A Novel Non-Viral Vector for DNA Delivery Based on Low Molecular Weight, Branched Polyethylenimine: Effect of Molecular Weight on Transfection Efficiency and Cytotoxicity

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Purpose. Low molecular weight branched polyethylenimine (LMW-PEI) was synthesized and studied as a DNA carrier for gene delivery with regard to physico-chemical properties, cytotoxicity, and transfection efficiency.

Methods. The architecture of LMW-PEI, synthesized by acid catalyzed ring-opening polymerization of aziridine was characterized by size exclusion chromatography in combination with laser light scattering and ^{13}C-NMR-spectroscopy. In vitro cytotoxic effects were quantified by LDH and MTT assay and visualized by transmission electron microscopy. The potential for transgene expression was monitored in ECV304 cells using luciferase driven by a SV40 promotor as reporter gene system.

Results. LMW-PEI (Mw 11'900 D) with a low degree of branching was synthesized as a DNA carrier for gene delivery. In contrast to high molecular weight polyethylenimines (HMW-PEI; Mw 1'616'000 *D*), the polymer described here showed a different degree of branching and was less cytotoxic in a broad range of concentrations. As demonstrated by transmission electron microscopy the LMW-PEI formed only small aggregates which were efficiently taken up by different cells in the presence of serum, most likely by an endocytic pathway. LMW-PEI yielded transfection efficiencies measured via expression of the reporter gene luciferase which were up to two orders of magnitude higher than those obtained with HMW-PEI. The reporter gene expression was concentration dependent, but in contrast to lipofection independent of serum addition.

Conclusions. The LMW-PEI described here is a new, highly efficient, and non-cytotoxic vector with a favorable efficiency/toxicity profile for gene therapeutic applications.

KEY WORDS: gene transfer; cytotoxicity; polyethylenimine; polyfection.

INTRODUCTION

The transfer of exogenous DNA into eukaryotic cells for experimental or therapeutic purposes has turned out as the "Achilles' heel" of in vitro and in vivo gene therapy. Different viral and non-viral vector systems are currently under intensive investigation (1). Retroviral, adenoviral, and adeno-associated viral vectors produce highly efficient transfections regarding the number of transfected cells (2). However, drawbacks provoked by the viral elements such as immune and/or toxic reactions, recombination with the wild-type virus and insertional mutagenesis (3) have stimulated efforts to improve synthetic non-viral transfer systems based on cationic lipids (lipofection) or cationic polymers (polyfection) (4).

HMW-PEI, a high molecular weight, highly branched polymer with a high cationic charge density, attracts special interest due to its lysosomal buffering capacity; potentially protecting DNA from degradation by lysosomal enzymes. Since every third atom is nitrogen, the overall protonation level increases from 20% to 45% when pH 7 drops to pH 5 rendering the polymeric network to a proton sponge with the potential of causing lysosomal swelling and rupture (5,6).

Different commercially available PEIs in the range from 25'000 to 800'000 D have successfully been used for in vitro and in vivo gene delivery of DNA and RNA either alone or as complex with additional components such as viral proteins and targeting devices (7–11). More than 25 cell lines and primary cell culture systems were successfully transfected by DNA/800 kD PEI complexes. In serum-free medium more than 90% of murine 3T3 fibroblasts expressed the β-galactosidase reporter gene under optimized conditions (12). Transfection efficiencies in the same range as those reported for adenoviral gene transfer were obtained in the mature mouse brain in vivo, whereas the low molecular weight 25 kD PEI yielded higher transfer rates compared to the 800 kD polymer (13). Similar results were reported for the in vivo gene delivery to rat kidneys using PEIs of different molecular weights (14). However, polyethylenimine with molecular weights <2000 D failed to yield protein expression as described by several authors (10,15).

To clarify the effects of molecular structure and mass of polyethylenimine (PEI) on the transfection properties on one hand and cytotoxicity on the other hand we synthesized PEI of low molecular weight (LMW-PEI) and compared the LMW-PEI to commercially available PEI with 800 kD (HMW-PEI).

