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ORIGINAL ARTICLE

Gef gene therapy enhances the therapeutic efficacy of doxorubicin to combat growth of MCF-7 breast cancer cells

Jose Prados · Consolación Melguizo · Ana Rosa Rama · Rául Ortiz · Ana Segura · Houria Boulaiz · Celia Vélez · Octavio Caba · Juan Luís Ramos · Antonia Aránega

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Abstract
Purpose The potential use of combined therapy is under intensive study including the association between classical cytotoxic and genes encoding toxic proteins which enhanced the antitumour activity. The main aim of this work was to evaluate whether the *gef* gene, a suicide gene which has a demonstrated antiproliferative activity in tumour cells, improved the antitumour effect of chemotherapeutic drugs used as first-line treatment in the management of advanced breast cancer.
Methods MCF-7 human breast cancer cells were transfected with *gef* gene using pcDNA3.1-TOPO expression vector. To determine the effect of the combined therapy, MCF-7 transfected and non-transfected cells were exposed to paclitaxel, docetaxel and doxorubicin at different concentrations. The growth-inhibitory effect of *gef* gene and/or drugs was assessed by MTT assay. Apoptosis modulation was determined by flow cytometric analysis, DNA fragmentation and morphological analysis. Multicellular tumour spheroids (MTS) from MCF-7 cells were used to confirm effectiveness of combined therapy (*gef* gene and drug).
Results Our results demonstrate that combined therapy *gef* gene/drugs (paclitaxel, docetaxel or doxurubicin) caused a decrease in cell viability. However, only the *gef*–doxorubicin (10 μM) combination induced a greater enhancement in the antitumour activity in MCF-7 cells. Most importantly, this combined strategy resulted in a significant synergistic effect, thus allowing lower doses of the drug to be used to achieve the same therapeutic effect. These results were confirmed using MTS in which volume decrease with combined therapy was greater than obtained using the gene therapy or chemotherapy alone, or the sum of both therapies.
Conclusions The cytotoxic effect of *gef* gene in breast cancer cells enhances the chemotherapeutic effect of doxorubicin. This therapeutic approach has the potential to overcome some of the major limitations of conventional chemotherapy, and may therefore constitute a promising strategy for future applications in breast cancer therapy.

Keywords Gene therapy · *gef* gene · Cytotoxic therapy · Breast cancer · Multicellular tumour spheroid · Doxorubicin

J. Prados and C. Melguizo contributed equally to this work.

J. Prados (✉) · C. Melguizo · A. R. Rama · R. Ortiz · H. Boulaiz · C. Vélez · A. Aránega
Department of Human Anatomy and Embryology, School of Medicine, Institute of Biopathology and Regenerative Medicine (IBIMER), University of Granada, 18071 Granada, Spain
e-mail: jcprados@ugr.es

O. Caba
Department of Health Sciences, University of Jaén, Jaén, Spain

A. Segura · J. L. Ramos
Department of Environmental Protection, CSIC-Estación Experimental del Zaidín, 18008 Granada, Spain

Introduction

Metastatic breast cancer (MBC) remains an incurable illness. The primary goal of its management is palliative and to improve the quality of life, prolong disease-free survival and possibly overall survival [1]. Chemotherapy clearly provides tumour shrinkage and substantial clinical benefit in advanced breast cancer [2], and is accepted as standard treatment for hormone-resistant and rapidly progressive

disease. Several chemotherapeutic options have been shown to be effective as first- or second-line therapy in the management of MBC with taxanes, such as paclitaxel (Pacl) and docetaxel (Doc) and anthracyclines, such as doxorubicin (Dox), being the most active drugs, although with substantial side effects [3]. An important area of cancer research seeks to improve the effectiveness of these cytotoxic agents in breast cancer and to reduce their side effects [4].

Gene therapy has been proposed as a strategy to enhance the antitumour effect of drugs in the treatment of refractory or advance cancers. The majority of these studies have assessed classical gene therapy systems such as *herpes simplex virus thymidine kinase* (*HSVtk*) [5, 6] and *cytosine deaminase* (*CD*) genes [7, 8] from *S. coli*. However, their results had two important limitations, the need to convert a non-toxic prodrug into a toxic metabolite and the limited bioavailability of the active drug metabolite [9, 10]. A new strategy that may avoid this problem is the use of therapeutic genes that directly encode cytotoxic proteins. In contrast to classic 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 tumour cells and may be effective for aggressively growing tumours as well as for those that grow more slowly. The most recent experiences with genes from bacteria [11], bacteriophages [12], viruses [13] or plants [14] expressing toxins showed significant antitumour activity. Their potential use in combined chemotherapy and/or radiotherapy is now being assessed.

Our group has 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. 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. Activation of this protein induces arrest of cellular respiration and cell death [15]. In human tumour cells, *gef* gene induces cell cycle arrest and apoptosis [16, 17], which may be a complementary strategy for classic cancer treatments, since it has been demonstrated that apoptosis deficiency is a critical factor for treatment failure in advanced cancer [18]. In this context, *gef* gene may improve current chemotherapy options in cancer, including MBC, and could be of use as an adjuvant therapy to increase the effectiveness of conventional tumour treatment.

