Cationic Lipid-Mediated Transfection of Differentiated Caco-2 Cells: A Filter Culture Model of Gene Delivery to a Polarized Epithelium

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Purpose. The use of rapidly dividing *in vitro* cell culture systems to assess the efficiency of gene delivery is now recognised as a poor indicator of *in vivo* success. We investigated whether differentiated Caco-2 cell filter-cultures would make a more suitable model for studying gene transfer to an epithelium.

Methods. Caco-2 cells were cultured on semi-permeable membrane filters into differentiated polarised monolayers. Monolayer differentiation was assessed by monitoring the transport of taurocholic acid. Cells at different stages of differentiation were transfected with DNA/DOTAP lipoplexes and later analysed for reporter gene activity. The uptake of radiolabled DNA was also evaluated at various stages of differentiation.

Results. Caco-2 cultures developed a resistance to lipoplex-mediated transfection as early as three days, when some cells were still dividing and undifferentiated. As cultures matured, expression of reporter gene progressively decreased partly due to reduced internalisation of DNA. The resistance to transfection could be overcome in part by pre-treatment of monolayers with calcium chelating agents or surfactants. However, transgene expression in treated monolayers was still significantly lower than that in dividing cultures.

Conclusions. Differentiated Caco-2 cells are a more appropriate model for gene-transfer studies to the intestinal epithelium because they demonstrate a resistance to transfection similar to that observed *in vivo*.

KEY WORDS: lipoplexes; cationic lipids; differentiated Caco-2 cells; transfection enhancers; gene delivery.

INTRODUCTION

Transfection complexes comprising DNA and either cationic lipids (1–3) or polymers (4,5) have been used successfully to transfer heterologous DNA to mammalian cells in culture. The gene transfer efficiencies of these delivery systems, with respect to the number of cells transfected and the level of transgene expressed, is very much dependent on the cell line of study and the conditions of transfection. When tested for *in vivo* efficacy, the extent and duration of gene expression has been disappointing (6). In order to identify gene delivery systems that are more efficient, a fundamental understanding of the process of gene uptake, delivery and expression within cells is necessary. The majority of studies evaluating these factors have used established cell lines often in phases of exponential growth and in an undifferentiated state. Irrespective of whether viral or non-viral gene delivery systems are used, it has been difficult to predict *in vivo* efficacy from such *in vitro* studies (7). Indeed, systems that had good activity *in vitro* were subsequently found to be inefficient *in vivo* (8,9). The poor correlation relates to the choice of cell culture model which has little functional and morphological similarity to the target cell *in vivo*. Although it is impossible to emulate identically the conditions *in vivo*, studies with differentiated cell cultures should serve as a compromise, making it possible to better understand the molecular mechanisms and membrane barriers that influence gene delivery

As a step towards this goal, we used the well characterised adenocarcinoma cell line, Caco-2, to study cationic lipid-mediated gene transfer. Under appropriate conditions of culture, Caco-2 cells grow to confluence and undergo differentiation, ultimately exhibiting morphological characteristics (10), brush border enzymes such as alkaline phosphatase (11) and drug metabolising enzymes found in gastric enterocytes (for review see Semenza (12)). In addition, they exhibit an apically polarised efflux system functionally similar to P-glycoprotein observed in multidrug resistant tumour cells (13). This cell line represents an excellent *in vitro* model of the small intestinal epithelium and has been used extensively for elucidating the mechanism of drug transport and predicting the intestinal absorption of drugs (14). Due to these properties, differentiated Caco-2 cells were selected as a model; the human origin of this cell line makes it ideal for evaluating the feasibility of gene therapy to the gastro-intestinal tract for conditions such as cancer and irritable bowel disease. Since the Caco-2 monolayers are non-dividing, they may represent good models for the development of gene delivery systems to other epithelia or alternative targets *in vivo*. Strategies to overcome the resistance to transfection observed, as a result of cell differentiation, are also reported.