MATERIALS AND METHODS

Synthesis of Low Molecular Weight Polyethylenimine

4 g of aziridine were dissolved in 50 ml of distilled water, and under magnetic stirring 0.5 ml of 37% HCl were added. Polymerization was initiated at 50°C and continued for 4 days. After removal of water under reduced pressure, the polymer was dissolved in 25 ml ethanol, and precipitated in 200 ml of cooled ether (0°C). The isolated polymer was dried in vacuo (1 mbar) at 40°C. Low molecular weight polyethylenimine was stored in a desiccator over silica gel at room temperature.

Characterization of Polyethylenimine Polymers

HMW-PEI was purchased from Fluka (Neu-Ulm, Germany) as an aqueous 50% (w/v) solution. ^{13}C-NMR spectra were obtained from D_2O solution at 30°C on a JNMR500 (Jeol) spectrometer. Absolute molecular weights were determined by

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laser light scattering, carried out using a miniDawn light scattering detector (Wyatt Technology, Wellensieck, Minden, Germany) and a K5 cell in water. Size exclusion chromatography (SEC) was carried out on suprema linear columns (8 × 300 mm, 10 μm, Polymer Standards Services GmbH, Germany), thermostatted at 30°C using 0.01 M aqueous sodium hydroxide as solvent and a differential refractometer as detector (Merck RI 71, Merck, Darmstadt, Germany). At a wavelength of 690 nm, scattering angles were 45.6°, 90°, and 134.4°. Weight-averaged molecular weights were calculated from light scattering data using the method of Zimm (16). Calculations were performed using the ASTRA software (Wyatt Technology, Wellensieck).

MTT-Assay

L929 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (Gibco) and 2 mM glutamine without antibiotics at 37°C, 10% CO_2, and 95% relative humidity. The MTT assay was performed according to the method of Mosmann (17). Polymer solutions were prepared in serum supplemented tissue culture medium containing 2 mM glutamine and sterile filtered (0.2 μm). pH and osmolarity of the preparations were routinely measured and adjusted to pH 7.4 and 280–320 mosm/kg. L929 cells (8000 cells/well) were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany). After 24 h the culture medium was replaced with 100 μL serial dilutions of the polymers and cells were incubated for 3, 12, and 24 h. 20 μL sterile filtered MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Deisenhofen, Germany) stock solution in phosphate buffered saline (PBS) (5 mg/mL) were added to each well reaching a final concentration of 0.5 mg MTT/mL. After 4 hours unreacted dye was removed by aspiration, the formazan crystals were dissolved in 200 μL/well DMSO (Merck) and measured spectrophotometrically in an ELISA reader (Titertek Plus MS 212, ICN, Eschwege, Germany) at a wavelength of 570 nm and 690 nm. The spectrophotometer was calibrated to 0 absorbance using culture medium without cells. The relative cell growth [%] related to control wells containing cell culture medium without polymer was calculated by [A] test/[A] control × 100.

LDH-Assay

100 μl from a total of 2 ml culture medium from ECV 304 cells, which had been cultivated with PEI for different time periods, were collected for LDH measurement. 50 μl were mixed with 1 ml reaction solution containing 56 mM Tris/HCl pH 7.4, 5.6 mM EDTA, 0.45 mM sodium pyruvate, and 282 μM NADH. Change of absorbance was read at room temperature for 3 minutes at 340 nm and LDH activity was calculated from the slope of the linear curve obtained. For total cellular LDH cells were worked with PBS, scraped in 1 ml PBS, and by freeze-thawing. 50 μl of the cell homogenate were disintegrated for the assay. Percentage of LDH-release was calculated as follows:

$$\%\text{LDH-release} = \frac{\text{total LDHmedium}}{\text{total LDHmedium} + \text{total LDHcells}} \times 100$$

Transmission Electron Microscopy

For electron microscopy cells were fixed as described by Ito and Karnovski (18), dehydrated, and embedded in Epon according to standard procedures (19) and analyzed on a Zeiss EM 109 electron microscope (Zeiss, Jena, Germany).

Gel Retardation Assay

10 μg plasmid and the appropriate amounts of the PEI stock solutions (0.9 mg/mL, pH 7.4) were each diluted to 250 μl 150 mM sodium chloride pH 7.4 and vortexed. After 10 minutes at room temperature, the diluted PEI was added to the DNA solution and the resulting mixture was vortexed again. After 10 more minutes, the complex was used for analysis. 50 μL aliquots of the complex solution were mixed with 5 μL loading buffer (50% (v/v) glycerol 85%, 1 mM EDTA, and 40 mM Tris/HCl pH 7.4) and loaded onto an ethidium-bromide containing 1% agarose gel. Electrophoresis (Blue Marine 200, Serva, Heidelberg, Germany) was carried out with a current of 80 V (LKB 2197 Power Supply, Pharmacia, Freiburg, Germany) for 2 h in TAE running buffer solution (40 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA).

