Original Contributions

Gene Transfer Into Normal and Atherosclerotic Human Blood Vessels

Mark D. Rekhter, Robert D. Simari, Carolyn W. Work, Gary J. Nabel, Elizabeth G. Nabel, David Gordon

Abstract—Gene transfer to blood vessels is a promising new approach to the treatment of the vascular diseases, but the feasibility of gene transfer to adult human vessels has not been explored. We introduced an adenovirus vector encoding a marker gene human placental alkaline phosphatase into normal and atherosclerotic human vessels in organ culture. In the normal vessels, recombinant gene was expressed preferentially in the endothelial cells (≈100%), intimal smooth muscle cells (1.3±0.4%, 1.4±1.0%, and 3.8±0.8% in the internal mammary arteries, saphenous veins, and normal coronary arteries, respectively), and various adventitial cells. Advanced, complicated atherosclerotic plaques demonstrated a similar efficiency of recombinant gene expression (3.1±0.5% and 3.8±0.3% of nonendothelial intimal cells in the coronary artery and carotid artery plaques, respectively). Of these intimal cells, macrophages and smooth muscle cells expressed a transgene, identifying them as targets for gene transfer. Areas of plaque rupture and thrombus are sites of predilection for expression of recombinant genes. Collagenase and elastase treatment increased the percentage of transgenic alkaline phosphatase–positive cells 7 times (P<0.001), suggesting that the pattern of gene expression was affected by the amount of surrounding extracellular matrix. These studies demonstrate the feasibility of gene transfer to human blood vessels. However, these studies also highlight important barriers to adenoviral gene delivery to the actual normal and atherosclerotic human vessels of clinical interest. (Circ Res. 1998;82:1243-1252.)

Key Words: gene transfer ■ smooth muscle cell ■ macrophage ■ arteriosclerosis ■ human

Gene transfer to blood vessels has potential application to the treatment of vascular diseases, as demonstrated in animal experiments,1–4 but the feasibility of molecular genetic approaches to human adult vessels is unknown. Specifically, human atherosclerotic plaques demonstrate certain advanced features, such as ruptures, erosions, and hemorrhages, that are not presently mimicked by an animal model. It is unknown, however, whether these unique features of human vessels may influence the outcome of gene transfer. Therefore, as a initial step, we explored organ cultures of human arteries and veins, because this controlled experimental system preserves in vivo structural relationships between various elements of the vascular wall and permits various manipulations.9

We introduced an adenovirus vector (ADV-hpAP) encoding a marker gene human placental alkaline phosphatase (hpAP) into normal and atherosclerotic human arteries and veins. Vessels were then maintained in organ culture, followed by histochemical staining for alkaline phosphatase. The goals of the study were to determine the following: (1) Can significant gene transfer be achieved with normal human blood vessels? (2) Is there a difference between normal and atherosclerotic vessels in susceptibility to a gene transfer? (3) What cell types can be readily infected in normal versus atherosclerotic vessels? (4) Do anatomic barriers (eg, extracellular matrix) influence the percentage of transgene-expressing cells in atherosclerotic plaques? These investigations were conducted to determine the feasibility of gene transfer into human blood vessels. Particular vessels were chosen because potential clinical targets of human gene therapy include normal coronary arteries of the donor heart to inhibit formation of transplant arteriosclerosis or transplant rejection; normal saphenous veins and internal mammary arteries to inhibit coronary bypass graft intimal hyperplasia; advanced atherosclerotic plaques to stabilize and promote regression of occlusive inoperable arteriosclerosis; and advanced, mechanically injured atherosclerotic plaques to prevent restenosis after the angioplasty. Although we described the overall patterns of transgene expression in human blood vessels, the main emphasis of this study was put on the transfectability of nonendothelial intimal cells (specifically, SMCs and macrophages) as the most probable direct participants of pathological intimal growth and therefore attractive targets for gene therapy.

