Multigene adenoviral therapy for the attenuation of ischemia-reperfusion injury after preservation for cardiac transplantation

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Objective: The protective effect of adenovirus-mediated ex vivo multigene transfer with superoxide dismutase, a free radical scavenger, and nitric oxide, a vasodilator with anti-inflammatory properties, was examined in the rat heart during experimental ischemia-reperfusion mimicking preservation for cardiac transplantation.

Methods: Donor rat hearts (n = 6 per group) were perfused with solution containing adenoviral vector carrying genes for β -galactosidase (group A), endothelial nitric oxide synthase (group B), manganese superoxide dismutase (group C), or both endothelial nitric oxide synthase and manganese superoxide dismutase (group D). Hearts were then implanted heterotopically into the abdomens of recipient rats. Four days later, transplanted hearts were collected, connected to a Langendorff perfusion apparatus, and subjected to 6 hours of ischemia followed by 1 hour of reperfusion. Cardiac function was evaluated with an intraventricular balloon at the beginning of Langendorff perfusion and after ischemia-reperfusion.

Results: Effective gene transfection was confirmed with *X-gal* staining in group A hearts. Positive immunoreactivity for endothelial nitric oxide synthase, manganese superoxide dismutase, or both was present predominantly in cardiomyocytes in group B, C, and D hearts. Percentage recovery of preischemic left ventricular developed pressure was $62.1\% \pm 7.36\%$ in group A; recoveries were increased to $79.6\% \pm 6.4\%$, $86.8\% \pm 9.1\%$, and $79.4\% \pm 6.2\%$ in groups B, C, and D, respectively.

Conclusion: These results indicate that adenoviral gene transfer of manganese superoxide dismutase and endothelial nitric oxide synthase can attenuate myocardial ischemia-reperfusion injury, with the former providing the most significant protection. Combined overexpression of manganese superoxide dismutase and endothelial nitric oxide synthase did not enhance myocardial recovery any further.

Oxidative stress, which is associated with increased formation of reactive oxygen species, plays a major part in the pathogenesis of myocardial ischemia-reperfusion injury.^{1,2} Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to hydrogen peroxide. Three isoforms of SOD exist; copper/zinc SOD, which has a cytoplasmic location, extracellular SOD, present outside the cell, and manganese SOD (Mn-SOD), which is found in the mitochondrial matrix.

This last isoform can be induced by compounds that increase the production of intracellular superoxide anion.^{3,4} It has been demonstrated that Mn-SOD is particularly important for myocardial protection because hearts of transgenic mice overexpressing Mn-SOD demonstrate a remarkable increase in resistance to ischemia-reperfusion injury.⁵

Nitric oxide synthase is an enzyme that exists in different types of cells in several constitutive and inducible isoforms. The endothelium is a major site for production of nitric oxide, which is a potent vasodilator^{6,7} and exerts antineutrophil actions that reduce the inflammatory components of ischemia-reperfusion injury.⁸ Several studies suggest that early damage to the coronary endothelium impairs nitric oxide production.⁹ It has been shown that nitric oxide may play an important protective role against cardiac reperfusion injury after ischemia.^{10,11} On the other hand, nitric oxide is reported to react with the superoxide anion to form peroxynitrite,¹² which exerts deleterious effects leading simultaneously to inactivation of nitric oxide,¹³ such that during reperfusion neutrophil aggregation and adherence are enhanced. These interactions make a strong case for attempting to overexpress both endothelial nitric oxide synthase (eNOS) and Mn-SOD together in cardiac cells, which to our knowledge has not been studied before.

This study was performed in a rat heart model involving hypothermic coronary perfusion for donor heart gene delivery, cardioplegic arrest, prolonged hypothermic ischemia, and reperfusion. We investigated whether multigene adenoviral-mediated gene transfer of recombinant genes for Mn-SOD, eNOS, or both would lead to better myocardial recovery after ischemia-reperfusion.

Material and Methods

Animals

Male Sprague-Dawley rats (weight 250-300 g) were used as donors and recipients in this study. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996, and the "European Convention on Animal Care" guide. This study was approved by the institutional ethics committee on animal research.