MATERIALS AND METHODS

Materials

Lipofectin™, Lipofectamine™ (both from Life Technologies, Paisley, UK) and DOTAP™ (Boehringer Mannheim, East Sussex, UK) were used as supplied. Tfx-50™ (Promega, Madison, WI, USA) was re-hydrated as a 3.5 mg/ml solution in nuclease free water. Poly-lysine hydrobromide (M_r 55,000) and transferrin-polylysine (both from Sigma, Dorset, UK) were prepared as 2.5 mg/ml and 1 mg/ml solutions respectively, in HEPES buffered saline (HBS: 20 mM HEPES and 150 mM NaCl, pH 7.4). DOTAP and DOPE powder were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Reagents used for cell culture were obtained from Life Technologies and all other reagents were obtained from Sigma unless stated otherwise.

Preparation of Liposomes

Large multilamellar liposomes were prepared by the solvent phase evaporation method. Briefly, DOTAP liposomes were prepared by evaporating a stock solution of the lipid in chloroform and then placing the dried lipid under vacuum overnight to remove residual amounts of solvent. The lipid film was re-suspended in water at a concentration of 1 mg/

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ml followed by vortexing at full speed for 1 minute at room temperature. The lipid solution was allowed to sit for 30–60 minutes under ambient conditions before use in transfection experiments. DOTAP:DOPE 1:1 (mol:mol) liposomes were prepared similarly.

Plasmid Preparation

Plasmids pRSVlacZ (7.8 Kb), containing the β-galactosidase (β-gal) gene under the promotional control of the Rous sarcoma virus long terminal repeat (a gift from Dr Ogilvie, Zeneca Pharmaceutical, UK) and pCMVlacZ (7.2 Kb) also containing β-gal driven by the cytomegalovirus early promoter enhancer element (Clontech, Palo Alto, CA) were used in the gene transfer experiments. Both viral promoters were found to be equi-potent in Caco-2 cells. Plasmids were propagated in *E. coli* XL-1 blue, isolated by the alkaline lysis method and purified by anion-exchange chromatography (Qiagen, Dorking, UK). The quantity and quality of the purified DNA was assessed by absorption measurements at 260 nm and 280 nm as well as electrophoresis on a 1% (w/v) agarose gel.

Preparation of Radiolabeled (^{32}P) DNA

Plasmid DNA, pCMVlacZ, was end labelled with α-^{32}P-ATP. Briefly, pCMVlacZ, was linearised with EcoR V and then purified by ethanol precipitation. One μg (1 μl) of linear DNA was radiolabeled using Klenow (Boehringer Mannheim) for 30 minutes at room temperature. Un-incorporated α-^{32}P-ATP was removed by two rounds of ethanol precipitation and the labelled DNA was re-dissolved in TE buffer (Tris 10mM, EDTA 1 mM), pH 8.

Preparation of Transfection Complexes

Plasmid DNA (6.4 μg) was diluted with HBS to a volume of 800 μl in a Falcon tube (Becton Dickinson Labware, Lincoln Park, NJ, USA). The appropriate quantity of gene delivery agent was separately diluted to 800 μl and then added to the DNA solution followed by gentle mixing with a pipette (final DNA concentration of 4 μg/ml). Complexes were formulated at a DNA:lipid/peptide mass ratio of 1:0.6 for pLL; 1:3 for TfpL; 1:9 for Lipofectin; 1:6 for DOTAP; 1:6 for Tfx-50 and 1:5 for Lipofectamine. After mixing, samples were incubated for 15–30 minutes before use in transfection experiments.

Particle Size Measurement

The sizes of DOTAP liposomes and lipoplexes in water were measured by laser dynamic light scattering using a Malvern PCS 4700 (Malvern, UK) equipped with a 100 mW laser.

Cell Culture

Caco-2 cells were maintained in MEM supplemented with foetal calf serum (20% v/v heat-inactivated), nonessential amino acids (1% v/v), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Transfection of Dividing Cell Cultures

Caco-2 cells were seeded at a density of 4×10^5 cells per 35 mm diameter well and incubated for 16–20 hours at 37°C. Immediately before transfection, cells were rinsed once with OptiMEM (Life Technologies) and then 1.5 ml of this medium was added to each well. A volume of 500 μl of transfection complex containing 2 μg of plasmid DNA complexed with either 1.2 μg pLL, 6 μg TfpL, 19.4 μg Lipofectin, 11.3 μg DOTAP™, 12.6 μg Tfx-50 or 10 μg Lipofectamine was added to each well. Cells were then incubated at 37°C for 4 hours. When either pLL or TfpL were used, transfection was carried out in the presence of 100 μM chloroquine. After four hours, the transfection medium was removed and replaced with 2 ml of complete culture medium. Incubation was continued for a further 44 hours and then cells were harvested for analysis of β-galactosidase activity.

