Angiogenic pretreatment to enhance myocardial function after cellular cardiomyoplasty with skeletal myoblasts

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Supplemental material is available online.

Objective: Improvements in ventricular function after cellular cardiomyoplasty appear to be limited by the poor survival of the cellular implants. Angiogenic pretreatment of infarcted myocardium may improve implanted cell survival and consequently myocardial function.

Methods: Fischer 344 rats underwent coronary artery ligation and injection of an adenovirus encoding vascular endothelial growth factor 121 or of saline solution at increasing intervals after ligation. Myocardial perfusion and mass preservation were assessed. On the basis of these data, four groups of animals underwent coronary ligation and adenovirus with or without syngeneic skeletal myoblast administration: (1) adenovirus at ligation and myoblasts 3 weeks later ($n = 7$), (2) saline solution at ligation and myoblasts 3 weeks later ($n = 8$), (3) saline solution at ligation and 3 weeks later ($n = 8$), and (4) saline solution at ligation and adenovirus with myoblasts 3 weeks later ($n = 5$). Left ventricular ejection fraction was analyzed by echocardiography before coronary ligation and 3 and 5 weeks later, after which cell survival was assessed in harvested tissues.

Results: Myocardial infarct perfusion was at least 50% greater in animals treated with adenoviral vector than with saline solution immediately after ligation ($P < .02$). In comparison, delayed adenovirus administration did not significantly diminish infarct perfusion but resulted in decreased myocardial preservation ($P < .05$). Accordingly, adenovirus administration nearly tripled implanted myoblast survival relative to saline solution-treated animals ($P = .004$). Left ventricular ejection fraction was improved, however, only after cell implantation with adenovirus pretreatment ($P = .027$).

Conclusion: Angiogenic strategies can help to preserve myocardium jeopardized by acute coronary occlusions. Angiogenic pretreatment enhances the efficacy of cellular cardiomyoplasty.

Despite progress during the last several decades in the treatment of coronary atherosclerosis, coronary artery disease remains the leading cause of death worldwide, resulting in approximately 7 million deaths each year. More specifically, the delayed treatment of acute coronary occlusion and consequent myocardial infarction typically lead to myocardial dysfunction

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Abbreviations and Acronyms

AdVEGF	= adenovirus encoding vascular endothelial growth factor
CSI	= cell survival index
LVEF	= left ventricular ejection fraction
PBS	= phosphate-buffered saline solution
T ₀	= time 0, coronary ligation and pretreatment time
T ₁	= time 1, 3 weeks after coronary ligation

and, potentially, congestive heart failure—the leading cause of death from coronary artery disease.

Although myocardial injury has conventionally been considered to be irreversible beyond 6 hours after acute coronary occlusion, data from a number of percutaneous coronary intervention trials suggests that delayed reopening of occluded coronary vasculature may lead to the preservation of myocardial function and improved long-term cardiac-related prognosis.¹ Such beneficial results are thought to be related to salvage of the ischemic border zone surrounding the central area of infarction, which may also progress to infarction depending on the adequacy of perfusion to these regions.

Therapeutic angiogenesis describes the strategy of administering angiogenic growth factors or other mediators to enhance the tissue of ischemic tissues, typically when an occluded arterial vasculature can not be reopened through conventional strategies.²⁻⁴ Although much remains unknown regarding the optimal administration of angiogenic agents, angiogenic strategies have already been applied with some success experimentally and clinically in enhancing the function of ischemic but viable myocardium. In contrast, angiogenic strategies would be expected to be less effective in improving the function of already infarcted myocardium. The implantation of myogenic or undifferentiated stem cells (cellular cardiomyoplasty) has been demonstrated in a number of animal and early clinical trials to effect salvage of such areas of myocardial infarction.⁴⁻¹⁶

A major limitation of cellular cardiomyoplasty techniques has been the poor survival (<10%) of cellular implants.¹⁴⁻¹⁷ We have surmised that cellular implant loss may be related to the relatively ischemic environment of the infarct tissue, and we and others have demonstrated that pretreatment with angiogenic mediators improves implanted cell survival and consequent physiologic outcomes.^{17,18} In contrast, others have advocated the concurrent administration of cells and angiogenic agents.¹⁹⁻²²

We analyzed the interval for administering angiogenic treatment after an acute coronary occlusion necessary to adequately salvage myocardium. With these data, we demonstrated that angiogenic pretreatment of infarcted myocardium before skeletal myoblast implantation, as opposed to

simultaneous angiogenic treatment and cell implantation, is critical for enhancing ventricular function.

