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Transfer into a mesothelioma cell line of tumor suppressor gene p16 by cholesterol-based cationic lipids

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Abstract

In this work, the tumor suppressor gene p16 was efficiently transferred into FR cells isolated from a patient with malignant mesothelioma using cationic liposomes prepared from trimethyl aminoethane carbamoyl cholesterol (TMAEC-Chol) and triethyl aminopropane carbamoyl cholesterol (TEAPC-Chol). This transfer was performed after preliminary assays were undertaken to find the optimal transfection conditions. Results showed that an efficient transfer of plasmids containing the reporter gene pCMV-β galactosidase vectorized by TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes into mesothelioma FR cells was obtained as assessed by luminometric measurements of β-galactosidase activity. Cytotoxicity studied by MTT test showed that at concentrations used for this study, the cationic liposomes have no effect on cell growth. Transfer into mesothelioma FR cells of a plasmid construct containing the tumor suppressor gene p16 was carried out with these liposomes. Western blotting and immunofluorescence showed the presence of p16 in treated cells. An inhibition of cell growth was observed, indicating that efficient tumor suppressor gene transfer can be performed by using cationic liposomes.

Keywords: Mesothelioma; Tumor suppressor gene p16; Cationic lipid; β-galactosidase; Gene therapy

1. Introduction

Malignant pleural mesothelioma (MPM) is an almost universally fatal neoplasm that is unresponsive to current treatment procedures including surgery, chemotherapy and radiation therapy [1]. Because of the dismal prognosis for patients with MPM, new treatment modalities are needed [2,3], among them, a promising area of research is gene therapy.

Actually, a variety of studies on tumor suppressor gene replacement has been developed for the treatment of malignancies. The association of mutated tumor suppressor genes (*p16*, *BRCA1*, *BRCA2*) with diverse cancers has focused their potential replacement with wild-type versions of suppressor genes, with the same goals as any anti-cancer therapies: selective targeting of tumor cells and optimal therapeutic index. Among the expressed proteins, $p16^{INK4a}$

Abbreviations: AMPGD, 3-4(-methoxyspiro[1,2-dioxetane-3,2′-tricyclo(3.3.1.1)decane]-4-yl)phenyl-β-D-galactopyranoside; DMSO, dimethylsulfoxide; DTT, dithiothreitol; FCS, fetal calf serum; Lip(+), cationic lipid; FTIR, Fourier transform infrared spectroscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffer saline; pCMV-β, plasmid containing β-galactosidase gene, promotor cytomegalovirus; QLS, quasi-elastic light scattering; RLU, relative light unit; TEAPC-Chol, (3β(*N*-(*N*′,*N*′,*N*′-triethylaminopropane)-carbamoyl)cholesterol iodide); TMAEC-Chol, (3β(*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)-carbamoyl)cholesterol iodide); X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

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is found to be mutated or deleted in many transformed cell lines and some primary tumors derived from multiple human tissues, including pancreas, skin, brain, bladder, lung and pleura [4]. $p16^{INK4a}$ participates in controlling the G1-S cell cycle checkpoint through its indirect interaction with pRb protein [5–8]. This $p16^{INK4a}$ gene represents a logical candidate for tumor suppressor replacement therapy for $p16^{INK4a}$-negative tumors. In some previous papers, p16 gene transfer in mesothelioma cells has been reported. Experiments by viral delivery have shown that the expression of the p16 protein can inhibit the progression of the cell cycle and reduce the growth of human cancer cell lines, including leukaemic [9] or mesothelioma cells [10].

A major problem in gene therapy concerns the method of DNA delivery knowing that generally naked DNA does not penetrate into cells, so a carrier is needed. In the last decade, there were intensive investigations for the transfer of DNA into living cells or into animal by using viral or nonviral vectors. Viral vectors are efficient but some hazards may exist for the host cells, then nonviral vectors appear as an alternative [11,12].

Although generally less efficient than the viral systems in delivery of the transgene to target cells, the main advantages of nonviral methods include an apparent tolerance by the immune system and less concern by safety issues. Among the nonviral carriers, cationic lipids are the most intensively studied, designed and tested [13–16]. By their positive charges, cationic lipids can form complexes with negatively charged DNA, carry the latter and help them to pass across cell barriers. Several assays of DNA transfer in cells and in animal models used this method [17–19].

So far, the suppressor gene $p16^{INK4a}$ had been transferred using viral vectors [5,10,20,21]. The use of cationic liposomes to mediate the p16 gene transfer has still been limited. Besides the use of lipofectamine [22], one cationic liposome has been used, not as a mere carrier but to enhance the rate of transfection of target cells with retroviral vectors. Porter et al. [21] complexed a virus with DC-Chol/DOPE 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol liposomes to transduce human mesothelioma xenografts and found that the transfection was enhanced four- to fivefold compared to that by the virus alone.

