

**Increased gene expression after liposome-mediated arterial gene transfer associated with intimal smooth muscle cell proliferation. In vitro and in vivo findings in a rabbit model of vascular injury.**

S Takeshita, ... , L Weir, J M Isner

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**Research Article**

Arterial gene transfer represents a novel strategy that is potentially applicable to a variety of cardiovascular disorders. Attempts to perform arterial gene transfer using nonviral vectors have been compromised by a low transfection efficiency. We investigated the hypothesis that cellular proliferation induced by arterial injury could augment gene expression after liposome-mediated gene transfer. Nondenuded and denuded rabbit arterial strips were maintained in culture for up to 21 d, after which transfection was performed with a mixture of the plasmid encoding firefly luciferase and cationic liposomes. In non-denuded arteries, the culture interval before transfection did not affect the gene expression. In contrast, denuded arteries cultured for 3-14 d before transfection yielded 7-13-fold higher expression (vs. day 0;  $P < 0.005$ ). Transfection was then performed percutaneously to the iliac arteries of live rabbits with or without antecedent angioplasty. Gene expression increased when transfection was performed 3-7 d postangioplasty ( $P < 0.05$ ). Proliferative activity of neointimal cells assessed in vitro by [<sup>3</sup>H]thymidine incorporation, and in vivo by immunostaining for proliferating cell nuclear antigen, increased and declined in parallel with gene expression. These findings thus indicate that the expression of liposome-mediated arterial gene transfer may be augmented in presence of ongoing cellular proliferation.

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# Increased Gene Expression after Liposome-mediated Arterial Gene Transfer Associated with Intimal Smooth Muscle Cell Proliferation

## In Vitro and In Vivo Findings in a Rabbit Model of Vascular Injury

Satoshi Takeshita, Dov Gal, Guy Leclerc, J. Geoffrey Pickering, Reimer Riessen, Lawrence Weir, and Jeffrey M. Isner  
Departments of Medicine (Cardiology), Pathology, and Biomedical Research, St. Elizabeth's Medical Center,  
Tufts University School of Medicine, Boston, Massachusetts 02135

### Abstract

Arterial gene transfer represents a novel strategy that is potentially applicable to a variety of cardiovascular disorders. Attempts to perform arterial gene transfer using nonviral vectors have been compromised by a low transfection efficiency. We investigated the hypothesis that cellular proliferation induced by arterial injury could augment gene expression after liposome-mediated gene transfer. Nondenuded and denuded rabbit arterial strips were maintained in culture for up to 21 d, after which transfection was performed with a mixture of the plasmid encoding firefly luciferase and cationic liposomes. In nondenuded arteries, the culture interval before transfection did not affect the gene expression. In contrast, denuded arteries cultured for 3–14 d before transfection yielded 7–13-fold higher expression (vs. day 0;  $P < 0.005$ ). Transfection was then performed percutaneously to the iliac arteries of live rabbits with or without antecedent angioplasty. Gene expression increased when transfection was performed 3–7 d postangioplasty ( $P < 0.05$ ). Proliferative activity of neointimal cells assessed in vitro by [<sup>3</sup>H]thymidine incorporation, and in vivo by immunostaining for proliferating cell nuclear antigen, increased and declined in parallel with gene expression. These findings thus indicate that the expression of liposome-mediated arterial gene transfer may be augmented in presence of ongoing cellular proliferation. (*J. Clin. Invest.* 1994, 93:652–661.) Key words: gene expression • cellular proliferation • liposomes • transfection • luciferase

### Introduction

Beginning with the report by Nabel et al. (1), work from several laboratories (2–5) has convincingly demonstrated the feasibility of using catheter delivery systems to transfer recombinant genes to normal and/or atherosclerotic arteries of experimental animals. All of these previous demonstrations, however, have been compromised by low transfection efficiencies regardless of the vector used. While the basis for such low-level gene expression is likely multifactorial, it is noteworthy that nearly all attempts to achieve arterial transfection to date have involved normal arteries with quiescent, nonproliferating cell populations.

Address correspondence to Dr. Jeffrey M. Isner, St. Elizabeth's Medical Center, 736 Cambridge Street, Boston, MA 02135.

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This feature of previous arterial gene transfer experiments is of interest for two reasons. First, gene transfer studies performed in vitro using nonvascular cell lines have suggested that transfection efficiency may be increased up to 20-fold in mitotic cells (6). Second, recent analyses of tissue specimens retrieved by directional atherectomy from restenotic and even primary sites of arterial narrowing have disclosed evidence of ongoing cellular proliferation (7). Thus, for those applications of gene therapy that have been designed to treat primary or recurrent arterial narrowing, it is possible that the proliferative milieu of certain vascular lesions might favorably influence the transfection efficiency associated with arterial gene transfer. Accordingly, the purpose of this study was to investigate the hypothesis that cellular proliferation in the vascular wall resulting from arterial injury could augment gene expression after liposome-mediated arterial gene transfer. For in vitro studies, we used an organ culture model described previously (5) in which rabbit aortic strips were transfected with or without antecedent endothelial denudation. For in vivo studies, we performed percutaneous arterial gene transfer in rabbit iliac arteries with or without antecedent balloon angioplasty. The results of both the in vitro and in vivo studies indicate that gene expression after liposome-mediated, site-specific arterial gene transfer may be augmented by ongoing cellular proliferation.

### Methods

#### *In vitro studies*

**Arterial organ culture.** In vitro analysis of gene expression as a function of intimal proliferation was performed using strips of descending thoracic aorta harvested from male New Zealand White rabbits (Pine Acre Rabbitry, Norton, MA) and maintained in organ culture using a modification of techniques described previously (8, 9). The protocol for this and all subsequent series of experiments was approved by St. Elizabeth's Institutional Animal Care and Use Committee. Rabbits were anesthetized with a mixture of ketamine and acepromazine after premedication with xylazine. The descending thoracic aorta was exposed, ligated at both ends, dissected from mediastinum, and immediately immersed in sterile PBS. Adherent perivascular connective tissue was gently dissected away, and the aorta was carefully cut along the longitudinal axis. The artery was then cut into identically sized strips, 3 mm in width and 9 mm in length. At the time of harvest, some strips were denuded by gently scraping the endothelial surface with a sterile scalpel blade; care was taken to preserve the remaining strips intact. Before transfection, all arteries, denuded and nondenuded as well, were cultured for up to 21 d in 2 ml of DME (GIBCO BRL, Gaithersburg, MD) containing 2 mM L-glutamine (GIBCO BRL), 100 IU/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL), which was supplemented with 20% FCS (GIBCO BRL), in an atmosphere of 95% air and 5% CO<sub>2</sub> at 97% humidity.

