Cardiac-Selective Expression of Extracellular Superoxide Dismutase After Systemic Injection of Adeno-Associated Virus 9 Protects the Heart Against Post–Myocardial Infarction Left Ventricular Remodeling

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Background—Cardiac magnetic resonance imaging has not been used previously to document the attenuation of left ventricular (LV) remodeling after systemic gene delivery. We hypothesized that targeted expression of extracellular superoxide dismutase (EcSOD) via the cardiac troponin-T promoter would protect the mouse heart against both myocardial infarction (MI) and subsequent LV remodeling.

Methods and Results—Using reporter genes, we first compared the specificity, time course, magnitude, and distribution of gene expression from adeno-associated virus (AAV) 1, 2, 6, 8, and 9 after intravenous injection. The troponin-T promoter restricted gene expression largely to the heart for all AAV serotypes tested. AAV1, 6, 8, and 9 provided early-onset gene expression that approached steady-state levels within 2 weeks. Gene expression was highest with AAV9, which required only 3.15×10¹¹ viral genomes per mouse to achieve an 84% transduction rate. AAV9-mediated, cardiac-selective gene expression elevated EcSOD enzyme activity in heart by 5.6-fold (P=0.015), which helped protect the heart against both acute MI and subsequent LV remodeling. In acute MI, infarct size in EcSOD-treated mice was reduced by 40% compared with controls (P=0.035). In addition, we found that cardiac-selective expression of EcSOD increased myocardial capillary fractional area and decreased neutrophil infiltration after MI. In a separate study of LV remodeling, after a 60-minute coronary occlusion, cardiac magnetic resonance imaging revealed that LV volumes at days 7 and 28 post-MI were significantly lower in the EcSOD group compared with controls.

Conclusions—Cardiac-selective expression of EcSOD from the cardiac troponin-T promoter after systemic administration of AAV9 provides significant protection against both acute MI and LV remodeling. (Circ Cardiovasc Imaging. 2013;6:478-486.)

Key Words: AAV ■ gene therapy ■ LV remodeling ■ MI ■ MRI

In clinical gene therapy applications, therapeutic gene expression should be confined to the organ or cell type of interest to avoid potential side effects attributable to gene expression in off-target organs. Previously, several cardiac-specific promoters have been shown to provide cardiac-restricted gene expression after direct injection or by pressure-regulated retroinfusion of the anterior interventricular vein. Recently, we demonstrated that a truncated chicken cardiac troponin-T (cTnT) promoter confers a high degree of cardiac selectivity, while maintaining ≥40% of the strength of cytomegalovirus (CMV) promoter in 1-week-old mice after systemic administration of highly efficient AAV serotypes 1, 6, 8, and 9.

The present study describes a side-by-side comparison of AAV serotypes with regard to the time course, magnitude, and distribution of reporter gene expression...
in the heart after systemic administration in 5-week-old mice. We demonstrate that the cTnT promoter provides cardiac-restricted gene expression in mouse hearts when administered in combination with all the AAV serotypes tested. We show that serotype AAV9, followed closely by AAV8, provides superior transduction to cardiomyocytes compared with the other serotypes tested. Furthermore, we demonstrate that a single intravenous injection of an AAV9 vector expressing extracellular superoxide dismutase (ECsOD) from the cTnT promoter is adequate to both reduce infarct size and curtail subsequent left ventricular (LV) remodeling. ECsOD is 1 of 3 isoforms of SOD responsible for scavenging superoxide in mammals. ECsOD is a glycosylated homotetrameric enzyme (155 kDa) that is secreted from cells to bind heparin sulfate proteoglycans on cell surfaces and in the extracellular matrix, where it converts superoxide into less toxic hydrogen peroxide. In addition to its antioxidant functions, ECsOD plays a key role in preserving the bioavailability of nitric oxide by protecting it against destruction by superoxide. We suggest that the cardioprotective effects of ECsOD overexpression may be related to increased capillary area fraction, as well as reductions in neutrophil recruitment.

