

## ***Basic Science Review***

# **Enhancing Myocardial Plasmid Expression by Retrograde Coronary Venous Delivery**

**Eyas Al-Shaykh Youssef,<sup>1</sup> MD, Ping Zhang,<sup>1</sup> MD, Pamela I. Rogers,<sup>1</sup> RATG, Patrice Tremble,<sup>2</sup> PhD, Joe Rokovich,<sup>2</sup> PhD, Brian H. Johnstone,<sup>1</sup> PhD, Keith L. March,<sup>1,3</sup> MD, PhD, and Dongming Hou,<sup>1\*</sup> MD, PhD**

Myocardial delivery of genes holds great promise for treating many heart diseases; however, the optimal delivery technique, which maximizes safety and efficacy, has not been established. Two delivery techniques were evaluated in swine; percutaneous retrograde coronary venous delivery (RCVD) and direct intramyocardial injection (IM). RCVD was performed in the anterior interventricular vein (AIV) with an end-hole occlusion balloon catheter. The plasmid gWiz, encoding  $\beta$ -galactosidase (10 ml; 1 mg/ml), was injected using either manual high pressure (HP-RCVD;  $n = 5$ ) or pressure wire-guided low pressure (LP-RCVD;  $n = 4$ ). For the IM group ( $n = 4$ ),  $\beta$ -Gal plasmid (5 mg/ml) was injected at 10 sites (200  $\mu$ l/site) in the anterior left ventricular wall. Animals were euthanized after 5 days. The percentage of  $\beta$ -Gal expressing cells in the delivered region was higher in the HP-RCVD (0.26%  $\pm$  0.05%) than the LP-RCVD (0.05%  $\pm$  0.03%;  $P = 0.07$ ) and IM groups (0.02%  $\pm$  0.01%;  $P = 0.01$ ). Myocardium from the HP-RCVD group contained 7- and 17-fold higher levels of  $\beta$ -Gal activity than either LP-RCVD and IM groups, respectively ( $P = 0.05$  for both). The results of this study confirm the safety and efficacy of RCVD for myocardial gene delivery. © 2005 Wiley-Liss, Inc.

**Key words:** myocardium; coronary venous; gene delivery; plasmid

## **INTRODUCTION**

Coronary artery disease continues to be a major cause of morbidity and mortality worldwide [1]. Currently, standard therapies for this disease include medical therapy and revascularization either by percutaneous angioplasty or coronary bypass surgery [2]. Therapeutic angiogenesis has emerged as a potential therapeutic modality for a subset of the patient population possessing occlusive vessel disease that is untreatable by standard revascularization procedures [3–5]. In principle, revascularization of ischemic heart tissues could be promoted by delivering one or multiple angiogenic growth factors (either as proteins or genes) to the myocardium near the arterial blockage [6,7]. Thus far, the promise of this strategy has not been realized in controlled randomized clinical trials [8]. One major obstacle to realizing the full potential of this therapy is the lack of an optimal modality for localized delivery of therapeutic agents. Intracoronary infusion and direct myocardial injection are presently the only techniques used in pilot human studies [9–15]. However, the former, although convenient and making use of routine

procedures and devices, is limited by rapid washout, poor localization of delivery area, systemic exposure, and limited applicability to patients with complete arterial occlusion [16,17]. Direct intramyocardial (IM) injection, on the other hand, requires either open heart surgery (CABG) or expensive and time-consuming electromechanical NOGA mapping techniques [18]. Thus, there is a need for additional delivery modalities

<sup>1</sup>Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana

<sup>2</sup>Edwards Lifesciences, Irvine, California

<sup>3</sup>Richard L. Roudebush Veterans Administration Medical Center, Indianapolis, Indiana

\*Correspondence to: Dr. Dongming Hou, Indiana Center for Vascular Biology and Medicine, 975 W. Walnut Street, IB 441, Indianapolis, IN 46202. E-mail: dmhou@iupui.edu

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that increase local delivery to the target tissue and are easily implemented in the clinical setting.