Culture of Differentiated Caco-2 Cells

To enable differentiation, cells were seeded at a density of 3×10^5 cells per 24 mm diameter collagen coated PTFE filter with a mean pore size of 0.4 μm (Transwell-COL™, Costar, Cambridge, WI, USA). Culture inserts were placed in 6-well plates where 1.5 ml and 2.6 ml of cell culture medium was added to the apical and basolateral compartments, respectively. The cells were fed every second day and allowed to grow and differentiate for up to 30 days.

Use of Polarised Taurocholate Transport to Validate the Filter-Culture Model

For transport studies, 0.05 μCi of ^{14}C taurocholic acid (NEN Life Science Products) was added to either the apical or basolateral chamber. Samples were removed from each chamber after a 1h incubation period at 37°C and analysed by liquid scintillation counting. This allowed the apparent permeability of the monolayers in either direction over the 1h period to be calculated. ^{3}H mannitol (NEN Life Science Products) was used to validate the existence of an intact monolayer.

Transfection of Differentiated Caco-2 Cells

Caco-2 cell monolayers were first rinsed with OptiMEM on both apical and basolateral surfaces and 1.5 ml of transfection complex (2–6 μg DNA) was added to the apical compartment whilst the basolateral surface was bathed in 2.6ml OptiMEM. Cells were then transfected as previously described.

Transfection Enhancer Studies

Caco-2 cell monolayers were washed 5 times with phosphate buffered saline (PBS) and then a 1.5 ml volume of a solution of either EGTA (2.5 mM), polysorbate 80 (0.05%w/v), taurocholic acid (1 mM), taurodeoxycholic acid (1.2 mM) or PBS was added to the apical compartment. Monolayers were incubated for 30 minutes at 37°C after which time the monolayer was rinsed twice with PBS before transfecting as previously described.

Cellular Uptake of Radiolabeled DNA

Unlabelled pCMVlacZ (165 μg) was mixed with 2 μg of ^{32}P-pCMVlacZ, complexed with DOTAP^{MLV} liposomes and the radioactivity of the solution measured after incubating for 15 minutes. Where appropriate, cells were pre-treated with 0.05%

w/v polysorbate 80 and then transfected from the apical surface as described previously. Cells were allowed to incubate for 4 hours at 37°C after which time monolayers were washed twice with PBS, overlaid with lysis buffer (0.1% Triton X-100, 250 mM Tris, pH 8) and subjected to two freeze-thaw cycles. The cells were scraped and the lysate collected and centrifuged for 5 minutes at 1300 rpm. The radioactivity in the supernatant was measured on a β counter (1215 Rackbeta, Wallac, UK) and the results normalised to total protein. Data represent the mean of triplicate samples ± SEM.

Cell Harvesting and Analysis of β-Galactosidase Activity

Cell monolayers were washed twice with PBS, overlaid with lysis buffer and frozen at −70°C. After thawing, the cell lysate was centrifuged for 5 minutes at 13000 rpm to pellet cell debris. β-galactosidase activity in the soluble cell fraction was measured using either σ-nitro-phenyl-β-D-galactopyranoside or methyl-umbelliferyl-β-D-galactopyranoside. Purified *E. coli* β-galactosidase was used to generate calibration curves. Each cell lysate was also analysed for total protein (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard. All data are presented as β-galactosidase activity per milligram protein of the mean of triplicate samples ± SEM.

RESULTS

Transfection of Caco-2 Cells During Exponential Growth Phase

In preliminary experiments, exponentially growing Caco-2 cells were transfected with several commercially available cationic lipids and synthetic polypeptides. When cells were treated with either pLL or TfpL polyplexes, there was no detectable β-galactosidase activity in the cell extracts despite the use of 100 μM chloroquine (Figure 1). We used chloroquine because it buffers endosomal pH and promotes escape of DNA into the cytoplasm. This effect is probably due to the rupture of the endosomal membrane caused by an increased osmotic pressure. The fact that neither pLL nor TfpL showed transfection activity here may indicate that the concentration of chloroquine used was not sufficient to exert an endosomolytic effect in Caco-2 cells; higher concentrations were inappropriate as

