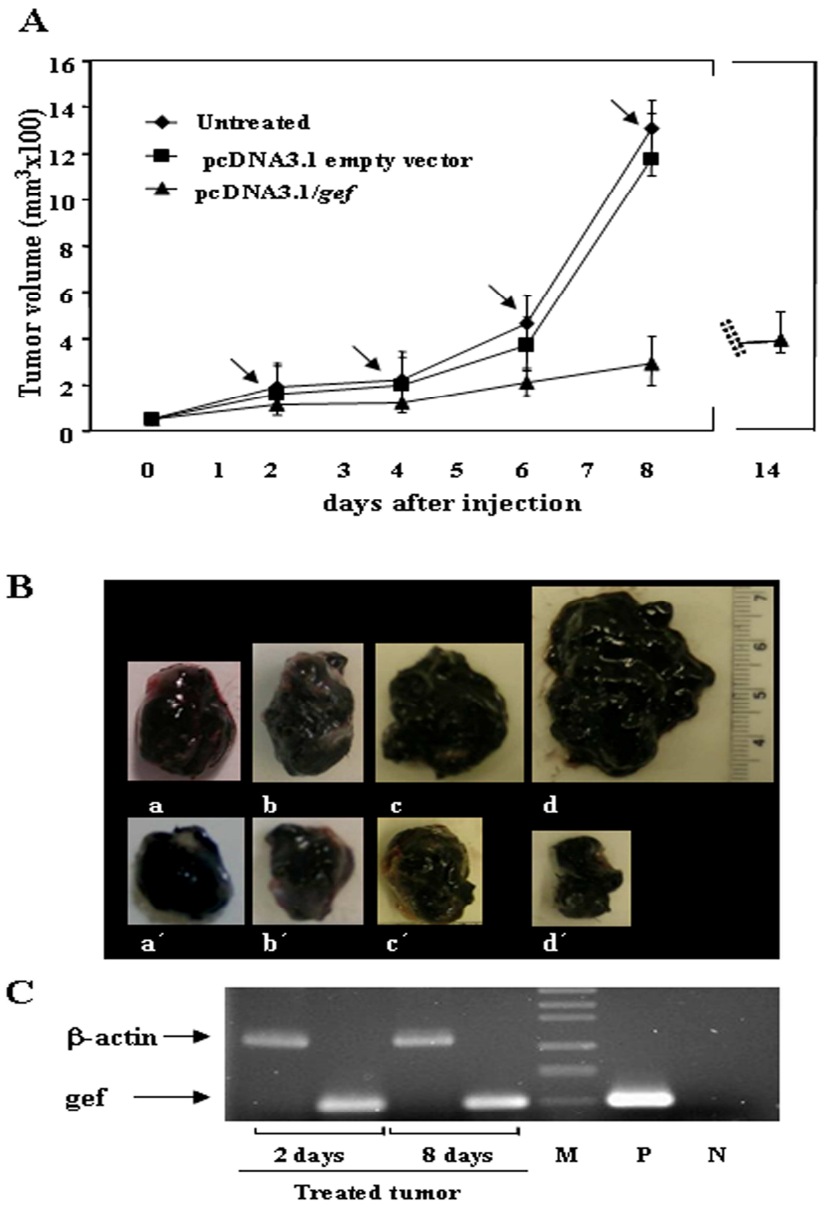

Figure 4. Effect of direct intratumoral injection of the *gef* gene on the growth of subcutaneous tumor induced by B16-F10 tumors cells in mouse. A, Tumor volume variation after *gef* gene treatment. Time of injection is indicated through arrows; treatment began (day 0) when tumors had reached a volume of 75 mm^3. The plot shows a significant tumor volume reduction in the treated group (n = 14) as compared with the control groups, transfected with empty vector (n = 7) and without treatment (n =7). B, Representative gross appearance of tumors excised from mice sacrificed during treatment at 2 (a´), 4 (b´) 6 (c´) and 8 (d´) days and tumors obtained from mice without treatment at the same time intervals (a, b, c and d, respectively). C, Determination of *gef* gene expression in tumor mice after 2 and 8 days of pcDNA3.1/*gef* treatment. The integrity of the RNA tissue used was determined using β-*actin* primers. M, molecular weight. P, positive control (pcDNA3.1/*gef)*. N, negative control (mouse tumors without treatment).

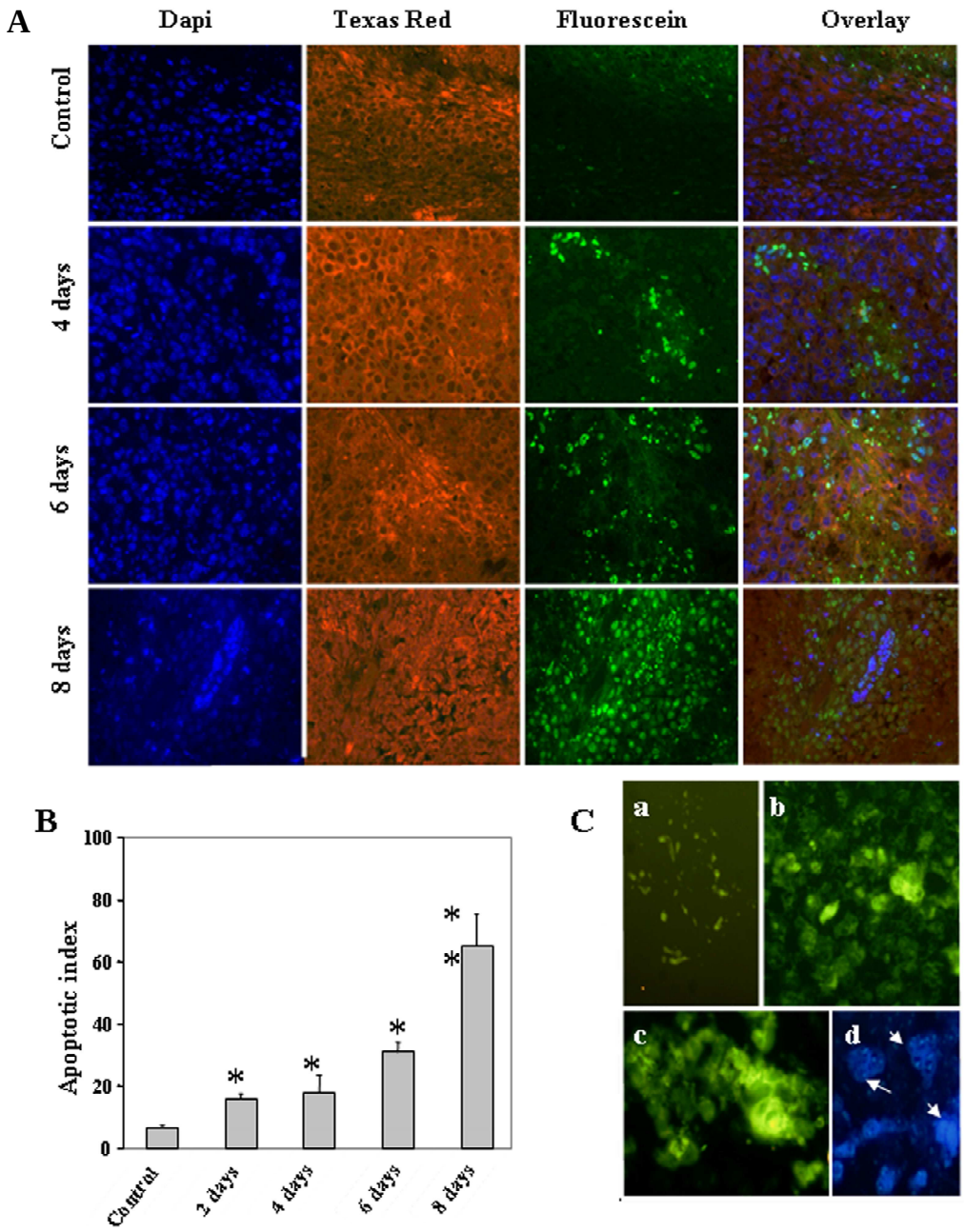

Figure 5. Histological evaluation of apoptosis and caspase activity of melanoma cells *in vivo.* A. Representative photographs of tumor sections showing TUNEL-positive cells (green). Apoptotic cells increased in tumors treated with pcDNA 3.1/*gef* at 4, 6 and 8 days compared to untreated tumors (control) (x20). B. Percent apoptosis in each group. Level of significance compared to control cells: *P<0.05; **P<0.01. C. Caspase expression in melanoma tumor was detected using immunofluorescence staining. Microscopic analysis showed that melanoma tissue after treatment with pcDNA3.1/*gef* was strongly caspase-9-positive. Untreated tumors (a) and melanoma tumor after 4 (b) (40x) and 6 (c) (60x) days of treatment. Cell nuclei counterstained with DAPI showed that some cells displayed apoptotic morphology

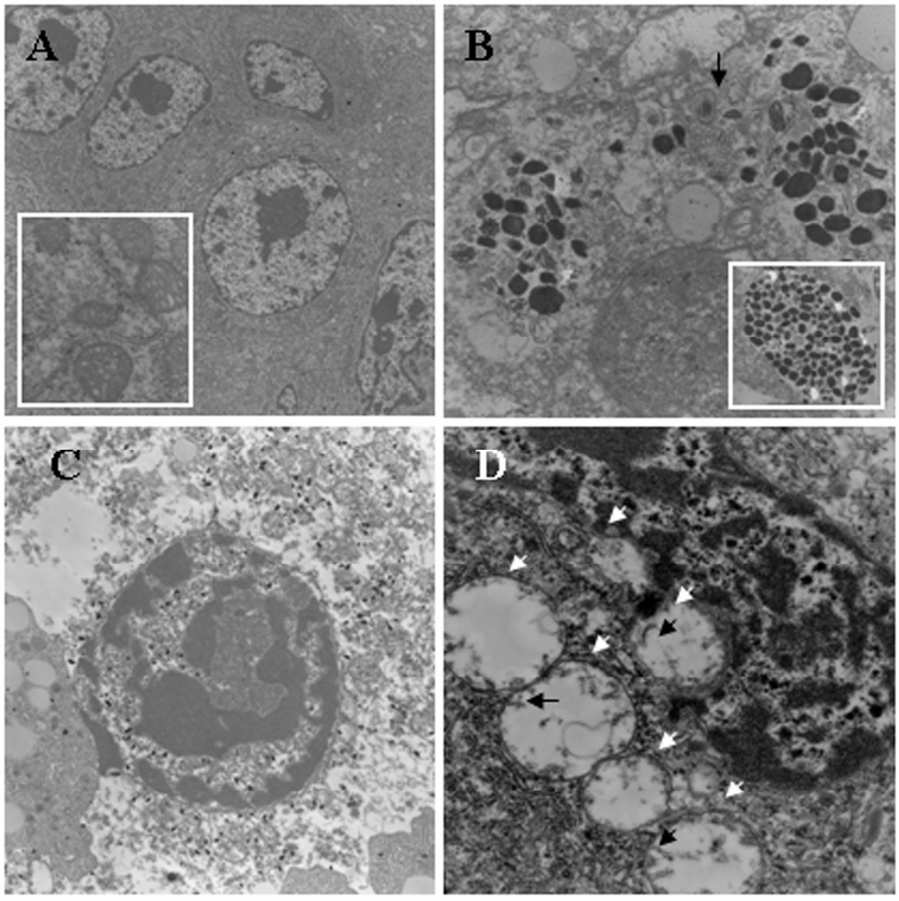

Figure 6. Transmission electron microscopy of melanoma tumors without treatment showed typical tumour cells with polygonal shape, large nucleus, light cytoplasmic complexion containing well preserved organelles (A) including mitochondrias (A insert) (1100x) and a large amount melanosomes (B insert) including premelanosomes (arrows) (B) (x4000). Representative photomicrograph of melanoma treated with pcDNA3.1/gef (6 days) showing ultrastructural characteristics of apoptosis such as chromatin condensation, crescent formation, and margination (C) (x6300). Note the nucleus near of the swollen mitochondrias (white arrows) with disrupted cristae (black arrows) (D) (12000x). Data were obtained from the study of at least three tumors.

Acknowledgments

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"**THE CYTOTOXIC ACTIVITY OF THE PHAGE E PROTEIN SUPPRESS THE GROWTH OF MURINE B16 MELANOMAS *IN VITRO* AND *IN VIVO*"**

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3.1.- ABSTRACT

Novel treatment modalities, including gene therapy, are needed for patients with advanced melanoma. The *E* gene from the phage ΦX174 encodes a 91 aa protein which lyses *Escherichia coli* by formation of a transmembrane tunnel structure. To evaluate whether this *E* gene has a cytotoxic impact on melanoma cells *in vitro* and *in vivo*, and could therefore be used as a new therapeutic strategy for this tumor type, we selected the B16-F10 murine melanoma cell line as a model. We used a nonviral gene delivery approach (pcDNA3.1/E plasmid) to study the inhibition of melanoma cells' proliferation *in vitro* and direct intratumoral injection of pcDNA3.1/E complexed with jetPEI to deliver *E* cDNA to rapidly growing murine melanomas, and found that the *E* gene has both a strong antiproliferative effect in B16-F10 cells *in vitro* and induces an efficient decrease in melanoma tumor volume *in vivo* (90% in 15 days). Interestingly, the GFP-E fusion protein expressed in melanoma cells was located in the mitochondria. *In vitro* and *in vivo* analysis demonstrated significant functional and morphological mitochondrial alterations accompanied by a significant increase of cytochrome c and active caspase-3 and -9 in transfected cells, which suggests that tumoral cell death is mediated by the mitochondrial apoptotic pathway. These results show that *E* gene expression in melanoma cells has an extraordinary antitumor effect, which means it may be a new candidate for an effective strategy for melanoma treatment.

Key Words Melanoma - E gene - Gene therapy - Apoptosis - Caspase – Mitochondria

3.2.- INTRODUCTION

Although cancer rates remain stable, the number of invasive melanoma cases continues to rise. Melanoma represents only 4% of all skin cancers but nearly 80% of total skin cancer deaths, predominantly because of metastatic spread. Apart from surgery, the treatment options for melanoma, particularly metastatic melanoma, are relatively limited and emphasize the need for the development of novel efficacious therapies. As melanoma is a highly therapy-refractory tumor, it demands effective therapeutic combinations [1]. Suicide gene therapy has been proposed as a strategy for the treatment of intractable cancers and has been assayed in some clinical trials alone or in combination with other therapies (tumor irradiation or chemotherapy). Strategies to facilitate apoptosis by gene therapy in melanoma may be an alternative or complementary strategy for its treatment since it has been demonstrated that apoptosis deficiency is a critical factor for therapy resistance in this tumor [2]. Classical cancer suicide gene therapy employs genes which encode enzymes that convert nontoxic prodrugs into cytotoxic compounds which preferentially affect rapidly growing cells such as those found in cancers [3]. The two most widely used prodrug systems, namely herpes simplex virus thymidine kinase/gancyclovir (HSV*tk*/GCV) and bacterial cytosine deaminase/5-fluorocytosine (CD/5FU), have been assayed in melanoma *in vitro* and *in vivo* with limited results [4, 5]. Therapeutic genes which encode cytotoxic proteins directly could be an attractive alternative to this strategy. In contrast to classical suicide genes, which act by disrupting DNA synthesis and therefore target only rapidly dividing cells, these new toxins may act by killing both quiescent and rapidly dividing tumor cells and may be effective for aggressively growing tumors as well as for those that grow more slowly. The most recent experiences with genes expressing toxins from bacteria such as diphtheria toxin [6] or streptolysin O [7], plants such as saporin (SAP) [8], viruses such as the matrix protein of vesicular stomatitis virus [9], and bacteriophages such as alpha-holin [10], have shown a high cytotoxicity for tumoral cells derived from

different tissues.

In this context, the *E* gene is another potentially interesting bacteriophage lysis gene for cancer therapy. In contrast to most double-stranded DNA phages, which generally encode two genes that elicit host cell lysis (endolysin and holing protein), the small single-stranded DNA phage ΦX174 has only one lysis gene. The 91 aa E protein encoded by this causes cell lysis at concentrations of 100–300 molecules per cell [11], although its mechanism of action is controversial. Gene fusion analysis has revealed that only the 29 amino-terminal amino acids of the E polypeptide encompassing the putative transmembrane domain are required for lytic activity [12, 13]. However, this polypeptide has no detectable cell-wall degrading activity, and given its simple primary structure it is unlikely to have any enzymatic activity at all. Scanning electron microcopy images of cells undergoing E-mediated lysis have shown discrete 50- to 200-nm holes in the cell membrane. This observation has led to the proposal of a model in which the E protein oligomerizes to form a ‘‘transmembrane tunnel’’ spanning the entire cell envelope, thereby releasing the cytoplasmic content [14].

In light of the above, we decided to investigate the potential of the native *E* gene in cancer gene therapy approaches by testing this gene in both *in vitro* and *in vivo* systems to determine its tumoral cell-killing efficiency. We selected B16-F10 murine melanoma cells because this tumor cell line is a very good model for many human malignancies due to its highly invasive and metastatic nature. Our results demonstrate that *E* gene expression induces a significant decrease in melanoma cell viability and a spectacular reduction of melanoma tumor growth rates *in vivo* by inducing apoptosis in tumoral cells via the mitochondrial pathway. These growth-inhibitory and cell-killing effects strongly suggest that the *E* protein may have a potential use in cancer gene therapy.

3.3.-MATERIAL AND METHODS

Cell Culture: The B16-F10 murine melanoma cell line (CRL6475) was obtained from American Type Culture Collection (ATCC) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, 40 mg/l gentamicin and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37℃ in an atmosphere containing 5% CO_2.

Transfection. The *E* gene (Dr. J.L. Ramos, Zaidín Experimental Station, CSIC, Granada, Spain) was amplified from the pMC22 plasmid with primers (sense 5'-ATGAAGCAGCATAAGGCGATG-3' and antisense 5'-TTACTCGGATTCGTAAGCCGTC-3') and subcloned into the pcDNA3.1-TOPO vector following the manufacturer's instructions (Invitrogen, Barcelona, Spain). The resulting plasmid pcDNA3.1/*E* was transformed into the subcloning efficiency DH5 alpha chemically competent *E. coli* (Invitrogen). The correct DNA sequence was confirmed by sequence analysis using the T7 primer. We used jetPEI DNA transfection reagent (PolyPlus Transfection, Inc, NY, USA) for cell transfection, according to the manufacturer's instruction. The efficacy of cell transfection was checked using pcDNA3.1/GFP (Green Fluorescent Protein) provided by Dr. G. Ortiz (IBIMER, Granada, Spain).

Reverse Transcription-PCR (RT-PCR). RNA was extracted from transfected and parental cells with the Rneasy Mini kit **(**Qiagen). cDNA was generated by means of the Promega Reverse Transcription System (Promega, Madrid, Spain) using total cellular RNA (1 µg). PCR amplification of the *E* gene was performed under the following conditions: 94 ℃ for 1 min, 35 cycles at 94℃ for 1 min, 55℃ for 30 s and 72 ℃ for 30 s, a nd 72℃ for 10 min. The sense primer 5'-GCTTTCCTGCTCCTGTTGAG-3' and the antisense primer 5'-TTGACGCACGTTTTCTTCTG-3' were used for RT-PCR. RNA integrity was

assessed by amplification of β-actin mRNA (sense: 5'-ATCATGTTTGAGACCTTCAA-3' and antisense 5'-CATCTCTTGCT CGAAGTCCA-3'). PCR products were analyzed by standard agarose gel electrophoresis. Images were scanned and analyzed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative *E* mRNA expression was calculated as the ratio of *E* to β-actin. RNA from B16-F10 melanoma induced in mice was obtained with the QIAamp RNAasy Fibrous Tissue Mini Kit (Qiagen) and RT-PCR was performed as described above.

Proliferation Assays. Parental and transfected cells were seeded in a 96-well plate at 6 x 10^3 cells per well. After 24, 48, and 72 h, 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well and incubated at 37 ℃ for a further 4 h. Then, 200 µl of dimethyl sulfoxide (DMSO) was added to each well after removal of the medium. The optical density was then determined using a Titertek multiscan colorimeter (Flow, Irvine, California) at 570 and 690 nm. The linearity of the MTT assay with cell number was tested for each B16-F10 cell stock before each cell growth experiment. B16-F10 cells transfected with empty vector were used in the proliferation assay as control.

Generation of the GFP-E Fusion Protein. The creation of a fusion protein between protein lysis E and the GFP was chosen as the method for studying intracellular localization. The plasmid pcDNA3.1/GFP was used to perform the subcloning. The E cDNA was obtained from pMC22-E by PCR using 5'- GCTATGGTACGCTGGACTTTG-3' as the forward primer and 5'-GCTCTAGACTCTCCTTCCGCA-3' as the reverse primer. The latter was engineered to eliminate the stop codon from the E cDNA clone containing GFP so that it could be expressed as a fusion protein. A PCR reaction with pMC22-E as the template was performed under the following conditions: 1 × 94℃ for 1 min, 30 × (94℃ for 1 min; 55℃ for 90 s; 72℃ for 90 s), 1 × 72℃ for 10 min. Amplification of the target sequence of the correct size was confirmed by gel

electrophoresis. The PCR product was ligated into pcDNA3.1/GFP vector following the manufacturer's protocol (Invitrogen). The resulting plasmid (pcDNA3.1/GFP-E) was transformed into subcloning efficiency DH5 alpha chemically competent *E. coli* (Invitrogen).The correct DNA sequence was confirmed by DNA sequencing analysis.

Microscopy Analysis. B16-F10 cells were transfected with the pcDNA3.1/GFP-E construction as described above. For mitochondrial staining, the medium was changed to DMEM containing 500 nM MitoFluor Red (MitoTracker, Invitrogen), incubated for 15 min, and then replaced with normal medium. For nuclear staining, DAPI (Invitrogen) was diluted 1:1000 in a 1:1 solution of sterile water and PBS to a final concentration of 100 nM. DAPI solution (1 ml) was added to fixed cells in a 60-mm dish and incubated for 20 min at room temperature. The cells were then rinsed briefly with PBS and mounted. GFP was excited at 488 nm, DAPI nuclear stain at 364 nm, and MitoFluor Far Red at 588 nm. Fluorescent microscopy analysis was carried out with a Nikon Eclipse Ti (Nikon Instruments Inc. NY, USA.). Alternatively, the fluorescence was detected by confocal microscopy using a Leica DMI6000 microscope (Heidelberg, Germany).

Apoptosis Analysis. For analysis of the cell-cycle distribution, parental and transfected cells (pcDNA3.1/GFP-E construction) were harvested, washed twice with sample buffer (100 mg glucose; 100 ml PBS without Ca^{2+} or Mg^{2+}), and fixed in 70% (v/v) cold ethanol for at least 1 h before staining. The cells were pelleted, washed once with sample buffer, and resuspended in PI (propidium iodide) solution (50 µg/ml PI, 0.5 mg/ml RNase in sample buffer, pH 7.4) for 30 min in the dark. A fluorescence-activated cell sorter analysis was performed 24, 48, and 72 h after transfection. Transfected cells treated with the pan caspase inhibitor ZVAD-FMK (BD Pharmingen, San Diego, CA) were also analyzed. Controls were realized with pcDNA3.1 and pcDNA3.1-GFP. The data were collected and analyzed using the Cellfit program with a FACScan flow

cytometer (Becton Dickinson, San Jose, CA, USA). To confirm apoptosis, cells transfected with pcDNA3.1/*E* (without GFP) were washed twice with PBS and incubated in binding buffer containing annexin V-FITC (25 µg/ml) and PI (25 µg/ml) in the dark for 15 min at room temperature (Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA, USA). Microscopy analysis was carried out with a Leica DMI6000 confocal microscope.

Measurement of the Mitochondrial Membrane Potential ($\Delta\Psi_m$). To measure levels of $\Delta\Psi_m$ disruption, parental cells and cells transfected with pcDNA3.1/E) were washed twice with cold PBS and incubated with 40 nM DiOC6(3) (Invitrogen) for 15 min at 37℃. They were then washed with ice-cold PBS and resuspended in 500 µl of PBS. The fluorescence intensities of DiOC6(3) were analyzed on a FACScan flow cytometer with excitation and emission settings of 484 and 500 nm, respectively.

Western Blotting. Thirty micrograms of protein extracts from parental and transfected B6-F10 cells were used for SDS-PAGE in a Mini Protean II cell (Bio-Rad, Hercules, CA). The protein extract from pcDNA3.1/GFP-E transfected cells treated with the pan caspase inhibitor ZVAD-FMK (BD Pharmingen) was also analyzed. The caspase inhibitor (100 µM) was applied 24 h before transfection. The separated proteins were transferred to a nitrocellulose membrane by applying a current of 20 V at room temperature for 30 min. The blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h and then incubated with primary antibodies [rabbit polyclonal IgG anti-caspase-3 (1:1000 dilution), anti-caspase-8 (1:200 dilution), and anti-caspase-9 (1:500 dilution), mouse monoclonal anti-cytochrome c antibody (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal anti-□-actin antibody (1:5000 dilutions; Abcam, Cambridge, MA)] overnight at 4 ℃. After addition of peroxidase-conjugated secondary antibody, proteins were detected by enhanced chemiluminescence (ECL, Bonus, Amersham, Little Chalfont, UK). GFP-E fusion protein was detected

with an Anti-GFP N-terminal antibody (Sigma, St. Louis, MO). Samples were checked for mitochondrial contamination with mouse monoclonal anti-COX IV antibody (1:5000 dilutions; Abcam). The mitochondrial fraction from B16-F10 cells (Mitochondria isolation kit, Sigma) was used as positive control.

Tumor Induction and Measurement. Female C57BL/6 mice (Scientific Instrumentation Centre, Granada University) were used for the *in vivo* study. All mice (weight: 25–30 g) were maintained in a laminar air-flow cabinet in a room kept at 37℃ and 40–70% relative humidity with a 12 -hour light/dark cycle under specific pathogen-free conditions. All studies on animal models were approved by the Ethical Committee of the Medical School of Granada University and performed according to its guidelines. Tumors were induced by subcutaneous injection of $5x10^5$ B16-F10 cells into the left flanks of C57BL/6 mice. Tumors were allowed to grow to the appropriate size (75 mm^3, the ideal minimum size for intratumoral injection) before treatment. After reaching this volume, the tumors were measured at periodic intervals following treatment using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a). The tumor volume was calculated from these measurements using the formula $V = ab^2\pi/6$.

Intratumoral Treatment. *In vivo* JetPEI (Polyplus Transfection, Inc) was used as a transfection enhancer reagent. PEI/DNA complexes with a ratio of 1:6 were prepared in a solution of 10% w/v glucose. This was carried out in a two-step procedure for the preparation of a standard quantity of 20 µg of PEI/DNA complex, according to the manufacturer's instructions. Tumors were then treated intratumorally during 15 days with pcDNA3.1/*E* plasmid or empty vector. A control group, which was not treated, was included. The *in vivo* experiments were conducted twice with a total n =14, with the exception of the control group (n =7).

Immunohistochemistry. Tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 3–5-μm sections. Apoptosis was evaluated by the TUNEL technique using the In Situ Cell Death Detection Kit (Roche). Cell nuclei of cultures were counterstained with DAPI and fluorescence images were captured using a Leica DMI6000B inverted microscope. For measuring proliferation, sections were probed with biotinylated Ki-67 antibody (1:50) (Dako, Spain). After deparaffinization and rehydration, the tissue sections were incubated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase. The sections were blocked for 30 min with goat serum and incubated overnight with the primary antibody at 4 ℃. The sections were then washed with PBS and incubated with a biotinylated secondary antibody for 30 min. After several washes with PBS, the products were visualized using streptavidin horseradish peroxidase with diaminobenzidine as chromogen and hematoxylin as the counterstain. The percent apoptosis and Ki-67 labeling index were determined by counting the number of labeled cells and dividing by the total number of cells in the field (5 high-power fields/slide). Values were presented as the mean ± SD (standard deviation).

Transmission Electron Microscopy (TEM). Melanoma tumors grown in mice were collected, cut into small pieces, and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 1 h. After post-fixation with 1% OsO_4 in cacodylate buffer (room temperature, 2 h), sections were dehydrated through graded ethanol concentrations with a final propylene oxide dehydration. Samples were then embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H7000 transmission electron microscope.

