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Efficient in Vivo Catheter-Based Pericardial Gene Transfer Mediated by Adenoviral Vectors

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Summary: Adenoviral vectors are promising agents for a number of in vivo gene therapy applications including diseases of the heart and coronary vessels. Efficient intravascular gene transfer to specific sites has been achieved in occluded vessels, but otherwise is hampered by the effect of blood flow on localized vector uptake in the vessel wall. An alternative delivery approach to coronary arteries is the expression of diffusible gene products into the pericardial space surrounding the heart and coronary arteries. However, in vivo pericardial access is comparatively difficult and has been limited to surgical approaches. We hypothesized that efficient adenovirus-mediated gene expression in pericardial lining mesothelium could be achieved by transmyocardial vector delivery to the pericardium. To evaluate this concept, a hollow, helical-tipped penetrating catheter was used to deliver vector-containing fluid directly into the intrapericardial space. The catheter was introduced percutaneously in anesthetized mongrel dogs, advanced into the right ventricle, and the tip passed through the apical right ventricular myocardium under direct radiographic visualization until the open end of the catheter tip resided in the intrapericardial space. Adenoviral vectors expressing either nuclear-localizing beta-galactosidase, cytoplasmic luciferase, or secreted human α1AT reporters (Av1nBg, Av1Lu, or Av1Aa, respectively) were instilled through the catheter into the intrapericardial space. Three days later the animals were sacrificed and reporter gene expression was evaluated in pericardium, epicardium, and multiple other tissues. In animals receiving Av1nBg, beta-galactosidase activity was evident in most of the pericardial lining endothelium, up to 100% in many areas. In animals receiving Av1Lu, luciferase reporter activity was abundant in pericardial tissues, but near-background levels were observed in other organs. In animals receiving Av1Aa, human α1AT was abundant (16–29 mg/ml) in pericardial fluid, but was undetectable in serum. All animals tolerated the procedure well with no electrocardiographic changes and no clinical sequelae. These observations demonstrate highly efficient adenovirus vector delivery and gene transfer and expression in the pericardium and support the feasibility of localized gene therapy via catheter-based pericardial approaches. We suggest that the pericardial sac may serve as a sustained-release protein delivery system for the generation of desired gene products or their metabolites for diffusion into the epicardial region.

Introduction

Heart disease is one of the most important clinical problems in humans and is a leading cause of death and morbidity. Adenovirus-mediated gene transfer to the myocardium and vasculature is a promising new treatment approach for some of these diseases in humans and offers a method to test specific hypotheses regarding the function of certain genes within cardiac and vascular tissues in animal models. Myocardial ischemia due to coronary insufficiency is a disease for which gene therapy may be useful, and one potential therapeutic approach could be to enhance collateral coronary circulation through induction of coronary angiogenesis. Recent studies have also suggested the feasibility of augmenting angiogenesis in models of peripheral ischemia by direct arterial gene transfer.[1]

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Gene transfer to the vascular wall has been approached using a variety of vectors including retroviruses, DNA-liposomes, and adenovirus vectors, as well as by reimplantation of genetically modified vascular cells.[2] Although adenovirus appears to be the most efficient of these vectors in vivo, high-frequency transduction has been accomplished in the vasculature only by isolation of a vascular segment for 20 to 45 min to allow for vector uptake into the cell of the vascular wall.[3–5] Poloxamer 407 offers one possible strategy to reduce this requirement for a prolonged contact time by increasing the apparent adenovirus vector transduction rate by as much as 30 to 100 fold.[6–8]

Another hurdle for localized therapeutic delivery to specific intravascular sites of disease is the difficulty in achieving high-efficiency local gene delivery without significant systemic delivery to many other sites. This difficulty is due to rapid blood flow and the finite time required for efficient vector uptake.[9] Intravascular administration has been associated with extensive systemic gene transfer due to circulation of vector beyond the target tissue.[10] Myocardial gene transfer has been performed using a variety of methods, including the delivery of adenovirus by arterial infusion[11] or direct myocardial injection.[12] Techniques employing direct intramyocardial injection limit systemic vector spread, but also restrict the area of cardiac transduction or grafting to only a few millimeters surrounding the injection site.[13–15] Clinically significant levels of myocardial gene expression may require an approach that allows for more widespread transduction.

