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Gene Transfer in Differentiated Primary Rat Tracheal Epithelial Cells by Non-Viral Vectors

Valeria Casotti[1] • Marie-Luise Syren[1] • Giorgia Dina[2] • Elena Copreni[1] • Sante Di Gioia[1] • Donatella Piro[3] • Massimo Conese[1,3*]

[1] Institute for Experimental Treatment of Cystic Fibrosis, San Raffaele Scientific Institute, 20132 Milano, Italy
[2] Neuropathology Unit, Department of Neuroscience, San Raffaele Scientific Institute, 20132 Milano, Italy
[3] Department of Biomedical Sciences, University of Foggia, 71100 Foggia, Italy

Corresponding author: * **m.conese@unifg.it**

ABSTRACT

The cellular barriers to efficient gene transfer into the airways are still poorly understood. Rat tracheal epithelial (RTE) cells faithfully reproduce the mucociliary, pseudostratified characteristics of the *in vivo* human airway epithelium and are a suitable model for studying major pathways of airway cell differentiation. We studied the efficiency of various non-viral cationic vectors in the primary culture of RTE cells grown in different conditions. When we grew RTE cells on collagenated plastic dishes for 4 days in basal conditions (25 ng/ml EGF), a poorly-differentiated (PD) phenotype appeared, whether or not retinoic acid (RA) was present in culture medium. The cells were transfected with a plasmid encoding a luciferase reporter gene under the control of the cytomegalovirus promoter. The highest luciferase levels were obtained with a cationic lipid (DOTAP) and a cationic polymer (PEI). When cells were grown for 12 days on collagen-coated inserts (Transwells®) at the air-liquid interface in the presence of RA, the differentiation was towards a well-differentiated phenotype, as assessed by electron microscopy. Conversely, in the absence of RA, the cells acquired a flattened undifferentiated phenotype. In the absence of RA, the transfection levels were 3-8.8–fold higher than in the presence of RA. However, in differentiated cells, PEI gave transfection levels closer to those obtained in PD cells than DOTAP. These results support our view that PEI can overcome the barriers imposed by a differentiated phenotype.

Keywords: differentiation, non-viral, polyethylenimine, reporter gene
Abbreviations: CK, cytokeratin; **DMPE**, dimyristoylphosphatidylethanolamine; **DOPE**, oleoyl-phosphatidylethanolamine; **DOTAP**, N-(1-(2,3-dioleoyloxy)-propyl)-N,N,N-trimethyl ammonium methylsulfate; **EGF**, epidermal growth factor; **PD**, poorly-differentiated; **PEI**, polyethylenimine; **RTE**, rat tracheal epithelial

INTRODUCTION

Gene transfer into the airways could be the definitive way of treating congenital diseases, like cystic fibrosis (CF) and α_1-antitrypsin deficiency (Driskell and Engelhardt 2003), but it is hampered by an array of barriers (Ferrari *et al*. 2002; Conese *et al*. 2007). Gene transfer vectors are blocked by the blanket-like mucus, by apical membrane glycocalyx, by the lack of appropriate receptors in the same location, by the tight junctions between the cells, by the intracellular endosomo-lysosomal compartments where their degradation eventually occurs, and finally by the nuclear membrane. In CF airways inspissated mucus and mucus plaques (Worlitzsch *et al*. 2002) will enhance the barrier to airway gene transfer even further. Although the mucus represents the major extra-cellular barrier, it is the apical membrane of the airway lumen-facing columnar cell, the predominant cell type that must be transfected *in vivo* in CF, which constitutes the primary hurdle to an efficient gene transfer in the context of a differentiated airway epithelium. The apical plasma membrane is relatively resistant to viral and non-viral transfer agents, due to the paucity or the lack of internalizing receptors or a low rate of endocytosis from this compartment (Matsui *et al*. 1997; Jiang *et al*. 1998; Pilewski 2002). Several agents have been shown to enhance delivery of genes to intact airways including the divalent cation chelator ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Wang *et al*. 1998, 1999; Chu *et al*. 2001; Johnson *et al*. 2003; Meng *et al*. 2004), the short chain fatty acids sodium caprate (Johnson *et al*. 2003) and sodium laurate (Johnson *et al*. 2003), the detergents polidocanol (Parsons *et al*. 1998) and α-L-lysophosphatidylcholine (Limberis *et al*. 2002; Kremer *et al*. 2007). In alternative, researchers have been pursuing the scope of having an efficient gene transfer into airway epithelial cells by pseudotyping viral vectors (reviewed in: Anson *et al*. 2006; Conese *et al*. 2007; Flotte *et al*. 2007) or by adding ligands to the non-viral backbone to expolit receptors expressed on the apical plasma membrane (Ziady *et al*. 2002; Grosse *et al*. 2004).