**In vitro arterial gene transfer in organ culture.** Two different plasmids were used for the in vitro experiments. The plasmid pRSVLUC (courtesy of Dr. Allan R. Brasier, University of Texas, Galveston, TX)

containing the firefly luciferase coding sequence (10) coupled to the Rous sarcoma virus long terminal repeat promoter (11) was used to serve as a quantifiable measure of transfection efficiency (12). The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot, Institut Pasteur, Paris, France) containing a nuclear targeted  $\beta$ -galactosidase sequence coupled to the simian virus 40 early promoter was used as well to permit identification of cellular locale resulting from successful transfection (13). The expression of this plasmid cannot result from endogenous  $\beta$ -galactosidase activity; accordingly, histochemical identification of  $\beta$ -galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered nonspecific for the purpose of this study. The plasmid DNA and liposome, a 1:1 (wt/wt) formulation of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride and dioleoyl phosphatidylethanolamine (Lipofectin Reagent; GIBCO BRL) (14), were diluted separately in reduced serum media (OPTI-MEM; GIBCO BRL). Six different ratios of DNA to liposome were tested in vitro. The highest level of expression was observed at a ratio of 1:4 ( $28.7 \pm 6.5 \times 10^3$  Turner light units [TLU]<sup>1</sup>/g of tissue), which was significantly higher than either a ratio of 1:1 ( $1.5 \pm 0.9 \times 10^3$  TLU/g;  $P < 0.05$ ) or 1:6 ( $7.9 \pm 3.5 \times 10^3$  TLU/g;  $P < 0.05$ ). This DNA/liposome ratio (1:4) was next optimized for the content of DNA, varying the amount of DNA used from 2.5 to 15  $\mu$ g/ml. Specifically, mixtures prepared with 2.5:10, 5:20, 7.5:30, 10:40, 12.5:50, and 15:60 of DNA/liposomes ( $\mu$ g/ml), respectively, were tested for optimal gene expression. Highest gene expression was achieved using a mixture of 10  $\mu$ g/ml of DNA with 40  $\mu$ g/ml of liposomes. The two solutions of plasmid DNA and liposomes therefore were mixed together in a petri dish to a final concentration of 10  $\mu$ g/ml DNA and 40  $\mu$ g/ml liposomes. Cultured strips of rabbit aorta were transferred into this transfection medium and incubated for 30 min on a rotating plate. Upon completion of transfection, the medium was changed again to 2 ml of DME with 20% FCS. 3 d after transfection, all arteries were harvested for the purpose of determining the magnitude of gene expression. Luciferase activity was quantified using the Luciferase Assay System (Promega Biotec, Madison, WI) as described previously (5, 15). Briefly, the artery to be analyzed was ground to a powder, resuspended in 1 $\times$  cell culture lysis reagent, and homogenized (Virtis Co., Gardiner, NY). The resulting mixture was spun for 5 min. A 20- $\mu$ l aliquot of the cell extract was then mixed in a sample tube with 100  $\mu$ l of luciferase assay reagent at room temperature and inserted into a luminometer (20e; Turner Designs, Sunnyvale, CA) that reports results on a scale established to yield as low as  $10^{-3}$  TLU. Three different aliquots for each specimen were analyzed in this manner. The specimen's total luciferase activity was derived from the mean of the aliquot results. The light unit values obtained were within the linear range of a dilution curve established by analysis of light units produced from a known amount of luciferase (Sigma Chemical Co., St. Louis, MO). Although assays of different dilutions of luciferase standards constantly yielded linear curves, conversion into picograms resulted in important level variations depending on the source of the standard, as others have also reported (16). Thus, results are expressed as TLU. As a reference, under our conditions, transfection of rabbit vascular smooth muscle cells (80% confluent) in 60-mm dishes, for 30 min, using 15  $\mu$ g of pRSVLUC and 45  $\mu$ g of Lipofectin yielded  $431.3 \pm 49.1$  TLU per dish (total from all cells, five dishes) when assayed 3 d later. Background level was always measured by analyzing a sample consisting only of cell culture lysis reagent and luciferase assay reagent but lacking homogenized tissue; all such analyses consistently produced readings of 0 TLU.  $\beta$ -Galactosidase activity was determined by incubation of arterial strips with 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside chromogen (X-Gal; Sigma Chemical Co.) as previously described (17). After staining with X-Gal solution, tissues were paraffin embedded, sectioned, and counterstained with nuclear fast red.

1. *Abbreviations used in this paper:* PCNA, proliferating cell nuclear antigen; TLU, Turner light units; X-Gal, 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside chromogen.

*Assessment of proliferative activity of arteries in organ culture.* Proliferative activity of intimal cells was evaluated using [<sup>3</sup>H]thymidine (2.5  $\mu$ Ci/ml, 6.7 Ci/mmol; New England Nuclear, Boston, MA). Non-denuded and denuded arteries were pulse labeled by [<sup>3</sup>H]thymidine for 24 h. After labeling, tissues were washed and fixed in formalin. Paraffin-embedded sections were cut on to glass slides. Deparaffinized sections were dipped in Kodak NTB-2 emulsion, air dried, and exposed at 4°C in the dark for 7 d, and then stained with hematoxylin and eosin. The total cellular content of the entire section was manually counted under an  $\times 40$  objective. In denuded arteries, the mean number of thymidine-labeled neointimal smooth muscle cells per field was determined; in nondenuded arteries, the mean number of thymidine-labeled endothelial cells per field was evaluated as well. To identify cells in the neointima as smooth muscle cells, immunohistological staining with a monoclonal antibody to smooth muscle  $\alpha$ -actin (HHF-35; Enzo Diagnostics, New York, NY) was applied to paraffin-embedded sections cut on to a poly-L-lysine-coated slides. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxidase in PBS. Sections were pretreated with normal horse serum and then incubated with monoclonal antibody to smooth muscle  $\alpha$ -actin for 30 min at room temperature. Bound primary antibody was detected using an avidin-biotin-immunoperoxidase method according to the supplier's guidelines (Signet Laboratories, Dedham, MA). Sections were lightly counterstained with hematoxylin.

#### *In vivo studies*

*Animal model.* In vivo analysis of gene expression as a function of intimal proliferation was investigated using the New Zealand White rabbits described above for in vitro studies. In preparation for arterial gene transfer, rabbits were fed a 0.5% hypercholesterolemic diet for 2 wk, after which balloon endothelial denudation of both external iliac arteries was performed as previously described (5, 18). 5–6 wk after denudation, one external iliac artery of each rabbit was dilated using a 2.5-mm standard percutaneous transluminal coronary angioplasty balloon catheter (Boston Scientific, Watertown, MA) under fluoroscopic guidance. The catheter was introduced through the right common carotid artery, and advanced to one external iliac artery; balloon inflation was performed at 8 atmospheres for 60 s, and after deflation, repeated a second time. The contralateral external iliac artery was not treated with balloon angioplasty in order to serve as a control.