The aim of this study is to investigate whether *gef* gene enhances the antitumour effect of drugs used as a first-line therapy in MBC. Our results suggest *gef* gene could be a new tool in the design of gene therapy strategies against MBC, by increasing the effectiveness of classic drugs. The chemotherapeutic potential of *gef* gene/Dox combination in human breast cancer treatment merits a thorough evaluation.

Methods

Cell culture

The MCF-7 breast carcinoma cell line (European Collection of Cell Culture) was grown with DEMEM (Sigma Chemical Co., St. Louis, MO), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 40 mg/l gentamicin and 500 mg/l ampicillin (Antibióticos S.A, Madrid, Spain). Cells were maintained in monolayer culture at 37°C in an atmosphere containing 5% CO_2.

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-TOPO vector following manufacturer's instructions (Invitrogen, Barcelona, Spain). 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). To optimise transfection conditions, the pcDNA3.1-TOPO/lacZ (encoding β-galactosidase) and X-GAL staining was used. To detect *gef* protein, a fusion protein (*gef*-GFP) was generated using the plasmid pcDNA3.1/GFP (provided by Dr. G. Ortiz). The gef cDNA was obtained by PCR, modifying the reverse primer to eliminate the stop codon, and ligated into pcDNA3.1/GFP vector following the manufacturer's protocol (Invitrogen). The resulting plasmid pcDNA3.1/GFP-*gef* was confirmed and amplified as described above.

Transfection

One day before transfection, confluent cells were seeded into 6-well plates (2×10^5 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 incubation at room temperature, 2 µg of plasmid DNA were added (ratio 1:3). MCF-7 cells, yielding approximately 70% confluence, were transfected pcDNA3.1/*gef*. 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-TOPO plasmid in which the *gef* gene was absent was used as a negative control. The transfection of pcDNA3.1/GFP-*gef* to determine gef protein was realized as described above.

Reverse transcription-PCR (RT-PCR)

Upregulation of mRNA expression of *gef* cDNA was determined by RT-PCR. Total RNA was extracted from transfected and parental cells with the Rneasy Mini kit (Qiagen, Madrid, Spain), and cDNA was generated by means of the Promega reverse transcription system (Promega, Madrid, Spain) using total cellular RNA (1 μg). PCR amplification of *gef* gene took place under the above-described conditions and was run on a 1.8% agarose gel and visualized by ethidium bromide staining. RNA integrity was assessed by amplification of β-actin mRNA. Images were scanned and analysed using the Quantity One Analysis Software (Bio-Rad Laboratories, Inc., Barcelona, Spain).

Microscopic analysis and western blotting

Expression of gef protein was confirmed in MCF-7 pcDNA3.1/GFP-*gef* transfected cells with excitation at 488 nm. Fluorescent microscopy analysis was carried out with a Nikon Eclipse Ti (Nikon Instruments Inc. NY, USA). Protein extracts (30 μg) from parental and transfected cells were used for SDS-PAGE in a Mini Protean II cell (Bio-Rad, Hercules, CA). The separated proteins were transferred to a nitrocellulose membrane (20 V at room temperature for 30 min) and the blots treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h. GFP-gef fusion protein was detected with an anti-GFP N-terminal antibody (Sigma, St. Louis, MO).

Proliferation assay

Parental and transfected MCF-7 cells were seeded in 48-well plates at a density of 5×10^3 cells per well. In order to determine the effect of the combined therapy (*gef* gene and drugs: Pacl, Doc and Dox) they were treated with gradient concentrations (0.1, 1 and 10 μM) according to the recommended dose for patients [19]. After 24, 48 and 72 h, 20 μL 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 4 h. Then 200 μl of dimethylsulphoxide (DMSO) was added to each well after the medium was removed. Absorbances were then measured using a Titertek multiscan colorimeter (Flow, Irvine, CA) at 570 and 690 nm. Linearity of the MTT assay with cell number was tested for each MCF-7 cell stock before each cell growth experiment.

Apoptosis analysis

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). Binding buffer (500 μl) was then added and cells were immediately processed with a FACScan flow cytometer (Becton–Dickinson, San Jose, CA). Microscopy analysis was carried out in a Leica DMI6000 confocal microscope with an Argon/Krypton laser (Heidelberg, Germany).

DNA fragmentation assay

After the treatments, MCF-7 cells were lysed in a buffer containing 20 mM Tris pH 8.5, 5 mM EDTA, 10% sodium dodecyl sulphate (SDS), and 100 μg/ml proteinase K and incubated overnight at 37°C. Lysates were cleared by centrifugation at 12,000*g* for 20 min. DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1), precipitated with 95% ethanol for 2 h at −80°C and analysed by agarose gel electrophoresis.

MTS initiation

To generate multicellular tumour spheroids (MTS), exponentially growing monolayer MCF-7 cells were harvested by trypsinisation. Then 2×10^3 cells/well were grown in a 48-well plate (BD) previously coated with 200 μl 0.75% agarose type II in FBS-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 into a rocker designed for three-dimensional agitation (70 cycles/min) as described previously [20]. A single MTS of approximately 100 μm of diameter was obtained in each well. Growth of the spheroids was monitored and measured using an inverted phase-contrast microscope to obtain a median relative volume (volume at day *x*/volume at day 0), which was calculated as previously described by Monazzam et al. [19]. When the MTS grow to a considerable size they also become asymmetric, thus the time for their evaluation was set for 7 days.

