

Basic Investigation

Widespread Regional Myocardial Transfection by Plasmid Encoding Del-1 Following Retrograde Coronary Venous Delivery

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This study quantifies myocardial transfection following percutaneous retrograde coronary venous delivery (RCVD) of a plasmid encoding human Del-1. RCVD of Del-1, GFP plasmid, or marker dye was conducted in 14 pigs. After selective cannulation of a coronary vein, a delivery site was confirmed by contrast injection and myocardial blush. Ten milliliters of plasmid hDel-1 or GFP was administered. Animals were euthanized 3 and 7 days post-RCVD. hDel-1 gene expression was evaluated by quantitative RT-PCR. An average myocardial expression of 4.5×10^5 copies hDel-1/ μg total RNA was observed within the approximately $5 \times 5 \text{ cm}^2$ target tissue of the left ventricle. GFP expression was detected by fluorescent microscopy. hDel-1 protein expression was confirmed by immunohistochemistry. Regionalized myocardial expression was found in all pigs. hDel-1 RNA was not found in distant tissues except in the three pigs with prominent venovenous washout (PVW). These levels were 3 to 4 log units lower than those found in myocardium. Single retrograde coronary venous administration resulted in efficient regional myocyte transfection of hDel-1 and GFP. This method may be useful and clinically feasible for myocardial angiogenesis therapy. *Cathet Cardiovasc Intervent* 2003;58: 207–211. © 2003 Wiley-Liss, Inc.

Key words: gene therapy; myocardium; drug administration

INTRODUCTION

Therapeutic myocardial angiogenesis through gene transfer is a promising approach to ischemic heart disease, but the ideal route of delivery, vector, and gene to employ are yet to be determined. Gene transfer to the heart has been approached using viral and nonviral vectors. Although adenovirus is the most efficient of these vectors in vivo, the potential of viral vectors to induce adverse inflammatory and host immune responses has led to a renewed interest in the use of nonviral vectors for cardiac gene transfer [1]. Direct intramyocardial injection of plasmid is being pursued in both animal models and clinical trials [2], though the level of gene expression achievable from plasmid-based vectors has generally been found to be relatively low [3].

Retrograde coronary venous delivery (RCVD) of marker and therapeutic agents has been shown to provide for relatively high intramyocardial concentrations within substantial volumes of tissue [4,5]. This study was designed to

evaluate whether single-dose RCVD of a formulated plasmid would provide efficient selective myocardial transgene

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expression. Human Del-1 (developmentally regulated endothelial locus-1) [6] was chosen for delivery because of its angiogenic activity in angiogenesis assays and in transfected limb muscle [7,8].

MATERIALS AND METHODS

The plasmid hDel-1 administered to all animals is comprised of a eukaryotic expression cassette encoding the human Del-1 gene, transcriptionally regulated by the cytomegalovirus (CMV) enhancer promoter and containing a synthetic intron 5' to the Del-1 coding sequence (Valentis). Another plasmid-expressing green fluorescent protein (GFP) was constructed similarly. Plasmids were formulated at a concentration of 1 mg/ml, with 5% Poloxamer 188 in isotonic saline.

Animal care and handling followed the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (revised 1985). The Animal Care and Use Committee of the Indiana University School of Medicine approved the protocols. Fourteen juvenile domestic pigs (30–40 kg) were sedated with i.m. ketamine (20 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg), followed by i.v. sodium pentothal (25 mg/kg). After intubation, anesthesia was maintained with isoflurane (2.5%).

Venous access was obtained via the jugular vein. After selective cannulation of the great coronary vein by a modified 8 Fr multipurpose guiding catheter, a 5 Fr occlusion balloon catheter was advanced into the anterior interventricular vein (AIV). The balloon catheter was inflated temporarily to occlude the AIV. Delivery site was determined by injection of 1 to 1.5 ml of contrast (iohexol), resulting in a myocardial blush (Fig. 1A). If a blush was not observed, the catheter was repositioned to another site. Also noted was the presence and extent of venovenous anastomoses. The retrograde infusion volume was 10 ml and infusion time was 7.5 ± 1.2 sec in all animals. Balloon occlusion was maintained for 10 min.

Three pigs received a mixture of a blue tissue marking dye (TMD) with iohexol (1:1) to determine the distribution of solute delivery. Nine pigs were given the hDel-1 plasmid, and two pigs received GFP plasmid. Animals were either euthanized acutely (TMD pigs), 3 (GFP), or 7 days (del-1) post gene delivery.