*Percutaneous arterial gene transfer.* The same plasmids (pRSVLUC, pGSVLacZ) used for arterial gene transfer in organ culture were used for in vivo arterial gene transfer. Between 0 and 14 d after balloon angioplasty, percutaneous arterial gene transfer was performed. Rabbits were anesthetized with ketamine and acepromazine, intubated, and maintained with nitrous oxide and isoflurane anesthesia. A 5-Fr. double-balloon catheter (Boston Scientific), designed to infuse a solution of DNA and liposomes into a 2-cm long segment of artery isolated between the two balloons, was introduced through the right common carotid artery, and advanced to one external iliac artery. The proximal balloon was inflated at the origin of external iliac artery, after which the distal vessel was washed sequentially with 5 ml of saline and 5 ml of reduced-serum medium (OPTI-MEM). Next, 0.5–1.5 ml of the DNA/liposome solution (20  $\mu$ g/ml of DNA, 80  $\mu$ g/ml of liposome) was infused through the catheter via a perfusion port positioned midway between the two inflated balloons. An infusion of 0.2 ml of OPTI-MEM was used to advance any DNA/liposome solution remaining in the catheter lumen proximal to the balloons. The two balloons remained inflated for 30 min. The contralateral external iliac artery, in which primary balloon denudation had been performed but balloon angioplasty had not, was also transfected with an identical amount of DNA/liposome solution using the double-balloon catheter in an identical fashion.

Transfected arterial segments of both external iliac arteries were harvested 3 d after transfection for analysis of gene expression. Quantitative analysis of gene expression was performed by measurement of luciferase activity as described above. Identification of the site and the number of cells successfully transfected in vivo was performed in two

ways. The first involved the protocol for nuclear-targeted  $\beta$ -galactosidase described above. In addition, sections taken from the mid-portion of atherosclerotic arteries transfected with pRSVLUC were hybridized with antisense-radiolabeled probes generated from a riboprobe vector, pOLUC, containing the same firefly luciferase cDNA as that found in pRSVLUC. To rule out false-positive results, sense-radiolabeled probes for luciferase were used on the same series of slides under identical conditions. Due to the fact that frozen sections typically disrupt the cytoarchitecture of cells in atherosclerotic specimens, it is frequently problematic to determine with certainty that silver grains are exclusively limited to the boundaries of the cytoplasm as opposed to the extracellular matrix. Accordingly, we used the convention adopted by previous investigators (19–21) in which positive hybridization is determined by the finding of silver grain clusters in relation to the cell nucleus. This interpretation is further supported by previous work indicating that the mRNA for certain proteins is preferentially limited to a perinuclear site (22). Probe preparation and in situ hybridization were performed as described previously (5, 23).

#### Assessment of intimal proliferative activity after angioplasty in vivo.

To evaluate the time course of intimal proliferative activity postangioplasty, external iliac arteries subjected to the above-described sequence of balloon denudation and angioplasty were studied by histochemical analysis for proliferating cell nuclear antigen (PCNA) as previously described (7). Briefly, the presence of PCNA was evaluated using a monoclonal antibody to human PCNA (Signet Laboratories). Tissues were fixed overnight with 100% methanol. After paraffin embedding, sections were cut on to poly-L-lysine-coated slides and air dried overnight. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxidase in PBS. Sections were pretreated with normal horse serum and then incubated with anti-PCNA antibody (1:50 dilution) overnight at 4°C. Bound primary antibody was detected using a streptavidin-biotin-alkaline phosphatase method according to the supplier's guidelines (StrAvidGen Super Sensitive; Biogenex, San Ramon, CA). Sections were lightly counterstained with either methyl green or hematoxylin. Cells within the atherosclerotic plaque were counted under an  $\times 40$  objective to determine the mean percentage of cells expressing PCNA relative to the total number of neointimal cells examined.

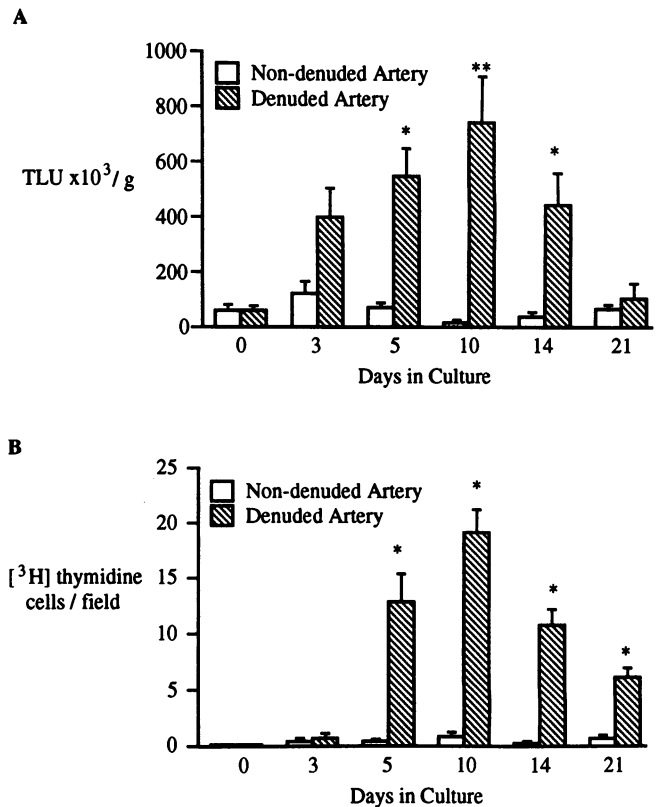
#### Statistics

Results were expressed as mean  $\pm$  SEM. Statistical significance of obscured differences was evaluated using the Student's *t* test for unpaired observations. A value of  $P < 0.05$  was accepted to denote statistical significance.

