Liposome-Mediated Gene Transfer Into Human Vascular Smooth Muscle Cells

J. Geoffrey Pickering, MD, PhD; Jaclynn Jekanowski, BS; Lawrence Weir, PhD; Satoshi Takeshita, MD; Douglas W. Losordo, MD; Jeffrey M. Isner, MD

**Background** Complexing recombinant DNA with cationic liposomes is a convenient means of introducing foreign genes into cells (lipofection) and could potentially form the basis for genetically modifying diseased blood vessels in patients. The mechanism of lipofection is incompletely understood, but it is recognized that the degree of successful gene transfer is highly dependent on cell type. To date, there has been no reported experience with lipofection of human vascular smooth muscle cells.

**Methods and Results** Primary cultures of human vascular smooth muscle cells were transfected under optimized conditions with a plasmid expressing either firefly luciferase (Luc) or nuclear-localized β-galactosidase (NL-β-gal). Cells were derived from either normal human internal mammary arteries (n=6), fragments of primary atherosclerotic plaque (n=4), or fragments of restenotic lesions (n=5). Concurrent lipofection of rabbit vascular smooth muscle cells and NIH 3T3 cells was performed as well. Cultures derived from 15 patients all demonstrated positive expression of the reporter gene. Compared with NIH 3T3 cells, however, expression in human vascular smooth muscle cells was markedly reduced: in cells derived from internal mammary artery, Luc expression, normalized for protein content, was 123-fold lower than in NIH 3T3 cells, whereas the proportion of cells expressing NL-β-gal was 30-fold lower. Luc expression in cells derived from restenotic tissue was significantly greater than from cells derived from primary plaque (P<.03). Within a given population of cells, the mitotic index of cells expressing the recombinant gene was significantly higher than the mitotic index for the total population of cells (P<.05). Finally, cotransfection experiments, in which lipofection of smooth muscle cells was performed using genes for NL-β-gal and for human growth hormone, showed that among positive transfectants, a high proportion of cells (23% to 36%) coexpressed both genes.

**Conclusions** The efficiency of successful lipofection in human vascular smooth muscle cells in vitro is low. Transfection appears to be preferentially facilitated in cells derived from restenotic tissue, and specific properties of smooth muscle cells, including growth rates, appear to be critical for successful transfection. Further elucidation of cell properties that promote transfection is required to augment the efficiency of liposome-mediated gene transfer in human vascular cells. (Circulation. 1994;89:13-21.)

**Key Words** • vessels • cells • atherosclerosis • genetics • lipids

The ability to express recombinant genes in the blood vessel wall has raised prospects for gene therapy of vascular disease. Two general approaches to introducing genes into the vessel wall have been studied. In one approach, referred to as indirect gene transfer, target cells are first isolated and gene transfer is done in vitro; cells that express the recombinant gene product are then selected and transplanted into the host vessel wall. In the second approach, genes are delivered "in situ" to cells within the vessel wall; this direct, in vivo approach to delivery of genes is attractive as a therapeutic modality because it obviates the need to remove vascular cells from the patient. Because direct gene transfer precludes the opportunity to select for positive transfectants, however, it is essential that an adequate amount of DNA be introduced and expressed by the target tissue. The efficiency with which cells of human vascular tissue may be transfected has, to date, not been evaluated.

Vascular smooth muscle cells may be suitable targets for the direct gene transfer approach because of their proximity to the lumen surface and abundance in the vessel wall. Furthermore, abnormal accumulation of smooth muscle cells is a feature of atherosclerosis and of certain accelerated forms of vascular disease, such as restenosis after balloon angioplasty. One potential means of transfecting smooth muscle cells within the vessel wall is through the use of cationic liposomes. Liposome-mediated gene transfer is a convenient method of transferring recombinant DNA into cells and has been used to directly transfec the arterial wall of live animals. The efficiency of successful gene transfer using cationic liposomes, however, is variable and highly dependent on the cell type. Most in vitro experience to date has been with continuous/immortal animal cell lines. The results of studies using these types of cells, however, have uncertain implications for the likelihood of success of direct arterial gene transfer in patients.