**Methods**

**AAV Vector Production**

The AAV vectors bearing the cTnT promoter driving the expression of firefly luciferase (AcTnTLuc), enhanced green fluorescent protein (eGFP; AcTnTeGFP), and mouse ECsOD (AcTnTeEcSOD), as well as an AAV vector with the CMV promoter directing expression of firefly luciferase (ACMVLuc), have been described previously. AAV vectors were packaged in HEK293 cells by the double or triple transfection method and purified by ammonium sulfate fractionation and iodixanol gradient centrifugation. Titters of the AAV vectors (viral genomes [vg]/mL) were determined by quantitative real-time polymerase chain reaction.

**Animal Procedures**

Animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Virginia and conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised 1985). C57Bl/6 mice (5 weeks old weighing 15 g) were purchased from The Jackson Laboratories (Bar Harbor, ME). For intravenous injection, mice were anesthetized with 1% to 1.2% isoflurane in oxygen, while viral solution was slowly injected via the jugular vein. For all procedures, depth of anesthesia was monitored using an IVIS 100 imaging system (Caliper Life Sciences, Hopkinton, MA), as described previously. Results are presented as relative light units per 1 mg protein (RLUs/mg protein).

**Bioluminescence Imaging and Quantitative Luciferase Activity Assays**

All in vivo and ex vivo bioluminescence imaging was performed using an IVIS 100 imaging system (Caliper Life Sciences, Hopkinton, MA), as described previously. After bioluminescence imaging and euthanasia at 6 weeks post vector injection, the protein extracted from each organ was measured using luciferase assay reagents from Promega Corp (Madison, WI), as detailed previously. Results are presented as relative light units per 1 mg protein (RLUs/mg protein).

**Fluorescence Microscopy**

Four weeks after vector administration, animals were euthanized and hearts were processed for cryosectioning and fluorescence microscopy, as described previously. Six-micrometer cryosections were used for documenting eGFP expression by fluorescence microscopy. To determine the distribution of eGFP expression in the myocardium, a minimum of 4 fields at ×20 magnification were captured from midventricular sections of each heart. The percentage of the total tissue fractional area showing eGFP expression was calculated using the threshold function in Photoshop CS2 (Adobe Systems, San Jose, CA).

**Determination of AAV Vector Genome Copy Number**

Four weeks after vector administration, total genomic DNA was prepared from the mouse hearts by standard phenol-chloroform extraction. AAV vector genome copy numbers were determined by real-time quantitative polymerase chain reaction, as detailed previously and in the online-only Data Supplement.

**Immunohistochemistry**

Immunostaining for ECsOD protein was performed on 6-µm fixed-frozen sections using rabbit anti-ECsOD antibody (2.5 µg/mL, Stressgen Bioreagents, Victoria, Canada). Sections were counterstained with hematoxylin before photography. Capillaries were stained with biotinylated GSL I–isolectin B4 (5 µg/mL, Vector Laboratories). Neutrophils in heart sections were immunostained using rat monoclonal antimouse neutrophil antibody (Serotec, Raleigh, NC). Neutrophil counts and capillary fractional areas were assessed using ImageJ (NIH, Bethesda, MD), as detailed in the online-only Data Supplement.

**SOD Zymography**

Flash-frozen tissue samples were homogenized in radioimmunoprecipitation assay buffer, and protein content was determined for each sample using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 µg) were electrophoresed under reducing conditions on 12.5% polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. ECsOD was detected by standard protocol using rabbit anti-ECsOD antibody (Stressgen Bioreagents). Results were expressed relative to the average signal intensity obtained from the hearts of AcTnTeGFP-injected control mice.

**EcSOD Activity Assay**

EcSOD enzymatic activity in hearts was measured after separation of EcSOD from copper-zinc SOD (CuZnSOD) and manganese SOD (MnSOD) by passing the tissue homogenate through a concanavalin A-Sepharose column, as described previously. SOD activity in the effluent was measured using an SOD assay kit (Cell Technology, Mountain View, CA).