## Results

### Gene expression and intimal proliferative activity in organ culture

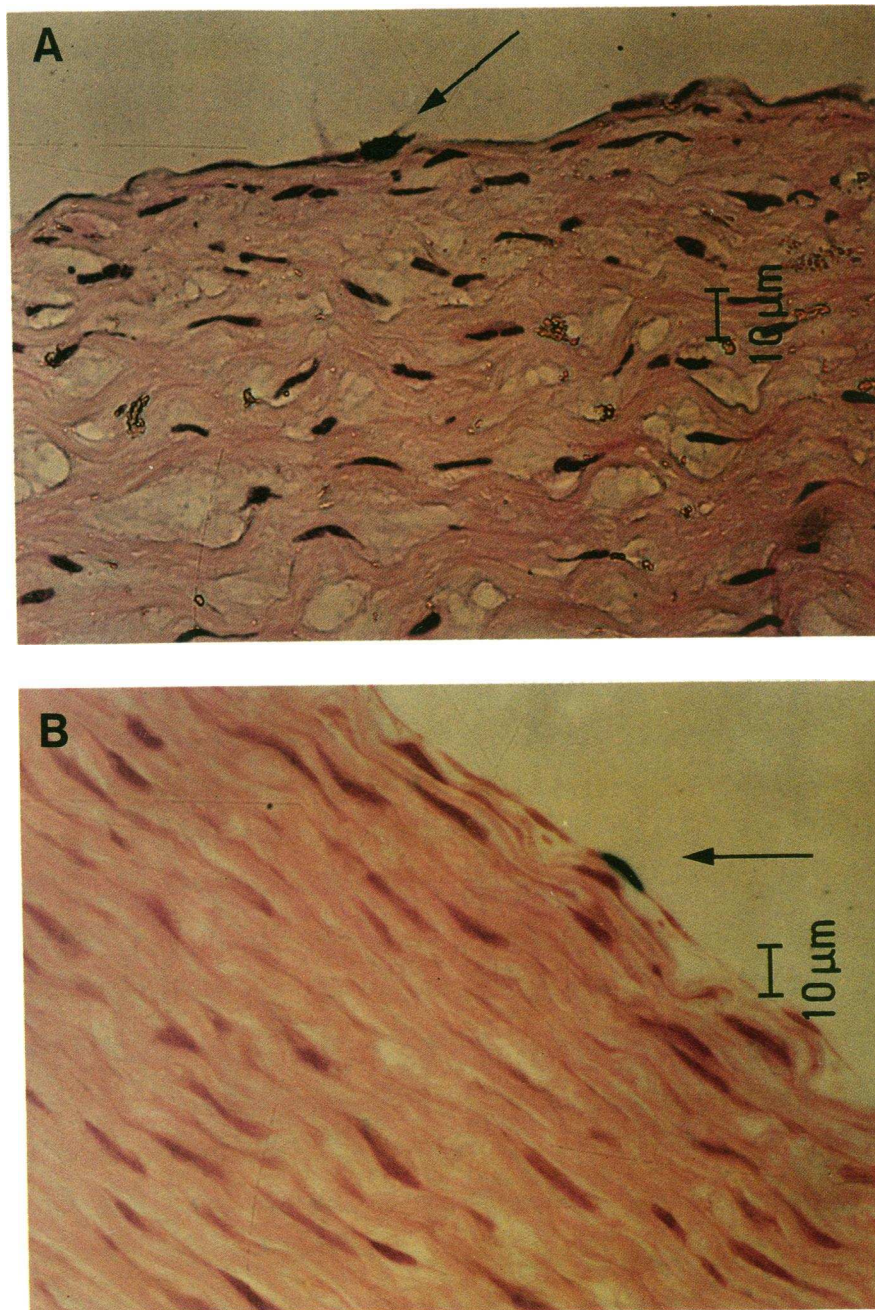
**Luciferase activity (Fig. 1A).** Nondenuded and denuded arterial strips were transfected with pRSVLUC at days 0, 3, 5, 10, 14, and 21. At each time point, transfection was performed in four or more nondenuded and four or more denuded arterial strips. For nondenuded arteries, the length of time in organ culture before transfection had no effect upon the level of gene expression. Specifically, luciferase activity of nondenuded arteries at day 0 ( $57.1 \pm 21.9 \times 10^3$  TLU/g) was not different from those at subsequent timepoints including day 3 ( $120.5 \pm 43.2$ ); day 5 ( $69.1 \pm 18.2$ ); day 10 ( $16.2 \pm 3.5$ ); day 14 ( $38.5 \pm 12.7$ ); and day 21 ( $66.4 \pm 17.2$ ) ( $P = \text{NS}$  for all). In contrast, denuded arteries transfected between 3 and 14 d postdenudation yielded 7–13-fold higher expression compared with day 0; maximum expression was observed at day 10. Specifically, for denuded arteries, luciferase activity increased significantly from that measured in strips transfected at day 0 ( $58.5 \pm 20.5 \times 10^3$  TLU/g) to that measured in strips transfected at day 3 ( $398.6 \pm 104.0$ ;



**Figure 1.** Effect of cell proliferation on gene expression in arterial organ culture. (A) Gene expression, indicated by luciferase activity measured in TLU/g of tissue, remains low in all nondenuded arterial strips, regardless of the number of days strips were cultivated before transfection with pRSVLUC. In contrast, gene expression increased in denuded arterial strips, particularly those maintained in organ culture for  $\geq 3$  d before transfection. Asterisks denote statistically significant increase in luciferase activity in denuded vs. nondenuded strips transfected after 5 ( $*P < 0.05$ ), 10 ( $**P < 0.01$ ), and 14 d ( $*P < 0.05$ ) in culture. (B) Proliferative activity indicated by [<sup>3</sup>H]-thymidine labeling remains low in all nondenuded arterial strips. Among denuded arterial strips, the number of labeled cells increased by day 5 and reached a maximum at day 10. The difference in the number of thymidine-labeled cells in nondenuded vs. denuded arteries was statistically significant at days 5, 10, 14, and 21 ( $*P < 0.001$  for all).

$P < 0.005$ ); day 5 ( $546.0 \pm 100.5$ ;  $P < 0.001$ ); day 10 ( $741.1 \pm 168.1$ ;  $P < 0.001$ ); and day 14 ( $443.8 \pm 113.3$ ;  $P < 0.005$ ). When luciferase expression of denuded arteries was compared with that observed in nondenuded arteries transfected at identical timepoints, luciferase activity of denuded arteries was significantly higher than that of nondenuded arteries at day 5 ( $P < 0.05$ ), day 10 ( $P < 0.01$ ) and day 14 ( $P < 0.05$ ).

