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.

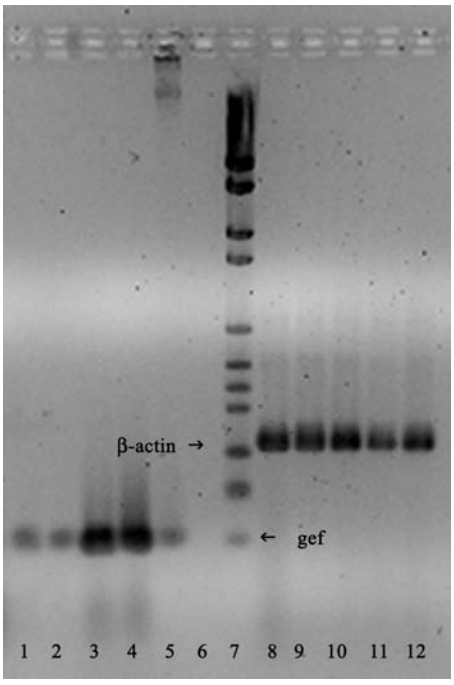

Figure 1. Determination of *gef* gene expression by RT-PCR. Total RNA isolated from transfected and parental A-549 lung cancer cells was transcribed to cDNA using reverse transcriptase PCR amplification as described in Materials and methods. Amplified PCR products of *gef* mRNA and ß-actin mRNA were 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.

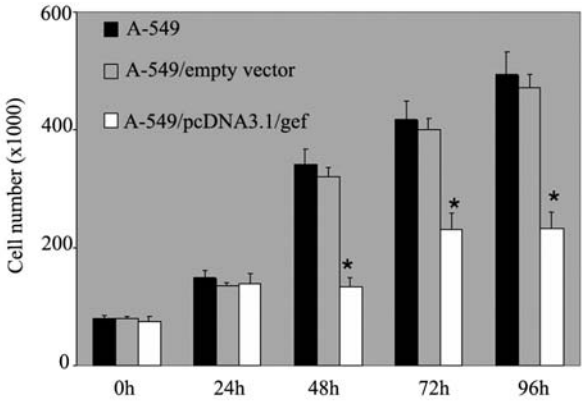

Figure 2. Effects of *gef* transfection on growth of A-549 cells. Parental A-549 cells and A-549 cells transfected with empty vector or pcDNA3.1/*gef* were seeded at a density of $8x10^4$ in plastic dishes and cultured for 4 days. Cell numbers were measured daily (24, 48, 72 and 96 h) by sulphorhodamine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

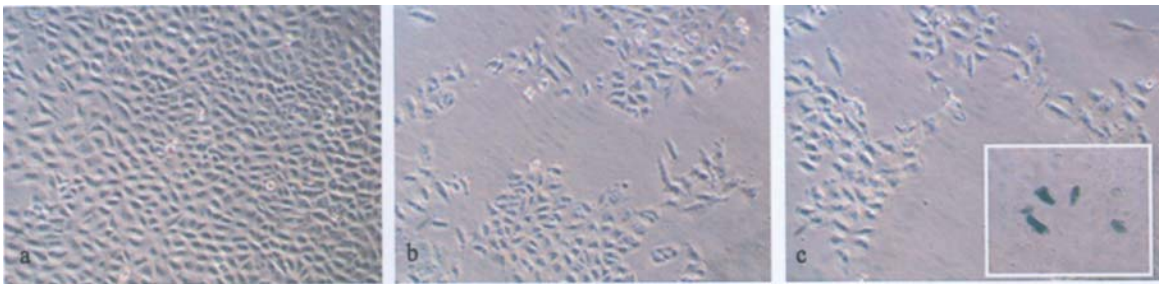

Figure 3. Phase-contrast photomicrographs showing morphology of parental and transfected A-549 cells. Parental A-549 cells (a) grew in clumps, were typically polygonal and formed a monolayer culture on the entire flask surface at 96 h. In contrast, A549 pcDNA3.1/V5/His-*gef* transfected cells at 48 h (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.

