Polyelectrolyte Nanoparticles Mediate Vascular Gene Delivery

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Purpose. The purpose is to develop a non-viral gene delivery system that meets the requirements of colloidal stability of DNA complexes expressed in terms of no particle aggregation under physiologic conditions. The system should be used to transfect cardiovascular tissues.
Methods. We used a strategy based on the formation of polyelectrolyte nanoparticles by deposition of alternatively charged polyelectrolytes onto a DNA core. Polyelectrolytes were transfer RNA as well as the synthetic polyanion, polyvinyl sulfate (PVS), and the polycation polyethylenimine (PEI). The PEI/DNA complex formed the DNA core.
Results. We observed that the DNA is condensed by polycations and further packaged by association with a polyanion. These nanoparticles exhibited negative surface charge and low aggregation tendency. *In vivo* rat carotid artery experiments revealed high transfection efficiency, not only with the reporter gene but also with the gene encoding human urokinase plasminogen activator (Hu-uPA). Hu-uPA is one of the proteins involved in the recovery of the blood vessels after balloon catheter injury and therefore clinically relevant.
Conclusions. A strategy for *in vivo* gene transfer is proposed that uses the incorporation of polyanions as RNA or PVS into PEI/DNA complexes in order to overcome colloidal instability and to generate a negative surface charge. The particles proved to be transfectionally active in vascular gene transfer.

KEY WORDS: β-gal expression; human urokinase plasminogen activator; nanoparticle-mediated gene transfer; vascular gene transfer.

INTRODUCTION

Efficient delivery of nucleic acids remains a major obstacle in non-viral gene therapy. Colloidal particles (polyplexes and lipoplexes) carrying DNA serve as non-viral gene vectors. However, their efficiency is poor. The high tendency to aggregate in physiologic salt solutions and inability of cells to take up large aggregates *in vivo* have been major hindrances (1–3). Low dissemination in tissues and induction of thrombosis provide additional problems (4,5). Recent data suggest that coating the polycation/DNA complexes with polyanions can diminish particle aggregation (increasing colloidal stability) (6,7). The strategy was originally based on studies by Sukhorukov and colleagues (8). They showed that the development of nanoparticles by stepwise deposition of alternatively charged polyelectrolytes onto a charged core leads to polyelectrolyte nanoparticles that are able to encapsulate different pharmacological agents, proteins, and so forth. Highly efficient interaction between DNA and polycation in the core results in formation of a condensed particle that could be further packaged by association with a polyanion. We reasoned that this approach would allow the formation of compact particles with low aggregation capacity. Moreover, additional charged layers might protect encapsulated plasmid DNA both from substitution by the charged plasma components from the DNA-polycation complex, and from nuclease degradation. This approach was first used for DNA encapsulation using a DNA/polylysine core (6). Transfection activity of these particles was shown by these authors in a later paper (7). Here, we created polyelectrolyte nanoparticles that exhibited colloidal stability and high transfection efficiency in an *in vivo* model. In opposite to other authors (7), we demonstrated by measuring of the ζ-potential a negatively charged particle surface and could exclude aggregation.

MATERIALS AND METHODS

Plasmids

Full-length cDNA for human uPA (1.6 kb) which was generously donated by R. Beabealashvilly (Cardioloy Research Center of Russia, Moscow, Russia) and cDNA for E.coli β-gal (3.4 kb) was directionally inserted into the pcDNA3 expression vector (Invitrogen, Frederick, MD, USA). The expression of uPA, β-gal and pcDNA3 is transcriptionally regulated by the CMV IE promoter/enhancer. pCMV Luc was a gift of V. Sandig (MDC).