Adenoviral Vector

A serotype 5 adenovirus encoding for nonnuclear targeted *Escherichia coli* β -galactosidase under the control of the cytomegalovirus promoter was used in the control group (AdCMVLacZ; provided by James Wilson, Institute for Gene Therapy, University of Pennsylvania, Philadelphia, Pa). This vector has been rendered replication defective by replacing the entire E1a region and most of the E1b region of the adenoviral genome with the complementary DNA expression cassette. Adenoviral vector carrying the gene for eNOS was generated as previously described elsewhere.¹⁴ In

brief, bovine eNOS complementary DNA was cloned into the shuttle plasmid pACCMVpLpA. The resulting plasmid was linearized with NruI and cotransfected with d1309 into 293 cells by calcium phosphate-DNA coprecipitation. D1309 is a biologically selected restriction enzyme site loss variant of wild-type adenovirus type 5, which retains only a single XbaI site at nucleotide 1339. The 293 cells were human embryonic kidney carcinoma cells that had been transformed with the left end of human adenovirus type 5 DNA. Recombinant adenovirus vectors were generated by homologous recombination. Viral plaques were picked and propagated in 293 cells. Viral DNA was enriched by Hirt extraction and screened by restriction mapping and polymerase chain reaction for the presence of eNOS complementary DNA. Positive plaques underwent two further rounds of plaque purification in 293 cells. Stocks were prepared from positive plaques, and these were used to generate high-titer preparations. Viral preparations were prepared by infecting a confluent monolayer of 293 cells in T175 flasks with viral stock at a multiplicity of infection of 1 to 10. Virus was purified by double cesium gradient ultracentrifugation and was dialyzed against 10-mmol/L tris(hydroxymethyl)aminomethane, 1.0-mmol/L magnesium chloride, 1.0-mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, and 10% glycerol for 4 hours at 4°C. Viral titer was determined by plaque assay. Mn-SOD recombinant adenoviral construct was generated according to a previously described method. Briefly, Mn-SOD constructs were generated by cloning of an *EcoRI/PvuII* fragment from the pRK5 Mn-SOD construct.¹⁵ Recombinant adenoviral plasmid constructs were generated by cloning transgene into pAd.CMVlink, which contains the cytomegalovirus enhancer-promoter and an SV40 polyadenylation site for efficient expression of the transgene.¹⁶ Recombinant virus was generated by cotransfection of *NheI*-cut pAd plasmid with *ClaI*-cut Ad5.sub360 (E3-deleted) viral DNA.¹⁷ After transfection, plates were overlaid with agar, and initial plaques were harvested for screening by enzymatic activity. This recombinant virus was screened for Mn-SOD activity by secondary infection on 293 cells. Initial plaques that expressed functional enzyme were further purified through two subsequent rounds of plaque purification. Viral titer was determined by assessing plaque-forming units (pfu) on 293 cells.

Experimental Groups

Rats were divided into four groups according to transfected genes: group A (control, transfected with AdCMVLacZ) as control, group B (eNOS, transfected with AdeNOS), group C (Mn-SOD, transfected with AdMnSOD), and group D (both eNOS and Mn-SOD, transfected with AdeNOS and AdMnSOD). There were 6 rats in each group. Hearts collected from donor rats were globally transfected by the perfusion method and transplanted into the abdomens of recipient rats as described in detail. Four days later, a delay necessary for gene expression, transplanted hearts were excised, connected to the Langendorff system, and subjected to cold cardioplegic arrest and reperfusion accompanied by myocardial function monitoring and analysis of samples collected at the end by immunostaining (Figure 1).

Donor Operation and Gene Transfer

Donor rats (250-275 g) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). A median laparotomy was per-

Figure 1. Experimental protocol. Four days after gene transfection, hearts were subjected to 6 hours of global ischemia and 1 hour of reperfusion. Hearts were assessed for efficacy of gene transfection with immunohistochemical methods and for function by intraventricular balloon.

formed to expose the abdominal aorta. After injection of 200 U aqueous heparin into the inferior vena cava, the aorta was cannulated with a 24-gauge cannula, and the heart was arrested with an infusion of cold University of Wisconsin solution into the aorta. A median sternotomy was performed, venae cavae and pulmonary veins were ligated with 4-0 silk, and the heart was harvested and stored in the same cardioplegic solution at 4°C.