We hypothesized that an effective alternative delivery strategy would be catheter-based local gene transfer to pericardial mesothelium and high-level, localized expression of a potentially therapeutic, diffusible protein into the subepicardial interstitium and pericardial fluid compartment surrounding the heart and coronary arteries.[16] To test this hypothesis, we employed a percutaneous approach using a hollow, helical-tipped catheter positioned transmurally across the right ventricular wall to deliver adenoviral vectors to the intrapericardial space. The results demonstrate virtually complete transduction of the parietal pericardium and, to a slightly lesser extent, the epicardium, with minimal to no systemic delivery. This strategy could have significant implications for gene therapy applications for cardiovascular disease.

Materials and Methods

Adenoviral Vectors

Replication-deficient recombinant E1-deleted adenovirus vectors Av1nBg, Av1Lu, Av1Aa, encoding either a nuclear-targeted histochemical reporter [beta-galactosidase (β-gal)], a readily quantifiable cytoplasmic reporter (firefly luciferase), or a readily quantifiable secreted protein reporter [human α1-antitrypsin (α1AT)], respectively, were constructed previously and vector stocks were prepared, quantified, and stored as previously described.[17,18]

Animals

Adult mongrel dogs (n = 11) were studied under protocols approved by the Institutional Animal Care and Use Committee, Indiana University School of Medicine, in accordance with the "Guide for the Care and Use of Laboratory Animals" [Department of Health and Human Services, publication No. (National Institutes of Health) 86-23, revised 1985]. Animals were maintained on a normal diet before and after vector delivery. Both surgical (n = 4) and percutaneous transventricular (n = 7) approaches were employed to evaluate pericardial gene transfer. Both procedures were performed under general anesthesia with thiopental-sodium (25 mg/kg). Following induction, animals were intubated and ventilated with oxygen containing 2% isoflurane for maintenance of anesthesia.

Percutaneous Delivery Approach

Percutaneous deliveries were performed using a hollow, helical-tipped catheter designed for controlled penetration into or through the myocardium during fluoroscopic visualization. Following placement of a 7 French sheath into the right jugular vein, a catheter was placed through the sheath and advanced under fluoroscopic guidance into the right ventricle to the cardiac apex, with the catheter tip directed inferiorly. An infusion of saline through the delivery lumen was maintained at 0.5–1 cc/min throughout this procedure in order to avoid clogging of the helical penetration tip with blood elements. Upon firm contact with the myocardium, the catheter tip was advanced through the myocardium using a gentle turning motion. After advancement over several mm, hand infusion of a 2:1 meglumine/normal saline mixture was initiated and contrast location was monitored fluoroscopically. Successful intrapericardial tip placement was identified by accumulation of contrast in the pericardium, at which point the catheter was fixed in position and flushed with 2 ml of saline prior to delivery of suspended vector. Vector suspension ($1–5 \times 10^9$ plaque forming units) was then delivered in 8 ml of Dulbecco's modified eagle's medium (DMEM). Following delivery, final catheter position was confirmed by fluoroscopic visualization of a bolus of air instilled into the pericardial space, after which the catheter was removed.

Surgical Delivery Approach

As a control for vector delivery, adenovirus vector was also directly instilled into the pericardial sac using a surgical approach. Anesthetized animals were given 1.5 mg of pancuronium-bromide intravenously as a neuromuscular paralyzer. The chest was opened ventrolaterally in layers at the sixth intercostal space on the left side by electrocautery. The pericardial sac was grasped with an Allis clamp to dissect a small window through the pericardial fat pad.[19] A circumferential suture was sewn into the pericardial sac, followed by insertion of a 24-gauge plastic catheter and tightening of the suture about the catheter. The adenovirus vector was diluted to 8 ml in DMEM (pH 7.4) containing 2% fetal bovine serum (FBS)

and was instilled through the catheter over several seconds. The suture was then tightened as the catheter was withdrawn. A chest tube was temporarily placed to expand the lung postoperatively and then withdrawn, and the wound in closed with sutures. A pleural suction trap filled with viricidal solution was used to prevent contamination. Animals were subsequently isolated and given free access to food and water.