Primary human epithelial cells have been used in gene delivery experiments, either when grown on collagenated plates or allowed to polarize on filters (Caplen *et al*. 1995; Fasbender *et al*. 1995; Fajac *et al*. 2003). However, the results were poorly reproducible, due to intra- and inter-individual diversity, and a limited supply of these cells is generally available. Air–liquid interface cultures of primary rat tracheal epithelial (RTE) cells faithfully reproduce the mucociliary, pseudostratified characteristics of the *in vivo* human airway epithelium (Yoon *et al*. 1997). Furthermore, RTE cell cultures have been shown to be a model for studying major pathways of airway cell differentiation (Kaartinen *et al*. 1993; Andrews *et al*. 2000; Kim *et al*. 2002). They are well characterized and the variability should be limited by using cogenic animals. Although RTE cells have been used in earlier studies for gene transfer with viral vectors (adenovirus and lentivirus) (Pickles *et al*. 1998; Kobayashi *et al*. 2003), they have not yet been evaluated for gene transfer with non-viral vectors. In order to dissect those differentiative and proliferative conditions that might influence cationic vector-mediated gene delivery into primary respiratory cells, we have undertaken a study making use of non-viral cationic vectors belonging to various types and RTE cells grown under different culture conditions. Our data show

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that when RTE cells are grown at the air-liquid interface in the presence of retinoic acid (RA), a mucociliary phenotype occurs. Under these conditions, cells are less amenable to transfection than cells grown in the absence of RA. Nevertheless, polyethylenime shows to be efficient also under these conditions.

MATERIALS AND METHODS

RTE cell culture

RTE were isolated and cultured as previously published (Kaartinen *et al*. 1993) with modifications. Female Sprague-Dawley rats (100-125 g) (Charles River, Italy) were euthanized with CO_2 asphyxiation. Tracheas were excised and filled with a 0.1% solution of protease type 14 (Sigma, St. Louis, MO, USA) in DMEM/F12 medium and incubated for 24 hours at 4°C. Epithelial cells were removed from the trachea by flushing with media, counted, and plated either on collagenated 24-well (2×10^4 cells/well) or 6-well plates (2×10^5 cells/well). The culture medium was Dulbecco's modified Eagle's medium/F12 medium supplemented with insulin (10 µg/ml), hydrocortisone (0.1 µg/ml), transferrin (5 µg/ml), epidermal growth factor (25 ng/ml), penicillin (50 units/ml) and streptomycin (50 µg/ml). Experiments were performed on the fourth day of culture, when 30-50% of the surface was covered by cells, as judged by the staining with Harris Hematoxylin (Sigma).

In another set of experiments, RTE were plated onto the apical surface of Transwell-Col® (Corning, Acton, MA, USA) tissue culture inserts (pore size 0.4 µm, either 12 or 24 mm diameter). At the seventh day of culture, the apical medium was removed and cells were grown at the air-liquid interface with the growth factors supplemented only from the baso-lateral medium. After 12 days in culture on Transwells, cells exhibited an average transepithelial resistance of 1000 Ω/cm^2, as measured by an epithelial voltmeter (millicell-ERS; Millipore, Bedford, MA, USA).