Statistical Analysis. The SPSS 14 software package (SPSS, Chicago, IL, USA) was used for all statistical analyses. Results were compared by using Student's *t* test. All data are expressed as means ± SD. Differences were

considered statistically significant at a *P* value of less than 0.05

3.4.- RESULTS

Inhibition of B16-F10 Cell Growth In Vitro by E Gene. *E* expression in B16-F10 transfected cells was assessed by RT-PCR. As shown in Fig. 1a, an amplification fragment of 223 bp was found in B16-F10 cells transfected with pcDNA3.1/*E* at different time periods, thus indicating the effectiveness and ability of the construction for use in the subsequent experiment. Analysis of the bands, which were normalized by comparison with the β-actin signal, showed a progressive increase of *E* expression (3- and 4.3-fold higher at 48 and 72 h versus B16-F10 cells at 24 h). The B16-F10 cells transfected with pcDNA3.1/*E* showed a significant and time-dependent decrease in cell viability (Fig. 1b), with a 17.2% decrease versus control cultures being observed 24 h after transfection. The decrease in cell viability was 50.3% at 48 h, although the main decrease occurred at 72 h (75.4%). In contrast, the growth of B16-F10 cells transfected with the empty vector (control group) was similar to that of the parental cells.

Subcellular Localization of E Protein and Changes in Cell Morphology. B16-F10 cells were transfected with pcDNA3.1/GFP-E to determine the localization of E protein. After 24 h, expression of the GFP-E fusion proteins showed a clear signal in the cell cytoplasm with a dotted fluorescence pattern (Fig. 2a). Analysis at different post-transfection times showed characteristic changes in the tumor cell morphology, with the main feature after 24 h being the appearance of cytoplasmic extensions (Fig. 2b,c). Many of these cells were swollen and appeared to be vacuolated. The number of rounded cells increased rapidly after 72 h, and they began to progressively detach from the surface of the culture dish and die (Fig. 2d,e,f). The mitochondrial localization of the GFP/E fusion protein was confirmed by dual monitoring of the red fluorescence of Mitofluor, which specifically stains

mitochondria in live cells, and the green fluorescence of GFP. The yellow color in the merged images confirms that GFP-E co-localizes with the MitoTracker dye in the mitochondria (Fig. 3a, b).

Expression of E Gene Induced Apoptosis in B16-F10 Cells. To determine whether *E* gene expression induced apoptosis, B16-F10 cells transfected with pcDNA3.1/GFP-E were analyzed by FACScan. As can be seen in Fig. 4a, the apoptosis fractions were 3.7±0.2% for the parental cells (control) and 4.1±0.34% for the cells treated with pan caspase inhibitor. Similar results were obtained for pcDNA3.1 and pcDNA3.1-GFP transfected cells (2.2±0.2% and 1.9±0.3%, respectively). In contrast, cells transfected with pcDNA3.1/GFP-E for 24 h showed an apoptosis fraction of 23.2±0.81%, significantly higher than that of the control group. After 48 and 72 h of transfection, the percentage of apoptotic cells increased to 30.4±0.52% and 58.3±0.69%, respectively. An annexin V and PI study confirmed the induction of apoptosis by the *E* gene in melanoma cells (Fig. 4b,c).

Expression of E Gene-Induced Modulation of Mitochondrial Membrane Potential. As shown in Fig. 5a significant decrease in $\Delta\Psi_m$, as measured by DiOC6(3) dye staining, was detected in transfected cells, thus indicating an increase in mitochondrial membrane permeability after *E* gene treatment. After 24 h transfection, 27.5% of cells showed a decrease in $\Delta\Psi_m$. This percentage increased progressively with transfection time, and the most prominent $\Delta\Psi_m$ dissipation was observed at 72 h, with 51.8% of cells having altered mitochondrial membrane permeability. No changes in $\Delta\Psi_m$ were detected in B16-F10 transfected with the empty vector or parental cells.

Apoptotic Signaling Pathway Induced by E Gene Expression. Western blot analysis showed that alteration of the $\Delta\Psi_m$ in transfected cells was accompanied by the release of cytochrome c (Fig. 5b). Determination of the caspase expression in the same cells showed enhanced caspase-9 and -3

activation. In contrast, caspase-8 showed no expression modulation (Fig. 5b). Treatment of B16-F10 cells with the pan caspase inhibitor Z-VAD-FMK efficiently inhibited pcDNA3.1/*E*-induced caspase-3 and -9 activation and release of cytochrome c, which appeared at similar levels to those in the control cells (Fig. 5b). All the data indicate that *E* transfection induces mitochondria-mediated apoptosis in melanoma cells.

E Gene Effects on Melanoma Growth In Vivo. The potential of the *E* gene to promote tumor cell death *in vivo* was evaluated by direct injection of the plasmid complexed with jetPEI into B16-F10 subcutaneous mice tumors. Fig. 6a,b show that injection of pCDNA 3.1/*E* gene (20 µg) was able to inhibit tumor growth. The tumor volumes of mice treated with pcDNA 3.1/E were significantly smaller than those of control mice ($p < 0.05$). At the end of observation (day 15), tumor growth was inhibited by up to 90.6% in pcDNA 3.1/E-treated mice compared with control mice. Mice treated with *E* gene showed no evidence of systemic toxicity (i.e. animal death, loss of body weight, other tissue damage, or changes in behavior or aspect). ***After empty vector injection*** with jetPEI, no tumor growth modifications were observed. RT-PCR was performed to ensure the *E* gene was over-expressed in the tumors of mice treated with pcDNA 3.1/E but not in control mice treated with pcDNA 3.1 (Fig. 6c).

In Vivo Analysis of E-Induced Apoptosis. To gain further insight into the mechanism of melanoma growth inhibition by *E* gene *in vivo*, we analyzed the apoptosis-linked DNA fragmentation and the mitotic index using the TUNEL assay and the detection of Ki-67, respectively. As shown in Fig. 7, the number of apoptotic cells (green) was significantly higher in tumors treated with pcDNA3.1/E than in control tissue. As regards the Ki-67 analysis assay, only small differences in nuclear staining could be observed between E-gene-treated (15 days) and untreated tumors. Melanoma tumors after 4 and 8 days of treatment showed similar nuclear staining for Ki-67 to control tumors (Fig. 7). These tumors only showed a small but non-significant decrease in the mitotic

index after 15 days of treatment (data not shown).

Transmission Electron Microscopy. The most important morphological change in the melanoma tumors after pcDNA3.1/E treatment was the pronounced swelling in the mitochondria seen from 2-4 days until the end of treatment. Cells with altered mitochondria exhibited an apoptosis-like aspect consisting of a reduced cell size, formation of vacuoles, and nuclear condensation with chromatin aggregated in large, dense, granular masses which abutted on the nuclear membrane (Fig. 8). In contrast, the control tissue showed giant malignant cells with an intact cell membrane, single- or multiple-nuclei cells, well-preserved organelles, and the presence of typical melanosomes. The mitochondria in control cells and cells treated with vehicle alone remained unaffected (Fig. 8).

3.5.-DISCUSSION

Gene therapeutic approaches which involve genes encoding cytotoxic proteins for tumor cells are being developed as a promising alternative cancer treatment. This is the first study in which the bacteriophage lysis gene *E* was evaluated for its ability to kill melanoma cells *in vitro* and *in vivo.*

New treatment strategies for malignant melanoma are urgently needed because conventional approaches, like chemotherapy and radiation, have little impact on patient survival in the advanced stages of the disease. To date, classical suicide gene therapy systems have not guaranteed the successful treatment of melanoma and have induced only a partially positive response. Recently, it was demonstrated that the administration of GCV in B16-HSV-tk tumors induced in mice was completely ineffective and that GCV may have therapeutic value only as an adjuvant for other T-cell therapies [15]. On the other hand, to increase the low effect of the CD/5-FU system in melanoma, a fusion protein based on the human single chain fragment variable (scFv) human antibody (E8) specific for CEA and yeast cytosine deaminase (yCD) has

been assayed [16]. One of the main limitations of these indirect action systems, however, is the need to use prodrugs [17]. During the last few years, antitumoral strategies based on transfection of the cDNA constructs encoding toxins with a direct action have been developed. The nonsystemic administration of a prodrug in these systems reduces its side effects, its bioavailability limitations, and the need for two consecutive applications of vector and prodrug. Moreover, these genes can be directly expressed in the cytosol of the target cells, thus overcoming the problems (cytotoxicity, internalization efficiency, and resistance acquired by cancer cells) originating from their use as components of immunotoxins or recombinant chimeras [18]. In this context, and as we pointed out previously, some toxic genes have demonstrated their efficacy in cancer gene therapy [6-10]. In melanomas, for example, viral genes encoding toxins such as viral protein R and some plant genes such as SAP have been applied and been found to induce tumoral cell death [19, 20]. We have recently shown that a suicide gene from *Escherichia coli* known as *gef* has a therapeutic effect against these cells [21], and we have now demonstrated that the *E* gene from the fX174 phage not only inhibits melanoma proliferation *in vitro* but is also highly toxic for tumors *in vivo* (growth arrest of more than 90%). Other phage proteins, such as *λ*-holin [22], have led to a substantial reduction in the viability of breast cancer cells *in vitro* and in their growth rates *in vivo*. However, as discussed in more detail below, their effects are not as pronounced as those obtained with *E* gene.

The cytotoxic effects of the *E* protein include alterations in cellular morphology preceding cell death. For example, forty eight hours after induction the cells become rounded and in some cases extensively vacuolated, and they finally become detached from the surface of the culture dish. This abnormal cell morphology strongly suggests the development of apoptosis. Interestingly, an inactivation of apoptotic programmes has been linked to the pronounced clinical chemoresistance of melanoma. Defects in proapoptotic signaling pathways and enhancement of antiapoptotic pathways may contribute synergistically to this

apoptosis deficiency [3, 23]. In fact, some toxic genes, such as SAP, have been found to induce cell death in B16-F10 melanoma cells by direct DNA fragmentation [20]. Analysis of our transfected B16-F10 melanoma cells by FACScan and confocal laser-scanning microscopy clearly showed the ability of the *E* gene to stimulate apoptosis in a time-dependent manner, although its specific mechanism of action remains unclear

The molecular target for the E protein in prokaryotic cells is the enzyme phospho-MurNAc-pentapeptide translocase (MraY), an integral membrane protein involved in bacterial cell wall peptidoglycan biosynthesis, with an essential role being played by peptidyl-prolyl isomerase SlyD [13]. Eukaryotic cells differ fundamentally from prokaryotic cells in terms of their cellular structure, organization, metabolism, and membrane composition. However, since the eukaryotic endomembrane system, including mitochondria, arose in an ancestral prokaryotic lineage [24], bacteriophage genes, including E, might act in cell organelle membranes. In order to analyze this connection, we decided to investigate the possible mitochondrial alterations and the molecular events underlying the apoptosis induced in our transfected B16-F10 cells.

Apoptosis may occur via either death-receptor-dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is triggered by the activation of death receptors such as Fas and TRAIL receptors (DR4, DR5). These go on to activate initiator caspase-8, which then cleaves executioner caspase-3. The mitochondrial pathway is mediated by Bcl-2 family proteins, which disrupt the mitochondria membrane potential and result in the release of apoptogenic factors, such as cytochrome c, from the mitochondria into the cytosol. These factors, in turn, form an apoptosome with apoptosis activating factor 1 and caspase-9 [25]. Treatments that modulate apoptosis, for example with bcl-2-targeted antisense, are a promising new strategy in melanoma treatment [26]. Assays with drugs such as hydroquinone or thiobenzanilides in this tumor have demonstrated caspase-9 activation [27, 28]. This caspase-mediated tumoral cell injury can also be induced by suicide genes. The CD/5FU

system induces activation of caspases-3 and -9 but not caspase-8 in human malignant glioma cells [29], while a modification (the bifunctional *E. coli* CD and uracil phosphoribosyltransferase fusion) is able to induce caspase-3 activation only [30]. HSV*tk*/GCV, on the other hand, activates caspase-3, -8, and -9 in rat bladder carcinomas [31] and a variant (thymidylate kinase) induces apoptosis in Jurkat cells by activation of caspase-3 only. Our studies in B16-F10 cells expressing *E* showed changes to the integrity of the mitochondrial membrane and a significant increase of cytochrome c. This protein is able to activate caspase-9, which in turn activates caspase-3 and other downstream caspases [25]. A western blot analysis showed an increase in active caspase-3 and -9 in transfected B16-F10 cells, which strongly suggests that the cell death induced by the *E* gene is related to the mitochondrial apoptotic pathway. We cannot, however, exclude the possible participation of other apoptosis-mediated molecules in treated B16-F10 induced tumors, such as endonuclease G, Smac/DIABLO, and HtrA2 [32].

Having shown that the *E* gene effectively mediates the killing of melanoma tumor cells *in vitro* and that the mechanism of action involves induction of apoptosis, we further investigated its ability to affect the growth of an *in vivo* model. Statistical evaluation of tumor growth rates obtained from mice treated with pcDNA3.1/*E* complexed with cationic lipids revealed a significantly reduced growth rate in comparison to the untreated mice (90.6% relative volume reduction after 15 days of treatment). This reduction was remarkably superior to that obtained with other phages, such as alpha-holin, in breast cancer (50% at 15 days), with the HSV-tk/GCV system (40-50%) [25] or, more recently, with the SAP gene (67%) [23] in the same tumor. In addition, our results are also superior to those of McCray *et al.* [19], who used the Vpr gene integrated in the pcDNA3.1 vector in melanoma tumor from B16-F10 culture cells to achieve an 86% reduction of tumor volume, although only after 25 days. Suspension of the pcDNA3.1/E treatment again caused tumor growth. This strong *in vivo* antitumoral effect of the *E* gene is consistent with the apoptosis-

inducing ability of this gene demonstrated *in vitro*. TUNEL staining confirmed a significant increase in the number of apoptotic cells in the experimental group treated intratumorally with pcDNA3.1/E. The mitochondrial-mediated apoptotic pathway *in vivo* was also strongly supported by our ultrastructural findings in the induced B16-F10 tumors in mice, which showed dilated mitochondria with disrupted cristae. Finally, we analyzed Ki-6, an antigen which is overexpressed in G1 and S phases but absent in resting cells, to estimate the proliferation intensity [33]. Treated tumors (at different times) did not show significant Ki-67 staining differences in comparison to untreated tumors. Although these results suggest that the *E* gene induces a growth delay in melanoma by inducing tumor cell apoptosis rather than by acting negatively on tumor cell division, further studies will be necessary to support this hypothesis.

In summary, we have reported, for the first time, the ability of the *E gene* to induce the death of melanoma cells *in vitro* and *in vivo.* The successful use of this gene as a new anticancer gene therapy system may establish a role for it in cancer treatment. However, as *E* gene binds to the mitochondrial membrane and its activity is not tumor-specific, adenoviral vectors modified by attaching tumor-specific promoters should be used to assay melanoma treatment. On the other hand, the shorter latency of the *E* gene for effective antitumoral action may be due to a bystander effect, although this has not been demonstrated. We are currently working on experiments to enhance *E* gene activity by combining it with cytotoxic drugs [34] or, as described by Fecker *et al.* [35], by using specific enhancer/promoter genes (such as tyrosinase) to induce tissue-specific expression. We are also working on the integration of therapeutic genes (such us *λ*-holin) into new vectors (such as ReCon) to improve the tumoral response, as reported previously by Brandtner *et al.* [36]. Our results suggest that *E* is a candidate gene for *in vivo* oncologic applications and that it may contribute to the eradication of tumor mass in combination with surgery or classic radio- or chemotherapy.

3.6.- FIGURES

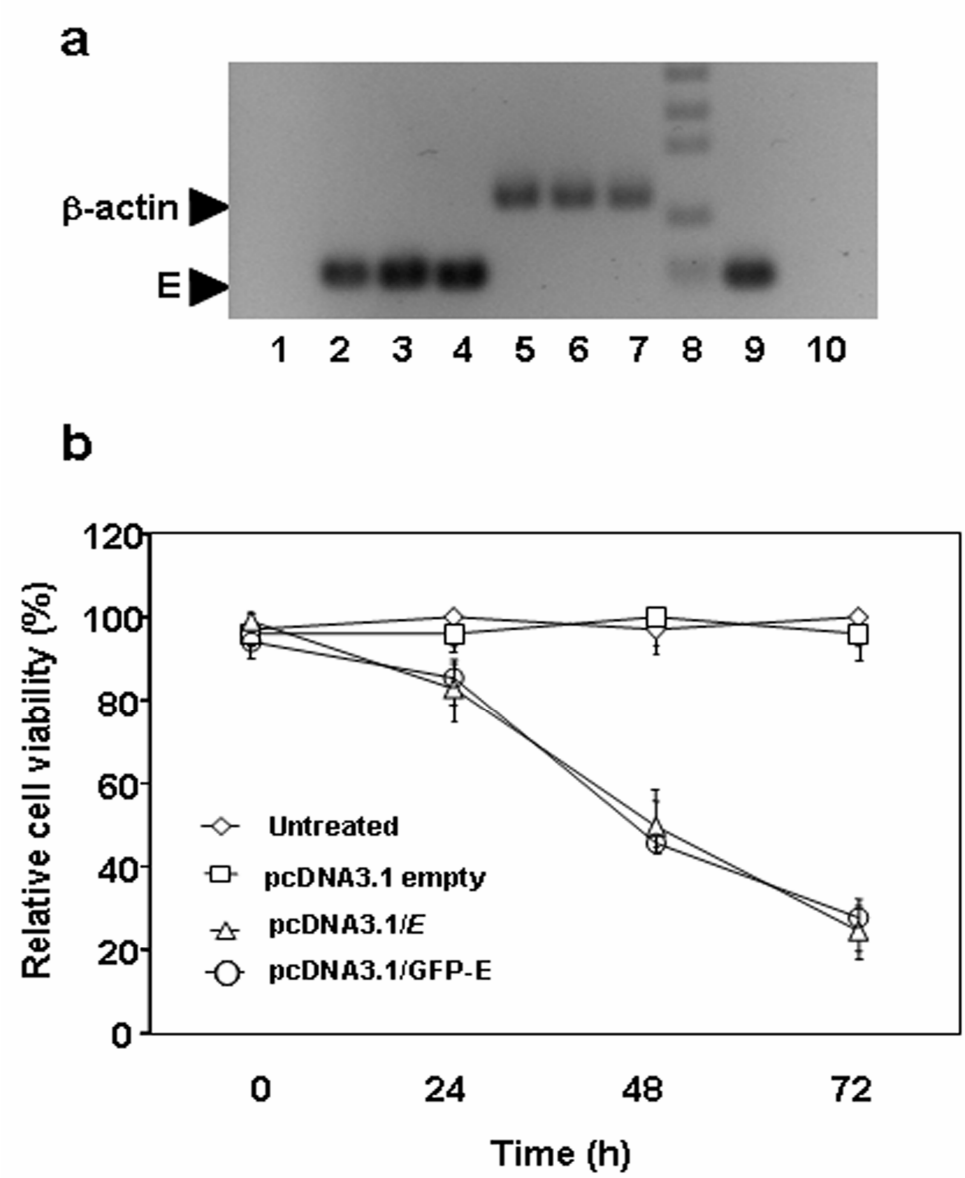

Fig. 1 *E* gene expression and growth rate inhibition in B16-F10 cells. **a** RT-PCR showing *E* gene expression. Lane 1: negative control (RT-). Lanes 2-4: B16-F10 transfected cells at different time periods (24, 48, and 72 h, respectively). Lanes 5-7: the integrity of the RNA was demonstrated using β-actin primers in B16-F10 transfected cells at the same times. Lane 8: molecular weight. Lane 9: positive control (pcDNA3.1/E). Lane 10: negative control (non-transfected cells). **b** Growth of B16-F10 cells expressing *E* was detected by MTT assay. Cells transfected with pcDNA3.1/E and with pcDNA3.1/GFP-E showed a clearly increased cell death compared to the control cells and cells transfected with empty pcDNA3.1 vector ($P < 0.05$). Values represent means ± SD of quadruplicate cultures.

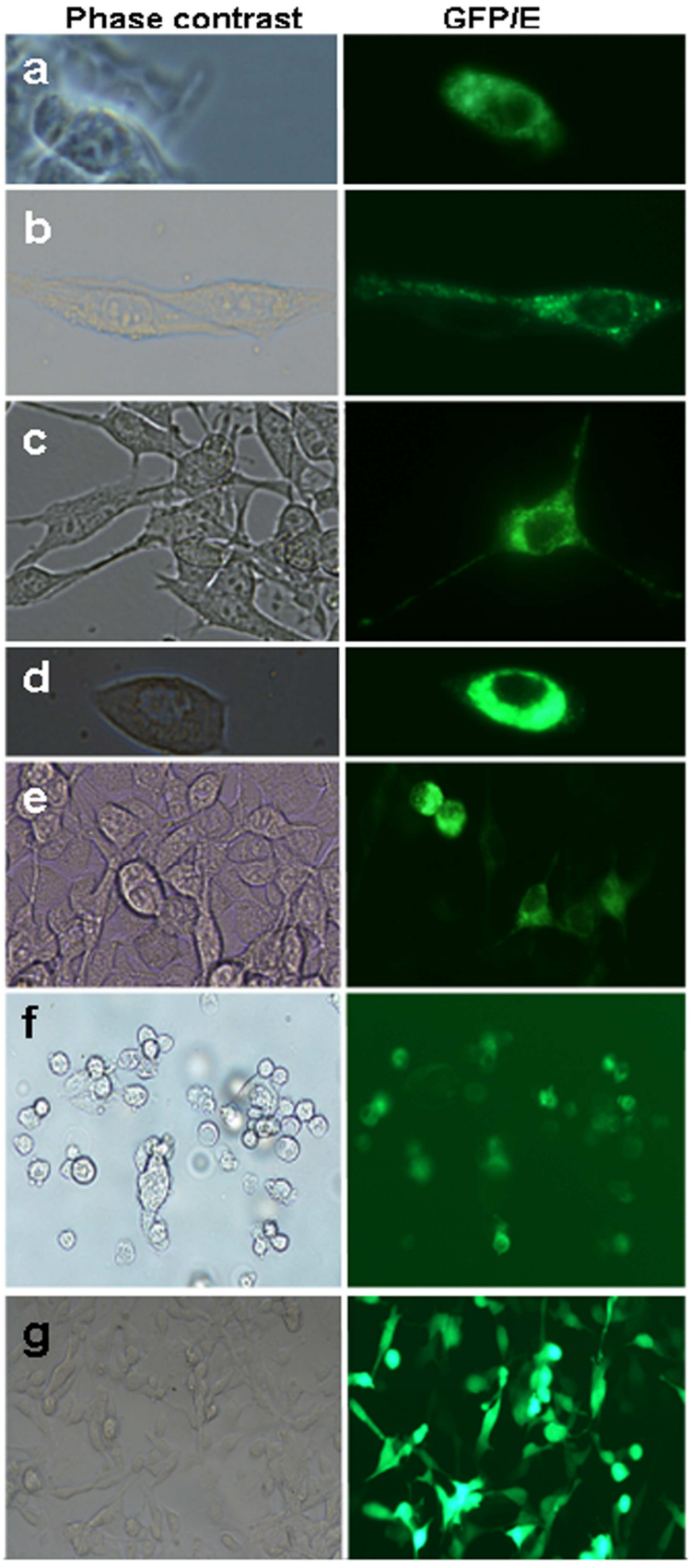

Fig. 2 Subcellular localization of the GFP/E fusion protein expressed in B16-F10 cells. The cells were transfected with a GFP/E fusion construct as indicated in the ‘‘Materials and Methods’’ section. Twenty four hours after transfection (**a**, x40) the fluorescence pattern was dotted and localized in the cell cytoplasm .Cytoplasmic extensions, which progressively increased in length, appeared after 48 h (**b** and **c**, x40). A rapid increase in the number of rounded cells detached from the surface of the culture dish was observed after 72 h (**d**, x40). The cultures contained cells with different morphologies (**e**, x20), although the majority of cells were rounded (**f**, x20). Cells transfected with GFP (without E) show no morphological changes (72 h) (**g**, x20).

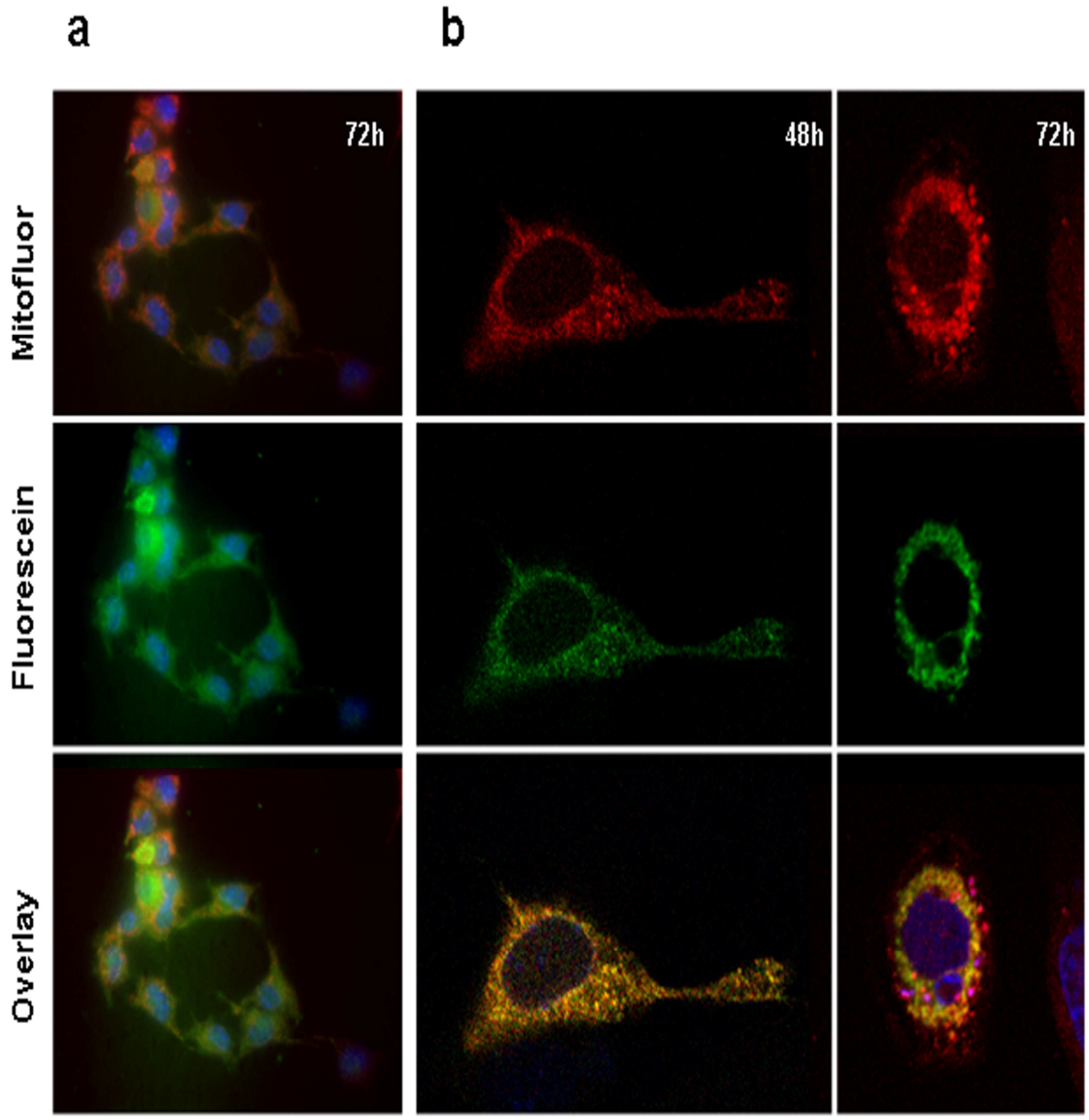

Fig. 3 Mitochondrial localization of the GFP/E fusion protein in B16-F10 cells. Representative image of transfected B16-F10 cells expressing E-GFP at different times taken using fluorescent (**a**) and confocal (**b**) microscopy. The dotted pattern of GFP-E fluorescence is shown in green. The majority of GFP-E expressed co-localizes with MitoFluor, which is shown in red. Co-localization appears in yellow. Cell nuclei were counterstained with DAPI. **a**: x40; **b**: x100.

