Transfection Efficiency and Toxicity of Polyethylenimine in Differentiated Calu-3 and Nondifferentiated COS-1 Cell Cultures

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ABSTRACT In the present study, we evaluated polyethylenimine (PEI) of different molecular weights (MWs) as a DNA complexing agent for its efficiency in transfecting nondifferentiated COS-1 (green monkey fibroblasts) and well-differentiated human submucosal airway epithelial cells (Calu-3). Studying the effect of particle size, zeta potential, presence of serum proteins or chloroquine, it appeared that transfection efficiency depends on the experimental conditions and not on the MW of the PEI used. Comparing transfection efficiencies in both cell lines, we found that PEI was 3 orders of magnitude more effective in COS-1 than in Calu-3 cells, because Calu-3 cells are differentiated and secrete mucins, which impose an additional barrier to gene delivery. Transfection efficiency was strongly correlated to PEI cytotoxicity. Also, some evidence for PEI-induced apoptosis in both cell lines was found. In conclusion, our results indicate that PEI is a useful vector for nonviral transfection in undifferentiated cell lines. However, results from studies in differentiated bronchial epithelial cells suggest that PEI has yet to be optimized for successful gene therapy of cystic fibrosis (CF).

KEYWORDS: Nonviral transfection, Polyethylenimine, COS-1 cells, Calu-3 cells, Apoptosis.

INTRODUCTION

Gene delivery focuses on the therapeutic use of genes and promises considerable advances in the treatment of several important diseases. Easily accessible epithelial tissues from the air-exposed side of the lungs offer an unique opportunity for the treatment of cystic fibrosis (CF). CF is a lethal, autosomal recessive clinical disorder that has been prompted as a model disease for gene therapy because the delivery of the correct cystic fibrosis transmembrane regulator (CFTR) gene to the bronchial epithelium in vitro and in vivo would result in restoration of Cl⁻ channel activity and abolish many of the clinical symptoms in the lung.¹ Despite promising results in vitro, the transfection efficiency in vivo has proven to be low

and transient. Two major problems have to be addressed before successful gene delivery in vivo can be accomplished. There is a need for an in vitro model that represents the situation in vivo accurately and for an appropriate gene delivery vector system. This system can then be used to test gene delivery vectors for gene therapy in CF. In the present study, we have chosen the human, mucus-producing submucosal-gland carcinoma cell line Calu-3 as a model for the airway mucosa and polyethylenimine (PEI) as a nonviral polycationic polymer for the condensation and delivery of DNA.

PEI is obtained by acid-catalyzed polymerization of aziridine,^{2,3} yielding a highly branched network with a high cationic charge-density potential that can ensnare DNA. Since 1995, PEI has been found to be a versatile polymeric vector for gene delivery that tightly condenses plasmid DNA and is able to promote transgene delivery to the nucleus of mammalian cells.⁴⁻⁷ A merely mechanistic study⁸ has shown that cationic lipid-DNA complexes can dock and interact with proteoglycans expressed on the surface of mammalian cells, promoting their intracellular uptake, a possible route that might also be followed by PEI/DNA complexes. The high transfection efficiency of PEI in vitro has been ascribed to its ability to act as a proton sponge that buffers the low pH in the endolysosomal compartments and potentially induces ruptures of the endolysosomal membrane, resulting in the release of PEI/DNA complex into the cytoplasm.⁴ Furthermore, Lechardeur et al⁹ have shown that upon entry into the cytoplasm, naked plasmid DNA (pDNA) undergoes a rapid turnover because of degradation by cytosolic nucleases. Moret et al¹⁰ have shown that PEI is able to protect pDNA against degradation by serum DNases. Although the proton-sponge effect and the ability to deliver DNA to the nucleus enhance transgene expression, the presence of PEI in the cellular nucleus may interfere with transcriptional and translational processes and even induce cell death.¹¹ Efforts have been undertaken to diminish the high cationic charge density of PEI to a magnitude that promotes DNA delivery but decreases the adverse effects of PEI on cell viability.¹²

Calu-3 cells have been suggested as an appropriate model for the nasal and bronchotracheal airway epithelium^{13,14} because they form tight monolayers, expressing the tight junction-associated protein ZO-1 and the adherin protein E-cadherin; present apical villi; and produce mucous secretions. Recently, the P-glycoprotein activity and the barrier function of the Calu-3 cells have

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been further evaluated.^{15,16} However, their potential use as a model for gene delivery has not yet been investigated. As a comparison, we have also used the COS-1 (green monkey fibroblasts) cell line as a model for a nondifferentiated, fast-growing, and relatively easily transfectable cell line.