Nanoparticle Assembly

The reporter plasmids pcDNA3 β-gal, pCMV Luc and the plasmid pcDNA3 Hu-uPA were grown in *Escherichia coli* DH5, isolated, and purified using standard methods (Qiagen Plasmid Maxi Kit, Hilden, Germany). Transport RNA Type V from wheat germ and pluronic F127 were from Sigma (St. Louis, MO, USA). PEI was from Aldrich (Steinheim, Germany), catalog number 40872-7. It has 55,800 Da and an oligomerization level of approx. 7 (information of C. Plank gratefully acknowledged). Polyvinylsulfate (PVS) was from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Nanoparticles were prepared in Hepes buffer (20 mM, pH 7.4) by adding the polyanion to the preformed core. The PEI/DNA core complex with N/p = 8 was prepared as given in (9). N/p = 8 corresponds with PEI/DNA = 1:1 (w/w). The following con-

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ABBREVIATIONS: PVS, polyvinyl sulfate; PEI, polyethylenimine; Hu-uPA, human urokinase plasminogen activator; N/P, nitrogen/phosphate ratio; RLU, relative light units; PBS, phosphate-buffered saline; BSA, bovine serum albumin; R_i, weight ratio polyanion/DNA; PCS, photon correlation spectroscopy; VSMC, vascular smooth muscle cell.

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ditions which were established as described in the Results section were selected for the *in vivo* experiments: DNA/PEI/polyanion (1:1:6 wt/wt/wt).

In vitro Transfection Measurements

About 2×10^5 ECV 304 cells per well of a 24-well plate grown in RPMI 1640 medium with L-glutamin (Gibco BRL) supplemented with 10% FCS were used for transfection. Nanoparticles on the basis of 2 μg pCMV Luc, PEI/DNA 1:1 w/w and tRNA as above in R_i ratios (tRNA/DNA w/w) of 1–6 were produced by adding the tRNA to the PEI/DNA core complex in 20 mM Hepes and brought to 0.15 M NaCl in 200 μl volume. The nanoparticles were mixed with 0.8 ml culture medium and added to the cells. In another experimental setup 0.1 mM chloroquine was present in the transfection mixture. Transfection and luciferase activity were performed as described in (3).

Physicochemical Measurements

Size and Zeta potential measurements were performed using a Malvern Zetasizer (Malvern Ind., Malvern, England). Turbidity was obtained by absorbance measurements of nanoparticles in 0.15 M NaCl at 400 nm in a 1 cm cuvettes.

Animals

Male Wistar-Kyoto rats (4 to 5 months old) were obtained from a colony maintained at the Cardiology Research Center, Moscow, Russia. Their left common carotid artery was subjected to balloon catheter injury using surgical procedures approved by the Cardiology Research Center's Animal Experimentation Committee.

Surgery and Gene Delivery into the Arterial Wall

Rat carotid arteries were injured with an inflated balloon catheter as described previously (10). Briefly, after anesthetizing the rats with ketamine (100 mg/kg body wt, intraperitoneally, Astrapin Pharma EmbH, Pfaffen-Schwabenheim, Germany), a midline incision was made in the neck to expose the left external carotid artery. A 2 F Fogarty arterial embolectomy catheter (Baxter Healthcare, Deerfield, IL, USA) was introduced into this artery through an arteriotomy and passed into the common carotid artery to the aortic arch. The balloon was inflated and then slowly rotated while pulling the catheter back toward the external carotid artery. This was repeated three times and then the external carotid artery was ligated. The contralateral right carotid artery as well as uninjured left carotid arteries from sham-operated rats served as controls. For periadventitial administration of shells, the arteries were gently dissected free of their surrounding connective tissue and then 0.5 ml Pluronic gel (40%) in PBS containing preformed nanoparticles or not covered cores was placed around the injured artery. Pluronic F127 (50%) gel was prepared in PBS at 4°C and mixed with the nanoparticles (100 μl particle solution plus 400 μl of gel). After mixing salt concentration was adjusted to 0.15 M NaCl. The gel polymerized once loaded on the vessel segment of about 1.5 cm length. The incisions were closed and the animals were allowed to recover. β-galactosidase reporter gene and Hu-uPA gene activities were assessed in the vessel segment after 7 days.

Tissue Collection and Processing

The animals were deeply anesthetized with sodium pentobarbital (100 mg/kg body wt; Sanofi, Sante Animale), and Evans blue (60 μg/kg body weight; intravenously) was administered, so that removal of endothelium in the damaged vessels could be confirmed. Then the animals were perfused (120 mmHg) with saline solution, followed by 4% formaldehyde solution for 10 min. Left and right common carotid arteries were removed, cleaned of extraneous material and cut into three equal segments before embedding in paraffin or Tissue-Tek (Sakura Finetek, Mijdrecht, The Netherlands). Cross-sections (7 μm for immunohistochemistry), were cut from each block, at 100- to 200-μm intervals.