Transfection Experiments

For gene transfer studies cells (2.4×10^4 cells per cm^2) were grown in DMEM supplemented with 10% FCS and gentamycine at 37°C and 5% CO_2 in a humidified chamber. 24 h after seeding, the medium was changed to a volume of 2.5 mL. For three dishes 10 μg plasmide (pGL3-vector, Promega, Mannheim, Germany) and the appropriate amount of PEI stock solution (0.9 mg/mL, pH 7.4, sterile filtered) were diluted into 750 μL each with 150 mM sodium chloride. PEI/DNA ratios were calculated on the basis of PEI nitrogen per DNA phosphate and expressed as PEI/DNA equivalents (7). After 10 minutes at room temperature the PEI solution was pipetted to the DNA. After 10 more minutes at room temperature 500 μL of the PEI-DNA solution was given to the 2.5 mL medium and cells were incubated 1–6 hours. Medium was changed again and cells were incubated for 36–48 hours. Luciferase gene expression was quantified by using a commercial kit (Promega) and photon counting (Philipps scintillation counter PW 4700, Philipps, Eindhoven, Netherlands). All experiments were run in triplicate and data were expressed as relative light units (RLU).

RESULTS

Synthesis and Characterization of the LMW-PEI

LMW-PEI was synthesized by acid catalyzed ring-opening polymerization of aziridine in aqueous solution at elevated temperatures (20). Structure and composition of the polymers were characterized by size exclusion chromatography in combination with a light scattering detector and ^{13}C-NMR spectroscopy. Since tertiary amino groups represent the branching points of the polyethylenimine polymers, the degree of branching can be calculated from the secondary/tertiary amino group ratio. The ratio of ^{13}C-signals associated with primary, secondary, and tertiary aminogroups for LMW-PEI are 28.4:49.8:21.8, whereas HMW-PEI yields a ratio of 34.0:35.4:30.59, indicating that LMW-PEI is less branched than HMW-PEI. HMW-PEI showed a very broad distribution of the molecular weights in the range from 10^4 to 10^7 D in agreement with product specifications. In

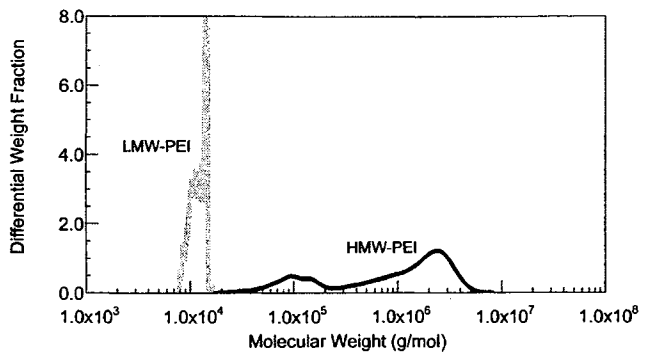

Fig. 1. Physicochemical characterization of HMW and LMW polyethylenimine. Determination of the molecular weight (Mw) by size exclusion chromatography in combination with laser light scattering. HMW-PEI: Mw 1,616 × 10^3 D, LMW-PEI: Mw 11.89 × 10^3 D.

contrast, LMW-PEI showed a very narrow polydispersity and an average molecular weight of 11,900 D (Fig. 1).

In Vitro Cytotoxicity

The in vitro cytotoxicity of LMW-PEI was compared to HMW-PEI using the lactate dehydrogenase (LDH) release (Fig. 2a, b) and the MTT-assay (Fig. 2c, d). The PEIs were added to the culture medium in different concentrations and the LDH release into the medium as a marker for the degree of damage of the cellular membrane was measured after 30 or 60 minutes, respectively. Under the experimental conditions LMW-PEI did not cause a release of LDH after 30 and 60 minutes indicating that it had no membrane damaging effect (Fig. 2a). In contrast, HMW-PEI increased the level of LDH in the culture medium at a concentration of 500 μg/mL after 30 minutes and at a concentration of 5–50 μg/mL after 60 minutes incubation time (Fig. 2b). These observations were obtained both with ECV 304 cells and L929 fibroblasts.

