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Enhancement of Nonviral Gene Transfer to Endothelial Cells Using Lipofection of Histone-Complexed DNA

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ABSTRACT

Arterial gene transfer represents a novel approach for the treatment of a variety of vascular diseases. Lipofectamine (DOSPA/DOPE)-mediated transfer of pCMVβgal (7.2 kbp) to subconfluent bovine aortic endothelial cells (BAEC) resulted in a transfection efficiency of 5.7 ± 2.35% of cells staining positive for β-galactosidase for a 10:1 ratio by weight of lipid/plasmid. Using histones up to 1 histone per 5 bp increased efficiency over 3-fold higher to a level of 18.2 ± 1.8%. In confluent BAEC, which are more difficult to lipofect, the use of histones increased the transfection efficiency over 10-fold to a level of 4.5%. Histones form a tight compact structure with the DNA and also protect DNA from DNase degradation. Using histones as a compacting agent while lowering the amount of lipofectamine resulted in a large drop in transfection efficiency, demonstrating that a critical liposome/plasmid ratio had to be maintained. When histones were not used, posttransfection treatment of endothelial cells with 100 μM chloroquine, which helps in disruption of the early endosome by increasing endosome pH, caused a 2.5-fold increase over transfection levels without chloroquine. However, chloroquine provided no benefit when plasmids were histone compacted. Thus, the use of chloroquine is not required. Since the improvements in transfection efficiency with histones (via improved DNase protection) and with chloroquine (via improved endocytotic escape) were not additive, the final rate limit to transfection may be plasmid entry into the nucleus. Ligands that facilitate receptor binding or fusigenic agents that promote endosome escape can aid in lipofection. Still, such approaches will not fully overcome the limits on efficiency that exist for nondividing endothelial cells *in vivo*.

INTRODUCTION

ARTERIAL GENE TRANSFER has been proposed for the treatment of atherosclerosis and restenosis following angioplasty. The two major strategies of arterial gene transfer are (1) the cultivation, transfection, and reintroduction of autologous endothelial cells to the recipient[1,2] and (2) the direct catheter delivery of genetic material with transfection vehicle or virus to the cells of the artery.[3–9] Several challenges exist in implementing arterial gene therapy (Table 1). A major technical challenge during coronary artery gene transfer, whether liposome mediated or virus mediated, is the slow uptake process that requires about 15–30 min

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TABLE 1. CHALLENGES IN ARTERIAL GENE TRANSFER

Problem	*Comment*	*Reference*
Packaging	Cationic lipid, histones, polylysine	10,20,24,25,29,37
Intraluminal delivery limits and washout	Flow-through double balloon catheter Polymer coating (?)	13,17,19
Boundary layer to cell surface	Enhanced mixing needed; perfusion catheter	
Binding to cell surface	Adenovirus, LDL, peptides-polylysine transferrin.	24,42,432,45
Endocytosis	Fusion peptides	43,44
Endocytotic degradation	Histones	
Endocytotic escape	Chloroquine, fusion peptides, proton pump inhibitors	20,21,30,31,32,35,36,39,42,44
Cytoplasmic degradation	Histones	42
Nuclear import	Adenovirus: mechanism unknown Liposomes: poor; requires cell division Histones not fully efficient	25,41,42,43
Genetic stability of expression	?	
Transport to vessel media	Poor; Wolinsky catheter	13,14,17,19
Adverse phenotype	Tissue factor induction Excess adenovirus is toxic and interferes with adhesion *Toxicity of lipofection reagents*	14

incubation under no flow conditions before any level of gene expression can be observed.[10,11] Methodologies that stop blood flow for excessive times are not likely to be clinically relevant because of the risk of ischemia. Flow-through double balloon catheters may reduce the risk of ischemia while providing sufficient time for incubation. Alternatively, the use of porous balloon catheters for gene transfer into the canine femoral artery results in expression of luciferase ranging from 0.1 to 22 pg of luciferase for exposure time ranging from 1 to 10 min as compared with a value of 42.3 pg using a surgical technique in which the artery is occluded for 30 min.[8]

Both liposome-mediated routes[6] and retroviral vectors are more efficient in mitotic cells.[5] *In vivo*, endothelial cells have a very low mitotic rate, and hence these methods have resulted in low levels of transfection efficiency. Adenoviral vectors efficiently transduce nondividing cells and are well suited for arterial gene transfer. In fact, high levels of expression of over 90% have been achieved with adenoviruses *in vitro*[12] and *in vivo*.[4,13–15] Although adenovirus vectors do not integrate into the genome and generate little risk of insertional mutagenesis (in contrast to retroviruses), expression is lost in a matter of weeks. With adenovirus, a level of 100% transduction of cultured endothelial cells has been achieved with a 24-h incubation, with as many a 60% of cells transduced with only a 15-min incubation.[4] Removal of the E1 region of the adenovirus genome has been shown to greatly hinder replication of adenoviruses. However, with high infection levels, the E1 region has been found to be dispensable for replication.[16]