We have studied the expression of foreign DNA that has been introduced, via liposomes, into primary cultures of human vascular smooth muscle cells. Plasmids encoding either firefly luciferase or nuclear-localized β-galactosidase were transfected into smooth muscle cells derived from normal arteries, from human atherosclerotic plaque, and from restenotic human atherosclerotic lesions. Furthermore, cotransfection of vascular smooth muscle cells with two reporter genes, both of which when expressed may be detected histochemically,
was performed with genes encoding for nuclear-localized β-galactosidase and human growth hormone.

Methods

Cell Culture

Primary cultures of human arterial smooth muscle cells were initiated by explant outgrowth with unused segments of internal mammary artery retrieved at the time of coronary artery bypass surgery. Vascular smooth muscle cells derived from human atheroma were also cultivated according to methods previously described. These cells were grown from fragments of either primary atherosclerotic plaque or restenotic vascular tissue retrieved from the peripheral arteries of patients by directional atherectomy. Rabbit arterial smooth muscle cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular smooth muscle cells was confirmed morphologically by phase-contrast microscopy and by positive immunostaining with a monoclonal antibody to smooth muscle α-actin (Clone 1A4, Sigma). Cells were grown in medium (M199, Gibco, Gaithersburg, Md) supplemented with 10% fetal bovine serum. All transfections were performed exclusively with smooth muscle cells in the second subculture.

NIH 3T3 cells, a continuous cell line derived from mouse fibroblasts, were also studied. This enabled general comparisons of transfection efficiency to be made between vascular smooth muscle cells and a well-characterized continuous cell line.

Transfection Procedure

Positively charged liposomes (Transfection-reagent, Boehringer Mannheim) containing the cationic lipid analogue 1,2-dioleoyloxy-3-(trimethylammonio)propyl)-N,N,N',N'-tetramethyl-ammonium methylsulfate were used for all transfections. Plasmid DNA was purified by centrifugation through a cesium chloride gradient. DNA (5 μg) was mixed with 30 μg of liposomes in 200 μL of Hanks' balanced salt solution (HBSS, Gibco). These levels of DNA and liposomes proved to yield the highest expression of marker protein in all cell lines used, under the conditions used in the study. Liposome/DNA complexes were diluted with 1.5 mL serum-supplemented medium and added to subconfluent cells growing in 21-cm² culture dishes. After 5 hours, a further 3.5 mL of serum-supplemented medium was added to the culture dishes. The transfection mixture was removed and replaced with fresh serum-supplemented medium after a further 15 hours. Cells were then allowed to grow for 72 hours, after which they were studied for gene expression. Although the use of serum has been reported to inhibit liposome-mediated transfection of some cell lines, we have found that human vascular smooth muscle cells tolerate serum-free conditions poorly and with differing sensitivities (eg, cells derived from primary atheroma survive less well than those derived from restenotic plaque or from arterial media). In the presence of liposome/DNA complexes, cell survival is further reduced. Therefore, to increase cell survival and minimize variations in cell loss between cell lines, transfections were performed in the presence of 10% fetal bovine serum.

 Cotransfection experiments using two different plasmids were also performed. In these experiments, the amount of DNA/liposome complex was reduced by one half (2.5 μg plasmid DNA, 15 μg transfection reagent) so that the final concentration of DNA and liposome would be the same as for single transfections. The plasmid/DNA mixtures were prepared separately and diluted separately before being added to the cultures.

Expression Vectors and Analysis of Recombinant Gene Expression

Transfections were performed using three different plasmids, containing sequences that encode for one of three marker proteins: firefly luciferase, nuclear-targeted Escherichia coli β-galactosidase, or human growth hormone.