Analysis of Gene Expression

Three days after vector delivery, animals were necropsied and gene transfer and expression in pericardial, epicardial, and other tissues were evaluated in animals (n = 7) receiving a mixture of Av1nBg and Av1Lu, using the histochemical chromogen substrate 5-bromo-4-chloro-3-indolyl-1-β-d-galactopyranoside (X-gal) as previously described.[20] Following removal of the heart and sampling for luciferase assay (see below), the majority of the organ was rinsed in ice-cold phosphate-buffered saline (PBS) and fixed at 4°C for 4 h in a solution of PBS, 2% formaldehyde, and 0.02% glutaraldehyde. Tissues were then rinsed twice in PBS and stained for 4 h at 37°C in a buffered X-gal solution [3 mM K3Fe(CN)6, 3 mM K4Fe(CN)6, 2 mM MgCl2, and 1 mg/ml X-gal]. Successful gene transfer and expression of nuclear localizing beta-galactosidase was indicated by cells with blue nuclei.

Relative gene transfer to pericardium or potentially to other organs was evaluated by luminometry in dogs (n = 6) receiving Av1Lu using a quantitative cytoplasmic luciferase reporter. Briefly, samples of each tissue were removed and stored in ice-cold PBS prior to homogenization in lysis buffer (25 mM tris-phosphate pH 7.8, 2 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1% triton X-100, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.75 mM phenylmethylsulfonyl fluoride (PMSF), in PBS. Homogenates were centrifuged at 4,000 rpm for 10 min at 4°C, and the resulting supernatants centrifuged for an additional 10 min at 14,000 rpm and 4°C. Duplicate 20 μl samples of the cleared supernatant were evaluated for luciferase enzymatic activity with an Opticomp II luminometer (Gem Scientific, Hamden, Connecticut, USA). A 100 μml aliquot of assay reagent [20 mM Tricine, pH 7.8, 1.07 mM (MgCO3) 4Mg(OH)2•5H2O, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM D-luciferin, and 530 mM ATP] was added to each 20 ml sample, and light production was measured over a 10-s period using the microprocessor-controlled photon counter. Background luminescence values were subtracted from sample values to arrive at relative light unit (RLU) values from each sample. Using the Bradford method,[21] protein assays were performed on aliquots of the same tissue extracts to permit expression of these results as RLU normalized for protein concentration.

The secretion of adenovirus vector-mediated reporter protein into the pericardial space was evaluated in dogs (n = 3) following intrapericardial delivery of the Av1Aa vector. The total volume of intrapericardial fluid found at necropsy was approximately 2–3 ml. This pericardial fluid was evaluated for human α1-antitrypsin content using a standard radial immunodiffusion assay which has demonstrated lack of cross-reactivity with the canine protein.[22]

Results

Feasibility of Pericardial Gene Delivery

Percutaneous delivery of the adenoviral vectors to the pericardium and subsequent gene transfer and expression in endothelial lining cells was well tolerated clinically in all animals over the time evaluated in this study. Because of the helical nature of the penetrating wound through the ventricular wall, overt bleeding into the pericardial sac did not occur upon removal of the catheter, as evidenced by evaluation of pericardial fluid at necropsy. Specifically, there were no hemodynamic changes or clinical sequelae at any time, and all animals were well and without complications at necropsy 3 days after vector administration. Twelve-lead electrocardiograms (ECGs), obtained before vector instillation (baseline), immediately after instillation and at necropsy, revealed no changes in ECG rhythm or morphology or findings consistent with pericardial inflammation or myocardial damage (data not shown). Intrapericardial delivery via the percutaneous approach was demonstrated by fluorographic imaging of instilled contrast media (Fig. 1). As a control, adenovirus vectors were also administered to the pericardium via a surgical approach with a pericardiotomy. All animals tolerated the surgical procedure well clinically.

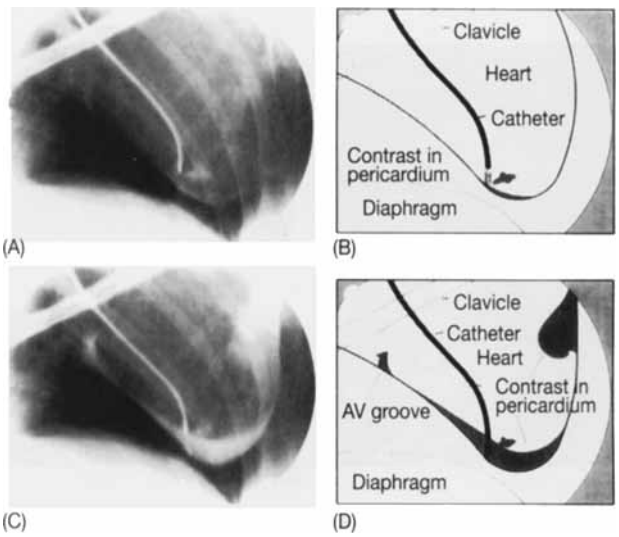

Fig. 1 Sequential fluorographic images, obtained during a percutaneous delivery procedure, from the right anterior oblique projection. (A) Cardiac silhouette, with the helix catheter in place transmurally in the right ventricular wall. The instillation of contrast had just begun at the time of angiography; a thin layer of contrast is seen outlining the cardiac edge, confirming pericardial loculation. This image is represented as a line drawing in (B) for clarity. (C) The same projection after the infusion of approximately 15 cc of a mixture of radiographic contrast and vector suspension, with a line representation in (D).