Another problem that arises with respect to gene transfer to the arterial wall is the problem of poor medial penetration. In uninjured arteries, gene transfer is restricted to the endothelium layer,[13] whereas in injured or denuded arteries, the smooth muscle cells are transfected to a higher extent.[13,14] Smooth muscle cells in injured vessels can be transfected with an efficiency of 29% *in vivo*, with gene transfer confined to the vascular media.[14] Nabel et al., however, have reported both plasmid and retroviral vector-mediated gene

transfer in all layers of the normal pig iliofemoral artery wall,[17] whereas Leclerc et al. found liposome-mediated transfer only to the neointima.[18] To study whether liposome and viral particles can penetrate through the various layers of the arterial wall, Rome et al.[19] used collodial carbon particles (120–500 nm) similar in size to liposomes, fluorescent latex spheres (93 nm) similar in size to adenoviral and retroviral particles, and finally horseradish peroxidase (HRP), which is about 4 nm in diameter. Infusion of colloidal carbon particles into carotid arteries of sheep using a double balloon perfusion catheter resulted in 70% of injured and uninjured arteries being stained in the intima, and 19–29% of arteries showed staining of adventitia. No staining of media was observed in any of the uninjured arteries, whereas 4% of injured arteries showed medial staining. The results using latex spheres were essentially the same. Rome et al.[19] found that 98% of uninjured and 86% of injured sections had fluorescent particles in the intima, and 47% of uninjured and 55% of injured vessels had fluorescent particle accumulation in the adventitia. In both injured and uninjured vessels, only 5% of sections had any fluorescent particles in the media. When HRP was used, however, the amount of particles in the media increased dramatically, and the amount in the adventitia of injured vessels decreased. These results show that direct penetration of liposome or viral particles cannot occur through the intima into the media, whereas transfer into the adventitia can occur likely through the vaso vasorum. Hence, the specificity of gene transfer to the endothelium can be achieved, and this has been confirmed by Schulick et al., who found that more than 90% of transduced cells were endothelial cells of rat carotid artery.[9]

Liposome-mediated gene transfer is a simple alternative to viral routes, as there is little risk of immune response. Also, from a regulatory, manufacturing, economic, and ease-of-use standpoint, liposomal routes are presumed to be advantageous. For lipofection routes to succeed, a major problem to overcome is low transfection efficiency of nondividing cells. In some cell types, lipofection reagents can be associated with some cellular toxicity. As with most existing gene therapy approaches, the transience of expression will remain a challenge regardless of transfection or transduction route. In the present study, using histones for plasmid complexation in conjunction with lipofectamine, a cationic liposome, we significantly increased the levels of transfection efficiency in endothelial cells.

MATERIALS AND METHODS

Cell Culture

Bovine aortic endothelial cells (BAEC) (Cell Systems Corporation, Kirkland, WA) were grown to confluence, passed to a 6-well culture dish at a split ratio of 1:3, and then grown to 70–80% confluence. Growth medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated newborn calf serum, 0.30 mg/ml of glutamine, 150 U/ml penicillin, and 0.15 mg/ml streptomycin (GIBCO, Grand Island, NY). Cultures were maintained in an incubator maintained at 37°C and 95% humidity with 5% CO_2.

Transfection Procedure

Cationic liposomes (Lipofectamine Reagent, Gibco-BRL), containing a 3:1 by weight mixture of polycationic lipid 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1 propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) were used in all transfections. For each transfection, 10 μl of liposome (2 μg/μl) and 2 μg of DNA ($\pm$histones) were diluted into 100 μl of serum-free medium, OPTIMEM Reduced Serum Medium (Gibco-BRL). A mammalian expression vector pCMVβ (7.2 kbp) was obtained from Clontech laboratories (Palo Alto, CA). pCMVβ contains a cytomegalovirus promoter, the *Escherichia coli* β-galactosidase (β-gal) gene, and a polyadenylation sequence. The plasmid was grown in *E. coli* and purified by phenol-chloroform extractions, followed by ethanol precipitation, and finally polyethylene glycol precipitation. DNA for transfection was resuspended in TE buffer (10 m*M* Tris, pH 8.0, 1 m*M* EDTA). In some experiments, dialyzed type IIIS calf thymus histones (Sigma) in PBS were added to the DNA and incubated for 30 min to allow complexation. Varying amounts of histones were added to the DNA to optimize the amount of histones required for highest

transfection. Pure liposome (10 μl) diluted to 100 μl was added to the DNA/histone complex and incubated for 45 min, and then 0.8 ml of OPTIMEM medium was added to this mixture, which was then overlaid on the cells. The cells were incubated for 2 h at 37°C, after which the mixture was aspirated, and 4% serum in OPTIMEM medium was added, and the cells were allowed to grow for 2 days as recommended by the manufacturer and in compliance with standard incubation times used in other studies.[20,21] The cells were then fixed and assayed for β-gal activity. In some experiments, following transfection of cells for 2 h, 100 μM of chloroquine (Sigma) in OPTIMEM medium was added to the cells