The luciferase expression vector used was pRSVLUC (gift of Dr A. Brasier, Galveston, Tex). This contains a 5' deletion of firefly luciferase cDNA, with transcription under the control of the Rous sarcoma virus long-terminal repeat promoter. Use of this reporter gene allowed for quantification of gene expression in cell lysates. Cells were washed three times with calcium-free HBSS, and extracts were prepared by use of a cell lysis reagent (Promega, Madison, Wis) containing 1% Triton-X 100. Half of the extract was taken for analysis of total protein content by the Biorad Microassay procedure. Bovine serum albumin (1 mg/mL) was added to the other half as a carrier protein, and luciferase activity was measured. For this, a 20-μL aliquot was mixed at room temperature with 100 μL of luciferase assay reagent (Promega) containing beetle luciferin. Emission of light, integrated over 10 seconds, was measured with a luminometer (model 20e, Turner Designs, Sunnyvale, Calif). Results, read as light units, were within the linear range of the detection system as evaluated with serial dilutions of a known amount of luciferase (product No. L-9009, Sigma). Background activity, measured with phosphate-buffered saline or lysates of nontransfected cells, was consistently zero.
pGSVLac-Z (kindly provided by Dr C. Bonnerot, Pasteur Institute, Paris, France) was used for transfecting cultures with the gene encoding for β-galactosidase. This vector contains the Lac-Z gene fused at its amino terminus to the simian virus 40 (SV40) large tumor nuclear localization signal. Expression of the corresponding protein has previously been demonstrated to be localized to the cell nucleus. The Lac-Z gene used contains the SV40 small T antigen intron and an SV40 polyadenylation signal. Transcription is driven by the SV40 early region promoter.

β-Galactosidase expression was assessed histochemically according to previously described methods. Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed for 10 minutes with 0.5% glutaraldehyde in PBS. Cells were then incubated for 2 hours at 37°C with a reaction mixture containing 4-Cl-5-Br-3-indolyl-β-D-galactopyranoside (X-gal, 1 mg/mL), potassium ferricyanide (5 mmol/L), potassium ferrocyanide (5 mmol/L), and MgCl₂ (2 mmol/L). The proportion of cells with blue-stained nuclei was then counted under phase-contrast microscopy.

The plasmid pXGH52' was used to transfect cultures with the gene for human growth hormone. This plasmid contains the mouse metallothionein-1 promoter fused to the full-length human growth hormone gene. Although this promoter is inducible with heavy metals such as zinc, zinc is toxic to cultured cells and is furthermore not required for growth hormone expression. A heavy metal supplement was therefore not used in these studies. Growth hormone expression was assessed immunohistochemically. Cells were fixed for 10 minutes with acetone at -20°C and incubated for 30 minutes...
with monoclonal antibody to human growth hormone (clone 54/9 2A2, BioGenix, San Ramon, Calif). Bound primary antibody was detected by use of a biotin-streptavidin amplification system and the chromogen 3,3'-diaminobenzidine according to the supplier's guidelines (BioGenix).

Cell cultures were also cotransfected with pGSVLac-Z and pXGH5. We found that when cells were fixed with 0.5% glutaraldehyde (a fixative suitable for the in situ detection of β-galactosidase activity with X-gal), immunodetection of growth hormone was markedly reduced. In contrast, fixation of cells with acetone allowed the detection of both growth hormone and β-galactosidase. Acetone was therefore used as the fixative for these experiments. Immediately after fixation, the cells were incubated with the X-gal solution as described above. Cells were then washed with PBS and immunostained with anti-GH antibody. The fast red TR chromogen (BioGenix) was used in the immunolabeling reaction to maximize color contrast between the two labels.

Results were compared by one-way ANOVA with Sheffé's post hoc test.

**Results**

**Luciferase Expression in NIH 3T3 Cells and in Primary Cultures of Vascular Smooth Muscle Cells**

Cultures of NIH 3T3 cells (n=3), rabbit vascular smooth muscle cells (n=2), and human vascular smooth muscle cells cultured from tissues of 13 patients (internal mammary artery, n=4; primary atherosclerotic plaque, n=4; restenotic lesions, n=5) were studied. All
values for luciferase expression were normalized for total protein content. Seventy-two hours after transfection, luciferase activity could be detected in lysates from all cultures. Expression in NIH 3T3 cells, however, exceeded that observed in vascular smooth muscle cells cultured from either rabbits or humans (Fig 1). Specifically, light emission per weight of protein from lysates of NIH 3T3 cells was 64-fold greater than that obtained from rabbit medial smooth muscle cells and 123-fold higher than that obtained from human medial smooth muscle cells. Among plaque-derived smooth muscle cells, expression in cells derived from restenotic tissue was significantly greater than that in cells derived from primary lesions ($P<.05$) (Table).