Histochemistry and Microscopy

Electron micrographs of nanoparticles were obtained with a Zeiss EM 910 electron microscope at an accelerating voltage of 80 kV after negative staining of the samples with 1% uranyl acetate on a formvar coated grid. For the detection of β-galactosidase expression, the excised aorta segments were fixed in PFM, stained with β-gal assay, cut into slices of 7 μm in deeply frozen state, lightly stained with hematoxylin and analyzed by microscopy. The immunohistochemical detection of Hu-uPA was performed on frozen sections (7 μm) according to the standard method used in our laboratory. The sections were fixed in 10% solution of formalin in PBS for 2 min at room temperature, than they were fixed in 96% ethanol for 30 min at –20°C. The activity of endogenous peroxidase was blocked by incubation of sections in 3% solution of hydrogen peroxide in PBS for 30 min at room temperature. The nonspecific staining caused by secondary antibodies was blocked by incubation of sections in 10% solution of normal horse serum in 1% BSA/PBS for 30 min at room temperature. The sections were immunoreacted with solution of mouse monoclonal antibodies (Ab) against Hu-uPA (Loxo, Germany) in 1% BSA/PBS, concentration 6.7 μg/ml for 1 h at room temperature. Bound primary Ab were detected with biotin-conjugated horse anti-mouse antibodies in 1% BSA/PBS (Vector Laboratories, Germany), concentration 5.0 μg/ml and with Vectastain ABC Kit (Vector Laboratories, Germany). The staining was visualized with the diaminobenzidine reaction (DAB chromogen system; Immunotech, Hamburg, Germany). Sections were lightly counterstained by Mayer's hematoxylin and analyzed by microscopy. Total cell numbers in the neointima (i.e., hematoxylin stained nuclei in the neointima) were determined by counting (three sections per rat).

All results are mean ± SE of mean (SEM.). Comparisons between groups were performed using the one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using 'Jandel SigmaStat'.

RESULTS

Nanoparticle Assembly, Characterization, and Transfection Measurements

The polycationic core consisted of polyethylenimine (PEI) and a plasmid DNA. Fixed ratio N/p = 8 was used in all experiments (9). Covering of the core was performed with either natural polyanions such as transfer-RNA (tRNA) or

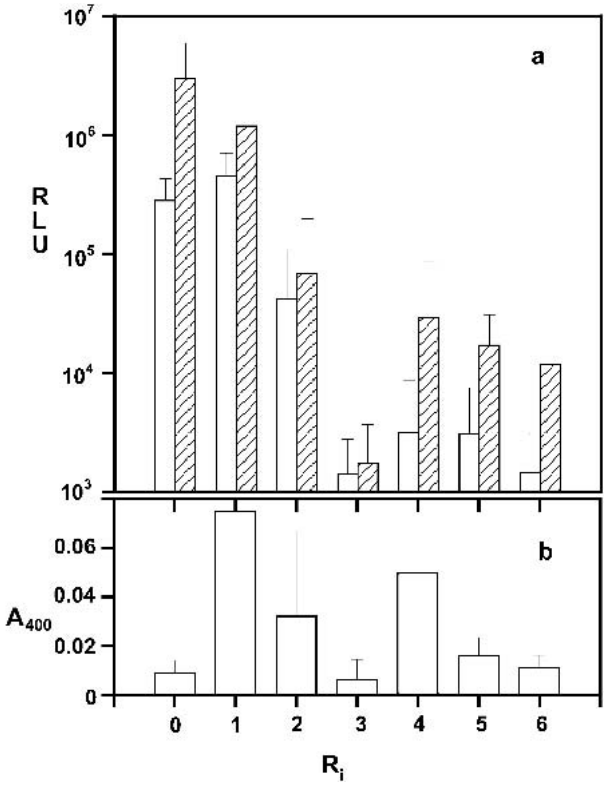

Fig. 1. Transfection activity on ECV 304 cells (a) and turbidity (b) of nanoparticles in a range of R_i values (weight ratio polyanion/DNA, here tRNA/pCMV Luc). Nanoparticles were produced on the basis of 2 μg DNA per sample and PEI/DNA = 1 wt/wt. White columns are without chlorquine, hatched columns with 0.1 mM chloroquine. Turbidity was measured as A_{400} in Hepes buffer, 0.15 M NaCl.