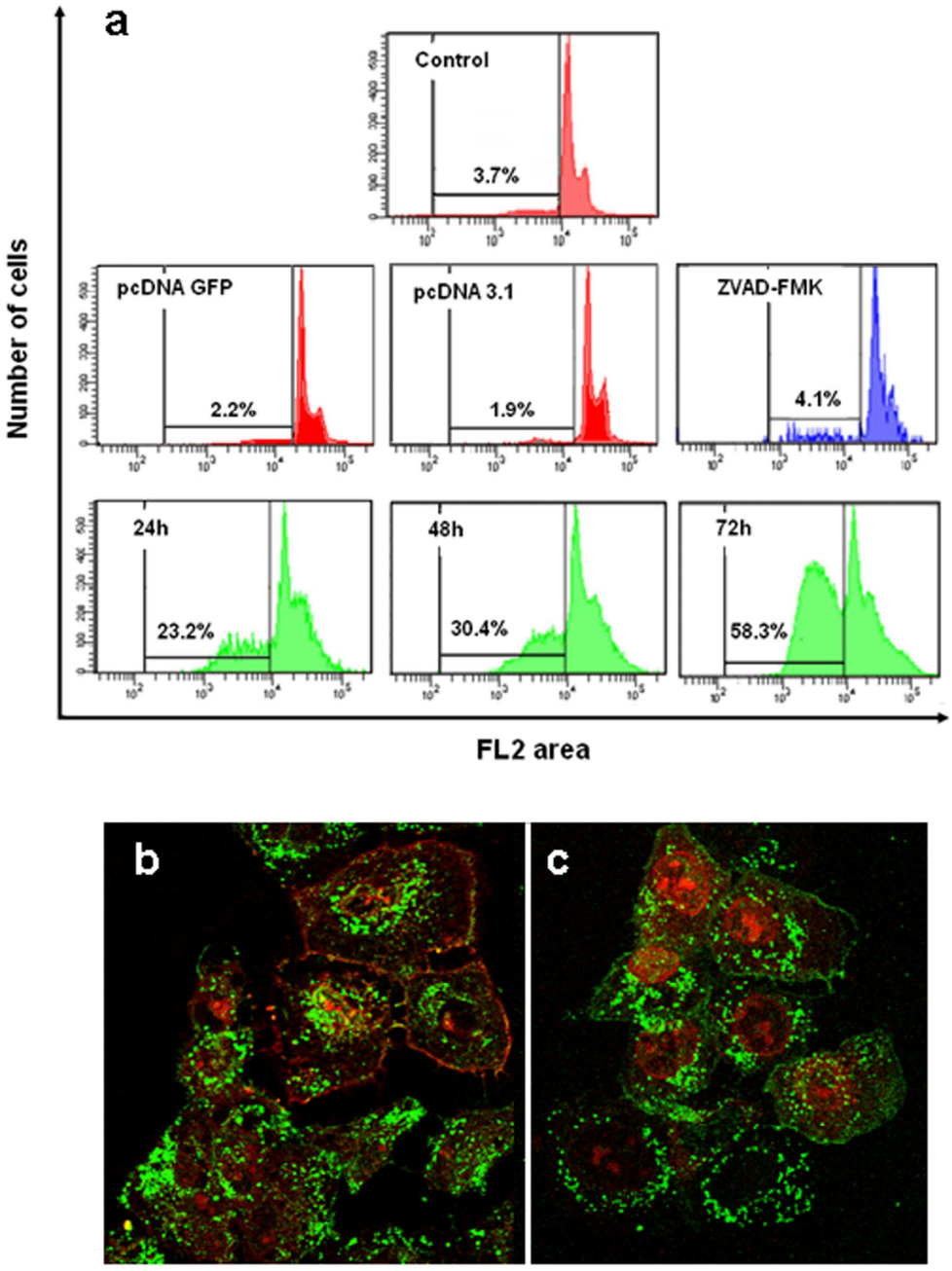

Fig. 4 Analysis of apoptosis induction by the *E* gene in B16-F10 cells. The apoptosis was assessed by PI staining by calculating the percentage of cells in the sub-G1 fraction. Transfected cells treated with ZVAD-FMK were also analyzed at 72 h. These data are representative results from four separate experiments (**a**). To confirm apoptotic induction by E gene expression, B16-F10 cells were transfected with pcDNA3.1/*E* vector and analyzed by annexin V-FITC staining and confocal microscopy. Cell nuclei were counterstained with PI. The figure shows representative images of a stronger staining when the study was carried out at 48 (**b**) and 72 h (**c**) after *E* transfection; magnification, x40.

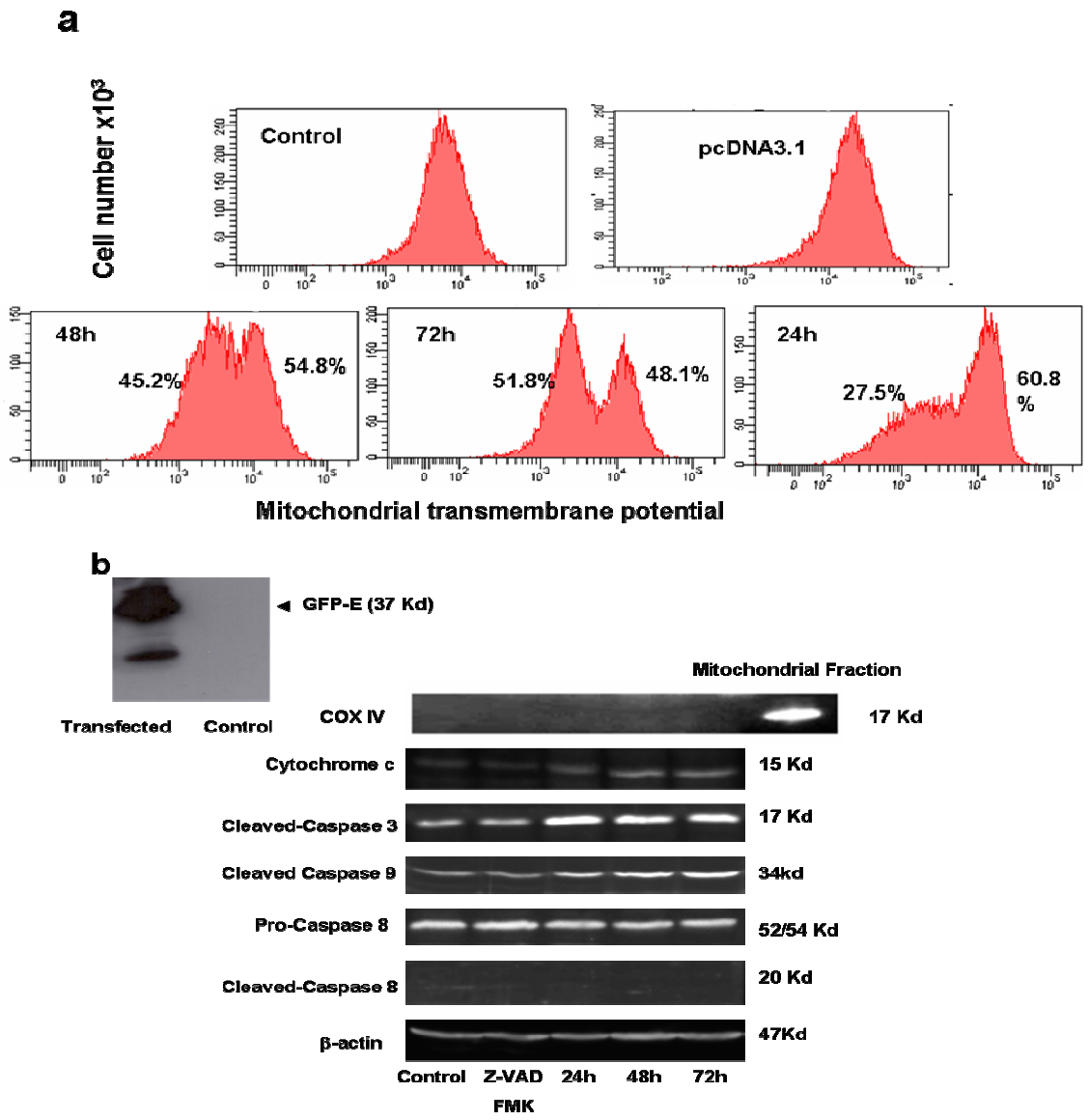

Fig. 5 Apoptotic mechanism induced by *E* gene expression. **a** Mitochondrial membrane potential ($\Delta\Psi_m$) disruption induced by E gene expression in B16-F10 cells. DiOC6(3) was added to the cell suspension (500 µl PBS) for 15 min at a final concentration of 40 nM. The fluorescence intensity of DiOC6(3) was analyzed by flow cytometry. The data shown are representative of three independent experiments. **b** Western blotting analysis of apoptotic signals. GFP-E fusion protein was detected in the B16-F10 transfected cells (24h). Cytoplasmic extracts from B16-F10 parental (control) and transfected cells at 24, 48, and 72 h were prepared to determine cytochrome c and caspase proteins. The pan-caspase inhibitor z-VAD-FMK was applied to determine whether caspases were involved in this process, and the filter was probed with β-actin antibody to determine whether the amount of proteins in each lane was comparable. Mitochondrial contamination was estimated using anti-COX IV antibody. Immunoblots were visualized with an enhanced chemiluminescence detection system.

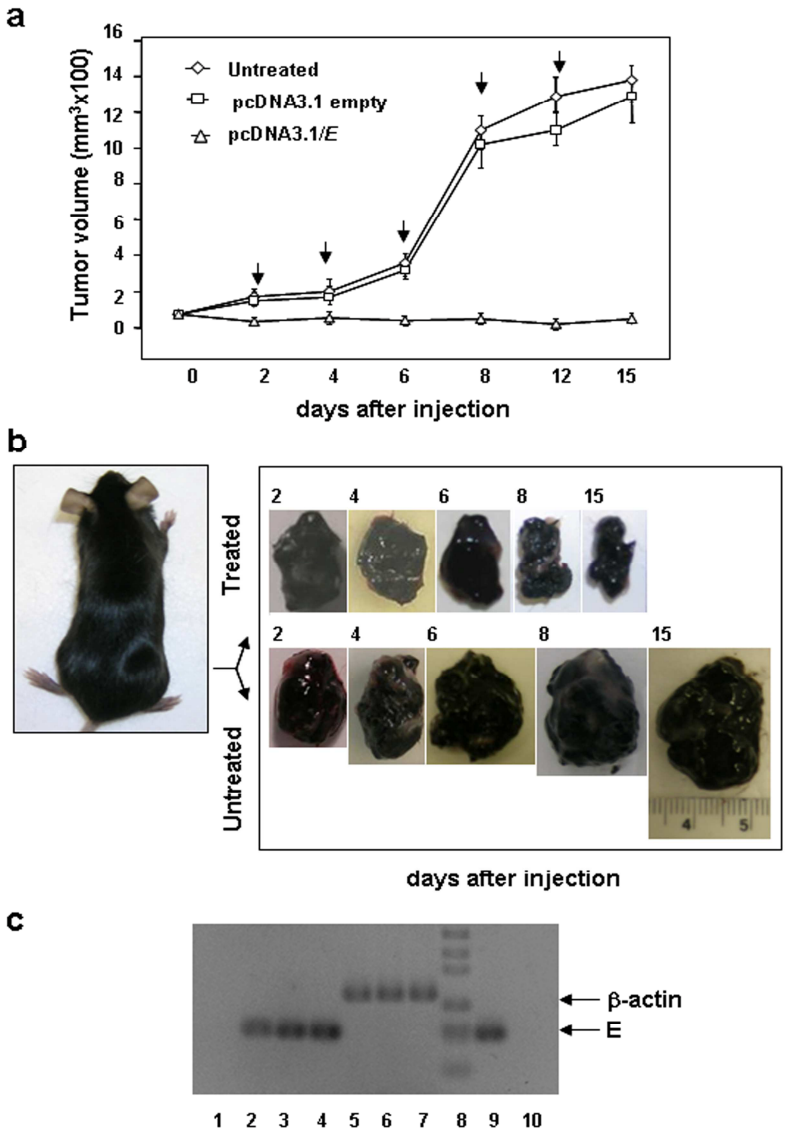

Fig. 6 Effect of direct intratumoral injection of the *E* gene on the growth of subcutaneous tumor induced by B16-F10 murine tumor cells. **a** Tumor volume variation after *E* gene treatment (time of injection is indicated with arrows). The plot shows a significant reduction in tumor volume with respect to untreated tumors or those transfected with empty vector. **b** Representative gross appearance of tumors excised from mice sacrificed during treatment at 2 ,4, 6, 8 and 15 days and tumors obtained from untreated mice at the same time intervals. **c** Representative image of the determination of *E* gene expression in tumor mice. Lane 1: negative control (RT-). Lanes 2-4: *E* gene expression at 4, 8, and 15 days of treatment, respectively. Lanes 5-7: the integrity of the RNA tissue used was determined using β-actin primers. Lane 8: molecular weight. Lane 9: positive control (pcDNA3.1/E). Lane 10: negative control (untreated tumors).

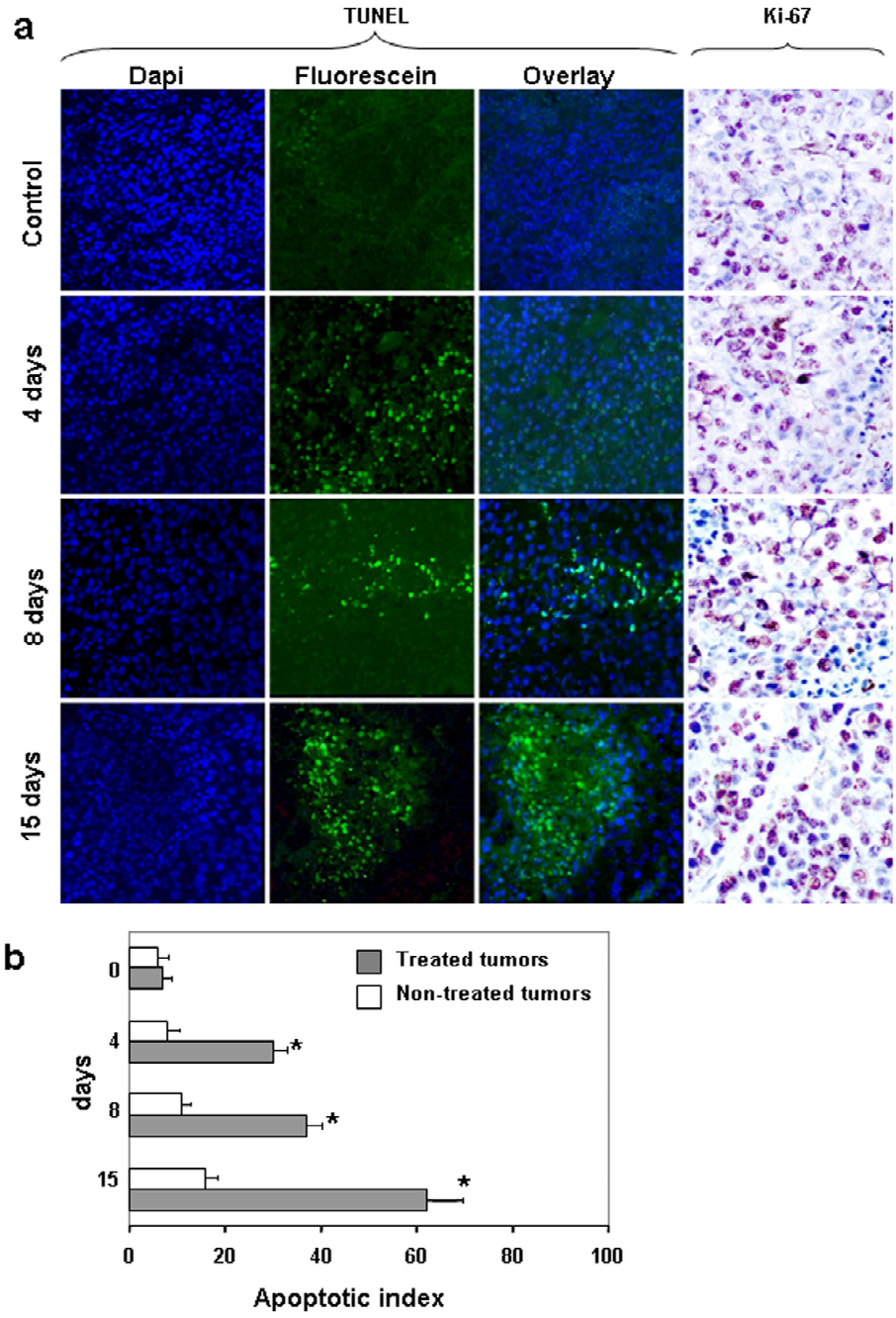

Fig. 7 Histological evaluation of apoptosis and proliferation activity of melanoma cells *in vivo.* **a** Representative photographs of tumor sections showing TUNEL-positive cells (green). Sections were counterstained with DAPI (blue). Apoptotic cells increased in tumors treated with pcDNA 3.1/E at 4, 8, and 15 days compared to untreated tumors (control); magnification, x20. Proliferative activity, as detected by Ki-67 staining, was not significantly modulated. Nuclei were counterstained with hematoxylin; magnification, x40. **b** Percent apoptosis (apoptotic index) in each group. Values are expressed as means ± SD. Level of significance compared to control cells; *P < 0.05 compared with control (day 0).

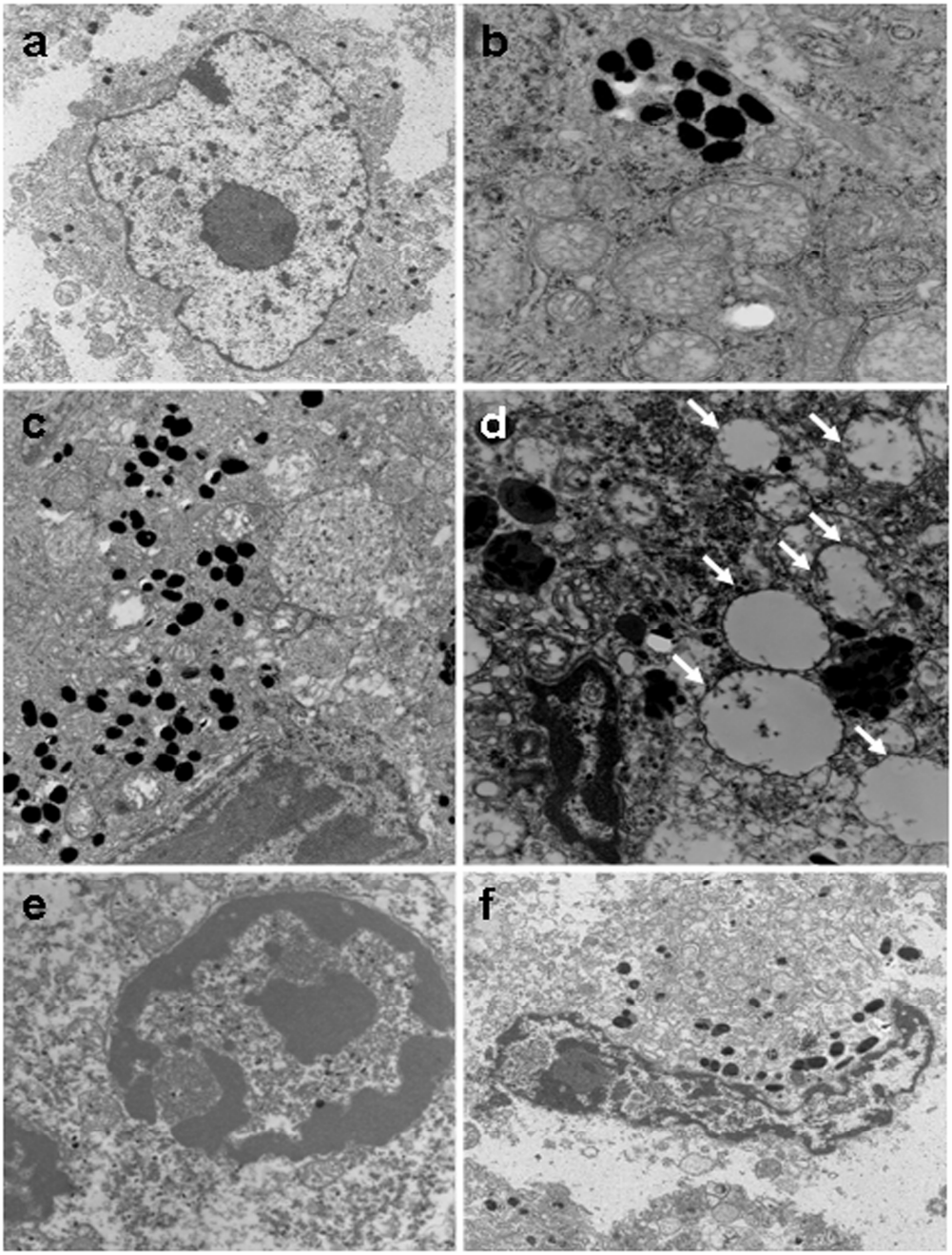

Fig. 8 TEM images of melanoma tumors. Untreated tumors show typical tumor cells with a polygonal shape, a large nucleus, and a light cytoplasmic complexion containing well-preserved organelles (**a**) (x1100), including mitochondria and a large number of melanosomes (**b**) (x4000). Tumors transfected with pcDNA3.1/E also show a large number of melanosomes (**c**) (x2100) but their mitochondria are swollen with no, or with disrupted, cristae (**d**, arrows) (x4000). Ultrastructural characteristics of apoptosis, such as chromatin condensation, crescent formation, and margination, were also observed in the nuclei of melanomas treated with pcDNA3.1/E after 4 days (**e**) (x6300). Necrotic nuclei were observed at the end of the treatment (15 days) (**f**) (x6300).

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V.- DISCUSIÓN

La morbilidad y mortalidad que presentan diferentes patologías, entre las que se encuentran las tumorales malignas, para las que se dispone de terapias más paliativas que curativas, han sido la base para el desarrollo de nuevas formas de tratamiento entre las que incluyen lo que se ha dado en llamar estrategias biológicas terapéuticas. Uno de sus mayores exponentes, la terapia génica somática, combina la utilización de conocimientos de biología molecular, genética clásica y farmacología, con el objetivo de modificar la composición génica de las células y corregir su defecto ya sea éste hereditario o adquirido (Taneja y cols., 1995). El campo de desordenes en el que es aplicable esta nueva vía de tratamiento es muy amplio, siendo uno de los más prometedores el oncológico (Farzaneh y cols., 1998). En él, la terapia génica se dirige fundamentalmente hacia cuatro objetivos terapéuticos: 1) la transferencia de genes supresores de tumores; 2) la transferencia de genes que potencian la inmunidad; 3) la transferencia de genes de resistencia a drogas; y 4) la transferencia de genes suicidas. Los genes suicidas, una más dentro de estas estrategias, una vez transfectados en la célula tumoral, actúan lesionándola ya sea por un mecanismo de acción directa o bien modificando una prodroga para producir una agente terapéutico eficaz. Inicialmente, fueron usados como marcadores fenotípicos de la eficacia de los métodos de transfección, pero su capacidad para generar sustancias tóxicas y lesivas, propicia el desarrollo de los primeros ensayos experimentales. No obstante, son numerosos los factores de los que depende su eficacia como la elección de la célula diana sobre la que se va a actuar; la combinación de promotor/enhancer para la óptima expresión del gen terapéutico; el control de la expresión génica del sistema utilizado y, la eficacia del procedimiento de transfección génica (Gogev y cols., 2003).

.-Diferentes sistemas de genes suicidas han sido aplicados al tratamiento del cáncer

Han sido diferentes los sistemas que han intentado lesionar las células tumorales y también diferente el éxito que han tenido en las diferentes experiencias llevadas a cabo. Uno de los sistemas más explorados ha sido el que se basa en la idea central de que si la activación genes suicidas puede prevenir la formación de tumores inducidos por vectores virales que han sido transfectados en células normales, el mismo gen insertado en el genoma de una célula tumoral puede, tras la administración de la adecuada prodroga, inducir la destrucción de la célula. Así, el gen de la timidín-kinasa del virus herpes simplex (HSV-tk) transfectado en células normales y tumorales y, tras la exposición a drogas anti-herpes como aciclovir (ACV) o ganciclovir (GCV), conduce indefectiblemente a la muerte celular in vitro e in vivo (Van Dillen y cols., 2002). La acción de esta enzima sobre GCV es más efectiva, provocando la fosforilación de la prodroga en metabolitos di (GCV-DP) y trifosfato (GCV-TP) que inhiben la acción de la ADN polimerasa (Singhal y Kaiser, 1998).

De forma casi paralela a la utilización del sistema HSV-tk, una nueva enzima la citosin-desaminasa (CD) comenzó a utilizarse con la esperanza de una mejora en el pronóstico del tratamiento del cáncer. Esta enzima presente en bacterias y hongos (también en cantidades no significativas en células de mamífero) es capaz de convertir una droga inerte, la 5-Fluorocitosina (5-FC), en 5-Fluorouracilo (5-FU), que fosforilado a forma monofosfato y trifosfato, se convierte en un agente tóxico (Smythe, 2000) capaz de inhibir irreversiblemente la timidilato-sintetasa y la transcripción del RNA, lo que conlleva a la muerte celular. La eficacia de este sistema fue demostrada en tres líneas de ratón (NIH-3T3 de fibroblastos, 207-10 de sarcoma y 38-2 de adenocarcinoma de colon) transfectadas con CD y posteriormente, en células humanas (Austin, 1993) de cáncer colorrectal (línea WiDR) en las que se observó un incremento de 560 veces de la toxicidad de 5-FC.

Aunque los citados anteriormente son los más investigados, en los últimos años han aparecido nuevos sistemas que intentando mejorar los resultados previamente obtenidos, han tenido diferente suerte en los ensayos in vitro e in vivo realizados. Así, la enzima nitrorreductasa de Escherichia Coli es capaz de convertir la prodroga CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamida] en un derivado 4-hidroxilamino que después de acetilarse, se transforma en un potente agente tóxico con acción sobre los mecanismos de reparación de DNA, generando lazos cruzados ("crosslinks"). La expresión de este gen en diferentes líneas celulares provoca su sensibilización al CB1954 (McNeish, 1998), presentando una gran ventaja respecto a otros sistemas de genes suicidas, su independencia del ciclo celular, por lo que potencialmente puede actuar sobre las células quiescentes. El gen de la enzima desoxicitidina kinasa (dCK) ha sido ensayada en terapia génica, por su capacidad para fosforilar algunos análogos de nucleótidos. La inserción de dCK en una línea de glioma aumenta la toxicidad de los antimetabolitos generados (Hapke, 1996). En este mismo tipo de tumor (líneas MCF-7 y 9L) la transfección del gen la enzima citocromo P-450 provoca un aumento de la sensibilidad a la ifosfamida tanto in vitro como in vivo, según experiencias realizadas por Chen y cols. (1996). El gen gtp de Escherichia Coli que codifica para la enzima xantina/guanina fosforribosil-transferasa (XGPRT) es capaz de fosforilar la prodroga 6-tioxantina convirtiéndola en 6-tioxantina monofosfato (6-XMP). Ésta, una vez convertida en 6-tioguanina monofosfato se transforma en un agente altamente tóxico, habiendo sido demostrado su efecto en células tumorales de glioma (Tamiya y cols., 1996).

.-Terapia antitumoral con genes suicidas de acción directa

Hasta la fecha, los sistemas de terapia génica basada en genes suicidas que precisan de la utilización de una prodroga, presentan importantes problemas, entre los que cabe destacar los relacionados con la toxicidad, la

liberación y la bioviabilidad de la droga activa una vez generada. De hecho, éstas son algunas de las causas de los diferentes fracasos cosechados en la realización de experiencias preclínicas y clínicas en los últimos años. Como alternativa, se han desarrollado sistemas con genes suicidas independientes de la administración de una prodroga o, dicho de otra forma, sistemas que sean capaces de inducir directamente la muerte o la disminución de la proliferación de las células tumorales. Así, desde hace bastante tiempo se conoce el sistema linamarasa/linamarina se basa en la hidroxilación de la linamarina por una glucosidasa (linamarasa) que provoca la liberación de glucosa y cianuro, elemento altamente tóxico para la célula (Cortés y cols., 1997). Mediante la transfección del gen del factor de transcripción E2F-1, que estimula la progresión del ciclo celular de la fase G1 a la fase S, se ha conseguido inducir apoptosis en células de cáncer de mama (Hunt y cols., 1997). Sistemas como el del gen de la apoptina, una proteína derivada del virus de anemia de pollo, tienen efecto lesivo sobre células de osteosarcoma humano induciendo apoptosis (Zhuang y cols., 1995). El gen que codifica la metioninasa de la Pseudomonas Putidia ha sido utilizado en terapia génica. Esta enzima degrada la metionina extracelular, esencial para el crecimiento de la mayoría de los tumores. Varios estudios han demostrado que la infección de las células tumorales mediante adenovirus con este sistema tiene un efecto antitumoral in vitro e in vivo (Yoshioka y cols., 1998).

En este contexto, nosotros hemos analizado el gen E perteneciente a una familia de genes de lisis y localizado en el fago ΦX174, como posible candidato a ser usado en la terapia génica antitumoral debido a su acción como formador de poros en la membrana celular. Junto a él, hemos estudiado el efecto de Gef, un gen de Escherichia Coli que codifica para la muerte celular al igual que otros como hok, flmA, srnB, y pndA que aparecen normalmente en plásmidos. Este último codifica para proteínas pequeñas y muy parecidas en el extremo C-terminal hidrofílico (50 aminoácidos) que “agujerea” la membrana, provocando un flujo saliente de Mg^{2+} y entrante de moléculas periplásmicas

como la RNasaI. No se conoce su mecanismo íntimo de acción, aunque se han considerado dos modelos: la interacción de la proteína con una proteína compleja de membrana y, la formación de un oligómero que abre poros en ésta, sin que exista un receptor específico. Lo que si se ha podido demostrar es que, seis horas después de su expresión, el gen Gef hace que la célula procariota aparezca como una célula "fantasma" no viable, con un centro translúcido y un material celular condensado en los polos. Experiencias previas con este gen han demostrado su acción sobre la membrana de células eucarióticas pero no se conoce su efecto in vivo (Boulaiz y cols. 2003).