In this context, we used original cationic liposomes for a transient model of $p16^{INK4a}$ expression in $p16^{INK4a}$ defective MPM cells. Cationic lipids were synthesized with cholesterol as the hydrophobic part, linked to the polar head by a carbamide bond, but containing a ramified quaternary ammonium polar head and different lengths of the spacer. These cationic lipids, trimethyl aminoethane carbamoyl cholesterol (TMAEC-Chol) and triethyl aminopropane carbamoyl cholesterol (TEAPC-Chol), form with the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) very stable liposomes with well-defined size and are able to carry oligonucleotides or plasmids into several cell lines [23,24]. In these previous works, the transfection level by TMAEC-Chol/DOPE liposome has been compared with other carriers. For 9L and MCF7 cell lines, comparison with other available cationic liposomes showed that TEAPC-Chol/DOPE liposomes have advantages compared to some known transfection reagents [23,25]. Therefore, we propose to use these liposomes to deliver a plasmid encoding p16 in a mesothelioma cell line. In this work, we determined the optimal transfection conditions of these cationic liposomes and tested in vitro their ability to deliver wild-type p16 expression plasmids. We report here the successful import of the tumor suppressor gene p16 in a human cultured pleural mesothelioma cell line. This successful delivery was demonstrated by the internalization of p16, its functionality and its effect on the cell proliferation. This might represent a first step for setting up future therapy of mesothelioma using cationic liposomes.

2. Materials and methods

2.1. Materials

DOPE was obtained from Aventi-Polar Lipid (Alabaster, AL) and used without other purification. Antibodies against p16 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). For light microscopy observation, secondary antibodies were labelled with fluorescein isothiocyanate (FITC). Reagents are analytical grade and purchased from Aldrich, Carlo Erba and Fluka.

2.2. Cationic lipids

Cationic lipids TMAEC-Chol and TEAPC-Chol were synthesized and characterized by Fourier transform infrared spectroscopy (FTIR). The synthesis of these lipids was described in previous papers [24,25].

2.3. Liposome preparation

Cationic lipids (TMAEC-Chol, TEAPC-Chol) and DOPE in the weight ratio 1:1 were dissolved and mixed in chloroform and the solution was dried in a rotating evaporator. Solvent trace was removed under vacuum overnight. The following day, sterile water (Millipore) was added and the mixture was sonicated for 1 h in cycles of 15 min (13 min followed by 2 min rest) to clarity. The liposome preparations were controlled by negative staining followed by transmission electron microscopy (TEM). Their size was characterized by quasi-elastic light scattering (QLS). Polystyrene latex spheres of 109 nm in aqueous solution were used for the calibration.

2.4. Plasmids

pCMV-β is a 7.2-kb plasmid encoding β-galactosidase under the control of the cytomegalovirus promotor and was purchased from Promega (Madison, WI). pCMV-p16, a 5.4-kb plasmid encoding p16 under the control of the same

promotor, was a gift of Dr. C.J. Larsen (Institut de Génétique Moléculaire, Paris). All plasmids were conditioned in Tris–EDTA buffer (TE), pH 8.

2.5. Formation of complexes

For complex forming, plasmid DNA (1.5 to 4.5 mg) and cationic liposomes at desired molar charge ratios over DNA (number of positive charges of cationic lipid per negative charge of DNA) were separately diluted in equal volume (10 μl) of sterile water and then mixed. Complexes were formed instantaneously after mixing and let at room temperature for 15 mn before transfection. Preliminary assays showed that transfection of mesothelioma FR cells with cationic liposomes still has small effect on the proliferation. This protocol has been then modified to enhance the transfection level by using spermine to condense plasmids prior to the formation of complexes with cationic liposomes. Indeed, this polycation is well known to condense DNA and to enhance the transfection level [26]. Control transfection with pCMV-βgal or pCMV-p16 precondensed by spermine but unvectorized by cationic liposomes gave a negligible level β-galactosidase or p16 genes. These assays indicated that β-galactosidase activity was optimal when spermine was added to plasmids in the ratio of 3 or 4 nmol for each milligram of DNA before complexing with TMAEC-Chol/DOPE or with TEAPC-Chol/DOPE liposomes, respectively. These spermine/DNA ratios were used for complex preparations presented in the following.

2.6. Cell culture

A mesothelioma cell line, FR, was established in one of our laboratories. It was obtained from a pleural fluid in a patient with malignant mesothelioma. The cell line characteristics were investigated by immunocytochemistry, ultrastructural and chromosome analysis [27–29].

Cells were grown at 37 °C under 5% CO_2 in RPMI 1640 L-glutamine medium (Life Technologies, Inc.), supplemented with fetal calf serum (10%), penicillin/streptomycin (50 U/ml) and Hepes (10 mM). The medium was changed twice weekly. This cell line was used between passages 7 and 21.

2.7. Transfection protocol

The day before transfection, cells were seeded onto microscope four-well Lab-Tek plates (typically 5×10^4 cells/well) or onto six-well plates (5×10^5 cells/well). The following day, the cells were washed, the culture medium was removed and replaced with serum-free OPTI-MEM (500 μl in Lab-Tek wells or 1000 μl in six wells). Plasmids or liposome/plasmid complexes prepared in sterile water were gently dropped for transfection. After 6 h, OPTI-MEM was replaced with culture medium supplemented with serum for the defined incubating time.

2.8. Transfection efficiency evaluated by X-gal test

The transfection efficiency was estimated by using pCMV-β followed by a coloration with X-gal reagent (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). For each well in Lab-Tek plates, complexes of pCMV-β (3 μg) and liposomes (18 nmol) were used. The X-gal test was performed after 48 h of incubation. For this, the cells were washed with PBS solution and fixed in 1% formaldehyde, 0.2% glutaraldehyde, in phosphate buffer saline (PBS), for 2 min. After washing twice with PBS, the X-gal solution (1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM $MgCl_2$ in PBS) was added and the cells were incubated for a further 12 h before microscopic observation. The transient transfection efficiency was estimated by the percentage of blue-stained cells over the total number of cells.