synthetic polyanions, for example, polyvinylsulfate (PVS). Optimal colloidal stability was empirically determined by a indirect method. We compared *in vitro* transfection and turbidity of nanoparticles at a series of polyanion (here tRNA) concentrations, given as weight ratio polyanion/DNA R_i (Fig. 1). DNA and PEI concentration were kept stable at 1/1 wt/wt. Turbidity (measured in 0.15 M NaCl) and transfection changed in parallel (Fig. 1). Highest transfection was found at a ratio $R_i = 1$ where also highest turbidity, that is, aggregation was seen (nearly electroneutrality). At $R_i = 6$ no turbidity and only very small *in vitro* transfection was observed. As expected, the nanoparticles were more transfectionally active if 0.1 mM chloroquine was added to transfection mixture. From findings in literature (1–3) that transfection is depending on the size of the complexes and that small complexes do only poorly transfect, but are colloidally stable, we concluded that optimal colloidal stability was achieved at the following ratio: $R_i = 6$ or DNA/PEI/polyanion 1:1:6 wt/wt/wt.

Colloidally stable nanoparticles were characterized in Hepes buffer and physiologic salt solution according to size/aggregation by PCS and surface charge by ζ-potential (Table I) as well as by electron microscopy (Figs. 2D, 2E, and 2F). The nanoparticles showed a spherical shape, negative surface charge and low aggregation tendency under physiologic conditions. In opposite to these nanoparticles, the DNA/PEI complexes were positively charged and aggregated with increasing time (see also Ref. 9). Thus, the transfection efficiency of PEI/DNA complexes was possibly due to aggregation and to the positive ζ- potential in sharp difference to the aggregation-free and negatively charged nanoparticles.

Vascular Gene Delivery

For vascular gene delivery, we devised an application method using pluronic gel F127 as a bio-tolerated adhesive (10,11). Our purpose was to keep nanoparticles in contact with the targeted vessel for a longer time in order to facilitate uptake. Briefly, the carotid artery was injured using a balloon catheter, and 0.5 ml pluronic gel (40%) in PBS containing either preformed nanoparticles or uncovered PEI/DNA cores were placed around the injured artery segment. The nanoparticles contained 6.6 μg pcDNA3 β-gal plasmid or 3.3 μg per animal for tRNA- or PVS nanoparticles, respectively. After operation, the gel kept the nanoparticles in close contact with the vessel and provided effective perivascular gene delivery. We repeated the experiments of Edelman *et al.* (13) and found that after 3 days the gels had completely dissolved whereas the release of naoparticles was observed to occur after 1 day. The vessel segments were assayed for β-galactosidase gene expression after 7 days. Figure 2 shows β-galactosidase expression in the rat carotid artery using pcDNA3 β-gal containing tRNA shells (Fig. 2A) or the corresponding PVS shells (Fig. 2B). Figure 2C shows a PEI/DNA control. β-galactosidase was expressed in more than 50% of the cells in the periadventitia and in 20% of the cells in the adventitia. In the media, a few cells showed expression. The DNA/PEI (core) control containing 6.6 μg DNA was virtually inactive. We also performed controls with DNA (pcDNA3 β-gal) and pluronic gel as above in order to show that the β-gal expression was indeed due to the nanoparticles. In the case of high DNA amount (150 μg) in pluronic we obtained an expression comparable to nanoparticles, in the case of low amount (6.6

Table I. Physical Characterization of Nanoparticles (Mean ± SEM from at Least 3 Experiments)

Type of particles	Size (nm) 20 mM Hepes pH 7.4	Size (nm) 150 mM NaCl	Polydispersity 20 mM Hepes pH 7.4	Polydispersity 150 mM NaCl	ζ-Potential (mV)
DNA/PEI N/P = 8	61.8 ± 5.2	*169.2 ± 21.3	0.24 ± 0.03	0.20 ± 0.06	+35.1 ± 1.9
tRNA shells $R_i = 6$	90.0 ± 10.3	153.0 ± 16.8	0.23 ± 0.03	0.35 ± 0.04	–38.4 ± 2.2
PVS shells $R_i = 6$	70.9 ± 6.7	135.4 ± 9.3	0.20 ± 0.04	0.58 ± 0.05	–46.0 ± 4.9

* Immdiately after mixing, >2000 nm after 2–4 hours.