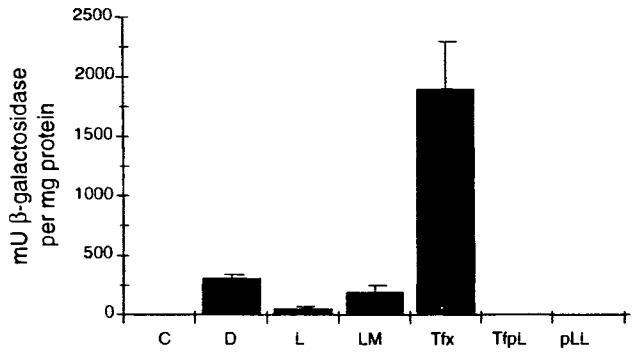

Fig. 1. Comparative transfection studies in dividing Caco-2 cells. Caco-2 cells in exponential growth phase were transfected with pRSVlacZ complexed with either DOTAP (D), Lipofectin (L), Lipofectamine (LM), Tfx-50 (Tfx), Transferrin-polylysine (TfpL) or polylysine$_{267}$ (pLL). C represents control cells transfected with naked DNA.

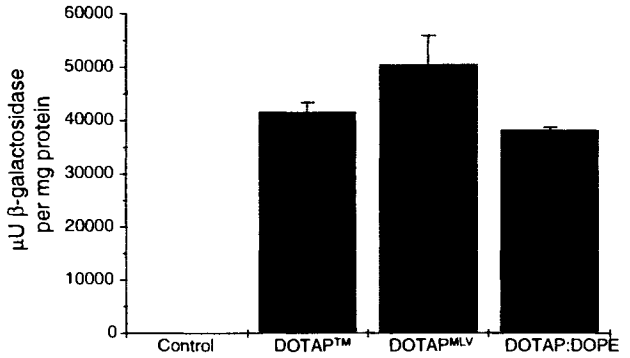

Fig. 2. Effect of DOTAP formulation on transfection of Caco-2 cells. One day old filter cultures of Caco-2 cells were transfected with pRSVlacZ complexed with either DOTAP™, DOTAP^{MLV} or DOTAP:DOPE^{MLV}.

they are associated with chloroquine-induced cellular toxicity. All of the cationic lipids tested were effective in achieving transgene expression but with differing efficacy. Tfx-50 demonstrated the highest transfection activity with a specific β-galactosidase activity 4, 5 and 7 fold higher than DOTAP, Lipofectamine and Lipofectin, respectively. We concluded that any of these materials would be suitable models for the study in filter-cultured cells. Though Tfx-50 appeared to be the most efficient, DOTAP was selected for use in subsequent studies because formulations could be prepared at a fraction of the cost of the other materials.

The transfection efficiencies of three DOTAP formulations were evaluated; commercially supplied liposomes, DOTAP™, and large multilamellar liposomes prepared using either DOTAP (DOTAP^{MLV}) or a 50:50 molar mixture of DOTAP:DOPE. The comparative studies were carried out at a net positive charge of 2.4. Figure 2 shows that all three formulations were equivalent in terms of transfection activity. Co-formulation of DOTAP with the neutral lipid, DOPE, did not influence transfection activity. For the filter-culture studies, the most cost-effective formulation was selected; DNA/DOTAP^{MLV} lipoplexes.

The sizes of DOTAP™ and DOTAP^{MLV} liposomes and lipoplexes formed with plasmid DNA were measured by dynamic light scattering. Table 1 shows that DOTAP™ liposomes had a mean diameter of 181 nm compared with 253 nm for DOTAP^{MLV}. Following complexation with DNA, DOTAP™ lipoplexes were found to be significantly larger than DOTAP^{MLV} lipoplexes and well in excess of 400 nm. DOTAP^{MLV} lipoplexes, in contrast, were not substantially bigger than the uncomplexed liposome (292 nm compared to 253 nm).

Table 1. The Size of DOTAP™ and DOTAP^{MLV} Liposomes and Lipoplexes

Sample	Size of liposome (nm)	Size of lipoplex (nm)
DOTAP™	181 ± 16	424 ± 14
DOTAP^{MLV}	253 ± 28	292 ± 4

Note: Data represent the mean ± SEM.