2.9. Transfection level

The level of the transfection was estimated by measuring the quantity of the expressed protein. Cells were seeded in six-well plates and the chemiluminescence of β-gal was measured 48 h after transfection in the presence of the substrate 3-4(-methoxyspiro[1,2-dioxetane-3,2′-tricyclo(3.3.1.1)decane]-4-yl)phenyl-β-D-galactopyranoside (APMGD) by using the Tropix kit Galactolight Plus (Tropix, Bedford, MA). Following the procedure of the supplier, after lysing the cells, 20 μl of the extract was incubated with 200 μl of the reagent (diluted to 1%). After 1 h, 300 μl of the accelerator was added and luminometric measurement was made using a BCL luminometer (Gouteyron, Val le Puy, France). Protein was titrated by using a Bio-Rad kit in order to normalize the transfection level and expressed in relative light units (RLU) per milligram of total protein.

2.10. Western blot analysis

Cells were grown in the presence of pCMV-p16/liposome complexes in six-well plates. After 24 or 48 h, cells were detached using a nonenzymatic cell dissociation solution (Sigma) and treated with cold lysis buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM PMSF, 1 mM $NaHCO_3$, pH 8) containing antiproteases cocktail. The protein concentrations were measured by BSA protein assay kit quantification (Bio-Rad, Hercules, CA) and standardized by adjusting lysis buffer volumes. Fifty microliters per lane were separated by 10% SDS-PAGE and trans-blotted onto nitrocellulose membrane (Schleicher and Schull, Dassel, Germany) for p16. Nitrocellulose filters were blocked for 1 h using 5% fat dry milk in 1× PBS. The membranes were incubated with anti-p16 antibodies diluted at 1:2500 in PBS containing 0.5% Tween 20 (PBS-T) for 1 h. After three washes in PBS-T, membranes were incubated with horseradish peroxidase-conjugated donkey anti-mouse diluted at 1:10 000, followed by development

with an enhanced chemiluminescence Western blot detection kit ECL (Amersham, Buckinghamshire, UK).

As positive control for $p16^{INK4a}$, HBL100 cells (human normal breast cell line) have been used and untransfected FR cells were taken as negative control.

2.11. Immunofluorescence localization of p16

Transfected and untransfected cells were grown in four-well Lab-Tek plates (Nalgen Nunc, Napperville, IL). After 24 or 48 h, cells were washed three times with PBS, followed by fixation in acetone for 10 min. After rehydration, the nonspecific sites were blocked for 30 min with PBS containing 0.1% BSA. The cells were then incubated for 1 h with anti-p16 diluted at 1:25 in PBS. The plates were washed again and incubated for 1 h with FITC-conjugated secondary antibodies (Immunotech, Marseille, France) diluted 1:20 in PBS. After additional washing, the plates were mounted using Mowiol (Calbiochem, La Jolla, CA) and then visualized using a Reichert fluorescence microscope.

2.12. Cytotoxicity

Cellular toxicity was investigated by studying the viability of cells, using the MTT method. Cells were seeded in 96-well plates. After defined times in the presence or not of TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes, cells were washed twice with PBS and then 100 μl of RPMI medium supplemented with 10% serum and containing 1 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added. The coloration was performed after 4-h incubation, cells were lysed with dimethylsulfoxide (DMSO) and the optical density at 570 nm of the solution was read by a Bio-Rad plate Reader (Model 450).

2.13. Cell proliferation assay

In order to determine the effect of restoration of p16 on cell proliferation, cell growth was investigated after transfection. FR cells in 10% FCS/RPMI were seeded into T25 tissue culture plates at a density of 3×10^5 cells/plate (Falcon, Strasbourg, France) and allowed to adhere to the plastic for 24 h. Cells were then transfected following the protocol described above. After the desired time (3, 6, 9 days), they were washed with PBS, dissociated with 0.025% trypsin–EDTA (Life Technologies) and counted using a Coulter counter (Coultronics, Margency, France).

3. Results

3.1. Characterization of TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes

Liposomes prepared from TMAEC-Chol (or TEAEC-Chol) lipid and DOPE were previously characterized by QLS and TEM as described previously [23,31]. Electron microscopy showed that liposomes are unilamellar vesicles and QLS indicated a monomodal distribution of size with the mean value of 124 and 104 nm for, respectively, TMAEC-Chol/DOPE (1:1) and TEAPC-Chol/DOPE (1:1) liposomes in water. The polydispersity factors were 0.33 and 0.23, respectively. The liposome size was practically independent on the preparation conditions; there is only a small difference in these characteristics from one preparation to another, and liposomes were very stable, preserved at many months without noticeable alteration in size distribution.

3.2. Characteristics of the FR cell line

Characteristics of FR cells were previously investigated [26–28]. This cell line exhibited the characteristics of mesothelial cells as determined by immunocytochemistry and ultrastructural studies. Cells coexpressed cytokeratin and vimentin, but no CEA expression was found. Ultrastructural features agreed with the typical characteristics of mesothelial cells, which are occurrence of desmosomes, long and slender microvilli and perinuclear organization of intermediate filaments. Cells were hyperdiploid since 64% of the metaphases contained between 51 and 75 chromosomes per metaphase. The remaining metaphases were tetraploid or nearly tetraploid.