Gene transfer was achieved through the perfusion method as described previously elsewhere.¹⁸ Briefly, 5 mL of University of Wisconsin solution containing a viral titer of 1.0×10^9 pfu/mL in group A, B, or C. Group D hearts had a total viral dose of 2.0×10^9 pfu/mL (1.0×10^9 pfu/mL of eNOS and 1.0×10^9 pfu/mL of Mn-SOD) in 5 mL of University of Wisconsin solution. This solution was circulated through the coronary vasculature of the donor heart for 15 minutes by means of a peristaltic pump (Rainin Instrument, LLC, Oakland, Calif). The viral solution was infused into the donor organ through the cannula inserted into the aorta and was collected by a 14-gauge catheter placed into the pulmonary artery. Both catheters were connected by means of polyvinyl chloride tubing to the vial containing the viral solution. The flow rate was 0.75 mL/min. During the perfusion period, the container with the heart and the vial with the vector were kept on ice, and temperatures of both solutions did not exceed 4°C.

Heterotopic Heart Transplantation

Heterotopic abdominal heart transplantation was performed with standard microsurgical techniques.¹⁹ In brief, rats (275-300 g) were anesthetized by intraperitoneal administration of pentobarbital (70 mg/kg). The donor hearts were transplanted into the recipients by end-to-side anastomoses of the aorta and the pulmonary artery to the abdominal aorta and inferior vena cava, respectively, with 8-0 monofilament sutures. During surgery the heart was wrapped in gauze and kept cold by use of topical ice-cold saline solution. After the operation, all rats recovered with oxygen in a warm environment. Viability of the grafts was verified daily by palpation of the beating transplanted heart.

Global Ischemia-Reperfusion

On the fourth day after gene transfer, animals were anesthetized with diethyl ether and anticoagulated by intravenous injection of heparin (500 U). Transplanted hearts were quickly excised and perfused with modified Krebs-Henseleit buffer (120.0-mmol/L sodium chloride, 4.5-mmol/L potassium chloride, 20.0-mmol/L

sodium hydrogen carbonate, 1.2-mmol/L potassium phosphate, 1.2-mmol/L magnesium chloride, 2.5-mmol/L calcium chloride, and 10.0-mmol/L glucose, gassed with 95% carbon dioxide to obtain pH 7.4 at 37°C) at a pressure equal to 1 cm H₂O by means of a Langendorff apparatus. A thin-walled balloon was inserted into the left ventricle through the left atrium to monitor left ventricular pressure and control left ventricular volume. After stabilization, left ventricular developed pressure (LVDP), minimum dP/dt, maximum dP/dt, and rate-pressure product (RPP, heart rate $\times$ LVDP) were measured with left ventricular diastolic pressure stabilized at 10 mm Hg. The hearts were then subjected to global ischemia after infusion of cold (4°C) crystalloid (St Thomas No. 1) cardioplegia for 6 hours followed by 1 hour of reperfusion. Then the same measurements of the left ventricle were repeated.

X-gal Staining

Group A hearts were dissected, embedded in ornithine carbamoyl-transferase medium (Miles Inc, Elkhart, Ind), and frozen in liquid nitrogen. Frozen sections (6 μ m thick) were fixed in 2% paraformaldehyde and 0.125% glutaraldehyde in phosphate-buffered saline solution (PBS) for 5 minutes, washed three times in PBS with 2-mmol/L magnesium chloride, then incubated in three changes of PBS containing 2-mmol/L magnesium chloride, 0.01% sodium deoxycholate, and 0.02% NP-40. Sections were then incubated in staining buffer (30-mmol/L potassium ferrocyanide and 30-mmol/L potassium ferricyanide in PBS containing 2-mmol/L magnesium chloride, 0.01% sodium deoxycholate, and 0.02% NP-40) for 2 minutes before incubation in fresh staining buffer containing 1-mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (*X-gal*) in a moist chamber overnight at 37°C. Subsequently, sections were rinsed in PBS, counterstained with neutral red, and rinsed in water before mounting. Blue-stained cells indicated the presence of β -galactosidase expression.