Effect of Differentiation on Transgene Expression and DNA Uptake

The intestinal epithelial transport system for taurocholic acid was used to validate the differentiation of Caco-2 cultures. Figure 3 compares the flux of taurocholate (TC) across the monolayer in either direction as a function of age. As the monolayers polarised and began to express transporters for TC, the rate of apical-to-basolateral transport increased. Transport in the opposite direction decreased, presumably due to the generation of tighter junctions between cells in filter culture. By day 8 the transport of TC was twice as great in the apical-to-basolateral direction, and by day 16 the selectivity had increased, so that apical-to-basolateral transport was 26.2 times faster than basolateral-to-apical transport. Mannitol flux or electrical reistance measurements indicated that in common with other reports our cultures did not change significantly over the 16–28 day period.

To evaluate the effects of differentiation on transfection, cells at different ages were transfected with DNA/DOTAP lipoplexes. Figure 4a shows cells at 1, 10 or 30 days treated with either naked DNA or with DNA/DOTAP lipoplexes. The specific β-galactosidase activity in 1 day old filter-cultures transfected with DNA/DOTAP was five-fold higher than control cells transfected with naked DNA. In contrast, there was no significant difference in 10 and 30 day cultures transfected with either naked DNA or DNA/DOTAP lipoplexes. The specific β-galactosidase activity in 1 day old cultures transfected with DNA/DOTAP was 36 and 53 fold higher than 10 and 30 day cells, respectively. In a similar experiment, cultures were examined at more frequent time points (1, 3, 6, 13, 21 and 27 days; Fig. 4b). Again, only 1 day old cells demonstrated appreciable levels of β-galactosidase expression following transfection. Surprisingly at 3 days, when some of the cells were expected to be dividing, as confluence throughout the area of the filter had not been established, the specific β-galactosidase activity was seven-fold lower than observed in 1 day old cells. Hence, due to this early change in the transfection behaviour, experiments were carried out with monolayers between days 7 and 30.

To investigate whether the resistance to transfection observed in differentiating Caco-2 cells was a consequence of

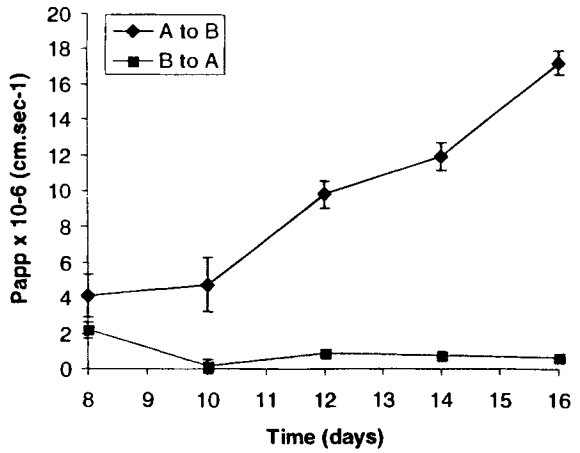

Fig. 3. Transport of radiolabelled taurocholate across filter-cultures of Caco-2 cells, in both the apical-to-basolateral (AB) and basolateral-to-apical (BA) directions, as a function of time in culture. The figure shows mean permeability coefficients (Papp) ± S.D.