Several additional percutaneous techniques for local delivery to the myocardium are being developed in animal models, such as intrapericardial [19–22] and retrograde venous delivery [23–28]. The retrograde coronary venous delivery (RCVD) approach holds great promise for obtaining enhanced myocardial delivery, using moderate pressures and volumes. In this study, we compared myocardial gene delivery and safety between RCVD and direct IM in a porcine model.

Effective delivery by RCVD depends on limited disruption of the venocapillary bed, thus allowing exposure of the myocardium to the agent. Based on our preliminary studies with fluoroscopic imaging of injected contrast dye at various pressures and volumes, we determined that a pressure higher than baseline distal coronary venous pressure under occlusion ( $46 \pm 7$  mm Hg after balloon inflation) is necessary to achieve observable extravasation. Therefore, we chose to evaluate two different retrograde venous injection pressures: the predicted minimal pressure to achieve significant delivery while minimizing excessive damage (150 mm Hg or three times baseline distal coronary venous pressure), and a higher-pressure delivery group (six times higher than baseline distal pressures).

## MATERIALS AND METHODS

Animal handling and care followed the recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (revised 1985). All protocols were approved by the Animal Care and Use Committee at the Indiana University School of Medicine.

Thirteen Yorkshire domestic pigs (25–35 kg) of mixed sex were randomly assigned to one of three groups: RCVD using maximum (peak pressure = 300 mm Hg) hand pressure ( $n = 5$ ); RCVD with pressure wire-guided low pressure (peak pressure = 150 mm Hg;  $n = 4$ ); or IM injections ( $n = 4$ ). Pigs were sedated with IM ketamine (20 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg), followed by IV sodium pentothal (25 mg/kg). After intubation, anesthesia was maintained with isoflurane (2.5%).

gWiz-Beta-gal reporter plasmid encoding the bacterial gene for  $\beta$ -galactosidase ( $\beta$ -Gal; Aldevron, ND) was used as a marker for plasmid delivery. Plasmid DNA for injection was formulated in a mixture of 0.15 M NaCl, 2 mM Tris, pH 8.0, and 5% Poloxamer 188 (w/v; BASF, NJ) with concentrations of 1 mg/ml used for RCVD and 5 mg/ml for IM.

### High-Pressure RCVD

Venous access was obtained via the internal jugular vein and the coronary sinus was cannulated using a

modified 8 Fr multipurpose guide catheter (Cook, IN). A 0.014" flexible guidewire was advanced through the catheter into the anterior interventricular vein (AIV) before advancing an end-hole occlusion balloon catheter (Meditech, MA) into the AIV. The balloon was expanded in the AIV to allow maximum local delivery and minimize spillage of gWiz-Beta-gal solution backward into the systemic circulation. Catheter placement was confirmed angiographically by injection of 1–2 ml of diluted nonionic contrast into the AIV, and proper positioning was associated with a blush in the area of myocardium surrounding the catheter (Fig. 1A). In the event that blush was not observed, the catheter was repositioned to another site and then retested. A coronary pressure wire (RADI medical systems) was introduced through the catheter lumen to measure distal coronary venous pressures during all retrograde deliveries. After inflating the balloon, plasmid gWiz-Beta-gal (10 ml at 1 mg/ml) was injected into the AIV using rapid bolus injection by the hand over an approximately 5-sec period (average distal venous pressure was  $220 \pm 23$  mm Hg and the peak pressure was 300 mm Hg). Following complete delivery of the agent, the occlusion balloon remained inflated for 10 min.