Materials and Methods
Recombinant ADVs
Two replication-deficient, recombinant ADVs were constructed and purified as described.7 These vectors were prepared from adenovirus...
Gene Transfer Into Human Vessels

Selected Abbreviations and Acronyms

ADV = adenoviral vector
hpAP = human placental alkaline phosphatase
M199 = medium 199
pfu = plaque-forming units
poly d(T) = polythymidine
SMC = smooth muscle cell

5 serotype and contain deletions in E1 and E3 regions, rendering them replication deficient. An ADV lacking a cDNA insert, ADV-ΔE1, was used for control experiments. The second ADV, ADV-hpAP, encodes for an hpAP reporter gene driven by a β-actin promoter and cytomegalovirus enhancer with an SV40 polyadenylation sequence. Viral stocks were sterilized with a 0.45-micron filter and evaluated for the presence of replication-competent virus as previously described.33 None of the stocks used in these experiments yielded replication-competent virus. Viral stocks were diluted to titers of 1×10^9 pfu/mL, stored at −20°C, and thawed on ice for 5 minutes before use.

Tissue Collection

Rabbit Aortas

Thoracic aortas were obtained from intact New Zealand White rabbits fed a regular chow. At euthanasia, aortic segments were immediately immersed in M199 with 1% penicillin and streptomycin and 10% FCS. After rinsing, these segments were cut into rings 3 to 4 mm thick. Aortic rings were then transferred into 24-well plastic dishes (1 ring per well) and maintained in the same culture medium.

Human Blood Vessels

Samples of normal and atherosclerotic human coronary arteries were obtained from diseased hearts removed at the time of heart transplantation. These hearts fell into two categories: (1) idiopathic dilated cardiomyopathy, in which the arteries display only normal diffuse intimal thickening with occasional small atherosclerotic plaques, and (2) severe end-stage coronary artery disease with myoccardial infarctions in which hemodynamically significant coronary atherosclerosis is found. We also studied samples of carotid artery plaques removed at the time of endarterectomy surgery, because these frequently display many advanced features of interest, such as thrombus organization. Portions of internal mammary artery and saphenous vein left over from coronary bypass surgery were obtained as an example of normal human blood vessels. The final study set consisted of 14 coronary arteries (6 plaques and 8 normal arteries with diffuse intimal thickening obtained from 5 hearts), 15 carotid plaques, 5 internal mammary arteries, and 4 saphenous veins. Vessels were cut into rings 3 to 4 mm thick and handled as described above for rabbit aortas. Time between surgical removal of the tissues and beginning of gene transfer experiments did not exceed 2 hours. These studies were approved by the Human Subjects Review Committee at the University of Michigan.

Gene Transfer in Organ Culture

Rabbit Aorta

In our pilot experiments with the rabbit aortic rings, the specimens were incubated with various dilutions of ADV-hpAP or ADV-ΔE1 (2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4 pfu/mL) in DMEM containing 10% FCS, 1% glutamine, and 1% penicillin-streptomycin for 30 minutes and 1, 3, 6, 12, and 24 hours at 37°C. After incubation with adenovirus, the specimens were washed in M199, then maintained in M199 with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin for 2 days, and finally fixed in 10% formalin overnight. It was determined (see “Results”) that incubation of rabbit aorta with adenovirus at a concentration 2×10^7 pfu/mL for 3 hours allowed the maximal efficiency of transgene expression. Therefore, this protocol was used for the experiments with human blood vessels.

Human Vessels

Rings of human vessels (3 to 5 per vessel) were incubated with ADV-hpAP at a concentration of 2×10^7 pfu/mL for 3 hours, maintained in M199 with 10% FCS for 2 days, and fixed in 10% formalin overnight. A set of carotid plaque samples (n=5) was fixed in methacarn (methanol:chloroform:glacial acetic acid in a 60:30:10 volume ratio) immediately after incubation with ADVs.