The aim of this study was to investigate the transfection efficiency of commercially available PEI with molecular weights (MWs) of 600-1000, 60, and 25 kDa as polycationic nonviral gene delivery vectors in Calu-3 and COS-1 cells. Further emphasis was given to the comparison between differentiated Calu-3 and nondifferentiated COS-1 cells as in vitro models and the development of the Calu-3 cell line as a model for gene delivery to the bronchial epithelium. The α 3-luciferase plasmid driven by an SV40 promoter was used as a reporter gene for assessment of the transfection activity, and the cell survival was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The effects of fetal calf serum (FCS) and the lysosotropic compound chloroquine on the transfection efficiency and the cytotoxicity were also investigated. The particle size and zeta potential of PEI/DNA complexes were measured because these parameters play an important role during the endocytosis process. Finally, cell death was further assayed by Hoechst staining of the nuclei in order to visualize apoptotic nuclei that could be induced by the presence of PEI in the nucleus.

MATERIALS AND METHODS

Preparation of plasmid

pRSV- α 3-Luc plasmid (Promega, Leiden, The Netherlands) consists of 5.6 kbp and contains the firefly luciferase gene and an ampicillin resistance gene that are controlled by a SV40 promoter/enhancer. The plasmids were replicated in the high-copy DH5- α Escherichia coli strain grown in selective ampicillin (50 μ g/mL) supplemented Luria-Bertani medium, isolated by alkaline lysis followed by anion exchange chromatography using the Giga Qiagen kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Purity of the plasmid and integrity of the cDNA insert were determined by agarose gel electrophoresis and UV spectroscopy (E 260/280 nm ratio). After isolation, the DNA was dissolved to an end concentration of 1.2 μ g/ μ L TrisHCl buffer (pH 8.0). The purity of the plasmid was assayed by 0.8% agarose gel electrophoresis.

Preparation of plasmid/PEI complexes

The commercially available PEI used in these experiments was branched and came from different suppliers. The PEI with the highest MW (600-1000 kDa) was purchased from Fluka (New York, NY); the PEI with MWs of

60 kDa and 25 kDa was obtained from Sigma/Aldrich (St Louis, MO). The PEI were aseptically dissolved to a concentration of 500, 200, 100, 50, and 5 μ g/mL, respectively, in sterile Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/L glucose, pH 7.4) from Gibco BRL (Basel, Switzerland). Exactly 6 μ g RSV-Luc plasmid in 5 μ L Tris-EDTA buffer (Qiagen) was pipetted in sterile "DNase/RNase free" Eppendorf vials (Eppendorf, Hamburg, Germany) and 200 μ L PEI solution was gently added, vortexed for 10 seconds, and incubated at room temperature for 30 minutes to allow spontaneous PEI/DNA complex formation. After 30 minutes, 1.3 mL DMEM, cell culture medium (DMEM/FCS 10%), or 100 μ M chloroquine (Sigma/Aldrich) solution in DMEM, was added to each Eppendorf, yielding the following PEI/DNA ratios: 16.7, 6.7, 3.3, 1.7, and 0.3 μ g/ μ g.

Particle size and zeta potential determination by photon correlation spectroscopy

Particle size and zeta potential measurements of PEI/DNA complexes were performed by photon correlation spectroscopy and electrophoretic mobility, respectively, on a Zeta Sizer 3000 (Malvern Instruments, Southborough, UK) instrument bearing integrated size and zeta modules, equipped with a 10-miliWatt helium neon laser producing light at a wavelength of 633 nm and the Malvern PCS version 1.41 (1992) software. For particle size determination, complexes of PEI with DNA were prepared as described in the previous section and measured in glass cuvettes at 25°C and a fixed scattering angle of 90°. The media used were filtered through a 0.2- μ m filter before the measurements. We also performed size measurements of the complexes in the presence of 30 and 60 μ M chloroquine concentration in DMEM. The calibration of the instrument was checked using 200-nm Nanosphere Size Standard reference latex beads (Duke Scientific, Palo Alto, CA) at 25°C in DMEM. Zeta potential determination is based on the electrophoretic mobility of the complexes in an aqueous medium. For this reason, the polyplexes were measured in a low-salt environment consisting of 10mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES, Sigma/Aldrich) buffer adjusted to pH 7.4 with 1M NaOH. For these measurements, we used the same complexation procedure as described in the previous section, with the exception that DMEM was replaced by 10mM HEPES buffer. The pH of the formulations was checked; it was not changed by the presence of PEI. The zeta potential was measured in glass cuvettes, using the dip-in cell and Zeta Potential Transfer Standard (-25 mV) (Malvern Instruments). All measurements were carried out at least in triplicate.