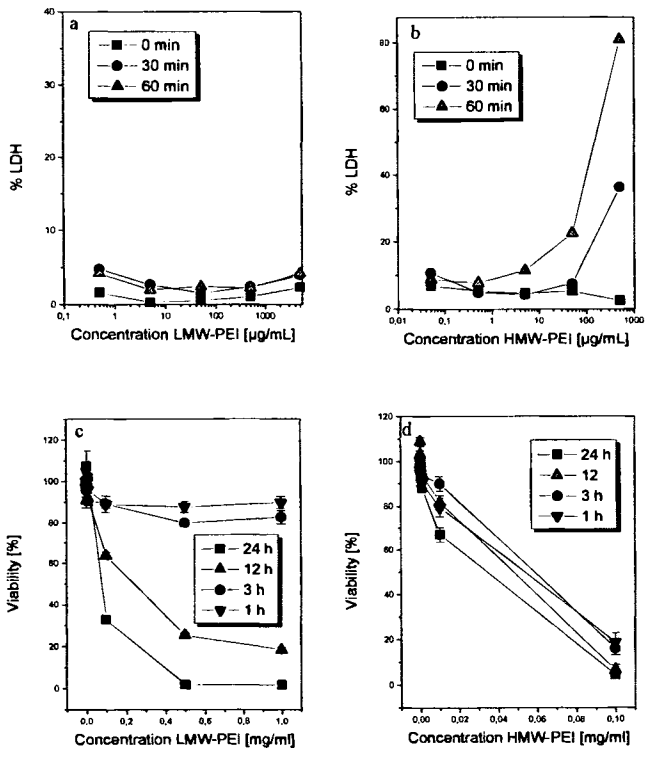

Fig. 2. Viability of ECV304 cells (a, b) and L929 mouse fibroblasts (c, d) after application of either HMW-PEI or LMW-PEI in various concentrations. LMW-PEI did not alter the viability of cells as shown by LDH release (a). Using the MTT assay (c) only at high concentrations and long incubation times a significant toxic effect on mitochondrial activity could be observed using LMW-PEI. In contrast, both assays showed a concentration and time dependent cell damage when HMW-PEI was used (b,d). Final concentrations of HMW-PEI used in cell transfection experiments as described below were maximal 6 μg/mL during a 1 hour incubation time.

By using the MTT-assay, similar results were obtained with L929 mouse fibroblasts recommended by many standard institutions as reference cell line for testing cytotoxicity of polymers. HMW-PEI exposure caused a rapid and complete loss of cell viability at an IC_{50} > 35 μg/mL independent from the time of incubation (Fig. 2d). In contrast, significant toxic effects on mitochondrial activity could not be observed using LMW-PEI at concentrations up to 1 mg/mL after exposure for up to 3 h (Fig. 2c). With longer exposition times up to 24 h, the IC_{50} of HMW-PEI is one order of magnitude lower than LMW-PEI.

Visualization of the Cytotoxic Effects

The high cytotoxicity of HMW-PEI is caused by a high affinity binding on the outer surface of the plasma-membrane. As demonstrated by transmission electron-microscopy (Fig. 3a, b) this polymer precipitates in huge clusters (2–6 μm) adhering to the cell surface. HMW-PEI binding caused a massive necrosis which is already seen after 30 minutes by phase contrast microscopy.

In contrast, when cells were treated with LMW-PEI only small aggregates in the range of 10–50 nm could be detected on the outer surface of the plasma-membranes (Fig. 3c). In accordance with the LDH- and MTT-assay the morphology of these cells did not differ from untreated cells. Ultrastructurally, several LMW-PEI particles were enclosed by forming endocytic vesicles of about 350 nm in size, which would be compatible with a macropinocytotic uptake mechanism (Fig. 3d, e). These data demonstrate the cytotoxicity and uptake of PEI is affected by polymer size and structure. High cationic charge densities, a compact and highly branched structure as well as high molecular weights affect the biocompatibility of PEI in a negative sense.