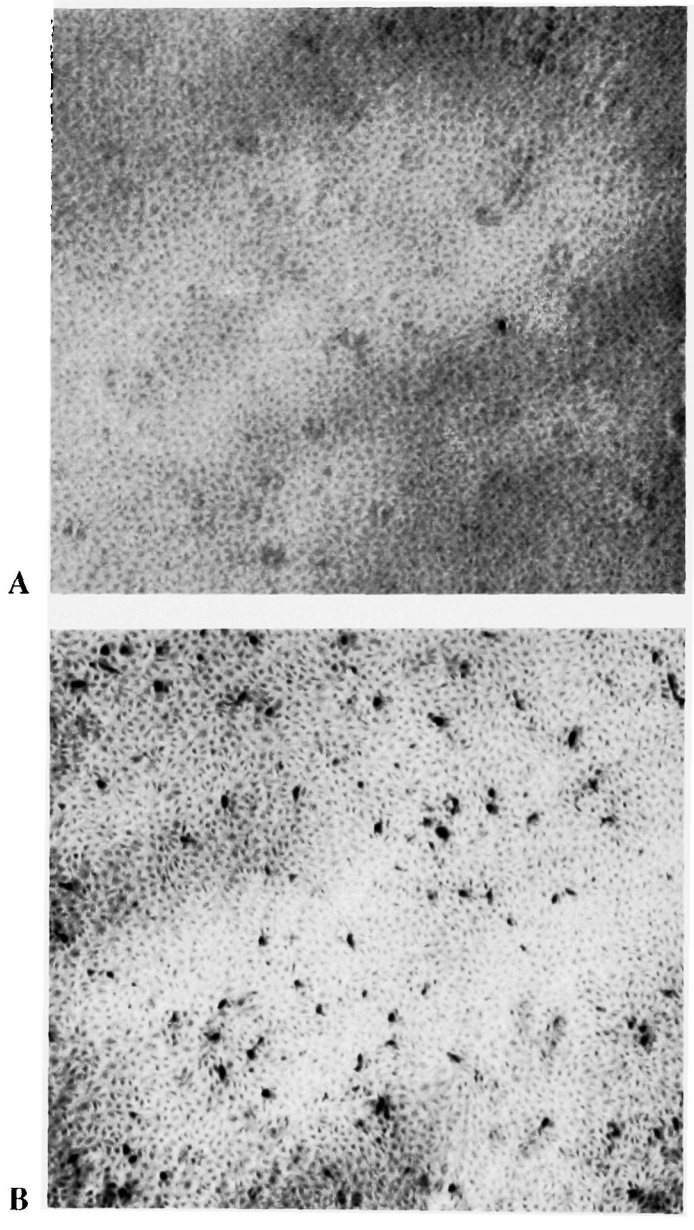

FIG. 1. Histones enhance efficiency of lipofection of confluent bovine aortic endothelial cells. Confluent endothelium were lipofected at 5 days postseeding with pCMVβ in the absence (A) or presence (B) of histones. Endothelial cells were transfected with lipofectamine (20 μg lipid per 2 μg DNA) ± histones (10 μg histone per 2 μg DNA). Cells were stained for β-galactosidase expression. Transfection of confluent monolayers resulted in 0.42 ± 0.36% efficiency without histones and 4.5 ± 1.08% efficiency with histones.

and incubation continued for 2 h. During the transfections, the cells were continuously monitored for cell viability and adhesion.

Exposure of endothelial cells to defined levels of laminar shear stress during lipofection used a parallel flow chamber as previously described,[22,23] with fluid flow driven by a Harvard syringe pump. A glass slide with endothelium was mounted on the liquid-primed polycarbonate chamber in close proximity to an opposing parallel face of the chamber. The wall shear stress imposed on the monolayer was calculated by solution of the Navier-Stokes equation for laminar flow of a newtonian liquid where the wall shear stress (τ_w = dynes/cm^2) was defined as $\tau_w = 6\ Q\mu/(B^2W)$, where flow rate, Q = cm^3/sec, visosity, μ = 0.01 poise, total gap thickness, B = 0.02 cm, and width, W = 2.49 cm. The entrance length needed for steady parabolic flow to be established was less than 1 mm. The Reynolds number for fluid flow was less than 50, indicating that the flow was laminar.

To assay for β-gal, cells were washed once with 2 ml of phosphate-buffered saline (PBS) and fixed with 1 ml of 4% paraformaldehyde in PBS. A staining solution (1 ml) containing 1 mg/ml bromochloroindolyl-galactoside (Xgal, Sigma), 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, and 2 m*M* $MgCl_2$ was added to the cells and incubated for 4 h. The cells were then rinsed with 2 ml of PBS. The percent of cells stained per field of view was counted using a Nikon phase-contrast microscope. The count was repeated with at least four fields of view for each transfected monolayer and averaged.

RESULTS

To find the optimal ratio of lipofectamine/DNA, a level of 0, 14, 20, 30, or 40 μg of lipofectamine (2 μg/μl) was complexed with 2 μg of pCMVβ. Subconfluent BAEC were then transfected and assayed for β-gal activity 48 h posttransfection. The transfection efficiency was found to be 0%, 3.46 $\pm$ 1.4%, 7.15 $\pm$ 0.7%, and 3.4 $\pm$ 1.8% at 0, 14, 20, and 30 μg lipofectamine, respectively. A level of 40 μg of lipofectamine was cytotoxic to the cells. Thus, the optimal lipofectamine/pCMVβ ratio was 10:1 by weight, where 10 μl of lipofectamine was used to transfect 2 μg of pCMVβ into subconfluent BAEC. Using this 10:1 ratio, we proceeded to transfect confluent BAEC (at 5 days postseeding) to understand lipofection in nondividing endothelial cells, a situation more typical of *in vivo* endothelium with very low mitotic rates. Direct lipofection of confluent BAEC was quite inefficient, with only 0.42% of cells transfected (Fig. 1A). When