Enzymatically Digested Human Carotid Plaques

To evaluate the importance of extracellular connective tissue matrix as a determinant of gene transfer efficiency into human atherosclerotic lesions, 6 carotid plaques were gently deendothelialized with a cotton swab and minced into small pieces (10 to 20 pieces per plaque) of ~3×3×3 mm in size. All of these pieces were treated with a mixture of 0.15% collagenase, type 4 (isolated from Clostridium histolyticum, 213 U/mg dry wt, obtained from Worthington) and 0.01% pancreatic elastase (90 U/mg dry wt, obtained from Sigma) dissolved in M199 for 1 hour at 37°C.31 No manipulations to remove other enzymatic activities (eg, addition of specific enzyme inhibitors) were performed. The other half was incubated in M199 under the same conditions and was used as a control. After the enzymatic treatment, minced tissue was washed in M199, incubated with ADV-hpAP, maintained in organ culture, and fixed in 10% formalin overnight. A separate set of carotid plaques (n=5) was subjected to the same procedure but was fixed in methacarn immediately after incubation with adenovirus.

Tissue Processing

Fixed specimens were dehydrated and embedded in paraffin. All samples from the same blood vessel were placed into one block. Thus, each tissue block contained several (minimum, 3) pieces from the same vessel.

Histochemistry

Expression of recombinant hpAP protein was detected by histochemical analysis of infected, formalin-fixed vascular samples. The slides were deparaffinized and incubated in PBS at 65°C for 30 minutes to inactivate endogenous alkaline phosphatase. The sections were incubated in PBS containing a chromogenic substrate of 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine (1 mg/mL, Gibco BRL) and nitro blue tetracium chloride (1 mg/mL, Gibco BRL) for 19 hours. This substrate yields a dark purple-to-brown stain in the presence of alkaline phosphatase. Pellets of 293 cells infected with either ADV-hpAP or ADV-ΔE1, fixed in formalin, and processed in the same fashion as the organ culture samples were used as positive and negative tissue controls, respectively.

Immunocytochemistry

Single-Label Immunocytochemistry

Carotid plaque sections fixed in methacarn immediately after the incubation with ADV-hpAP were used for immunocytochemical analysis of the tissue distribution of adenoviral particles (immediate results of adenoviral infection). Sections were deparaffinized and goat anti-adenovirus (anti-hexon) antibody (dilution 1:100, Biodien) was applied overnight at 4°C. The secondary antibody incubation (biotinylated horse anti-goat antibody, 1:200 dilution, Vector Laboratories) was for 30 minutes at room temperature, followed by streptavidin–alkaline phosphatase (dilution 1:1000, Vector Laboratories) for 30 minutes, then developed with alkaline phosphatase substrate (alkaline phosphatase substrate kit I, Vector Laboratories), which produced a red reaction product. Adenovirus-positive and -negative control slides were obtained from Chemicon International, Inc.