### Low-Pressure RCVD

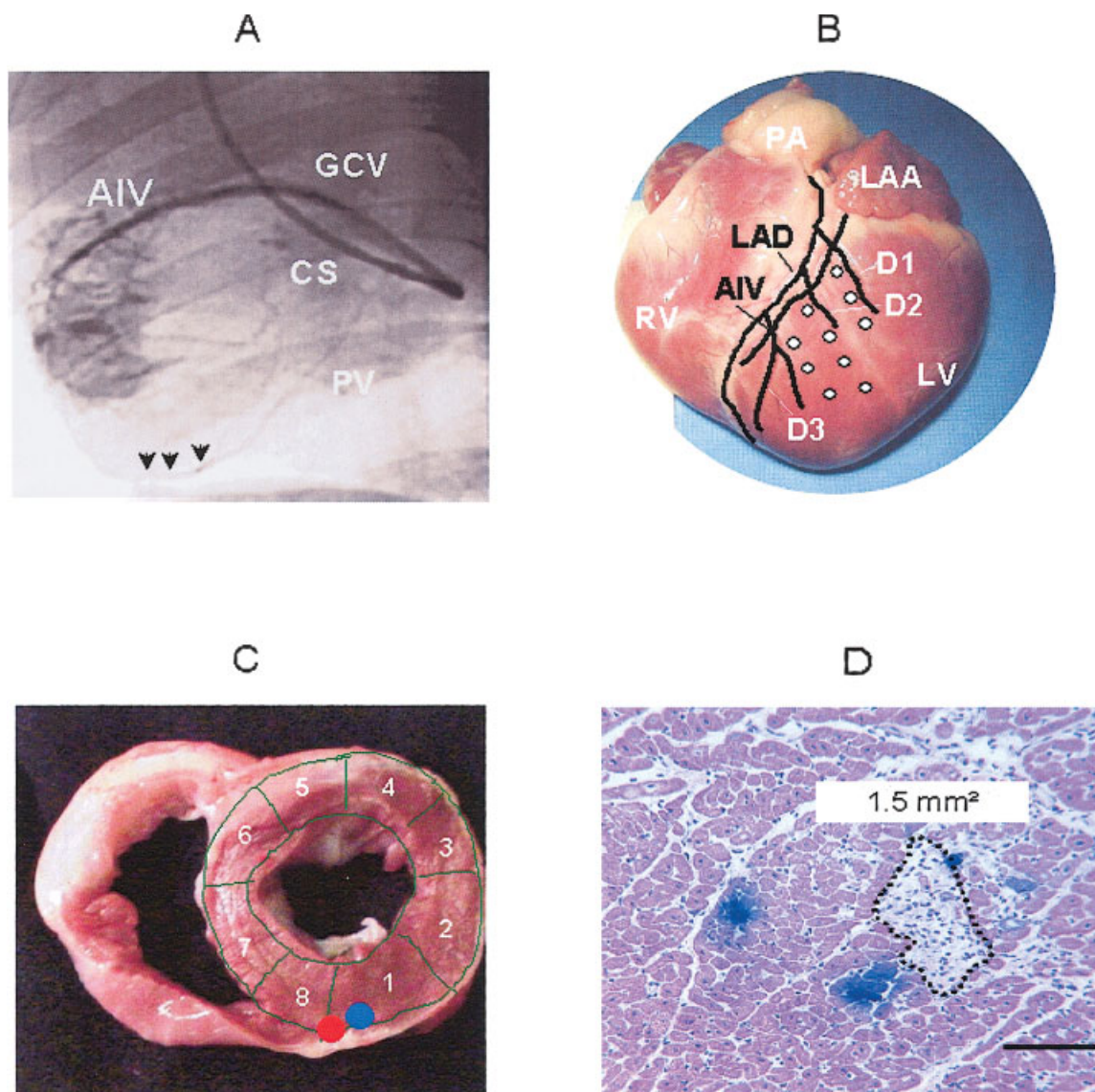
The AIV was cannulated and the balloon catheter was inserted in the AIV as described above. After inflating the balloon, 10 ml gWiz-Beta-gal plasmid (1 mg/ml) was infused, with the average injection pressure at  $110 \pm 18$  mm Hg and the peak pressure at 150 mm Hg. The average time for delivery of 10 ml was about 10 sec. Balloon occlusion was maintained for 10 min after all solution was delivered.