Transfection in MCF-7 MTS

MTS cells were transferred using a Pasteur pipette from the 48-well microplate to a 96-well plate (one MTS per well) coated with agarose and containing 100 μl of medium. To perform the combined therapy experiments, the MTS were transfected with pcDNA3.1/*gef* in two different ways, on the first day of the experiment only (MTS-FD) and every

2 days during the experiment (days 1, 3 and 5) (MTS-ETD). MCF-7 spheroids transfected with empty vector were used as a control. The response to treatment was evaluated by measuring MTS volume as described above.

Combined therapy in MCF-7 MTS

MTS in a 96-well plate microplate (one MTS per well) were analysed in four groups: non-treated MTS, drug-treated non-transfected MTS, drug-treated transfected MTS-FD and drug-treated transfected MTS-ETD. The drugs (Pacl, Doc and Dox) were used at the same doses described above. The response to each anticancer treatment was evaluated by measuring MTS volume during treatment.

Statistical analysis

SPSS 7.5 software (SPSS, Chicago, IL) 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 <0.05.

Results

In vitro evaluation of *gef* gene expression

In vitro evaluation of *gef* gene expression was performed by RT-PCR. As shown in Fig. 1, an amplification fragment of 154 bp was found in the MCF-7 cells transfected with pcDNA3.1/*gef*, 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). GFP-gef protein was detected in the cytoplasm after transfection of MCF-7 cells with pcDNA3.1/GFP-*gef* (Fig. 1).

Effects of the combined therapy on MCF-7 cell proliferation

The main goal of this study was to evaluate the therapeutic potential resulting from the combination of two antitumour strategies, suicide gene therapy (*gef* gene) and conventional chemotherapy (Pacl, Doc and Dox), in breast cancer cells. For this purpose, MCF-7 cells were exposed to classic chemotherapy agents for breast cancer treatment in non-transfected and transfected cells. As shown in Fig. 2, *gef* gene was able to induce a continuous decrease in the MCF-7 cell viability in the absence of drugs. After 72 h transfection a 31% decrease in cell viability was observed.