Immunocytochemistry of epithelial antigens

The following monoclonal antibodies were used to stain rat epithelial cell antigens: anti-cytokeratin 14, dilution 1:50 (NCL-LL002, from Novocastra Laboratories, Newcastle, UK) that recognizes basal cells in the pseudostratified epithelium, anti-CK 18, dilution 1:100 (Roche Diagnostics S.p.A., Monza, Italy), identifying columnar cells; and anti-cytokeratin 13, dilution 1:10 (clone AE8, from ICN, Aurora, OH, USA), specific for squamous cells. Day 4 RTE cells were fixed with 100% methanol for 10 minutes at –20°C, treated with PBS containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1% H_2O_2 for 10 minutes. Membrane permeabilization was carried out by soaking cells in PBS containing 1% Triton X-100. Cells were subsequently challenged for 1 hour with the appropriate dilution of monoclonal anti-cytokeratin antibodies (in PBS containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.2% gelatin). The detection was carried out by a biotin/avidin system using the ABC Vectastain Elite kit (Vector Laboratories, Burlingame, CA, USA). When RTE cells were grown on Transwells, cells were detached, cytospun on coverslips, and processed as described above. Coverslips were then mounted with aqueous mounting media (Electron Microscopy Science, Washington, PA, USA). Observations were carried out on a Zeiss Axioskop microscope (Thornwood, NY, USA). Negative control included the omission of primary antibodies and showed no staining.

Electron microscopy

RTE grown on Transwells for 12 days were fixed *in situ* for 2 hours at room temperature with 2% glutaraldehyde in 120 mM phosphate buffer and then washed with the buffer. The layers were postfixed with 2% OsO_4 in 120 mM cacodylate buffer, dehydrated in ethanol and infiltrate in mixture of Epon and ethanol 100% (1:2, 1:1, 2:1 for 1 hour in each). At the end the cultures were embedded in Epon overnight at 60°C. Ultrathin sections, doubly stained with uranyl acetate and lead citrate, were examined in a Hitachi (San Jose, CA, USA) H-7000 electron microscope.

Plasmid

Plasmid pCLuc encodes the *P. pyralis* luciferase coding region under the control of the cytomegalovirus (CMV) immediate early enhancer/promoter region (Bragonzi *et al*. 2000). Plasmid DNA was purified on double gradient of cesium chloride (Sambrook and Russell 2001). A_{260}/A_{280} was determined spectrophotometrically and was between 1.8 and 2.0. Purity was checked by agarose gel electrophoresis.

Cationic vector-DNA complexes formation and transfections

Branched PEI 25 kDa (Sigma-Aldrich) was used as a 100 mM (4.5 g/l) aqueous stock solution. DOTAP (Roche, Italy) was purchased as a 1 mg/ml acqueous solution. FuGENE™ 6 was purchased from Roche. GL-67/DOPE/DMPE/PEG5000 (GL67-PEG5000; kindly provided by SH Cheng, Genzyme, Framingham, MA, USA) was formulated at 1:2:0.05 molar ratio (Eastman *et al*. 1997) and reconstituted as 2 mM aqueous solution. For complex formation, the following amounts of each vector were used per 2 µg of DNA: 2.5-20 equivalents of PEI nitrogen per DNA phosphate, that is 1.5-12 µl of 10 mM PEI solution; for DOTAP, a 3 to 9-fold w/w excess, that is 3-9 µl of 1 mg/ml stock solution; for FuGENE™, 3-9 µl; for GL67-PEG5000, from 0.25 to 4 N/P equivalents were used, that is from 0.75 to 12 µl of 2 mM GL67-PEG5000 solution. Complexes were formed by adding the solution of the transfection reagent into the DNA solution and waiting 15 minutes at room temperature. DNA complexes with PEI and GL67-PEG5000 were formed in 150 mM NaCl; with DOTAP in 20 mM Hepes buffer, pH 7.4; with FuGENE™ in OPTI-MEM (Gibco BRL). RTE cells were incubated with the complexes for 24 hours and then evaluated for transgene expression.