Cell lines and culture conditions

Calu-3 cells (#HTB-55) were purchased from the American Type Culture Collection (ATCC, Rockville, MD) at

passage number (PN) 19. The cells were grown in 75-cm² flasks for 7 days in DMEM supplemented with 10% FCS (Hyclone, Logan, UT) and 50 µg/mL penicillin (pen) and streptomycin (strep) (Sigma/Aldrich) at 37°C in a 90% humidified incubator and 5% CO₂ until 80% confluency was reached. Then, cells were either passaged to new flasks or seeded on 24-well or 96-well plates for transfection and toxicity experiments, respectively. The cell culture medium was changed every 2 days. The experiments were performed in 18-day-old confluent, differentiated, and polarized Calu-3 cells of PN 25 to 45. The COS-1 cells (PN 7-15), a generous gift from Dr C.M.P. Backendorf (Department of Molecular Genetics, Leiden University, Leiden, The Netherlands), were grown in culture medium and the transfection and toxicity experiments were performed 7 days after seeding in confluent monolayers.

Transfection experiments

Calu-3 cells were seeded at a seeding density of 1×10^5 cells/cm² on 24-well plates and grown in DMEM/FCS 10%, pen strep at 37°C in a 90% humidified incubator and 5% CO₂ for 18 days.¹⁷ COS-1 cells were seeded at 1×10^4 cells/cm² and used 7 days after seeding. Prior to starting the transfection experiment, the cells were rinsed twice with warm phosphate-buffered saline (PBS, pH 7.4), and every well was supplied with 500 µL DMEM, DMEM/FCS, or DMEM/chloroquine (100µM), respectively. Exactly 500 µL of the PEI/DNA complex formulations, as described under "Preparation of plasmid/PEI complexes," was added. The negative control group consisted of naked DNA in DMEM. The final pDNA concentration was 2 µg/well. The cells were transfected for 8 hours and mildly shaken every hour, rinsed with warm PBS, supplied with 1 mL culture medium, and allowed 48 hours for luciferase protein expression. After 48 hours, the cells were rinsed and lysed in 250 µL cold (4°C) reporter lysis buffer, the cellular debris was pelleted by cold centrifugation at 13 000 rpm for 5 minutes, and the luciferase activity of 5 µL cell lysate was measured with a 50-µL luciferase assay kit (Promega, Leiden, The Netherlands) in a Biolumat Luminometer (Berthold, Woerden, The Netherlands). The relative light units (RLU) were standardized to the total amount of protein present in the cell lysate that was measured with the Bradford colorimetric assay (BioRad, Alphen aan den Rijn, The Netherlands).

Cell survival assay

Cell survival of confluent 18-day-old Calu-3 cells or 7-day-old COS-1 cells was assessed by an MTT colorimetric assay in 96-well plates.^{18,19} Prior to the 3-hour MTT (5 mg/mL in HBSS/HEPES [Hank's Balanced Salt Solution/HEPES] [Sigma/Aldrich]) treatment, the Calu-3 cells were exposed for 5 hours to the PEI formulations at the same end concentrations used for the transfection ex-

periments. After lysis of the cells in NaOH/SDS (sodium dodecyl sulphate, Sigma/Aldrich), 0.01/1.0% (wt/vol), the absorbency was measured at 590 nm in a Bio-Rad 96-well plate reader. Values of 8 measurements were normalized to 100% for the control group (exposure to transport medium DMEM/FCS).

Mucus staining

Calu-3 cell monolayers were fixed overnight in 4% paraformaldehyde in PBS (pH 7.4). The fixative was replaced by 70% ethanol, and the monolayers were transferred to a Shandon Hypercenter 2 (Pittsburgh, PA) tissue processor for automated dehydration, clearing, and paraffin infiltration. Monolayers were successively embedded in paraffin, and sections of 5 µm were cut using a Leica Reichert-Jung 2030 Microtome (Leica, Rijswijk, The Netherlands). Staining with periodic acid-Schiff's reagent (PAS, Sigma-Aldrich) was used to detect intracellular mucus, and the slides were examined by light microscopy. These techniques have been used to detect mucins in cultured human lung adenocarcinomas and hamster tracheal epithelial cells.²⁰