### Direct Intramyocardial Injection

A left lateral thoracotomy was performed at the fifth intercostal space and the pericardium was opened to expose the anterior surface of the heart. A 1 ml tuberculin syringe with a 27 gauge needle was used to inject 2 ml of gWiz-Beta-gal plasmid (5 mg/ml) at 10 sites in the anterior wall (200  $\mu$ l injection volume at each site; Fig. 1B). Injection sites were labeled with biocompatible green dye to facilitate identification during processing. The chest was then closed and the pigs were allowed to recover.

### Definition of RCVD Delivery Area

In experiments reported elsewhere, tissue marking dye was infused into the AIV by RCVD [24]. Animals were sacrificed and hearts were removed and processed as described below. The volume of myocardium stained with the dye ( $25\% \pm 11\%$ ) was considered to represent the delivery area.



**Fig. 1.** Diagrams of treatment and harvesting schema. **A:** Angiogram showing typical vein architecture and catheter positioning for RCVD. The balloon-tipped catheter is inserted into the AIV and advanced to the distal region, which is confirmed by injecting 1–2 ml of diluted nonionic contrast after balloon inflation (note blush in the lower left corner). Minimal venovenous communication (black arrows) between the AIV and the posterior vein (PV) was noted in this heart. **B:** Direct IM injection into the anterior wall of the left ventricle (LV). White circles represent injection sites. **C:** Cross-section of left ventricle demonstrating harvest of wedges. Segment number-

ing begins at the LAD/AIV (red and blue dot, respectively). Sections 8 and 1 are anterior; 2 and 3 lateral; 4 and 5 posterior; and 6 and 7 septal. **D:** Typical microinfarction observed after X-Gal and hematoxylin-eosin staining. Note the inflammatory cells in the MI area. Scale bar = 0.5 mm. LAD, left anterior descending artery; LV, left ventricle; RV, right ventricle; LAA, left atrial appendage; PA, pulmonary artery; AIV, anterior interventricular vein; D1, D2, and D3, diagonal branches of the LAD; GCV, great cardiac vein; CS, coronary sinus; PV, posterior vein. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com)].

### Tissue Preparation and X-Gal Staining

All animals were euthanized by a lethal dose of pentobarbital (65 mg/kg) at day 5 after gene delivery. The hearts were explanted and placed on ice for processing. Each heart was transversely sliced into five sections of 1–1.5 cm thickness along the apical-basal axis before further subdividing into eight wedge-shaped pieces of

approximately equal dimensions (Fig. 1C). The basal half of each wedge piece was immediately used for histochemical staining and the apical half was stored at  $-80^{\circ}\text{C}$  for subsequent  $\beta$ -Gal enzymatic activity assays.

Histochemical staining with X-Gal was performed according to a standard protocol [29]. Briefly, myocardial sections were embedded in optimal cutting tem-

perature medium (OCT) and cryosectioned at 10  $\mu\text{m}$  thickness before fixing in 2% formaldehyde/0.2% glutaraldehyde. The sections were incubated in X-Gal solution for 2 hr, then stained with hematoxylin-eosin. The percentage of X-Gal-positive cells in the delivery area was determined by microscopic counting.

Postprocedural myocardial infarction (MI) area was quantified microscopically in each segment using planimetry. Due to the short-term nature of the experiments (5 days), MI area was identified as regions containing an increased density of inflammatory cells. MI regions in each section were traced manually and areas were calculated using NIH imaging software and expressed as a percentage of the total left ventricular area (Fig. 1D).

