Mechanistic Studies on Nonviral Gene Delivery to the Intestine Using *in Vitro* Differentiated Cell Culture Models and an *in Vivo* Rat Intestinal Loop

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Purpose. To identify factors influencing nonviral vector transfection in differentiated CaCo-2 and mucus-secreting coculture, CaCo-2:Ht29GlucH, cell culture models and to compare these *in vitro* results with *in vivo* transfection efficiency in rat intestine.

Methods. A range of nonviral vectors including DOTAP, Lipofectin, Superfect, PEI, and polylysine were investigated. CaCo-2 and a mucus-secreting coculture were used at 21 days. Transfection efficiency was assessed using pCMV*luc* (firefly luciferase) plasmid, and radiolabeled plasmid was used to determine the binding and internalization of plasmid DNA. The *in vivo* model used was a ligated rat intestinal loop.

Results. Transfection levels decreased by over 1000-fold in differentiated models relative to nondifferentiated COS-7 cells and were related to reductions in luciferase production by individual cells. Active internalization of DNA by the differentiated cells decreased. Removal of mucus by the mucolytic agent *N*-acetylcysteine, from the coculture system significantly reduced ($p < 0.05$) transfection efficiency. *In vivo* the transfection efficiency of PEI proved superior to DOTAP™.

Conclusions. Nonviral gene delivery to the hostile environment of the intestine is possible. Mechanistic studies using differentiated intestinal cell models aid identification of the rate-limiting steps to transfection and represent a more physiologically relevant approach to predict gene delivery to the intestine.

KEY WORDS: CaCo-2 cells; CaCo-2:Ht29GlucH coculture; mucus; intestinal gene delivery; nonviral vectors.

INTRODUCTION

Gene delivery to the intestinal epithelium has many potential advantages including ease of access, which would facilitate direct *in vivo* gene transfer by oral self-administration or standard endoscopic techniques (1). From a therapeutic aspect there are many disease targets in the intestine including inflammatory bowel disease (2), cystic fibrosis, colon cancer, and transporter deficiencies. In many cases local delivery and transfection may be adequate, thus avoiding the difficulties encountered on delivery through the vasculature including interactions with plasma proteins, aggregation, and poor extravasation. In addition, the intestine could be used as a site for genetic immunization or for the production and secretion of therapeutic proteins into the bloodstream to act at some distal site (3).

A number of studies have already confirmed that the intestine can be transfected (4,5). Retroviral (6–8) and adenoviral (2) vectors have been used successfully to transfect the intestine *in vivo*. MacLaughlin *et al.* (9), using chitosan as a nonviral vector, reported limited *in vivo* transfection in rabbit intestine; however, the results were very variable (9). If the oral route is to be a viable alternative, the factors influencing transfection in the intestine need to be investigated.

In this study fully differentiated intestinal cell culture models, including CaCo-2 and a CaCo-2:Ht29GlucH coculture, are used to investigate factors influencing transfection efficacy of a full range of lipid, polymeric, and peptide nonviral vectors. These models are more physiologically challenging compared to nondifferentiated models frequently used in gene delivery studies. They facilitate study of the mechanism of uptake and the pattern of transfection in differentiated cells. The CaCo-2 model has previously been used to screen nonviral vectors (10,11).

In addition to the barriers presented by the target cells, extracellular conditions *in vivo* such as the presence of mucus may influence the transfection efficiency of a given gene delivery vector (GDV) in the lung (12) and intestine. The mucus-secreting CaCo-2:Ht29GlucH coculture (13) was used to determine the effect of mucus on the transfection ability of the most efficient vectors. This model also allowed examination of the effect of mixed cell types on transfection levels; this more closely resembles the *in vivo* intestinal situation, where several cell types are present.

Finally, the ability of these cell culture models to predict *in vivo* transfection was assessed by comparison of the results to those obtained in an *in vivo* rat intestinal loop (2).

MATERIALS AND METHODS

Materials

DOTAP™ (*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammoniummethylsulfate) (Roche), Lipofectin™ (*N*-[1-(2,3-dioloeyloxy)propyl]-*N,N,N*-trimethylammonium chloride) (Gibco BRL), and Superfect™ Transfection Reagent (Qiagen) were used as supplied by the manufacturers. Poly-L-lysine hydrobromide (30,000–70,000 Da) was supplied by Sigma, UK and reconstituted in HEPES-buffered saline (HBS: 20 mM HEPES and 150 mM NaCl, pH 7.4) to produce a 2.5 mg/ml solution. Polyethylenimine (PEI), 25 kDa, was made up in 1 M NaCl–50 mM HEPES/HCl, pH 7, and dialyzed in tubing (with a molecular weight cutoff of 6000 to 8000) for 2 days with frequent changes of dialysate, before being lyophilized overnight. The PEI was reconstituted with nuclease-free water at a concentration of 10 mM. Cell culture reagents were obtained from Gibco BRL, and all other reagents were sourced from Sigma unless otherwise stated.