Luciferase assay

Twenty-four hours after transfection, cells were rinsed twice in PBS and then lysed with 100 µl of cell lysis buffer (25 mM Tris/HCl, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton X-100, pH 8.0). Twenty microliters of cell lysate were mixed with 100 µl of luciferase assay substrate (Promega, Madison, WI, USA) at room temperature and the light emission (integrated over 30 s) was quantified with a LB 9501 luminometer (Berthold, Bad Willbad, Germany). Light emission was normalized to the protein content of each sample, determined by Bradford's assay for protein concentration using a commercial kit (BioRad).

(^{3}H)-thymidine incorporation

Cells were plated according to the different protocols and incubated in complete medium containing 10 µCi/ml of (methyl-^{3}H) Thymidine (specific activity 185 GBq/mmol; Amersham) for 6 hours. Proteins were removed by adding ice-cold 5% TCA for 20 minutes and washing the cells in absolute ethanol. DNA with incorporated (^{3}H)-thymidine was then extracted in 0.1 M NaOH, 2% Na_2CO_3 and counted at a β-counter. Counts were normalized to protein concentration.

Statistical analysis

Statistical analysis of the results was done by analysis of variance (ANOVA). To calculate p values, Fisher's least-significant-difference test was used. All analyses were carried out with the StatView program (Abacus Concepts Inc., Berkeley, CA, USA) on a Macintosh computer. Differences with p values <0.05 were considered significant.

RESULTS

Proliferation and phenotypic characterization of RTE grown for 4 days

Retinoic acid (RA) is known to arrest growth and induce mucoid differentiation of airway epithelial cells. A thymidine incorporation assay showed that RTE cells proliferated

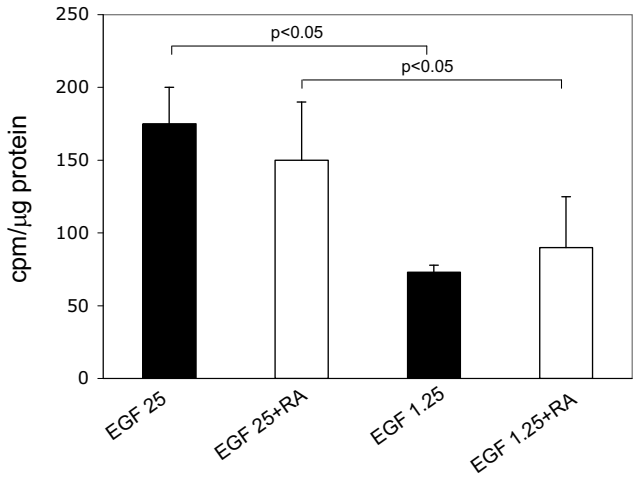

Fig. 1 Proliferation of RTE cells after 4 days in culture. Cells were cultured in the presence of 25 ng/ml EGF (EGF 25) or 1.25 ng/ml EGF (EGF 1.25) in the absence or presence of RA (10 ng/ml). (^{3}H)-thymidine incorporation was evaluated as described in Materials and Methods. Counts (cpm) were normalized to the protein content (µg). Data are expressed as means and standard errors of the mean (n=4).