Immunohistochemical Staining

Midventricular cross-sections of the transplanted hearts were embedded in OCT medium and frozen in liquid nitrogen. Frozen sections (5 μ m thick) were cut at 25- μ m intervals, fixed for 10 minutes in cold acetone (4°C), fan dried for 10 minutes, and further fixed in 1% paraformaldehyde and ethylenediaminetetraacetic acid for 3 minutes. Endogenous peroxidase activity was blocked with 0.1% sodium azide and 0.3% hydrogen peroxide for 10 minutes. Incubation of sections with 5% goat serum in PBS

with polysorbate 20 blocked nonspecific protein binding sites. Samples from groups A, B, and D then had (1:250) anti-eNOS monoclonal antibody (N30020; Transduction Laboratories, Inc, Lexington, Ky) added and were incubated for 60 minutes at room temperature. After rinsing, biotinylated rabbit antimouse F(ab')₂ (1:300) was added for 20 minutes. After further incubation for 20 minutes with peroxidase-conjugated streptavidin (1:300), the slides were incubated for 30 seconds in 0.1-mol/L sodium acetate buffer, pH 5.2. Then they were placed in 3-amino-9-ethylcarbazole substrate solution and incubated for 15 minutes at room temperature, counterstained in mercury-free hematoxylin for 1 minute, and further rinsed for 3 minutes under cold running tap water before being mounted. For Mn-SOD staining in groups A, C, and D, a similar protocol was used, with the primary antibody (K90096C; BioDesign, New York, NY) at a dilution of 1:200. The secondary antibody was 1:1000 of sheep/goat peroxidase (M15345).

Statistics

Values are presented as mean $\pm$ SD. One-way analysis of variance was used, followed by Bonferroni test to indicate individual significant differences.

Results

There were no technical failures or operative deaths in the 24 consecutive gene transfection experiments in the study. Positive cardiomyocyte cytoplasm staining with *X-gal* can be seen in cardiomyocytes from the AdCMVLacZ-transfected group but was not observed in nontransfected animals (Figure 2). Immunohistochemical staining for eNOS in groups A and B showed weak endothelial expression of native eNOS in group A and both endothelial and cardiomyocyte expressions of eNOS in group B (Figure 3). It also showed a number of inflammatory cells (immunologic reaction to the adenoviral vector). Figure 4 presents an immunohistochemical analysis of Mn-SOD expression in groups A and C, showing overexpression of Mn-SOD in myocytes of the group C hearts. Group A hearts showed weak staining for native Mn-SOD in cardiac myocytes. Inflammatory cells were found in these slides. Group D hearts were analyzed separately for eNOS and Mn-SOD; overexpression of each gene was detected in each sample tested (Figure 5), with overexpression of eNOS mainly in endothelial cells and a smaller expression in the cytoplasm of cardiomyocytes. Overexpression of Mn-SOD was seen in cardiomyocytes. Some inflammation was also seen in these samples.

In Langendorff perfusion experiments, no statistically significant difference was seen before ischemia among the groups ($n = 6$ in each group; Table 1) in terms of LVDP ($P = .9$), minimum dP/dt ($P = .274$), maximum dP/dt ($P = .106$), and RPP ($P = .885$). Percentage recovery of LVDP after global ischemia (4°C, 6 hours) is shown in Figure 6. The percentage recovery of LVDP after ischemia in the control transfected hearts (group A) reached a peak value of $62.1\% \pm 7.3\%$ after reperfusion. In comparison, statistically

Figure 2. Group A transgene expression. Demonstration of β -galactosidase transgene expression by histochemical staining with *X-gal* in heart transfected with AdCMVLacZ. Myocytes stain positively for β -galactosidase (yellow arrows). Original magnification $\times 600$.

significant improvements in the percentage recovery of LVDP were observed in the group transfected with eNOS (group B), which reached a peak level of $79.6\% \pm 6.4\%$ ($P = .009$ vs group A) after reperfusion, and in the group transfected with eNOS and Mn-SOD (group D), which reached a level of $79.4\% \pm 6.2\%$ ($P = .015$ vs group A). The percentage recovery of LVDP was better in Mn-SOD-transfected hearts (group C), reaching a level of $86.8\% \pm 9.1\%$ ($P < .001$ vs A). However, the differences between groups C and B and between groups C and D were not statistically significant ($P = .9$ and $P > .999$, respectively).