Method

Cell Line and Cell Culture

CaCo-2, passage 30–50, and COS-7 cells, passage 9–40 (ECACC, UK), were cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal calf serum (10% v/v), L-glutamine (1% w/v), gentamicin (100 mg/ml), and sodium pyruvate (1 mM). Ht29GlucH (a generous gift

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from Dr. Daniel Louvard, Pasteur Institute, Paris) were cultured, passage 12–32, in medium that was further supplemented with 5 μg/ml of transferrin. All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. COS-7 cells were seeded in six-well plates (35 mm diameter) at a density of 5.2×10^4 cells/cm^2 24 h before transfection. In order to allow differentiation, CaCo-2 cells are seeded on 24-mm-diameter, 0.4-μm pore size, polycarbonate filters, Transwells™, at a density of 63,000 cells/cm^2. The cocultures were seeded at a density of 4.25×10^5 cells/cm^2 onto collagen-coated Transwells™ at a ratio of CaCo-2:Ht29GlucH of 3:1 and maintained in transferrin-supplemented medium for 21 days. Ht29 cells were also seeded on collagen-coated Transwells™ at a density of 8.5×10^5 cells/cm^2. For the filter culture systems medium is added to the apical (1.5 ml) and basolateral (2.6 ml) chambers every 48 h for the first 7 days and then daily until day 19–21. These differentiated cultures were used between days 19 and 23 unless otherwise stated. Transepithelial electrical resistance values (TEERs) were measured using a WPI voltmeter/ohmmeter fitted with "chopstick" electrodes.

Plasmid

The pCMV*luc* plasmid contains the firefly luciferase gene under the control of the cytomegalovirus (CMV) enhancer/promoter. This pCMV*luc* plasmid was maintained and propagated in the *E. coli* strain DH5α. It was isolated by alkaline lysis and purified using anion-exchange chromatography using the Plasmid Maxi Kit (Qiagen Inc., USA) in accordance with the manufacturer's instructions and stored in TE buffer (Tris 10 mM, EDTA 1 mM), pH 8 at –20°C. Purity of the plasmid was assessed using 1.0% w/v agarose gel electrophoresis, and its concentration was determined from the absorbance at 260 nm according to established protocols (14).

Preparation of Radiolabeled [^{32}P]DNA

Plasmid DNA was end-labeled with [α-^{32}P]dCTP, as previously described (10). Briefly, the linearized (using the HindIII restriction enzyme) pCMV*luc* plasmid (25 ng) was denatured and mixed with 4 ml of High Prime Solution (Boehringer Mannheim) and 5 ml of [α-^{32}P]dCTP (50 mCi) on ice and reacted for 10 min at 37°C. Unincorporated [α-^{32}P]dCTP was removed using a freshly prepared Sepharose column.

Transfection Protocol

Before transfection the cells were rinsed twice with serum-free medium and then incubated in serum-free medium for up to 1 h (when pLL was used, chloroquine was added 10 min before transfection to give a final concentration of 100 μM). The complexes for transfection were formed by adding an equal volume of vector at the required concentration in HBSS to plasmid DNA (2 μg/well for COS-7 and 6 μg/well for filter cultures) made up in the same diluent. The optimum ratio of vector:DNA was determined for each system in each of the cell lines. Transfection complexes were left to complex for 30 min before being added to the cells. After 4 h of incubation, the transfection medium was removed, and full serum-containing medium was added to the cells. Incubation at 37°C continued for a further 20 h, at which time the medium was removed, and the wells were washed with PBS twice.

In order to determine the level of reporter gene expression, the cells were lysed using lysis buffer (Promega, USA) and a freeze–thaw cycle. The lysate was assayed using a luciferase assay kit (Promega, USA) according to manufacturer's instructions in a luminometer TD 20/20 (Promega). The relative light units (RLU) were compared against a calibration curve constructed using standard luciferase (Promega), and the results were expressed as RLU of luciferase/mg protein (Bio-Rad protein assay kit).

Removal of Mucus

N-Acetylcysteine (N-AC) (0.3% w/v) in HBSS was added to the apical chamber of the filter culture systems for 30 min before transfection, at which point it was removed, and the cells rinsed twice, before the transfection medium was added.

MTT Assay

COS-7 and CaCo-2 cells were seeded at densities of 65,000 cells and 35,000 cells per well, respectively, onto 96-well plates and cultured for 24–48 h in DMEM until they reached confluency. Subsequently, 100 μl of the transfection complexes in HBSS was added to the wells and incubated for 10 min at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (20 μl) was then added to each well, and the cells were incubated for a further 4 h at 37°C. The reaction product was then solubilized with 100 μl of sodium dodecyl sulfate (10% w/v) in 0.01 N HCl overnight before the reaction product was quantified using a Dynatec plate reader at 590 nm and compared with control cells.

Confocal Studies

Following transfection the cells were fixed with paraformaldehyde (3% w/v) and stained, first with a primary rabbit (1 in 300 dilution with 5% w/v BSA, 15 mM sodium azide in PBS from a stock of 50 μg/ml), antiluciferase (firefly) antibody, affinity purified (Europa Bioproducts Ltd.), and then with a fluorescently labeled secondary anti-IgG antibody (1 in 100 dilution with 5% w/v BSA, 15 mM sodium azide in PBS from a stock of 0.75 mg/ml), fluorescein isothiocyanate (FITC)-conjugated AffiniPure donkey antirabbit IgG (H+L) (Jackson ImmunoResearch). By counterstaining the cell walls with DHPE Texas Red® (Molecular Probes) individual cell transgene expression could be determined. The cells were mounted in DABCO stored at 4°C and viewed within one week using a BioRad Confocal Scanning Laser Microscope, Model MRC1024.

Cell Association and Internalization of Radiolabeled DNA

Transfection complexes were made up as for transfection, except that radiolabeled DNA was mixed with pCMV*luc* plasmid, to give approximately 40,000 CPM/μg DNA. The cells were rinsed twice and then incubated with Opti-MEM (unless otherwise stated) before studies. The complexes were added to the cells (to give a final concentration of 2 μg/well and 6 μg/well plasmid DNA for COS-7 cells and filter cultures, respectively) and were left in contact with the cells at 37°C (except in the case of experiments specifically carried out at 4°C) for 4 h. After this time, the transfection medium

was removed, and the cells were rinsed with PBS twice, lysis buffer was added, and the cells were freeze-thawed. The cells were scraped and centrifuged at 10,000 rpm for 10 min. The radioactivity of the supernatant was measured in disintegrations per minute (dpm) on a Tri-Carb 2100 series liquid scintillation counter and normalized to total protein. At 37°C the radioactivity represents the sum of plasmid bound and internalized. At 4°C, where all active processes are halted, the radioactivity represents bound plasmid only; by subtraction, the degree of active cellular uptake of plasmid can be quantified.