The hearts receiving dye were sectioned and stained with hematoxylin-eosin. Dye distribution area was measured by planimetry and expressed as a percentage of the LV area of the short-axis cross-section. In addition to the heart, the lung, liver, kidney, and spleen were harvested in gene-transferred pigs. The target region (based on the blush area identified by fluoroscope at the time of contrast infusion) of GFP hearts were sampled and embedded in optimal cutting temperature medium (OCT) and

subjected to cryosectioning (6 μ m), then nuclei counterstained with a DAPI solution (0.5 μ g/ml) and analyzed with both standard and confocal microscopy (Biorad 1000 series). Immunostaining was performed using standard techniques. Briefly, paraffin cardiac sections were cut at 5 μ m and affixed to glass microscope slides. Immunostaining was performed using a mouse antihuman Del-1 primary antibody (1:100; Valentis) after blocking with a biotinylated horse antimouse IgG (1:400). Secondary antibody binding was revealed by avidin complex, with a staining reaction performed using 3,3'-diaminobenzidine (DAB) solution (Vector Laboratories). Nuclei were counterstained with hematoxylin. To determine the quantity of Del-1 mRNA in total RNA isolated from the target myocardial tissue, quantitative RT-PCR was performed using the Superscript One-Step RT-PCR System (Invitrogen Life Sciences). PCR was accomplished using a 5' primer specific for sequence in the 5' UTR and a 3' primer specific to the human Del-1 coding sequence. The accumulation of fluorescence was measured in real time with a 7700 sequence detector (Applied Biosystems). Data are presented as mean \pm SEM and assessed by an unpaired (two-tailed) *t*-test. Significance was assumed at $P < 0.05$.

RESULTS

Retrograde injection was well tolerated without adverse hemodynamic effects. The arterial pressure and heart rate did not change significantly before or after delivery. Nonsustained ventricular tachycardia was typically noted to occur during retrograde infusion, comprising 5–10 beats and ceasing immediately following the injection.

Myocardial contrast blush was associated with minimal venous drainage in 11 (79%) pigs or PVW in 3 (21%) pigs. There was no blush obtained following delivery contrast into the AIV in two pigs, requiring gene delivery via the obtuse marginal vein.

Myocardial blue dye staining was demonstrated in all three TMD pigs. Gross examination revealed the stained area to be located in the anterior wall of the left ventricle (Fig. 1B). Cross-sections indicated transmural staining, which included the anterior wall, anterior interventricular septum, and anterior papillary regions. The TMD-stained area represented $25\% \pm 11\%$ of LV (Fig. 1C). Microscopy showed interstitial and vascular distribution of the blue pigment (Fig. 1D). Fluorescent microscopy following GFP transfection confirmed expression in cardiomyocytes (Fig. 1E). Evaluation of consecutive 6 μ m cryosections, the mean percentage of GFP-positive cells in the transduced region, was 3%. Positive immunostaining, denoting expression of hDel-1 protein, was seen in cardiomyocytes in the target region (Fig. 1F). By way of