μg) we obtained no expression. Thus, at least at low DNA concentration, pluronic gel as used herein is not able to enhance β-gal expression.

Human uPA Expression and Cell Accumulation in the Developing Neointima

Based on the fact that urokinase plasminogen activator (uPA) stimulates the migration and proliferation of vascular SMC, the key processes in vessel wall remodeling after injury (10,13), we next compared the effect of uPA gene delivery on accumulation of neointimal cells after balloon catheter injury with the effect of recombinant human uPA application. As we have shown previously, the recombinant uPA resulted in augmented neointimal cell accumulation after vessel injury (10). The tRNA shells containing 6.6 μg of the plasmid encoding human uPA (Hu-uPA) were applied periadventitially, as described above. Controls were pcDNA3 β-gal plasmid delivered in tRNA shells, pure pluronic F127 gel, and pluronic F127 containing 20 nmol/kg recombinant Hu-uPA. On the 7th day, animals were sacrificed and the left and right carotid arteries were subjected to histochemical analysis. Perivascular recombinant Hu-uPA application more than doubled the number of vascular smooth muscle cells (VSMC) in the neointima compared to control pure gel (Table II). The increases in neointima VSMC numbers were also observed after Hu-uPA cDNA application. However, the increase was less pronounced than the effect of recombinant uPA (Table II). Control application of pcDNA3 β-gal plasmid did not affect neointima cell numbers (Table II). Human uPA immunoreactive peptides were not detected in the uninjured and injured rat carotid arteries after application of pure gel or pcDNA3 β-gal plasmid (Figs. 2C and 2D). The expression of Hu-uPA was detected only in injured left carotid artery after Hu-uPA cDNA application and localized predominantly to periadventitial, adventitial and neointimal cells (Fig. 3A). Only unit cells in the media expressed Hu-uPA. Controls (Figs. 3B, 3C, and 3D) did not exhibit positive reaction with anti-Hu-uPA antibodies.

Table II. Neointimal Cell Number per Vessel Cross-Section (Mean ± SEM)

Hu-uPA cDNA	β-gal cDNA	rec Hu-uPa	Gel F127
862 ± 112*	536 ± 70	1337 ± 270†	507 ± 109
n = 8	n = 7	n = 6	n = 7

* $p < 0.05$ versus β-Gal.
† $p < 0.05$ versus Gel F127.

DISCUSSION

Our aim in this study was to create a new, highly effective, *in vivo* non-viral gene delivery system for vascular gene transfer. Such a system must have certain properties, namely nanosize, colloidal stability in physiologic salt solutions, and preferably also protein environment, as well as the ability to carry foreign DNA into the cells *in vivo* with subsequent gene expression. We based our work on the studies by Sukhorukov and colleagues who developed polyelectrolyte nanoparticles by stepwise deposition of alternatively charged polyelectrolytes on a charged core (8). Studies in this direction have been reported earlier by Trubetskoy *et al.* (6,7). In their recent paper they used the principle of recharging cationic DNA complexes with highly-charged polyanions and obtained efficient transfection *in vitro* and in the mouse lung at neutral or positive particle surface charge. ζ-potential was not measured. Under these conditions aggregation cannot be excluded. Plank's group also designed nanoparticles using protective copolymers, but they showed no *in vivo* application (9). Their particles had positive or slightly negative ζ-potential.

Since our aim was to assemble a transfection system that can work without any additional enhancing agents, we used polyethylenimine (PEI) as DNA compacting agent (15). PEI is known as an effective DNA carrier that, due to endosomolytic properties, can perform transfection without chloroquine or fusogenic peptides (9). N/p = 8 was chosen for two reasons. First, the transfection complexes with this ratio showed high efficacy in a wide spectrum of different cell lines (9). Second, being assembled in Hepes buffer, N/p = 8 has a small size, low polydispersity, and a positive charge (Table I).