Animal Model

All animal experiments were done at Trinity College Dublin in association with the BioResources Unit, which is registered with the Deparment of Health, the competent authority designated under EU directive 86/609, as implemented in Ireland by Statutary Instrument No. 17/94. The facility is under the full-time direction of a veterinary surgeon who maintains the health and welfare program. The provision of animal care is in compliance with appendix A of the Directive.

Male Wistar rats (250–350g) were fasted overnight and anesthetized using Sagatal (pentobarbital) delivered intraperitoneally (IP). All surgery was performed on a hot plate using aseptic techniques. A ¾–inch midline incision was made in the anterior abdominal wall of the anesthetized rat, and an appropriate segment of the jejunum was located and carefully brought to the surface of the cavity. A loop approximately 2 cm in length was ligated with Vascustatt™ Maxiim vascular ties and secured with thread. Care was taken to ensure that viability of normal blood supply and peristalsis were maintained. The appropriate vector/DNA complex (in 2 ml of fluid) was injected into the proximal end of the loop using a 30-gauge needle affixed to a 2-ml syringe. The loop remained outside the peritoneum for 3.5 h, and the rats were maintained under anesthetic. After 3.5 h, the ligation was released, and the incision was stitched. The animal was allowed to recover and was given Temgesic™ (0.01 ml) for analgesia. The rats were provided with water and heat overnight. After 24 h the rats were euthanized using carbon dioxide. The treated intestinal segment was extracted and rinsed thoroughly with ice-cold PBS. It was then frozen rapidly using liquid nitrogen and crushed using a precooled pestle and mortar. The frozen segment was then homogenized in 2 ml Lysis buffer on ice. The homogeneous slurry was incubated in ice for 15 min and then mixed. The sample was then centrifuged at 15,000 rpm for 5 min at 4°C. The cleared supernatant was then transferred to a clean microcentrifuge tube. Twenty microliters of supernatant was then assayed for luciferase using the Promega Luciferase Assay Kit and corrected for protein content using the Biorad DC Protein Assay Kit.

Complexes were prepared by mixing plasmid DNA with PEI [nitrogen/phosphate (N/P) ratio 10] or with DOTAP™ [charge ratio (CR) +/– 2.4] in HBSS to 2 ml final volume.

Statistics

Unpaired Student's *t* test (two-tailed) was used to test the significance of the difference between two means.

RESULTS

Transfection of Nondifferentiated and Differentiated Cell Culture Models

The degree of transfection produced (under optimized conditions) by each vector system in each of the cell culture models expressed as RLU/mg protein/μg pDNA (corrected for both protein and the quantity of plasmid DNA (pDNA) added] is shown in Fig. 1. The level of expression of the reporter gene produced by the range of vectors tested in COS-7 cells (day 1) was higher for all the systems compared to the three differentiated models, CaCo-2, Ht29GlucH, and coculture monolayers (day 21). In COS-7 cells that are proliferating, the best of the vector/DNA systems produced transfection with an order of magnitude of 10^7 RLU/mg protein, whereas the maximum level in the differentiated monolayers was approximately 10^4 RLU/mg protein. These figures demonstrate that in undifferentiated cells, microgram quantities of luciferase protein are expressed per well; in contrast, in the differentiated monolayers, only nanograms of luciferase are expressed by the best of the vector systems.

In order to investigate further the progressive effects of differentiation on transfection, CaCo-2 and coculture cells were transfected at day 1, 5, 10, 15, and 21 using DOTAP™/DNA complexes, charge ratio (CR +/–) 2.4. The level of luciferase expression at these different stages of differentiation is shown in Fig. 2. In the case of CaCo-2 cells the highest level of expression, similar to that seen previously with COS-7 cells, was produced in 1-day-old cells and decreased progressively as the cells aged. By day 10, when the CaCo-2 cells have reached confluency, the specific activity had dropped almost 30-fold. When the cells are deemed fully differentiated at day 15, the levels have dropped 100-fold. This drop continues, and at day 21 the level of transgene expression is 170-fold lower than that on day 1. Similarly for the coculture CaCo-2: Ht29GlucH monolayers, the highest level of luciferase expression was produced in 1-day-old cells and decreased progressively as the cells aged. At day 5, 10, 15, and 21 the level of expression dropped 10-, 16-, 25-, and 300-fold, respectively. The TEER values were monitored for CaCo-2 monolayers throughout the experiments (data not shown). As the TEER

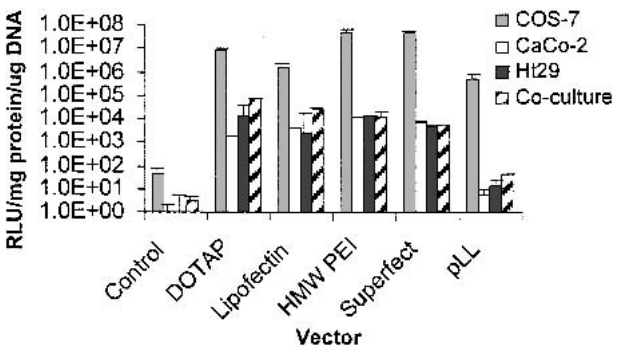

Fig. 1. Transfection efficiency (RLU/mg protein/μg pDNA) of different vector:DNA systems in nondifferentiated (COS-7) and differentiated (CaCo-2, Ht29GlucH, coculture) cell culture models. The systems were made up at the optimum ratios (vector:pDNA) for each vector in a given model. Control represents transfection with naked DNA.