Gel Retardation Assay

The interaction between the positively charged polymers and the plasmid at different charge ratios was analyzed by agarose gel electrophoresis (Fig. 4). The analysis of the free expression vector DNA (lane 6, 15) revealed two fluorescent bands corresponding to the supercoiled and circular forms of the plasmid. HMW-PEI (lane 7) and LMW-PEI (lane 16) gave no detectable signal. At the HMW-PEI/DNA ratio of 1 (lane 1) a fraction of plasmid was still free to migrate in the gel. Simultaneously, another fraction of plasmid DNA revealed a retardation in mobility indicating the complexes were larger in size and/or less negatively charged than free DNA. Increasing the HMW-PEI/DNA ratio from 1 (lane 1) up to 20 (lane 2–5) there was a drop in the total ethidium bromide staining, probably due to the exclusion of the dye by plasmid condensation. LMW-PEI showed a complete retardation of DNA at 2 to 26.67 PEI

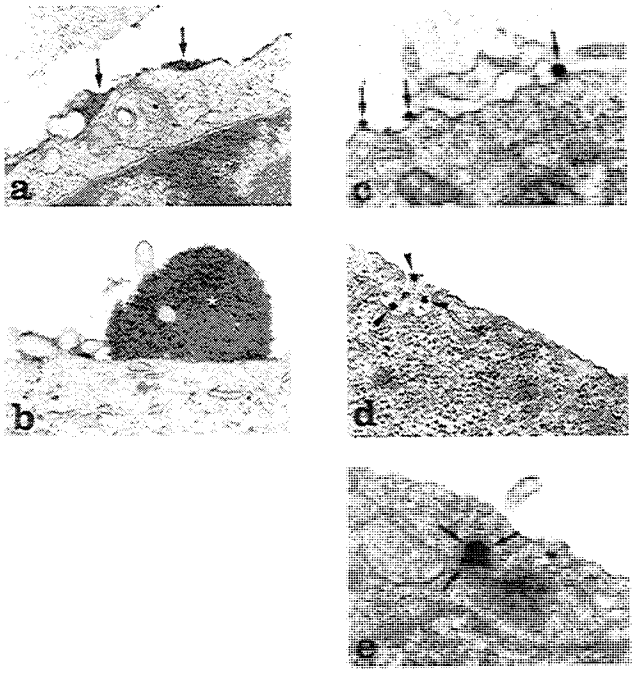

Fig. 3. Electron microscopy of ECV304 cells incubated with HMW-PEI and LMW-PEI. HMW-PEI covered the cell surfaces with a 10–20 nm thick sheet (a, arrows). In addition, huge complexes were formed which were also associated with the plasma-membrane (b, star). Most cells became necrotic when treated with higher concentrations as 5 μg/mL, even after incubation times as short as 30 minutes. When ECV304 cells were incubated with LMW-PEI only small aggregates were observed at the cell surface (c, arrows), while the morphology of cells was not different from control cells. LMW-PEI particles were enclosed by plasma membranes (d, arrowheads) and internalized in vesicles of about 350 nm in size (e, arrows). magnification a: ×40′000; b, c, d: ×50′000.

nitrogen per DNA phosphate (lane 8). A complete inaccessibility of complexed DNA to the intercalated dye could be achieved using LMW-PEI/DNA ratios of 40 and higher (lane 11–14).

Transfection Efficiency and Serum Dependency

To examine the potential of LMW-PEI for introducing exogenous DNA into eukaryotic cells we compared the transfection efficiency of LMW-PEI and HMW-PEI with regard to the PEI concentration and the serum stability of the complexes. As a reporter gene system we used the luciferase gene driven by a SV40 promoter. When the cell line ECV304 was transfected with pGL3/HMW-PEI complexes of varying PEI concentrations, transfection levels increased up to 1.8 μg PEI/μg plasmade (N/P = 13.33). Lower ratios were less efficient, whereas at higher ratios (N/P = 20) a significant decrease in transfection efficiency was observed most likely due to a cytopathic effect (Fig. 5a). Phase contrast microscopy revealed changes in the morphology of the cells. Strikingly, LMW-PEI was 100-fold more efficient at N/P = 26.67, and the expression of luciferase could even be increased using up to 66.66 LMW-PEI nitrogen per DNA phosphate without cytotoxic effects (Fig. 5b). Similar results were obtained with 3T3 fibroblasts (data not shown). When transfection experiments were performed with 3T3 cells using medium supplemented with serum, no significant difference in expression of luciferase could be observed compared with transfection experiments carried out without serum. Moreover, after a 6 h incubation with LMW-PEI/DNA complexes the serum treated cells yielded significantly higher expression levels of luciferase than serum-free treated cells (Fig. 6). Finally, transfection efficiency was dependent on the transfection time. This effect varied between cell lines used, but in all cell lines tested a maximum of transfection efficiency was achieved after 6 h incubation of cells with LMW-PEI/DNA complexes (data not shown). Prolonged exposure of cells to polycation-plasmid