.-Aplicación del gen Killer Gef en cáncer de pulmón

A pesar de los avances terapéuticos, la terapia convencional del cáncer de pulmón ha dejado de mejorar las tasas de supervivencia en pacientes con CPNM. El diagnóstico tardío y la habitual alta tasa de mortalidad del cáncer de pulmón, junto con la ineficacia y los efectos perjudiciales de la quimioterapia y la radioterapia, han desarrollado la necesidad de la adopción de nuevos enfoques de tratamiento. En este contexto, la terapia génica aparece como una nueva vía mediante la que diseñar protocolos de actuación que deberán primero ser testados a nivel experimental en cultivos celulares y luego en animales de experimentación, pero que pueden derivar en la realización de ensayos clínicos. Además, la expectativa que ha creado la utilización de terapia combinadas en las que la terapia génica es un adjuvante del tratamiento con citotóxicos, justifica la investigación en este campo (Fang y Roth, 2003). Durante el trabajo desarrollado en esta tesis se exploró una terapia combinada con el gen killer gef y el paclitaxel, como un nuevo enfoque para el tratamiento de cáncer de pulmón.

Cuando transfectamos el gen killer gef en la línea A-549 de cáncer de pulmón de células no pequeñas, la supervivencia celular fue significativamente menor frente a las células control entre las 48-96 h, con la mayor disminución

en la tasa de proliferación a las 72 h (55%). Efectos similares de inhibición sobre el crecimiento de la línea A-549 fueron reportados por Narumi y cols. (1998) mediante el uso de proteínas citolíticas formadoras de poros (perforinas), que también se unen a la membrana de las célula tumorales. Curiosamente, la disminución en el número de células del tumor observado en nuestros experimentos no se asoció a la apoptosis, a pesar de una pérdida progresiva de la cultura monocapa uniformidad. También se han encontrado estos mecanismos no apoptóticos en genes que no son de mamíferos, y que han sido usados en la terapia génica contra el cáncer (Katabi y cols., 2002). Recientemente se ha demostrado la inhibición del crecimiento en células de cáncer de mama mediante el uso de la proteína del bacteriófago λ-Holin, que es una proteína que puede permeabilizar la membrana bacteriana (Agu y cols., 2007). Mediante el uso de este gen se ha visto que a las 48 h de inducción, las células comenzaban a volverse multinucleadas, con gran contenido vacuolar y luego se despegaban.

Estos resultados indican que los efectos citotóxicos de la proteína λ-holin incluyen alteraciones en la morfología celular anterior a la muerte. El gen gef disminuye el potencial de membrana, provoca la pérdida de integridad de membrana, así como también provoca cambios morfológicos (Katabi y cols., 2002). Sin embargo, su mecanismo de acción específico en las células eucariotas no lo habíamos dilucidado aún. Las células eucariotas se diferencian fundamentalmente de las células procariotas en su estructura celular, la organización, el metabolismo y la composición de la membrana. A pesar de esto, ya que el sistema de endomembranas de las células eucariotas deriva de los procarióticas (Emelyanov, 2003), el gen gef podría actuar sobre las membranas de los orgánulos de las células eucariotas. De hecho, toxinas bacterianas, como la citolisina del Vibrio cholerae cytolysin o la proteína VacA de la Helicobacter pylori actúan directamente sobre la membrana citoplasmática de células eucariotas (Coelho y cols., 2000). Esta hipótesis con respecto al gen gef, está fuertemente apoyada por nuestros resultados, que

demuestran cambios ultraestrucuturales en las células A-549 transfectadas, con dilatación de las mitocondrias, ruptura de crestas mitocondriales, evaginaciones de la membrana citoplásmica y un menor contenido y tamaño de microvellosidades.

Después de establecer la eficacia del gen gef en la inhibición del crecimiento en células humanas de cáncer de pulmón, investigamos su uso en una terapia combinada con el paclitaxel, un fármaco de elección para el tratamiento del cáncer de pulmón. La combinación de estrategias de terapia génica con terapias convencionales puede mejorar la eficacia de estas últimas. Así pues, la respuesta anti-tumoral de fármacos quimiterápicos se ha mejorado con el uso combinado de HSV-tk en el cáncer de vejiga (Shieh y cols., 2006), con p53 en el cáncer de mama (Cristofanilli y cols., 2006) y con E2F-1 en el melanoma (Hao y cols., 2006). La terapia combinada con docetaxel o paclitaxel y los genes p53 o la IL-12 mejoran los resultados en el cáncer de pulmón (Nishizaki y cols., 2001). El paclitaxel, que presenta actividad contra un amplio rango de tipos tumorales, también mostró mayor eficacia cuando se combina con la terapia génica en cáncer de ovario y de mama metastásico (Janát-Amsbury y cols., 2006). Durante el trabajo de esta tesis nosotros hemos utilizado la combinación de paclitaxel y la terapia génica con el gen gef en MTS de la línea celular de cáncer A-549. Con este modelo hemos tratado de imitar la realidad del medio ambiente celular y dar una imagen más real de los efectos de los fármacos, mediante la inclusión de limitaciones en la penetración, distribución y mecanismos de señalización celular (Kostarelos y cols., 2004). El análisis de los resultados obtenidos tras el uso de la terapia combinada en los sistemas MTS de A-549 demostró una importante inhibición del crecimiento después de 96 h, en comparación con el control de MTS. La mayor reducción se obtuvo con el uso del gen gef y con una dosis de paclitaxel 1μM. Esta inhibición fue mayor que la obtenida por medio de la terapia génica o la quimioterapia solas y que la suma de ambas, de tal manera que se produce un

potenciamiento de la terapia convencional con el uso de la terapia génica. Esto sugiere su potencial terapéutico en el tratamiento del cáncer de pulmón.

.-Aplicación de los genes Killer E y Gef en melanoma

En otro de los objetivos desarrollado durante esta tesis, hemos demostrado el potencial del uso de los genes killer E y gef en el tratamiento in vitro e in vivo del melanoma.

Hasta la fecha, la terapia genética con genes killer clásicos no han garantizado el éxito en el tratamiento del melanoma y han inducido sólo parcialmente una respuesta positiva (Sanchez-Perez y cols., 2007; Zamboni y cols., 2008). Una de las principales limitaciones de estos sistemas de acción indirecta es la necesidad de utilizar prodrogas (McKeowny cols., 2004). Los resultados obtenidos durante esta tesis han demostrado que la transfección mediante el pcDNA 3.1/E-gef, no sólo inhibe la proliferación del melanoma in vitro (provocando una inhibición del crecimiento celular de un 75% y un 35% a las 72h, los genes E y gef respectivamente), sino también que es muy tóxico para los tumores in vivo. El tratamiento con el gen E indujo una disminución significativa en el crecimiento del tumor (en torno a un 70% de reducción del volumen después de 8 días de tratamiento), al igual que con el gen gef (en torno a un 80% de reducción del volumen después de 8 días de tratamiento). Otros tratamientos experimentales con HSV-tk/GCV (Soubrane y col., 1999) o más recientemente con el gen de SAP (Zarovni y cols., 2007) en el mismo tipo de tumor, inducian un 40-50% y 67% de reducción del volumen tumoral, respectivamente. McCray y cols., en 2006, utilizó el gen Vpr integrado en el pcDNA3.1 en tumores de la línea B16-F10 de melanoma, describiendo un 86% de reducción del volumen tumoral, pero requiriendo para ello de 25 días de tratamiento, mientras que nosotros obtuvimos con el gen E un 90,6% de reducción del volumen tumoral después de 15 días de tratamiento. Por lo tanto, la principal ventaja de los genes killer E y gef no es sólo su eficacia en las

células del melanoma, sino también un periodo de inducción más corto para que sea eficaz su acción antitumoral.

Nuestros resultados demuestran que aproximadamente a las cuarenta y ocho horas después de la inducción de los genes E y gef, las células de B16-F10 comenzaban a volverse multinucleadas, con gran contenido vacuolar y luego se despegaban. Experimentos de microscopía confocal con anexina V, han demostrado que tanto los genes E como gef son capaces de inducir apoptosis. Estos resultados son similares a los obtenidos con el gen de la Saponina, que también induce la muerte celular programada y mediante la fragmentación del ADN en células de B16-F10 (Zarovni y cols., 2007). Curiosamente, la pronunciada quimiresistencia que presenta el melanoma se debe a fallos en la ruta de señalización de apoptosis que dan lugar a que no se produzca, de tal manera que el uso de proapoptóticos que minimizan esos defectos en vías de señalización o que potencien la apoptosis pueden contribuir a esta deficiencia de la apoptosis (Soengas y Lowe., 2003). Análisis por técnicas de TUNEL por inmunohistoquímica en cortes de tumores tratados con pcDNA3.1/E-gef, pusieron de manifiesto que el tratamiento aumentó de manera significativa la apoptosis los tumores de melanoma in vivo. La incidencia de la apoptosis en el tumor se correlacionaba con el efecto de inhibición del crecimiento tumoral, lo que nos sugiere que nuestro tratamiento experimental provoca un regresión tumoral por el aumento significativo de la apoptosis.

El fenómeno de la modulación de la apoptosis se observar en muchos tratamientos, por ejemplo con moléculas antisense de bcl-2, que parece ser una prometedora nueva estrategia en el tratamiento del melanoma (Moreira y cols., 2006). Ensayos con drogas como la hidroquinona o tiobenzoanilidos en este tipo de tumor han demostrado un mecanismo de acción relacionado con la activación de la caspasa-9 (Fernandes y cols., 2008; Hu y cols., 2008). Esta lesión en células tumorales mediada por caspasas también pueden ser

inducidos por los genes killer, de hecho, el sistema CD/5FC induce apoptosis en células de glioma maligno humano por la activación de las caspasas-3 y -9, pero no la caspasa-8 (Kurozumi y cols., 2004). Sistemas como la citosina desaminasa y la uracil fosfotransferasa de E. coli, son capaces también de inducir apoptosis a través de la activación de la caspasa-3 sólo (Gopinath y cols., 2007). El HSVtk / GCV activan las caspasas-3, -8 y -9 en carcinomas de vejiga en ratas (Shibata y cols., 2003) y una variante, la timilidata quinasa, induce la apoptosis en células Jurkat por activación de caspasa-3 (Sato y cols., 2007). Nuestros estudios en las células B16-F10 al expresar la proteína E y gef demostraron alteración de la integridad de la membrana mitocondrial, lo que nos sugirió que la apoptosis podía estar mediada por la vía mitocondrial. Esta hipótesis está respaldada por el incremento de la caspasa-9 y -3 activada mientras que la caspasa-8 no sufría activación en las células B16-F10 transfectadas con E y gef. Además, los estudios con DIOC demostraron que el potencial de la membrana mitocondrial estaba alterado en la mayoría de la población celular transfectada, asi como los cambios ultaestrucuturales que muestra las mitocondrias (dilatadas y con las crestas rotas), lo que apoya la hipótesis sobre el posible efecto proapoptótico vía mitocondrial de los genes E y gef en melanoma in vitro. Por otro lado los ensayos in vivo demuestran que la caspasa-9 aumenta significativamente después del tratamiento con los genes E y gef, apoyando de nuevo la teoría de la apoptosis por la vía mitocondrial in vivo. Sin embargo, no podemos excluir la posible participación de otras moléculas de mediadoras de apoptosis como la endonucleasa G, SMAC / DIABLO y HtrA2 (Keeble y cols., 2007). De tal manera que se requieren más estudios para dilucidar los mecanismos exactos involucrados.

En resumen, hemos informado por primera vez de la capacidad de los genes E y gef para inducir la muerte de las células de cancer de pulmón in vitro (y en MTS) y en melanoma in vitro e in vivo. La utilización con éxito de estos genes podria establecer una nueva estrategia de terapia génica en el tratamiento del cáncer. Sin embargo, como genes E y gef se unen a los

sistemas de endomembranas celulares y su actividad no es específica del tumor, es necesario que se lleve a cabo su utilización en vectores adenovirales asociados a promotores tejido-específico del tumor, para ser usado de manera eficiente en el tratamiento del cáncer de pulmón y melanoma. Por otro lado, el efecto más rápido de los genes E y gef, que otros Killer hace pensar en un posible efecto bystander, aunque aun seguimos investigando para dilucidar si realmente se produce. Actualmente nos encontramos trabajando en experimentos para mejorar la actividad de los genes E y gef mediante la combinación con fármacos citotóxicos (Prados y cols., 2008), como se describe por Fecker y cols., en 2005, o mediante el uso específico de promotores tejido específico (como la tirosinasa) para inducir la expresión específica del tejido. También estamos trabajando en la integración de genes terapéuticos (por ejemplo los λ-Holin) en nuevos vectores (como Recon) para mejorar la respuesta tumoral, como se informó anteriormente por Brandtner y cols., en 2008. Nuestros resultados sugieren que tanto el gen E como el gef son buenos candidatos para aplicaciones oncológicas tanto in vitro como in vivo y que puede contribuir a la erradicación de la masa tumoral, en combinación con la cirugía clásica, la radio o la quimioterapia.

VI.- CONCLUSIONS

- Gene therapy by gef gene achieved a marked antitumor effect in lung cancer line A-549 which was observed in inhibition of cell survival from 55% at 72h and a reduction in the volume of spheroids tumor (MTS) of 35.2% at 92h of treatment.

.- Gene therapy by gef gene has an enhancer effect of the antitumor action of paclitaxel, cytotoxic agent of choice in the treatment of lung cancer. This effect was observed both in cell cultures and in MTS from A-549.

- The E and gef genes possess a potent cytotoxic effect on melanoma cells (B16-F10), causing an inhibition of cell growth by 75% and 35% at 72h, respectively

.- The genes gef and E have a potent in vivo antitumor effect on melanoma induced in mice causing a regression of the tumor volume of 70-80% after 8 days of treatment.

.- The expression of genes gef and E produces a morphological and functional alterations of mitochondria in cells from melanoma (B16F10). The morphological alteration is characterized by dilation and rupture of mitochondrial crests while the functional alteration involves a significant decrease of membranepotential.

.- The mechanism of action of E and gef genes in melanoma B16F10 line causes the activation of apoptosis through the mitochondrial pathway, raising the levels of activated caspase 3 and 9. This mechanism of action was confirmed for both genes in vivo studies using TUNEL.

.- The product of the E gene (E protein), when is expressed in melanoma cells (B16F10), tends to be located in the membrane of mitochondria. Its has been able to demonstrate its presence in this organelle.

.- The experimental results with killer genes gef and E indicate their effectiveness as tools for the development of gene therapy protocols that alone or in combination with cytotoxic agents can improve the response of patients with lung cancer and melanoma

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VIII.- PUBLICACIONES

Combined therapy using suicide *gef* gene and paclitaxel enhances growth inhibition of multicellular tumour spheroids of A-549 human lung cancer cells

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Abstract. The low efficiency of conventional therapies in achieving long-term survival of lung cancer patients calls for development of novel options. The potential use of combined gene therapy is under intensive study. One approach uses the expression of genes encoding cytotoxic proteins that affect cellular viability. The *gef* gene from *E. coli*, identified as a member of a gene family encoding homologous cell-killing functions, encodes for a membrane protein with a toxic domain which leads to a decrease in the rate of tumour cell growth. To improve the antitumoral effect of the paclitaxel in lung cancer cells, we investigated a combined suicide gene therapy using this drug and *gef* gene *in vitro*, using A-549 lung cancer cells in culture and forming multicellular tumour spheroids (MTS). Our results showed that *gef* expression in A-549 cells led to an ultrastructural changes, including dilated mitochondria with clear matrices and disrupted cristae and cell surface alterations such as reduction in length and number of microvilli and cytoplasmic membrane evaginations. The use of paclitaxel in A-549 lung cancer cells transfected with *gef* gene enhanced the chemotherapeutic effect of this drug. Volume analyses showed an 87.4% decrease in the A-549 MTS growth after 96 h in comparison with control MTS. This inhibition was greater than that obtained using the gene therapy or chemotherapy alone. In conclusion, *gef* gene has a cytotoxic effect in lung cancer cells and enhances cell growth inhibition when used with paclitaxel. These results indicate that this combined therapy may be of potential therapeutic value in lung cancer.

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Introduction

Lung cancer is the leading cause of cancer-related mortality in both men and women. Non-small cell lung cancer (NSCLC) represents about 75-80% of all lung cancers, and most of these patients are in advanced stage at diagnosis (1). Although chemotherapy has recently shown promising results in adjuvant strategies for early-stage patients (2) and some progress has been made in the treatment of locally progressive and advanced disease (3), latest studies suggest that a therapeutic plateau has been reached and that novel, more specific and less toxic therapeutic strategies are needed (4). A number of gene therapy techniques have been developed, but their safety and efficiency remain unsatisfactory. However, interest is growing in the development of combined approaches using gene therapy and local tumour irradiation or chemotherapy (5). The combination of gene therapy with various drugs has been shown to enhance tumour cell killing. Recently, novel advances in the combined use of suicide gene therapy and antitumour drugs have been reported in bladder cancer (6), pancreatic cancer (7) and breast or colorectal cancer (8). However, few studies of this type have been performed in lung cancer. In fact, classical strategies using a suicide gene e.g., herpes simplex virus thymidine kinase (HSV-tk), have shown beneficial effects but with some limitations (9). They are able to convert a non-toxic prodrug into a toxic metabolite, but the release of toxic metabolites and their bioavailability are two important shortcomings of the use of these systems (10). Therefore, increasing attention is being paid to the transfer of genes that are not dependent on the use of a prodrug. Our group recently developed a new cancer gene therapy strategy using a toxic gene from the chromosome of *E. coli* (*gef*) which does not need a prodrug to be effective in tumour cells

(11,12). The *gef* gene, a member of a gene family with homologous cell-killing functions, encodes a membrane protein of 50 amino acids that is anchored in the cytoplasmic membrane by the N-terminal portion. The C-terminal part is located in the periplasm (13). Mutagenesis studies have shown that this periplasmic portion encodes the toxic domain and that its dimerization is not essential for the toxic effect. Activation of this protein induces arrest of cellular respiration and cell death (14). Studies of suicide cassettes consisting of members of the gene family plus inducible promoters have documented their efficacy (15).

Based on the knowledge that the *gef* gene encodes a cytotoxic protein that binds to cell membranes, we investigated whether this gene can be used in a combined therapy with the antitumour drug paclitaxel in an experimental protocol to the treatment of lung cancer cells. Results obtained suggest that the combination of these treatments enhanced the anticancer effect and could be potentially used for cancer gene therapy approaches.

Materials and methods

Cell culture and MTS formation. The lung carcinoma cell line A549 (ATCC-CCL185) was grown with Ham's F12K (Sigma Chemical Co., St. Louis, MO), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 40 mg/l gentamycin and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37˚C in an atmosphere containing 5% CO_2. To generate multicellular tumour spheroids (MTS), exponentially growing monolayer A-549 cells were harvested by trypsinization and counted using a haemocytometer. Dead cells were excluded using trypan blue stain, then $10x10^3$ cells/well were grown in a 24-well microplate (BD Biosciences) previously coated with 400 μl 1.33% agarose type II in FCS-free medium and allowed to dry for 30 min. Plates were incubated at 37˚C in a 5% CO_2 atmosphere to promote aggregation and transferred onto a rocker designed for three-dimensional agitation (70 cycles/min) as described previously (16). Growth of the spheroids was monitored and measured to obtain a median relative volume (volume at day x/volume at day 0), as previously described by Boyd *et al* (17).

Vector construction. The *gef* gene was kindly provided by Dr J.L. Ramos from the Zaidín Experimental Station, CSIC, Granada, Spain. After its amplification using specific primers (sense 5'-ATGAAGCAGCATAAGGCGATG-3' and antisense 5'-TTACTCGGATTCGTAAGCCGTC-3') *gef* gene was subcloned into the pcDNA3.1 vector following manufacturer's instructions (Invitrogen). The resulting plasmid pcDNA3.1/*gef* was confirmed by sequence analysis using the T7 primer 5'-TAATACGACTCACTATAGGG-3'. Plasmid DNA was amplified in *E. coli* DH5α and purified by large-scale plasmid preparation using columns (Qiagen, Barcelona, Spain). DNA was dissolved in free TE buffer for storage. To optimize transfection conditions, the pcDNA3.1/lacZ encoding ß-galactosidase under the CMV promoter was used as a positive control vector for transfection and expression. A control pcDNA 3.1 plasmid in which the *gef* gene was absent was used as a negative control.

gef transfection in A-549. One day before transfection, confluent cells were seeded into 6-well plates ($0.8x10^5$ cells per well). Briefly, a transfection mixture was prepared by adding 94 μl of the serum-free medium and 6 μl FuGENE-6 reagent (Roche Diagnostic, Barcelona, Spain). After 5 min of incubation at room temperature, 2 μg of plasmid DNA (pcDNA3.1/*gef*) was added (ratio 2:6). The transfection mixture was incubated for 45 min at room temperature. A-549 cells, yielding approximately 70% confluence, were transfected with empty (control) or *gef* gene containing pcDNA vector. Cells were cultivated for 8 h at 37˚C, and the medium containing transfection mixture was then replaced with the growth medium. The ß-galactosidase-positive cells were counted microscopically to determine the transfection efficiency which was between 40 and 50%.

In vitro expression of gef gene. Upregulation of mRNA expression of *gef* cDNA was determined by RT-PCR. Total RNA was extracted from transfected (24, 48, 72 and 96 h) and parental cells with the Rneasy Mini kit (Qiagen), and cDNA was generated by means of the Promega reverse transcription system using total cellular RNA (1 μg). PCR amplification of *gef* gene took place under the above-described conditions and was run on a 2% agarose gel and visualized by ethidium bromide staining. RNA integrity was assessed by amplification of ß-actin mRNA (sense 5'-ATCATGTT TGAGACCTTCAA-3' and antisense 5'-CATCTCTTGCT CGAAGTCCA-3'). Images were scanned and analysed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative *gef* mRNA expression was calculated as the ratio of *gef* to ß-actin.

Proliferation assays. Haemocytometer analysis and sulphorhodamine B proliferation assay were performed to evaluate the effects of *gef* gene on cell growth. Parental and transfected cells (including cells transfected with empty vector) growing in well plates were trypsinized after 24, 48, 72 and 96 h and collected. Then, cells were counted with a haemocytometer. Trypan blue dye exclusion was used to determine cell viability. The same experiment was repeated using sulphorhodamine-B (SRB). Cells were fixed with 10% trichloroacetic acid for 60 min at 4˚C and stained with 0.4% sulphorhodamine B/1% acetic acid by incubating for 10 min with constant shaking. Cells previously washed with 0.1% acetic acid were left in 10 mM Trizma for 15 min at room temperature with constant shaking. Optical density was then determined using a Titertek multiscan (Flow, Irvine, CA) colorimeter at 492 nm. Linearity of the SRB assay with cell number was tested for each A-549 cell stock before each cell growth experiment. A-549 cells transfected with empty vector were used in the proliferation assay as controls.

Measurement of Annexin V and PI staining. Annexin V and PI staining was used to assess apoptosis (Pharmingen, San Diego, CA). Briefly, medium was removed, then cells were washed twice with PBS and incubated in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ 1.8 mM $CaCl_2$, pH 7.4) containing Annexin V-FITC (25 μg/ml) and PI (25 μg/ml) in the dark for 15 min at room temperature. Then, 500 μl binding buffer was added and cells

were immediately processed with a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analysis. Parental and transfected A-549 cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. Pellet and monolayer were post-fixed with 1% osmium tetroxide in 0.1 cacodylate buffer for 1 h at room temperature and dehydrated in ethanol. Cells were detached from culture vessel by rapid treatment with propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. Sections were contrasted with uranyl acetate-lead citrate and examined in a Hitachi H7000 transmission electron microscope. For SEM, adherent transfected and parental tumour cells on coverslips were fixed with 2% glutaraldehyde, dehydrated in graded concentrations of ethanol and dyed using the critical point method. These preparations were coated with platinum and observed under a Hitachi S-800 scanning electron microscope (Hitachi, Tokyo).

Combined therapy in MTS. MTS from A-549 cells were transferred, using a Pasteur pipette, from the 24-well microplate to a 96-well plate (one MTS per well) coated with agarose and containing 200 μl of medium. MTS were transfected with pcDNA3.1/*gef* as reported above. Four groups of MTS were analysed: control MTS, transfected MTS, paclitaxel-treated transfected MTS and paclitaxel-treated non-transfected MTS. Paclitaxel was used at 10 nM, 100 nM and 1 μM according to Monazzam *et al* (18). The experiment was carried out four times with six replicates in each group. The response to each anticancer treatment was evaluated by measuring MTS volume during treatment, as reported above.

Statistical analysis. SPSS 7.5 software (SPSS, Chicago, IL) was used for all statistical analyses. Results were compared by using the Student's t-test. All data are expressed as means ± SD. Differences were considered statistically significant at a P-value of <0.05.

Results

In vitro evaluation of gef expression. *In vitro* evaluation of *gef* gene expression was performed by RT-PCR. As shown in Fig. 1, an amplification fragment of 153 pb was found in A549 cells transfected with pcDNA3.1/*gef* for different times, indicating the effectiveness and ability of the construction to be used in the subsequent *in vitro* experiment. To demonstrate the integrity of the RNA preparations, PCR was performed using ß-actin primers (Fig. 1). Studies of the bands, normalized by comparison with the ß-actin signal, showed that the highest *gef* expression occurred at 72 and 96 h after transfection (6.8- and 9-fold higher, respectively, vs. A-549 cells at 24 h).

Inhibition of the A-549 growth rate by gef gene. After establishing that transfected A549 cells expressed *gef* transcripts, we analyzed the potential of *gef* gene to decrease the growth of lung cancer cells. Cell growth was measured in A-549 cells transfected by either pcDNA3.1/*gef* (experimental group) or empty pcDNA3.1 (control group) at 24, 48, 72 and 96 h. As shown in Fig. 2, the growth of A549 cells transfected with the empty vector was similar to that of the parental cells. In contrast, A-549 cells transfected with pcDNA3.1/*gef* showed a significant and time-dependent decrease in growth.

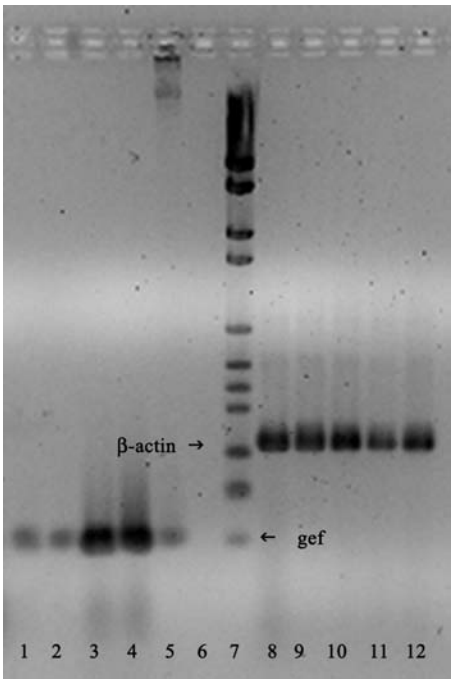

Figure 1. Determination of *gef* gene expression by RT-PCR. Total RNA isolated from transfected and parental A-549 lung cancer cells was transcribed to cDNA using reverse transcriptase PCR amplification as described in Materials and methods. Amplified PCR products of *gef* mRNA and ß-actin mRNA were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. PCR of *gef* gene: lanes 1-4, transfected A-549 (24, 48, 72 and 96 h respectively); lane 5, pcDNA3.1/*gef* (positive control); lane 6, parental A-549 cells (negative control); lane 7, molecular weight. PCR of ß-actin: lanes 8-11, transfected A-549 cells (24, 48, 72 and 96 h respectively); lane 12, parental A-549 cells.

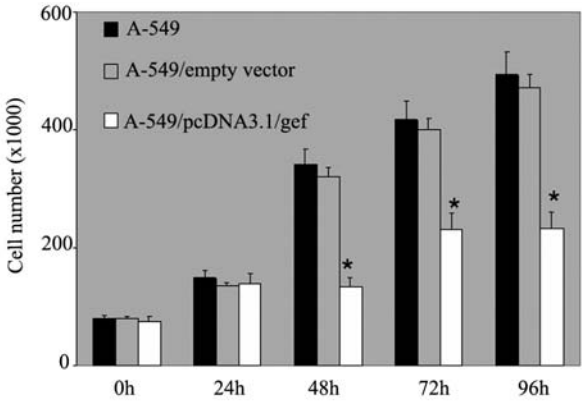

Figure 2. Effects of *gef* transfection on growth of A-549 cells. Parental A-549 cells and A-549 cells transfected with empty vector or pcDNA3.1/*gef* were seeded at a density of $8x10^4$ in plastic dishes and cultured for 4 days. Cell numbers were measured daily (24, 48, 72 and 96 h) by sulphorhodamine B assay. Values represent means ± SD of quadruplicate cultures (*P<0.05 compared with empty vector transfected cells).