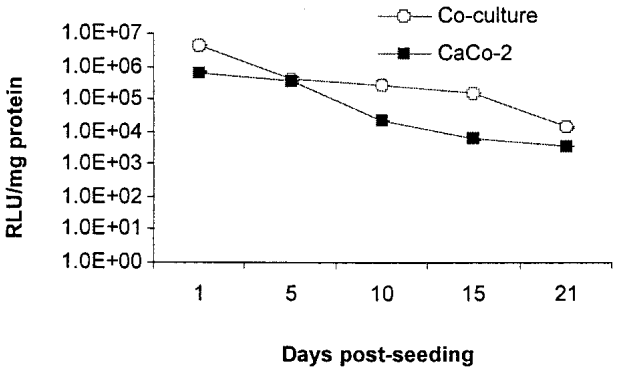

Fig. 2. Effect of differentiation of CaCo-2 and coculture (CaCo-2:Ht29GlucH) monolayers on luciferase expression (RLU/mg protein, mean ± S.D., n = 3). Monolayers were transfected on days 1, 5, 10, 15, and 21 with 6 μg pCMV*luc* complexed with DOTAP™ (+/–2.4).

values increased 14-fold from day 1 to day 21, the transfection levels decreased 170-fold.

MTT assays for the range of vectors in CaCo-2 cells and COS-7 cells showed a dramatic difference in the level of toxicity in each cell line. In the COS-7 cells (day 1) all the systems caused some degree of toxicity including naked DNA; in contrast, in the differentiated CaCo-2 cells (day 21) the same systems at even higher concentrations caused little if any decrease in dehydrogenase activity. The only system that displayed toxicity on the CaCo-2 cells was the pLL/pDNA complex, which was used in the presence of chloroquine.

Pattern of Transfection in Nondifferentiated vs. Differentiated Cells

In order to determine if the decrease in transfection on differentiation resulted from transfection of fewer cells or, in contrast, a general reduction in transfection intensity by all individual cells, the luciferase protein within each cell of a given sample (of nondifferentiated COS-7 and differentiated CaCo-2 cells), transfected using DOTAP™/pCMV*luc* complexes, was fluorescently stained. The confocal images shown in Figs. 3 and 4 are taken as approximating the center of the cells, from a series of horizontal *xy* sections through the cell. In the COS-7 cells (Fig. 3a), luciferase is located throughout the cell and has certain zones of increased concentration as indicated by increased intensity of the green FITC label (Fig. 3b). In the case of the differentiated CaCo-2 cells, luciferase expression again occurred in almost all cells (Fig. 4a), but not to the same extent as in COS-7 cells (Fig. 4b). Small speckles

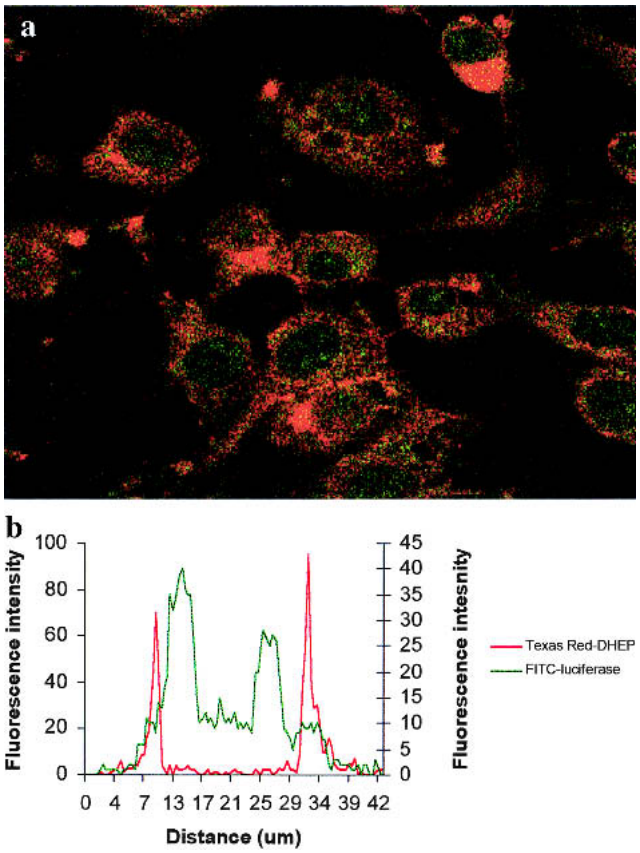

Fig. 3. Confocal scanning image of the *xy* cross section of COS-7 (day 1 postseeding) after transfection with pCMV*luc* plasmid complexed with DOTAP™ CR +/–2.4. (a) Scan taken approximately half-way through the cells with the laser intensity set at 30% (Gain 1500, Iris 2) and (b) fluorescence intensity of stains with the red peaks representing the position of the cell walls while the green line represents the location of luciferase in the cell.