Methods**Model Preparation and Vector Administration**

Adult male Fischer 344 rats (275-300 g; Harlan Sprague Dawley, Inc, Indianapolis, Ind), treated in accordance with protocols approved by the Evanston Northwestern Healthcare Institutional Animal Care and Use Committee, were anesthetized with intraperitoneally administered ketamine and xylazine (85 and 12 mg/kg body weight, respectively), intubated, and placed on a rodent ventilator (Harvard Instruments, Holliston, Mass). A left fourth intercostal space thoracotomy was then performed, and the left coronary artery was found and ligated 1 to 2 mm from its origin. At the same time, five uniformly distributed 20- μ L injections each containing 2.0×10^9 particle units of replication-defective adenovirus encoding the 121-amino acid isoform of vascular endothelial growth factor (AdVEGF),²³ an equal volume of phosphate-buffered saline solution (PBS), or no injections ($n = 8$ per group) were administered into the area of myocardium subtended by the ligated coronary artery, readily identifiable as an area of blanching on the anterolateral aspect of the left ventricle. The thoracotomy was then closed in layers, and the animal was allowed to recover under supervision. Rethoracotomy was performed 2 or 7 days later, and delayed vector or PBS administration was performed analogously.

Evans Blue Perfusion Analysis

Animals were killed by pentobarbital overdose (150 mg/kg) 21 days after coronary ligation, and the heart was excised. The ascending aorta was cannulated and perfused with 15 mL Evans blue dye (Sigma, St Louis, Mo) in PBS (0.25 mg/mL).²⁴ The infarct region, which was readily delineated by the gross absence of blue staining, was resected from the stained left ventricle with a Leica dissection microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). The wet weight of the excised segments was measured, and the epicardial surface area was determined with Metamorph software (Universal Imaging, Downingtown, Pa). The infarcted myocardium was then homogenized in PBS and extracted with formamide at 60°C. Evans blue content was then measured in the supernatant of each sample at 578 nm, as previously described.²⁴

Microsphere Analysis

In separate confirmatory experiments, rats treated as described were subjected to thoracotomy 21 days after ligation for microsphere perfusion analysis ($n = 5$ per group). An aliquot of 1×10^6 red fluorescent polystyrene microspheres (15 μ m diameter; Molecular Probes, Inc, Eugene, Ore) in 1 mL PBS was injected directly into the left atrium. After 15 minutes, the animal was killed and the heart was excised. The thinned infarct region was identified grossly and excised under a dissection microscope, as previously described.⁷ After wet weights and infarct surface areas were measured, microsphere content in each infarct sample was determined in triplicate according to manufacturer recommendations.

Histologic Evaluation

In another set of animals treated as described here, whole hearts were excised and then fixed with zinc-formalin before paraffin embedding. Sections were taken from the ventricular apex to the base at 500- μ m intervals with a 10- μ m thickness and prepared for histochemical staining with hematoxylin and eosin (Sigma).

Isolation of Transgenic Myoblasts

Skeletal myoblasts were harvested from adult syngeneic Fischer 344 rats expressing the gene encoding for human alkaline phosphatase. (Transgenic rats were a gift from E. Sandgren, University of Wisconsin.)²⁵ Briefly, 0.5 mL of 0.5% bupivacaine (Abbott Laboratories, Abbott Park, Ill) was injected in the rat hind limbs, and skeletal muscle biopsy specimens (approximately 1 cm³) taken 72 hours later were minced and digested with trypsin 0.025% ethylenediaminetetraacetic acid (Gibco BRL, Carlsbad, Calif) and collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ). Primary harvests were expanded on laminin-coated (Gibco) plates (equivalent to 1×10^8 cells after 7 days) and passaged before confluence. The cultured cells were 95% to 99% viable (by trypan blue exclusion) and approximately 85% pure (fluorescein isothiocyanate-labeled desmin monoclonal antibody). Cells were distributed into freezing media and stored (1.5×10^6 cells/vial).