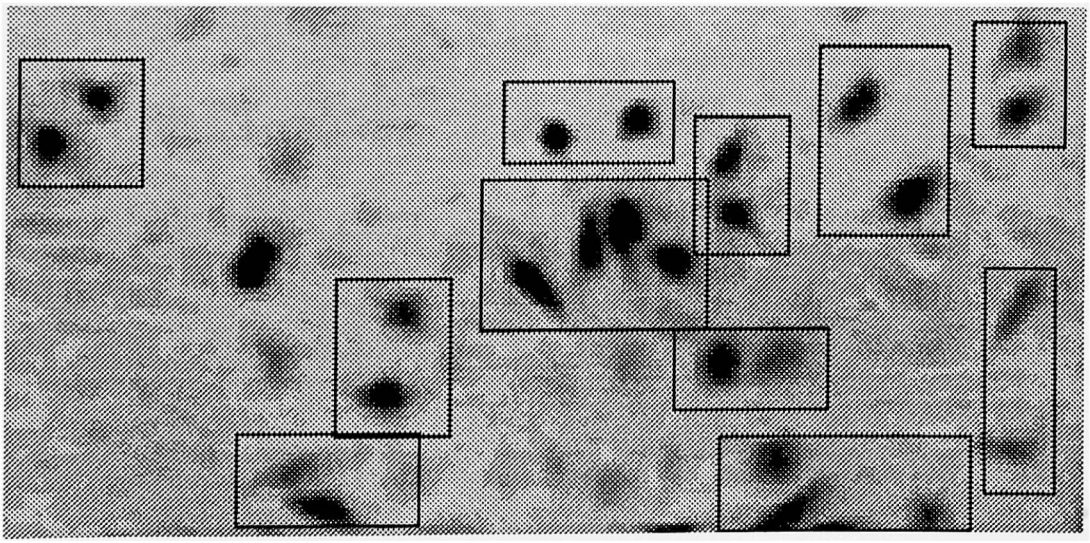

FIG. 2. The abundance of doublets of β-gal-expressing cells indicates that cellular division was the dominant mode of successful lipofection.

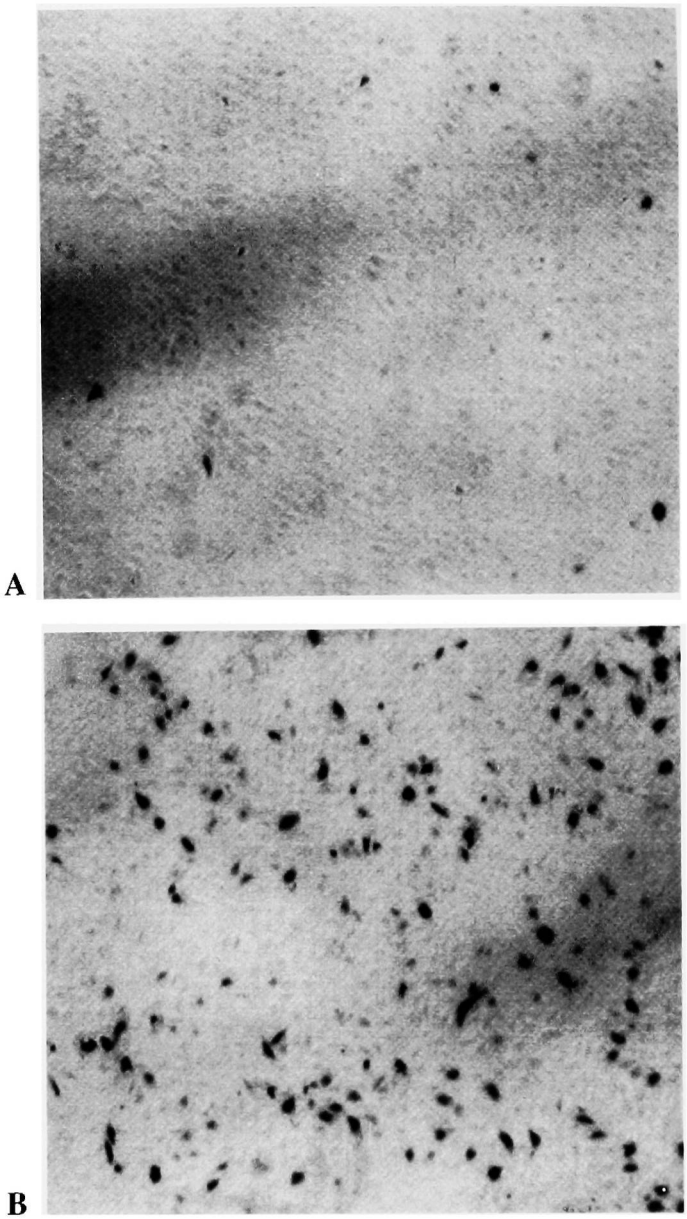

FIG. 3. Effect of histones and chloroquine on lipofection of subconfluent bovine aortic endothelial cells (2 days post-seeding) at 80% substrate coverage. Lipofection was conducted with 20 μg lipofectamine to compact 2 μg DNA alone (A) or 2 μg DNA complexed with 10 μg histones with postincubation with 100 μM chloroquine (B).

histones were used to complex the DNA, transfection efficiency increased over 10-fold to a level of 4.5% (Fig. 1B). As only 4.5% of the confluent BAEC were transfected with the histone-DNA-lipofectamine, the targeting of plasmid by histones through nuclear pores of the nucleus was not a highly efficient process. The importance of cellular division on the efficiency of lipofection can be seen in the abundance of doublets of transfected cells in a rapidly dividing population (Fig. 2). Much less frequently were single transfected cells seen with no obvious transfected neighboring cell.

Lipofection of subconfluent BAEC resulted in higher transfection efficiency than was found in confluent BAEC. The lipofection efficiency with subconfluent BAEC was of the order of a few percent (Fig. 3A) and could be enhanced severalfold with the use of histones as a plasmid-packaging agent. The highest transfection efficiency of 20 $\pm$ 1.15% was found when 10 μg histones (1 histone per 5 bp) was used for packaging 2 μg DNA (Figs. 3B and 4). Histones from calf thymus contain 27.8 wt% lysine and 2.1 wt% arginine. At one histone molecule per 15 bp, 3.09 μg of histone can neutralize 2 μg of 7.2 kb pCMVβ plasmid.

Thus, histones in 3-fold excess of the amount needed to neutralize the plasmid gave the maximum transfection efficiency (Fig. 4). Wagner et al.[24] found that neutralizing amounts of polylysine gave maximum transfection efficiency, which suggests differences between the function of DNA complexes with histones and polylysine. We have found that increasing the amount of histones above 10 μg per 2 μg DNA resulted in cell detachment, possibly by disruption of adhesion.