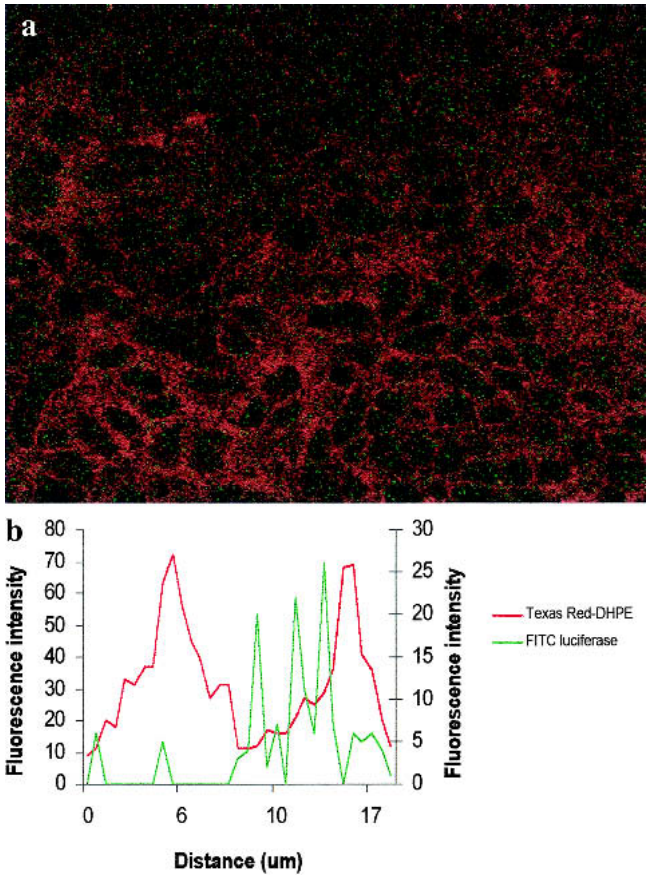

Fig. 4. Confocal scanning image of the *xy* cross section of CaCo-2 cells (day 21 postseeding) after transfection with pCMV*luc* plasmid complexed with DOTAP™ CR +/–2.4; scan was taken approximately half-way through the cells with the laser intensity set at 30% (gain 1500, iris 2) and (b) fluorescence intensity of stains, with the red peaks representing the position of the cell walls while the green line represents the location of luciferase in the cell.

of FITC are seen in the cytoplasm of the CaCo-2 cells (Fig. 4a).

Mechanism of DNA Uptake by Nondifferentiated and Differentiated Cells

To study the roles of active and passive mechanisms in DNA uptake, experiments using [^{32}P]DNA were carried out at two different temperatures: 37°C (cell bound plus internalization) and 4°C (cell bound only). The levels of DNA bound and internalized (at 37°C) are compared to the level of DNA bound (at 4°C) in Fig. 5. The difference between these two levels is deemed to be DNA actively internalized by cells (dpm at 37°C – dpm at 4°C). When the results for each cell line were normalized for protein and for the quantity of DNA added per well, the levels of pDNA bound to the cells at 4°C (areas in gray) were similar for each cell line, with differences seen between vector systems. In contrast, when the levels of pDNA actively internalized are compared (the white areas on the graph), there is a very significant drop ($p < 0.05$) in active internalization of DOTAP™ and PEI/DNA complexes in the differentiated cell monolayers (CaCo-2 and coculture) compared to the nondifferentiated cells (COS-7). This decreased capacity for active internalization may partly explain why differentiated cells, which were found to have a significant amount of DNA associated with them, are more difficult to transfect.

Similarly, when nondifferentiated CaCo-2 cells (day 1) were compared with differentiated CaCo-2 (day 21) at 37°C, there was a 10-fold drop in the level of total cell-associated (bound plus internalized) DNA between day 1 and day 21 after treatment of the cells with pDNA alone or with DOTAP/DNA complexes, and a fivefold drop for PEI/DNA and Superfect/DNA complexes.

Mucus as an Extracellular Barrier to Intestinal Transfection

To investigate mucus as a potential barrier to intestinal transfection, cocultures were grown, and the mucus layer reduced using N-AC 0.3% w/v (the concentration was chosen after extensive testing to balance toxicity and mucolytic efficiency) before transfection. The removal of mucus significantly ($p < 0.05$) decreased the transfection efficiency in the control and with all three vectors tested (Fig. 6). Additional controls were run in which CaCo-2 monolayers (mucus-free)

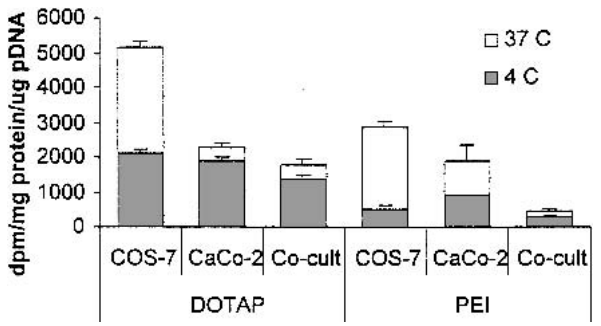

Fig. 5. Cell binding and/or uptake (dpm/mg protein/μg pDNA, mean ± S.D., $n = 3$) of pDNA at 4°C or 37°C by nondifferentiated COS-7 (day 1 postseeding) and differentiated CaCo-2 and coculture cells (day 21 postseeding) following exposure to GDV/[^{32}P]pCMV*luc* complexes for 4 h.

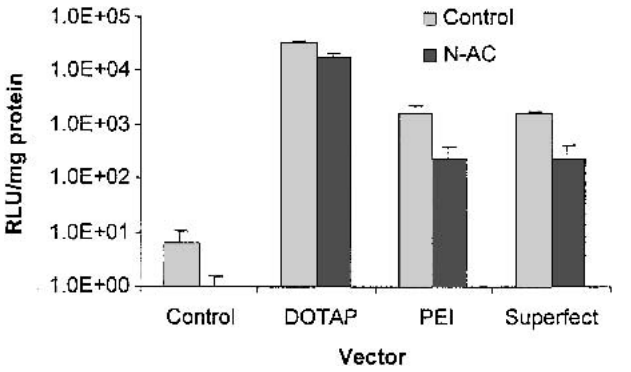

Fig. 6. The effects of 0.3% w/v *N*-acetylcysteine (N-AC) on the transfection efficiency (RLU/mg protein, mean ± S.D., $n = 3$) of DOTAP™, PEI, and Superfect™ pCMV*luc* complexes in CaCo-2: Ht29GlucH coculture monolayers. Control represents transfection with naked pDNA.

were treated with 0.3% w/v N-AC, and subsequently these and untreated control monolayers were transfected with naked plasmid DNA and DOTAP™/pCMV*luc* complexes. No significant difference in luciferase expression was found between treated and untreated monolayers. This indicates that the effect of N-AC is related to its effect on the mucus output of the Ht29GlucH cells in the coculture model.