Hoechst 33258 staining of nuclei

For the assessment of cell death by apoptosis, the Hoechst 33258 assay was used. Calu-3 or COS-1 cells were seeded as described in "Transfection experiments" and were used at 18 or 7 days, respectively. The PEI formulations were prepared as described in "Preparation of plasmid/PEI complexes" except that no pDNA was added. Some 5% dimethyl sulfoxide (DMSO, Sigma/Aldrich) in DMEM served as positive apoptotic control. Incubation in PBS is a model for serum starvation (positive control), and DMEM, DMEM/FCS, and DMEM/chloroquine were used as negative controls. The cells were rinsed with PBS, incubated with the respective formulations for 8 hours, scraped in the medium, and gently pelleted by centrifugation at 150g for 5 minutes. The medium was removed, and the cells were resuspended in 250 µL of 3.7% (vol/vol) formaldehyde/PBS solution containing 2 µg/µL Hoechst 33258 (Hoechst AG, Frankfurt am Main, Germany) dye and incubated for 30 minutes at room temperature in the dark. Sequentially, the cells were pelleted, washed with 1 mL PBS, resuspended in 50 µL PBS, spotted on an objective glass, and dried at 37°C for 15 minutes. Prior to visualization they were stored at 4°C. Nuclear condensation was visualized by optical imaging of the fluorescent staining using a Zeiss IM 35 inverted video microscope (Zeiss, Oberkochen, Germany) equipped with a Nikon fluor 40x oil immersion objective (Nikon Europe BV, Badhoevedorp, The Netherlands), filter set of 360-nm excitation and longpass filter 470-nm emission (Zeiss), CH220 camera (Photometrics, Tucson, AZ), and Image Pro 4.1 software (Media Cybernetics, Leiden, The Netherlands).

RESULTS

PEI/DNA complex size and zeta potential

The purity of plasmid DNA was confirmed by agarose gel electrophoresis and is presented in Figure 1. A strong band is present at 5.6 kbp, indicating the presence of supercoiled plasmid, and a faint band of relaxed DNA is visible below. The size of PEI/DNA complexes with PEI of varying MWs are presented in Figure 2. Formulations in DMEM and culture medium resulted in complex particle sizes between 200 and 350 nm, with small interexperimental variations. We also performed size measurements in PBS; the values were in accordance with previously reported data²¹ (data not shown). This indicates that PEI/DNA complex formation is an efficient, spontaneous process driven by electrostatic interactions. Figure 2 and 3 show that chloroquine seems to perturb the formation of complexes, as the sizes of the complexes are unexpectedly high and show high variability. Addition of FCS or chloroquine to the culture medium also induced a high polydispersity (0.8-1.0) of the polyplexes. The presence of small (200 nm), medium (700 nm), and large complexes (> 1000 nm) indicates that fetal serum proteins and chloroquine disrupt the PEI-DNA interactions by either aggregate formation or electrostatic interactions. It is important to bear in mind that size measurements of complexes exceeding 1 μ m become inaccurate, but the observation that chloroquine induces aggregation remains unchanged.

The zeta potential of polyplexes in a low-salt environment (10mM HEPES) decreases proportionally with lower PEI concentrations (Figure 4). The negative potential at an N/P ratio of 0.2 is due to an excess of negative charges from unbound DNA. The polydispersity of the polyplexes over the whole range of N/P ratios was small (0.05-0.2), indicating that discrete particle sizes were present in solution. Measurement of zeta potential in high-ionic-strength culture medium was impossible because of inhibition of the electrophoretic mobility of relatively large polyplexes by an excess of small and fast ions.

Transfection efficiency and cell survival

Figure 5 shows the transfection efficiency and the toxic effects of polyplexes of different N/P ratios and different formulations in COS-1 cells. In general, the transfection efficiency in COS-1 cells was high (106-107 RLU/mg protein) and showed a typical bell-shaped profile with the optimum around N/P ratios of 3.3 and 1.7 μ g/ μ g. It is interesting to note that at an N/P ratio of 16.7 the polyplexes are less effective than at lower ratios, apparently because of increased toxic effects of PEI. The sharp drop at an N/P ratio of 0.2 can be explained by a relatively

Figure 1. RSV- α 3-luciferase plasmid (Promega) integrity assessed by 0.8% (wt/vol) agarose gel electrophoresis, isolated as described under "Preparation of plasmid," and visualized by ethidium bromide staining. Lane 1: 36 ng plasmid; lane 2: 72 ng plasmid; lane 3: 120 ng plasmid; M: 1 kbp marker (BioRad). The plasmid has a size of 5.6 kbp, and 2 bands are distinguished, the bright supercoiled plasmid and a faint open circular band.

much lower concentration of polyplexes than at higher N/P ratios, resulting in the presence of uncomplexed DNA that has a low transfection efficiency. A striking effect of the presence of FCS proteins during the transfection experiments is the increase in cytotoxicity. Apparently, the relatively large polyplex/protein complexes induce cell death probably either via more efficient uptake of polyplexes and also unbound PEI or a more intimate interaction and disruption of the plasma membrane resulting in higher toxicity. PEI III exerts the highest toxicity, probably because of the smaller MW of PEI but also because the transfection efficiency increases slightly compared to DMEM formulations. As expected, the lysotropic compound chloroquine did not increase the luciferase gene expression; its effect on the disruption of the lysosomes is already accomplished by the proton-sponge effect of the PEI. Noteworthy is the higher transfection efficiency of the 25-kDa PEI accompanied by an increased cytotoxic effect.