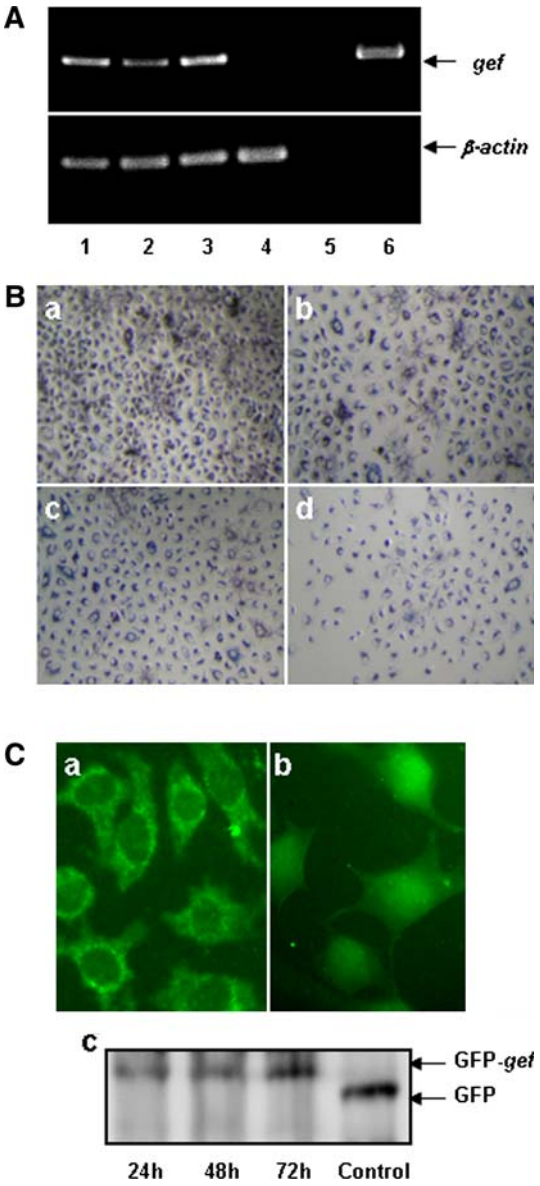

Fig. 1 *gef* gene expression in MCF-7 cells. **A** Total RNA isolated from transfected and parental MCF-7 was transcribed to cDNA using RT-PCR amplification as described in "Methods". The integrity of the RNA was demonstrated using β-actin primers. Amplified PCR products of gef mRNA and β-actin mRNA were separated by 1.8% agarose gel electrophoresis and visualized with ethidium bromide. Lanes *1–3* MCF-7 pcDNA3.1/gef transfected cells at different time periods (24, 48, and 72 h, respectively); lane *4* non-transfected MCF-7 cells (negative control); lane *5* negative control (empty vector); lane *6* positive control (pcDNA3.1/gef). **B** Representative images of MCF-7 cells growth (MTT assay) after 24 h (*b*), 48 h (*c*) and 72 h (*d*) gef transfection compared to control cells (*a*). **C** Representative images of the GFP-gef fusion protein expression in MCF-7 cells. MCF-7 cells were transfected with a GFP-gef fusion construct, as indicated in the "Methods" section. Twenty-four hours after transfection (*a* ×40), the fluorescence pattern was *dotted* and localized in the cell cytoplasm. Cells transfected with GFP (without gef) showed a typical diffuse pattern of GFP fluorescence (24 h) (*b* ×40). Western blot analysis confirmed the presence of the GFP-gef fusion protein (34 kDa) at 24, 48 and 72 h in cells transfected with pcDNA3.1/GFP-gef. Cells transfected with GFP (without gef) were used as control (*c*)

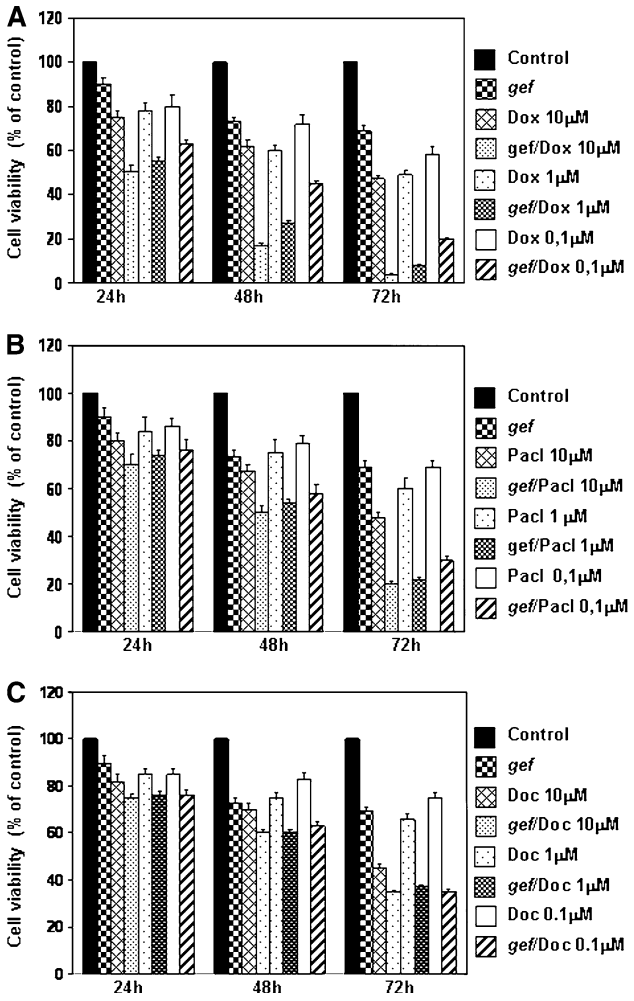

Fig. 2 Effect of combined therapy on MCF-7 cell proliferation. An initial population (*black bars*) of MCF-7 cells was transfected with *gef* gene, treated with drugs or both (combined therapy) at 24, 48 and 72 h. Drugs used were doxorubicin (**A**), paclitaxel (**B**) and docetaxel (**C**) at the indicated concentrations. Cell proliferation was estimated using the MTT assay, as described in "Methods". Results are expressed as percentages of viable cells by comparison to the maximal (100%) cell proliferation of normal cultures. Values are means ± SE of six measurements in four separate experiments

Non-transfected cells treated with Dox, Pacl and Doc at different concentrations also showed a significant decrease in cell viability (Fig. 2). However, transfected MCF-7 cells in the presence of drugs showed a greater decrease in survival population compared to the results obtained with drugs or *gef* gene alone. The most interesting finding was obtained when Dox was used in transfected cells, as this combined therapy not only induced just the sum of drug and *gef* individual treatments, but there was also a significant enhancement of the antitumour effect. As shown in Fig. 2A, the combined therapy *gef*/Dox (10 μM) produced a 50, 83 and 96% decrease in MCF-7 cell viability at 24, 48 and 72 h, respectively, when compared to control cells. Therefore, this combined therapy had 15% more antitumour effect at 24 h than the sum of both *gef* gene (12%) and Dox 10 μM (23%) treatments together (Fig. 2A). This enhancer phenomenon was also observed at 48 h (18% more effect) and 72 h (10% more effect). Similar results were obtained with Dox at 1 μM although the enhancer effect on cell viability was slightly less than the sum of the both treatments (Fig. 2A). Finally, Dox at 0.1 μM only produced a mild enhancer effect (5–7%) which was not significant. As shown in Fig. 2B, Pacl at different concentrations in transfected cells induces a significant decrease in the cell viability that represents the sum of the effect of both treatments. A similar finding was obtained with Doc although at 1 μM or 0.1 μM this drug produced a slight greater effect in transfected MCF-7 cells (Fig. 2C).