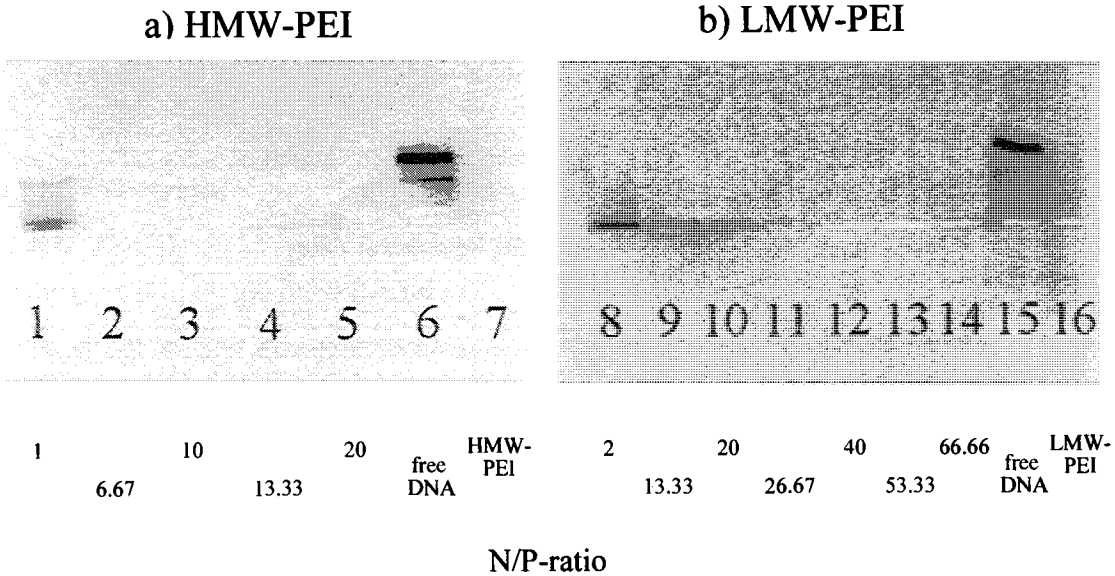

Fig. 4. The effect of increasing concentrations of HMW-PEI (a) and LMW-PEI (b) on the electrophoretic migration of plasmid DNA through an agarose gel. Increasing amounts of PEI were added to a constant amount of 10 μg DNA as described under "Materials and methods." The complex composition was calculated on the basis of PEI nitrogen per DNA phosphate and expressed as PEI/DNA equivalents according to Boussif *et al.* (7).