Gene Transfer to Pericardial Surface Mesothelium

Following intrapericardial adenovirus vector delivery, X-gal staining (n = 7) revealed widespread adenovirus-mediated gene transfer and expression over much of the visceral (epicardial) and parietal pericardium (Fig. 2). Low power en face examination of both surfaces showed diffuse blue staining indicating transgene expression in most of the surface [shown for parietal pericardium (Fig. 2A)]. Epicardial staining was typically most intense in the areas overlying the atria and auricular appendages. Microscopic inspection of parietal pericardium en face revealed nuclear histochemical staining in nearly 100% of cells in many areas, demonstrating efficient transduction of the mesothelial cell lining (Fig. 2B). Transgene expression was limited to the single cell layer immediately lining the pericardial space on the surface of the parietal pericardium (Fig. 2C) as well as the epicardium (Fig. 2D). These findings were not appreciably different between animals following percutaneous vector delivery and following surgical vector delivery, as also described previously.[23]

To estimate the ratio of infectious vector particles to target cells lining the pericardial space, for example, the multiplicity of infection (MOI), the pericardial and epicardial surface cell density was examined microscopically and the cell density was determined to be approximately 3–4 × 10^5 cells per cm^2. Using a value of 132 cm^2 for the exposed intrapericardial surface area in dogs,[24] the MOI was calculated to be about 20–100 pfu/cell assuming even spread of the vector in the pericardial sac.

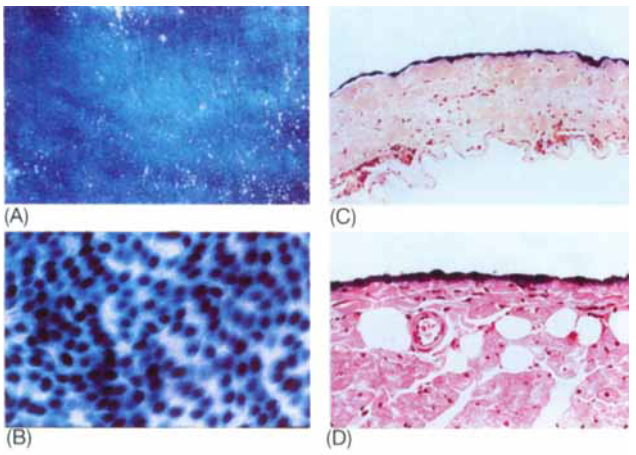

FIG. 2 (A) Parietal pericardium stained with X-gal following in vivo exposure to the Av1nBg vector (16 × original magnification); punctate blue staining demonstrating the presence of transgene expression over the surface of the parietal pericardium. (B) Microscopic inspection of parietal pericardium en face (400 ×), demonstrating high-frequency transduction of the mesothelial cell lining. (C) Cross-sectional view of the parietal pericardium (250 ×); and (D) cross-sectional view of the epicardium following Av1nBg exposure, stained with X-gal as well as hematoxylin and eosin to demonstrate tissue morphology (250 ×).

Percutaneous Catheter-Mediated Adenovirus Delivery Is Localized to the Pericardium

To evaluate the relative amount of gene transfer locally into pericardial tissues versus systemically into noncardiac tissues following percutaneous vector delivery, Av1Lu was administered to dogs (n = 6). This particular vector was used because the luciferase enzyme reporter is confined to the cytoplasm of the gene-targeted cell and luciferase transgene expression is accurately and readily quantifiable in tissues by luminometry.[25] Evaluation of cardiac tissues at several sites as well as lung, spleen, liver, and periaortic lymph nodes (n = 6) demonstrated that gene transfer was highly localized to the pericardium (Fig. 3). As expected from the X-gal staining results after the Av1nBg vector administration, gene transfer and expression was highest in the parietal pericardium and epicardium, but markedly reduced by 500 to 1000-fold in endocardial and noncardiac tissues (Fig. 3). Thus, gene transfer after percutaneous, transventricular, helical-tipped catheter vector delivery is highly localized and targeted to pericardial mesothelial cells. Similar expression levels and localization were found 7 days following administration of Av1nBg and Av1La (n = 1) with pericardial luciferase expression of 258,800 light U/mg protein.