Apoptosis in MCF-7 cells by combined therapy

As only the *gef*/Dox combination produced a significant decrease in the viability of MCF-7, the apoptosis experiments were conducted with this combination. Individual treatment with both *gef* gene and Dox induced apoptosis in MCF-7 cells. As shown in Fig. 3A, a significant increase in apoptosis was found at 24, 48 and 72 h after *gef* gene transfection (13, 28 and 38%, respectively) in relation to non-transfected cells used as a control. This apoptosis induction was confirmed by a typical DNA ladder fragmentation pattern (Fig. 3B) and by confocal laser-scanning microscopy (Fig. 3C). On the other hand, MCF-7 cells treated with Dox showed a time- a concentration-dependent apoptosis increase (Fig. 4). However, transfected MCF-7 cells exposure to Dox showed a significant increase in apoptosis level in comparison to cells treated with *gef* or Dox alone (Fig. 4). The most significant findings were obtained with combined therapy (*gef*/Dox 10 μM) which caused 61, 88 and 95% apoptosis at 24, 48 and 72 h, respectively. These results indicated that *gef* gene was able to enhance the apoptotic effect of Dox in MCF-7 more than the sum of apoptosis induced by *gef* and Dox individually. Similar findings were observed with lower doses of Dox although the apoptosis increase was lower (1 μM) or not significant (0.1 μM).

Growth modulation of *gef* gene-transfected MTS-MCF-7 cells

Volume rate of MTS derived from MCF-7 cells was measured after *gef* transfection during 7 days in order to analyse the potential of this gene to decrease the growth of breast cancer cell spheroids. Volume rate of MTS transfected with empty vector was similar to that of non-transfected MTS (data not shown). In contrast, *gef* gene-transfected MTS showed a significant and time-dependent decrease in growth rate in both MTS-FD

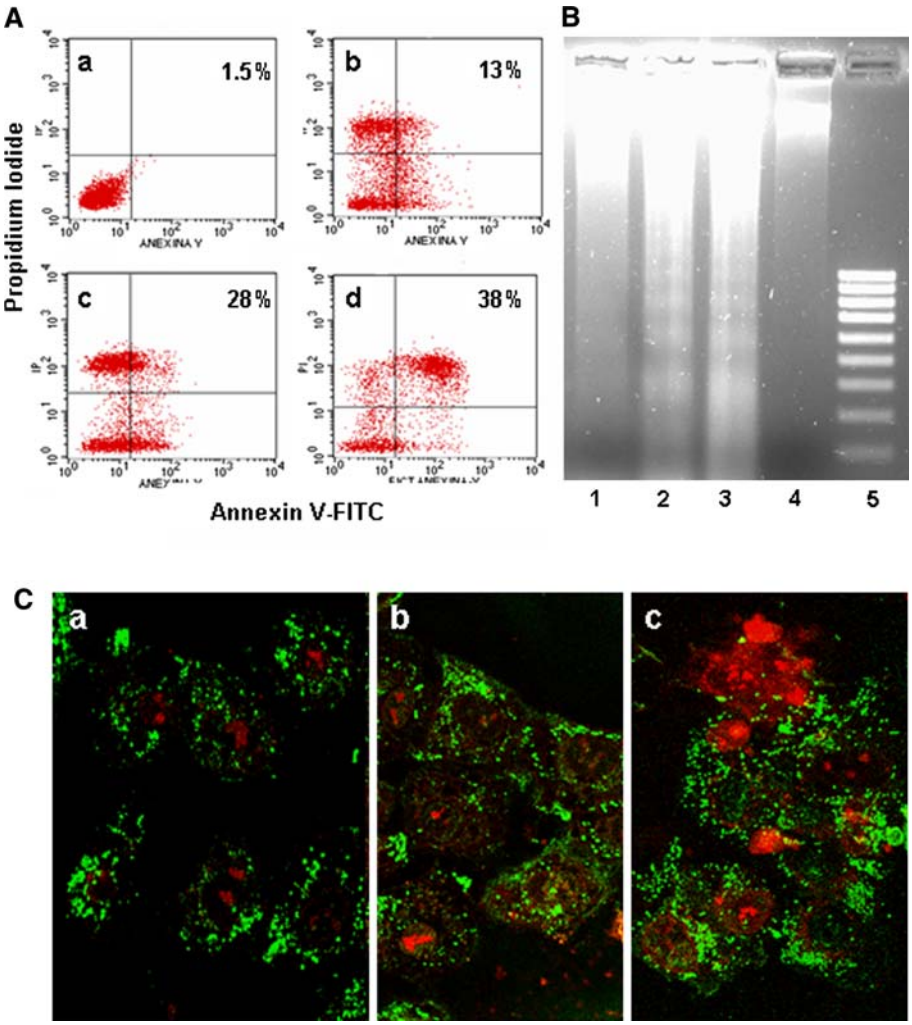

Fig. 3 Analysis of apoptosis induced by *gef* gene in MCF-7 cells. **A** Fluorescence-activated cell sorting realized in MCF-7 parental cells (*a*) and MCF-7 cells after *gef* gene transfection at 24 h (*b*), 48 h (*c*) and 72 h (*d*). Cells were stained with annexin V and propidium iodide to evaluate apoptotic cell death, as described in "Methods". **B** Representative image of DNA fragmentation analysis of MCF-7 cells transfected with *gef* gene at 24 h (lane *1*), 48 h (lane *2*) and 72 h (lane *3*) in comparison to DNA from MCF-7 parental cells (lane *4*). Cellular DNA was isolated and subjected to agarose gel electrophoresis, followed by visualization of bands as described in "Methods". DNA size marker (lane *5*). **C** Representative image (confocal laser-scanning microscopy) of apoptosis induced in MCF-7 cells after transfection of *gef* gene at 24 h (*a*), 48 h (*b*) and 72 h (*c*)