Myoblast Preparation

Stored myoblasts were thawed and collected in PBS (1 mL) immediately before myocardial administration. Cells used for survival studies were treated with a 1- μ mol/L solution of Hoechst 33342 (Sigma) and incubated at 37°C in 5% carbon dioxide for 15 minutes. The cells were subjected to three washes in PBS at 5 times the initial media volume and then allowed to recover for 45 minutes in Ham F12 media. The cells were re-suspended in 100 μ L PBS. An aliquot of 1×10^6 red fluorescent 2.0- μ m microspheres (Molecular Probes) was added to each cell injectate as an internal delivery and sampling standard.¹⁷ Cells used for functional and echocardiographic studies were likewise prepared, except without the addition of Hoechst 33342 or microspheres.

Myoblast Administration Studies

The human alkaline phosphatase-negative rats underwent initial coronary ligation and adenoviral vector or PBS administration at time 0 (T₀), followed by myoblast administration through a rethoracotomy 3 weeks later (T₁), as follows: group 1 (n = 7) received AdVEGF at coronary ligation (T₀) and myoblasts 3 weeks later (T₁); group 2 (n = 8) received PBS at T₀ and myoblasts at T₁; group 3 (n = 8) received PBS at T₀ and T₁; and group 4 (n = 5) received PBS at T₀ and AdVEGF with myoblasts at T₁. The myoblast aliquot or PBS was administered as five equidistant subepicardial 20- μ L injections throughout the infarcted myocardium, which was identified as a grayish-white area on the anterolateral surface of the left ventricle (approximately 20%-30% of the left ventricle). The thoracotomy was then closed, and the animals were allowed to recover.

Figure 1. Myocardial infarct perfusion. Perfusion is depicted by Evans blue concentration in region of myocardial infarction 21 days after coronary ligation as function of interval between coronary ligation and adenoviral vector (VEGF) versus saline vehicle (PBS) administration.

Functional Assessment by Echocardiography

Echocardiographic analysis was performed at three points: before coronary ligation, 3 weeks after ligation (T₁), and 5 weeks after ligation (2 weeks after cell implantation). Left ventricular ejection fraction (LVEF) was measured from apical end-systolic and end-diastolic points in the 2-dimensional echocardiographic long-axis view with a 14-MHz pediatric probe (Acuson Corporation, Mountain View, Calif). All measurements were performed off-line by using the cine-loop feature to retrospectively visualize the beating heart at the maximal end-diastolic and end-systolic volumes required for endocardial area tracing (leading edge method). Final analysis included the averaged measurements of three consecutive cardiac cycles per animal by an experienced observer (B.S.) blinded to the treatment groups.

Cell Survival Analysis

Animals were killed 2 weeks after cell implantation. The hearts were then harvested, and myocardial tissues were analyzed for cellular implant survival as previously described.¹⁷ A cell survival index (CSI) was calculated as the ratio of blue-stained nuclei (cellular implants) divided by the total number of red microspheres (control for injectate delivery and tissue sampling) in five separate fields per animal. A mean CSI was calculated from these measurements for each animal.

Statistical Analysis

Data are expressed as means $\pm$ SD. Two-sided Fisher exact test or analysis of variance was used to show significance. Post hoc tests with Bonferroni correction were used for pairwise comparisons.

Results

Perfusion Analyses

Infarct perfusion was significantly greater in animals that received AdVEGF rather than PBS immediately after ligation, as demonstrated by Evans blue uptake in the region of

Figure 2. Myocardial infarct mass. Infarct wet weight measured 21 days after coronary ligation is depicted as function of interval between coronary ligation and adenoviral vector (*VEGF*) versus saline vehicle (*PBS*) administration as either absolute weight divided by area of infarct (A) or relative (percentage) difference in infarct mass (B).

infarction ($180 \pm 30 \mu\text{g}/\text{mg}$ tissue vs $120 \pm 20 \mu\text{g}/\text{mg}$ tissue, respectively, $P = .003$) and by microsphere perfusion studies (14 ± 2 spheres/mg tissue vs 8 ± 3 spheres/mg tissue, $P = .016$). AdVEGF administration delayed until 7 days after ligation did not significantly alter infarct perfusion (Figure 1) but did result in decreased myocardial mass preservation (Figure 2). This did not appear to be related to changes in infarct area (Figure 3) but did appear to be associated with myocyte preservation and prevention of fibrosis, rather than increased myocardial edema (Figure 4).