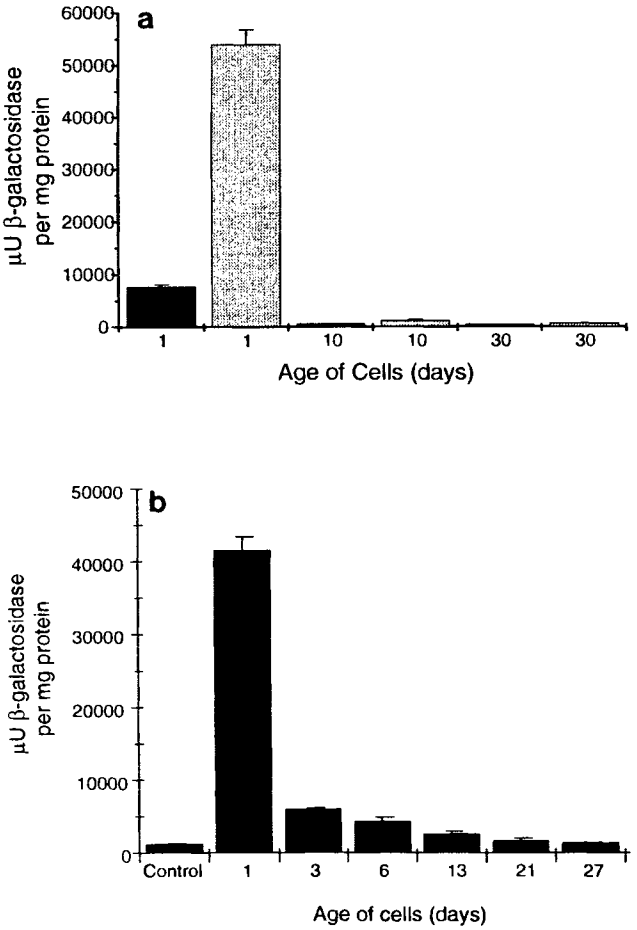

Fig. 4. Effect of differentiation of Caco-2 cells on β-galactosidase expression. Filter cultures of cells at (a) 1, 10 and 30 days transfected with either naked DNA (solid bars) or DNA/DOTAP (hatched bars) and (b) 1, 3, 6, 13, 21 and 27 days transfected with DNA/DOTAP. Control represents cells at 1 day in culture transfected with naked DNA.

reduced internalisation, the uptake of radiolabelled DNA over a 4 hour incubation period was studied. Figure 5 shows that the uptake of naked DNA was negligible in all cells irrespective of age. However, on complexation with DOTAP, cellular uptake of DNA was significantly increased, consistent with the proposed mechanism of action of cationic lipid-mediated gene delivery. Hence, in 1 day old cells, 10% of the input dose of DNA was recovered in cell extracts compared with only 3% and 2% in 12 and 15 day old cells, respectively. These data clearly demonstrate that a significant factor affecting transfection in differentiating Caco-2 cells is inefficient internalisation of DNA delivery systems.

Effect of Membrane Disrupting Agents on Gene Transfer

The effect of four membrane disrupting agents were evaluated for their ability to influence transfection in the Caco-2 cell culture model. The agents selected have been reported to enhance the absorption of hydrophilic drugs across the Caco-2 monolayer.(15,16) Ethylene glycol-bis-β-aminoethyl ether (EGTA), polysorbate 80 (Tween 80), taurocholic acid and taurodeoxycholic acid were used at concentrations expected to be

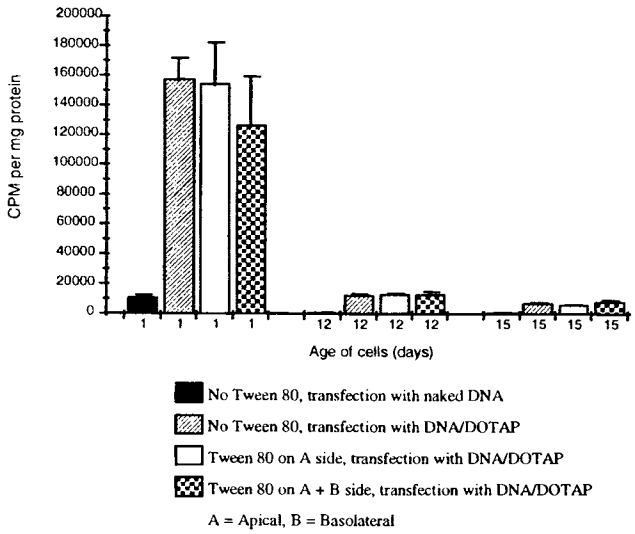

Fig. 5. Uptake of ^{32}P-radiolabeled DNA in to Caco-2 cells. Caco-2 cells at 1, 12 or 15 days in culture were transfected with ^{32}P-DNA/DOTAP^{MLV} lipoplexes and after 4 hours, the amount of radioactivity in the soluble cell fraction was measured. Control cells were transfected with naked DNA.

non-toxic and reversible. The specific β-galactosidase activity in monolayers treated with membrane-active agents was significantly higher than untreated controls (Fig. 6). The specific β-galactosidase activities were 4444, 2780, 2960 and 2691 μU/mg for EGTA, polysorbate 80, taurocholic acid and taurodeoxycholic acid, respectively, versus an apparent mean activity of 835 μU/mg for control cultures. In repeat experiments, the precise level of transfection enhancement was variable but was always 3–10 fold higher than observed in untreated controls.