Lipofection of ~ 80% subconfluent endothelial cells with histone-DNA complex resulted in higher levels of transfection when compared with lipofection with DNA alone (Figs. 3 and 4). We conducted an experiment with chloroquine with and without histones to explore the relative roles of these agents on such processes of endosomal escape, plasmid stability in the cytoplasm, and nuclear import. Incubation with chloroquine of cells lipofected with DNA alone increased transfection efficiency over 2.5-fold from 3.33 ±

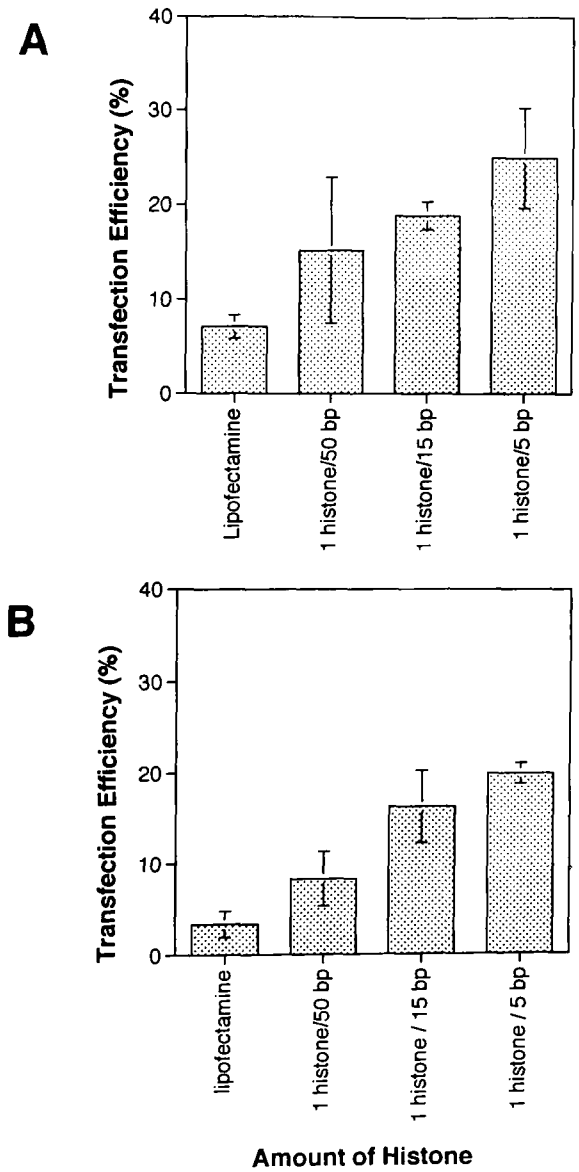

FIG. 4. Effect of histone per basepair (bp) on lipofection of subconfluent bovine aortic endothelial cells. Lipofection was conducted with 20 μg lipofectamine to compact 2 μg DNA that had been complexed with up to 10 μg histones. (A and B) Two independent experiments conducted with independent passages of BAEC.

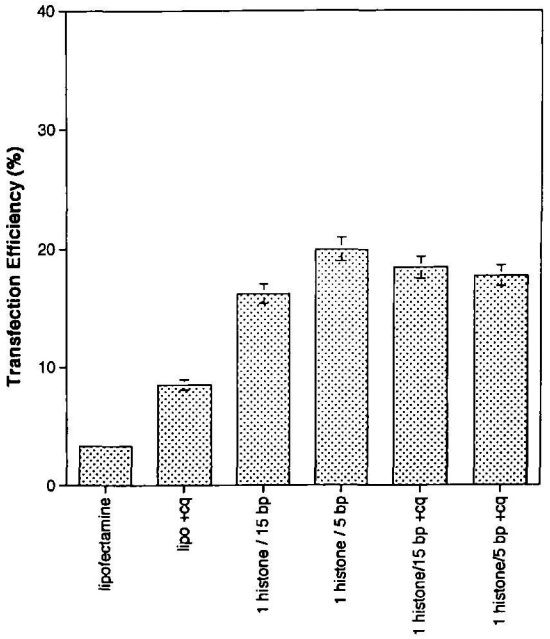

FIG. 5. The enhancement of lipofection by either histones or chloroquine is not additive. Subconfluent BAEC were transfected with lipofectamine (20 μg per 2 μg DNA) in the presence of increasing amounts of histones per DNA base-pair (bp) with and without postincubation with 100 μM chloroquine (cq).

1.45% to 8.53 ± 3.53% (Fig. 5). However, the addition of chloroquine to cells transfected with DNA-histone/lipofectamine complex did not further increase the transfection efficiency significantly (Fig. 5).

If histones had facilitated nuclear transport because of their nuclear targeting sequences, the use of chloroquine and histones would be at least partly additive, yet this was not found. It is possible that chloroquine leads to DNase inhibition, and thus histones and chloroquine would not be additive in their enhancement of lipofection efficiency. However, it is not clear why chloroquine only gives about 8% transfection effi-