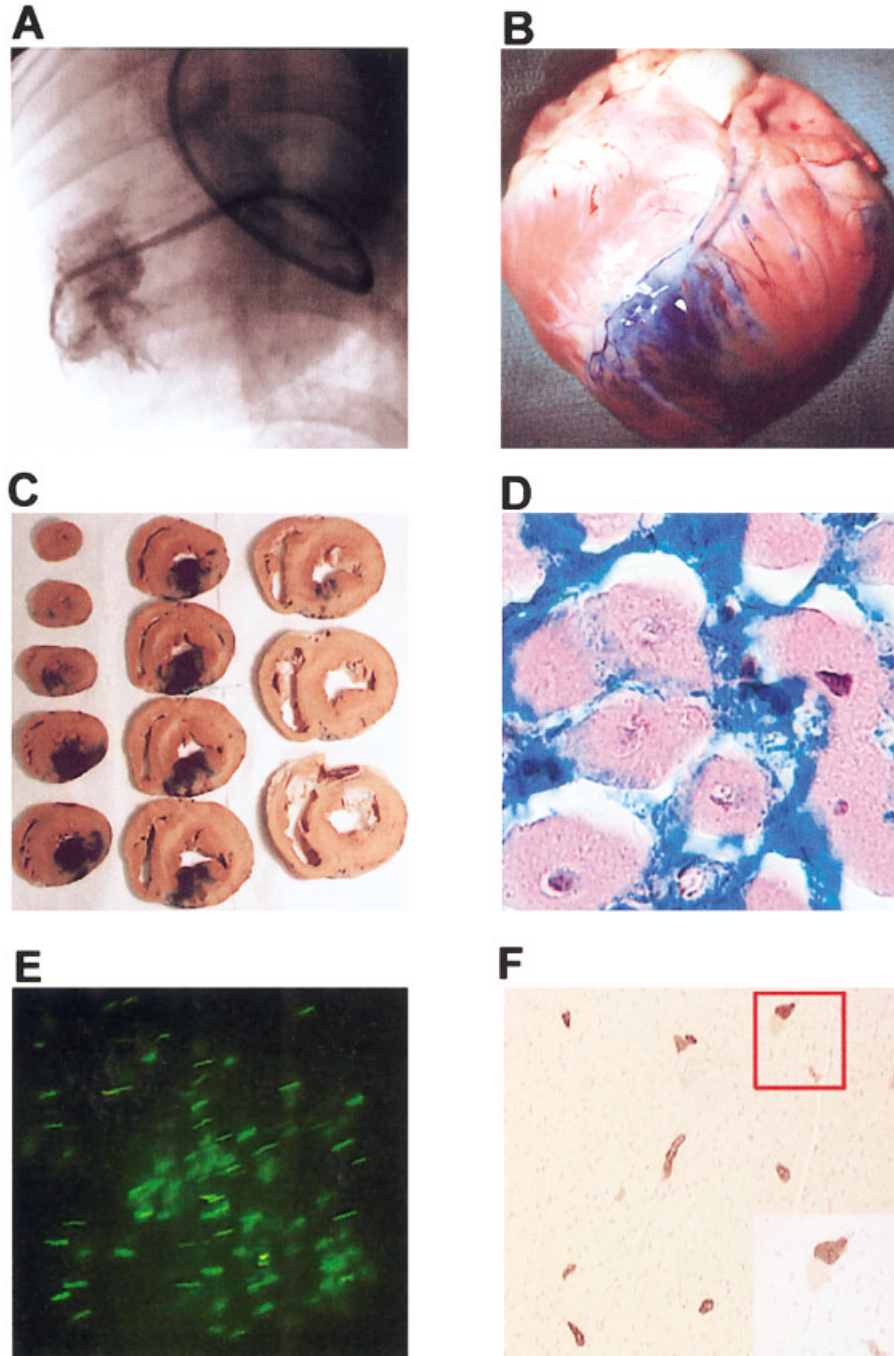


Fig. 1. A: Coronary venogram demonstrating myocardial blush. **B:** Gross specimen with tissue marker dye staining from RCVD. **C:** Cross-sections from apex to base of a TMD-delivered heart. **D:** Interstitial extravasation of TMD in myocardial tissue (100 × magnification). **E:** Fluorescence microscopy showing multiple layers of myocyte expression GFP (4 × magnification). **F:** Immunostaining of hDel-1 protein in myocardial tissue (10 ×; 40 × magnification).

comparison, in more prolonged studies of pigs with myocardial ischemia, the mean percentage of hDel-1–positive cells in the transduced region has been seen to be 1.21% ± 0.7% 3 weeks after delivery (data not shown).

The target myocardial tissue was determined based on angiography typically to cover an area of 5 × 5 cm² and was divided at harvest into 22 segments that were then equally divided along the endocardial/epicardial axis.

TABLE I. Level and Distribution of hDel-1 Transgene Expression in Myocardium and Other Organs Following RCVD (Copies hDel-1/ μ g Total RNA)

Tissue	Average	Maximal
Endocardium	$5.5 \times 10^5 \pm 2.6 \times 10^5$	$1.4 \times 10^6 \pm 7.5 \times 10^5$
Epicardium	$3.6 \times 10^5 \pm 1.6 \times 10^5$	$1.2 \times 10^6 \pm 5.4 \times 10^5$
PVW absent (n = 6)	$6.0 \times 10^5 \pm 2.1 \times 10^5$	$1.7 \times 10^6 \pm 7.4 \times 10^5$
PVW present (n = 3)	$1.7 \times 10^5 \pm 7.8 \times 10^{4a}$	$2.9 \times 10^5 \pm 1.2 \times 10^{5b}$
Lung	ND	
Liver	1/9	
Kidney	ND	
Spleen	2/9	

*Values are mean \pm SEM. ND: denotes not detected in any sample from specified tissue; 1/9, 2/9: fractions denote fraction of samples of specified tissue exhibiting detectable transgene mRNA but below the limit of quantitation in the assay (500 copies).