3.3. Cytotoxicity study

The cytotoxicity of TMAEC-Chol/DOPE liposome and TEAPC-Chol/DOPE liposome was determined with the MTT assay. Fig. 1 represents the viability of mesothelioma FR cells incubated with and without cationic liposomes as a

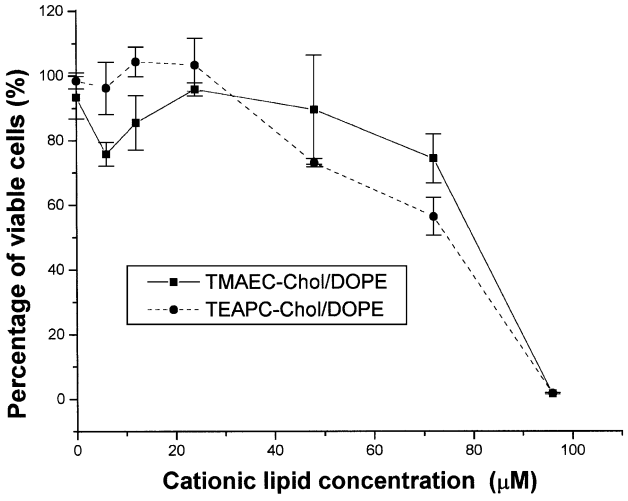

Fig. 1. Dose-dependent viability of mesothelioma FR cells transfected with TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes after 48 h of incubation. Percentage of viable cells was obtained by MTT assay as described in Materials and methods and referred to the viability of control cells taken as 100%.

function of the cationic lipid concentration. From these plots, the concentration corresponding to half the maximum growth TC_{50} was estimated to be 70 μM for TEAPC-Chol/DOPE liposomes. This TC_{50} is slightly higher for TMAEC-Chol/DOPE liposomes. With the cationic lipid/DNA molar charge ratio $X=2$, this would allow the use of TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes to transport plasmids up to DNA concentration of 35 μM (in nucleotides).

3.4. Transfection efficiency with plasmids vectorized by TMAEC/DOPE and TEAPC/DOPE liposomes

The transfection efficiency evaluated by the test X-gal can be seen in Fig. 2. The blue-colored cells, indicating the presence of expressed β-galactosidase, were observed by light microscopy and counted. Controls of endogenous β-galactosidase and the effect of the liposome carrier were evaluated using untransfected cells (Fig. 2A) which do not show any detectable blue spot. Very few blue-stained cells were observed after transfection with of unvectorized plasmids (Fig. 2C). With the same amount of DNA carried by TMAEC-Chol/DOPE liposomes, the efficiency of the transfection for mesothelioma FR cells can reach 70% (Fig. 2B).

3.5. Transfection level

The results on transfection level, estimated by luminometric measurements of expressed β-gal, are shown in Fig. 3. For this purpose, the total chemiluminescence intensity (in RLU), including that of endogenous β-gal, was measured. The endogenous β-gal, estimated from the intensity due to the untransfected cells, was then suppressed and the results were normalized for every milligram of total protein.

In these assays, we have explored the effect of the molar charge ratio cationic lipid/DNA and found that the optimal level was observed when the molar charge ratio cationic

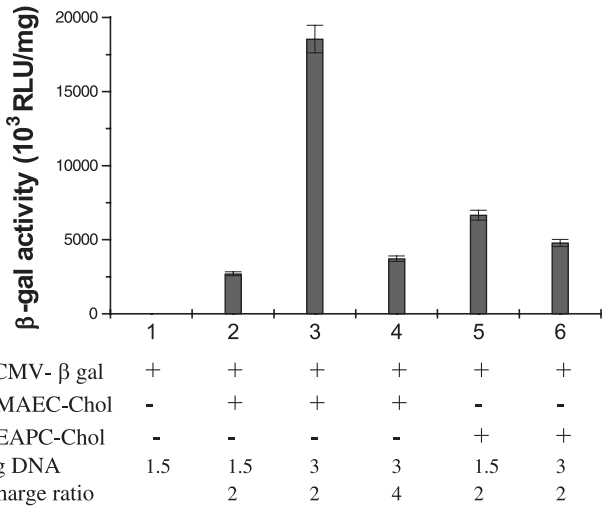

Fig. 3. Transfection level of plasmid pCMV-β precondensed with spermine (3 nmol/mg DNA) delivered into FR cells by cationic liposomes TMAEC-Chol/DOPE (1:1) and TEAPC-Chol/DOPE (1:1), evaluated by β-gal activity 48 h after transfection. The effects of the lipidic nature and of the molar charge ratio cationic/DNA are shown. Cells: 5×10^5/well. Transfection medium for the first 6 h: OPTIMEM without serum. After 6 h, OPTIMEM was replaced with the serum-containing culture medium RPMI 1640. (1) Cells transfected with unvectorized pCMV-β. (2) Cells transfected with TMAEC-Chol/DOPE carrying 1.5-μg pCMV-β, $X=2$. (3) and (4): with the same liposome carrying 3-μg pCMV-β, charge ratios $X=2$ and $X=4$, respectively. (5) and (6): with TEAPC-Chol/DOPE liposomes carrying 1.5- and 3-μg pCMV-β, respectively; charge ratio $X=2$.