We focused on a variety of nanoparticles with different compositions. Two different anionic polymers were tested, namely, natural anioinic polymers such as transfer RNA type

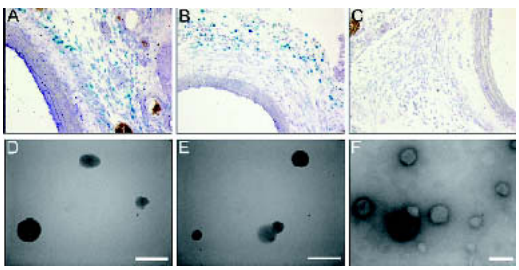

Fig. 2. β-galactosidase (β-gal) expression in rat carotid artery after gene delivery by polyelectrolyte nanoparticles. tRNA nanoparticles (A) and PVS nanoparticles (B) containing a β-gal encoding plasmid show high β-gal expression, compared with the DNA/PEI (core) control (C). Electron micrographs of the respective shells (D, E, F). Bars on micrographs indicate 100 nm.

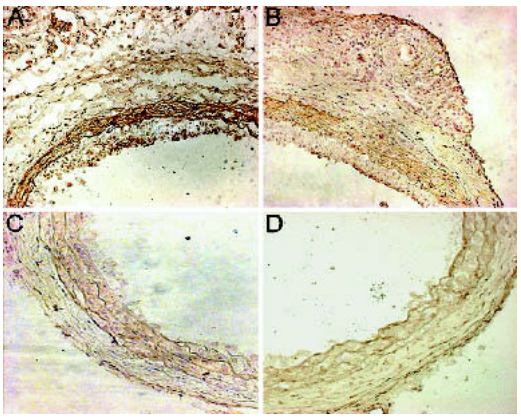

Fig. 3. Hu-uPA expression in rat carotid artery after gene delivery by tRNA nanoparticles. tRNA nanoparticles containing Hu-uPA encoding cDNA (A), an isotypic control (recombinant Hu-uPA) (B), or an irrelevant β-gal encoding plasmid (C), and empty Pluronic F127 gel (D) were transferred to the rat carotid artery as described. Immunohistochemistry was done with mouse monoclonal antibody (Ab) against Hu-uPA. Bound primary Ab was detected with biotin-conjugated horse anti-mouse Ab and stained.

V (tRNA) from wheat germ, and synthetic anionic polymers such as polyvinylsulfate (PVS). Coating was performed by adding different amounts of polyanions to the preformed PEI/DNA cores. The ratio of polyanion/DNA w/w (R_i) was varied in a stepwise fashion from 0 to 6.0. Crude turbidity experiments were performed spectrophotometrically by measuring the absorption of particle containing solutions at $\lambda = 400$ nm. Using the selection criterion of low *in vitro* transfection and turbidity, we found that the desired colloidal stability of the nanoparticles was achieved at a mass ratio polyanion/DNA equal to 6.0 (Fig. 1). Transfection could only be observed in the presence of chloroquine at this high negative particle charge. We assume that relatively low transfection efficacy was due to the small particle size and the anticipated loss of cell interaction *in vitro*. It is also reported that at such a high negative charge of the particles and physiologic salt concentration the nanoparticles become physically unstable. Polyanions may dissociate DNA complexes by competing with DNA for binding to the polycation (16). However, there was no indication for such an effect under the conditions studied here.

The properties of these complexes were then more thoroughly investigated by electron microscopy, dynamic light scattering, and measurement of the ζ-potential (Figs. 1 D and 1E and Table I). The staining pattern of the particles with uranyl acetate and negative surface charge clearly demonstrate that we actually obtained an encapsulation of the PEI/DNA core by polyanions into a shell-like structure. The absence of aggregation and the negative surface charge (ζ potential) as demonstrated in Table I were good prerequisites for *in vivo* gene delivery. Son *et al.* (17) demonstrated efficient *in vivo* gene delivery for colloidally stable negatively charged complexes of cationic liposomes and plasmid DNA (17). As well as free plasmid DNA which is strongly negatively charged, nanoparticles could be taken up by cells *in vivo* via a scavanger receptor (18). Scavanger receptors bind a variety of polyanionic ligands including polynucleotides. An unspecific sticking due to aggregates to small blood vessels and capillaries can be excluded (15). Because of their negative charge at this mass ratio polyanion/DNA, interaction with serum components should be small or not exist. Trubetskoy *et al.* (7) suggest that the ternary polyelectrolyte complexes used by them are resistant to serum inhibition. We, therefore, suggest that the relatively high negative charge of the nanoparticles could have a beneficial effect on *in vivo* gene transfer.