Cellular Implant Survival

In contrast to our expectations, regardless of whether AdVEGF was administered as pretreatment 3 weeks before cell implantation (group 1) or concurrently with cell implantation (group 4), the (implanted) CSI was increased relative to the PBS-treated control group (group 2; CSI 1.9 ± 0.7 and 1.6 ± 0.6 vs 0.7 ± 0.4 , respectively; $P = .004$). Histologic analysis of implanted tissues clearly found human alkaline

Figure 3. Myocardial infarct area. Infarct area measured 21 days after coronary ligation is depicted as function of interval between coronary ligation and adenoviral vector (*VEGF*) versus saline vehicle (*PBS*) administration.

phosphatase-positive cells in the infarcted myocardium at densities consistent with CSI determinations (Figure E1).

Echocardiography

LVEF was similar in all experimental groups at T0 immediately before coronary ligation and at T1 3 weeks later (Figure 5). LVEF decreased by approximately 33% during this interval, consistent with a large area of anterolateral myocardial thinning noted by echocardiographic imaging (Figure E2).

LVEF was significantly improved 2 weeks after cell implantation in (pretreated) group 1 relative to (control) groups 2 through 4 ($P = .0003$). LVEF 2 weeks after cell implantation approached baseline values for group 1 ($62\% \pm 14\%$) and was significantly greater than values in control groups 2 through 4 ($38\% \pm 10\%$, $49\% \pm 19\%$, and $42\% \pm 9\%$ respectively, $P = .027$). Finally, whereas 5 of 7 group 1 animals (71%) demonstrated an improvement in LVEF, improvements were seen in only 1 of 8 group 2 animals (13%), 1 of 8 group 3 animals (13%), and 2 of 5 group 4 animals (40%, $P = .047$ for group 1 vs groups 2 and 3 and $P = .04$ for group 1 vs group 4; Figure E3).

Discussion

In this study, we demonstrated that salvage of myocardial function appears to be optimized if angiogenic treatment is delivered soon after an acute coronary occlusion and if angiogenic pretreatment of myocardium is provided before cell implantation. In contrast to our previous studies involving fetal cardiomyocyte implants,¹⁷ the functional benefit of this pretreatment strategy was not directly correlated with improved survival of (skeletal myoblast) implants.

Figure 4. Morphology of myocardial scar as function of various treatments. (A) Saline vehicle administration at time of coronary ligation (day 0); (B) adenoviral vector administration at time of coronary ligation (day 0); (C) saline vehicle administration 7 days after coronary ligation; (D) adenoviral vector administration 7 days after coronary ligation. LV, Left ventricle.

Several alternative strategies have previously been reported to improve the survival of cells implanted into regions of myocardial infarction, including the ex vivo transfection of these cells with angiogenic mediators, the simultaneous administration of cells and angiogenic agents, and the endowment of cellular implants with antiapoptotic properties through ex vivo transfection.^{18-22,26} Although it is not yet clear whether any of these approaches will ultimately yield superior cell survival benefits, and although the mechanisms whereby cell implantation enhances cardiac function remain controversial (autocrine and paracrine biologic activities vs passive geometric and compliance properties vs contractile contributions), it is notable that improved cell survival appears fairly consistently to improve the outcomes of cellular cardiomyoplasty.^{8,14-22,26,27}

It is conceivable that the effectiveness of various implant survival strategies may be a function of the susceptibility of the respective implants to ischemia and the robustness or rapidity of effect of these survival strategies. For example, it is possible that the apparent inconsistency in terms of cell survival between this study and our previous study may be related to the relative sturdiness of myoblasts versus fetal cardiomyocytes. In this regard, it is conceivable that although myoblasts are sufficiently resistant to ischemia to survive in the infarct milieu, they may nevertheless survive only in a state of functional hibernation, failing to contribute significantly to improved overall left ventricular function. Enhanced ventricular function with AdVEGF pretreatment could therefore be the result of the interval provided for angiogenesis and increased infarct perfu-

sion to develop and thereby support enhanced (implanted) cell function in group 1 relative to the simultaneous treatment group 4 in this study. It can not be ruled out, however, that the antiapoptotic effects of vascular endothelial growth factor may also underlie these observations.