**Myocardial Infarction and Size Measurement**

In the acute myocardial infarction (MI) study, a total of 15 mice were used. At 5 weeks of age, 8 mice were injected with AcTnTeGFP and 7 mice with AcTnTeEcSOD at a dose of 3.15×10⁹ vg/mouse to achieve steady-state gene expression by 9 weeks of age. Of these 15 mice, a total of 12 (6 in each group) completed the following protocol. Four weeks after vector administration, mice underwent surgical induction...
28 after surgery. CMR was performed as described previously, and on the day before MI surgery (baseline) and again on days 1, 7, and (EF) were assessed by cardiac magnetic resonance (CMR) imaging study of LV remodeling. Mouse LV volumes and ejection fraction effects of EcSOD and to establish large infarcts suitable for the coronary occlusion was used to overcome the cardioprotective above for MI were used to induce MI, except that a 60-minute imaged at baseline, but only a subset (n=8) were imaged at subsequent served as untreated controls. All 12 of the untreated controls were were similarly injected with AcTnTeGFP; and the remaining 12 mice old mice were injected with 3.15×10¹¹ vg/mouse of AcTnTEcSOD; 5 A total of 23 mice were used in the LV remodeling study. Six 5-week-old mice were injected with 3.15x10¹² vg/mouse of AcTnTcEcSOD; 5 were similarly injected with AcTnTcGFP; and the remaining 12 mice served as untreated controls. All 12 of the untreated controls were imaged at baseline, but only a subset (n=8) were imaged at subsequent time points. The same anesthetic and surgical procedures described above for MI were used to induce MI except that a 60-minute coronary occlusion was used to overcome the cardioprotective effects of EcSOD and to establish large infarcts suitable for the study of LV remodeling. Mouse LV volumes and ejection fraction (EF) were assessed by cardiac magnetic resonance (CMR) imaging on the day before MI surgery (baseline) and again on days 1, 7, and 28 after surgery. CMR was performed as described previously, and infarct size was determined on day 1 post-MI using late gadolinium-enhanced CMR, as described previously. LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), and LVEF were calculated, as described previously. Mice were euthanized with an overdose of pentobarbital at the end of the study.

Statistics
Parametric (Student t test, ANOVA) and nonparametric (Mann-Whitney, Kruskal-Wallis) statistical analyses were performed as appropriate on the basis of sample size and distribution, with values of P<0.05 considered significant.

Results
Cardiac-Selective Gene Expression From AAV Serotypes Harboring the cTnT Promoter
An initial comparison of the CMV and cTnT promoters was made using AAV6 and AAV9 serotypes (Figure I and Figure I in the online-only Data Supplement). In vivo bioluminescence imaging of mice injected with ACMVLuc or AcTnTLuc indicated that the CMV promoter programmed luciferase expression throughout the body in both AAV6- and AAV9-injected mice (Figure IA and IB in the online-only Data Supplement, CMV). In contrast, the cTnT promoter largely restricted luciferase expression to the heart in both AAV6 and AAV9 vector–injected mice (Figure IA and IB in the online-only Data Supplement, cTnT). Bioluminescence signals were stronger in AAV9- compared with AAV6-injected mice, regardless of the promoter used. Quantitative luciferase assays revealed that expression from the cTnT promoter in hearts from mice treated with AAV9 was 7.9-fold higher than in those treated with AAV6 (Figure 1A). The heart:liver ratios of luciferase activity (Figure 1B) were calculated and used as composite indices of the cardiac specificity provided by AAV6 and AAV9 in combination with CMV or cTnT promoters. For the non–tissue-specific CMV promoter, the heart:liver ratio of luciferase activity was higher in mice treated with AAV6 than with AAV9 (7.3 versus 1.4; Figure 1B). In contrast, luciferase expression from the cTnT promoter in the AAV6 and AAV9 groups was 578- and 441-fold higher, respectively, in the heart than in liver (Figure 1B). This comparison reveals that, although AAV9 is more efficient than AAV6 for cardiac gene delivery, the ratio of heart:liver gene expression from AAV6 is nevertheless higher than AAV9 when using a non–tissue-specific promoter.

Time Course and Magnitude of Cardiac-Selective Gene Expression From AAV Serotypes
To compare the kinetics of cardiac-selective gene expression after systemic administration, each of the available AAV serotypes harboring AcTnTLuc was administered to mice at 5 weeks of age (1x10¹² vg/mouse intravenously). Starting 3 days after vector injection, D-luciferin–dependent bioluminescence signals appeared and were confined to the left side of the thoracic cavity in all groups throughout the study period (Figure II in the online-only Data Supplement). Serotypes AAV1, 6, 8, and 9 showed robust gene expression within the first week that approached a steady-state plateau by 2 weeks after administration (Figure 2A). In the AAV2 group, light output increased slowly throughout the study but remained low compared with all other serotypes (Figure 2A). Light output was strongest in the AAV9 group, followed closely by AAV8. Light output was significantly lower in AAV1 and AAV6 groups as compared with AAV8 or 9. On day 42 postinjection, light output from AAV1 and
AAV6 was 7.4- and 5.3-fold lower, respectively, as compared with AAV9.