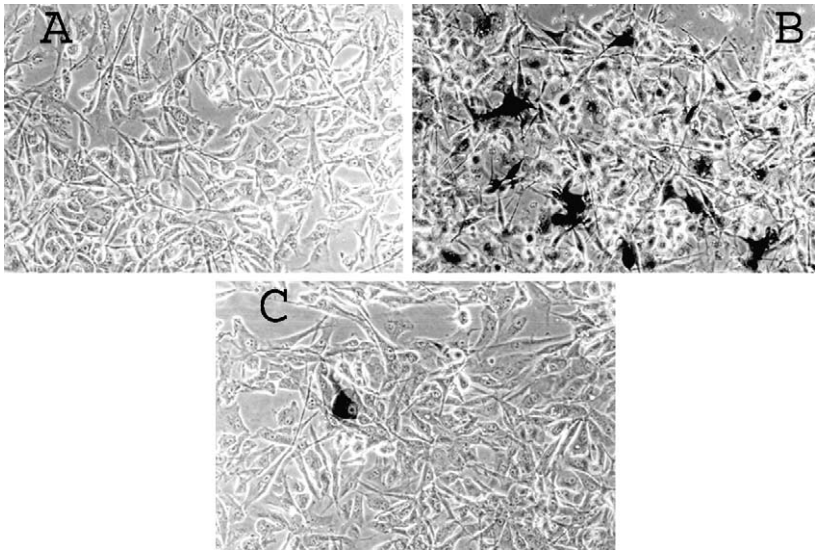

Fig. 2. Transfection efficiency of plasmid pCMV-β delivered into FR mesothelioma cells by TMAEC/DOPE liposomes. Staining was obtained with X-gal assay 48 h after transfection. (A) Untransfected cells. (B) Cells transfected with pCMV-β vectorized by TMAEC-Chol/DOPE liposomes. (C) Cells transfected with free pCMV-β.

lipid/DNA was $X=2$. For comparison, we present the transfection levels in mesothelioma FR cells when TMAEC-Chol/DOPE and TEAPC-Chol/DOPE were used. Fig. 3 shows that the transfection level of TMAEC-Chol/DOPE is higher than that of TEAPC-Chol/DOPE liposomes.

3.6. Delivery and persistence of gene p16 observed by immunofluorescence

Mesothelioma cells transfected with pCMV-p16 plasmids complexed with TMAEC-Chol/DOPE and TEAPC-Chol/