equally well in the presence of RA, when grown in basal conditions (25 ng/ml EGF) (**Fig. 1**). To see whether a decrease in EGF might allow RA to inhibit growth, cells were incubated in the presence of 1/20 of EGF concentration (1.25 ng/ml) and RA. Also in this case, however, cell proliferation did not change as compared to RA deprivation. Keratinocyte growth factor (KGF), a member of the acidic fibroblast growth factor family of polypeptides, has been shown to be highly mitogenic for respiratory epithelial cells *in vitro* and *in vivo* in rats and mice (Wang *et al*. 1999; Zsengeller *et al*. 1999). Under our experimental conditions, KGF (1-100 ng/ml) did not induce thymidine incorporation at levels higher than 25 ng/ml EGF. To characterize the phenotype of RTE cells grown for 4 days on collagenated plastic dishes under different culture conditions, cytokeratin expression was checked by immunocytochemistry. RTE cells grew as islands and expressed strongly cytokeratin (CK) 14 and moderate to low levels of CK 13 (**Fig. 2**), but not CK 18. This phenotype did not substantially change when RTE cells were grown in the presence of RA. To rule out whether these cells were expressing any of the markers of differentiated mucous cells, RT-PCR was used to evaluate MUC5 mRNA presence. Day 4 RTE cells did not express MUC5 under all the culture conditions (not shown). Overall, these results show that RTE grown on collagenated plastic for 4 days presented a poorly-differentiated (PD) phenotype. To study the non-viral-mediated gene transfer, we used cells grown in the presence of 25 ng/ml EGF.

Transfection of day 4 RTE cells

Non-viral-mediated gene transfer into RTE cells was investigated comparing various cationic vectors. The vectors were optimized as regard to their ratio to DNA. An optimal ratio for each vector corresponded to a low positive charge of the complexes (0.8-1.2 +/-), a result compatible with our previous experience with *ex-vivo* models of human nasal and bronchial epithelial cells (Biffi *et al*. 1999; Sersale *et al*. 2001, 2002). As shown in **Fig. 3**, a cationic lipid (DOTAP) and a cationic polymer (PEI) gave the highest luciferase levels, as compared to the other cationic vectors FuGENE and GL67-PEG5000. Further attempt to increase the transfection mediated by DOTAP or PEI adding either neutral lipids (e.g. DOPE) or polycations (e.g. poly-L-lysine) did not achieve better results. The presence or absence of RA in the medium culture was irrelevant.