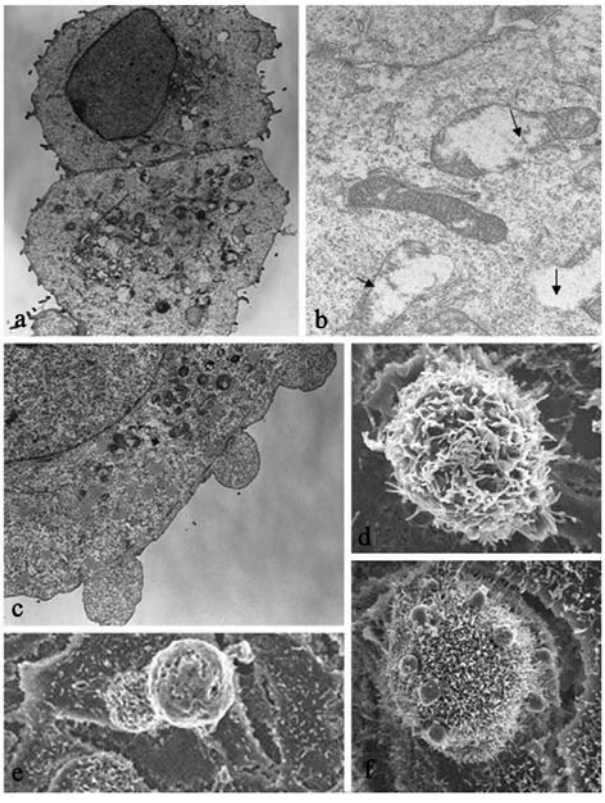

Figure 4. TEM and SEM analyses of A-549 cells. Conventional electron microscopy of parental A-549 cells (a) showed typical tumour cells with polygonal shape, large nucleus and light cytoplasmic complexion (x1100). Transfected A-549 cells showed dilated mitochondria with disrupted cristae (arrows) (b) (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).

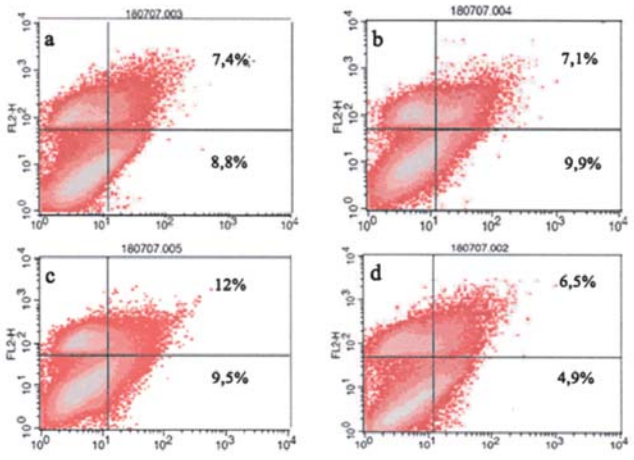

Figure 5. Fluorescence-activated cell sorting analysis of apoptosis induction by *gef* gene in A-549 lung cancer cells. Cells were stained with Annexin V and propidium iodide to evaluate apoptotic cell death, as described in Materials and methods. Representative images for comparisons between parental 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

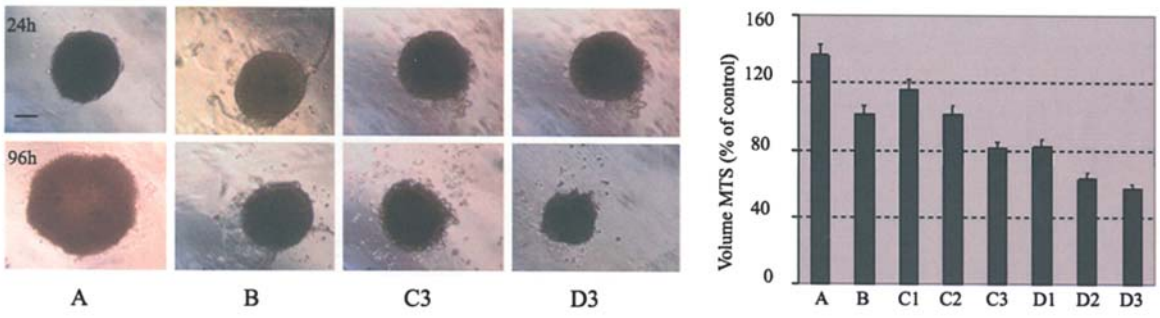

Figure 6. Analysis of the combined therapy (pcDNA3.1/*gef*/paclitaxel) in A-549 cells, using multicellular tumour spheroids (MTS). Growth of MTS was monitored by measurement of their cross-sectional area, calculating the median relative volume (volume at day x/volume at day 0). The graph depicts percentage 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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