Percentage recoveries of minimum and maximum dP/dt after global ischemia are shown in Figures 7 and 8. Group B, C, and D hearts showed statistically significantly better recoveries of minimum and maximum dP/dt than did group A hearts ($P < .001$ and $P < .001$ vs group A, $P < .001$ and $P < .001$ vs group A, respectively). Better recoveries of both minimum and maximum dP/dt were seen in group C than in groups B and D, but the differences were not statistically significant ($P = .061$ and $P = .374$, respectively, vs group B and $P = .112$ and $P = .606$, respectively, vs group D). Values for minimum and maximum dP/dt were $52.3\% \pm 4.4\%$ and $51.3\% \pm 7.2\%$, respectively, in group A, $72.2\% \pm 6.5\%$ and $71.5\% \pm 7.2\%$, respectively, in group B, $81.2\% \pm 3.7\%$ and $78.3\% \pm 3.3\%$, respectively, in group C, and $72.8\% \pm 4.4\%$ and $72.3\% \pm 4.6\%$, respectively, in group D.

As shown in Figure 9, group B and C hearts had statistically significantly better percentage recovery of preischemic RPP than did group A and D hearts ($78.88\% \pm 9.0\%$ in group B, $P = .003$ vs group A, and $76.53\% \pm 8.1\%$ in

Figure 3. Immunohistochemical staining for eNOS. **A**, Immunohistochemical staining of midventricular section in heart transfected with AdeNOS with monoclonal antibody to eNOS (counterstained with mercury-free hematoxylin) showing endothelial (yellow arrows) and cardiomyocyte (red arrows) expressions of eNOS. Some inflammatory cells can be seen (bright green arrow). Original magnification $\times 400$. **B**, Immunohistochemical staining of midventricular section of heart transfected with AdCMVLacZ with monoclonal antibody to eNOS (counterstained with mercury-free hematoxylin) showing some endothelial expression of native eNOS (yellow arrows). Original magnification $\times 200$.

Figure 4. Mn-SOD immunohistochemical staining. **A**, Immunohistochemical staining of midventricular section of heart transfected with AdMnSOD with monoclonal antibody to Mn-SOD (counterstained with mercury-free hematoxylin). Expression is present in cardiomyocytes (red arrows). Original magnification $\times 200$. **B**, Immunohistochemical staining of midventricular section in heart transfected with AdCMVLacZ with monoclonal antibody to Mn-SOD (counterstained with mercury-free hematoxylin). Some staining of native Mn-SOD can be seen in cardiac myocytes (red arrows). Original magnification $\times 300$.

group C, $P = .011$ vs group A, compared with $56.5\% \pm 7.1\%$ in group A and $64.6\% \pm 7.1\%$ in group D, $P = .93$ vs group A). Although we did not use pacing on the Langendorff system, heart rate was relatively consistent in the preischemic period in all four groups studied here. Percentage recovery of heart rate after ischemia differed among groups (group A $70\% \pm 4.4\%$, group B $83.3\% \pm 4.3\%$, group C $78.4\% \pm 6.4\%$, and group D $71.5\% \pm 5.1\%$). Group B showed statistically significantly higher percentage recovery of heart rate after ischemia than did groups A and

D ($P = .003$ and $P = .013$, respectively). Differences were not statistically significant between groups B and C ($P = .602$), C and A ($P = .187$), C and D ($P = .559$), and D and A ($P > .999$).

Discussion

This study demonstrated statistically significant improvement in postischemic recovery of mechanical function in hearts transfected with genes for eNOS or Mn-SOD relative to those with control gene transfection in a protocol mim-

Figure 5. Immunohistochemical staining for eNOS and Mn-SOD. **A**, Immunohistochemical staining of midventricular portion in heart transfected with AdeNOS and AdMnSOD with monoclonal antibody to eNOS (counterstained with mercury-free hematoxylin). Most eNOS overexpression can be seen in endothelial cells (*yellow arrows*) with smaller expression in myocytes (*red arrows*). Original magnification $\times 200$. **B**, Immunohistochemical staining of midventricular portion in same heart (transfected with AdeNOS and AdMnSOD) with monoclonal antibody to Mn-SOD (counterstained with mercury-free hematoxylin). Overexpression of Mn-SOD can be seen in cardiomyocytes (*red arrows*). Original magnification $\times 200$.