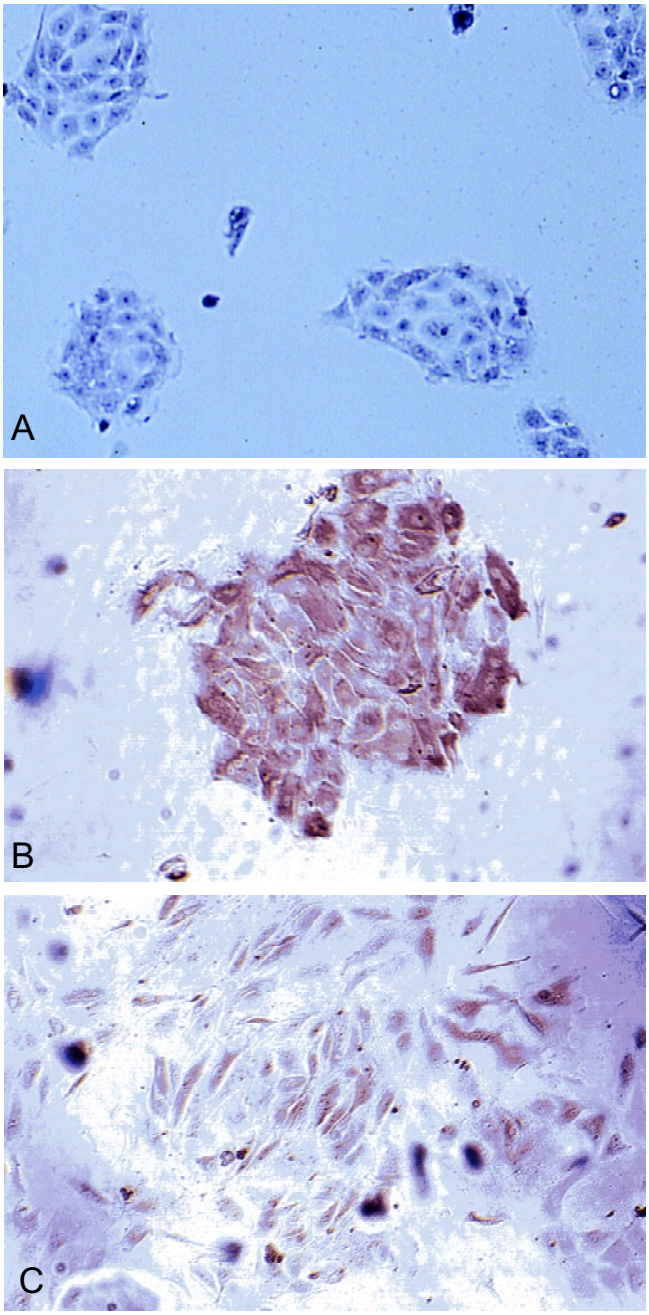

Fig. 2 Cytokeratin expression of RTE cells after 4 days in culture. RTE cells were either stained with Harris Hematoxylin (A) or decorated with antibodies directed against CK 14 (B) or CK 13 (C). Representative pictures obtained from three cultures are shown. Original magnification: A, X 20, B and C, X 40.

Proliferation and phenotypic characterization of RTE cells grown on Transwells

It has been described that RA, collagen gel substratum, and an air-liquid interface are required for expression of a mucociliary phenotype which most closely approximated the morphology of the tracheal epithelium *in vivo* (Kaartinen *et al*. 1993). RTE cells were grown on Transwells at the air-liquid interface for 12 days in the presence or the absence of RA. CK 14 was highly expressed in both conditions; CK 13 was slightly expressed in some cells or not expressed at all in the absence of RA, while in the presence of RA CK 13 was not expressed (not shown). Electron microscopy showed that RTE grew in the absence of RA in a multilayered fashion and displayed a flattened undifferentiated phenotype (**Fig. 4**). In the presence of RA, cells with mumerous microvilli, ciliated and fewer secretory cells could be observed. RT-PCR showed that MUC5 gene was

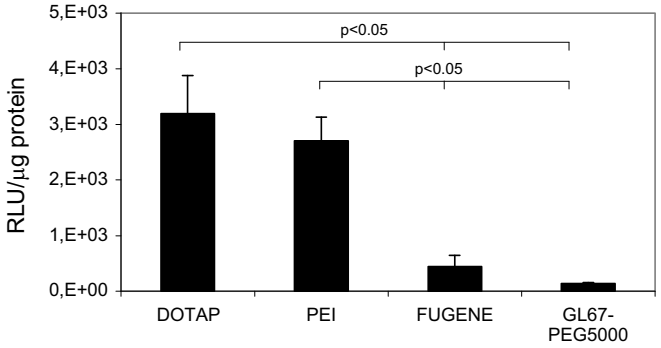

Fig. 3 Transfection of RTE cells after 4 days in culture. Cells were transfected with DNA (2 µg) complexed to DOTAP (6 µl), PEI 25 kDa (6 µl), Fugene 6 (6 µl), or GL67-PEG5000 (6 µl). Luciferase levels were evaluated after 24 hours the addition of the complexes. Luciferase expression is shown as Relative Light Units (RLU)/µg protein. Data are expressed as means and standard errors of the mean (n=4-6).

transcribed only in the presence of RA (data not shown), a result compatible with those previously published (Guzman *et al.* 1996). Moreover, the thymidine incorporation did not change if RA was withdrawn from the culture medium.

Transfection of RTE cells grown on Transwells

These results indicated that RTE grown for 12 days on collagenated inserts at the air-liquid interface in the presence of RA were differentiated to a mucociliary phenotype, while in the absence of RA approximated to a squamous phenotype. Under these culture conditions, the transfection efficiency was evaluated with DOTAP and PEI, the gene transfer agents which were more efficient in day 4 RTE cells. The luciferase levels obtained with both reagents in the presence of RA (i.e. with differentiated cells) were significantly lower than those obtained in the absence of RA (i.e. with undifferentiated cells). The luciferase levels obtained in RA-deprived cells were 8.8- and 3.0-fold higher than in cells grown in the presence of RA with DOTAP and PEI, respectively (**Fig. 5**). However, while DOTAP-mediated transfection dropped almost to background levels, the luciferase levels mediated by PEI were closer to those measured in PD cells.