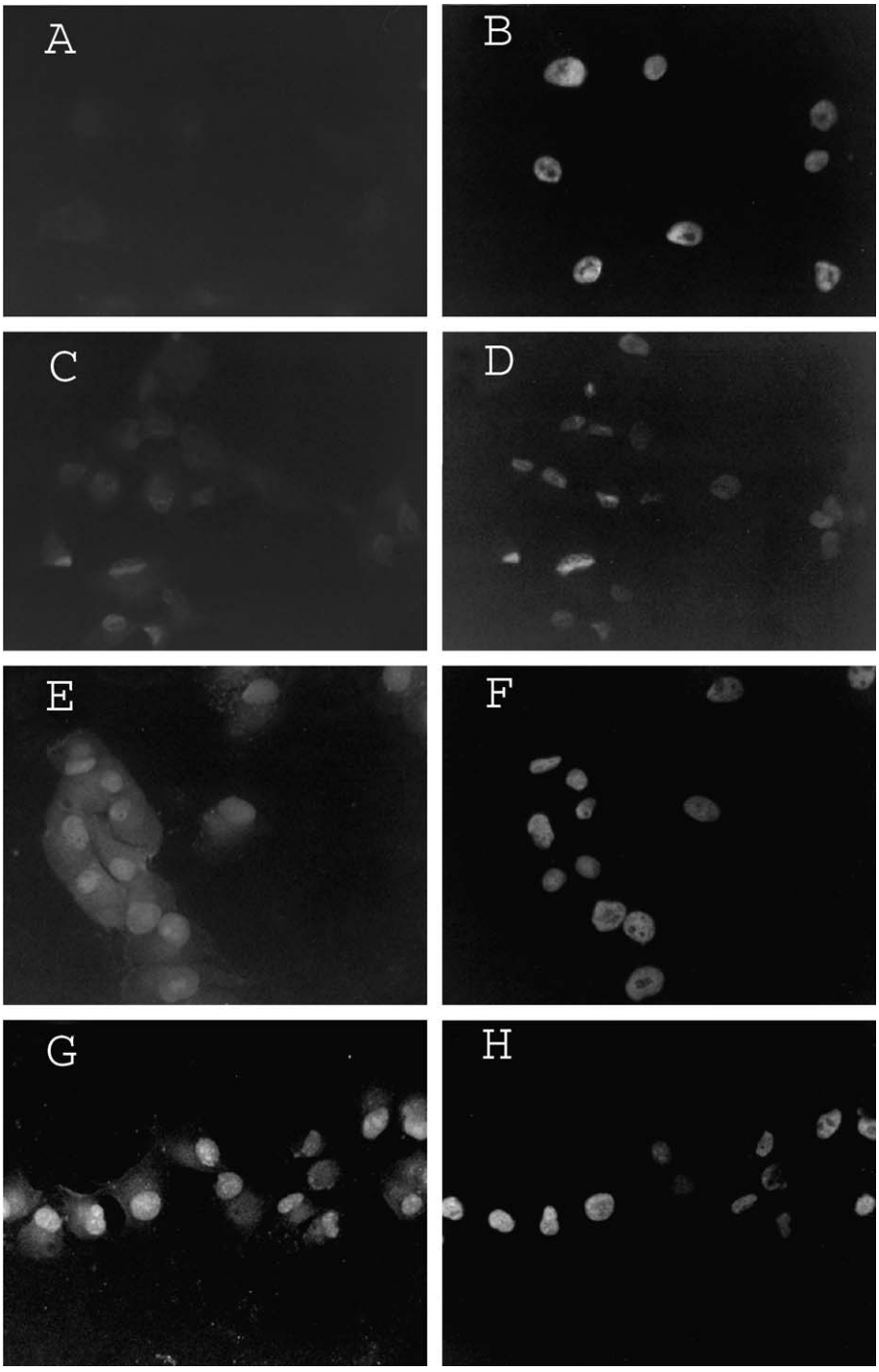

Fig. 4. Transfer of p16 into mesothelioma cells FR, observed by immunofluorescence using antibodies against anti-p16, labelled with fluorescein. Cells were transfected in OPTIMEM with pCMV-p16 complexed with cationic liposomes for 24 h. (A) Control untransfected cells FR. (C) Control FR cells transfected with pCMV-p16 unvectorized by liposomes. (E) FR cells transfected with pCMV-p16 complexed with TMAEC-Chol/DOPE liposomes. (G) FER cells transfected with pCMV-p16 complexed with TEAPC-Chol/DOPE liposomes. Amount of plasmids: 3 μg. Molar charge ratio cationic lipid/DNA: $X=2$. (B, D, F, H): same fields observed with DAPI staining.

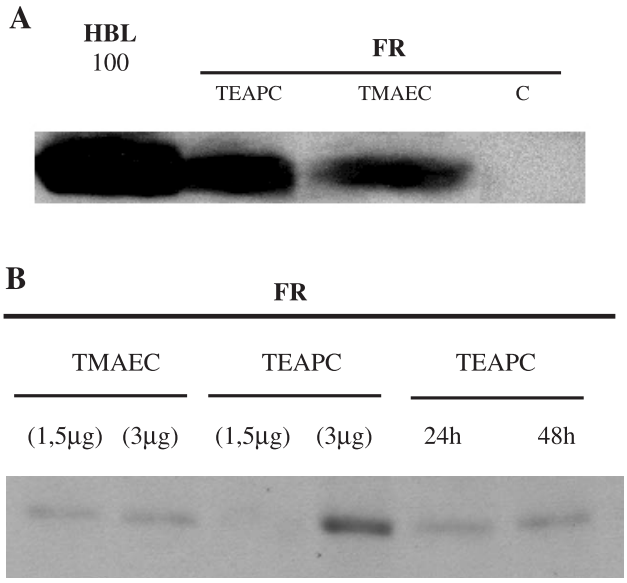

Fig. 5. Western blot analysis of pCMV-p16 transfer into FR mesothelioma cells by cationic liposomes TMAEC-Chol/DOPE and TEAPC-Chol/DOPE. (A) Expression of the suppressor gene p16. The positive control was indicated by the spot in lane 1 obtained with endogenous p16 of untransfected mammary carcinoma HBL 100 cells (left lane). The negative control was indicated by the right lane C obtained with untransfected FR cells. The two middle lanes respectively show the expression of exogenous p16 introduced by TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes. (B) Effect of the incubation time (24 or 48 h), of the DNA amount (1.5 or 3 µg) and of the nature of cationic lipid (TMAEC-Chol or TEAPC-Chol).

DOPE liposomes are illustrated in micrographs of Fig. 4. Untransfected cells did not show any presence of p16, indicating that mesothelioma FR cells do not express p16 (Fig. 4A). Cells transfected with unvectorized pCMV-p16 showed only low level of p16 in the nucleus (Fig. 4C) whereas cells transfected with pCMV-p16 vectorized by TMAEC-Chol/DOPE and TEAPC-Chol/DOPE cationic liposomes showed higher labelling (Fig. 4E and G, respectively). Comparison with cells stained with DAPI, a nuclear staining (Fig. 4B, D, F, H), shows that the expression was rather nuclear. The fluorescence is only slight, diffuse in the cytoplasm and much more intense in the nucleus as expected. It seems that there is slightly more expression of p16 with TEAPC-Chol/DOPE liposomes than with TMAEC-Chol/liposomes. These observations were confirmed by Western blot analysis. Pictures made after 24 and 48 h showed that the expression of p16 was maintained at 48 h (data not shown).