Expression of Nuclear-Targeted β-Galactosidase in NIH 3T3 Cells and in Primary Cultures of Vascular Smooth Muscle Cells

Quantification of light emission from cells transfected with luciferase cDNA is an extremely sensitive detection method but does not yield information on the proportion of positive transfectants. We therefore transfected cells with a plasmid containing the nls-Lac-Z gene and determined the proportion of transfected cells histologically. Cells expressing the nls-Lac-Z gene were clearly identified by dark blue staining of the nucleus (Fig 2a). Although faint cytoplasmic staining was also occasionally seen, this pattern was observed in some control cultures incubated with liposomes but no DNA. In contrast, nuclear staining was never seen in control cultures. A mean of 3975±257 cells were counted in each of two experiments with NIH 3T3 cells. Of these cells, 3.0±0.7% expressed the transgene. Cultures of three primary cell lines of rabbit vascular smooth muscle cells were transfected. Among 3523±489 cells counted per experiment, the mean proportion of positive cells, 1.2±0.5%, was significantly lower ($P<.05$) than for NIH 3T3 cells. Among 3268±448 cells counted from human vascular smooth muscle cells cultured from two patients, the mean proportion of positive cells was lower still at 0.10±0.05% ($P<.05$). These data are summarized in Fig 2b.

Cotransfection With Plasmids Encoding nls-Lac-Z Gene and Human Growth Hormone Gene

We considered the possibility that, within a given population of cells, there might be a subpopulation that was more likely to be successfully transfected. To assess this, we cotransfected cultures with two different plasmids. If there were no preferential transfection events and all cells had an equal probability of being transfected, then the likelihood of a cell expressing both reporter genes would be approximated by the product of the individual transfection proportions. Rabbit vascular smooth muscle cells were used for this evaluation, as opposed to human cells, since the greater number of positive transfectants would allow a more reliable assessment of the proportions of single- and double-transfected cells.

Transfection with the growth hormone-encoding plasmid (pXGH5) alone yielded 0.9% positive cells. Positive transfectants were generally scattered throughout the culture area, and staining within a positive cell was distributed throughout the cytoplasm (Fig 3a). Transfection of a parallel culture with the nuclear-localized β-galactosidase-encoding plasmid (pGSVLac-Z) alone yielded 0.8% positive transfectants. If all cells had an equal likelihood of being transfected, when cells were incubated with two plasmids, the proportion of double-transfected cells would be approximately 0.9%×0.8%, which is 0.0072%, or <1 in 13 000 cells. In fact, in two separate experiments, the proportion of cells expressing both marker proteins was 0.18±0.02%, or >1 in 560 cells. An example of a double-transfected cell is shown in Fig 3b, and the proportion of single- and double-transfected cells is summarized in Fig 3c.

Transfection Rate and Mitotic Index

In all transfection experiments described above, cells that either were undergoing mitosis or had recently completed mitosis consistently constituted a fraction of the positive transfectants. This was observed in vascular smooth muscle cells transfected with pXGH5 alone, with pGSVLac-Z alone, and in cells cotransfected and expressing both plasmids. By counting cells from four separate experiments, we ascertained that among rabbit vascular smooth muscle cells growing in serum-supplemented medium, the overall proportion of mitotic and postmitotic cells (ie, cells undergoing mitosis or pairs of recently divided daughter cells) was 0.9±0.1%. The proportion of mitotic and postmitotic cells among the subpopulation of positive transfectants, however, was significantly higher at 3.1±2.2% ($P<.05$) (Fig 4).

Discussion

Vascular smooth muscle cells represent an important cellular target for arterial gene transfer.3-5 The poten-
tial utility of applying gene therapy to circulatory sites in human subjects would be strengthened, however, by demonstration that potential differences in species do not preclude successful gene transfer. Previous studies have established that the efficacy of liposome-facilitated transfection may vary up to 100-fold, depending on the cell line used. Therefore, to clarify the feasibility of adapting this mode of gene transfer to vascular smooth muscle cells of human subjects, we determined the extent to which cells derived from normal and pathological human arteries could be successfully transfected with foreign DNA.