The foregoing results from noninvasive bioluminescence imaging were validated by performing luciferase activity assays on protein extracts from the various organs after the final imaging session conducted on day 42 after vector administration (Figure 2B). Again, luciferase activity was lowest in the AAV2 group. In comparison with conventional AAV2, cardiac luciferase expression was 50-fold higher with AAV1, 63-fold higher with AAV6, 186-fold higher with AAV8, and 491-fold higher with the AAV9 capsid.

**Distribution of AAV Gene Delivery in the Myocardium**

To determine the minimum dose required to transduce the vast majority of cardiomyocytes in the heart, AAV8 and AAV9 carrying AcTnTluc were administered intravenously at incremental doses to 5-week-old mice. Four weeks after vector injection, eGFP expression in mouse hearts was documented by fluorescence microscopy (Figure 3). The results show that AAV9 transduces cardiomyocytes more efficiently than AAV8 at all doses tested. A transduction rate of 84% was observed with AAV9 at a dose of 3.15×10¹¹ vg/mouse (Figure 3A and 3B). With AAV8, the transduction rate was 37% at that same dose. Thus the cardiomyocyte transduction rate for AAV9 is 2.25-fold higher than AAV8 after intravenous administration to 5-week-old mice at a dose of 3.15×10¹¹ vg/mouse.

Mean vector copy numbers per 1 µg genomic DNA increased in a dose-dependent fashion for both serotypes over doses ranging from 3.15×10⁸ to 1×10¹² vg/mouse (Figure 3C). At every dose tested, vector genome copy numbers were higher with AAV9 than with AAV8.

**EcsOD Expression From the cTnT Promoter**

Western blot analysis of mouse hearts harvested 4 weeks after intravenous injection revealed dose-dependent increases of EcsOD expression in mice injected with AcTnTlucEcsOD (Figure 4A). EcsOD levels were 16-fold higher in mice treated with AcTnTlucEcsOD at a dose of 3.15×10¹¹ as compared with baseline physiological expression in control mice treated with the same dose of AcTnTlucGFP (the minimum dose required to achieve >80% transduction of cardiomyocytes). Immunohistochemical analyses confirmed EcsOD expression in cardiomyocytes from mice injected with AcTnTlucEcsOD (Figure 4B and 4C). Further, gel zymography revealed dose-dependent increases in SOD activity (Figure 4D) that comigrated with EcsOD in parallel gels subject to Western blot analysis (Figure 4E). There was no difference in SOD activity attributable to MnSOD between control and AcTnTlucEcsOD-treated mice (Figure 4D).

Quantitative analysis of EcsOD activity performed after separation from the other 2 isoforms (MnSOD and CuZnSOD) using Con-A Sepharose revealed that EcsOD activity in hearts from mice treated with AcTnTlucEcsOD was 5.6-fold higher than in control mice treated with AcTnTlucGFP (Figure 4F; P=0.015).

**Cardiac-Specific Expression of EcsOD From cTnT Promoter Reduces Infarct Size**

Four weeks after vector administration (3.15×10¹¹ vg/mouse intravenously), MI was induced by 30 minutes of left anterior descending occlusion. At 2 hours after reperfusion, the area at risk and infarct area were determined by Phthalo blue and triphenyltetrazolium chloride staining, respectively (Figure 5A). The area at risk was similar in hearts from mice injected with either AcTnTlucEcsOD (control) or AcTnTlucEcsOD (EcsOD-treated) (Figure 5B). However, infarct size as percent area at risk was reduced by 40% in the EcsOD-treated group (33.9±6.3%, mean±SEM, n=4) compared with the control group (56.3±5.8%, n=5; P=0.035; Figure 5C). As a percentage of LV mass, infarct size was 14.3±3.2% in the EcsOD-treated group compared with 21.4±2.1% in the control group.