3.7. 16^{INK4} expression observed by Western blotting

In order to assess the efficiency of the transfer of p16 by using cationic liposomes TMAEC-Chol/DOPE and TEAPC-Chol/DOPE, we have undertaken Western blot analysis. The results are given in Fig. 5. In the left lane of Fig. 5A, a positive control was made with HBL100 cells where a large amount of p16 was found, confirming the high level of expression in this cell line [5]. Negative control was obtained with untreated FR cells (right lane C) where p16 was not detected. On the contrary, the presence of p16 was observed in middle lanes with cells treated with TMAEC-Chol/DOPE liposomes and TEAPC-Chol/DOPE liposomes, indicating the efficiency of the transfection. Fig. 5B shows that the transfer of p16 is dose-dependent and the expression of p16 was slightly higher when TEAPC-Chol/DOPE liposomes were used than with TMAEC-Chol/DOPE liposomes. Fig. 5B reveals also that p16 was still observed 48 h after transfection.

3.8. Dose and time effect on proliferation of transfected FR mesothelioma cells

The functionality of delivered p16 in mesothelioma FR cells was investigated by proliferation assay. Cells treated with pCMV-p16 have been counted 3 days after the transfection. Data are given in Fig. 6. With 3, 5 or 8 µg of pCMV-p16, we observed an effect of 16%, 20% and 34%, respectively, on the inhibition of cell proliferation. Moreover, with the dose of 8 µg of pCMV-p16, the effect on cell growth was followed in time, by counting cells 3, 6 and 9 days after the transfection as indicated in Fig. 7. In first experiments, cells were transfected one time with 8 µg of pCMV-p16. An inhibition of proliferation of about 34% was observed after 3 days. However, after 6 days the proliferation reached the control level as indicated by the dashed plots. In the second experiments, cells were administered again with the same amount of pCMV-p16 after 3 and 6 days. In this case, an inhibition of 50% at day 6 and another of 63% were observed at day 9 for TMAEC-Chol/DOPE liposomes (solid plots). A similar but slightly smaller effect of 45% at day 6 and 53% at day 9 was obtained with TEAPC-Chol/DOPE liposomes. It is to note that in these assays, results were obtained by comparison to the controls

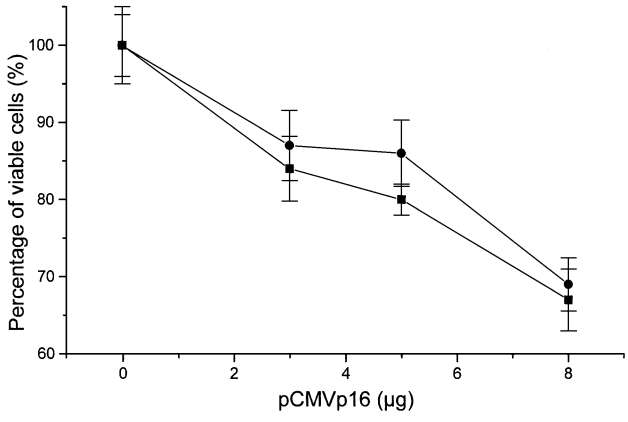

Fig. 6. Dose effect on proliferation inhibition of mesothelioma cells transfected by pCMV-p16 complexed with cationic liposomes TEAPC-Chol/DOPE (●) and TMAEC-Chol/DOPE (■). pCMV-p16 and cationic liposomes were complexed in the molar charge ratio cationic lipid/DNA, $X=2$. Results were obtained at day 3, with three sets of measurements in duplicate. Cell number of control untreated cells was taken as 100%.

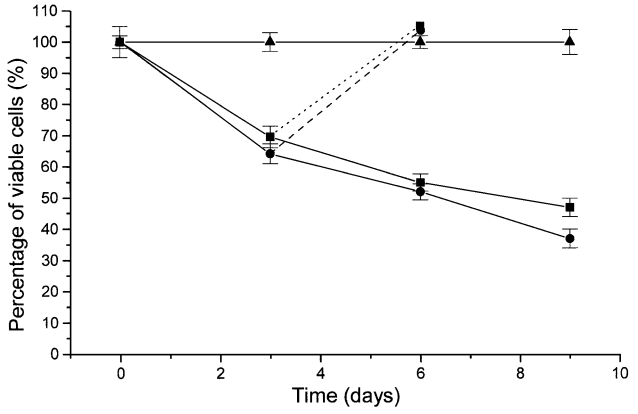

Fig. 7. Proliferation inhibition of mesothelioma cells under the effect of pCMV-p16 delivered by cationic liposomes TMAEC-Chol/DOPE and TEAPC-Chol/DOPE. (▲) Untreated cells; (■) cells transfected with pCMV-p16/ (TEAPC-Chol/DOPE) complexes; (●) cells transfected with pCMV-p16/(TMAEC-Chol/DOPE) complexes. Dashed lines: cells treated with one dose at day 0. Solid lines: cells treated with one dose at day 0 and with repeated doses at day 3 and day 6. Complexes were prepared with 8-μg pCMV-p16 and TMAEC-Chol/DOPE or TEAPC-Chol/DOPE liposomes in the molar charge ratio cationic lipid/DNA, $X=2$. Results were obtained with three sets of measurements in duplicate. Cell number of control untreated cells was taken as 100%.

obtained with untreated cells and cells incubated with liposomes alone or with plasmid alone. Moderate concentrations of liposomes alone slightly enhance cell growth and no change was observed with plasmid alone. So, the proliferation inhibition observed is only due to pCMV-p16 delivered by liposomes. This is clearly a proof of the ability of TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes to efficiently deliver cancer suppressor gene p16.