In Calu-3 cells, the transfection efficiency (Figure 6) was significantly lower than in COS-1 cells; although peaking at an N/P ratio of 16.7, the efficiency dropped to baseline

Figure 2. Size of PEI/DNA complexes in DMEM, DMEM/FCS (cell culture medium), and DMEM/chloroquine (100 μ M) at pH 7.4. The complexes were prepared by mixing 6 mg DNA with PEI to the indicated N/P ratio for 30 minutes at room temperature.

Figure 3. Size of PEI/DNA complexes in DMEM/chloroquine (60 and 30 μ M) at pH 7.4. The complexes were prepared by mixing 6 μ g DNA with PEI to the indicated N/P ratio for 30 minutes at room temperature.

values at lower PEI concentrations. The concentration-dependent effect on the transfection efficiency was also abolished. Furthermore, Calu-3 cells seemed to be more susceptible to the toxic effects of PEI compared to COS-1 cells. There is a clear inverse relationship between transfection efficiency and toxicity; hence higher N/P ratios induced more cytotoxicity but also increased the transfection efficiency. In the case of chloroquine addition, the cytotoxicity was reduced at N/P ratios below 16.7 μ g/ μ g and the transfection efficiency was much

lower than with the DMEM or the culture medium formulation. This phenomenon can be explained by the disruptive effect of chloroquine on the polyplexes, which leads to the formation of large aggregates that were excluded by size from intracellular uptake.

Mucus staining

Under air-interface culture conditions, mucus-containing vesicles were detected in Calu-3 cells by specific stain

Figure 4. Zeta potential of PEI/DNA complexes in low-salt environment (10 mM HEPES) at pH 7.4. The complexes were prepared by mixing 6 μ g DNA with PEI to the indicated N/P ratio for 30 minutes at room temperature.

ing with PAS (Figure 7). Mucus secretions were not quantified but appeared to be produced at higher amounts in cells cultured under physiological conditions at the air interface than under submerged conditions. As Calu-3 cells are known to express MUC1, MUC4, MUC5, and MUC5B genes,²² it is assumed that the mucins secreted from Calu-3 cells are comparable in structure to the situation in vivo. Calu-3 cells also express the wild-type CFTR protein,²³ which allows for the examination of the relationship between CFTR and mucin regulation, especially with respect to altered mucin sulfation occurring in cystic fibrosis.²⁴

Hoechst 33258 staining of nuclei

The effects of different PEI formulations on the nuclear structure of COS-1 cells is depicted in Figure 8. The effects are shown only for the highest PEI concentration corresponding with the PEI/DNA ratio of 16.7; at lower PEI concentrations no difference compared to the controls was detected. Panels 8A and 8B show the strong apoptotic response of the positive controls as 5% DMSO and serum starvation. The arrows indicate the presence of apoptotic nuclei where the chromatin structure in the nuclei is disrupted and the DNA is compacted in discrete compartments under the influence of apoptotic signals awaiting cell dismembering into apoptotic bodies. There is no evidence of apoptotic nuclei in the negative controls as DMEM, culture medium, and DMEM/chloroquine (8C, 8D, 8E, respectively). PEI I (8F, 8G, 8H) shows the presence of some apoptotic nuclei in the case of culture medium (8G) and DMEM/chloroquine (8H) treatment. In

DMEM (8F), however, we found no evidence of apoptosis. PEI II (8I, 8J, 8K) induces apoptosis in culture medium (8J) but not in DMEM (8I) or DMEM/chloroquine (8K). PEI III (8L, 8M, 8N) has the most profound influence on the cell viability, in that it induces apoptosis under all these conditions. Nevertheless, the overall induction of indisputable apoptotic nuclei formation by PEI was rather low (around 5%), even for the conditions where apoptosis was visualized.

The situation in Calu-3 cells is presented in Figure 9. Panels 9A and 9B show the positive controls, DMSO and PBS, respectively, and the obvious presence of apoptotic nuclei, while the negative controls (9C, 9D, 9E) show no sign of apoptosis. Some apoptotic nuclei are visible in all PEI formulations (panels 9F-N), indicating that also in Calu-3 cells apoptosis is induced by PEI treatment. Nevertheless, the levels of apoptotic nuclei are comparable to COS-1 cells around 5%.