Combination of Alkaline Phosphatase Histochemistry and Immunocytochemistry

Recombinant hpAP activity may localize separately or together with the immunocytochemical signal of interest. We used slightly different techniques for analysis of these two distinct types of spatial
associations. (1) Staining of separate tissue structures: To address the question about spatial relationships between local transgene expression and organized thrombi in advanced carotid atherosclerotic plaques, we combined alkaline phosphatase histochemical staining with fibrin II immunostaining, which was used as a marker of thrombus/hemorrhage-related fibrin deposition.12 Mouse anti–fibrin II β-chain antibody NYB-T2G112 was supplied by Accurate Chemical and Scientific Corp. Alkaline phosphatase staining was carried out as described above. The primary antibody (NYB-T2G1 at 1:50 dilution in 2% normal horse serum) was applied overnight at 4°C. The biotinylated horse anti–mouse IgM antibody (dilution 1:200) was then applied for 3 hours at 4°C, followed by another 30-minute incubation in streptavidin–alkaline phosphatase (dilution 1:1000, Vector Laboratories) for 30 minutes, then developed with alkaline phosphatase, which produced a red reaction product. (2) Staining of colocalized structures: To identify the cell types expressing the transgene hpAP, combined alkaline phosphatase histochemistry and cell type–specific immunocytochemistry was performed. The cell type–specific antibodies used on these tissues were as follows: a monoclonal mouse α-actin antibody (1:500 dilution, Boehringer Mannheim Biochemical) to identify SMCs, a monoclonal mouse CD68 antibody (1:1000 dilution, DAKO) to identify monocytes/macrophages, and goat polyclonal anti–von Willebrandt factor antibody (1:200 dilution, Atlantic Antibodies) to identify endothelial cells. All primary antibodies were diluted with 2% normal horse serum. The biotinylated secondary antibody was then applied for 3 hours at 4°C, followed by streptavidin–colloidal gold (1:1000 dilution, Goldmark Biologicals) for 30 minutes. Slides were then incubated with the silver enhancement kit (Goldmark Biologicals) for 10 to 15 minutes under microscopic control.

In Situ Hybridization Assay of Cell Viability

Rationale
Although various techniques are available for accurate evaluation of cell viability in tissue culture on a cell-by-cell basis,13 we are not aware of any reliable approach applicable to intact tissue in situ or in organ culture. We assume that translational activity may serve as an approximate index of cell viability (in other words, dead cells are not expected to make mRNA and synthesize proteins). Therefore, we propose to use in situ detection of polyadenylated mRNA sequences as a marker of viable cells within the tissue. Polyadenylated mRNA sequences are conserved in routine paraffin-embedded specimens expected to make mRNA and synthesize proteins. Therefore, we are not aware of any reliable approach applicable to intact tissue in situ or in organ culture. We assume that translational activity may serve as an approximate index of cell viability (in other words, dead cells are not expected to make mRNA and synthesize proteins). Therefore, we propose to use in situ detection of polyadenylated mRNA sequences as a marker of viable cells within the tissue. Polyadenylated mRNA sequences are conserved in routine paraffin-embedded specimens expected to make mRNA and synthesize proteins.

Methodology and Validation
In situ hybridization with the fluorescein-labeled poly d(T) oligonucleotide was performed using the probe and detection kit provided by Novocastra Laboratories. The slides were incubated with proteinase K in 0.05 mol/L Tris/HCl buffer (pH 7.6) for 30 minutes at 37°C, then were hybridized with oligonucleotide probe during 2 hours at 37°C. Detection of the hybridized probe included incubation with anti-fluorescein rabbit antibody conjugated to alkaline phosphatase (1:100 dilution in TBS) for 30 minutes and, finally, incubation in alkaline phosphatase–developing solution, as described above for alkaline phosphatase histochemical staining. As a control for specificity of in situ hybridization, serial sections were incubated with fluorescein-labeled random oligonucleotide cocktail provided by Novocastra Laboratories. To validate this novel assay of cell viability, cultured rat aortic SMCs were killed by incubation with 0.1% saponin for 10 minutes,13 which was confirmed by conventional cell viability assay (Life/Death kit, Molecular Probes), washed with PBS, and fixed 1, 2, and 4 hours later. Poly d(T) in situ assay revealed that virtually all untreated cells were poly A–positive. Notably, strong nuclear and cytoplasmic staining was found. In 1 hour, cytoplasmic staining completely disappeared, whereas ~80% of the nuclei exhibited faint signal. Two and 4 hours after treatment, only 20% of the nuclei were very weakly stained, whereas the rest of the cells were totally negative. Therefore, we concluded that mRNA loss is a fairly early indicator of cell death and therefore may be used to evaluate cell viability in organ culture.