### Measurement of Myocardial $\beta$ -Gal Enzyme Activity

Tissue samples were thawed and approximately 5 g of muscle was placed in 12 ml of prechilled lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% TritonX-100) with proteinase inhibitors added immediately prior to homogenization (0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g}/\text{ml}$  pepstatin A, 5  $\mu\text{g}/\text{ml}$  leupeptin, and 10  $\mu\text{g}/\text{ml}$  aprotinin). The tissue was homogenized on ice using a Brinkman Polytron tissue disrupter until no large pieces of tissue were visible. After centrifugation (2,000  $g$  for 25 min at 4°C), the supernatant was transferred to a 1.5 ml microcentrifuge tube, and again centrifuged (15,000  $g$  for 25 min at 4°C). The endogenous  $\beta$ -Gal activity was precleared by incubating with Chelex (BioRad, Hercules, CA) by adding 20  $\mu\text{l}$  of a 50% slurry of Chelex in lysis buffer to a microcentrifuge tube containing 50  $\mu\text{l}$  of lysate and mixing gently. The Chelex was pelleted by centrifugation at room temperature and the  $\beta$ -Gal enzyme activity measured in the supernatant. An aliquot of the Chelex-treated supernatant was saved to determine protein concentration of the solution (BCA assay; Pierce Chemical, Rockford, IL).

$\beta$ -Gal enzyme activity was measured using a Galacto-Light Plus chemiluminescent reporter system from Applied Biosystems (Emeryville, CA) using methods and standards supplied by the manufacturer. The amount of  $\beta$ -Gal protein was estimated by comparison to a standard curve generated using a  $\beta$ -Gal standard supplied with the kit. The data are expressed as ng  $\beta$ -Gal per  $\mu\text{g}$  of tissue.

### Statistics

Results are presented as mean  $\pm$  SEM. Data were compared using Student's *t*-tests (GraphPad InStat soft-

ware, San Diego, CA) with differences between data sets considered significant at  $P < 0.05$ .

### RESULTS

The coronary venous system was successfully cannulated in all hearts and all animals survived the 5-day study. Continuous monitoring of heart rate, rhythm, and blood pressure was maintained during all procedures. During delivery in both high-pressure (HP)-RCVD and IM groups, transient ventricular tachycardia (3–5 beats) was observed but did not require intervention as it resolved immediately after delivery was completed and did not result in hemodynamic compromise.

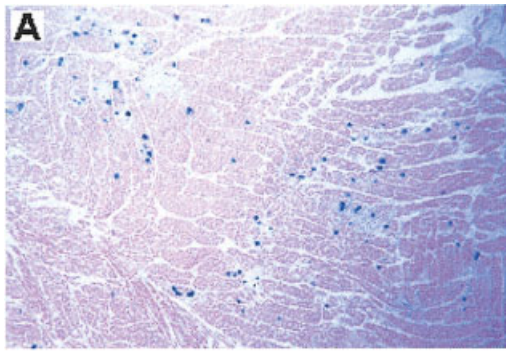
A radio-opaque region (blush), lasting 1–2 min and covering a large region of the anterior wall of the left ventricle, was observed after infusion of the contrast agent into the balloon-occluded vein (Fig. 1A). Comparisons of  $\beta$ -Gal transgene delivery efficiencies between the three different delivery groups were performed by both histochemical assessment of individual cells and quantitative enzymatic assays of tissue homogenates. Transgene expressing cells were widely distributed over the myocardium following RCVD delivery (Fig. 2A), whereas transfection was primarily observed around the needle track with IM injections (Fig. 2B). The histological sections stained with X-Gal were also quantitated (Fig. 3A). The density of  $\beta$ -Gal-positive cells was significantly higher ( $P = 0.01$ ) in the HP-RCVD group ( $0.26\% \pm 0.05\%$ ) than in the IM group ( $0.02\% \pm 0.01\%$ ). There was also a trend toward higher expression in the HP-RCVD group compared to the low-pressure (LP)-RCVD group ( $P = 0.07$ ). No difference was observed between LP-RCVD ( $0.05\% \pm 0.03\%$ ) and IM groups ( $P = 0.48$ ).