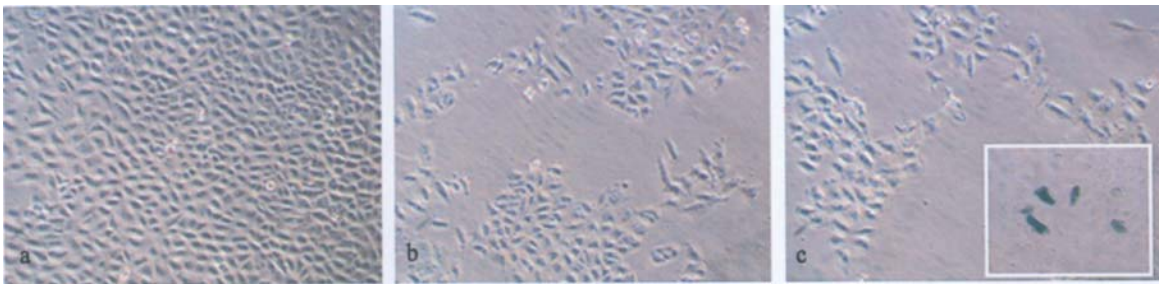

Figure 3. Phase-contrast photomicrographs showing morphology of parental and transfected A-549 cells. Parental A-549 cells (a) grew in clumps, were typically polygonal and formed a monolayer culture on the entire flask surface at 96 h. In contrast, A549 pcDNA3.1/V5/His-*gef* transfected cells at 48 h (data not shown), 72 h (b) (cells stained with X-gal in corner) and 96 h (c) (x40) formed an irregular monolayer culture with the progressive presence of zones without cells.

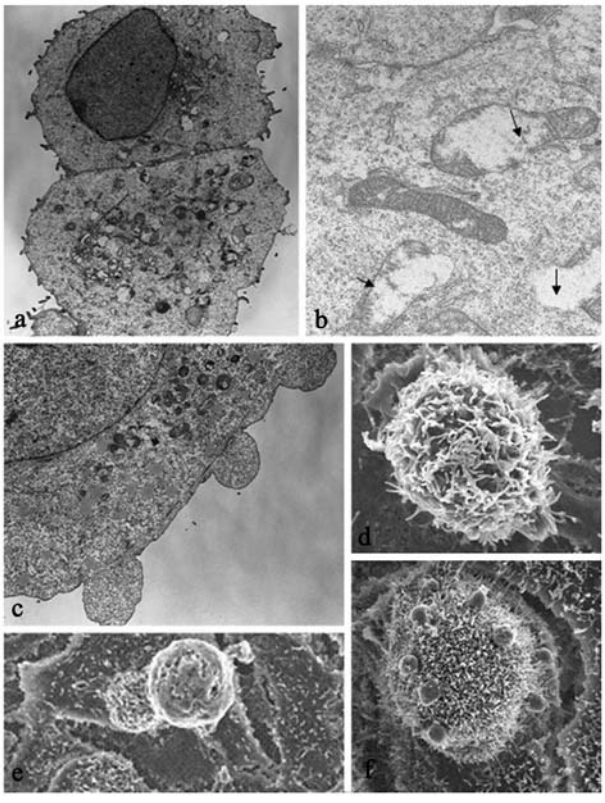

Figure 4. TEM and SEM analyses of A-549 cells. Conventional electron microscopy of parental A-549 cells (a) showed typical tumour cells with polygonal shape, large nucleus and light cytoplasmic complexion (x1100). Transfected A-549 cells showed dilated mitochondria with disrupted cristae (arrows) (b) (x12000) and cytoplasmic membrane evaginations (c) (x4400). Confocal microscopy of parental A-549 cells (d) showed numerous microvilli on cell surface. In contrast, transfected A-549 cells were characterized by progressive disappearance of microvilli (e and f) and membrane evaginations (f).

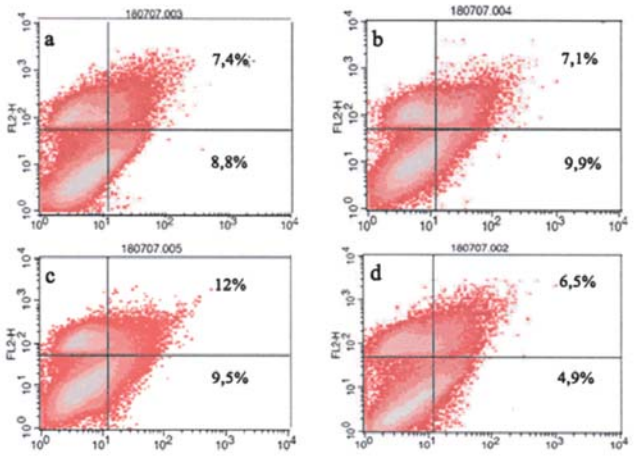

Figure 5. Fluorescence-activated cell sorting analysis of apoptosis induction by *gef* gene in A-549 lung cancer cells. Cells were stained with Annexin V and propidium iodide to evaluate apoptotic cell death, as described in Materials and methods. Representative images for comparisons between parental A-549 cells (a) and transfected A-549 cells at 48 h (b), 72 h (d) and 96 h (d). These data are mean results of four separate experiments.

No significant differences were found at 24 h after transfection, then transfected cells showed a significant decrease in growth rate (39%) vs. empty vector transfected cultures at 48 h, with the largest decrease in the proliferation rate observed at 72 and 92 h (55 and 42.2%, respectively).

Morphological characteristics of transfected A-549 cells. Light microscopy observations typically showed A549 lung cancer cells with polygonal shape and sheet-like pattern in normal monolayer culture, compatible with their epithelial origin. Cells were attached to the bottom of the flasks with an irregular arrangement in confluent cultures, although some cells showed short cytoplasmic projections. Cultures of transfected cells with empty vector showed no morphological changes with respect to the parental cell line (data not shown). However, microscopic comparisons between control group cultures and cultures of cells transfected with pcDNA3.1/*gef* over four days showed a progressive loss of monolayer culture uniformity, with the presence of irregular zones without cells (Fig. 3). Conventional electron microscopy and confocal microscopy were used for ultrastructural analyses of transfected A-549 cells. Control cells showed the characteristic features of undifferentiated cells, i.e., polygonal shape, large nucleus and scant cytoplasm (Fig. 4a). In transfected A-549 cells, the most relevant ultrastructural features were the presence of dilated mitochondrias with clear matrices and disrupted cristae and of cell surface alterations, i.e., reduction in length and number of microvilli and appearance of cytoplasmic membrane evaginations (Fig. 4b and c). No compaction or segregation of chromatin was observed, indicating absence of apoptosis (data not shown). Similar

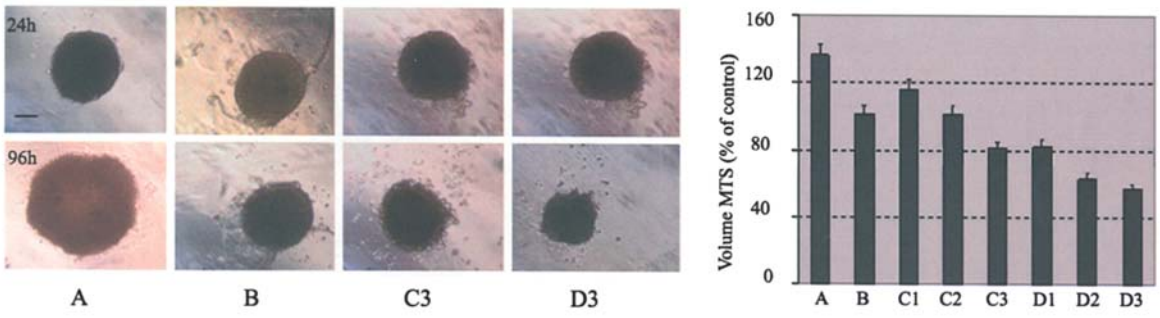

Figure 6. Analysis of the combined therapy (pcDNA3.1/*gef*/paclitaxel) in A-549 cells, using multicellular tumour spheroids (MTS). Growth of MTS was monitored by measurement of their cross-sectional area, calculating the median relative volume (volume at day x/volume at day 0). The graph depicts percentage volume changes in MTS after 96-h treatment in each experimental group. Group A, control A-549 MTS; Group B, A-549 MTS treated with pcDNA3.1/*gef*; Group C, A-549 MTS treated with 10 nM (C1), 100 nM (C2) and 1 μM (C3) paclitaxel; Group D, A-549 MTS treated with combined therapy pcDNA3.1/*gef* and 10 nM (D1), 100 nM (D2) and 1 μM (D3) paclitaxel; Bar, 300 μm. These data are mean results of four separate experiments. Light microscopic image represents A-549 MTS of the experimental group A, B, C3 y D3.

morphological alterations were observed in SEM images. Cells with both morphological changes (microvilli reduction and membrane evaginations) were observed (Fig. 4e and f). In contrast, A-549 parental cells were characterized by numerous microvilli on their surface (Fig. 4d).

Apoptosis analysis. A-549 cells were studied by means of an Annexin V-FITC apoptosis detection kit to determine possible apoptotic cell death resulting from *gef* gene transfection. Treatment with empty vector had no significant apoptotic effect on these cells (data not shown), and no significant differences in apoptosis level were observed between transfected (24-96 h in culture) and control A-549 cells (Fig. 5). No typical apoptotic changes were observed under microscopy, as reported above.

Combined therapy with gef gene and paclitaxel in A-549 cells. The therapeutic potential of combined *gef* gene and paclitaxel therapy was evaluated in A-549 lung cancer cells. The response to each anticancer treatment was evaluated by measuring A-549 MTS volumes, as described above. The largest decrease in growth rate after treatment with pcDNA3.1/*gef* or paclitaxel was observed at 96 h (Fig. 6). At this time, a 35.2% volume decrease was observed in *gef* gene transfected A-549 MTS. With paclitaxel treatment, a dose-dependent reduction in A-549 MTS volume was detected, with a decrease of 20.3, 35 and 54.6% vs. control MTS after administration of 10 nM, 100 nM and 1 μM of paclitaxel, respectively (Fig. 6). However, a more effective inhibition of MTS growth was obtained by the combined therapy (pcDNA3.1/*gef* and paclitaxel treatments) than by each treatment alone. The effect of paclitaxel at different concentration was enhanced by gef gene expression but the largest reduction in A-549 MTS volume was obtained with 1 μM paclitaxel. This treatment produced an 87.4% decrease in the MTS growth rate at 96 h vs. control MTS (Fig. 6).

Discussion

Despite therapeutic advances, conventional lung cancer therapy has failed to improve survival rates in NSCLC patients. The habitually late diagnosis and high mortality of lung cancer, alongside the ineffective and harmful effects of chemotherapy and radiotherapy, mandate the adoption of novel treatment approaches. Thus, research is in progress into combined modality treatment strategies for cancer, including gene therapy with anti-tumour drugs (19). The present study explored a combined therapy with *gef* suicide gene and paclitaxel as a new approach to the treatment of lung cancer.

To date, isolated gene therapy has not guaranteed the successful treatment of lung cancer. Gene therapy with anti-angiogenic factors, proapoptotic genes or autologous tumour cells modified with an adenovirus vector have induced a partial response (5). Thus, HSV-tk/ganciclovir (GCV) treatment was shown to selectively kill lung cancer cells (20). However, despite the use of new specific promoters (e.g., INSM1) (21), this approach is limited by the release of toxic metabolites and their bioavailability (10). Moreover, the development of chemoresistance in lung cancer cells (such as GLC4) significantly changes GCV sensitivity, reducing the efficacy of HSV-tk/GCV (22). Therefore, therapeutic systems are required that are not dependent on the use of a prodrug. In fact, bacterial genes that encode toxins, viral genes, and even plant genes have been shown to be able to induce tumour cell death (23-25).

With this background, we assayed the *gef* gene in A-549 lung cancer cells. The *gef* protein is known to form pores in bacterial cell membranes, promoting host cell lysis, and it has demonstrated a cytotoxic effect in melanoma and breast cancer cells (11,12). When we transfected *gef* gene in A-549 lung cancer cells, the number of surviving cells was significantly lower vs. control cells at 48-96 h, with the largest decrease in the proliferation rate observed at 72 h (55%). A similar growth inhibition effect was found in A-549 cells by Narumi *et al* (26) using a cytolytic pore-forming protein (perforin) that also binds to the tumour cell membrane. Interestingly, the decrease in tumour cell number observed in our experiments was not associated with apoptosis, despite a progressive loss of monolayer culture uniformity. Other non-mammalian genes used in cancer gene therapy induce cell death by a non-apoptotic mechanism (27). It was recently demonstrated that breast cancer cell growth is inhibited by bacteriophage λ-holin, a protein that can permeabilise the

bacterial membrane (24). Forty-eight hours after induction cells became multinucleated, in some cases extensively vacuolated, and finally detached from the culture dish surface. These findings indicate that the cytotoxic effects of the λ-holin protein include alterations in cellular morphology preceding cell death. The *gef* gene diminishes the membrane potential, leads to membrane leakiness and also induces morphological changes (28). However, its specific mechanism of action in eukaryotic cells has not been elucidated. Eukaryotic cells fundamentally differ from prokaryotic cells in terms of their cellular structure, organisation, metabolism and membrane composition. Nevertheless, because the eukaryotic endomembrane system arose in an ancestral prokaryotic lineage (29), *gef* gene may act in cell organelle membranes. In fact, bacterial toxins such as *Vibrio cholerae* cytolysin or *Helicobacter pylori* VacA protein directly interact with the eukaryotic cytoplasm membrane (30,31). This hypothesis is strongly supported by our ultrastructural findings in the transfected A-549 cells of dilated mitochondrias with disrupted cristae, cytoplasmic membrane evaginations and smaller and fewer microvilli.

After establishing the efficacy of the *gef* gene to affect the growth of human lung cancer cells, we investigated its use in a combined therapy with paclitaxel, a drug of choice for treating lung cancer. Addition of gene therapy strategies to conventional therapies appears to improve their effectiveness. Thus, the anti-tumour response was enhanced by combining chemotherapeutic drugs with HSV-tk in bladder cancer (6), with p53 in breast cancer (32) and with E2F-1 in melanoma (33). Combined therapy with docetaxel or paclitaxel and p53 or interleukin 12 genes improved outcomes in lung cancer (34). Paclitaxel, which has a known activity against a broad range of tumour types, also showed higher efficacy when combined with gene therapy in ovarian and metastatic breast cancers (35,36). We tested the combination of paclitaxel and gene therapy in lung cancer cells by using A-549 MTS. This model mimics the real biological environment and gives a more relevant picture of the drug effects by including limitations in penetration, distribution and feedback mechanisms in cell signalling (37). Volume analyses of the A-549 MTS showed that the combined therapy induced significant MTS growth inhibition after 96 h in comparison with control MTS. The largest reduction was obtained with the use of *gef* gene and paclitaxel 1 μM. This inhibition was greater than that obtained using the gene therapy or chemotherapy alone. These results showed that the combination of *gef* gene and paclitaxel enhanced cell growth inhibition in A-549 MTS, suggesting its therapeutic potential in lung cancer. However, as in most gene therapy systems, gene delivery and selectivity for cancer cells remain a challenge. In this respect, retroviral vectors have the advantage of selectively transducing dividing cells and of integrating into the genome of the infected target cell. Recent developments in vector design, such as the reconstituting retroviral vector system (39), allow the generation of high-titre vector viruses expressing genes that encode cytotoxic products. By replacing the constitutively active viral promoters with tissue- or tumour-specific promoters (40), a targeted delivery of cytoxically acting gene products appears to be feasible. In summary, our data demonstrate the potential clinical relevance of a new combined therapy which could be used for lung cancer gene therapy.

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Original Article

Regression of established subcutaneous B16-F10 murine melanoma tumors after *gef* gene therapy associated with the mitochondrial apoptotic pathway

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Abstract: Novel treatment modalities, including gene therapy, are needed for patients with advanced melanoma. We evaluated whether the *gef* gene, a suicide gene from *Escherichia coli,* had a significant cytotoxic impact on melanoma *in vivo*. First, we used a non-viral gene delivery approach (pcDNA3.1/*gef*) to study the inhibition of melanoma cells (B16-F10) proliferation *in vitro*. Secondly, we used direct intra-tumoral injection of pcDNA3.1/*gef* complexed with jetPEI to deliver *gef* cDNA to rapidly growing murine melanomas. We demonstrated that *gef* gene not only has an antiproliferative effect on B16-F10 cells *in vitro,* but also induces an important decrease in melanoma tumor volume (77.7% in 8 days) *in vivo*. Interestingly, after *gef* gene treatment, melanoma showed apoptosis activation associated with the mitochondrial pathway, suggesting that the induction of this death mechanism may be an effective strategy for its treatment. Our *in vivo* results indicate that *gef* gene might become a suitable therapeutic strategy for patients with advanced melanoma.

Key words: apoptosis – caspase – *gef* gene – melanoma – mitochondria

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Introduction

Melanoma represents only 4% of all skin cancers, but nearly 80% of skin cancer deaths, predominantly because of metastatic spread (1). Apart from surgery, treatment options for melanoma, particularly metastatic melanoma, are relatively limited**.** As melanoma is a highly therapy-refractory tumor, it demands effective therapies combinations (2). Suicide gene therapy has been proposed as a strategy for the treatment of intractable cancers and has been assayed in some clinical trials by itself or in combination with other therapies (tumor irradiation or chemotherapy). In melanoma, strategies to facilitate apoptosis by gene therapy may be an alternative or complementary strategy for its treatment (3) as it has been demonstrated that apoptosis deficiency is a critical factor for therapy resistance in this tumor (4).

Classical cancer suicide gene therapy employs genes encoding enzymes that convert non-toxic prodrugs into cytotoxic compounds (5). However, these prodrug systems have been assayed in melanoma both *in vitro* and *in vivo* with limited results (6,7). As an attractive alternative to this strategy, therapeutic genes that directly encode cytotoxic proteins could be used. In contrast to classical suicide genes that act by disrupting DNA synthesis (targeting only rapidly dividing cells) these new toxins may act killing both quiescent and rapidly dividing tumor cells and may be effective for aggressively growing tumors as well as for those that grow more slowly. Many genes encoding cytotoxic products have been evaluated as gene therapy approaches (8,9). The most recent experiences with genes expressing toxins from bacteria (10), from plants (11) or from bacteriophage (12) showed a high cytotoxic impact on tumoral cells derived from different tissues.

In this context, our group has developed last year a direct cancer gene therapy system based in the suicide gene named *gef*. The *gef* gene, member of a gene family with homologous cell-killing functions, encodes a membrane protein of 50 amino acids which is anchored in the cytoplasmic membrane by the N-terminal portion, whereas the C-terminal part is located in the periplasm (13). Although activation of this protein induces arrest of respiration and

death in bacterial cells the mechanism of action in tumoral cells is unclear. We have previously demonstrated that *gef* protein is able to induce changes in proliferation rate and differentiation degree of tumoral cells without having to use prodrugs (14,15). However, its possible *in vivo* application could not be demonstrated yet.

In this study, we have evaluated for the first time the potential use of the *gef* gene for the treatment of melanoma tumors *in vivo*. Moreover, based on the knowledge that the *gef* gene encodes a cytotoxic protein that binds to cell membranes, we analysed *gef*'s mechanism of action. We used the B16-F10 murine melanoma model because of its highly invasive and metastatic nature and the cationic lipids jetPEI to deliver *gef* cDNA to rapidly growing murine melanomas. Results obtained suggest that treatment with the *gef* gene significantly decreases tumor growth, inducing apoptosis in melanoma tumor cells by means of the mitochondrial pathway.

Methods

Cells and reagents

The B16-F10 murine melanoma cell line (CRL6475) was obtained from American Type Culture Collection and was grown in Dulbecco's modified eagle's medium (Sigma, St Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 40 mg/l gentamicin and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37°C in an atmosphere containing 5% CO_2.

gef transfection in B16-F10 cells

The *gef* gene was amplified using specific primers (sense 5′-ATGAAGCAGCATAAGGCGATG-3′ and antisense 5′-TTACTCGGATTCGTAAGCCGTC-3′) under the following conditions: 94°C for 1 min, 35 cycles at 94°C for 1 min, 53°C for 30 s and 72°C for 30 s and 72°C for 10 min and was subcloned into the pcDNA3.1-TOPO vector (Invitrogen, Barcelona, Spain) following the manufacturers' instructions. The resulting plasmid pcDNA3.1/*gef* was transformed into the subcloning efficiency DH5 alpha chemically competent *Escherichia coli* (Invitrogen). The correct DNA sequence was confirmed by sequence analysis using the T7 primer. One day before transfection, confluent cells were seeded into six-well plates (2×10^5 cells/well). Briefly, a transfection mixture was prepared by adding 94 μl of the serum-free medium and 6 μl FuGENE-6 reagent (Roche Diagnostic, Barcelona, Spain). After 5 min of incubation at room temperature, 2 μg of plasmid DNA (pcDNA3.1/*gef*) were added (ratio 1:3). B16-F10 cells, yielding approximately 70% confluence, were transfected with *gef* gene-containing pcDNA3.1 vector. Cells were cultivated for 8 h at 37°C, and the medium containing transfection mixture was then replaced with the growth medium. A pcDNA3.1 plasmid in which the *gef* gene was absent was used as a negative control. The pcDNA3.1/green fluorescent protein (GFP) (provided by Dr. G. Ortiz) was used to optimize transfections conditions.

Proliferation assays

Parental and transfected cells growing in well plates were trypsinized after 24, 48, 72 and 96 h and collected. Cells were fixed and stained with 0.4% sulphorhodamine B/1% acetic acid. Cells previously washed with 0.1% acetic acid were left in 10 mM Trizma for 15 min at room temperature. Optical density was then determined using a Titertek multiscan (Flow, Irvine, CA, USA) colorimeter at 492 nm. Linearity of the SRB assay with cell number was tested for each B16-F10 cell stock before each cell growth experiment. B16-F10 cells transfected with empty vector were used in the proliferation assay as controls.

In vitro and *in vivo* expression of *gef* gene

Upregulation of mRNA expression of *gef* cDNA was determined by RT-PCR. RNA was extracted from transfected and parental cells with the RNeasy Mini kit (Qiagen). RNA from tumor was obtained with the RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was generated by means of the Promega reverse transcription system using total cellular RNA (1 μg). PCR amplification of *gef* gene took place under the above-described conditions. RNA integrity was assessed by amplification of β-actin mRNA. Images were scanned and analysed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative *gef* mRNA expression was calculated as the ratio of *gef* to β-actin.

Annexin V and propidium iodide staining

Parental and transfected cells were washed twice with phosphate-buffered saline (PBS) and incubated in binding buffer containing annexin V-FITC (25 μg/ml) and propidium iodide (25 μg/ml) in the dark for 15 min at room temperature (Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA, USA). Then, binding buffer (500 μl) was added and cells were immediately processed with a FACScan flow cytometer. Microscopy analysis was carried out by Technical Services from the Granada University in a Leica DMI6000 (Heidelberg, Germany) confocal microscope with laser Argon/Krypton.

Assay for cytoplasmic mono- and oligonucleosomes

The Cell Death Detection ELISA Kit (Boehringer, Mannheim, Germany) was used for assessing apoptosis in transfected cells following the manufacturer's protocol. Parental and transfected cells (2×10^4) were lysed and the cell lysates were overlaid and incubated in microtitre plate

modules coated with antihistone antibody. Samples were then incubated with anti-DNA peroxidase followed by colour development with ABTS substrate. Samples absorbance was determined with Titertek multiscan at 405 nm.

Measurement of mitochondrial membrane potential

Parental and transfected cells were washed twice with cold PBS and incubated with 40 nM DiOC6(3) for 15 min at 37°C. Then, cells were washed with ice-cold PBS and resuspended in 500 ml of PBS. Fluorescence intensities of DiOC6(3) were analysed on a FACScan flow cytometer with excitation and emission settings of 484 and 500 nm, respectively.

Caspase activity assay

Caspase-9 and -8 activities were measured using caspase colorimetric assay kits (R&D Systems, Minneapolis, MN, USA). Briefly, parental and transfected cells were washed twice with cold PBS and resuspended in 50 μl of cold lysis buffer, incubated for 10 min and centrifuged for 1 min at 10 000 ***g*** to precipitate cellular debris. Assay was performed in triplicate on a 96-well plate following the manufacturers' protocol. Results are expressed as the fold increase in pcDNA3.1/*gef* treated cells over that of control cells. Etoposide (Sigma) (50 μmol/l) was used as positive control of caspase activities in B16-F10 cells.

Tumor induction and measurement

For *in vivo* study, female C57BL/6 mice (Scientific Instrumentation Centre, Granada University, Granada, Spain) were used. All mice (weighing 25–30 g) were maintained in a laminar air-flow cabinet at a room kept at 37°C temperature and 40–70% humidity with a 12-h light/dark cycle under specific pathogen-free conditions. All studies on animal models were approved by the Ethical Committee of the Medical School of Granada University and performed according to its guidelines. Tumors were induced by subcutaneous injection of 5×10^5 B16-F10 cells into the left flanks of C57BL/6 mice. Tumors were allowed to grow to the appropriate size (75 mm^3) before treatment (ideal minimal size for intra-tumoral injection). After reaching this volume (treatment day 0), tumors were measured at periodic intervals following treatment using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a). Using these measurements the tumor volume was calculated by the formula $V = ab^2 \times \pi/6$.

Intra-tumoral plasmid treatment

In vivo JetPEI (Polyplus-transfection Inc.) was used as a transfection enhancer reagent. PEI/DNA complexes with a ratio of 1:6 were prepared in a solution of 10% w/v glucose. This was carried out in a two-step procedure for the preparation of a standard quantity of 20 μg of PEI/DNA complex, according to the manufacturer's instructions. Tumors were then treated intra-tumorally with pcDNA3.1/*gef* plasmid. The pcDNA3.1 LacZ plasmid was used to normalize transfection efficiency. Moreover, control groups (without treatment and treated with empty vector) were included. Treatments were administered during 14 days. Comparative study between treated and non-treated groups was realized during the first 8 days because of the high rate of mortality in control group.

Histological analysis

Tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and cut into 3–5 μm sections. Cells were immunofluorescently labelled with primary antitubulin mouse monoclonal antibody (1:500) (Sigma) followed by Texas Red dye-conjugated affinitPure Goat Anti-Mouse IgG + IgM (1:500) (Jackson ImmnoResearch Laboratories, West Grove, PA, USA). The presence of apoptotic cells within the tumor sections was evaluated by the TUNEL technique using the In Situ Cell Death Detection Kit Fluorescein (Roche, Mannheim, Germany) according to manufacturers' recommendations. Cell nuclei were counterstained with DAPI. Per cent apoptosis (apoptotic index) was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high power fields/slide). Immunohistochemical analyses of caspases were realized using antiactive forms of caspase-9 (1:50) (Cell Signaling Technology, Inc) and caspase-8 (1:100) (Imgenex, San Diego, CA, USA). FITC-conjugated antirabbit secondary antibody at room temperature for 1 h was used for the detection. Cell nuclei of cultures were counterstained with DAPI. Fluorescence images were captured using an Olympus DP11 microscope with a Nikon Eclipse Ti digital imaging system.

Transmission electron microscopy

Melanoma tumors grown in mice were collected, cut up into small pieces and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 1 h. After postfixation with 1% OsO_4 in cacodylate buffer (room temperature, 2 h), sections were dehydrated through graded ethanol concentrations with a final propylene oxide dehydration. Samples were then embedded in Epon 812 resin. Ultrathin sections, were stained with uranyl acetate and lead citrate and examined in a Hitachi H7000 transmission electron microscope (TEM).

Statistical analysis

SPSS 14 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Results were compared by using

the Student's *t*-test. All data are expressed as mean ± SD. Differences were considered statistically significant at a *P*-value of <0.05.

Results

Expression of *gef* gene and inhibition of cell growth in B16-F10 cells in culture

In vitro evaluation of *gef* gene expression was performed by RT-PCR. As shown in Fig. 1a, an amplification fragment of 153 bp was found in B16-F10 cells transfected with pcDNA3.1/*gef* for different time periods, indicating the effectiveness and ability of the construction in order to be used in the subsequent experiment. To demonstrate the integrity of the RNA preparations, PCR was performed using β-actin primers. Analysis of the bands, normalized by comparison with the β-actin signal, showed a progressive increase of *gef* expression; this was 3.9- and 4.5-fold higher at 48 and 72 h vs B16-F10 cells at 24 h and was maximal at 96 h after transfection (sixfold higher vs B16-F10 cells at 24 h). Previously, cell transfection was optimized by pcDNA3.1/GFP (Fig. 1b). As shown in Fig. 1c, the B16-F10 cells transfected with pcDNA3.1/*gef* showed a significant and time-dependent decrease in growth. Twenty-four hours after transfection a 28% decrease in growth rate versus control cultures was observed. The decrease in proliferation was 45.5% at 48 h. The main decrease in proliferation rate occurred at 72 h and 96 h, when similar ratios of growth decrease were observed (64.6% and 69.7%, respectively). In contrast, the growth of B16-F10 cells transfected with the empty vector (control group) was similar to that of the parental cells.