Evaluation of Tissue Integrity
Advanced, complicated plaques often have complex geometry, determined by surface erosions and ruptures.16 We hypothesized that cells adjacent to such “openings” may have better accessibility to adenoviral particles and therefore a greater degree of the transgene expression. To label original contours of the plaques, we used the Davidson Marking System (Bradley Products).12 Carotid plaque rings were incubated with ADV-hpAP and maintained in organ culture for 2 days. Before formalin fixation, they were incubated with green tissue-marking dye for 5 minutes. After histochemical staining for alkaline phosphatase, spatial associations among the cells expressing a transgenic hpAP (purple cytoplasm) and bright green marks of the plaque openings were assayed.

Morphometric Analysis
Sections were studied by light microscopy with the Image 1 system of image analysis (Universal Imaging Corp). Nonoverlapping microscopic fields covering the whole section were analyzed. On the single-labeled slides, each field was scored for total number of nuclei and number of hpAP-positive cells. The average index per sample was then used to calculate the statistical comparisons between different normal and atherosclerotic blood vessels. With the double-labeled slides, the percentage of hpAP-positive cells that were associated with each cell type–specific marker was determined, with averages again computed on a per-sample basis. Average percentages of hpAP-positive cells for each group of blood vessels were obtained and compared either by Student’s t test or one-way ANOVA. Statistical analysis was performed with the statistical package Epistat (Epistat Services).

Results
Optimization of Experimental Conditions in Organ Culture of Rabbit Aorta
Gene transfer experiments involving human blood vessels are complex. Therefore, we optimized conditions of organ culture and adenovirus infection using freshly obtained rabbit aorta. Aortas fixed immediately after harvesting did not demonstrate any signs of endothelial desquamation or injury of medial SMCs. In situ hybridization with poly d(T) probe demonstrated homogeneous staining of all medial SMCs (data not shown). Moreover, aortic segments that underwent in vitro adenoviral infection and were maintained in organ culture for 2 days still exhibited excellent morphological preservation of cells and extracellular structures (Figure 1a). In situ hybridization revealed that all luminal endothelial cells, as well as the vast majority of medial SMCs and various adventitial cells, showed translational activity (Figure 1b). Reaction with scrambled (negative-control) oligonucleotide probe showed no hybridization signal. However, hybridization signal was somewhat stronger in the internal and external layers of medial SMCs compared with SMCs in the middle portion of the media, which may be reflective of a mild hypoxia or nutritional deficiency of SMCs in the very depth of the aortic slices. Thus, adenoviral infection and maintaining of normal artery in organ culture do not significantly impair the viability of vascular wall cells.

After experimenting with various concentrations of adenovirus (2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7, and 2×10^8 pfu/mL) and dwell time (30 minutes and 1, 3, 6, 12, and 24 hours)
hours), we concluded that incubation of rabbit aorta with adenovirus at a concentration of $2 \times 10^9$ pfu/mL for 3 hours allowed the maximal efficiency of transgene expression (Figures 1c, 1d, and 2). No toxic effects were observed in the range of vector concentrations used. Alkaline phosphatase histochemical staining revealed that all endothelial cells and various number of adventitial cells expressed a transgene. No medial SMCs showed alkaline phosphatase positivity. No alkaline phosphatase staining was observed in the mock-transfected specimens. Thus, rabbit experiments demonstrated the feasibility of the organ culture approach to evaluation of a transgene expression in vascular tissues.