To investigate the mechanism by which transfection activity was enhanced, we determined the uptake of radiolabelled DNA following treatment with polysorbate 80. Some monolayers were pre-treated either on the apical surface or on both apical and basolateral surfaces. The results in Fig. 5 demonstrate that in neither dividing nor quiescent cells did polysorbate 80 increase in the amount of radiolabelled material recovered in cell extracts.

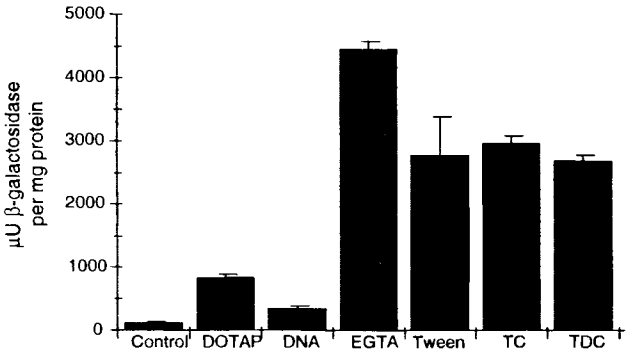

Fig. 6. Effect of transfection enhancers. Fourteen day old Caco-2 cells were incubated with EGTA, Tween 80, taurocholic acid (TC) or taurodeoxycholic acid (TDC) for 30 minutes prior to transfection with DNA/DOTAP lipoplexes. Control (untransfected cells), DNA (naked DNA) and DOTAP (DNA/DOTAP lipoplexes without pre-treatment with enhancer).

DISCUSSION

The Caco-2 filter-culture system is now well established and used extensively within the pharmaceutical industry as a screening tool for predicting the oral absorption characteristics of new drugs. Despite their colonic origin, differentiated Caco-2 cells have a phenotype which is closely related to the epithelium of the small intestines. Given the degradative environment within the lumen of the gut it is unlikely that gene therapy would be targeted at the apical surface of the gut epithelium. However, since there is considerable interest in treating lung diseases with gene therapy, we propose that the highly consistent Caco-2 model will present a similar resistance to the apical surface of the airway epithelium. The key advantage of using the Caco-2 monolayer is its ability to easily form a tight differentiated monolayer. Although lung epithelial cell lines can be cultured under conditions that permit differentiation, these monolayers are poorly characterised compared with Caco-2 and their culture is more labour-intensive.

One problem we observed with the Caco-2 model was a relatively high background level of galactosidase activity. Various brush border glycosidases are expressed by Caco-2 cells upon differentiation, such as sucrase-isomaltase and lactase, which have a potential to interfere with the analysis of reporter gene activity. Presumably these enzymes can hydrolyse β-galactosides, including the substrates used in this work. We suggest that in future luciferase, chloramphenicol acetyl transferase or green fluorescent protein may serve as better reporter genes.

Exponentially growing Caco-2 cells could be transfected readily with a variety of cationic lipids but were resistant to transfection with either pLL or TfpL. Our results with polyplexes are consistent with reports by Hart *et al.* (17) who observed that Caco-2 cells could only be transfected when pLL was conjugated to a cyclic-RGD peptide, enabling gene delivery via integrins. Experiments on differentiating cells showed that there was a resistance to transfection from as early as 3 days in culture (the earliest time point studied). What is remarkable about this finding is that at this stage, monolayers were not confluent and cells did not exhibit morphological markers of differentiation. At the density at which cells were seeded on filters, full differentiation of cultures would not be expected to occur until day 14. This suggest that from a very early stage, even before the morphological characteristics of differentiation become apparent, other biochemical and/or biophysical processes were occurring, sufficiently strong to make the process of gene uptake and/or expression inefficient. Similar observations have been reported in respiratory epithelial cells.(18,19) Fasbender *et al.* (18) observed a lower susceptibility to cationic lipid-mediated gene transfer in human lung epithelial cells at 24 hours compared with 6 hours after seeding and this correlated with a lower level of cell-associated transfection complexes and an increased trans-epithelial resistance of the monolayers. Matsui *et al.* (19) subsequently demonstrated that the increased resistance to transfection in differentiated cells was partly due to reduced negative surface charge of cell membranes. It is possible that a change in surface characteristics is a phenomenon operative in differentiating epithelial cells. This therefore represents a plausible explanation for our observations, whereby a lower level of cell associated cationic transfection complexes could result in a lower efficiency of transfection. With specific reference to Caco-2 cells, it is noteworthy that poor transfection

efficiency in differentiated cultures has also been observed with adenovirus (20).