(transfected only the first day) and MTS-ETD (transfected every 2 days) (see "Methods"). The greatest decrease in growth rate after treatment with *gef* gene was observed at 7 days (Fig. 5). At this time, a 17.2 and a 28.1% volume decrease was observed in MTS-FD and MTS-ETD, respectively.

Effects of the combined therapies on MCF-7-MTS growth

To confirm the therapeutic potential of combined *gef*/Dox therapy in breast cancer cells, we evaluated the modulation of MTS growth. Doxorubicin at 10, 1 and 0.1 μM induced a clear growth rate inhibition in non-transfected MTS (Fig. 6). However, the exposure of transfected MTS (both MTS-FD and MTD-ETD) to this drug induced a higher decrease in MTS volume than the treatment with *gef* or Dox alone. Moreover, the MTS growth inhibition was also greater that those obtained by the sum of both *gef* and drug treatments together, indicating a significant enhanced of antitumour effect. Treatment with Dox (10 μM) in

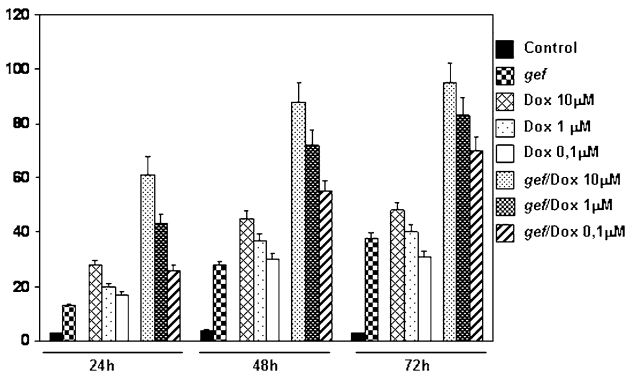

Fig. 4 Modulation of apoptosis by combined therapy *gef* gene–Dox in MCF-7 cells. Fluorescence-activated cell sorting was used to determine and compare the apoptosis induced by *gef* gene, Dox at different concentrations and combined therapy (*gef*/Dox). Values are means ± SE of six measurements in four separate experiments

MTS-ETD produced greater growth inhibition than MTS-FD. In MTS-ETD, the largest decrease in volume (63%) was obtained with Dox 10 μM compared to the volume of MTS control. *Gef* gene also enhanced MTS growth

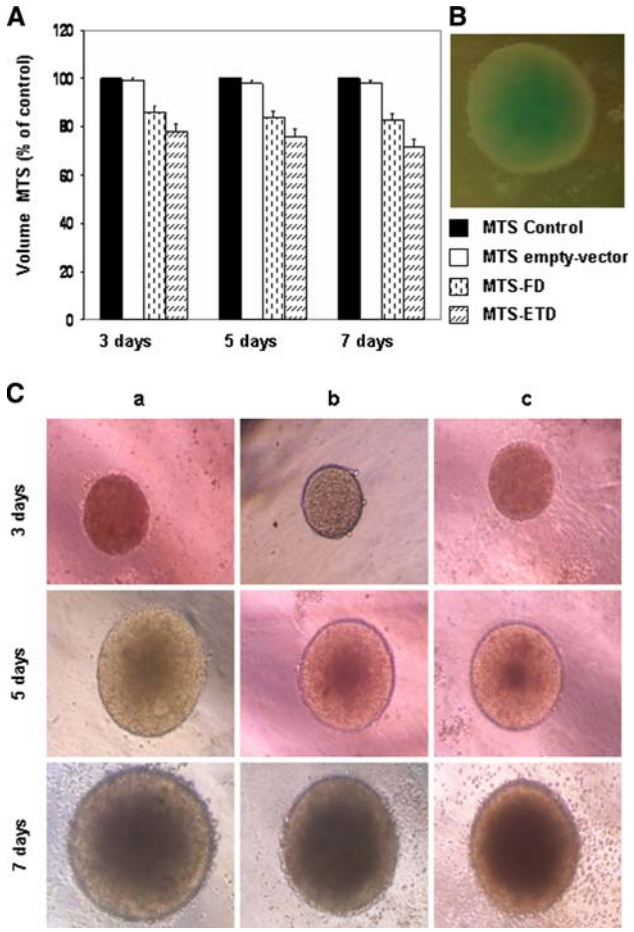

Fig. 5 Modulation of MTS-MCF7 cell growth after *gef* gene transfection. **A** The graph represents the effect of the *gef* gene expression on the MTS volume. Values are means ± SE of six measurements in three separate experiments. **B** Representative micrographs of X-GAL-stained MTS-MCF7 after transfection with pcDNA3.1-TOPO/lacZ (72 h). **C** Representative phase-contrast photomicrographs showing the growth of non-transfected MTS (control) (*a*), MTS-FD (*b*) and MTS-ETD (*c*)