4. Discussion

The results of this work showed that the tumor suppressor gene p16 could be transferred using cationic liposomes into FR cells deficient in $p16^{INK4a}$ expression.

To define the best conditions for gene transfer, we investigated the transfection efficiency with two different formulations of liposomes and DNA/liposome complexes. When evaluating the activity of the reporter gene encoding the β-galactosidase, results indicate that TMAEC-Chol/DOPE and TEAPC-Chol/DOPE liposomes are efficient to carry DNA plasmids into FR cells.

The most important finding is the transfer of tumor suppressor gene $p16^{INK4a}$ in cells by using cationic lipids. The cellular expression of the transgene p16 was demonstrated by immunofluorescence and Western blot. The protein coded by the transgene was strongly expressed in the nucleus and slightly in the cytoplasm as attested by a pattern that was much more fluorescent inside than outside the nucleus. Moreover, the nuclear fluorescence was diffuse and not punctate, suggesting that the protein is not located in limited areas of the nucleus. A slight difference between the expression profiles of the reporter gene and the p16 gene is observed. This could be explained by the fact that there is a difference in size of plasmids pCMVβ (7 kb) and pCMV-p16 (5 kb), which implies a difference in the number of plasmid copies corresponding to the same amount of DNA delivered. Therefore, the number of p16 gene copies is greater than that of β-gal gene. Moreover, this may due also to the fact that cytoenzymatic techniques, such as the X-gal assay used here, are not as sensitive as immunocytological methods [30].

From our results, if TMAEC-Chol/DOPE and TEAPC-Chol/DOPE are both able to deliver plasmids in mesothelioma FR cell, the efficiency and the resident time of DNA slightly depend on the nature of the cationic lipid. The difference between TMAEC-Chol and TEAPC-Chol stands in the structure of their cationic polar heads. This dependence on the nature of the carriers can be explained by the binding of DNA to the lipid molecules. A stronger binding is necessary to form the complex whereas a weaker is needed to release DNA in the cellular compartment. The resident time is an interesting characteristic of the transfection. With the cationic liposomes used here, immunofluorescence indicates that the resident time is at least 48 h. Such a relatively long time may be important for a gene therapy modality, although its lifetime is limited as shown by the proliferation assay.

The functionality of the delivered gene was assessed by the effect on cell proliferation. Three days after transfection, a dose-dependent effect was already observed as indicated by Fig. 6. An inhibition of about 30% was obtained in cells incubated with 8 μg of pCMV-p16. This inhibition was only transient because the cell growth took place again and attained the level of the control after 6 days. However, with repeated doses, the inhibition was maintained and reached about 50% and 63% after 6 and 9 days, respectively. This inhibition effect agreed with the deficiency of p16 in the studied FR cells, for which the delivery of p16 prevented cell growth. This inhibition rate is in the same order with that observed by Frizelle et al. [10] on cells incubated with p16 gene delivered virally. It is worth noting from the results of cytotoxicity study that the IC_{50} of cationic lipid concentration is about 70 μM, corresponding to 35 μM in nucleotide so that in the range of used concentrations (8-50 μM in cationic lipid), liposomes alone were not cytotoxic. This allowed repeated administrations of p16 gene in order to maintain its inhibition effect on cell growth.

It is also important to note some other advantages in the use of cationic liposomes prepared with TMAEC-Chol and TEAPC-Chol lipids. As shown from previous works, they are prefabricated, very stable and have well-defined sizes. Moreover, they are able to form complexes with oligonucleotides or plasmids and can be mixed with any desired DNA before transfection. With these lipids, it is easy to obtain the complexation with DNA with high yield which can reach 100% [31]. This lipid-mediated gene transfer is relatively nontoxic and nonimmunogenic. Consequently,

cationic liposomes may permit multiple administrations of the gene of interest, which is likely to be necessary for the successful treatment of advanced tumors [18].

It must be emphasized that $p16^{INK4a}$ is an important protein to be considered in cancer therapy, regarding its role in the control of cell proliferation, especially as an inhibitor of pRb phosphorylation. In many types of tumor, besides MPM, disrupted pRb function seems related to $p16^{INK4a}$ inactivation rather than to pRb alteration [10,22,32–34]. Reexpression of $p16^{INK4a}$ by gene transfer in p16-deficient tumor cells has been found to restore pRb functionality [10,22], suggesting that $p16^{INK4a}$ might play a role in the treatment of cancer cells using gene therapy. Transfer of a suppressor gene such as p16 can also be used for other types of cancer cells derived from various human tissues, including pancreas, skin, brain, bladder and lung [5]. A summary on the frequency of p16 inactivation in several primary tumours has been reviewed by Liggett and Sidransky [35] and Ruas and Peters [6], in particular the implication on mesothelioma frequently associated with asbestos exposure.

Now, most experimental studies have used viral carriers for gene transfer mainly because of the better efficiency of viruses to achieve gene transfer in comparison with nonviral vectors. However, there is some reluctance to use viral vectors, regarding an insert size limit or its hazards for the host such as stimulation of the immune system or possibility of oncogenic risks. Therefore, the development of nonviral methods is needed. In the present study, using original TMAEC-Chol/DOPE and TEAPC-Chol/DOPE cationic liposomes with low cytotoxicity, we demonstrated an efficient gene transfer in a human mesothelioma cell line.

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