Animal Model

Initially the jejunum and duodenum were both tested as sites of administration using PEI/pDNA, but only the jejunum showed expression above control. The loop itself (2 cm), as well as the segments of intestine 5 cm above (proximal) and 5 cm below (distal), were excised and analyzed. Only two of the six animals tested showed significant increases in luciferase expression in the gut above background (Fig. 7). In both animals the pattern of expression was similar. Compared to the control, there was a small but significant ($p < 0.05$) increase in transgene expression in the segment proximal to the loop but a much larger (threefold vs. proximal) and more significant level of expression in the loop itself. The distal segment failed to show any luciferase expression. DOTAP™, using a similar high dose of plasmid DNA, was also tested in

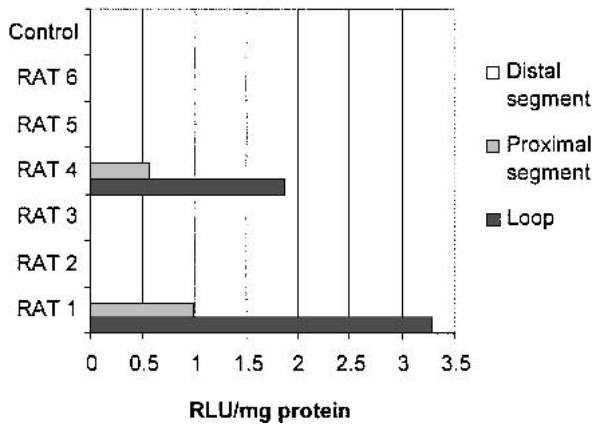

Fig. 7. Luciferase expression (RLU/mg protein) in the jejunal loop, and segments distal and proximal to the loop, for six rats transfected with 200 μg plasmid DNA complexed with PEI N/P 10 for 3.5 h and allowed to express overnight.

the jejunum but failed to produce any significant increase in transfection relative to control.

DISCUSSION

Differentiated cell culture models are more difficult to transfect efficiently and produce quantitatively less luciferase/mg protein for each microgram of plasmid DNA added to the cells. Proliferating, nondifferentiated models appear to give a falsely high determination of transfection efficiency, a problem that is evident in the poor correlation of these systems with *in vivo* data. The confocal images illustrate that this decreased level of luciferase expression in differentiated cells is not related to a decrease in the number of cells transfected but is associated with a general decrease in transfection intensity by all cells.

At day 10, when the filter cultures had reached confluency, there was a significant drop in transgene expression. A recent study by Zauner *et al.* (15) into the effects of cell cycling on transfection suggests that the reduction in gene expression observed in confluent cells is most probably due to the lower number of cycling cells. Brunner *et al.* (16) looked at a range of vector systems and determined that luciferase activity was 30- to 500-fold higher in cells transfected during S or G2 phase (just before the next cell division), compared with those transfected during G1 phase (beginning of the cell cycle). Confluent cell cultures become quiescent as a result of contact inhibition and fail to go through any further cell division cycles (17). Factors such as nuclear membrane permeability, plasmid uptake, protein synthesis activity, and plasmid inactivation are all cell cycle phase dependent. Consequently, confluency can affect transfection levels. However, in this study the levels of expression dropped before full confluency and long before full differentiation. This would indicate that there are biophysical and biochemical changes that occur within the cell and make the uptake and/or expression processes less efficient. For example, studies have shown that the cell surface of lung cells becomes less anionic as differentiation progresses (18). Molist *et al.* (19) found that there were differences in glycosaminoglycan (GAG) composition depending on the degree of differentiation of human colon carcinoma cells.

When the cells were fully differentiated at day 15, the level of expression, compared to day 1, had dropped 100-fold for CaCo-2 monolayers and 25-fold for cocultures. Other studies using viral gene delivery have similarly found that differentiated CaCo-2 cells were more resistant to transfection than undifferentiated CaCo-2 cells (20,21). One theory, for the increased resistance of differentiated cells to transfection, suggests that the low mitotic activity in fully differentiated cells leads to poor nuclear accessibility of the plasmid DNA (22–24).

DNA cell association studies carried out at day 1 and day 21 in CaCo-2 and coculture monolayers show the levels of total cell-associated (at 37°C) DNA decreased in the day 21 cells. These decreases are almost certainly a component in the overall effect of differentiation on transfection. The results from the uptake studies indicate clearly that there is a significant decrease in the level of active internalization of DNA by differentiated cells. Following differentiation, access to the more fluid and more easily transfected basolateral membrane is decreased. In addition, the presence of microvilli on the apical surface of fully differentiated enterocytes may restrict access to the absorptive membrane surface to smaller complexes, less than 30–40 nm. These factors may subsequently reduce the opportunities for active internalization. The presence of the glycocalyx and the brush border enzymes may also affect transfection levels by compromising the stability of certain gene delivery systems.