DISCUSSION

Several studies have indicated that gene delivery in cystic fibrotic tissues is able to restore the chloride flux via expression of the correct CFTR protein on the plasma membrane of the affected cells, as reviewed by Flotte and Laubbe.¹ The choice of the model cell line for in vitro prediction of the in vivo effects together with the choice and optimization of the delivery system are still disputed,

Figure 5. Transfection efficiency and toxicity of PEI at the indicated N/P ratios in confluent COS-1 (PN 7-15) that is 7 days old. DNA concentration was 2 µg/well; naked DNA was used as a negative control, and the cytotoxicity was determined by an MTT assay. (A) DMEM, (B) DMEM/FCS, (C) DMEM/chloroquine (100 µM).

Figure 6. Transfection efficiency and toxicity of PEI at the indicated N/P ratios in 18 days old confluent and differentiated Calu-3 (PN 25-45) that is. DNA concentration was 2 µg/well; naked DNA was used as a negative control, and the cytotoxicity was determined by an MTT assay. (A) DMEM, (B) DMEM/FCS, (C) DMEM/chloroquine (100 µM).

and further research is needed. In this study we have chosen the green monkey fibroblast-like COS-1 cell line as a model for nondifferentiated cells and the human bronchotracheal submucosal gland cell line Calu-3 as a model of a well-differentiated cell line. The aim was to develop the Calu-3 cell line as a reliable in vitro model for the assessment of gene delivery protocols to bronchotracheal tissues. PEI of high MW (600-1000 kDa, PEI I), medium MW (60 kDa), and low MW (25 kDa) was chosen as a nonviral transfection agent, since the use of viral vectors is limited by inflammatory response in the host. Furthermore the ability to control the size, potential, and sterility of non-viral the delivery vehicle is of major benefit for pharmaceutical formulations. PEI is a branched synthetic polymer that present a high cationic charge-density potential since every third atom is an amino nitrogen that can be protonated.⁴

The size of the PEI/DNA complexes presented in Figure 2 is clearly dependent on the conditions used for the preparation of the formulations. Polyplexes prepared in DMEM display a typical particle size between 250 and 300 nm with small variations and low polydispersity (0.05-0.2), and no clear differences were noticed between the PEI formulations of different MWs. We checked for possible interference effects of DMEM by measuring the size of standard beads in both PBS and DMEM and found no discrepancies (data not shown), meaning that DMEM does not interfere with data acquisition. In DMEM/FCS (cell culture medium) the sizes of the polyplexes were in the same range as in DMEM but the polydispersity of the system was clearly increased (0.1-0.5). Serum proteins present in FCS adhere to the polyplexes and result in the formation of aggregates, but small and discrete particles are predominant. Again, no

Figure 7. Lightmicroscopic photograph (100x magnification) of the specific staining (PAS) of intracellular mucus vesicles (blue) in Calu-3 cells at an air interface. Clearly visible is the expression of villi at the apical membrane.

clear differences were detected between PEI formulations of different MWs. When the lysosotropic compound chloroquine was added to the DMEM formulations, a dramatic increase in particle size and polydispersity index was detected. Olavarrieta et al²⁵ have shown that chloroquine is a planar molecule that interacts with DNA by intercalation between the two strands of the DNA double helix. This intercalation causes a reduction in DNA twist by first removing the natural negative supercoiling, resulting in relaxed pDNA and at high concentration even add net positive supercoiling. The chloroquine concentrations tested in these studies were 0.5, 10, 20, 40, and 100 $\mu\text{g/mL}$, corresponding with concentrations of 8, 19, 38, 78, and 190 μM . The results clearly demonstrate that the increasing chloroquine concentration indeed influences the electrophoretic mobility of plasmid DNA. Boussif et al⁴ postulate that PEI ensnares DNA via electrostatic interactions, leading to spontaneous complex formation, indicating that PEI/DNA complexes are not rigid but flexible systems that can be influenced by a DNA intercalator. Chloroquine-induced DNA relaxation drastically increases the shape and molecular radius of DNA molecules, enabling multiple interaction with different PEI molecules, usually present in excess, and in this way promotes the formation of larger aggregates. This process accounts for the increase in size that occurred during the size measurements in the presence of 100- μM chloroquine. Additional size measurements of PEI/DNA complexes at concentrations of 30- and 60- μM chloroquine are presented in Figure 3. We found that chloroquine has the most profound impact on the complex formation between DNA and PEI III. This might be explained by the relatively smaller MW of PEI III compared to PEI I and II, resulting in a higher number of free PEI molecules and leading to a more progressive aggregation with the relaxed DNA. Furthermore, the smaller PEI III molecules experience less electrostatic and steric hindrance in each other's vicinity, promoting extensive aggregation. The chloroquine effect was also visible at lower chloroquine concentrations (30 and 60 μM) and was pronounced at an N/P ratio of 1.7 for all three PEI formulations. At this N/P ratio, the zeta potential data show that there is only a slight net positive charge (± 10 mV). Such systems are prone to aggregation phenom-