Normal Human Blood Vessels

A difference between rabbit aorta and normal human vessels is the presence of distinct subendothelial intima (Figure 3a). It consists of several layers of SMCs and extracellular matrix. The number of subendothelial cells varies in different human vessels (from 1 to 3 layers in the internal mammary arteries to 5 to 10 layers in the coronary arteries). In situ hybridization assay demonstrated very good preservation of these cells in organ culture conditions (Figure 3b). The patterns of transgene expression were very similar in all types of normal human vessels. Typically, hpAP was expressed by luminal endothelial cells and scattered subendothelial cells (Figure 3c and 3e). Despite $\approx 100\%$ of luminal endothelial cells being hpAP-positive (when optimal vector concentration was used), $<5\%$ of subendothelial cells expressed the transgene (Figure 4), and expression was typically found adjacent to endothelium. However, this pattern was reproducibly altered at the sites near the side branches, where several layers of intimal cells exhibited hpAP positivity (Figure 3d). Combination of alkaline phosphatase histochemical staining and cell-specific immunogold labeling demonstrated that the vast majority of hpAP-expressing subendothelial cells were also $\alpha$-actin positive (Figure 3f, Table). There were no statistically significant
differences in the overall numbers of hpAP-positive intimal cells among normal human blood vessels (Figure 4). We also did not find any difference in adventitial expression of hpAP, which spanned from 10% to 21% in different preparations.

Human Atherosclerotic Plaques

Atherosclerotic plaques typically consist of a lipid/necrotic core and an overlying fibrous cap that isolates this core from the arterial lumen (Figure 5a). In this study, coronary plaques were usually less advanced, whereas carotid plaques frequently displayed erosions, hemorrhages, and organized thrombi. Both morphological analysis and in situ hybridization assay demonstrated excellent tissue preservation of human atherosclerotic plaque explants (Figure 5a and 5b).

The pattern of a transgene expression in atherosclerotic plaques was very similar to that in normal arteries; transgenic hpAP was expressed by almost all luminal endothelial and scattered subendothelial cells (Figure 5c). Clusters of hpAP-positive intimal cells were occasionally seen at sites of artificial endothelial desquamation (data not shown). However, the absence of endothelium was not a sufficient predictor of increased transgene expression by the intimal cells. At the same time, numerous hpAP-positive cells were associated with the sites of plaque erosions and organized thrombi (Figure 5d). This association was confirmed by use of the combination of alkaline phosphatase histochemical staining and fibrin immunocytochemistry (Figure 5e). Despite such differences in the localization of the transgene-expressing cells, statistical analysis did not reveal significant differences between the overall numbers of hpAP-positive intimal (non-endothelial) cells in human plaques versus normal blood vessels (Figure 4). Eight percent to 20% of the adventitial cells expressed hpAP, which also did not differ from normal vessels.

As described above in normal vessels, hpAP expression was observed in SMCs (Figure 5f). In contrast to the pattern...
of expression observed in normal vessels, hpAP expression was also seen in macrophages (Figure 5g). Foamy macrophages, a hallmark of atherosclerosis, also often expressed hpAP (Figure 5h). Of all hpAP-positive plaque intimal cells, 30% to 60% were SMCs, 25% to 50% were macrophages, and up to 20% did not express either of those cell type–specific markers (Table).

Plaque Integrity and Efficiency of Gene Transfer
We hypothesized that the pattern and efficiency of transgene expression was affected by the physical accessibility of the plaque cells to the vector. To test this hypothesis, we labeled original contours (and therefore the openings) of the plaques with a dye. It was evident that hpAP-expressing cells, regardless of the cell type, were localized within the labeled (open) regions on both luminal and abluminal sides of the carotid plaques (Figure 6a and 6b). This pattern corroborated the pattern of distribution of adenoviral particles immediately after infection (Figure 6c).

To address the question about the role of extracellular matrix as an anatomic barrier for adenovirus penetration, we gently deendothelialized carotid plaques, minced them into small pieces, treated them with a mixture of collagenase and elastase, and transfected the tissue. Comparison of relative areas occupied by hexon-immunostainable material immediately after infection revealed that enzymatic pretreatment increased the area containing hexon staining 6 times (P<0.01) (Figures 6d, 6e, and 7). Accordingly, 2 days after transfection, a 7-fold increase in the percentage of alkaline phosphatase–positive cells was observed in digested versus untreated plaques (P<0.001) (Figures 6f, 6g, and 8). Thus, not only did more efficient gene transfer correlate with the natural plaque openings, but artificial exposure of plaque cells further increased the number of transgene-expressing cells.