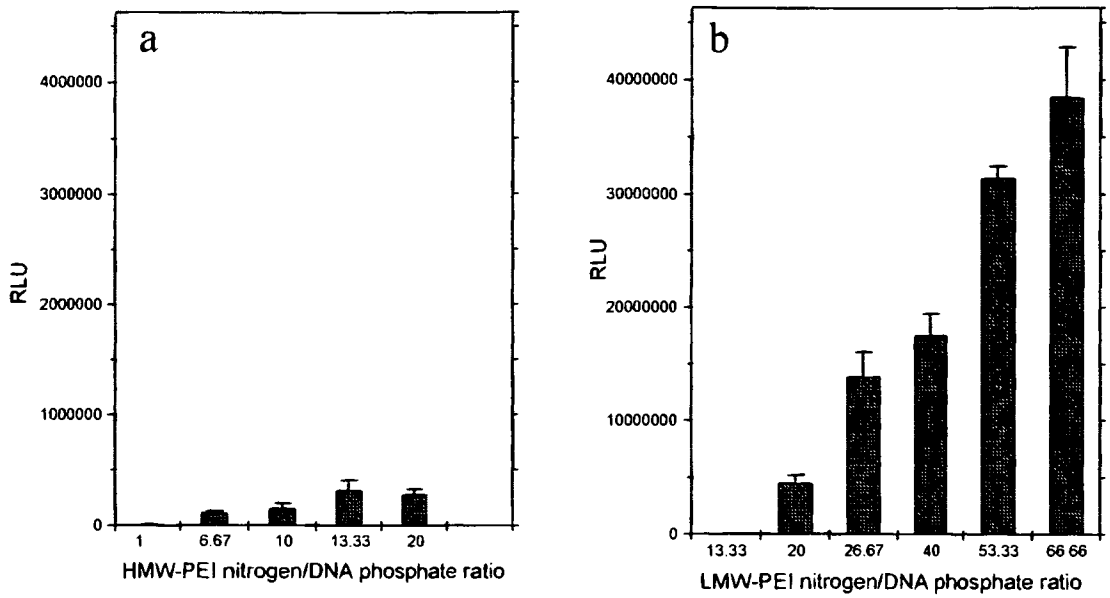

Fig. 5. Luciferase reporter gene expression under the SV40 promotor as a function of the PEI nitrogen/plasmid phosphate ratio. Each bar is the mean value of RLUs obtained from three independent culture dishes. Comparison between HMW-PEI (a) and LMW-PEI (b) using ECV304 cells. LMW-PEI could be used over a broad concentration range without inducing cytopathic effects, but with a 100fold higher transfection efficiency than HMW-PEI.

complexes increases both the probability of complex uptake and cytotoxic effects. This balance seems to be quite favorable for LMW-PEI complexes.

DISCUSSION

The development of a non-toxic and highly efficient non-viral gene delivery system applicable for human use is one of the major goals for human gene therapy. In this report, we describe a new polyethylenimine derivative with (i) a low molecular weight and narrow size distribution, (ii) a low degree of branching due to a high amount of secondary amino residues, (iii) relatively low cytotoxicity, and (iv) a high efficiency for the transfer of plasmid DNA into higher eukaryotic cells under in vitro conditions.

Commercially available HMW-PEI has successfully been used in various in vitro and in vivo experiments as a vector for DNA and RNA delivery (7). However, in vitro cytotoxicity studies demonstrated toxic effects for HMW-PEI compared to other polycationic macromolecules such as DEAE-dextran, polyvinyl pyridinium bromide, and cationized human serum albumin (21). In our preliminary experiments we observed highly cytotoxic effects of different PEIs depending on the molecular weight, the polydispersity and the surface charge density of the polymers (22). Similar observations were reported for poly-L-lysine (23). In addition, HMW-PEI may accumulate during in vivo applications, since there is neither a degradation pathway nor a mechanism of excretion for such molecules. In contrast to HMW-PEI, the LMW-PEI described here showed an acceptable cytotoxicity profile under in vitro conditions. At incubation times and polymer concentrations significantly higher than used for transfection experiments no loss of cell viability could be detected either by LDH-release or by the MTT-assay. It should be noted that the cytotoxicity test carried out using cationic polymers not complexed with DNA gives a worst case estimate for the interaction with cells and tissues. The cytotoxicity of non-viral transfectants, such as cationic lipids or polymers is usually decreased when DNA is added. To allow a direct comparison of the cytotoxic effects of polycations we report here studies in a mouse fibroblast cell line which is an established model to characterize the biocompatibility of polymers (21) as well as cell viability in a endothelial cell line ECV 304 which is considered to be relevant for the parenteral application of PEI/DNA complexes. The physicochemical characteristics of polymer-plasmid complexes and studies characterizing uptake and intra-cellular trafficking will be reported separately.