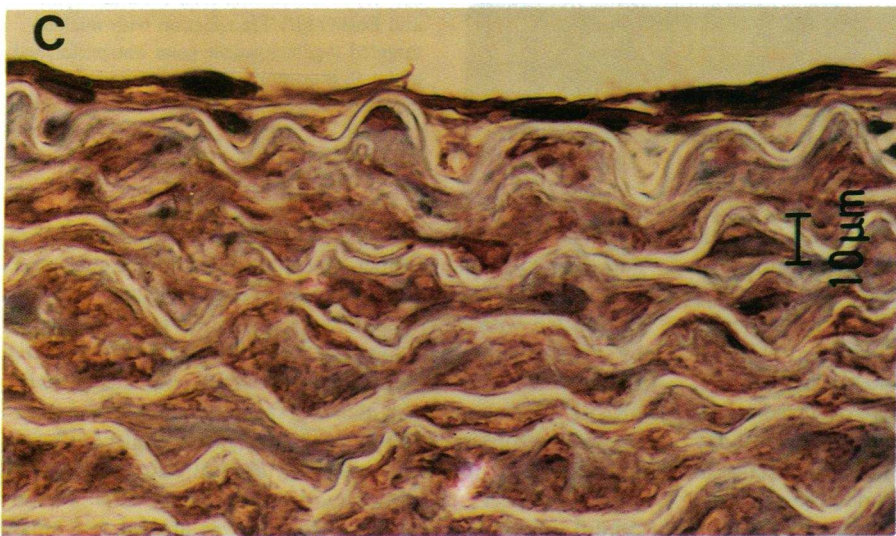
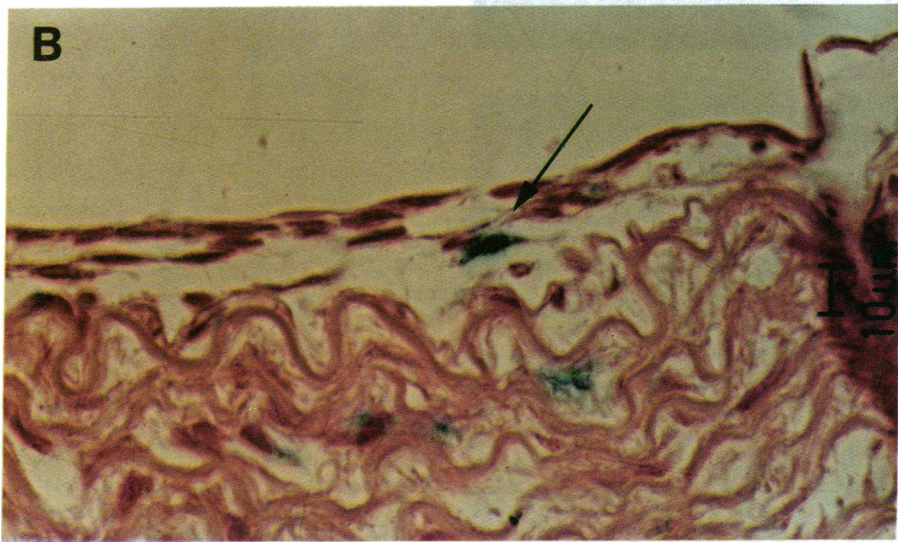
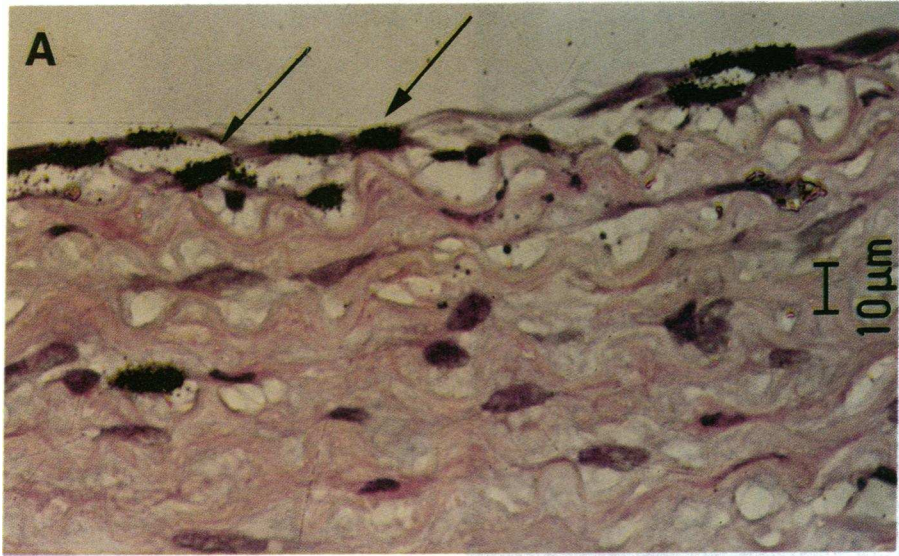
**Proliferative activity (Fig. 1B).** Nondenuded and denuded arterial strips were pulse labeled with [<sup>3</sup>H]thymidine for 24 h at days 0, 3, 5, 10, 14, and 21. For each time point, six or more nondenuded and six or more denuded strips were studied. Among strips labeled at day 0, no cells were labeled with thymidine in either nondenuded or denuded arteries. In nondenuded arteries, a small number of thymidine-labeled endothelial cells was observed at day 3, and the number of these cells remained stationary over the 21 d in culture: day 3 ( $0.3 \pm 0.3$ ); day 5 ( $0.3 \pm 0.2$ ); day 10 ( $1.0 \pm 0.3$ ); day 14 ( $0.2 \pm 0.1$ ); and day 21



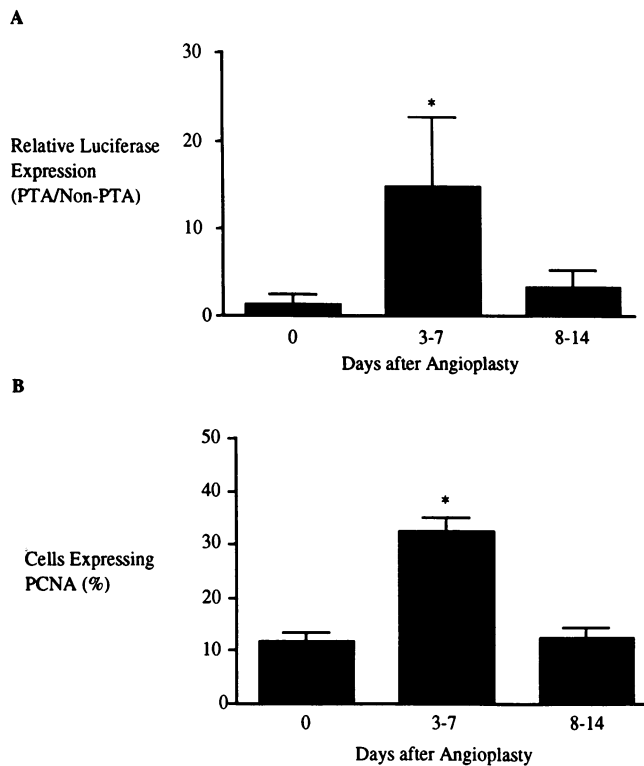
**Figure 2.** Light photomicrographs of non-denuded arterial organ culture. (A) [<sup>3</sup>H]-Thymidine labeling (arrow) of non-denuded artery at day 10 indicates low proliferative activity of endothelial cells. (Hematoxylin and Eosin) (B) Transfection with nuclear-targeted  $\beta$ -galactosidase gene was performed at day 0. Blue-stained nucleus (arrow) indicates successfully transfected endothelial cell (nuclear fast red).