The results presented here document that human vascular smooth muscle cells from normal arteries, primary atherosclerotic plaque, and restenotic lesions can all be successfully transfected with liposomes containing the cationic lipid DOTAP. The efficiency of lipofection, as assessed by use of three different indicator genes, however, was relatively low. Among cultures of either rabbit or human vascular smooth muscle cells, the foreign genes appeared to be preferentially expressed in a subpopulation of cells with a higher rate of mitosis. In addition, and perhaps related, the efficiency of gene transfer into smooth muscle cells derived from restenotic atherosclerotic plaque was higher than that in cells derived from primary plaque.

Cells in culture may be transfected with foreign DNA by a variety of techniques, including calcium phosphate precipitation, DEAE-dextran, electroporation, lipofection, virus-mediated delivery, and receptor-mediated delivery methods. For a gene transfer method to be suitable for in vivo application, it must be associated with low toxicity and high efficiency. The use of cationic liposomes (lipofection) to transflect cells is a relatively
straightforward approach that satisfies the criterion of low toxicity. Furthermore, lipofection has been successfully used to deliver marker genes to the vascular wall.4-5,8 The efficiency with which lipofection may be achieved in human vascular tissue is not known, but this is likely to be a key determinant of the success of direct gene transfer as a therapeutic modality for vascular disease.26 Using an alternative liposome preparation (DOTMA-containing liposomes), Felgner and coworkers12 demonstrated that lipofection is significantly more effective than either calcium phosphate or the DEAE-dextran transfection techniques,11 and up to 25% of cells in culture may be transfected. The present study indicates that in primary cultures of human vascular smooth muscle cells, this degree of success may not be attainable. Transfection efficiency in vivo was not assessed; however, given the relatively optimized conditions associated with transfections performed in vitro and the potential for inactivation of liposomes by tissue extracellular matrix,14 it appears reasonable to expect that lipofection in vivo would be no more, and probably less, effective than lipofection in vitro.

Serum has been shown to inhibit liposome-mediated transfection of some cell lines11 and may have contributed to the low degree of expression in the present study. Nevertheless, when NIH 3T3 cells were transfected under identical conditions, a >120-fold increase in luciferase activity was observed. Furthermore, in our experience, the use of serum-free conditions seriously compromises the viability of human smooth muscle cells exposed to liposomes in culture; consequently, reporter gene expression was generally no greater and often less than when lipofection was used under serum-free conditions.

The low expression of luciferase and the low proportion of cells expressing either β-galactosidase or growth hormone could result from incorporation of plasmid DNA into the nucleus of only a small number of cells; alternatively, DNA uptake may have involved a greater number of cells, but expression of these particular genes may have been relatively inefficient. Studies with cationic liposomes containing fluorescent-labeled lipids have shown that these lipid preparations fuse with virtually all cells in culture.11,15 It has been suggested, however, that plasmid uptake is, in part, independent of liposome fusion16; whether or not all cells actually take up plasmid is not known.

The relatively high proportion of cells coexpressing the genes for both growth hormone and β-galactosidase suggests that not all cells in a given culture have the same probability of being successfully transfected. Similar observations of double-transfected cells after lipofection have been noted in Xenopus brain organ culture experiments.14 Although we cannot exclude the possibility of physical association between the two different plasmids within a liposome/DNA complex, we attempted to minimize this possibility by preparing the DNA/liposome complexes separately and prediluting the complexes before adding them to the culture wells. As well, previous light-scattering experiments have suggested that formation of liposome complexes containing multiple plasmids is unlikely.13 The consistent presence of double-transfected cells suggest, therefore, that within a population of cells there may be a subpopulation with specific properties that increase the likelihood of incorporating and/or expressing DNA delivered by liposomes.