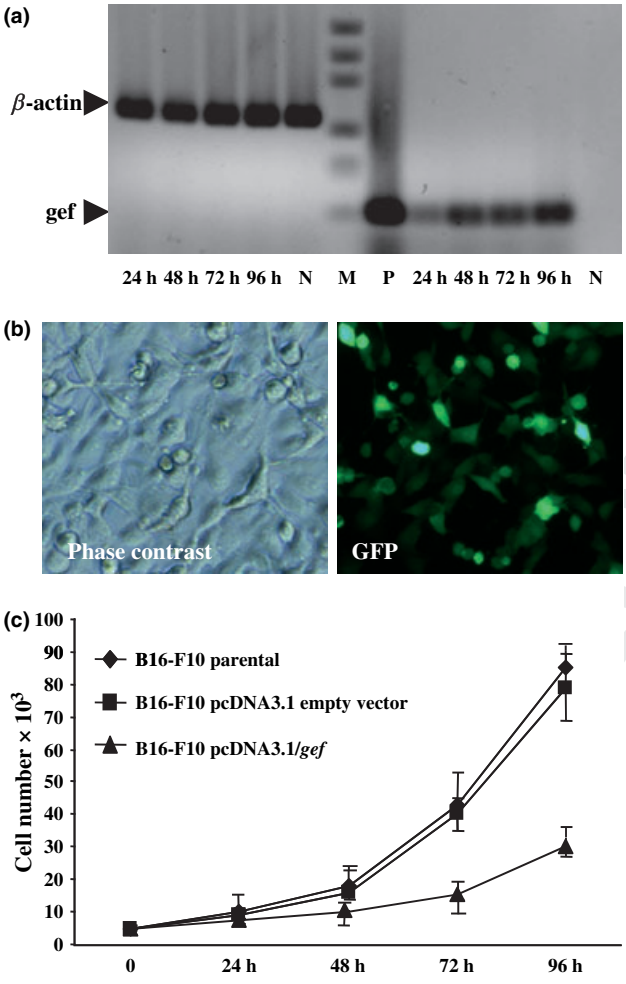

Figure 1. *gef* gene expression and growth rate inhibition in B16-F10 cells. (a) RT-PCR showing *gef* gene expression in B16-F10 transfected cells at different time periods. The integrity of the RNA was demonstrated using β-actin primers. N, negative control (B16-F10 non-transfected cells); M, molecular weight; P, positive control (pcDNA3.1/*gef*). (b) Representative photomicrograph (phase contrast and fluorescent images) of B16-F10 cells transfected with pcDNA3.1/GFP to optimize transfections conditions. (c) Growth of B16-F10 cells expressing *gef* was detected by sulphorhodamine B assay. Cells transfected with pcDNA3.1/*gef* showed a clearly decreased growth rate compared to the control cells and cells transfected with pcDNA3.1 empty vector ($P < 0.05$). Data represent the mean ± SD of four independent experiments.

Expression of *gef* gene-induced apoptosis in B16-F10 cells

Apoptotic rates of B16-F10 cells untreated or transfected with empty vector revealed no significant difference. Only 8.7% of the pcDNA3.1/*gef* transfected cells showed apoptosis after 24 h. However, at 48 and 72 h a significant increase was found (19% and 34%, respectively) (Fig. 2a). At 96 h apoptosis was similar to that found at 72 h (data not shown). These results indicated the ability of *gef* gene to stimulate apoptosis in B16-F10 melanoma cells after *in vitro* transfection. The induction of apoptosis by *gef* gene was also evident by confocal laser-scanning microscopy (Fig. 2b). Furthermore, to confirm whether the growth inhibitory effects of *gef* gene are related to the induction of apoptosis, we used an ELISA-based assay. Amounts of cytoplasmic oligonucleosomes (an indicator of apoptosis) increased between 24 and 96 h after *gef* transfection as compared with untreated cells. As shown in Fig. 2c, the strongest enrichment factor was obtained at 72 and 96 h (3.6 and 5.4, respectively). These results provide convincing data that up-regulation of *gef* induces apoptosis in B16-F10 cancer cells.

Gef gene therapy-induced modulation of mitochondrial membrane potential and caspase-9 activation

To determine if induced apoptosis by *gef* gene in B16-F10 cells is mediated via the mitochondrial pathway, mitochondrial membrane integrity was measured by DiOC6(3) dye staining. As shown in Fig. 3a, a significant decrease in

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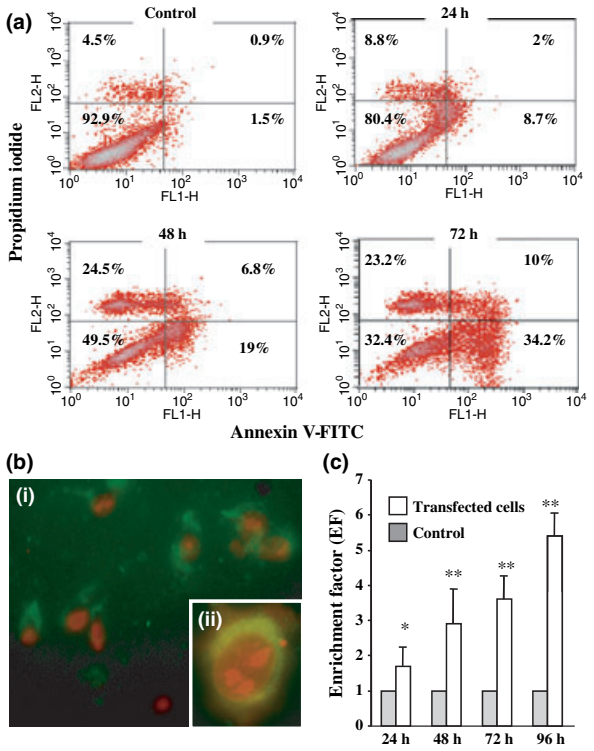

Figure 2. Analysis of apoptosis induction by *gef* gene in B16-F10 cells. (a) Fluorescence-activated cell sorting. Cells were stained with annexin V and propidium iodide (PI) to evaluate apoptotic cell death, as described in Methods. These data are representative results from four separate experiments. (b) Annexin V-FITC staining and confocal microscopy. The annexin V-FITC fluorescence localized at the periphery of the cells consequent to the translocation of phosphatidylserine (PS) residues from the inner leaflet of the plasma membrane to the outer leaflet. B16-F10 cells 48 h after transfection (a). Cell nuclei were counterstained with PI before examination under a confocal laser-scanning microscope. A stronger binding was observed when the study was carried out 96 h after *gef* transfection (b). The experiment was performed three times with identical results. Magnification: (a) 20×; (b) 40×. (c) ELISA apoptosis assay of cytoplasmic nucleosomes. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the formula: mean of absorbance of transfected cells/mean of absorbance of control cells = enrichment factor (EF). The EF was calculated relative to the control value (=1) of untreated cells. *$P < 0.05$; ** $P < 0.01$ compared to control cells.

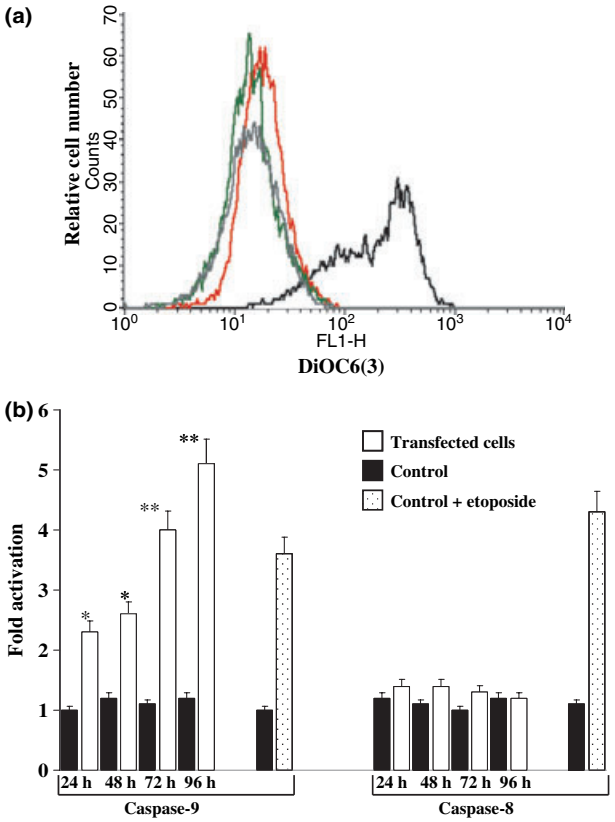

Figure 3. Mitochondrial membrane potential and caspase activity. (a) Reduction of mitochondrial transmembrane potential ($\Delta\psi_m$) in B16-F10 cells (black) after 24 h (red), 48 h (grey) and 72 h (green) of *gef* transfection. DiOC6(3) was added to the culture medium during the last 15 min of treatment at a final concentration of 40 nM. The fluorescence intensity of DiOC6(3) was analysed by flow cytometry. Data shown are representative of three independent experiments. (b) Caspase-9 and -8 activities were determined (as described in Methods) in pcDNA 3.1/*gef* transfected B16-F10 cells at indicated time points in comparison with parental cells (percentage values). Etoposide treatment (6 h) was used to demonstrate caspase 8 and caspase 9 activities in B16-F10 cells. Experiments were performed four times with identical results. *$P < 0.05$; ** $P < 0.01$ compared to control cells.

membrane potential was detected in transfected cells at 24, 48 and 72 h compared with parental cells (control) indicating a mitochondrial membrane permeability increase after *gef* gene treatment. At 96 h membrane potential modulation was similar to that found at 72 h (data not shown). Caspase-9 activity was induced after *gef* gene treatment in B16-F10 cells. Although its activity was modulated at different times, the largest increase was observed at 72 and 96 h (three- and 4.1-fold; respectively). The caspase-8 activity remained unchanged (Fig. 3b). These data support the hypothesis that *gef* gene-induced apoptosis through the mitochondrial-mediated pathway.

Gef gene effects on melanoma growth *in vivo*

The potential of *gef* gene to promote tumor cell killing *in vivo* was evaluated by direct injection of the plasmid complexed with jetPEI in B16-F10 subcutaneous mice tumors. Figure 4 shows that *gef* gene was able to inhibit tumor growth. During the first 2 days following treatment, tumor volume decreased by 40.4% in the *gef* gene treated group, as compared with the control group. On post-treatment days 4 and 6, the observed reduction was 45.2% and 54.3% respectively. Following a similar trend, a 77.7% volume reduction was observed on day 8. After this time, the control group showed a high mortality rate (Fig. 4). Mice treated with *gef* gene showed no evidence of systemic toxicity (i.e. animal death, loss of body weight, other tissue damage

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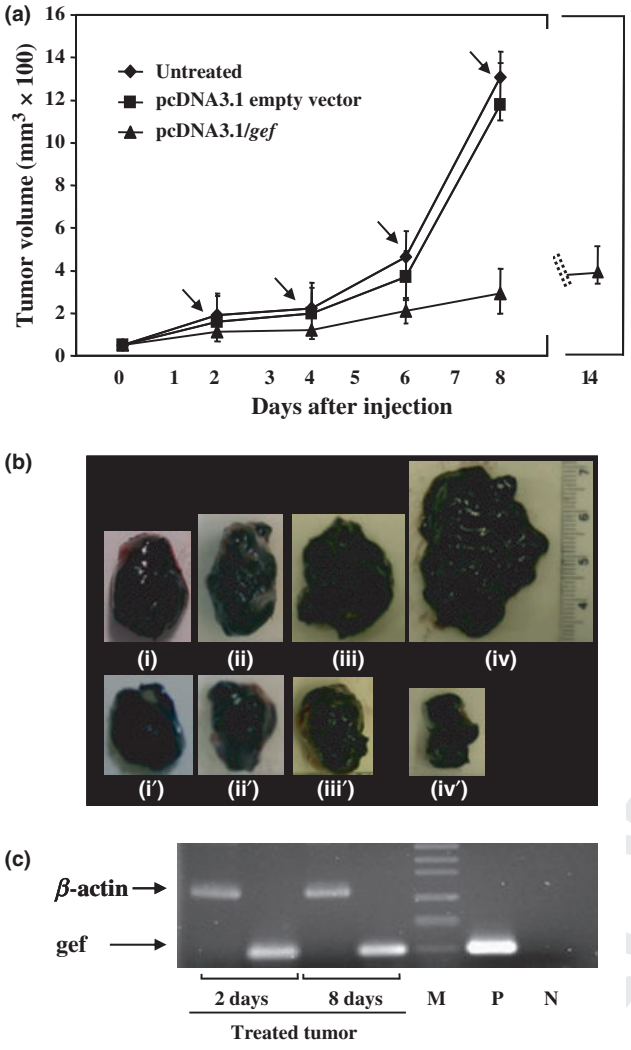

Figure 4. Effect of direct intra-tumoral injection of the *gef* gene on the growth of subcutaneous tumor induced by B16-F10 tumors cells in mouse. (a) Tumor volume variation after *gef* gene treatment. Time of injection is indicated through arrows; treatment began (day 0) when tumors had reached a volume of 75 mm^3. The plot shows a significant tumor volume reduction in the treated group (n = 14) as compared with the control groups, transfected with empty vector (n = 7) and without treatment (n = 7). (b) Representative gross appearance of tumors excised from mice sacrificed during treatment at 2 (a′), 4 (b′) 6 (c′) and 8 (d′) days and tumors obtained from mice without treatment at the same time intervals (a, b, c and d, respectively). (c) Determination of *gef* gene expression in tumor mice after 2 and 8 days of pcDNA3.1/*gef* treatment. The integrity of the RNA tissue used was determined using *β-actin* primers. M, molecular weight; P, positive control (pcDNA3.1/*gef*); N, negative control (mouse tumors without treatment).

or changes in behaviour or aspect). After empty vector injection with jetPEI, no tumor growth modifications were observed (Fig. 4a,b). RT-PCR was performed to ensure *gef* gene expression in all treated tumors. Figure 4c shows a comparison of *gef* gene expression in melanoma tissue between days 2 and 8 after the treatment.

gef gene-induced apoptosis in melanoma cells *in vivo*

To determine the *gef* expression efficiency to induce apoptosis *in vivo* we analysed established subcutaneous B16-F10 tumors with a TUNEL reaction mixture. As showed in Fig. 5a, the number of apoptotic cells (green) was significantly higher in tumors treated with pcDNA3.1/*gef* in comparison with the tissue control. Analysis of the melanoma sections showed a progressive increase of per cent apoptosis which was maximal at 8 days after treatment (Fig. 5b). To examine possible caspase-9 or -8-activation by *gef* gene treatment we used immunohistochemistry. Weak or absent expression of caspase-8 protein was detected in tissue samples (data not shown). However, all samples of melanoma tissue treated with pcDNA3.1/*gef* at different times showed a clear caspase-9 activation, with the strongest staining observed from the 4th day of treatment until the end of the experiment. Caspase-9 expression was not seen in any of the controls or in melanoma tissue treated with the empty vector (Fig. 5c).

Transmission electron microscopy

To further investigate the nature of *gef* gene-mediated cytotoxicity, B16-F10 mouse melanoma-induced tumors treated with *gef* gene *in vivo* were analysed by TEM. The control tissue showed giant malignant cells with an intact cell membrane and single- or multiple-nuclei cells. The first identifiable morphological change after treatment was the pronounced swelling in the mitochondria seen within 2 days of treatment. Mitochondria in control cells and cells treated with vehicle alone remained unaffected. Forty-eight hours later, apoptosis ultrastructural characteristics, such as chromatin condensation, crescent formation and margination were seen by electron microscopy in the treated melanoma, but not in the control group. Similar pictures were observed 6 and 8 days after treatment (Fig. 6).

Discussion

New treatment strategies for malignant melanoma are urgently needed because conventional approaches like chemotherapy and radiation have little impact on patient survival in the advanced stages of the disease. As a promising alternative, gene therapeutic strategies based on suicide gene expression in tumor cells have been developed. In this study, we have demonstrated the *in vivo* potential use of the *gef* cDNA as a suicide gene in a new melanoma gene therapy approach.

To date, classical suicide gene therapy systems have not guaranteed the successful treatment of melanoma and have

COLOUR

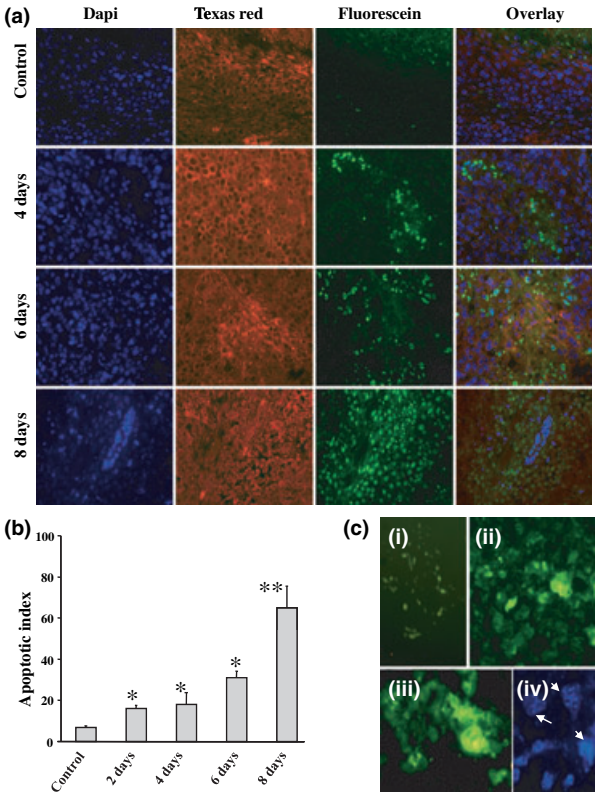

Figure 5. Histological evaluation of apoptosis and caspase activity of melanoma cells *in vivo*. (a) Representative photographs of tumor sections showing TUNEL-positive cells (green). Sections were counterstained with DAPI (blue) and anti-tubulin (red). Apoptotic cells increased in tumors treated with pcDNA 3.1/*gef* at 2 (data not shown), 4, 6 and 8 days compared to untreated tumors (control) (×20). (b) Per cent apoptosis in each group. Values were expressed as mean ± SD. Level of significance compared to control cells: $*P < 0.05$; $**P < 0.01$. (c) Caspase expression in melanoma tumor was detected using immunofluorescence staining. Microscopic analysis showed that melanoma tissue after treatment with pcDNA3.1/*gef* was strongly caspase-9-positive. Untreated tumors (a) and melanoma tumor after 4 (b) (40×) and 6 (c) (60×) days of treatment. Cell nuclei counterstained with DAPI showed that some cells displayed apoptotic morphology and nuclear segmentation (arrows) (d, 60×). All data were obtained from the study of at least three tumors.

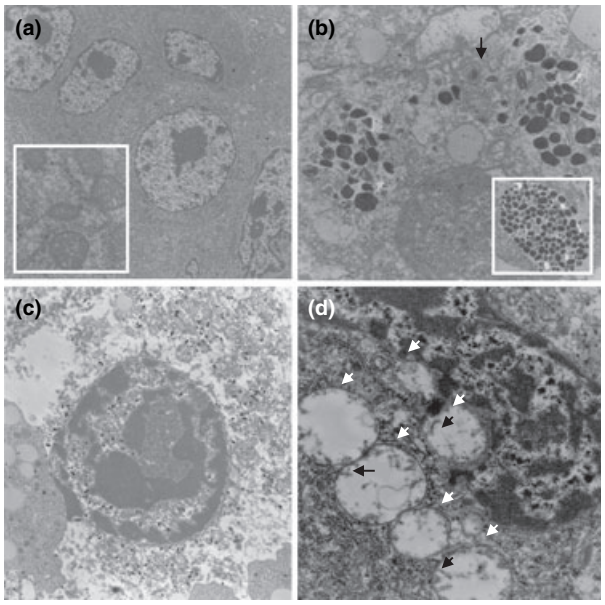

Figure 6. Transmission electron microscopy of melanoma tumors without treatment showed typical tumor cells with polygonal shape, large nucleus, light cytoplasmic complexion containing well preserved organelles (a) including mitochondrias (A insert) (1100×) and a large amount melanosomes (B insert) including premelanosomes (arrows) (b) (×4000). Representative photomicrograph of melanoma treated with pcDNA3.1/gef (6 days) showing ultrastructural characteristics of apoptosis such as chromatin condensation, crescent formation and margination (c) (×6300). Note, the nucleus near of the swollen mitochondrias (white arrows) with disrupted cristae (black arrows) (d) (12000×). Data were obtained from the study of at least three tumors.

induced only a partially positive response (16,17). One of the main limitations of these indirect action systems is the need to use prodrugs (18).The use of genes encoding toxins avoids the administration of a prodrug, eliminating its side-effects, its bioavailability limitations and the consecutive applications of vector and prodrug. Moreover, these genes can be directly expressed in the cytosol of the target cells, thus overcoming the problems (cytotoxicity, internalization efficiency and resistance acquired by cancer cells) originated by their use as components of immunotoxins or recombinant chimeras (19). In melanomas, viral genes encoding toxins such as viral protein R and some plant genes such as saporin (SAP) have been applied with a significant result to induce tumoral cell death (20,21). Our previous results *in vitro* showed that *gef* is effective in melanoma MS-36TG cells, modulating their proliferation capacity, differentiation degree and tumor malignancy (22). In the present study, we have demonstrated that the transfection of the pcDNA 3.1/*gef* not only inhibits *in vitro* melanoma proliferation but also it is highly toxic for tumors *in vivo*. The *gef* gene treatment induced a significant decrease in tumor growth (77.7% relative volume reduction after 8 days of treatment), an effect that was clearly improved upon repeated administrations. Experimental treatment with HSV-tk/GCV (23) or more recently with the SAP gene (21) in the same tumor type, induced a 40–50% and 67% relative volume reduction, respectively. McCray et al. (20), who used the Vpr gene integrated in the pcDNA3.1 (100 μg) vector in melanoma tumor from B16-F10 culture cells, described an 86% of tumor volume reduction which required 25 days of treatment. Therefore, the main advantage of the *gef* gene is not only its efficacy in melanoma cells but also the shorter latency for effective antitumoral action.

Although we have demonstrated the cytotoxic effect of *gef* gene in cancer cells, the specific mechanism of action has remained unclear so far. In prokaryotic cells, the *gef* gene diminishes the membrane potential, leads to membrane leakiness and also induces morphological changes (24). Eukaryotic cells fundamentally differ from prokaryotic cells in terms of their cellular structure, organization, metabolism and membrane composition. Nevertheless, as the eukaryotic endomembrane system arose in an ancestral prokaryotic lineage (25) *gef* gene might act in cell organelle membranes. Recently, it was demonstrated that breast cancer cells growth was inhibited by bacteriophage λ-holin, a protein that can permeabilize the bacterial membrane (12). Our results showed that 48 h after induction B16-F10 cells become multinucleated, in some cases extensively vacuolated and finally detached from the culture dish surface. Experiments with annexin, confocal laser-scanning microscopy and nucleosomes clearly showed that the *gef* gene is able to induce apoptosis in a time-dependent manner. These results are similar to those obtained with the SAP gene which also induces programmed cell death and direct DNA fragmentation in B16-F10 cells (21). Interestingly, the pronounced clinical chemoresistance of melanoma is strongly suggestive of an inactivation of apoptotic programmes. Defects in proapoptotic signalling pathways and enhancement of antiapoptotic pathways may synergistically contribute to this apoptosis deficiency (26). Immunohistochemical analysis by TUNEL assay revealed that pcDNA3.1/*gef* treatment significantly increased apoptosis in established subcutaneous B16-F10 tumors *in vivo*. The incidence of apoptosis in the tumor almost corresponded to the effect of tumor growth inhibition, suggesting that our experimental treatment resulted in tumor regression by significant augmentation of apoptosis.

Apoptosis may occur via death-receptor dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is triggered by the activation of death receptors, such as Fas and TRAIL receptors (DR4, DR5) activating initiator caspase-8, which then cleaves executioner caspase-3. The mitochondrial pathway of cell death is mediated by Bcl-2 family proteins, which disrupt the mitochondria membrane potential and result in release of apoptogenic factors, such as cytochrome *c*, from the mitochondria into cytosol; in turn, these factors would form an apoptosome with apoptosis activating factor 1 and caspase-9 (27). Treatments modulating apoptosis phenomenon, for example with bcl-2-targeted antisense, are a promising new strategy in melanoma (28). Assays with drugs such as hydroquinone or thiobenzanilides in this tumor type have demonstrated an action mechanism related to caspase-9 activation (29,30). This tumoral cellular injury mediated by caspases may also be induced by suicide genes and it may be relevant in relation to their application in tumors. In fact, CD/5FC system induces apoptosis in human malignant glioma cells by the activation of caspases-3 and -9 but not caspase-8 (31) while a certain modification, the bifunctional *E. coli* cytosine deaminase and uracil phosphoribosyltransferasefusion, is able to induce caspase-3 activation only (32). HSV*tk*/GCV activates caspase-3, -8 and -9 in rat bladder carcinomas (33) and a variant, the thymidylate kinase, induces apoptosis in Jurkat cells by activation of caspase-3 only (34). Our studies in B16-F10 cells expressing *gef* showed alteration of the mitochondrial membrane integrity suggesting that apoptosis is mediated by the mitochondrial pathway. This hypothesis is supported by the caspase-9 activity increase in B16-F10 transfected cells. Moreover, the mitochondrial transmembrane potential is altered in most of the cellular population, supporting the hypothesis on the possible effect of *gef* once it is released from the apoptotic cells. The mitochondrial-mediated apoptotic pathway is strongly supported by our ultrastructural findings in the induced B16-F10 tumors in mice which showed dilated mitochondria with disrupted cristae. Moreover, the *in vivo* assay shows that caspase-9 activity increases significantly after *gef* gene treatment, supporting the participation of a mitochondrial-mediated apoptotic pathway in our gene therapy system. However, we can not exclude the possible participation of other apoptosis-mediated molecule in treated B16-F10-induced tumors such as endonuclease G, Smac/DIABLO and HtrA2 (35). Further studies are required to elucidate the exact mechanisms involved.

We have reported the successful use of the *gef* gene as an anticancer gene therapy system, not only in melanoma cells in culture but in melanoma tumors *in vivo*. Our *in vivo* experiments show that *gef* gene has a rapid and efficient activity in relation to tumor volume decrease. However, *gef* gene binds to the mitochondrial membrane and its activity is not tumor-specific. Therefore, it will be necessary to create this specificity as in most of the toxic genes used in gene therapy (8–12). We have injected intra-tumorally the pcDNA3.1/gef plasmid to observe its activity in melanoma cells. Adenoviral vectors modified by attaching tumor-specific promoters should be used to assay metastatic melanoma treatment. Currently, we are using specific enhancer/promoter genes (such as tyrosinase) (36), new vectors (such as ReCon) (37) and combined therapy with cytotoxic drugs (38) to improve the tumoral response against *gef* gene. Moreover, it will be necessary to demonstrate the apoptosis induction in human melanoma by the extopic *gef* gene expression. In summary, our results suggest that *gef* is a suicide gene candidate for oncologic *in vivo* applications and that it may contribute to eradicate tumor mass in combination with surgery or classic radio- or chemotherapy.

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ORIGINAL ARTICLE

The cytotoxic activity of the phage E protein suppress the growth of murine B16 melanomas in vitro and in vivo

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Abstract Novel treatment modalities, including gene therapy, are needed for patients with advanced melanoma. The *E* gene from the phage φX174 encodes a 91-aa protein which lyses *Escherichia coli* by formation of a transmembrane tunnel structure. To evaluate whether this *E* gene has a cytotoxic impact on melanoma cells in vitro and in vivo, and could therefore be used as a new therapeutic strategy for this tumor type, we selected the B16-F10 murine melanoma cell line as a model. We used a nonviral gene delivery approach (pcDNA3.1/E plasmid) to study the inhibition of melanoma cells' proliferation in vitro and direct intratumoral injection of pcDNA3.1/E complexed with jetPEI to deliver *E* cDNA to rapidly growing murine melanomas, and found that the *E* gene has both a strong antiproliferative effect in B16-F10 cells in vitro and induces an efficient decrease in melanoma tumor volume in vivo (90% in 15 days). Interestingly, the GFP-E fusion protein expressed in melanoma cells was located in the mitochondria. In vitro and in vivo analysis demonstrated significant functional and morphological mitochondrial alterations accompanied by a significant increase of cytochrome *c* and active caspase-3 and -9 in transfected cells, which suggests that tumoral cell death is mediated by the mitochondrial apoptotic pathway. These results show that *E* gene expression in melanoma cells has an extraordinary antitumor effect, which means it may be a new candidate for an effective strategy for melanoma treatment.