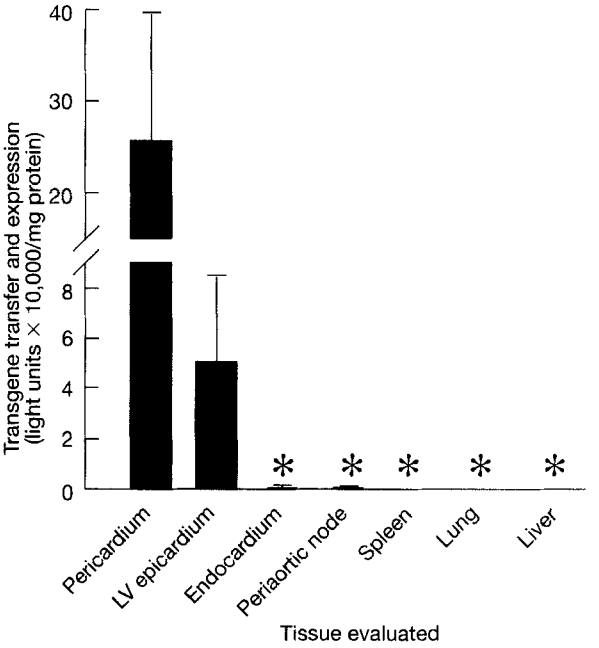

FIG. 3 Cardiac and extracardiac distribution of luciferase expression. Tissue sampling was performed from numerous sites to permit evaluation of the amount of systemic vector distribution and concomitant gene expression found subsequent to the intrapericardial installation. Expression in various tissues is indicated. Luciferase activity given as relative light units/mg tissue protein (RLU/mg) normalized per 10^7 pfu administered. The asterisks indicate a mean luminometry of < 1000 RLU/mg. LV = left ventricular.

Adenovirus-Mediated Protein Expression into the Intrapericardial Compartment

To evaluate the efficiency of adenovirus-mediated delivery of a diffusible protein into the intrapericardial lining fluid, Av1Aa was administered to dogs (n = 3) via the percutaneous catheter route. This vector expresses human α1-antitrypsin, which is distinguishable from canine α1-antitrypsin by ELISA. An equal amount (10^9 pfu) of Av1Lu was coadministered with Av1Aa in each animal to permit parallel quantification of local and potential systemic gene transfer. Three days after administration, animals were evaluated for human α1-antitrypsin by ELISA in pericardial fluid and serum. At necropsy, approximately 2 to 3 ml of pericardial fluid was detected for each animal. Human α1-antitrypsin protein levels in pericardial fluid were 15, 19, and 26 μg/ml, respectively, in the three animals. However, no human α1-antitrypsin was detectable in the serum.

Discussion

Although gene therapy for cardiovascular disease is promising, especially with high-efficiency in vivo vectors such as adenovirus, localized delivery to the heart and vessel wall remains a challenge. Previous attempts to deliver genes to vessels or myocardium have generally been based on surgical isolation of a vessel or direct myocardial injection. While these approaches limit systemic spread, they are not yet entirely satisfactory for clinical application in humans. Direct instillation of adenoviral vectors into the intrapericardial space from either percutaneous catheter or surgical pericardiotomy approaches demonstrated very high efficiency gene transfer to the pericardial mesothelium. Gene transfer was localized to pericardial tissues with very little transduction of extracardiac tissues. This study thus demonstrates the feasibility of using a catheter-based approach to the pericardium as a route for adenovirus-mediated cardiac gene transfer, analogous to its use for targeted drug delivery.[26–28] These results also demonstrate for the first time the possibility of pericardial gene transfer as an approach to sustained-release protein delivery, generating sufficiently high concentrations of desired gene products or their metabolites to result in diffusive transport into the epicardial region to an extent potentially sufficient to produce therapeutic biologic effects.