Quantitative enzymatic assays for  $\beta$ -Gal activity in tissue homogenates were also performed (Fig. 3B). Levels of  $\beta$ -Gal activity in myocardial tissues of the HP-RCVD group ( $108.7 \pm 22.4$  ng  $\beta$ -Gal/ $\mu\text{g}$  of myocardium) were 17- and 7-fold higher than LP-RCVD ( $15.6 \pm 12.2$  ng  $\beta$ -Gal/ $\mu\text{g}$  of myocardium) and IM ( $6.5 \pm 1.9$  ng  $\beta$ -Gal/ $\mu\text{g}$  of myocardium) groups, respectively ( $P = 0.05$  for both), confirming the histological data.

Postprocedural myocardial injury (MI area 0.12–1.1% of the total LV area) occurred in all treatment groups. The extent of myocardial injury was more in the HP-RCVD group ( $1.12\% \pm 0.19\%$ ) compared to the LP-RCVD ( $0.12\% \pm 0.04\%$ ;  $P = 0.002$ ) and IM ( $0.31\% \pm 0.09\%$ ;  $P = 0.009$ ) groups. The MI area was not statistically different between LP-RCVD and IM groups ( $P = 0.11$ ) (Fig. 4).

### DISCUSSION

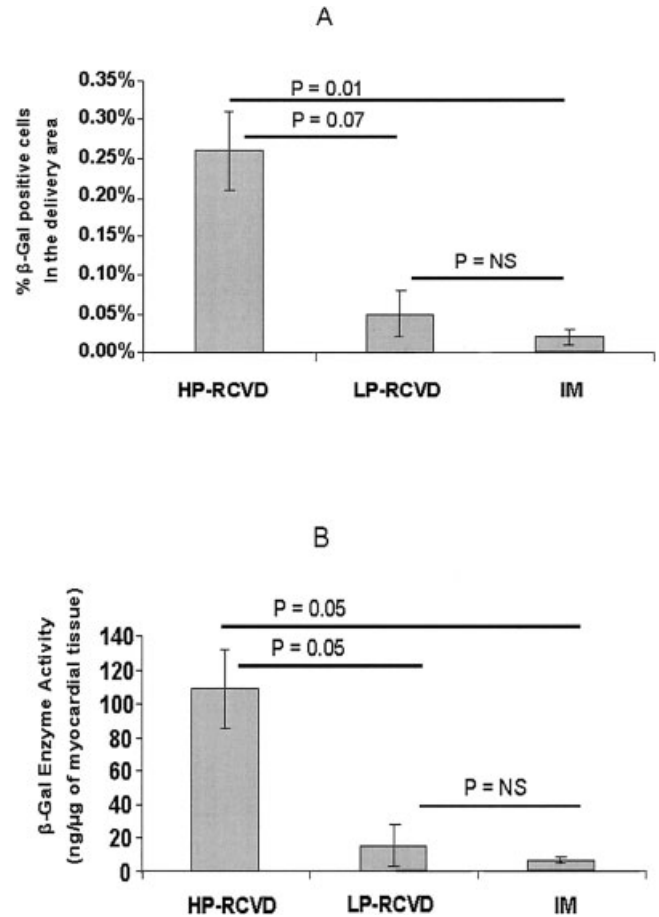
Retrograde coronary venous delivery provides an attractive alternative to either intracoronary or intra-



**Fig. 2.** Distribution of transgene expression in the myocardium following HP-RCVD and IM injections.  $\beta$ -Gal expression after RCVD is widespread (A), whereas cardiomyocytes expressing transgene (black arrows) are fewer in number and localized to small areas immediately adjacent to the needle entry site (dotted outline) after IM injection (B). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com)].

myocardial routes of administration. The coronary venous system is easily cannulated through the coronary sinus using off-the-shelf equipment and the procedure has a low incidence of reported complications. Moreover, local complications from venous access are far less than those of arterial accesses used in coronary angiography [30]. RCVD can be done on an outpatient basis, the same as diagnostic coronary or peripheral vascular angiography, and is a practical delivery modality for patients with total coronary artery occlusion, which would otherwise not be candidates for percutaneous intracoronary delivery of proangiogenic growth factors or their genes.