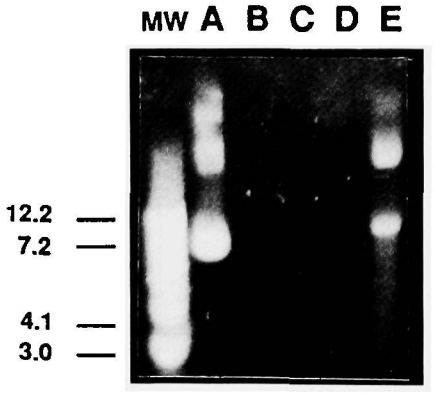

FIG. 6. Complexation of pCMVβ with histones provides DNase protection. MW marker (lane 1, kbp on left); pCMVβ (lane A); pCMVβ in Tris buffer pH 7.4 after 2 min treatment with 1 U DNase (lane B); pCMVβ in Tris buffer pH 8 after 2 min treatment with 1 U DNase (lane C); pCMVβ in Tris buffer pH 7.4 containing 100 μM chloroquine after 2 min treatment with 1 U DNase (lane D); pCMVβ-histone complex after 2 min treatment with 1 U DNase (lane E). The reactions were stopped by adding EDTA; 10 μl proteinase K (1 μg/μl) was then added to all the reaction mixtures, followed by phenol/chloroform reaction. Comparison of lanes A and E demonstrate the DNase protection effect of the histones, and comparison of lanes A and D show that chloroquine does not inhibit DNase activity. All samples were run on 0.7% agarose gel.

ciency, whereas histones give about 20% transfection efficiency if the two agents work by the same mechanism. From our experiments, we concluded that histones did not dramatically aid nuclear transport but rather provided protection against DNase degradation in either the endosome or cytoplasm. To study these issues in a purified system, we measured the DNase degradation of pure plasmid. DNA without histones was completely degraded in 2 min by 1 U of DNase at pH 7.4 (Fig. 6, lane B). To test if DNase activity is modulated by the basic conditions found in chloroquine-treated endosomes or by chloroquine directly, we measured DNase activity on plasmids at pH 8.0 and in 100 μM chloroquine (Fig. 6, lanes C and D). Basic conditions or high levels of chloroquine had no effect on the rapid degradation of plasmid by DNase. In lane E of Figure 6, the histones provided marked DNase protection of the plasmid. Although basic conditions and chloroquine did not inhibit DNase, histone complexation of plasmid provided substantial resistance to degradation.

When liposome is added to DNA, the DNA causes fusion of the liposomes and, above a critical ratio, the DNA is packaged into a tight complex[25] suitable for endocytosis. As histones act as a packaging agent, it was thought that the amount of lipofectamine reagent (an expensive reagent) could be reduced if the lipofectamine needed only to serve as an endosomal disruption agent. The transfection efficiency dropped over 90% when the amount of lipofectamine was lowered from 10 μg lipofectamine to 1 μg lipofectamine per μg of DNA (Fig. 7). An amount of lipofectamine above the critical value needed for complexation had to be present for high transfection efficiency. This indicated that cationic liposomes not only provided packaging but also played a critical role in endothelial endosomal escape, which cannot be replaced with chloroquine.

The variation of transfection efficiencies with time of incubation was also studied, as this is a critical issue in arterial gene transfer by intraluminal catheter. The minimum time of exposure to achieve a reasonable amount of expression was found to be 2 h (Fig. 8). Under 2 h, the transfection efficiency dropped below 1%. As with the other cell types, endothelial cell uptake of the complex is a slow process. Under flow conditions, the complex may be washed easily from the surface of the cell. We conducted transfection of endothelial cells under flow conditions to study this issue. Endothelial cells were grown to 80% confluence on glass slides and perfused with the DNA-lipid complexes (10 μg lipofectamine per 2 μg DNA) at a wall shear stress of 10 dynes/cm^2 for 5 min using a Harvard infusion pump and a parallel-plate flow chamber. The cells

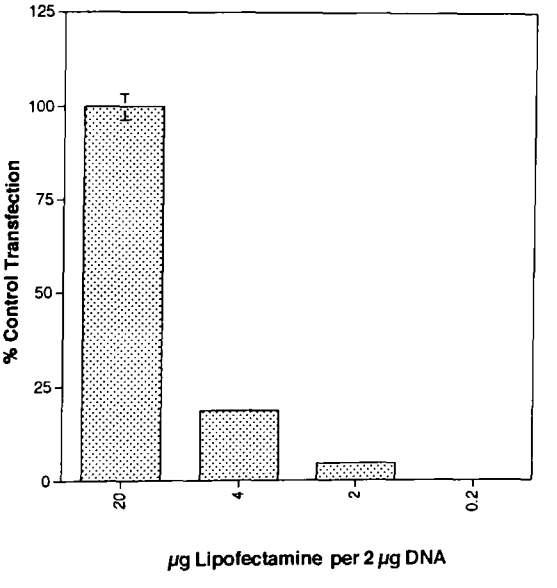

FIG. 7. Effect of lipofectamine reduction on transfection of DNA-histone complex with postincubation with chloroquine. DNA (2 μg) was packaged with 10 μg of histones and then transfected with reduced amounts of lipofectamine. The cells were incubated with the transfection mixture for 2 h and then treated with 100 μm chloroquine for an additional 30 min. Results are given relative to the maximum level of transfection efficiency achieved with a 10:1 ratio of lipofectamine/DNA.

were then allowed to grow for another 48 h and assayed for β-gal activity. Less than 1% of the cells were found to express β-gal, despite the fact that flow onset is known to enhance endocytosis. Improvement of binding of the transfection vehicle to the endothelium under flow remains an important issue of study.

Few agents are available for transient permeabilization of the nucleus in living cells. Interestingly, the addition of heparin to cells has been described as causing nuclear swelling.[26] We conducted an experiment to determine if incubating cells with heparin could increase transfection efficiency by improving nuclear import through nuclear pores due to nuclear swelling. We found no effect of heparin on transfection efficiency (Fig. 9). This *in vitro* finding, however, suggests that patients on heparin anticoagulation remain potential candidates for gene therapy via lipofection.