DISCUSSION

The non-viral vectors show some advantages respect to the viral vectors: they are non immunogenic, are poorly pro-inflammatory agents, and do not impose DNA size limitations. To achieve gene transfer into respiratory epithelial cells, three types of non-viral vectors have been used: cationic lipids, cationic polymers, and molecular conjugates. Although they are been investigating in gene therapy approaches, as for example in CF (Griesenbach *et al*. 2004; Konstan *et al*. 2004), the mechanisms underlying their modality of action and the influence of extracellular barriers and of the cellular phenotype are still being actively investigated (recently reviewed by Conese *et al* 2007).

Interaction of positively charged complexes with negatively charged macromolecules in serum and cell surfaces affects the efficiency of gene transfer (Plank *et al.* 1996). Normal mucus has been shown to inhibit cationic lipid- and PEI-mediated gene transfer into native sheep tracheal epithelium maintained at an air-liquid interface (Kitson *et al.* 1999; Ferrari *et al.* 2001). Similarly, sputum and bronchoalveolar lavage fluid recovered from CF patients were demonstrated to inhibit liposome- and PEI-mediated gene transfer (Stern *et al.* 1998; Rosenecker *et al.* 2003). In CF, these barriers are obviously even more important. The target respiratory epithelial cells are overlaid with a tenacious viscous mucus which is extremely thick and viscoelastic due to the presence of large amounts of DNA, proteins (albumin and mucin), phospholipids, and inflammatory products (Lethem *et al.* 1990; Sheils *et al.* 1996; Sanders *et al.* 2001).