Localized Gene Transfer to the Pericardium

The high transduction efficiency observed for the pericardial mesothelial cells is likely due to prolonged vector confinement within the pericardial space. Insofar as the vector particles are sufficiently large (100 nm in diameter) to render transpericardial diffusion minimal, the particles are restricted from systemic distribution and have no apparent route of elimination except that of uptake into the mesothelial lining cells. Other studies have evaluated a variety of enclosed cavities and potential spaces as targets for transduction, including the synovial capsule,[29] biliary system,[30] intrapleural cavity,[31] the intrathecal space,[32] the intraocular cavity,[33] and the pericardial space, using a surgical approach.[23] Gene transfer to the peritoneal cavity has been explored as a method of localized gene transfer for systemic protein delivery.[34] In this case, vector-derived human α1-antitrypsin was measurable in the systemic circulation at levels as high as 3.4 μg/ml.[34] Systemic delivery in this case is presumably related to the relatively high surface area of the mesothelial lining of the peritoneum, approximately 2 m^2, consistent with the common clinical use of the peritoneal cavity for systemic solute or fluid transfer in the context of peritoneal dialysis. In contrast to these findings in the peritoneum, the current study shows high local expression of α1-antitrypsin in the absence of measurable circulating levels. This distinction likely reflects the significantly reduced pericardial surface area and possibly a difference in the intrinsic diffusive exchange properties of these two membranes.

Implications for Human Gene Therapy

The measured average value of 20 μg/ml in pericardial fluid found for α1-antitrypsin is higher than that found in several studies of systemic α1-antitrypsin gene transfer,[3, 35] possibly as a consequence of the highly efficient transduction as well as localized secretion. This level is significantly greater than the levels required for many growth factors to exhibit physiologic effects. This suggests that the pericardial sac might potentially function as a sustained-release protein delivery system, generating sufficiently high concentrations of desired gene products or their metabolites to result in diffusive transport into the epicardial region to an extent sufficient to produce potentially therapeutic biologic effects. Such transport of macromolecules into the epicardial region has been measured[36, 37] and is consistent with observations of enhanced angiogenesis occurring in response to the epicardial placement of polymeric sustained release matrices containing basic fibroblast growth factor (bFGF).[38] In addition, efficient diffusion through the visceral pericardium has been demonstrated and appears to be the primary mechanism by which large amounts of atrial natriuretic peptide are conveyed from subepicardial atrial cardiomyocytes into pericardial fluid;[39] this fluid may in turn play a role as a physiologic reservoir for this and other endogenous compounds such as prostaglandins and peptide growth factors,[19, 39] much as is proposed for vector-encoded substances. Intrapericardial drug delivery has been described for several comparatively small compounds, including digoxin, lidocaine,[40] amiodarone[26] as well as other antiarrhythmic agents, and chemotherapeutic compounds.[41] Although these studies have successfully demonstrated delivery, single-dose administration of most agents might not necessarily bring about a prolonged therapeutic effect due to the loss of agents from the pericardial compartment. In contrast, transfer of genetic material into cells lining the pericardium for subsequent protein expression provides one method for sustaining the effects resulting from a single instillation. A number of proteins may be suggested as candidates for such delivery, with potentially therapeutic approaches including the delivery of genes encod-

ing angiogenic proteins to enhance the natural process of collateral vessel formation in response to ischemia. The collateralizing effects of repetitive or sustained dosing protocols of vascular endothelial growth factor (VEGF) and bFGF proteins, as well as others, have been described in the context of multiple animal models of ischemia.[42–45] The approach described here offers an alternative to delivery of these agents without the necessity for repetitive dosing or the implantation of sustained-release matrices.

Other potentially therapeutic candidates for pericardial expression include factors to promote vasodilation and smooth muscle quiescence, such as nitric oxide synthase (NOS) isoforms to enhance local NO production, or prostaglandin synthase to increase intrapericardial prostacyclin content. Locally enhanced expression of such genes might represent an antianginal or antirestenotic strategy of prolonged duration. In a similar fashion, it may be speculated that local synthesis of neuroactive peptides or other substances could act to modulate nerve conduction, thus affecting arrhythmogenesis or pain perception; and cardiotonic peptides have been described which might conceivably function to enhance myocardial contractility in a sustained fashion.

Finally, this study represents an initial description of a feasible and effective percutaneous approach for instillation of material into a normal pericardial space using a helical needle-tipped catheter. This method appears to be reasonably safe with no evidence of adverse sequelae seen over several days following the procedure. We suggest that percutaneous transluminal intrapericardial delivery using a range of devices may permit the minimally invasive instillation of nongenetic as well as genetic therapeutic agents with relative ease and safety for a variety of potential indications.

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