Keywords Melanoma · *E* gene · Gene therapy · Apoptosis · Caspase · Mitochondria

Introduction

Although cancer rates remain stable, the number of invasive melanoma cases continues to rise. Melanoma represents only 4% of all skin cancers but nearly 80% of total skin cancer deaths, predominantly because of metastatic spread. Apart from surgery, the treatment options for melanoma, particularly metastatic melanoma, are relatively limited and emphasize the need for the development of novel efficacious therapies. As melanoma is a highly therapy-refractory tumor, it demands effective therapeutic combinations [1]. Suicide gene therapy has been proposed as a strategy for the treatment of intractable cancers and has been assayed in some clinical trials alone or in combination with other therapies (tumor irradiation or chemotherapy). Strategies to facilitate apoptosis by gene therapy in melanoma may be an alternative or complementary strategy for its treatment since

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it has been demonstrated that apoptosis deficiency is a critical factor for therapy resistance in this tumor [2].

Classical cancer suicide gene therapy employs genes which encode enzymes that convert nontoxic prodrugs into cytotoxic compounds which preferentially affect rapidly growing cells such as those found in cancers [3]. The two most widely used prodrug systems, namely herpes simplex virus thymidine kinase/gancyclovir (HSV*tk*/GCV) and bacterial cytosine deaminase/5-fluorocytosine (CD/5FU), have been assayed in melanoma in vitro and in vivo with limited results [4, 5]. Therapeutic genes which encode cytotoxic proteins directly could be an attractive alternative to this strategy. In contrast to classical suicide genes, which act by disrupting DNA synthesis and therefore target only rapidly dividing cells, these new toxins may act by killing both quiescent and rapidly dividing tumor cells and may be effective for aggressively growing tumors as well as for those that grow more slowly. The most recent experiences with genes expressing toxins from bacteria such as diphtheria toxin [6] or streptolysin O [7], plants such as saporin (SAP) [8], viruses such as the matrix protein of vesicular stomatitis virus [9], and bacteriophages such as alpha-holin [10], have shown a high cytotoxicity for tumoral cells derived from different tissues.

In this context, the *E* gene is another potentially interesting bacteriophage lysis gene for cancer therapy. In contrast to most double-stranded DNA phages, which generally encode two genes that elicit host-cell lysis (endolysin and holing protein), the small single-stranded DNA phage φX174 has only one lysis gene. The 91-aa E protein encoded by this causes cell lysis at concentrations of 100–300 molecules per cell [11], although its mechanism of action is controversial. Gene fusion analysis has revealed that only the 29 amino-terminal amino acids of the E polypeptide encompassing the putative transmembrane domain are required for lytic activity [12, 13]. However, this polypeptide has no detectable cell-wall-degrading activity, and given its simple primary structure it is unlikely to have any enzymatic activity at all. Scanning electron microscopy images of cells undergoing E-mediated lysis have shown discrete 50- to 200-nm holes in the cell membrane. This observation has led to the proposal of a model in which the E protein oligomerizes to form a "transmembrane tunnel" spanning the entire cell envelope, thereby releasing the cytoplasmic content [14].

In light of the above, we decided to investigate the potential of the native *E* gene in cancer gene therapy approaches by testing this gene in both in vitro and in vivo systems to determine its tumoral cell-killing efficiency. We selected B16-F10 murine melanoma cells because this tumor cell line is a very good model for many human malignancies due to its highly invasive and metastatic nature. Our results demonstrate that *E* gene expression induces a significant decrease in melanoma cell viability and a spectacular reduction of melanoma tumor growth rates in vivo by inducing apoptosis in tumoral cells via the mitochondrial pathway. These growth-inhibitory and cell-killing effects strongly suggest that the *E* protein may have a potential use in cancer gene therapy.

Materials and methods

Cell culture

The B16-F10 murine melanoma cell line (CRL6475) was obtained from American Type Culture Collection (ATCC) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 40 mg/l gentamicin, and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37°C in an atmosphere containing 5% CO_2.

Transfection

The *E* gene (Dr. J.L. Ramos, Zaidín Experimental Station, CSIC, Granada, Spain) was amplified from the pMC22 plasmid with primers (sense 5′-ATGAAGCAGCA TAAGGCGATG-3′ and antisense 5′-TTACTCGGATTCG TAAGCCGTC-3′) and subcloned into the pcDNA3.1-TOPO vector following the manufacturer's instructions (Invitrogen, Barcelona, Spain). The resulting plasmid pcDNA3.1/*E* was transformed into the subcloning efficiency DH5 alpha chemically competent *E. coli* (Invitrogen). The correct DNA sequence was confirmed by sequence analysis using the T7 primer. We used jetPEI DNA transfection reagent (PolyPlus Transfection, Inc, NY, USA) for cell transfection, according to the manufacturer's instruction. The efficacy of cell transfection was checked using pcDNA3.1/GFP (Green Fluorescent Protein) provided by Dr. G. Ortiz (IBIMER, Granada, Spain).

Reverse transcription-PCR

RNA was extracted from transfected and parental cells with the Rneasy Mini kit (Qiagen). cDNA was generated by means of the Promega Reverse Transcription System (Promega, Madrid, Spain) using total cellular RNA (1 μg). Polymerase chain reaction (PCR) amplification of the *E* gene was performed under the following conditions: 94°C for 1 min, 35 cycles at 94°C for 1 min, 55°C for 30 s and 72°C for 30 s, and 72°C for 10 min. The sense primer 5′-GCTTTCCTGCTCCTGTTGAG-3′ and the antisense

primer 5′-TTGACGCACGTTTTCTTCTG-3′ were used for reverse transcription-PCR (RT-PCR). RNA integrity was assessed by amplification of β-actin mRNA (sense: 5′-ATCATGTTTGAGACCTTCAA-3′ and antisense 5′-CATCTCTTGCT CGAAGTCCA-3′). PCR products were analyzed by standard agarose gel electrophoresis. Images were scanned and analyzed using a Bio-Rad documentation system (Quantity One Analysis Software). Relative *E* mRNA expression was calculated as the ratio of *E* to β-actin. RNA from B16-F10 melanoma induced in mice was obtained with the QIAamp RNeasy Fibrous Tissue Mini Kit (Qiagen) and RT-PCR was performed as described above.

Proliferation assays

Parental and transfected cells were seeded in a 96-well plate at 6×10^3 cells per well. After 24, 48, and 72 h, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well and incubated at 37°C for a further 4 h. Then, 200 μl of dimethyl sulfoxide (DMSO) was added to each well after removal of the medium. The optical density was then determined using a Titertek multiscan colorimeter (Flow, Irvine, California) at 570 and 690 nm. The linearity of the MTT assay with cell number was tested for each B16-F10 cell stock before each cell-growth experiment. B16-F10 cells transfected with empty vector were used in the proliferation assay as control.

Generation of the GFP-E fusion protein

The creation of a fusion protein between protein lysis E and the GFP was chosen as the method for studying intracellular localization. The plasmid pcDNA3.1/GFP was used to perform the subcloning. The E cDNA was obtained from pMC22-E by PCR using 5′-GCTATGG TACGCTGGACTTTG-3′ as the forward primer and 5′-GCTCTAGACTCTCCTTCCGCA-3′ as the reverse primer. The latter was engineered to eliminate the stop codon from the E cDNA clone containing GFP so that it could be expressed as a fusion protein. A PCR reaction with pMC22-E as the template was performed under the following conditions: 1×94°C for 1 min, 30× (94°C for 1 min; 55°C for 90 s; 72°C for 90 s), 1×72°C for 10 min. Amplification of the target sequence of the correct size was confirmed by gel electrophoresis. The PCR product was ligated into pcDNA3.1/GFP vector following the manufacturer's protocol (Invitrogen). The resulting plasmid (pcDNA3.1/GFP-E) was transformed into subcloning efficiency DH5 alpha chemically competent *E. coli* (Invitrogen).The correct DNA sequence was confirmed by DNA sequencing analysis.

Microscopy analysis

B16-F10 cells were transfected with the pcDNA3.1/GFP-E construction as described above. For mitochondrial staining, the medium was changed to DMEM containing 500 nM MitoFluor Red (MitoTracker, Invitrogen), incubated for 15 min, and then replaced with normal medium. For nuclear staining, DAPI (Invitrogen) was diluted 1:1,000 in a 1:1 solution of sterile water and PBS to a final concentration of 100 nM. DAPI solution (1 ml) was added to fixed cells in a 60-mm dish and incubated for 20 min at room temperature. The cells were then rinsed briefly with PBS and mounted. GFP was excited at 488 nm, DAPI nuclear stain at 364 nm, and MitoFluor Far Red at 588 nm. Fluorescent microscopy analysis was carried out with a Nikon Eclipse Ti (Nikon Instruments Inc. NY, USA.). Alternatively, the fluorescence was detected by confocal microscopy using a Leica DMI6000 microscope (Heidelberg, Germany).

Apoptosis analysis

For analysis of the cell-cycle distribution, parental, and transfected cells (pcDNA3.1/GFP-E construction) were harvested, washed twice with sample buffer (100 mg glucose; 100 ml PBS without Ca^{2+} or Mg^{2+}), and fixed in 70% (*v*/*v*) cold ethanol for at least 1 h before staining. The cells were pelleted, washed once with sample buffer, and resuspended in propidium iodide (PI) solution (50 μg/ml PI, 0.5 mg/ml RNase in sample buffer, pH 7.4) for 30 min in the dark. A fluorescence-activated cell sorter analysis was performed 24, 48, and 72 h after transfection. Transfected cells treated with the pan-caspase inhibitor ZVAD-FMK (BD Pharmingen, San Diego, CA) were also analyzed. Controls were realized with pcDNA3.1 and pcDNA3.1-GFP. The data were collected and analyzed using the Cellfit program with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). To confirm apoptosis, cells transfected with pcDNA3.1/*E* (without GFP) were washed twice with PBS and incubated in binding buffer containing annexin V-FITC (25 μg/ml) and PI (25 μg/ml) in the dark for 15 min at room temperature (Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA, USA). Microscopy analysis was carried out with a Leica DMI6000 confocal microscope.

Measurement of the mitochondrial membrane potential ($\Delta\Psi_m$)

To measure levels of $\Delta\Psi_m$ disruption, parental cells and cells transfected with pcDNA3.1/E) were washed twice with cold PBS and incubated with 40 nM DiOC6(3) (Invitrogen) for 15 min at 37°C. They were then washed

with ice-cold PBS and resuspended in 500 µl of PBS. The fluorescence intensities of DiOC6(3) were analyzed on a FACScan flow cytometer with excitation and emission settings of 484 and 500 nm, respectively.

Western blotting

Thirty micrograms of protein extracts from parental and transfected B6-F10 cells were used for SDS-PAGE in a Mini Protean II cell (Bio-Rad, Hercules, CA). The protein extract from pcDNA3.1/GFP-E transfected cells treated with the pan-caspase inhibitor ZVAD-FMK (BD Pharmingen) was also analyzed. The caspase inhibitor (100 µM) was applied 24 h before transfection. The separated proteins were transferred to a nitrocellulose membrane by applying a current of 20 V at room temperature for 30 min. The blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h and then incubated with primary antibodies [rabbit polyclonal IgG anti-caspase-3 (1:1,000 dilution), anti-caspase-8 (1:200 dilution), and anti-caspase-9 (1:500 dilution), mouse monoclonal anti-cytochrome *c* antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal anti-β-actin antibody (1:5,000 dilutions; Abcam, Cambridge, MA)] overnight at 4°C. After addition of peroxidase-conjugated secondary antibody, proteins were detected by enhanced chemiluminescence (ECL, Bonus, Amersham, Little Chalfont, UK). GFP-E fusion protein was detected with an Anti-GFP N-terminal antibody (Sigma, St. Louis, MO). Samples were checked for mitochondrial contamination with mouse monoclonal anti-COX IV antibody (1:5,000 dilutions; Abcam). The mitochondrial fraction from B16-F10 cells (Mitochondria isolation kit, Sigma) was used as positive control.

Tumor induction and measurement

Female C57BL/6 mice (Scientific Instrumentation Center, Granada University) were used for the in vivo study. All mice (weight: 25–30 g) were maintained in a laminar air-flow cabinet in a room kept at 37°C and 40–70% relative humidity with a 12-hour light/dark cycle under specific pathogen-free conditions. All studies on animal models were approved by the Ethical Committee of the Medical School of Granada University and performed according to its guidelines. Tumors were induced by subcutaneous injection of 5×10^5 B16-F10 cells into the left flanks of C57BL/6 mice. Tumors were allowed to grow to the appropriate size (75 mm^3, the ideal minimum size for intratumoral injection) before treatment. After reaching this volume, the tumors were measured at periodic intervals following treatment using a digital caliper by measuring the longest diameter (a) and the next-longest diameter (b) perpendicular to (a). The tumor volume was calculated from these measurements using the formula $V = ab^2\pi/6$.

Intratumoral treatment

In vivo JetPEI (Polyplus Transfection, Inc) was used as a transfection-enhancer reagent. PEI/DNA complexes with a ratio of 1:6 were prepared in a solution of 10% *w*/*v* glucose. This was carried out in a two-step procedure for the preparation of a standard quantity of 20 µg of PEI/DNA complex, according to the manufacturer's instructions. Tumors were then treated intratumorally during 15 days with pcDNA3.1/*E* plasmid or empty vector. A control group, which was not treated, was included. The in vivo experiments were conducted twice with a total $n=14$, with the exception of the control group ($n=7$).

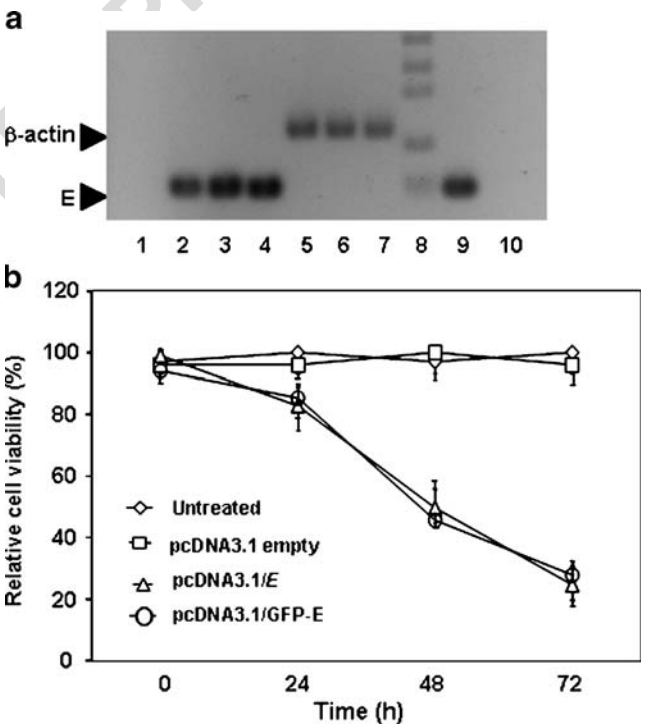

Fig. 1 *E* gene expression and growth rate inhibition in B16-F10 cells. **a** RT-PCR showing *E* gene expression. *Lane 1* negative control (RT−). *Lanes 2–4* B16-F10 transfected cells at different time periods (24, 48, and 72 h, respectively). *Lanes 5–7* the integrity of the RNA was demonstrated using β-actin primers in B16-F10 transfected cells at the same times. *Lane 8* molecular weight. *Lane 9* positive control (pcDNA3.1/E). *Lane 10* negative control (non-transfected cells). Raw data are given in as electronic supplementary material. **b** Growth of B16-F10 cells expressing *E* was detected by MTT assay. Cells transfected with pcDNA3.1/E and with pcDNA3.1/GFP-E showed a clearly increased cell death compared to the control cells and cells transfected with empty pcDNA3.1 vector ($P<0.05$). Values represent means ± SD of quadruplicate cultures. The complete data are shown as electronic supplementary material

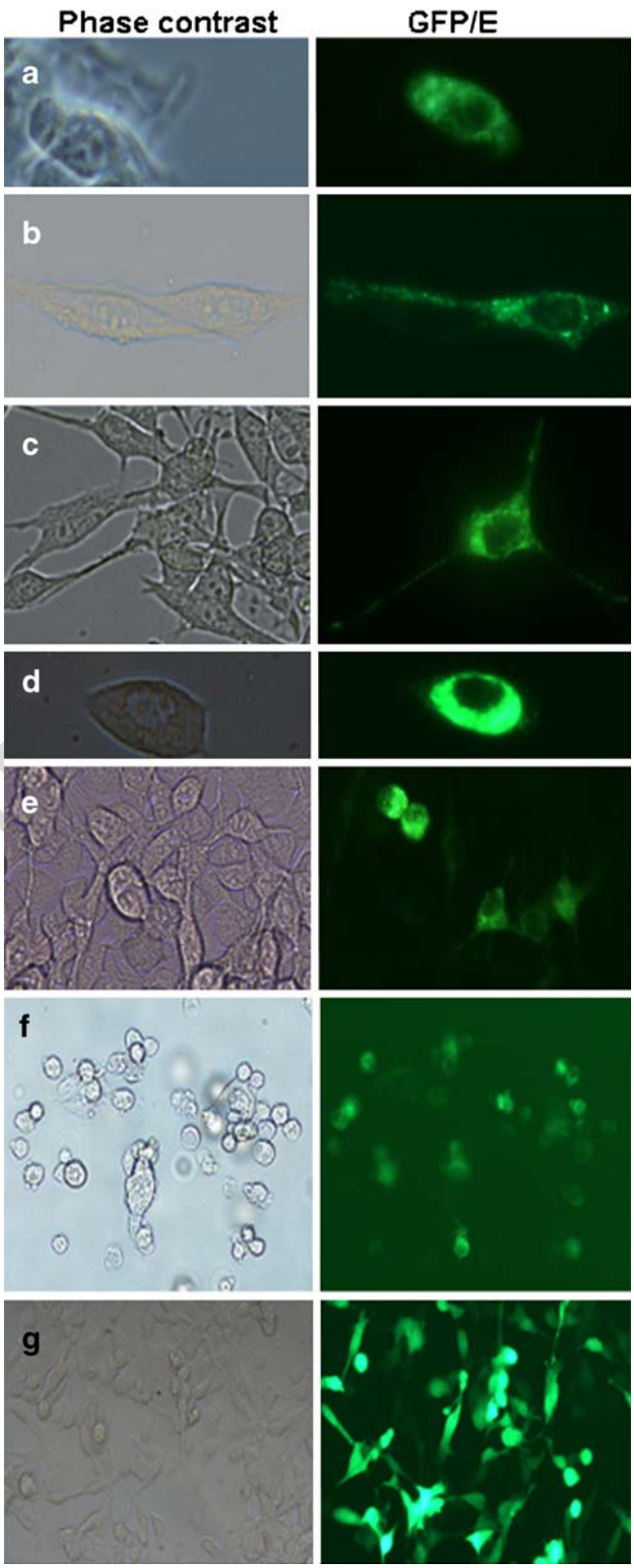

Fig. 2 Subcellular localization of the GFP/E fusion protein expressed in B16-F10 cells. The cells were transfected with a GFP/E fusion construct as indicated in the "Materials and methods" section. Twenty four hours after transfection (**a**, ×40), the fluorescence pattern was dotted and localized in the cell cytoplasm. Cytoplasmic extensions, which progressively increased in length, appeared after 48 h (**b** and **c**, ×40). A rapid increase in the number of rounded cells detached from the surface of the culture dish was observed after 72 h (**d**, ×40). The cultures contained cells with different morphologies (**e**, ×20), although the majority of cells were rounded (**f**, ×20). Cells transfected with GFP (without E) show no morphological changes (72 h; **g**, ×20)

Immunohistochemistry

Tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 3–5-μm sections. Apoptosis was evaluated by the TUNEL technique using the In Situ Cell Death Detection Kit (Roche). Cell nuclei of cultures were counterstained with DAPI and fluorescence images were captured using a Leica DMI6000B inverted microscope. For measuring proliferation, sections were probed with biotinylated Ki-67 antibody (1:50; Dako, Spain). After deparaffinization and rehydration, the tissue sections were incubated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase. The sections were blocked for 30 min with goat serum and incubated overnight with the primary antibody at 4°C. The sections were then washed with PBS and incubated with a biotinylated secondary antibody for 30 min. After several washes with PBS, the products were visualized using streptavidin horseradish peroxidase with diaminobenzidine as chromogen and hematoxylin as the counterstain. The percent apoptosis and Ki-67 labeling index were determined by counting the number of labeled cells and dividing by the total number of cells in the field (five high-power fields/slide). Values were presented as the mean ± SD (standard deviation).

Transmission electron microscopy

Melanoma tumors grown in mice were collected, cut into small pieces, and immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 1 h. After post-fixation with 1% OsO_4 in cacodylate buffer (room temperature, 2 h), sections were dehydrated through graded ethanol concentrations with a final propylene oxide dehydration. Samples were then embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H7000 transmission electron microscope.

Statistical analysis

The SPSS 14 software package (SPSS, Chicago, IL, USA) was used for all statistical analyses. Results were compared

by using Student's *t* test. All data are expressed as means ± SD. Differences were considered statistically significant at a *P* value of less than 0.05

Results

Inhibition of B16-F10 cell growth in vitro by *E* gene

E expression in B16-F10 transfected cells was assessed by RT-PCR. As shown in Fig. 1a, an amplification fragment of 223 bp was found in B16-F10 cells transfected with pcDNA3.1/*E* at different time periods, thus indicating the effectiveness and ability of the construction for use in the subsequent experiment. Analysis of the bands, which were normalized by comparison with the β-actin signal, showed a progressive increase of *E* expression (three- and 4.3-fold higher at 48 and 72 h versus B16-F10 cells at 24 h). The B16-F10 cells transfected with pcDNA3.1/*E* showed a significant and time-dependent decrease in cell viability (Fig. 1b), with a 17.2% decrease versus control cultures being observed 24 h after transfection. The decrease in cell viability was 50.3% at 48 h, although the main decrease occurred at 72 h (75.4%). In contrast, the growth of B16-F10 cells transfected with the empty vector (control group) was similar to that of the parental cells.

Subcellular localization of E protein and changes in cell morphology

B16-F10 cells were transfected with pcDNA3.1/GFP-E to determine the localization of E protein. After 24 h, expression of the GFP-E fusion proteins showed a clear signal in the cell cytoplasm with a dotted fluorescence pattern (Fig. 2a). Analysis at different post-transfection times showed characteristic changes in the tumor cell morphology, with the main feature after 24 h being the appearance of cytoplasmic extensions (Fig. 2b,c). Many of these cells were swollen and appeared to be vacuolated. The number of rounded cells increased rapidly after 72 h, and they began to progressively detach from the surface of the culture dish and die (Fig. 2d,e,f). The mitochondrial localization of the GFP/E fusion protein was confirmed by dual monitoring of the red fluorescence of Mitofluor, which specifically stains mitochondria in live cells, and the green fluorescence of GFP. The yellow color in the merged images confirms that GFP-E co-localizes with the Mito-Tracker dye in the mitochondria (Fig. 3a, b).

Expression of *E*-gene-induced apoptosis in B16-F10 cells

To determine whether *E* gene expression induced apoptosis, B16-F10 cells transfected with pcDNA3.1/GFP-E were analyzed by FACScan. As can be seen in Fig. 4a, the

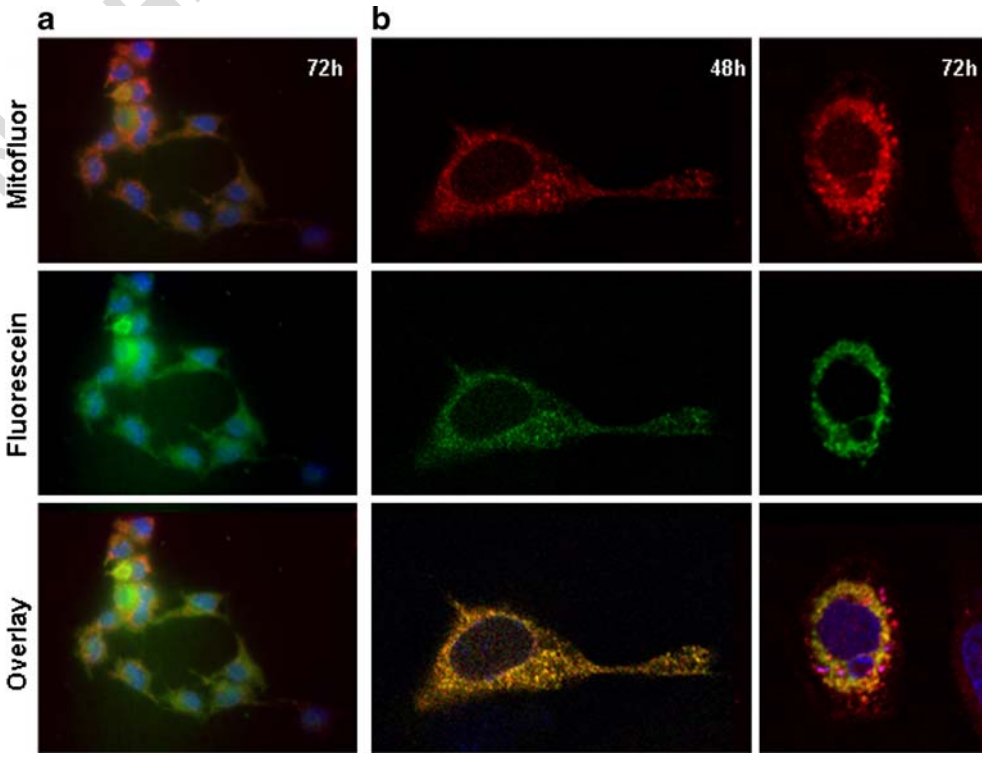

Fig. 3 Mitochondrial localization of the GFP/E fusion protein in B16-F10 cells. Representative image of transfected B16-F10 cells expressing E-GFP at different times taken using fluorescent (**a**) and confocal (**b**) microscopy. The dotted pattern of GFP-E fluorescence is shown in *green*. The majority of GFP-E expressed co-localizes with MitoFluor, which is shown in *red*. Co-localization appears in *yellow*. Cell nuclei were counterstained with DAPI. **a** ×40; **b** ×100

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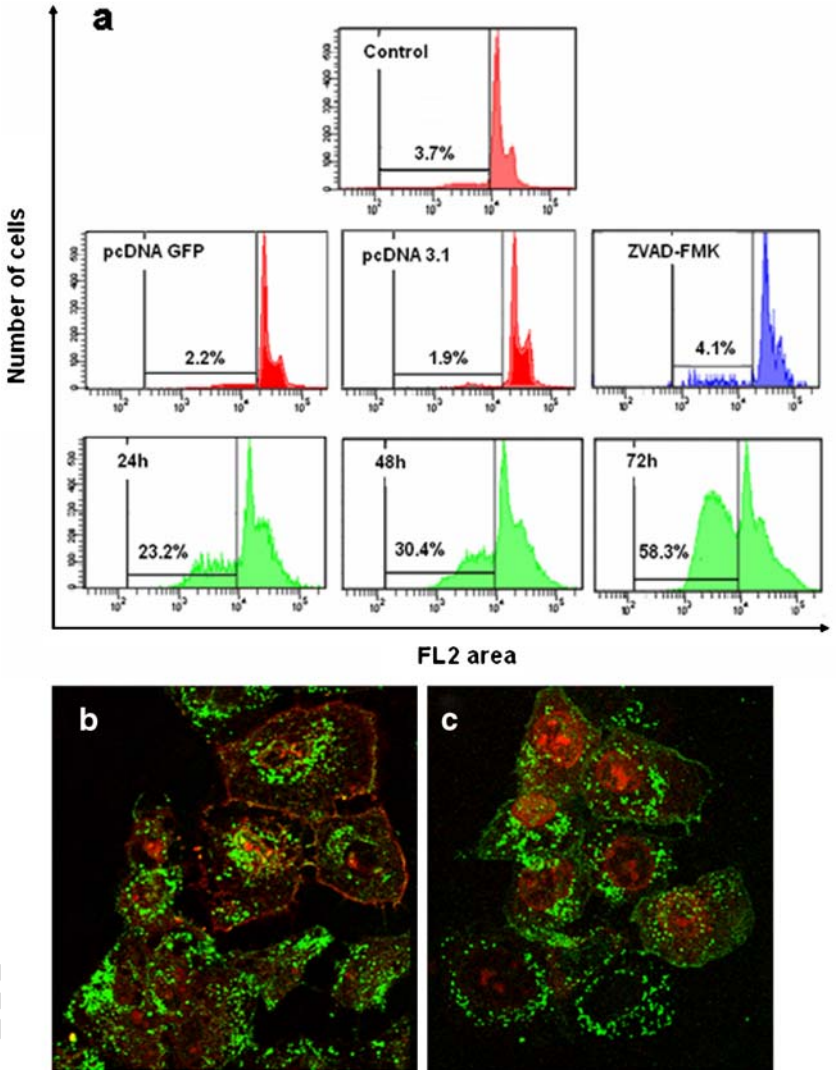

Fig. 4 Analysis of apoptosis induction by the *E* gene in B16-F10 cells. B16-F10 parental (control) and transfected cells (GFP/E fusion construct) were analyzed at indicated times by fluorescence-activated cell sorting to determine apoptotic cell death. The apoptosis was assessed by PI staining by calculating the percentage of cells in the sub-G1 fraction. Transfected cells treated with ZVAD-FMK were also analyzed at 72 h. These data are representative results from four separate experiments (**a**). To confirm apoptotic induction by E gene expression, B16-F10 cells were transfected with pcDNA3.1/*E* vector and analyzed by annexin V-FITC staining and confocal microscopy. Cell nuclei were counterstained with PI. The figure shows representative images of a stronger staining when the study was carried out at 48 (**b**) and 72 h (**c**) after *E* transfection; magnification, ×40

apoptosis fractions were 3.7±0.2% for the parental cells (control) and 4.1±0.34% for the cells treated with pan-caspase inhibitor. Similar results were obtained for pcDNA3.1 and pcDNA3.1-GFP transfected cells (2.2±0.2% and 1.9±0.3%, respectively). In contrast, cells transfected with pcDNA3.1/GFP-E for 24 h showed an apoptosis fraction of 23.2±0.81%, significantly higher than that of the control group. After 48 and 72 h of transfection, the percentage of apoptotic cells increased to 30.4±0.52% and 58.3±0.69%, respectively. An annexin V and PI study confirmed the induction of apoptosis by the *E* gene in melanoma cells (Fig. 4b,c).