( $0.6 \pm 0.3$  cells/field) (Fig. 2 A). In denuded arteries, neointimal cells were first observed at day 3, although the number of these cells was small; the number rapidly increased by day 5, and reached a plateau at day 10. Specifically, the number of thymidine-labeled neointimal cells increased from  $0.7 \pm 0.3$  cells/field at day 3, to  $12.9 \pm 2.4$  at day 5, and reached a maximum of  $19.1 \pm 2.1$  at day 10, after which there occurred a progressive decline in the number of thymidine-labeled cells: day 14 ( $10.8 \pm 1.4$ ); day 21 ( $6.0 \pm 0.9$ ) (Fig. 3 A). The difference in the number of thymidine-labeled cells in non-denuded vs. denuded arteries was significant at days 5, 10, 14, and 21 (all  $P < 0.001$ ). Virtually all cells forming the neointima of the denuded arteries stained positively with HHF-35, indicating the smooth muscle cell origin of these neointimal cells as previously reported by others (9) (Fig. 3 C).

**Locale and extent of cells transfected in vitro.** To localize and determine the number of cells successfully transfected,  $\geq 20$  histologic sections removed from each of 20 arterial strips transfected with pGSVLacZ were examined histochemically by staining with X-Gal solution. As negative controls, we used non-denuded and denuded arterial strips transfected with pRSVLUC plasmid in an identical fashion; blue nuclear staining was not observed in any of these negative control arteries. In both non-denuded and denuded arterial strips transfected with pGSVLacZ, dark blue nuclear staining indicative of successful transfection was observed, but was limited to a small number of cells (Figs. 2 B and 3 B). The number of endothelial and/or neointimal smooth muscle cells demonstrating evidence of successful transfection was  $< 1\%$  in all 20 strips examined in this manner.



*Figure 3.* Light photomicrographs of denuded arterial organ culture. (A) [<sup>3</sup>H]-Thymidine labeling (*arrows*) of denuded artery at day 10 indicates increased proliferative activity of neointimal cells. (Hematoxylin and Eosin) (B) Transfection with nuclear-targeted  $\beta$ -galactosidase gene was performed at 10 d postdenudation. Blue-stained nucleus (*arrow*) indicates successfully transfected neointimal cell (nuclear fast red). (C) Virtually all cells forming neointima of denuded arteries are stained positively with HHF-35, indicating the smooth muscle cell origin of these neointimal cells (Hematoxylin).



**Figure 4.** Effect of cell proliferation on gene expression in vivo. (A) Transfection with pRSVLUC was performed percutaneously in vivo using a double-balloon catheter to deliver a mixture of plasmid and liposomes to both external iliac arteries of a New Zealand White rabbit; one artery underwent balloon angioplasty (PTA) 0–14 d before transfection, while contralateral artery had no prior PTA. Relative luciferase activity (PTA-treated artery/non-PTA-treated artery) calculated for each rabbit increased significantly from group transfected at day 0 to group transfected at days 3–7 post-PTA ( $*P < 0.05$ ), then declined among group transfected 8–14 d post-PTA. (B) Percentage of neointimal cells expressing PCNA increased significantly from day 0 to days 3–7, and declined by days 8–14 ( $*P < 0.001$  vs. day 0 and days 8–14).

#### Gene expression and intimal proliferative activity in vivo

**Luciferase activity (Fig. 4A).** A total of 34 arteries was percutaneously transfected in vivo, either with pRSVLUC ( $n = 30$ ) or pGSVLacZ ( $n = 4$ ). In 17 arteries, no antecedent angioplasty was performed. In the remaining 17 arteries, transfection was performed immediately after angioplasty (day 0,  $n = 6$ ), 3–7 d after angioplasty ( $n = 7$ ), and 8–14 d after angioplasty ( $n = 4$ ). Transfection with pRSVLUC was technically successful in 29 of 30 external iliac arteries; in one case in which the artery was totally occluded, percutaneous transfection could not be completed. Luciferase activity was documented in 24 (82.8%) of 29 arteries. For each animal, luciferase activity in the angioplasty-treated artery was compared with that measured in the non-angioplasty-treated artery. The relative luciferase activity (angioplasty-treated artery/non-angioplasty-treated artery) calculated for each rabbit increased significantly from those in which transfection was performed at day 0 ( $1.3 \pm 1.2$ ) to animals transfected 3–7 d postangioplasty ( $14.4 \pm 8.0$ ;  $P < 0.05$ ); for animals in which transfection was performed on days 8–14 postangioplasty, analysis of relative luciferase activity disclosed

no significant increase compared to transfection on day 0 ( $3.3 \pm 1.9$  vs.  $1.3 \pm 1.2$ ;  $P = \text{NS}$ ).

**Proliferative activity (Figs. 4B and 5, A and B).** Iliac arteries in which initial balloon injury was followed 5–6 wk later by balloon angioplasty were then evaluated for the extent of proliferative activity at 0–14 d postangioplasty. The percentage of neointimal cells expressing PCNA increased significantly from day 0 ( $11.9 \pm 1.4\%$ ) to days 3–7 ( $32.2 \pm 2.7\%$ ), and returned to baseline levels by days 8–14 ( $12.3 \pm 2.1\%$ ) postangioplasty. Proliferative activity measured at days 3–7 was significantly higher than that measured either at day 0 ( $P < 0.001$ ) or at days 8–14 ( $P < 0.005$ ).