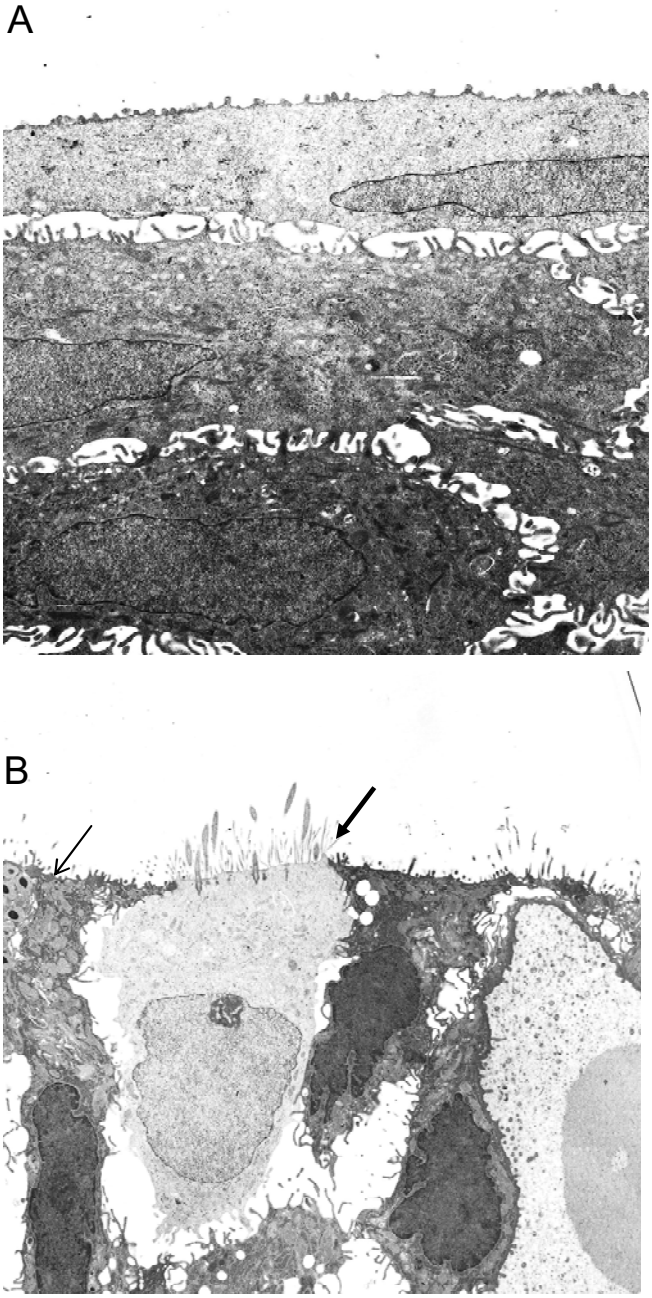

Fig. 4 Morphological evaluation of RTE cells grown on Transwells. RTE cells were grown in the absence (A) or in the presence (B) of RA for 12 days at air-liquid interface and then processed for electron microscopy studies. Representative sections obtained from three cultures are shown. (**A**) Flattened cells exhibiting microvilli and interdigitations with each other. (**B**) A cell showing cilia on the apical surface is indicated by a thick arrow. On the left margin, a cell with apical secretory vesicles is pointed by a thin arrow. Original magnification: X 5000.

Even if the gene transfer complexes could evade the mucus barrier, they have to face the cellular barrier represented by the apical portion of the plasma membrane. It has been shown that the differentiated mucociliary phenotype can inhibit gene delivery through cationic lipids by limiting the endocytic rate from the apical membrane (Fasbender *et al*. 1997; Chu *et al*. 1999). In our experimental conditions, transgene levels obtained in differentiated RTE cells (i.e. cells grown on Transwells in the presence of RA) with DOTAP were much lower than those obtained in PD RTE cells. These results confirm that the differentiated phenotype in the airways imposes some limit to the efficiency of cationic lipids. Interestingly, although PEI gave luciferase

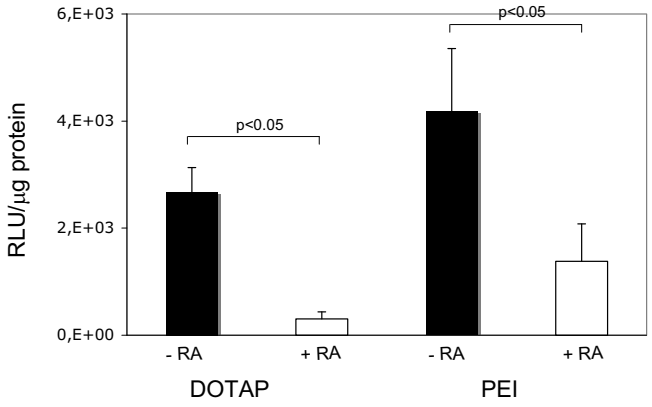

Fig. 5 Transfection of RTE cells cultured on Transwells. Cells were grown at air-liquid interface for 12 days in the absence or in the presence of RA and were transfected with DNA complexed to DOTAP or PEI. Luciferase expression is shown as Relative Light Units (RLU)/µg protein. Data are expressed as means and standard errors of the mean (n=3).

levels in differentiated RTE cells which were lower than those obtained in PD cells, nevertheless these levels were much higher than those attained by DOTAP. Our *in vivo* studies have shown that branched PEI 25 kDa is not only more efficient than DOTAP and GL67-PEG5000 but also it "targets" differentiated columnar epithelial cells in the murine bronchioles (Bragonzi *et al*. 2000). Thus, data present in this paper about PEI support what we have previously found in the mouse *in vivo*. PEI is a promising agent for gene delivery into the respiratory epithelial cells. PEIs have been shown to be very effective in achieving efficient transfection in *in vivo* animal models in the respiratory tract (Lemkine and Demeneix 2001). PEI can be successfully derivatized with different ligand moieties to give it more flexibility and targeting properties (Kichler 2004). Furthermore, PEI-DNA was proved to be less pro-inflammatory than lipid-DNA complexes *in vivo* (Gautam *et al*. 2001). In conclusion, PEI is a potentially powerful gene transfer agent that has to await further development before being widely applied as a useful gene therapy vector in untreatable disease like CF.

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