TABLE 1. Cardiac function parameters before ischemia during Langendorff perfusion

	LVDP (mm Hg)	Minimum dP/dt (mm Hg/s)	Maximum dP/dt (mm Hg/s)	RPP
AdCMV LacZ	73.53 $\pm$ 19	−845.3 $\pm$ 92	1156.3 $\pm$ 76	15461 $\pm$ 7202
eNOS	76.18 $\pm$ 22	−714.4 $\pm$ 123	1060 $\pm$ 64	15708 $\pm$ 6428
Mn-SOD	82.36 $\pm$ 16	−780.5 $\pm$ 131	1043 $\pm$ 72	14740 $\pm$ 6305
eNOS and Mn-SOD	77.07 $\pm$ 7	−860.8 $\pm$ 140	1111 $\pm$ 71	12686 $\pm$ 3871
P value*	.901	.274	.106	.885

Data are expressed as mean $\pm$ SD. There were no significant differences in any of the parameters among the groups before ischemia. n = 6 per group.
*Analysis of variance followed by Bonferroni test.

icking conditions of preservation for heart transplantation. However, combined overexpression of both genes did not lead to further improvement with statistical significance. These findings appear to provide evidence that overexpressions of both genes play a direct role in the enhancement of myocardial ischemia-reperfusion tolerance in vivo. However, combined overexpression of both genes needs further investigation.

The important role of Mn-SOD in protecting hearts against the detrimental effects of ischemia-reperfusion injury has been clearly shown by work with transgenic mice overexpressing Mn-SOD.⁵ These mice had better postischemic recovery of ventricular function after a period of ischemia and reperfusion; however, this protocol used a short period of ischemia, whereas our protocol used a prolonged period of hypothermic ischemia after cardioplegic arrest, which mimics clinical donor heart preservation. Moreover, in a transgenic animal the heart is genetically

altered to overexpress Mn-SOD; thus it may adapt itself and acquire a character different from that of the natural heart. Therefore our model with gene transfection may be more suitable for investigating the effect of Mn-SOD in potentially useful technique to test the effectiveness of adenoviral gene transfer. There are three isoforms of SOD: copper/zinc SOD, which has a cytoplasmic location, extracellular SOD, found in extracellular compartment, and Mn-SOD, which is found in the mitochondrial matrix.³ Woo and associates²⁰ found that adenoviral gene transfer of SOD and catalase attenuates postischemic contractile dysfunction. Recently, it was shown that in vivo adenoviral gene transfer of extracellular SOD alone provides the heart with substantial protection against myocardial stunning.²¹ We have already shown Mn-SOD to be superior in ameliorating regional ischemia reperfusion injury.²² In this experiment, we used a global ischemia-reperfusion injury model, which parallels clinical heart transplantation.

Figure 6. Recovery of LVDP after ischemia. Isolated hearts in four experimental groups ($n = 6$ in each group) were subjected to 6 hours of cold (4°C) global ischemia followed by 1 hour of reperfusion. Statistically significantly better recovery of LVDP after ischemia was shown in groups B, C, and D relative to group A. No statistically significant differences were found between groups B, C, or D, although group C hearts showed best recovery. Data are expressed as percentage of LVDP before ischemia. *Asterisk* indicates $P = .009$ versus group A; *double asterisk* indicates $P < .001$; *triple asterisk* indicates $P = .015$. *Bar heights* represent mean; *error bars* represent SD.

Figure 7. Recovery of minimum (*min*) dP/dt after ischemia ($n = 6$ in each group). Improved recoveries of minimum dP/dt after 6 hours of cold global ischemia and 1 hour of reperfusion were shown in groups B, C, and D relative to group A. No statistically significant differences were found between groups B, C, or D, although group C hearts showed best recovery. Data are expressed as percentage of basal minimum dP/dt before ischemia. All *asterisks* indicate $P < .001$ versus group A. *Bar heights* represent mean; *error bars* represent SD.