One property that appears to influence the transfection efficiency with liposomes is the growth rate of the individual target cells. The increased proportion of mitotic or postmitotic cells observed in the present study among cells expressing growth hormone or β-galactosidase is consistent with this hypothesis. With the exception of certain expression systems that use a recombinant vaccinia virus,27 uptake of plasmid into the nucleus is necessary for successful transfection. It has been suggested that nuclear uptake may occur preferentially in those cells that are entering mitosis because of enhanced permeability of the nuclear membrane in such cells.28 In the present series of experiments, cells were fixed 72 hours after transfection, so that the presence of mitotic figures at this point does not necessarily imply that the cells were undergoing mitosis at the

**Fig 4.** This and facing page. Photomicrographs of pairs of positively transfected cells, each with a morphology consistent with recently transfected cells. This finding was evident in cultures transfected with pGSVlac-Z alone (a), pXGH5 alone (b), and both plasmids (c).
time of transfection. The significant increase in the proportion of mitotic cells observed among positive transfectants, however, suggests that the subpopulation of “transfectable” cells was proliferating more rapidly (with a greater mitotic index) compared with the mean rate of proliferation among all cells.

The concept that cell proliferation augments lipofection efficiency is also supported by recent studies from our laboratory in which the effect of prior endothelial denudation on lipofection efficiency was studied. Takeshita and coworkers29,30 observed both in vascular organ culture29 and in vivo30 that endothelial denudation increased transfection expression and that the increase in transfection efficiency was accompanied by an increase in DNA synthesis. The latter was evidenced in vitro by thymidine incorporation and in vivo by immunostaining for proliferating cell nuclear antigen.

In the present study, the increased transgene expression in cells derived from restenotic plaque versus those from primary plaque may also be attributable to differences in proliferation rate. We have previously observed that the rate of DNA synthesis in smooth muscle cells cultured from restenotic lesions is higher than that of cells derived from primary lesions.16 Dartsch and coworkers31 have noted similarly increased growth rates of cells derived from restenotic lesions compared with cells derived from primary lesions. Furthermore, histological analysis of the presence of the proliferating cell nuclear antigen in tissue removed from lesions of patients undergoing directional atherectomy has provided direct evidence for increased cellular proliferation in restenotic versus primary lesions.32 Thus, restenosis after angioplasty might fortuitously represent a vascular condition that is inherently more amenable to gene therapy, since the proliferative milieu of the restenotic lesion may facilitate successful transfection.

It must be pointed out that transfection efficiency and therapeutic efficiency are not in fact synonymous; the differential between these two considerations may in fact vary substantially, depending on whether the protein product of the transgene includes a signal sequence for secretion. For gene products that are secreted, expression within even a small number of cells may have meaningful biological effects. This has been demonstrated by Nabel et al33,34 after liposome-mediated transfection of the arterial wall with the genes for fibroblast growth factor-1 and platelet-derived growth factor B.34 In the latter study, only 0.1% to 1% of cells in the transfected arterial segment were estimated to contain plasmid DNA. Likewise, we have recorded physiological levels of growth hormone in rabbits transfected with human growth hormone, despite immuno histochemical evidence of successful transfection in well under 1% of cells in the transfected segment.35

Finally, it must be acknowledged that the findings of the present study pertain exclusively to liposome-based transfection. Alternative transfection strategies, including the use of recombinant adenoviral vectors,36–38 liposome/virus complexes,39 and transferrin-polycation conjugates40,41 may yield transfection efficiencies superior to that described here. Whether lipofection itself may be modified to achieve more efficient transfection remains to be determined. The demonstrated safety of liposome-mediated arterial gene transfer40,41 represents sufficient reason to pursue this approach. Although the use of serial transfection has been reported to augment transfection efficiency in CMT cells,42 preliminary studies performed recently in our laboratory using a serial protocol (consecutive and/or alternative days) failed to augment the level of transfection achieved in human vascular smooth muscle cells in vitro (J.G. Pickering, J. Jekanowski, J.M. Isner, unpublished data). A more promising alternative may be the adjunctive use of reagents designed to inhibit lysosomal degradation. This strategy assumes that cellular uptake of liposomes occurs by receptor-mediated endocytosis. This well-defined pathway involves fusion of the resulting endosome with a lysosome, subjecting the incorporated DNA to possible degradation. Zenke et al44 and Wagner et al45 have in fact demonstrated that when gene transfer is performed with a system (transferrin-polycation conjugates) known to involve receptor-mediated endocytosis, chloroquine inhibition of lysosomal degradation successfully augments transfection efficiency. A similar strategy has yet to be tested for liposome-mediated transfection.

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