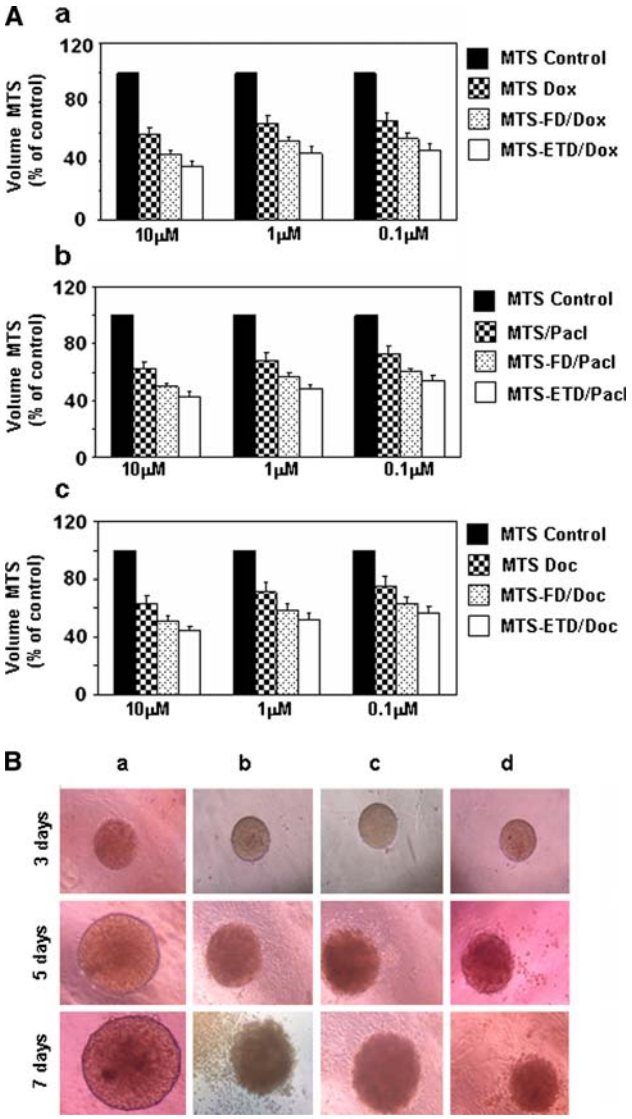

Fig. 6 Modulation of MTS-MCF7 cell growth after combined therapy. **A** The graphs represent the volume modulation of the transfected (MTS-FD and MTS-ETD) and non-transfected MTS after exposure to Dox (*a*), Pacl (*b*), Doc (*c*) at the indicated concentrations. Values are means ± SE of six measurements in three separate experiments. **B** Representative phase-contrast photomicrographs showing the growth of non-transfected MTS (control) (*a*), non-transfected MTS treated with Dox (10 μM) (*b*) transfected MTS (MTS-ETD) (*c*) and transfected MTS (MTS-ETD) treated with Dox (10 μM) (*d*)

inhibition when combined with Dox at 1 and 0.1 μM, although to a lesser extent (Fig. 6). Finally, *gef*/Pacl and *gef*/Doc combined treatment in MTS (MTS-FD and MTS-ETD) induced a volume reduction that represented the sum of the effect of *gef* and drug only, but did not enhance the antitumour effect (Fig. 6).

Discussion

The development of therapeutic strategies to improve overall survival in refractory advanced breast cancer remains a high priority. Drug combinations, including Pacl, Doc and Dox, are currently the most effective treatments in these patients, although with little impact in the prognosis and with substantial side effects [3]. In this study, we have demonstrated that the suicide *gef* gene combined with Dox, induced an adjuvant effect that enhances its antitumour effect in breast cancer cells. In addition, these results raise the possibility of a chemotherapy dose reduction when used in combination with gene therapy.

It has been demonstrated that the association of a taxane with an anthracycline improve treatment response of patients with MBC. Recent studies showed that patients receiving Doc after Dox had a 5-year disease-free survival

rate higher than those receiving a combination of the two drugs at the same time [21]. Phase I and II studies with the Dox-Pacl sequence defined by preclinical studies have reported objective responses above 40% [22, 23]. These results are higher than those obtained with other drugs, including fluorouracil, epirubicin and cyclophosphamide (FEC) [24, 25]. However, due to the patient response failure, it remains a priority to increase drug effectiveness against breast cancer cells. In this context, gene therapy has been proposed as a strategy to enhance drug activity and reducing drug doses in advanced cancer [4].