Electroporation was also done with endothelial cells to determine if bypassing the endosomal pathway can increase the transfection efficiency. Electroporation of 6×10^6 cells resulted in only 40% viable cells after electroporation at 400 V and 1180 μF (Cell Porator, Gibco-BRL). The cells were grown for another 48 h and assayed for β-gal activity. Less than 10% of viable cells were found to stain blue, which was in agreement with the work done by Kotnis et al. with endothelial cells.[27] In preliminary studies, we have directly microinjected pCMVβ-gal/histones into the cytoplasm of over 100 endothelial cells. We found no β-gal-positive cells, indicating that direct bypass of the endosomes does not result in high expression of a plasmid, consistent with earlier studies by Capecchi.[28]

DISCUSSION

Cationic liposomes are commonly used as a means of delivering DNA to cells.[20,29–32] DNA/lipid complexes form spontaneously after mixing aliquots of the liposome reagent with an aqueous solution of DNA, trapping nearly all of the DNA in the interior.[20,29,33] The major drawback of this method is that the efficiency of transfection is low with nondividing cells and certain target cells, such as endothelium. For example, COS-7 cells lipofected with chloramphenicol acetyl transferase (CAT) gene are nearly 80% CAT positive compared with essentially no CAT expression in endothelial cells.[34]

We report that histones can provide a marked enhancement of lipofection efficiency of confluent or subconfluent endothelial cells. Since confluent endothelium at 5 days postseeding was difficult to lipofect com-

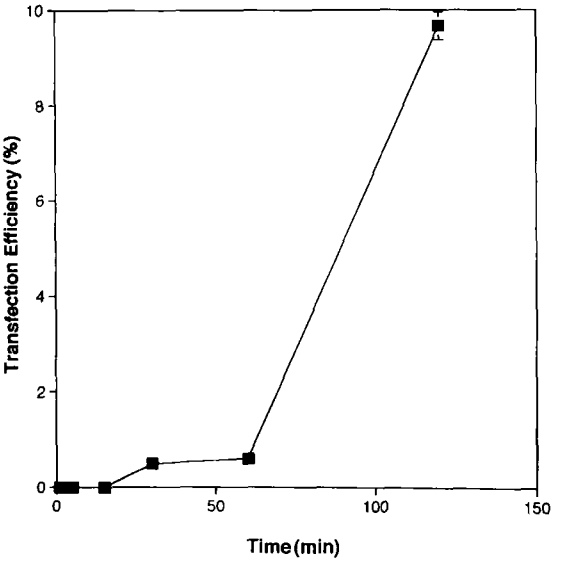

FIG. 8. Effect of incubation time on lipofection efficiency of subconfluent BAEC. DNA (2 μg) was packaged in 20 μg of lipofectamine, and then 80% confluent BAECs were incubated with the mixture for varying amounts of time. The cells were grown for 48 h and then assayed for β-gal.

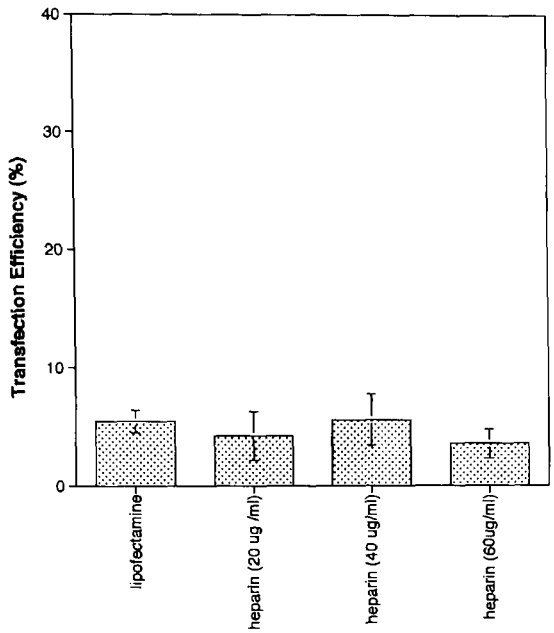

FIG. 9. Effect of heparin on lipofection efficiency of subconfluent BAEC. DNA (2 μg) was packaged in 20 μg of lipofectamine, and then 80% confluent BAEC were incubated with the mixture for 2 h. Varying amounts of heparin were added to the cells and incubated for another 1 h. The cells were assayed for β-gal activity after 48 h.

pared with actively dividing subconfluent endothelium, nuclear disruption during mitosis represented an important route for plasmid entry into the nucleus. Histones enhanced the transfection efficiency most likely by providing DNase protection, with a resulting high probability of transfection of the dividing cells in the population. Given the relatively low lipofection efficiency of confluent endothelium with histone-compacted plasmids compared with levels achieved with subconfluent endothelium, it is unlikely that histones facilitated the transport of a 7.2-kbp plasmid across nuclear pores. When histones were used in lipofection, chloroquine provided no benefit (Fig. 5). Since we found that the transfection efficiency does not increase with methods, such as electroporation or microinjection, that directly bypass the endosomal pathway for entry of plasmid into the cytoplasm, we conclude that transport across the nucleus is the final rate-limiting step for nonviral transfection techniques. Entry of genetic material into the cytoplasm is necessary but not sufficient for fully effective endothelial gene transfer.