For the vascular gene delivery, we relied on pluronic F127 gel as a adhesive which can be eliminate from the body (10). This method allowed us to keep the nanoparticles in contact with the targeted vessel up to 3 days and therefore gave us an opportunity to apply low doses of foreign DNA. The efficacy of this delivery method was proved by us (10) and other authors (12). The method was used for the first time by Rosenberg's group (11) in order to introduce antisense oligonucleotides into vessels from the periadventitial side. Here we refer to Edelman *et al.* (13), who measured the release of oligonucletides from polymer formulations. They found complete release from pluronic gel after 1–2 days. We repeated these measurements with nanoparticles and found complete release after 3 days (not shown here). We were able to obtain high gene delivery efficiency even with as little as 3.3 μg of plasmid DNA with PVS nanoparticles and 6.6 μg DNA with tRNA nanoparticles per animal. Experiments with the β-gal reporter gene revealed that β-galactosidase was expressed in >50% of the cells in the periadventitia and in up to 20% cells in the adventitia. Expression was observed up to a depth of about 15 cell layers. Therefore, the nanoparticles showed high gene delivery efficiency and were able to deeply penetrate into the tissue. The DNA/PEI (core) control containing 6.6 μg DNA was nearly inactive (Figs. 1A, 1B, and 1C), just as free DNA in the same concentration. However, free DNA was described to be transfectionally active in the presence of a combination of pluronics (19). Although similar amounts of DNA were used as in (19), we were not able to obtain DNA delivery in the presence of pluronic F127.

Unfortunately, we found β-galactosidase only in a few media VSMC. Perhaps more DNA is required. In any event, we believe that this difficulty can be overcome if the product encoded by the plasmid is secreted by transfected cells and then distributed within the vessel wall.

The urokinase plasminogen activator (uPA) can be secreted by many types of cells and is capable of stimulating VSMC migration and proliferation *in vitro* and *in vivo*. uPA is expressed early after injury, when the neointima develops and the medial VSMC are proliferating. (10,14,20,21) As we have shown previously, periadventitial application of recombinant Hu-uPA to the injured vessel substantially stimulates VSMC accumulation in the developing neointima (10). In this study, we showed that the application of tRNA nanoparticles containing 6.6 μg of Hu-uPA encoding plasmid imitates the effect of recombinant uPA to the injured vessel although the expression does not reach the extent of recombinant protein. This could be due to the small amount of DNA used. Histochemical analysis revealed the distribution of transgene Hu-uPA expression over the entire rat vessel wall. This state-of-affairs evidently results in a significant increase in the accumulation of VSMC in the neointima as a result of increased cell migration and proliferation (Fig. 2).

Concerning the toxicity of polyelectrolyte nanoparticles, experiments are in progress. However, because of the low DNA doses diminishing the toxic effect of PEI and because PEI is presumably hidden inside the nanoparticles, toxicity should be small. Trubetskoy *et al.* (7) found only very small toxic effects.

Polyethylene glycol (PEG) coupled to polycations such as PEI or pegylated block copolymers have been used to generate surface-shielded DNA delivery systems which improve the colloidal stability of DNA complexes. Measurements of ζ potential proved the shielding of the surface charge. Such systems are transfection active *in vitro* (9) and *in vivo* (22,23). However, the use of such hydrophilic systems cannot produce negative charged delivery systems. For this purpose a polyanion has to be integrated into the DNA complex. We have shown here that such negative systems are efficient in vascular gene transfer. A possible step to improve this system could be to couple PEG to the polyanion to make the nanoparticles more symmetrical, in the ideal case round, and slippery. A smooth surface could also be useful (besides negative charge) for efficient transport in the blood.

These results and data on direct injection of nanoparticles free of Pluronic F127 gel into transplanted tumors (unpublished data) suggest that this system is suitable, not only for gene delivery to blood vessels, but also for efficient gene

transfer to other organs and tissues. We therefore believe that this method represents a significant advance in the development of non-viral gene delivery systems for *in vivo* gene transfer. The method deserves further study.

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