**Discussion**

With freshly obtained rabbit aorta as a reference, we used organ culture techniques to study the feasibility of gene transfer into normal and atherosclerotic human blood vessels. Although it is not an in vivo system (eg, with blood flow, arterial pressure, etc), this approach provides a simplified reliable study system to begin to understand the intrinsic transfsectability of human vascular tissues.

Our results showed that almost all luminal endothelial cells and a variable number of adventitial cells within the explants of normal human coronary arteries, internal mammary arteries, and saphenous veins express recombinant genes after adenoviral infection. This pattern was very similar to findings observed in organ culture of rabbit aorta and to previous studies of in vivo transfection of normal animal arteries. Interestingly, up to 5% of subendothelial smooth muscle–like cells also actively expressed a transgene. This observation is important because these cells are the most likely source of intimal growth in atherosclerosis, restenosis, and bypass graft intimal hyperplasia. Moreover, in our experiments, subendothelial transduction was greater at branch points. The exact nature of this phenomenon is unknown. However, it may be determined by increased endothelial permeability and/or different architecture of subendothelial extracellular matrix, resulting in the better access of ADVs to subendothelial smooth muscle–like cells. Thus, the ability to target intimal cells without intentional endothelial denudation in organ culture conditions suggests the feasibility of gene therapy for human transplant arteriosclerosis and bypass graft failure.

The organ culture approach is attractive for analysis of gene transfer into human atherosclerotic plaques, because certain advanced features of human lesions, such as plaque ruptures, erosions, and hemorrhages, are difficult to reproduce in the animal models. At the same time, advanced, complicated plaques may be the most appealing target for human gene therapy. We demonstrated that the overall efficiency of recombinant gene expression in nonendothelial intimal cells in human plaques is similar to that of their normal counterparts. However, cells adjacent to the loci of plaque rupture and cells associated with organized thrombi were more prone to gene transfer. Unfortunately, we were unable to analyze potential relationships between the type of lesion and efficiency of gene transfer, because fresh human tissues with early atherosclerotic lesions like fatty streaks were not available for this study.

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**Cell Type Composition of Transgenic hpAP-Expressing Cells in the Intima of Human Blood Vessels in Organ Culture**

<table>
<thead>
<tr>
<th>Category in hpAP-Expressing Cells, %</th>
<th>α-Actin (Smooth Muscles)</th>
<th>CD68 (Macrophages)</th>
<th>Undefined Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal mammary artery</td>
<td>85.7</td>
<td>0</td>
<td>14.3</td>
</tr>
<tr>
<td>Saphenous vein</td>
<td>53.8</td>
<td>0</td>
<td>46.2</td>
</tr>
<tr>
<td>Normal coronary artery</td>
<td>75.5</td>
<td>1.9</td>
<td>22.6</td>
</tr>
<tr>
<td>Coronary atherosclerotic plaque</td>
<td>57.4</td>
<td>26.2</td>
<td>16.4</td>
</tr>
<tr>
<td>Carotid atherosclerotic plaque</td>
<td>33.0</td>
<td>47.1</td>
<td>19.9</td>
</tr>
</tbody>
</table>

*Percentage of undefined cells is estimated by subtracting the actual percentage of hpAP-expressing α-actin–positive cells and macrophages. Luminal endothelial cells were not counted.