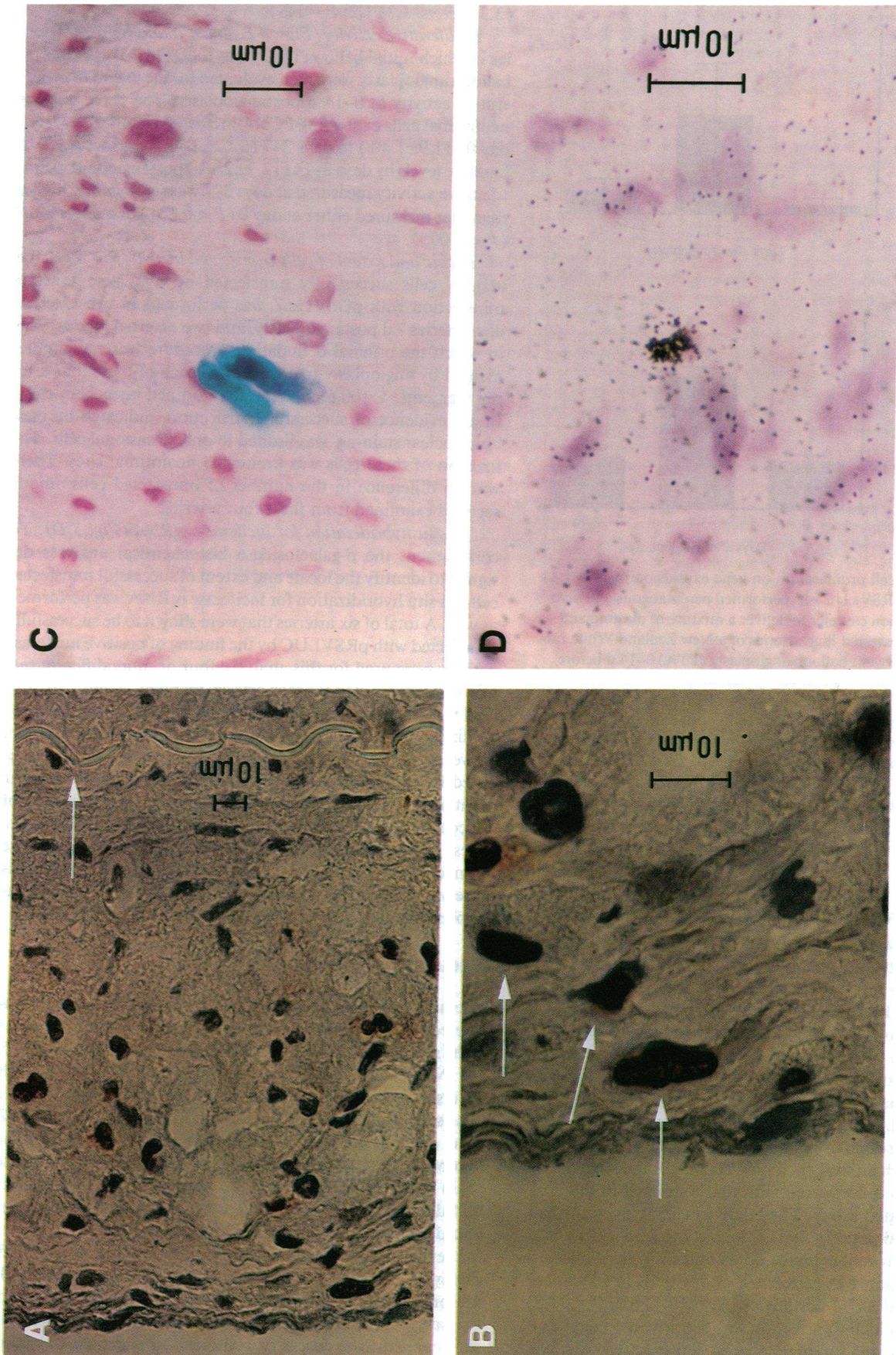
**Locale and extent of cells transfected in vivo (Fig. 5C).** To identify cells successfully transfected in vivo, percutaneous transfection with pGSVLacZ was performed in two external iliac arteries 7 d postangioplasty, and two external iliac arteries in which the original denudation procedure was not supplemented by angioplasty treatment. For each of these four arterial segments,  $\geq 100$  sections were examined histochemically. In all, evidence of successful transfection, indicated by dark blue nuclear staining, was limited to only occasional cells. The location of these cells was limited to neointimal sites. There was no difference in the number of transfected cells among sections examined from these four arteries.

**In situ hybridization for luciferase mRNA (Fig. 5D).** To complement the  $\beta$ -galactosidase histochemical analyses designed to identify the locale and extent of successful transfected cells, in situ hybridization for luciferase mRNA was performed as well. A total of six arteries that were shown to be successfully transfected with pRSVLUC by the finding of positive luciferase activity were used for this analysis; four or more different sections were examined for each arterial segment. Arteries hybridized with the sense-radiolabeled probe showed no positive hybridization signal. Arteries hybridized with the antisense probe, however, showed the evidence of positive transfection, as indicated by the finding of silver grain clusters in a perinuclear location (5). As was the case with pGSVLacZ, evidence of successful transfection was limited to only occasional cells. These cells were again limited to neointimal lesions, and the number of these cells was estimated to be  $< 0.1\%$  of total cells observed in the neointima of the non-angioplasty- and/or angioplasty-treated arterial segments.

#### Discussion

Attempts to perform arterial gene transfer have been repeatedly compromised by low transfection efficiencies, regardless of the site, species, gene construct, or delivery device used (1–5, 24). Nabel et al. (1), for example, estimated that  $< 1\%$  of cells were successfully transfected when a double-balloon catheter was used to transfect porcine ilio-femoral arteries with the *lacZ* gene, either in a retroviral vector or encapsulated by cationic liposomes. Likewise, Flugelman et al. (3) using the polymerase chain reaction to investigate the efficiency of retrovirus-mediated gene transfer in rabbit aortas, found that  $< 100$  transfected cells were present in a 2-cm length of artery.

Several factors may account for the low transfection efficiency observed in these previous experiments. These include not only technical factors, but certain biological properties of the target cells and/or organisms themselves that may represent constitutive sources of resistance to successful transfection.



**Figure 5.** Light photomicrographs of external iliac arteries postangioplasty (PTA). (A) Evidence of cellular proliferation indicated by immunostaining for PCNA in artery 3 d post-PTA. Arrow indicates internal elastic membrane (Hematoxylin). (B) High-power magnification of the same arterial segment. Arrows indicate positive nuclear staining for PCNA (Hematoxylin). (C) Transfection with nuclear-targeted  $\beta$ -galactosidase gene was performed at 7 d post-PTA. Dark blue-stained nucleus indicates successfully transfected neointimal cell (nuclear fast red). (D) In situ hybridization for luciferase mRNA indicates cellular locale of successful transfection with pRSV-LUC in vivo, performed 7 d post-PTA. Silver grain clusters in a perinuclear location indicates positive transfectant (Hematoxylin and Eosin).



tion. It is noteworthy, for example, that all of the above-cited attempts to perform arterial gene transfer involved arteries with a stationary cell population. Previous applications of gene transfer to nonvascular organ systems have indicated that the proliferative state of the target cells may have a profound impact upon the extent of gene expression. Miller et al. (25), for example, established that gene transfer was inhibited 100-fold in stationary vs. replicating cells. This effect, demonstrated with a replication-defective retroviral vector applied to rat fibroblasts, could not be obviated even when cells that were stationary at the time of gene transfer were stimulated to divide 6 h to 10 d posttransfection. For retroviral-mediated transfection, it thus appeared that infected cells were required to be replicating at the time of transfection.