Factors other than reduced internalisation of DNA must have played a contributory role in the resistance of differentiated cultures to transfection. For instance, the rate of cell proliferation has been found to be important for successful gene delivery, (21–23) probably because it dictates nuclear accessibility (a significant barrier for cationic lipid-mediated gene delivery (24)) of the plasmid following internalisation. Indeed, Wilke *et al.* (21) demonstrated that mitotic activity was a pre-requisite to efficient transfection probably due to the temporarily discontinuation of the nuclear membrane. From a mechanical perspective, because the apical surface of differentiated Caco-2 cells is dominated by microvilli (estimated diameter of 50 nm), the delivery of transfection complexes in excess of 30–40 nm is unlikely to be efficient.

Whilst we observed a 5-fold lower level of internalised DNA in day 10 cells compared with cells at day 1, this subsequently translated to a 36-fold difference in transfection activity. This disparity may be due to factors unrelated to gene delivery such as differences in the rates of transcription and translation and the cytoplasmic stability of the protein following synthesis. It is conceivable that these may differ between cycling-undifferentiated and quiescent-differentiated cells.

In an attempt to overcome the observed resistance to transfection in differentiating cells, we used agents known to enhance the absorption of hydrophilic drugs (15,16). The rationale for the use of a calcium chelator is that the formation of the intercellular junctional complex, is a calcium dependent mechanism which can be reversibly inhibited by treatment with EGTA, a specific chelator of Ca^{++} ions. Exposure of cells to EGTA for less than 45 minutes results in a reversible opening of tight junctions without inducing cellular toxicity (15). Following such treatment, a 5-fold enhancement in specific β-galactosidase activity was observed. It is not clear whether the effects of EGTA were due entirely to the opening of tight junctions, although this in itself would serve to increase the total cell surface area available for interaction with transfection complexes. It is also possible that the opening of the tight junctions enables access of complexes to the basolateral surface of the Caco-2 cells from which endocytosis may then occur.

The effects of two types of surfactants on the Caco-2 cell culture model were tested; polysorbate 80, a non-ionic surfactant and two bile acids; taurocholic and taurodeoxycholic acid. At appropriate concentrations, surfactants have been shown to increase the fluidity of membranes by integrating within the phospholipid bilayers (25) although high concentrations cause irreversible cell damage (26). Polysorbate 80, for instance, integrates within cell membranes, altering their microviscosity (27). Whilst an increase in membrane fluidity might be expected to favour endocytosis, (conversely, increased membrane rigidity when cells are incubated at 4°C must account, in part, for the corresponding inhibition of endocytosis), studies on DNA uptake into differentiated cells indicated that the beneficial effects of polysorbate 80 were not due to an increased rate of DNA internalisation. It is possible that increased fluidity of cell and hence endosomal membranes, may facilitate disruption by the lipidic transfection complexes.

The short exposure (30 minutes) of Caco-2 monolayers to calcium chelators and surfactants did not appear to cause toxicity; there was no vacuolisation of cells or detachment from filter supports after treatment. In addition the uptake of naked DNA was minimal. However, more sensitive techniques would be needed to confirm that these reagents are indeed innocuous.

In conclusion, differentiated cell cultures should serve as a better platform for routine screening of new transfection reagent and in addition represent a better model for studying and understanding the molecular mechanisms that govern gene delivery. The Caco-2 model is unique in that it is a human cell line that can be relied upon to generate differentiated monolayers routinely. Whether it will be possible to extrapolate data obtained from such systems to *in vivo* still remains to be determined. The fact that several groups have now demonstrated that differentiated cells do not undergo an appreciable extent of endocytosis probably also holds true for many *in vivo* target organs. This knowledge should aid in the design of gene delivery vectors that function by an alternative pathway.

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