Isolated gene therapy has not led to the successful treatment of breast cancer. Classic suicide genes such as CD and HSVtk, are able to decrease breast cancer cell proliferation, but the need to use a prodrug is its main limitation [26]. During the last few years, new genes that encode toxins with a direct antitumour action have been developed and are being studied as single treatments in tumour therapy and in combination with conventional tumour treatment strategies. These toxic genes have been successfully used in a wide variety of tumours such as pancreas cancer (diphtheria toxin gene) [11], lung cancer, glioma and hepatocellular carcinoma (streptolysin O gene) [27] and cervical cancer (λ-holin gene) [28]. Some of these toxics genes, such as saporin, showed synergistic effects when used with a cytotoxic drug [14]. We have previously demonstrated that *gef* gene showed antitumour activity in breast cancer cells by inducing apoptosis [18]. Moreover, it has been demonstrated that *gef* gene is able to enhance the effect of drugs in lung cancer cells [20]. In fact, the increase of Pacl antitumour effect has been also demonstrated when combined with IL12 gene therapy in breast and in ovarian cancers [29]. Novel advances in the combined use of gene therapy and cytotoxic drugs have recently been reported in bladder cancer [7], pancreatic cancer [30], and colorectal cancer [31]. Gene therapy with a caspase activator (Smac/DIABLO] has been used in breast cancer to enhance apoptosis induced by paclitaxel, doxorubicin, etoposide and tamoxifen [32]. These findings suggest that the antiproliferative effect of some drugs may be enhanced by the use of the gene therapy.

Our results showed that a combination of *gef* with Pacl, Doc and Dox produced a decrease in MCF-7 cell viability higher than that obtained with individual treatments. However, the most significant finding was related to the *gef* gene capacity to enhance Dox antitumour effect. Doxorubicin at 10 μM produced a decrease in cell viability of MCF-7 transfected cells greater (15% more) than the sum of the individual effects of *gef* and Dox when used separately. In fact, genes such as P450 3A4 were able to induce antitumor activity potentiation of doxorubicin when used in ovarian (CHO) and lung cancer cells (A-549) [33]. Interestingly, lower concentrations of Dox (1 μM) induced a similar antiproliferative enhancer effect to that of higher concentrations of this drug, suggesting that *gef*/Dox combination may be useful for reducing the cytotoxic concentrations needed in breast cancer treatment. *gef* gene alone was able to inhibit cell proliferation via apoptosis, as evidenced by MTT assay, flow cytometry analysis, DNA fragmentation and apoptotic morphology analysis. Our results support previous studies which have demonstrated that Dox, as a classical DNA-damaging agent, induced apoptosis in a variety of tumour cell lines, including breast cancer cells [34]. The in vitro experiments designed to investigate whether *gef* gene could enhance the apoptosis of MCF-7 cells induced by Dox were positives, resulting in a decrease of MCF-7 cell viability in a similar way that pro-apoptotic *mPNAS-4* gene enhanced the cisplatin activity in lung, colon and ovarian carcinoma cells [35]. In contrast, although combined *gef*/Pacl or *gef*/Doc therapy also produced a decrease in cell viability, this was equal to the sum of the effects when gene and drug were used separately. Similar results were obtained for all Doc and Pacl concentrations tested. It remains unclear why *gef* does not enhance the antitumor activity of taxanes despite the fact that their apoptotic effects in breast cancer cells have been confirmed in numerous studies [36]. Future studies will therefore be necessary to determine the apoptosis induction pathway for *gef*, which is probably related to potentiation of the effect of Dox but not Doc or Pacl.

To confirm the effectiveness of *gef*/Dox combined therapy in breast cancer, we used MTS from MCF-7 cells as an experimental system which mimics the real biological environment of a tumour, including limitations in drug or gene penetration [37] and distribution and feedback mechanisms in cell signalling [38]. In fact, MTS has been previously used to determine apoptosis and proliferation of MCF-7 cells after treatment with Dox, Doc and combined Dox/Doc therapy [39] and to study the efficacy of suicide gene therapy systems such us HSVtk [40]. Volume analyses of the MTS showed that combined *gef*/drug therapy caused significant growth inhibition in comparison with MTS control. As in cell cultures, the most significant results were obtained with the use of Dox (10 μM) after *gef* gene transfection. This combined therapy caused a volume reduction greater than the sum of individual *gef* and Dox treatments. These results confirmed a significant enhancement in MTS growth inhibition. Experiments using MTS with a continuous transfection (MTS-ETD) demonstrated that high-pressure sensitive *gef* gene expression increased the efficacy of the combined therapy by reducing the MTS volume more than using transfection only (MTS-FD). Moreover, as in culture cells, Dox at 1 μM after *gef* gene transfection also inhibited MTS growth more than the exclusive use of higher doses of Dox, supporting the hypothesis that the use of *gef* could reduce drug

concentrations while obtaining the same antiproliferative result. However, as is the case with most of the toxic genes used in gene therapy, it will be necessary to create tumour specificity using enhancer/promoter genes, new vectors and direct in vivo administration (intratumoral injection). Future studies with *gef* gene integrated into an optimised vector with targeted cellular promoters, similar to that described by Brandtner et al. [28] to λ-holin toxic gene, will be necessary to improve the effectiveness of this combined therapy in breast cancer.

In summary, we have reported the ability of the *gef* gene to enhance the antiproliferative effect of Dox in MCF-7 cells. In addition, combined *gef*/Dox therapy enhanced cell growth inhibition of MTS derived from these breast cancer cells. The successful use of this experimental combined therapy in enhancing the anticancer effect suggests a potential application in patients with advanced breast cancer.

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