Similarly favorable effects of cell proliferation upon transfection efficiency have been described *in vitro* using SV-40 DNA-calcium coprecipitates to transfect synchronized cultures of CV-1 cells (6), and *in vivo* using protein conjugates to facilitate transfection of rapidly dividing hepatocytes after partial hepatectomy (26, 27).

For liposome-based transfection, published data regarding the relationship of transfection to the host cell cycle is limited to studies performed *in vitro* in which neutral liposomes were used to transfect HeLa cells with  $\beta$ -lactamase (28); a twofold increase in exogenous DNA associated with nuclear DNA was observed among mitotic cells, compared with cells synchronized in G<sub>1</sub>. In the current series of experiments, transfection of stationary cells, even when transfection conditions were rigorously optimized for DNA-to-liposome ratio, and DNA concentration, continued to yield low levels of gene expression *in vitro*, as well as *in vivo*. In contrast, when gene transfer was directed to arteries in which cellular proliferation had been provoked by antecedent balloon injury, gene expression, judged by the magnitude of measured luciferase activity, increased by > 10-fold.

The mechanism by which cellular proliferation augments expression of liposome-mediated gene transfer remains enigmatic. Nicolau and Sene (28) suggested that nuclear uptake may occur preferentially in those cells that are entering mitosis, consequent to breakdown of the nuclear membrane. Strain et al. (6) proposed that nuclear factors responsible for preserving stability of DNA may be dispersed throughout the cell upon breakdown of the nuclear membrane during mitosis; these as yet unnamed factors might then act to stabilize foreign genetic material in the same way that such factors are presumed to protect and/or stabilize endogenous chromosomal DNA during mitosis (29). Reports of gene transfer performed *in vivo* using protein conjugates support this concept. Specifically, Wilson et al. (27) demonstrated that enhanced transfection efficiency observed in rapidly dividing hepatocytes was not the result of incorporation of the transgene into chromosomal DNA; they proposed instead that the foreign DNA was rendered resistant to nuclease digestion by alterations induced in its structure and/or protective intracellular partitioning.

The basis for the salutary effect of cellular proliferation on gene expression thus requires further clarification. The fact that we observed little effect on the number of cells transfected, yet measured a 10-fold increase in luciferase activity, leaves open the possibility that the favorable effect of cell proliferation may be conferred at a transcriptional or posttranscriptional level. In this regard, neither enhanced transcriptional activity

of the promoter nor posttranscriptional modulation of luciferase steady-state levels has yet been excluded.

The findings of this study have potential implications for previously proposed applications of arterial gene transfer (30, 31) using cationic liposomes. The first, and perhaps most important, concerns the proposed use of gene therapy for the treatment of restenosis. Using *in situ* hybridization and immunohistochemistry, we recently identified the mRNA for PCNA or the protein itself in  $20.6 \pm 18.2$  and  $15.2 \pm 13.6\%$ , respectively, of cells in restenosis lesions excised by directional atherectomy (7). Even for primary lesions, the extent of proliferative activity indicated by identification of PCNA mRNA *in situ* ( $7.2 \pm 10.8\%$ ), or by immunostaining for PCNA ( $3.6 \pm 3.5\%$ ), exceeded that previously observed in end-stage atherosclerotic lesions (32). Demonstration of evident ongoing proliferative activity at the time of percutaneous revascularization thus implies that the target cells themselves at the site of arterial narrowing may serve to augment the expression of gene transfer beyond levels previously reported in quiescent arteries. The observation that proliferative activity is typically higher in restenotic vs. primary lesions suggests that the former may be more suitable targets for liposome-mediated arterial gene transfer. It must be acknowledged, however, that the degree of proliferative activity necessary to augment gene expression to any given level will likely vary with the nature of the transgene and the putative role of that transgene in the pathogenesis of restenosis. Furthermore, the advent of novel liposome preparations, including those with poly- as opposed to monocationic lipid formulations (33), suggests that current experience with Lipofectin may not be representative of what can ultimately be achieved with second and third generation liposomal reagents (34).

Second, the demonstration that gene expression may be increased among proliferating cells, even though the number of successfully transfected cells per artery does not appear increased, is encouraging for those applications of gene therapy involving gene products that are either secreted or expressed at the cell surface. We have recently observed, for example, local levels of human growth hormone that were in a physiologic range in rabbit ear arteries transfected with pXGH5 (35), even though necropsy examination disclosed immunohistochemical evidence of successful transfection in well under 1% of cells in the transfected arterial segment (36). Likewise, Nabel et al. documented evidence of extensive vasculitis in arteries transfected with the gene for the foreign class I major histocompatibility complex HLA-B7 (37), development of intimal thickening in arteries transfected with the gene for platelet-derived growth factor-B (38), intimal hyperplasia and angiogenesis in arteries transfected with a secreted form of the FGF-1 gene (39), and intimal hyperplasia with substantial extracellular matrix production in arteries transduced with an expression vector for secreted, active TGF- $\beta$ 1 (40), in spite of the fact that in all cases successful transfection appeared limited to < 1% of cells. Thus, in the case of a transgene encoding for a secreted protein, a 10-fold increase in gene expression may well have significant biological consequences, even if the number of transfected cells remains limited.

Conversely, the current findings may not have favorable implications for gene transfer strategies that do not involve a secreted protein. Because such cases would require that a cell be successfully transfected in order for the gene product to

achieve its desired effect, a low number of successfully transfected cells might continue to compromise the intended therapeutic goal. To optimize transfection efficiency under these circumstances may require alternative modes of gene delivery, including modifications in catheter design (41) and/or the use of alternative vectors (42, 43). While safety issues regarding the use of retroviral and adenoviral vectors remain to be fully resolved, both have been shown capable of achieving superior transfection efficiencies (24, 43). As indicated above, previous work has established that retroviral infection requires active cell proliferation. Although this is clearly not the case for the adenovirus (24), the possibility that active cell proliferation may augment the efficiency of adenoviral gene transfer beyond that achieved thus far in stationary cell populations remains to be tested.

The results of these experiments also address the effect of arterial injury upon the expression of gene transfer. Gene expression was not compromised by either aggressive endothelial disruption *in vitro*, or balloon angioplasty *in vivo* performed immediately before transfection. These results thus suggest that the arterial injury performed immediately before transfection is unlikely to independently diminish the expression of liposome-mediated arterial gene transfer.

Finally, in contrast to the previously reported observations involving normal arteries (1, 37), the cellular locale for successful transfection was not observed in the medial layer of atherosclerotic arteries, but was instead limited to sites within the atherosclerotic plaque. Although this investigation was not designed to address this issue, it is entirely possible that the thickness and/or certain constituents (e.g., extracellular lipid) of the atherosclerotic lesion may potentially interfere with successful transfection of medial cells.

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