Macromolecules, which cause biological effects in cells, usually attach to the cell surface (24). The adsorption of HMW-PEI to the plasma-membrane was extraordinarily high and resulted in the formation of a polymer layer on the cell surface

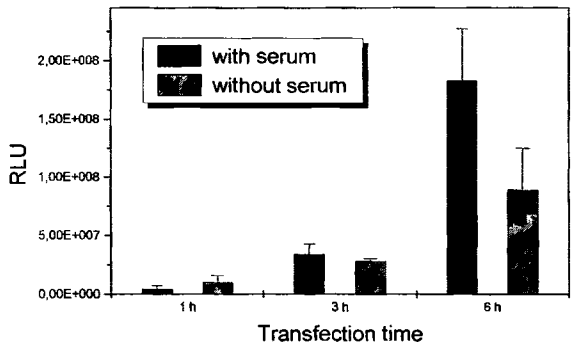

Fig. 6. Comparison of transfection efficiencies in serum-free and serum supplemented DMEM medium using 3T3 fibroblasts with pGL3/LMW-PEI complexes (10 μg PEI/μg plasmid). All experiments were run in triplicate. Cells were incubated for 1, 3, and 6 h, respectively.

as revealed by electron microscopy. Moreover, large HMW-PEI aggregates were formed, also decorating the cell surfaces. We speculate that HMW-PEI deposition impairs plasma membrane functions dramatically leading to cell death by necrosis. By contrast, LMW-PEI did not associate with the plasma-membrane leading to large aggregates on the cell surface. Small aggregates in the range of 10–60 nm in diameter were found inside ECV304 cells by electron microscopy. This size of these aggregates is compatible with an endocytic uptake of LMW-PEI. Further experiments are necessary to elucidate the endocytic uptake mechanism of PEI and PEI/DNA complexes.

The importance of cytotoxicity is further underlined by our results from cell transfection experiments. A significant decrease in transfection efficiency was observed using HMW-PEI/DNA at N/P ratios higher than 13.33. This is most likely due to cytopathic effects as well as exclusion of the huge aggregates from endocytic pathways and has also been reported by others for 3T3 cells (7). Thus HMW-PEI can be used for cell transfection only in a narrow therapeutic concentration range. In contrast, a 100x higher expression of luciferase using LMW-PEI as vector system was observed, most likely due to its low cytotoxicity and the formation of microaggregates capable of being internalized by endocytosis. Since in vivo high doses of DNA are often necessary to reach a pharmacological response, our PEI derivatives offers a possibility to avoid toxic side effects by the carrier systems especially with regard to high doses and multiple applications. These findings are in agreement with the observation that linear, low molecular weight (22 kD) PEIs are efficient vectors for gene delivery both under in vitro and in vivo conditions (25–27). Detailed studies on effects of the PEI structure and molecular weight on DNA complexation, transfection, and in vivo distribution have not been reported so far. The hypothetical mechanism for endosomal release of PEI/DNA complexes, also designated as the "proton sponge trick" (5), seem to be associated with the physico-chemical properties of PEI. Further studies are necessary to elucidate these effects.

Many cationic non-viral vectors are known to be sensitive to serum. In contrast to cationic liposomes such as Lipofectin (28) and Transfectam (12), the transfection efficiency of LMW-PEI was serum independent, an important prerequisite for in vivo gene delivery. When 10% fetal calf serum were added to the transfection medium, the activity of the LMW-PEI/DNA complexes was not inhibited. Obviously, the complexes tolerate the presence of other negatively charged macromolecules such as serum proteins, and the plasmid DNA is also protected from degradation by nucleases in the serum. In contrast, Boussif *et al.* (12) reported a decrease of transfection efficiency using HMW-PEI in serum supplemented instead of unsupplemented medium. Taken together this means the favorable properties of our LMW-PEI concerning cytotoxicity and transfection efficiency depends on its structural properties like size with low polydispersity, branching, and surface charge density. Interestingly, we were able to induce the expression of luciferase with our LMW-PEI derivative (10 kD), whereas different authors observed gene expression only with higher molecular weights. Using 2–10 kD PEI, gene delivery could not be achieved and was dependent on the presence of additional components such as viral elements (10,15).

In summary, LMW-PEI with a low degree of branching and a narrow molecular weight distribution, is an extremely efficient non-viral transfection vector. In contrast to commercially available high molecular weight PEI, no cytotoxicity but a highly efficient gene delivery to different cell types could be observed with LMW-PEI under in vitro conditions. The ability of LMW-PEI to form stable complexes with DNA in the presence of serum and the potential of its reactive amino groups for further modifications with the opportunity of targeted gene delivery are attractive features meriting further investigations as a in vivo gene delivery system.

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