Figure 8. Hoechst 33258 staining in COS-1: 5% DMSO in DMEM (A); serum deprivation in PBS (B); negative controls DMEM, DMEM/FCS, DMEM/chloroquine (100 μM) (C, D, E, respectively); PEI I in DMEM, DMEM/FCS, DMEM/chloroquine (F, G, H, respectively); PEI II in DMEM, DMEM/FCS, DMEM/chloroquine (I, J, K, respectively); PEI III in DMEM, DMEM/FCS, DMEM/chloroquine (100 μM) (L, M, N, respectively). The assayed N/P ratio was 16.7 and 40x magnification. Arrows indicate apoptotic nuclei.

ena because of diminished electrostatic repulsion between the molecules. The DNA relaxation event additionally increases the aggregate formation, probably by bridge formation between the PEI/DNA complexes.

These data shed new light on the previously postulated proton-sponge effect,⁴ which implies that chloroquine does not increase the transfection efficiency of PEI because PEI as such is able to buffer the pH in the lysosomal compartments. Nevertheless, the polydispersity of the gene delivery system might also account for loss in transfection efficiency.

The zeta potential of the polyplexes in 10-mM HEPES, low-salt medium (Figure 4), shows no significant differences between polyplexes prepared with PEI of different MWs. It is clear that decreasing PEI concentration also decreases the net positive charge of the polyplexes and at an N/P ratio of 0.2 even a slight negative charge is present because of an excess of DNA. The polydisper-

sity of the measured particles was small (0.05-0.2), indicating that PEI/DNA complex formation is a spontaneous process induced by electrostatic interactions between the positive amine moieties in PEI and the negative phosphate groups in DNA.

In COS-1 cells, it is noticeable that there is an optimal PEI concentration at which successful transfection and toxicity are in balance (Figure 5). The lower transfection efficiency at an N/P ratio of 16.7 is reduced because of toxic effects of free PEI molecules, although the size of the particles did not differ significantly from those at lower N/P ratios, excluding the DMEM/chloroquine data. At an N/P ratio of 0.2, the zeta potential measurements show a net negative charge, indicating that there is insufficient PEI available for complexation, delivery, and protection of DNA against nucleases, resulting in an inefficient gene transfer. The net positive charge at N/P ratios above 0.2 is necessary for increased transfection efficiency. An interesting fact is that the presence of serum proteins increases the toxic effects of PEI (Figure 5B). The polydispersity seen in the size data indicates that polyplexes of different sizes are formed, and it might well be that not only PEI/DNA particles but also PEI/protein complexes are present. In this case, the cells are exposed to a much higher PEI concentration because more "free" PEI is complexed by proteins and internalized by the cells, compared to for the DMEM formulations, resulting in an increased toxic effect. Nevertheless, the presence of serum did not decrease the transfection efficiency; in the case of PEI III, a slight increase could even be noticed, but the toxicity was also more evident than with PEI I and II. Addition of chloroquine (Figure 5C) did not increase the transfection efficiency of the polyplexes, and the toxicity was also low. The question arises whether this effect is caused by the proton-sponge effect, the measured increase in particle size, or a combination of the two. Von Harpe et al²⁶ suggested after potentiometric determinations that the optimal buffering capacity of PEI lies above the physiological pH, and for this reason another effect might play a role. Our size measurements indicate that this is the case given the dramatic increase in size when chloroquine was added to polyplexes formed in DMEM. However, PEI III, which had a lower MW, displayed an apparently better cellular uptake, leading to increasing toxicity.