Expression of *E*-gene-induced modulation of mitochondrial membrane potential

As shown in Fig. 5a, a significant decrease in $\Delta\Psi_m$, as measured by DiOC6(3) dye staining, was detected in transfected cells, thus indicating an increase in mitochondrial membrane permeability after *E* gene treatment. After a 24-h transfection, 27.5% of cells showed a decrease in $\Delta\Psi_m$. This percentage increased progressively with transfection time, and the most prominent $\Delta\Psi_m$ dissipation was observed at 72 h, with 51.8% of cells having altered mitochondrial membrane permeability. No changes in $\Delta\Psi_m$ were detected in B16-F10 transfected with the empty vector or parental cells.

Apoptotic signaling pathway induced by *E* gene expression

Western blot analysis showed that alteration of the $\Delta\Psi_m$ in transfected cells was accompanied by the release of cytochrome *c* (Fig. 5b). Determination of the caspase expression in the same cells showed enhanced caspase-9 and -3 activation. In contrast, caspase-8 showed no expression modulation (Fig. 5b). Treatment of B16-F10 cells with the pan-caspase inhibitor ZVAD-FMK efficiently inhibited pcDNA3.1/*E*-induced caspase-3 and -9 activation

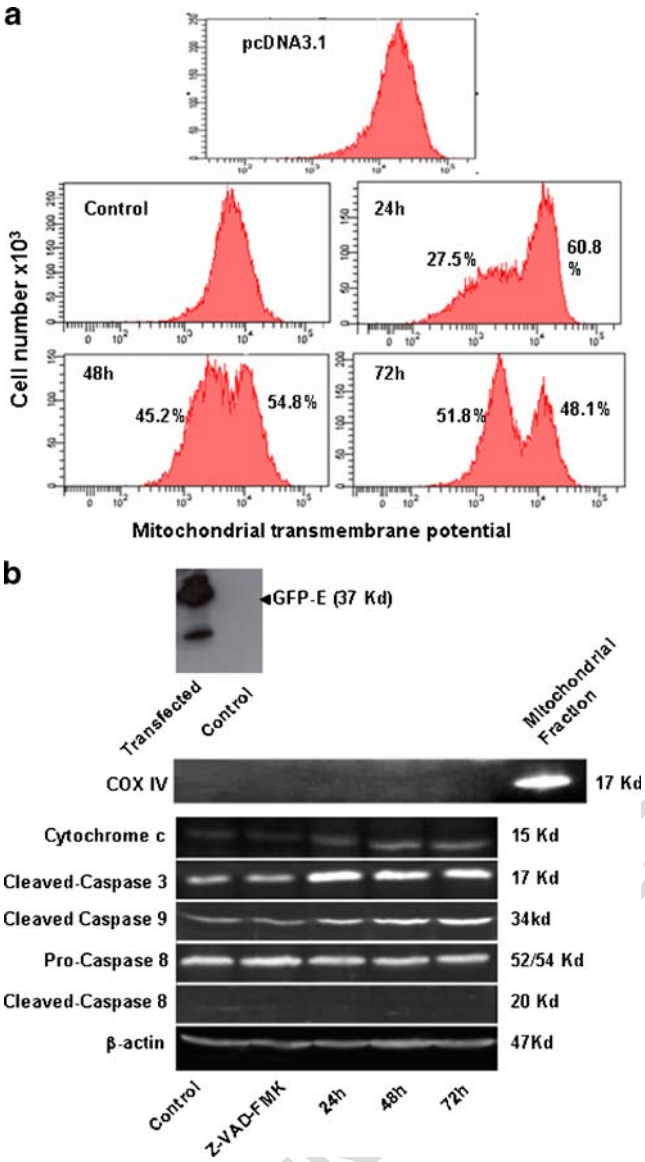

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Fig. 5 Apoptotic mechanism induced by *E* gene expression. **a** Mitochondrial membrane potential ($\Delta\Psi_m$) disruption induced by E gene expression in B16-F10 cells. DiOC6(3) was added to the cell suspension (500 µl PBS) for 15 min at a final concentration of 40 nM. The fluorescence intensity of DiOC6(3) was analyzed by flow cytometry. The data shown are representative of three independent experiments. **b** Western blotting analysis of apoptotic signals. GFP-E fusion protein was detected in the B16-F10 transfected cells (24 h). Cytoplasmic extracts from B16-F10 parental (control) and transfected cells at 24, 48, and 72 h were prepared to determine cytochrome *c* and caspase proteins. The pan-caspase inhibitor ZVAD-FMK was applied to determine whether caspases were involved in this process, and the filter was probed with β-actin antibody to determine whether the amount of proteins in each lane was comparable. Mitochondrial contamination was estimated using anti-COX IV antibody. Immunoblots were visualized with an enhanced chemiluminescence detection system. The complete data are shown as electronic supplementary material

and release of cytochrome *c*, which appeared at similar levels to those in the control cells (Fig. 5b). All the data indicate that *E* transfection induces mitochondria-mediated apoptosis in melanoma cells.

E gene effects on melanoma growth in vivo

The potential of the *E* gene to promote tumor cell death in vivo was evaluated by direct injection of the plasmid complexed with jetPEI into B16-F10 subcutaneous mice tumors. Figure 6a,b show that injection of pCDNA 3.1/*E* gene (20 µg) was able to inhibit tumor growth. The tumor volumes of mice treated with pcDNA 3.1/E were significantly smaller than those of control mice ($P<0.05$). At the end of observation (day 15), tumor growth was inhibited by up to 90.6% in pcDNA 3.1/E-treated mice compared with control mice. Mice treated with *E* gene showed no evidence of systemic toxicity (i.e., animal death, loss of body weight, other tissue damage, or changes in behavior or aspect). After empty vector injection with jetPEI, no tumor growth modifications were observed. RT-PCR was performed to ensure the *E* gene was overexpressed in the tumors of mice treated with pcDNA 3.1/E but not in control mice treated with pcDNA 3.1 (Fig. 6c).

In vivo analysis of *E*-induced apoptosis

To gain further insight into the mechanism of melanoma growth inhibition by *E* gene in vivo, we analyzed the apoptosis-linked DNA fragmentation and the mitotic index using the TUNEL assay and the detection of Ki-67, respectively. As shown in Fig. 7, the number of apoptotic cells (green) was significantly higher in tumors treated with pcDNA3.1/E than in control tissue. As regards the Ki-67 analysis assay, only small differences in nuclear staining could be observed between E-gene-treated (15 days) and untreated tumors. Melanoma tumors after 4 and 8 days of treatment showed similar nuclear staining for Ki-67 to control tumors (Fig. 7). These tumors only showed a small but non-significant decrease in the mitotic index after 15 days of treatment (data not shown).

Transmission electron microscopy

The most important morphological change in the melanoma tumors after pcDNA3.1/E treatment was the pronounced swelling in the mitochondria seen from 2–4 days until the end of treatment. Cells with altered mitochondria exhibited an apoptosis-like aspect consisting of a reduced cell size, formation of vacuoles, and nuclear condensation with chromatin aggregated in large, dense, granular masses which abutted on the nuclear membrane (Fig. 8). In contrast, the control tissue showed giant malignant cells

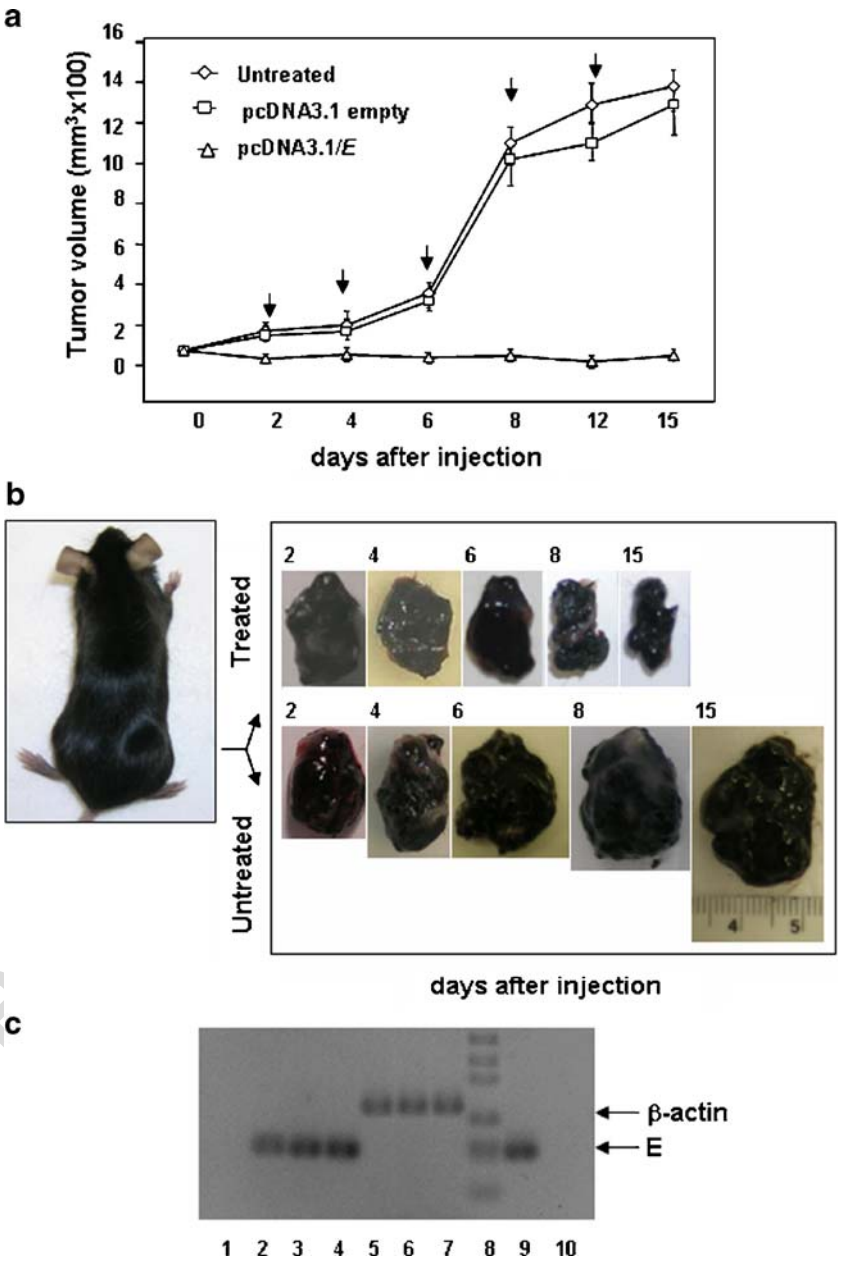

Fig. 6 Effect of direct intratumoral injection of the *E* gene on the growth of subcutaneous tumor induced by B16-F10 murine tumor cells. **a** Tumor volume variation after *E* gene treatment (time of injection is indicated with *arrows*). The plot shows a significant reduction in tumor volume with respect to untreated tumors or those transfected with empty vector. **b** Representative gross appearance of tumors excised from mice killed during treatment at 2 ,4, 6, 8, and 15 days and tumors obtained from untreated mice at the same time intervals. **c** Representative image of the determination of *E* gene expression in tumor mice. *Lane 1* negative control (RT−). *Lanes 2–4* *E* gene expression at 4, 8, and 15 days of treatment, respectively. *Lanes 5–7* the integrity of the RNA tissue used (obtained at the same time periods) was determined using β-actin primers. *Lane 8* molecular weight. *Lane 9* positive control (pcDNA3.1/E). *Lane 10* negative control (untreated tumors). The complete data are shown as electronic supplementary material

with an intact cell membrane, single- or multiple-nuclei cells, well-preserved organelles, and the presence of typical melanosomes. The mitochondria in control cells and cells treated with vehicle alone remained unaffected (Fig. 8).

Discussion

Gene therapeutic approaches which involve genes encoding cytotoxic proteins for tumor cells are being developed as a promising alternative cancer treatment. This is the first study in which the bacteriophage lysis gene *E* was evaluated for its ability to kill melanoma cells in vitro and in vivo.

New treatment strategies for malignant melanoma are urgently needed because conventional approaches, like chemotherapy and radiation, have little impact on patient survival in the advanced stages of the disease. To date, classical suicide gene therapy systems have not guaranteed the successful treatment of melanoma and have induced only a partially positive response. Recently, it was demonstrated that the administration of GCV in B16-HSV-tk tumors induced in mice was completely ineffective and that GCV may have therapeutic value only as an adjuvant for other T-cell therapies [15]. On the other hand, to increase the low effect of the CD/5-FU system in melanoma, a fusion protein based on the human single-chain fragment variable (scFv) human antibody (E8)

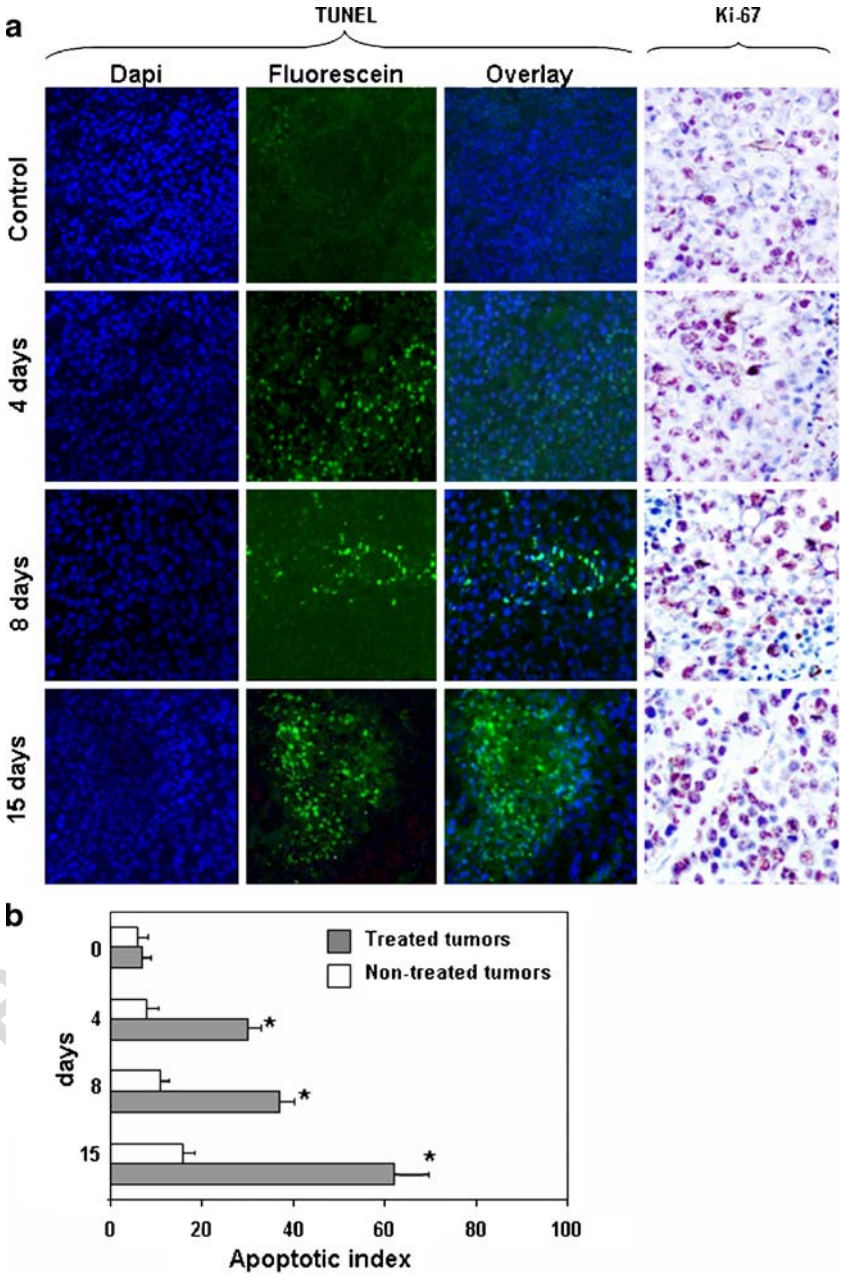

Fig. 7 Histological evaluation of apoptosis and proliferation activity of melanoma cells in vivo. **a** Representative photographs of tumor sections showing TUNEL-positive cells (*green*). Sections were counterstained with DAPI (*blue*). Apoptotic cells increased in tumors treated with pcDNA 3.1/E at 4, 8, and 15 days compared to untreated tumors (*control*); magnification, ×20. Proliferative activity, as detected by Ki-67 staining, was not significantly modulated. Nuclei were counterstained with hematoxylin; magnification, ×40. **b** Percent apoptosis (apoptotic index) in each group. Values are expressed as means ± SD. Level of significance compared to control cells; **P*<0.05 compared with control (day 0)

specific for CEA and yeast cytosine deaminase (yCD) has been assayed [16]. One of the main limitations of these indirect action systems, however, is the need to use prodrugs [17]. During the last few years, antitumoral strategies based on transfection of the cDNA constructs encoding toxins with a direct action have been developed. The nonsystemic administration of a prodrug in these systems reduces its side effects, its bioavailability limitations, and the need for two consecutive applications of vector and prodrug. Moreover, these genes can be directly expressed in the cytosol of the target cells, thus overcoming the problems (cytotoxicity, internalization efficiency, and resistance acquired by cancer cells) originating from their use as components of immunotoxins or recombinant chimeras [18]. In this context, and as we pointed out previously, some toxic genes have demonstrated their efficacy in cancer gene therapy [6–10]. In melanomas, for example, viral genes encoding toxins such as viral protein R and some plant genes such as SAP have been applied and been found to induce tumoral cell death [19, 20]. We have recently shown that a suicide gene from *Escherichia coli* known as *gef* has a therapeutic effect against these cells [21], and we have now demonstrated that the *E* gene from the fX174 phage not only inhibits melanoma proliferation in vitro but is also highly toxic for tumors in vivo (growth arrest of more than 90%). Other phage

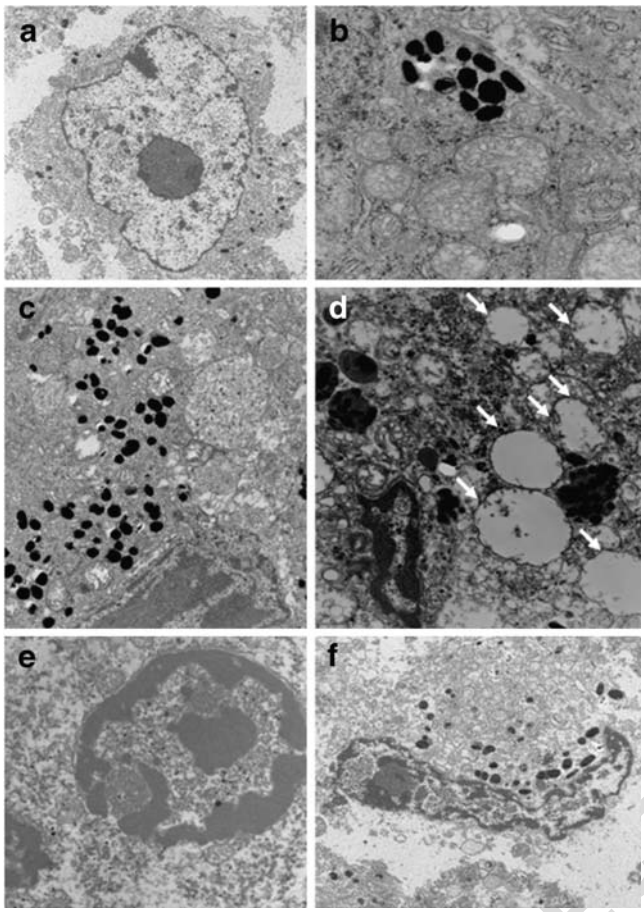

Fig. 8 TEM images of melanoma tumors. Untreated tumors show typical tumor cells with a polygonal shape, a large nucleus, and a light cytoplasmic complexion containing well-preserved organelles (**a**; ×1,100), including mitochondria and a large number of melanosomes (**b**; ×4,000). Tumors transfected with pcDNA3.1/E also show a large number of melanosomes (**c**; ×2,100) but their mitochondria are swollen with no, or with disrupted, cristae (**d**, *arrows*; ×4,000). Ultrastructural characteristics of apoptosis, such as chromatin condensation, crescent formation, and margination, were also observed in the nuclei of melanomas treated with pcDNA3.1/E after 4 days (**e**) (×6,300). Necrotic nuclei were observed at the end of the treatment (15 days; **f**; ×6,300)

proteins, such as λ-holin [22], have led to a substantial reduction in the viability of breast cancer cells in vitro and in their growth rates in vivo. However, as discussed in more detail below, their effects are not as pronounced as those obtained with *E* gene.

The cytotoxic effects of the *E* protein include alterations in cellular morphology preceding cell death. For example, forty eight hours after induction the cells become rounded and in some cases extensively vacuolated, and they finally become detached from the surface of the culture dish. This abnormal cell morphology strongly suggests the development of apoptosis. Interestingly, an inactivation of apoptotic programs has been linked to the pronounced clinical chemoresistance of melanoma. Defects in proapoptotic signaling pathways and enhancement of antiapoptotic pathways may contribute synergistically to this apoptosis deficiency [3, 23]. In fact, some toxic genes, such as SAP, have been found to induce cell death in B16-F10 melanoma cells by direct DNA fragmentation [20]. Analysis of our transfected B16-F10 melanoma cells by FACScan and confocal laser-scanning microscopy clearly showed the ability of the *E* gene to stimulate apoptosis in a time-dependent manner, although its specific mechanism of action remains unclear

The molecular target for the E protein in prokaryotic cells is the enzyme phospho-MurNAc-pentapeptide translocase (MraY), an integral membrane protein involved in bacterial cell wall peptidoglycan biosynthesis, with an essential role being played by peptidyl-prolyl isomerase SlyD [13]. Eukaryotic cells differ fundamentally from prokaryotic cells in terms of their cellular structure, organization, metabolism, and membrane composition. However, since the eukaryotic endomembrane system, including mitochondria, arose in an ancestral prokaryotic lineage [24], bacteriophage genes, including E, might act in cell organelle membranes. In order to analyze this connection, we decided to investigate the possible mitochondrial alterations and the molecular events underlying the apoptosis induced in our transfected B16-F10 cells.

Apoptosis may occur via either death-receptor-dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is triggered by the activation of death receptors such as Fas and TRAIL receptors (DR4, DR5). These go on to activate initiator caspase-8, which then cleaves executioner caspase-3. The mitochondrial pathway is mediated by Bcl-2 family proteins, which disrupt the mitochondria membrane potential and result in the release of apoptogenic factors, such as cytochrome *c*, from the mitochondria into the cytosol. These factors, in turn, form an apoptosome with apoptosis-activating factor 1 and caspase-9 [25]. Treatments that modulate apoptosis, for example with bcl-2-targeted antisense, are a promising new strategy in melanoma treatment [26]. Assays with drugs such as hydroquinone or thiobenzanilides in this tumor have demonstrated caspase-9 activation [27, 28]. This caspase-mediated tumoral cell injury can also be induced by suicide genes. The CD/5FU system induces activation of caspases-3 and -9 but not caspase-8 in human malignant glioma cells [29], while a modification (the bifunctional *E. coli* CD and uracil phosphoribosyltransferase fusion) is able to induce caspase-3 activation only [30]. HSV*tk*/GCV, on the other hand, activates caspase-3, -8, and -9 in rat bladder carcinomas [31] and a variant (thymidylate kinase) induces apoptosis in Jurkat cells by activation of caspase-3 only. Our studies in B16-F10 cells expressing *E* showed changes to the integrity of the mitochondrial membrane and a significant increase of cytochrome *c*. This protein is able to

activate caspase-9, which in turn activates caspase-3 and other downstream caspases [25]. A Western blot analysis showed an increase in active caspase-3 and -9 in transfected B16-F10 cells, which strongly suggests that the cell death induced by the *E* gene is related to the mitochondrial apoptotic pathway. We cannot, however, exclude the possible participation of other apoptosis-mediated molecules in treated B16-F10 induced tumors, such as endonuclease G, Smac/DIABLO, and HtrA2 [32].

Having shown that the *E* gene effectively mediates the killing of melanoma tumor cells in vitro and that the mechanism of action involves induction of apoptosis, we further investigated its ability to affect the growth of an in vivo model. Statistical evaluation of tumor growth rates obtained from mice treated with pcDNA3.1/*E* complexed with cationic lipids revealed a significantly reduced growth rate in comparison to the untreated mice (90.6% relative volume reduction after 15 days of treatment). This reduction was remarkably superior to that obtained with other phages, such as alpha-holin, in breast cancer (50% at 15 days), with the HSV-tk/GCV system (40–50%) [25] or, more recently, with the SAP gene (67%) [23] in the same tumor. In addition, our results are also superior to those of McCray et al. [19], who used the Vpr gene integrated in the pcDNA3.1 vector in melanoma tumor from B16-F10 culture cells to achieve an 86% reduction of tumor volume, although only after 25 days. Suspension of the pcDNA3.1/E treatment again caused tumor growth. This strong in vivo antitumoral effect of the *E* gene is consistent with the apoptosis-inducing ability of this gene demonstrated in vitro. TUNEL staining confirmed a significant increase in the number of apoptotic cells in the experimental group treated intratumorally with pcDNA3.1/E. The mitochondrial-mediated apoptotic pathway in vivo was also strongly supported by our ultrastructural findings in the induced B16-F10 tumors in mice, which showed dilated mitochondria with disrupted cristae. Finally, we analyzed Ki-6, an antigen which is overexpressed in G1 and S phases but absent in resting cells, to estimate the proliferation intensity [33]. Treated tumors (at different times) did not show significant Ki-67 staining differences in comparison to untreated tumors. Although these results suggest that the *E* gene induces a growth delay in melanoma by inducing tumor cell apoptosis rather than by acting negatively on tumor cell division, further studies will be necessary to support this hypothesis.

In summary, we have reported, for the first time, the ability of the *E gene* to induce the death of melanoma cells in vitro and in vivo. The successful use of this gene as a new anticancer gene therapy system may establish a role for it in cancer treatment. However, as *E* gene binds to the mitochondrial membrane and its activity is not tumor-specific, adenoviral vectors modified by attaching tumor-specific promoters should be used to assay melanoma treatment. On the other hand, the shorter latency of the *E* gene for effective antitumoral action may be due to a bystander effect, although this has not been demonstrated. We are currently working on experiments to enhance *E* gene activity by combining it with cytotoxic drugs [34] or, as described by Fecker et al. [35], by using specific enhancer/promoter genes (such as tyrosinase) to induce tissue-specific expression. We are also working on the integration of therapeutic genes (such as λ-holin) into new vectors (such as ReCon) to improve the tumoral response, as reported previously by Brandtner et al. [10]. Our results suggest that *E* is a candidate gene for in vivo oncologic applications and that it may contribute to the eradication of tumor mass in combination with surgery or classic radio- or chemotherapy.

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Conflict of interest statement The authors declare that they have no competing financial interests.

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