^a $P = 0.04$ vs. absent PVW.

^b $P = 0.02$ vs. absent PVW.

Transgene expression was then analyzed in each individual tissue sample. An average of 58% of these segments demonstrated hDel-1 transcript at levels quantifiable by the qRT-PCR assay.

All plasmid/hDel-1–delivered pigs demonstrated target myocardial tissue gene expression. The average transgene expression found in all quantifiable zones was $4.5 \times 10^5 \pm 1.5 \times 10^5$ copies of hDel-1 mRNA/ μ g total RNA, while the average of the maximum expression levels among the pigs was approximately threefold higher, 1.23×10^6 copies of hDel-1 mRNA/ μ g total RNA. There was no significant difference between endocardium mean ($5.5 \times 10^5 \pm 2.6 \times 10^5$) and epicardium ($3.6 \times 10^5 \pm 1.6 \times 10^5$; $P = 0.27$) expression (Table I).

Absence of PVW correlated with significantly higher average hDel-1 gene copy number (6.0×10^5 without PVW vs. 1.7×10^5 with PVW; $P = 0.04$; Table I). Expression of hDel-1 mRNA was highly correlated with the presence of residual plasmid. hDel-1 mRNA was not found in distant tissues except in the three pigs with PVW, which had expression lower than the limit of quantitation in the spleen (n = 2) and liver (n = 1), respectively.

DISCUSSION

This study is the first to document the feasibility of nonviral myocardial gene delivery by a retrograde coronary venous approach and demonstrate robust transgene expression. Our findings support potential clinical utility due to the relative safety of nonviral gene transfer in combination with a localized, percutaneous approach.

Prior studies of plasmid-mediated cardiac gene therapy have employed either epi- or endocardial approaches with multiple intramyocardial injections, or intracoronary administration. The latter is relatively easy and safe;

but limited by rapid washout, low localization of genes or proteins, and systemic exposure [1,9].

The use of the coronary venous system for agent delivery, specifically cardiac-selective gene therapy, has gained renewed interest [4,10]. This route is attractive for several reasons. Key requirements to achieve high-frequency transduction to solid tissues such as the myocardium are vector access to the cellular components and adequate contact time to permit vector uptake [11]. The coronary venous approach offers direct local delivery into the interstitium of the myocardium with minimal washout and allows for controlled dwell times for longer exposure depending on duration of balloon inflation. In addition, access to the coronary venous system is relatively easy, inexpensive, and requires conventional equipment.

RCVD exhibits efficient regional myocardial expression of the angiogenic gene hDel-1, as well as GFP, following single-dose plasmid delivery. Widespread transfection is apparent as a large proportion (58%) of tissue segments in the 5×5 cm² target zone contained transcript, with quantitatively similar expression demonstrated transmurally. Furthermore, the level of hDel-1 transgene mRNA expression observed within the myocardium was comparable to those identified previously to correlate with functional angiogenesis in mouse and rabbit models of hindlimb ischemia [7,8]. In distinct contrast with results previously reported for adenoviral transduction (7), it was not found necessary to occlude the arterial supply to the target region in order to achieve gene expression. This difference may be a consequence of the low pressures employed in the previous study.

This study is limited to gene transfer in normal pigs. Current work is aimed at determining functional significance and the angiogenic potential of hDel-1 expression in both normal and ischemic animals. Another potential problem is the possibility of systemic transgene expression in the presence of PVW, although levels in these animals were quite low. In addition, optimal conditions for retrograde venous delivery, including appropriate injection pressures, volume, dose, and dwell times, mandate further study.

This method may have significant general clinical utility in the catheterization laboratory by providing efficient delivery to relatively large areas of ventricular myocardium. Recently, human coronary venous anatomy has been investigated via retrograde venography. Although some variability of the coronary venous system among patients was found, the AIV is found in nearly every (99%) patient, suggesting that these veins indeed might be useful for therapeutic approaches [12]. In addition to therapeutic angiogenesis for chronic ischemia, a wide array of factors may be introduced during catheterization to treat acute coronary syndromes, heart failure, or myopathies.

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