The effect of mucus may be an important factor in the transfection of not only the lung but also the gut. To our knowledge, no studies have looked at the effect of gastrointestinal mucus on gene delivery, but several studies have looked at the effect of mucus on gene delivery to the airway epithelium. Conflicting results are found in the literature. In some studies no significant difference in transfection was observed when a mucolytic was used to remove mucus from human airway epithelial cells (25), but other studies have found that removal of mucus increased liposomal gene delivery but had no significant effect on adenoviral gene delivery to sheep airway epithelium (12). A study looking at the effect of mucolytics on gene transfer across a cystic fibrosis sputum barrier *in vitro* found that N-AC did not significantly affect transfection (26). The results of this work indicate that, for the vectors tested, the presence of mucus can enhance transfection efficiency. Binding of cationic transfection complexes by the sialic acid and sulfate groups of the mucin glycoproteins or immobilization of these large complexes within the mucus gel network may increase the contact time (transfection time) of complexes with the cells in culture. Drug–mucus interactions involving; electrostatic (13), hydrophobic, and hydrogen-bonding interactions (27) resulting in altered membrane permeability have previously been reported.

In addition, mucus has also been shown to act as a diffusional barrier. From the levels of DNA actively internalized (Fig. 5) by the differentiated monolayers, the cationic lipid DOTAP™ appears better able to overcome this diffusional barrier than the cationic polymer, PEI. The liposomal nature of DOTAP™ may enable it to more effectively diffuse through the mucus layer of the co-cultures and transfect more efficiently (Fig. 1). Another factor that may contribute to the effects of mucus is its protective function. This may reduce the toxicity associated with chloroquine used concomitantly with poly-L-lysine and may partly explain the increase in transfection efficiency of pLL/pDNA complexes in cocultures over the mucus-free CaCo-2 monolayers (Fig. 1).

The results of the animal studies indicate that although the intestine is a difficult site for transfection, transgene expression can be mediated successfully given the right formulation and site of administration. Very little has been published on nonviral gene delivery to the gut, with the emphasis to date being on viral-based systems. In the current work, expression levels achieved were variable; however, in both rats that were successfully transfected, the same pattern and level of transfection was obtained. MacLaughlin *et al.* (9), using chitosan and Lipofectin™ polymers as carriers for gene delivery to the rabbit intestine, reported similar high variability. Although PEI successfully produced transfection *in vitro* and *in vivo,* DOTAP™ failed to produce any significant luciferase expression *in vivo* despite its *in vitro* efficiency, indicating that success *in vitro* in differentiated cell models does not guarantee success *in vivo.* MacLaughlin *et al.* (9) similarly found that a polymeric system (which also included a lytic peptide) proved more successful than the lipid-based Lipo-

fectin™ system in the gut. Bragonzi *et al.* (28), looking at local *in vivo* intratracheal injection of a range of nonviral vector complexes, similarly found that the highest levels of transfection were obtained with PEI complexes, whereas DOTAP complexes gave no significant luciferase activity. Their results suggested that cationic polymers were better able to overcome the surfactant barrier of the lungs than cationic lipids, which tended to colocalize with surfactant protein A, forming aggregates in the lumen of the bronchi.

The poor activity of DOTAP™/pCMV*luc* complexes in the intestinal loop model may well be caused by the instability of the lipids in the hostile environment of the gut. The presence of intestinal digestive enzymes, such as lipases, and surfactants, such as bile salts, mean that liposomal systems are very unstable, particularly in the upper digestive tract. In the lower colonic area of the gut, where enzymatic activity is relatively decreased, following rectal infusion Lipofectin™ has been reported to achieve *in vivo* transfection (29).

Although the levels of transfection obtained *in vivo* were low and variable, the results show that transfection in the hostile gut environment is possible. Further work is necessary to optimize conditions such as dose and formulation. The differentiated intestinal cell culture models provide a more realistic prediction of the ability of a vector to transfect nonproliferating, differentiated cells *in vivo.* The models are a useful initial step to study factors influencing transfection in the gut such as differentiation, active uptake, and the presence of mucus. In future studies, the *in vivo*/*in vitro* correlation may be further improved by incubation of the vectors in simulated intestinal fluid before cellular transfection.