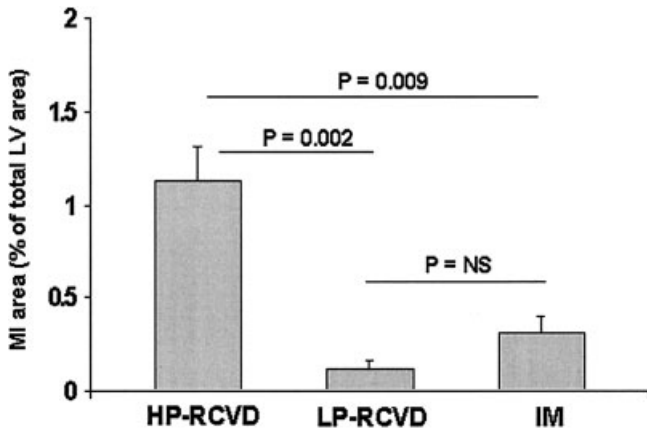
The two most critical parameters influencing RCVD efficiency are infusion pressures and residence time of agent in the interstitium [26]. Both variables are related in that intravenous pressures are largely dependent on anastomoses between major coronary veins (venove-



**Fig. 3.** A: Percentage  $\beta$ -Gal-positive cells in the delivery area. The percentage of myocardial cells transfected with  $\beta$ -Gal plasmid in the HP-RCVD group is approximately 10 times that observed in the IM group. There was a trend toward better transfection in the HP-RCVD compared to the LP-RCVD modality. B:  $\beta$ -Gal enzyme activity (ng/ $\mu$ g of myocardial tissue). The  $\beta$ -Gal enzyme activity was 7- and 17-fold higher in the HP-RCVD group compared to the LP-RCVD and IM groups, respectively.

nous communication), which lower venocapillary resistance and increase runoff of infusate. High-pressure injection is presumed to be necessary to attain limited disruption of the capillary bed, allowing penetration of agents into the interstitial tissue so that they may bathe myocardial cells [26]. However, ours is the first study to compare directly the influence of pressures on delivery efficiency. The results demonstrate a clear requirement for moderately high pressures (approximately 5- to 6-fold over basal venous pressures).

Coronary venous anatomy must be taken into account when translating RCVD to the clinical situation. In the clinical situation, it will be necessary to perform first a selective balloon-occlusion coronary venogram to evaluate potential anatomic venous variations and the presence of significant venovenous com-



**Fig. 4.** Postprocedural myocardial infarction (MI) area was quantified microscopically in each myocardial segment using planimetry (dotted line). MI regions in each section were traced manually and areas were calculated using NIH imaging software and expressed as a percentage of the total left ventricular area. Note the cluster of inflammatory cells within the dotted line representing inflammatory response within the recent MI area.

munications. Mapping the venous system allows positioning of the occluding balloon to minimize runoff. Optimal delivery pressures are obtainable by monitoring intravenous pressures while making adjustments by hand to compensate for variable resistances. However, occluding balloon positioning and pressure adjustments would not overcome runoff due to apical communications, which would require a novel two-balloon device with a distal as well as proximal balloon positioned on either end of the infusion hole. A final benefit of venography would be that other coronary veins would be mapped for potential use as secondary delivery conduits.

The precise pressure for optimal delivery will have to be determined by more thorough studies with pressures spanning those used here (150 and 300 mm Hg) and must also account for safety. Limited sustained myocardial injury (MI area of 0.12–1.1%) was observed with all three modalities used in this study. The clinical effects of this small MI area are not known but probably not significant given the limited size of myocardial injury. On the other hand, limited myocardial damage, resulting in extravasation of agent into the myocardium, is necessary for efficient delivery. Moreover, damage may synergize with the angiogenic agent to yield greater revascularization as a result of the inflammatory cell infiltration.

This study demonstrates the efficacy and relative safety of the RCVD technique. Given the less invasive nature and higher delivery efficiency of RCVD compared to the IM technique, the former would provide an attractive alternative in the clinical setting. How-

ever, the limited myocardial injury associated with these delivery techniques warrants further evaluation in future studies.

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