Because addition of chloroquine, ammonium chloride, or monesin increase the level of expression,[20,21] the endosome disruptive ability of DOPE alone may not be sufficient to deliver a sufficient amount of DNA into the cytoplasm. However, endosomal escape is not the final rate-limiting step. When gold-labeled DNA complexes were used, Zabner et al.[10] found there was a large concentration of these complexes in a perinuclear complex. Felgner and Ringold, using radioactively labeled complexes, showed that although 20–80% of the added polynucleotide was adsorbed by the cell, less than 1% of intact plasmid was found in the nuclear fraction.[29] Capecchi observed that plasmid DNA injected directly into the nucleus of mouse LTK cells resulted in 50–100% of the cells expressing thymidine kinase activity compared with no cells when the plasmid was injected into the cytoplasm.[28] In addition, Zabner et al. showed, using vaccinia virus for cytoplasmic transcription of lipofected plasmids, that nearly 98% of COS-1 cells have plasmid in the cytoplasmic compartment, whereas only 10% express β-gal.[10] These experiments point to the fact that delivery to the nucleus is the final rate-limiting step involved in nonviral transfection.

Chloroquine is an agent known to interfere with endocytosis by raising endosomal and lysosomal pH and thus inhibiting maturation of endosomes. Chloroquine can increase transfection efficiency with the use of DEAE-Dextran[35] but reduces adenovirus gene transfer. Because we found that chloroquine increased the transfection efficiency, the mechanism of endothelial entry of the DNA/lipofectamine complex was likely the endocytosis pathway, with DOPE helping in endosomal disruption. In endothelium, this process of en-

dosomal disruption by DOPE is evidently not fully efficient, as addition of chloroquine increased the transfection efficiency. However, enhancement of endosomal escape with chloroquine still left over 90% of the cells untransfected when DNA was lipofected without histones.

The ratio of DNA/lipid has been found to be an important factor that influences transfection efficiency.[29] Theoretically, the optimal ratio should be the number of positive charges contributed by the liposomes needed to exceed the number of negative charges on the DNA, so that the complex has a net positive charge to facilitate interaction with the net negatively charged cell surface. Based on the fact that 5000 negative charges are present on a standard 2500-bp plasmid, Felgner and Ringold hypothesized that four liposomes containing 50% DOTMA would be present in a DNA/lipid complex molecule. In practice, however, the optimum quantity of DNA is usually about half this amount. Using fluorescence quenching techniques, Gershon et al.[25] found that when the DNA/lipid ratio was lowered, the fluorescence decreased slowly initially and then dropped sharply at a DNA/lipid ratio of 1:1 with respect to the total charge of each species. Using electron microscopy techniques, they observed that at this ratio, the DNA/lipid form a very tight complex. Above this ratio, however, the DNA is not tightly compacted by the lipid, and the DNA has been found to protrude out of the complex, which might account for the lower transfection efficiency.[36]

Two pathways have been proposed for the entry of the DNA/lipid complex: (1) liposome-cell fusion with destabilization of the endosome[30–32,37] and (2) direct fusion with the plasma membrane.[33] Zhou and Huang,[38] using TEM, found that most of the DNA/lipid complexes were internalized by endocytosis, with endosomal disruption leading to transfer of complex into the cytoplasm. Only about 2% of the cells showed direct penetration across cell membrane. Chloroquine is an agent known to interfere with endocytosis by raising endosomal and lysosomal pH and thus inhibiting maturation of endosomes. In the case of liposome-mediated transfection, however, there are conflicting results with the use of chloroquine. Felgner et al., using a liposome containing DOPE and a cationic lipid DMRIE, showed that chloroquine treatment actually led to a decrease in the transfection efficiency,[20] but when they used a liposome with DORIE instead of DMRIE, an increase in transfection efficiency occurred. Farhood et al. reported a decrease in transfection efficiency when human epidermoid carcinoma cells transfected with DC-CHOL/DOPE liposomes were treated with chloroquine.[39] Legendre and Szoka, however, observed an increase in transfection efficiency when using DOTMA/DOPE liposomes.[21] The effect of increased transfection efficiency when cells were treated with chloroquine points toward a mechanism of entry of DNA/lipid complexes through endocytosis. The decrease in transfection efficiency was hypothesized by Felgner et al.[20] to result from chloroquine-mediated toxicity. Another hypothesis is that some lipid formulations fuse with early endosomes, releasing their contents into the cytosol, whereas others do not fuse until they reach the late endosomes.[20] Chloroquine can block maturation of the early endosome, preventing its maturation into the late endosome, thus inhibiting the path of those reagents that fuse with the late endosome. Hence, the pathway for entry into a cell line depends on the type of cell as well as the composition of the liposome. Based on this argument, endothelium likely uses the early endosome for release of lipofected DNA when DOSPA and DOPE are used. Given the potential for chloroquine cytotoxicity, its use can be avoided entirely if histones are used to package the plasmid prior to lipofection. The possibility exists that other more specific endosome-disrupting agents (e.g., bafliomyocin) may increase lipofection efficiency when used in conjunction with histone-complexed DNA.

Histones use lysine and arginine to bind DNA via charge-charge interactions to form a tight complex. Histones are actively transported across the nucleus. They have amino acid sequences, such as QAKKKKL or PKKKRKV, which are similar to the nuclear localization signals on other proteins like nucleoplasmin.[40] In rat liver cells, histones have been shown to enhance lipofection.[41] The addition of chloroquine to cells following lipofection of histone-complexed plasmid did not further increase transfection efficiency significantly, as compared with an increase when cells were treated with chloroquine following plasmid lipofection without histones. This upper bound on transfection efficiency with subconfluent BAEC lipofected with histone-complexed plasmid was about 20% of the population being β-gal positive and likely represented the number of cells that divided in the time before the plasmid was degraded. The nuclear import of plasmid during cell division will be inefficient *in vivo* with lipofection of nonmitotic cells. In the future, the tissue engineer must look to the adenovirus to understand how it has solved this problem of nuclear penetration in order for lipofection to be a more viable clinical route.

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