The situation in Calu-3 cells is completely different (Figure 6). MW and transfection vehicle do not seem to play a very important role, although again in the presence of FCS a slight increase in transfection efficiency was detected. Calu-3 cells that are 18 days old are fully differentiated and are not prepared to easily accept a foreign plasmid DNA, unless they are forced to do so by means of increased toxicity as seen at high PEI concentrations. The lower transfection efficiency in Calu-3 cells might also be caused by the exocrine activity of the cells. Calu-3 cells are able to produce mucus that consists of

Figure 9. Hoechst 33258 staining in Calu-3: 5% DMSO in DMEM (A); serum deprivation in PBS (B); negative controls DMEM, DMEM/FCS, DMEM/chloroquine (100 μ M) (C, D, E, respectively); PEI I in DMEM, DMEM/FCS, DMEM/chloroquine (F, G, H, respectively); PEI II in DMEM, DMEM/FCS, DMEM/chloroquine (100 μ M) (I, J, K, respectively); PEI III in DMEM, DMEM/FCS, DMEM/chloroquine (100 μ M) (L, M, N, respectively). The assayed N/P ratio was 16.7 and 40x magnification. Arrows indicate apoptotic nuclei.

glycosaminoglycans that can bind to the polyplexes in the medium, leading to aggregate formation and impeding the uptake of the polyplexes. It was also shown that anionically charged heparins are able to destabilize polyplexes, causing premature release of the DNA in the culture medium or in the cytoplasm. Microscopic studies (Figure 7) have clearly shown the presence of mucus-containing vesicles in the Calu-3 cells that might fuse with the lysosomal compartments where the PEI/DNA complexes are localized and disrupt the PEI/DNA interaction before the PEI is able to deliver the DNA into the nuclei of the cells. This hypothesis might also explain the higher toxicity seen in DMEM formulations in Calu-3 compared to COS-1. Complexes of PEI with mucins might lead to disruption of PEI/DNA interactions,¹⁰ and "free" PEI can be complexed and internalized by the cells, leading to higher toxicity profiles. The relatively lower toxicity profiles in the case of chloroquine addition can be explained by formation of such large complexes, which are virtually excluded from intracellular uptake because of their size. Next to PEI we have also investigated the transfection efficiency of DOTAP (N(1-(2,3-Dioleoyloxy)propyl)-N,N,N,-trimethylammonium phosphate) as a cationic lipid mi-

celle and chitosan as a cationic biopolymer, and we have found lower efficiencies than for PEI (data not shown). DOTAP exerts similar toxic effects as PEI, while chitosan is less toxic. These data underline the difficulty of transfecting well-differentiated cells, better comparable to the physiological situation than COS-1 cells, enforcing the strength of Calu-3 cells as a representative and promising in vitro model for the in vivo situation.

Finally, we tried to further elucidate the cellular events following the exposure of COS-1 and Calu-3 cells to PEI. Godbey et al⁶ have shown that PEI is taken up by the cells and trafficked to the nucleus. One could expect that the presence of a polycationic compound in the merely negatively charged environment of the nucleus might lead to transcriptional arrest processes or apoptotic signaling. An important cellular event during apoptosis is the cleaving of chromosomal DNA and packing in compartments, preparing the cell for breakdown into apoptotic bodies that can be efficiently cleared by adjacent cells without further damaging the tissue or triggering an immune response.^{27,28} When the cells are fixed with formaldehyde and the nuclei are stained with a fluorescent dye, the formation of apoptotic nuclei can be visualized by fluorescence microscopy. An often-used nuclear dye is Hoechst 33258, and incubation with DMSO (5% or more) or serum starvation is used as a positive control for apoptosis induction in cells. Assuming that PEI/DNA is transported into the cell nucleus and that Hoechst 33258 aspecifically stains DNA, we could not use PEI/DNA complexes for these studies, since the staining of DNA in polyplexes might induce artifacts. In Figures 8 and 9, panels A and B show these controls in COS-1 and Calu-3 cells, respectively. The presence of typical apoptotic nuclei is indicated by arrows. The negative controls did not show the presence of any apoptotic nuclei. Overall, the presence of apoptotic nuclei was low (about 5%), in COS-1 as well as in Calu-3, when the highest N/P (16.7) ratio was applied to the cells. At lower ratios, no apoptotic nuclei could be detected (data not shown). This result indicates that PEI toxicity leads to cell death via a signal different from the apoptotic signaling. Cellular plasma membrane disruption might be the cause of PEI toxicity.

CONCLUSION

From this study we can conclude that Calu-3 cells are a suitable model for the bronchotracheal epithelium that can be used for the development of more efficient nonviral gene delivery systems and thus meet the need for an in vitro system that accurately represents the in vivo situation, reducing the need to use laboratory animals. PEI is a highly efficient vector for delivery of plasmid DNA into nondifferentiated COS-1 cells, and for this reason it can be used as an inexpensive, universal nonviral

transfection agent for expression of transgenes for mechanistic studies in molecular biology. However, to examine transfection efficiency and toxicity (eg, in clinical trials for the treatment of a genetic disease such as CF), PEI will still have to be further developed in order to successfully overcome the barriers for gene transfer present in the cells affected by the disease.

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