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REFERENCES

1. J. W. Sandberg, C. Lau, M. Jacomino, M. Finegold, and S. J. Henning. Improving access to intestinal stem cells as a step toward intestinal gene transfer. *Hum. Gene Ther.* **5**:323–329 (1994).
2. M. A. Croyle, M. Stone, G. L. Amidon, and B. L. Roessler. *In vitro* and *in vivo* assessment of adenovirus 41 as a vector for gene delivery to the intestine. *Gene Ther.* **5**:645–654 (1998).
3. T. J. Sferra, D. McNeely, and P. R. Johnson. Gene transfer to the intestinal tract: a new approach using selective injection of the superior mesenteric artery. *Hum. Gene Ther.* **8**:681–687 (1997).
4. D. A. Sweetser, S. M. Hauft, P. C. Hoppe, E. H. Birkenmeier, and J. I. Gordon. Transgenic mice containing intestinal fatty acid-binding protein-human growth hormone fusion genes exhibit correct regional and cell-specific expression of the reporter gene in their small intestine. *Proc. Natl. Acad. Sci. USA* **85**:9611–9615 (1988).
5. S. N. Jones, M. Grompe, M. I. Munir, G. Veres, W. J. Craigen, and C. T. Caskey. Ectopic correction of ornithine transcarbamylase deficiency in sparse fur mice. *J. Biol. Chem.* **265**:14684–14690 (1990).
6. H. Soriano-Brucher, C. Lau, T. Hourigan, M. Finegold, F. Ledley, and S. J. Henning. Gene transfer into the intestinal epithelium. *Gastroenterology* **100**:A252 (1991).
7. C. Lau, H. E. Soriano, F. D. Ledley, M. J. Finegold, J. H. Wolfe, E. H. Birkenmeier, and S. J. Henning. Retroviral gene transfer into the intestinal epithelium. *Hum. Gene Ther.* **6**:1145–1151 (1995).
8. S. J. Henning. Gene transfer into the intestinal epithelium. *Adv. Drug Deliv. Rev.* **17**:341–347 (1995).
9. F. C. MacLaughlin, R. J. Mumper, J. Wang, J. M. Tagliaferri, I. Gill, M. Hinchcliffe, and A. P. Rolland. Chitosan and depolymerized chitosan oligomers as condensing carriers for *in vivo* plasmid delivery. *J. Control. Release* **56**:259–272 (1998).
10. A. N. Uduehi, S. H. Moss, J. Nuttall, and C. W. Pouton. Cationic lipid-mediated transfection of differentiated CaCo-2 cells: a filter culture model of gene delivery to a polarized epithelium. *Pharm. Res.* **16**:1805–1811 (1999).
11. C. W. Pouton, P. Lucas, B. J. Thomas, A. N. Uduehi, D. A. Milroy, and S. H. Moss. Polycation–DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J. Control. Release* **532**: 289–299 (1998).
12. C. Kitson, B. Angel, D. Judd, S. Rothery, N. J. Severs, A. Dewar, L. Huang, S. C. Wadsworth, S. H. Cheng, D. M. Geddes, and E. W. Alton. The extra- and intracellular barriers to lipid and adenovirus-mediated pulmonary gene transfer in native sheep airway epithelium. *Gene Ther.* **6**(4):534–546 (1999).
13. C. M. Meaney and C. M. O'Driscoll. Mucus as a barrier to the permeability of hydrophilic and lipophilic compounds in the absence and presence of sodium taurocholate micellar systems using cell culture models. *Eur. J. Pharm. Sci.* **8**:167–175 (1999).
14. J. Sambrook, E. F. Fritsch, and T. Maniatis. *Molecular Cloning-A Laboratory Manual*, Cold Spring Harbor Lab. Press, New York, 1989.
15. W. Zauner, S. Brunner, M. Buschle, M. Ogris, and E. Wagner. Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: a potential role of polylysine in nuclear transport. *Biochim. Biophys. Acta* **1428**:57–67 (1999).
16. S. Brunner, T. Sauer, S. Carotta, M. Cotten, M. Saltik, and E. Wagner. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Ther.* **7**:401–407 (2000).
17. D. Voet and J. G. Voet. *Biochemistry*, John Wiley & Sons, New York, 1990.
18. H. Matsui, L. G. Johnson, S. H. Randell, and R. C. Boucher. Loss of binding and entry of liposomes-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J. Biol. Chem.* **272**:1117–1126 (1997).
19. A. Molist, M. Romarís, U. Lindahl, J. Villena, M. Touab, and A. Bassols. Changes in glycosaminoglycan structure and composition of the main heparan sulphate proteoglycan from human colon carcinoma cells (perlecan) during cell differentiation. *Eur. J. Biochem.* **254**(2):371–377 (1998).
20. E. Walter, M. A. Croyle, B. J. Roessler, and G. L. Amidon. The absence of accessible vitronectin receptors in differentiated tissue hinders adenoviral-mediated gene transfer to the intestinal epithelium *in vitro*. *Pharm. Res.* **14**:1216–1222 (1997).
21. R. K. Batra, H. Berschneider, and D. T. Curiel. Molecular conjugate vectors mediate efficient gene transfer into gastrointestinal epithelial cells. *Cancer Gene Ther.* **1**:185–192 (1994).
22. M. Wilke, E. Fortunatt, M. Van de Breck, A. T. Hoogeveen, and B. J. Scholte. Efficacy of a peptide-based gene delivery system depends on mitotic activity. *Gene Ther.* **3**:1133–1142 (1996).
23. S. Takeshita, D. Gai, G. Lecierc, J. G. Pickering, R. Riessen, L. Weir, and J. M. Isner. Increased gene expression after liposome-mediated arterial gene transfer associated with intimal smooth muscle cell proliferation. *J. Clin. Invest.* **93**:652–661 (1994).
24. L. Vitiello, A. Chonn, J. D. Wasserman, C. Duff, and R. G. Worton. Condensation of plasmid DNA with polylysine improves liposome- mediated gene transfer into established and primary muscle cells. *Gene Ther.* **3**:396–404 (1996).
25. A. Fasbender, J. Zabner, B. G. Zeiher, and M. J. Welsh. A low rate of cell proliferation and reduced DNA uptake limit cationic lipid-mediated gene transfer to primary cultures of ciliated human airway epithelia. *Gene Ther.* **4**:1173–1180 (1997).
26. M. Stern, N. J. Caplen, J. E. Browning, U. Griesenbach, F. Sorgi, L. Huang, D. C. Gruenert, C. Marriot, R. G. Crystal, D. M. Geddes, and E. W. Alton. The effect of mucolytic agents on gene transfer across a CF sputum barrier *in vitro*. *Gene Ther.* **5**:91–98 (1998).
27. A. Wikman, J. Karlsson, I. Carlstedt, and P. Artursson. A drug absorption model based on the mucus layer producing human intestinal goblet cell line Ht29-H. *Pharm. Res.* **10**:843–852 (1993).
28. A. Bragonzi, G. Dina, A. Villa, G. Calori, A. Biffi, C. Bordignon, and B. M. Assael. Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. *Gene Ther.* **7**:1753–1760 (2000).
29. C. A. Westbrook and R. B. Arenas. Gene therapy of the gut: introduction of the APC tumor-suppressor gene for cancer prevention or treatment. *Adv. Drug Deliv. Rev.* **17**:349–355 (1995).