Our experiment suggests that enhanced enzymatic activity of eNOS can help to ameliorate the effects of ischemia-reperfusion in the myocardium. We have demonstrated

Figure 8. Recovery of maximum (*max*) dP/dt after ischemia ($n = 6$ in each group). Improved recoveries of maximum dP/dt after 6 hours of cold global ischemia and 1 hour of reperfusion were shown in groups B, C, and D relative to group A. Differences between B, C, and D were not statistically significant. Data are expressed as percentage of baseline maximum dP/dt measured before ischemia. All *asterisks* indicate $P < .001$ versus group A. *Bar heights* represent mean; *error bars* represent SD.

Figure 9. Recovery of RPP after ischemia ($n = 6$ in each group). Statistically significant improvements in recovery of RPP after 6 hours of cold global ischemia and 1 hour of reperfusion were shown in groups B and C relative to groups D and A. Difference between groups D and A was not statistically significant ($P = .93$). Data are expressed as percentage of RPP measured before ischemia. *Asterisk* indicates $P = .003$ versus group A and $P = .106$ versus group D; *double asterisk* indicates $P = .011$ versus group A and $P = .307$ versus group D. *Bar heights* represent mean; *error bars* represent SD.

overexpression of this gene in the cardiac endothelium and myocytes. On the other hand, in this study, eNOS was found to be less effective than Mn-SOD in improving myocardial recovery after ischemia and reperfusion. The transient nature of the eNOS-derived nitric oxide production is most

beneficial during the early phase of reperfusion, which may in part explain our results. In addition to that, nitric oxide interacts with superoxide anion to form peroxynitrite,¹² which in turn exacerbates the deleterious effects of free radicals and further reduces the ability of nitric oxide to ameliorate ischemia-reperfusion injury.

Although our results did not demonstrate that combined overexpressions of eNOS and Mn-SOD provide an additive protection relative to individual gene transfection, we must interpret these results with caution. One possible confounding factor is the use of higher virus dose in doubly transfected hearts. Adenovirus as a vector is known to trigger an inflammatory reaction, which may have been increased in doubly transfected hearts. Although a number of inflammatory cells were seen on histologic examination of singly and doubly transfected groups, these hearts did not seem markedly different. Future work is needed with the same vector carrying both genes together.

Continuous hypothermic perfusion of donor hearts compared with hypothermic immersion storage can be used for preservation of donor organs.^{23,24} Therefore a modified hypothermic perfusion technique could be applied for gene delivery to donor hearts. It was shown that when adenovirus is used as a vector, this technique would result in more efficient transgenic expression compared with that induced by a single bolus injection.¹⁸ Pellegrini and colleagues¹⁸ showed that adenoviral hypothermic perfusion model provided an 11- to 14-fold increase in transgenic expression compared with the high-pressure bolus injection. It allowed a 30-fold reduction in the viral dose compared with that found in other reports without affecting the level of transgene expression. Brauner and colleagues²⁵ increased adenoviral vector uptake into the donor organ to 80% with the slow infusion technique (compared with 10% with bolus injection). Pellegrini and colleagues¹⁸ achieved similar levels of gene transfection with lower dose of adenovirus with no significant inflammatory response. This model of vector delivery was followed in our experiments. We used positive staining of myocardial cells as proof of gene transfection. Inflammatory cells were not specifically stained for. We showed an application of this model in studying the interaction of eNOS and Mn-SOD as important therapeutic molecules in protecting the myocardium from global ischemia-reperfusion injury.

In this study we developed a novel system to study the cardioprotective roles of different genes in reperfusion injury. It consists of a combination of intracoronary perfusion of the adenovirus and transplantation to transfer the gene or genes of interest, followed by Langendorff perfusion to evaluate the effect on myocardial protection. We believe that this is a potentially useful technique to test the effectiveness of adenoviral gene transfer and to investigate the

effect of each molecule on myocardial ischemia-reperfusion injury.

Conclusion

This study demonstrated that gene transfection had potential to introduce supraphysiologic levels of protective enzymes such as eNOS and Mn-SOD and to enhance myocardial tolerance to ischemia beyond that provided by intrinsic factors. The combined gene transfer of recombinant Mn-SOD and eNOS in this model had no statistically significant additive protective effect relative to single gene transfer in attenuating myocardial damage under conditions of experimental heart transplantation. The value of multigene therapy needs to be investigated further.

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