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**Figure 4.** Percentage of intimal (nonendothelial) cells in normal human blood vessels expressing a recombinant hpAP gene. Data are expressed as mean±SEM. n=4 specimens in each group.
It is difficult to compare our results with the data obtained in animal experiments in vivo, because gene transfer has always been combined with some sort of mechanical injury of the preexisting atherosclerotic plaques. In other words, there are no data describing the patterns of transgene expression in intact animal atherosclerotic lesions. Previous studies using percutaneous delivery of ADVs encoding a lacZ reporter gene in atherosclerotic rabbit arteries with a channel...
balloon demonstrated transgene expression in ≈0.2% of intimal and medial cells. This result was ≈10-fold lower than observed in balloon-injured normal arteries. Recently, when delivering ADV-hpAP to atherosclerotic rabbit arteries after surgical exposure of arteries, we demonstrated that ≈32% of intimal and medial cells in balloon-injured atherosclerotic lesions expressed a transgene. In other studies, no differences in luciferase activity were observed between atherosclerotic balloon-injured and normal porcine coronary arteries after percutaneous delivery of ADVs encoding a luciferase gene with a porous balloon catheter. It is likely that such variability reflects the problems of catheter-based gene delivery. Organ culture experiments do not have this limiting step. Recent data of Ooboshi et al demonstrate that adenovirus-mediated gene transfer to the endothelium was much more effective in atherosclerotic than in normal monkey and rabbit arteries in organ culture.

It has also been reported that lacZ gene transfer into balloon-injured rabbit atherosclerotic arteries resulted in a transgene expression exclusively by SMCs. In our hands, however, a combination of histochemical alkaline phosphatase staining and immunogold cell type–specific labeling of human plaques in organ culture revealed hpAP gene expression in luminal endothelial cells, SMCs, and monocyte/macrophages. Moreover, ≈20% of the transgene-expressing cells did not express any of the above cell type–specific markers. These results corroborate our recent observations of different cell types expressing a transgene in rabbit atherosclerotic lesions. Thus, our data suggest a broad scope of cellular targets for gene therapy of human atherosclerosis.

There appeared to be no cell type in human atherosclerotic plaques that is intrinsically resistant to adenovirus-mediated gene transfer. We have also demonstrated that the vast majority of plaque cells are viable in organ culture. However,
not every viable cell expressed a transgene. Our results showed that adenoviral particles immediately after infection, as well as transgene-expressing cells (regardless of the cell type) 2 days after infection, tended to localize next to the plaque openings, such as erosions, ruptures, organized mural thrombi, etc. Taken together, these data imply the presence of permeability barriers, which limit adenoviral particles from reaching their cellular targets, otherwise potentially transfectable. The presence of anatomic barriers determining the pattern and efficiency of transgene expression has been established in animal experiments.1,27 No such data are available for advanced human atherosclerotic lesions. Because endothelial preservation in the explants of human atherosclerotic plaques is likely to be suboptimal, we decided not to analyze the role of endothelium (although potentially important), but rather to test a specific hypothesis that in advanced human atherosclerotic plaques, dense fibrous matrix serves as a barrier for adenovirus-mediated gene transfer. Carotid plaques were gently deendothelialized, minced, treated with a mixture of collagenase and elastase, then infected with ADV-hpAP and maintained in organ culture. Our results show that the efficiency of vector penetration was increased 6-fold, and accordingly, a number of transgene-expressing cells was increased 7-fold, compared with untreated plaque fragments. We suggest that the observed increase in the efficiency of transgene expression is caused primarily by barrier reduction, although the minor influence of extracellular matrix–dependent alterations in gene expression cannot be completely ruled out. Thus, having demonstrated that the highest efficiency of gene transfer was associated with either natural (erosions, ruptures, etc) or artificial (enzymatic treatment) plaque openings, we concluded that the actual pattern of gene expression depends on the exposure of individual cells to adenoviral particles, and this is affected by the amount of surrounding extracellular matrix. Moreover, these results show that the most vulnerable sites of atherosclerotic plaques are more prone to gene transfer than the rest of the plaque. Such plaques may be an excellent target for gene therapy aimed at stabilization of vulnerable atherosclerotic plaques.28

Thus, we have demonstrated the feasibility of gene transfer into both normal and atherosclerotic human blood vessels. Characteristics of the efficiency and patterns of a transgene expression in human vessels should support future efforts to design gene therapy for arteriosclerosis, restenosis, and graft failure.

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References
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