The clinical use of skeletal myoblasts has been discouraged in part because of data from initial clinical trials demonstrating increased incidence of ventricular arrhythmias after skeletal myoblast implantation, presumably on the basis of the electrical isolation of these cells from the host myocardium.²⁸ Subsequent clinical data have not confirmed these observations.^{29,30} Further, it is not clear that any candidate cell type will electrically integrate into the host myocardium such that potentially arrhythmogenic reentrant circuits can be avoided. If skeletal myoblasts possess superior survivability characteristics, then these cells may yet prove to be an important cardiomyoplasty candidate.

Study Limitations

This report does not address the possibility that the improvement in LVEF seen in (pretreatment) group 1 relative to (concurrent treatment) group 4 is simply the result of a greater time allowance for the development of angiogenesis in the former versus the latter group (5 vs 2 weeks, respectively), irrelevant to the implantation of cells. An AdVEGF-alone group would have been needed to rule out this possibility; numerous previous studies, however, including our previous work in the same model, tend to discount this premise.¹⁷⁻²⁰

Figure 5. Mean left ventricular (LV) ejection fraction (EF), as determined by echocardiographic analysis. Group 1 (n = 7) represents adenoviral vector at coronary ligation, myoblasts 3 weeks later. Group 2 (n = 8) represents saline vehicle at coronary ligation, myoblasts 3 weeks later. Group 3 (n = 8) represents saline vehicle at coronary ligation and 3 weeks later. Group 4 (n = 5) represents saline vehicle at coronary ligation, adenoviral vector with myoblasts 3 weeks later. T₀, Time of coronary ligation; T₁, 3 weeks after coronary ligation; T₂, 5 weeks after coronary ligation.

Another potential concern regarding these findings is in regard to our failure to detect a significant difference in LVEF with myoblast implantation relative to untreated, ligated controls. In this regard, previously reported improvements in LVEF in animal models have generally been relatively small (absolute change in LVEF <10%) although statistically significant. Although the failure to detect such changes with echocardiography in our small animal model may be considered a limitation of this study and may be related to the fairly large variability in the (no treatment) control group 3, our differentiation of the effects of angiogenic pretreatment from the control groups overall and as depicted in the same-animal improvement trends with pretreatment (Figure E3) can alternatively be viewed as highlighting the potential robustness of the pretreatment strategy.

Another limitation of this study was our use of staining techniques to assess implanted cell survival. An alternative approach would have been to use quantitative polymerase chain reaction assay to determine the number of surviving cells. However, we were unable to complete such studies. The previously validated staining assays¹⁷ ultimately used in this study represent a reasonable if cumbersome alternative.

Finally, we cannot rule out the possibility that the reported mass preservation reported after early postligation vascular endothelial growth factor treatment is related to changes in myocardial water content conferred by the vascular endothelial growth factor transgene, unrelated to myo-

cyte survival. Qualitative histologic analyses, however, suggest that this is not the case. Similarly, differences in tissue weight were observed at least 14 days after vector administration, well after any adenovirus-mediated gene expression effects would be expected to have subsided.

Conclusions

In conclusion, these studies support a role for angiogenic salvage of infarcting myocardium and for angiogenic pretreatment in enhancing the efficacy of cellular cardiomyoplasty, probably as a result of myocardial scar revascularization that supports the function of cellular implants in the infarct milieu. Theoretically, such angiogenic and cellular therapies could be administered in the setting of completed infarctions, early after the acute interval in which myocardial infarction is likely to occur, or in circumstances in which percutaneous coronary intervention results in the no-reflow phenomenon.

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Figure E1. Photomicrograph demonstrating human alkaline phosphatase–positive myoblasts treated with Elf 97 substrate (Invitrogen, Carlsbad, Calif) indicative of donor cells.

Figure E2. Two-dimensional echocardiographic images confirm preservation of left ventricular mass in (*left*) adenovirus-pretreated animals relative to (*right*) saline-pretreated animals. *Arrow* depicts anteroapical region receiving cell administration.

Figure E3. Improvements in ejection fraction in individual subjects at time of cell implantation (*Pre Cell*) versus 2 weeks later (*Post Cell*). Group 1 ($n = 7$) represents adenoviral vector at coronary ligation, myoblasts 3 weeks later. Group 2 ($n = 8$) represents saline vehicle at coronary ligation, myoblasts 3 weeks later. Group 3 ($n = 8$) represents saline vehicle at coronary ligation and 3 weeks later. Group 4 ($n = 5$) represents saline vehicle at coronary ligation, adenoviral vector with myoblasts 3 weeks later. $P = .04$ for group 1 versus groups 2 and 3.