**EcsOD Increases Myocardial Capillary Fractional Area and Suppresses Neutrophil Infiltration**

To explore the possibility that EcsOD overexpression might increase vascular density, we sought to determine capillary density in mice treated with AcTnTlucEcsOD. Four weeks after
vector administration, heart sections were examined for capillary fractional area by histochemical staining using biotinylated GSL I–isolectin B4. Representative sections are shown in Figure 5D. Capillary fractional area was found to be 17.7% higher (Figure 5E) in AcTnTEcSOD-treated mice (9.98% of tissue area) compared with control mice (8.48%; \( P = 0.029 \)).

To assess whether the expression of EcSOD impacted neutrophil infiltration into the myocardium after MI, parallel groups of mice were subjected to 30 minutes of left anterior descending occlusion and 24 hours of reperfusion before euthanasia. Hearts were then explanted for immunohistochemistry using an antineutrophil antibody (Figure 5F). Morphometric analysis revealed that neutrophil infiltration in AcTnTeGFP-treated mice was decreased by 60% compared with AcTnTeGFP-treated controls (10 038 neutrophils/mm\(^2\) [control] versus 4049 neutrophils/mm\(^2\) [EcSOD]; Figure 5G).

Cardiac-Specific Expression of EcSOD From the cTnT Promoter Inhibits Post-MI LV Remodeling

In a separate study, we examined the long-term benefits of cTnT-driven EcSOD expression on LV remodeling after myocardial ischemia/reperfusion injury. Five-week-old mice were injected with AcTnTeCSD or AcTnTeGFP packed in AAV9 capsids. Four weeks after vector administration, MI was induced by occluding the left anterior descending artery for 60 minutes. In contrast to the 30-minute occlusion chosen for the study of acute MI, a 60-minute occlusion was selected here to maximize ischemic injury relative to reperfusion injury, thus overcoming cardioprotection by EcSOD and producing large infarcts that were uniform in size between groups. Untreated and AcTnTeGFP-treated mice served as controls. LVEDV, LVESV, and LVEF were measured by CMR on the day before (baseline) and on days 1, 7, and 28 post-MI (Figure 6A). As expected, the extended duration of coronary occlusion (60 minutes) sufficed to overcome the cardioprotective effects of EcSOD because late gadolinium-enhanced CMR performed 1 day post-MI confirmed no significant difference in acute infarct size as percent LV mass between groups (untreated=38.1±1.5%, GFP=39.0±3.0%, EcSOD=39.5±1.1%; \( P = 0.59 \) by Kruskal-Wallis). Volumetric analyses at baseline and day 1 after MI showed no significant differences in LVEDV or LVESV among any of the 3 groups on either day. Further, LV volumes were not significantly different between untreated and eGFP groups at any time point (Figure 6B and 6C). However, in the EcSOD group, LVEDV was significantly smaller compared with the untreated group by day 7 after MI. Mean LVEDV at this time was 48.2±4.0 μL for EcSOD versus 68.6±3.5 μL for untreated (\( P = 0.041 \)). At 28 days after MI, both LVEDV and LVESV were significantly smaller than in either the untreated or eGFP group (LVEDV: 50.3±4.0 μL for EcSOD versus 85.0±3.5 μL for untreated and 74.4±4.4 μL for eGFP, \( P = 0.004 \) and 0.017, respectively; LVESV: 32.7±3.8 μL for EcSOD versus 61.2±3.3 μL for untreated and 50.5±4.2 μL for eGFP, \( P = 0.006 \) and 0.010, respectively). In the untreated and eGFP-treated groups, LV volumes progressively increased throughout the remodeling period, whereas in the EcSOD-treated group, neither LVEDV nor LVESV changed significantly after day 1. LVEF in the EcSOD group was numerically higher than either control group at every time point examined after MI. For example, day 28 post-MI LVEF for the EcSOD group was 35.7±2.9% versus 29.2±2.5% for untreated and 32.2±3.2% for the eGFP group (\( P = 0.457 \) for EcSOD versus either the untreated or eGFP-treated groups). Although this persistent trend toward improved EF did not reach statistical significance, EcSOD treatment clearly ameliorated the increases in both LVEDV and LVESV typically seen in control groups.

Discussion

Gene therapy strategies often seek to restrict the expression of therapeutic genes to the organ or cell type of interest because
that AAV9 in combination with the cTnT promoter provides the highest level of cardiac-selective gene expression among the serotypes tested. Using this potent combination, we then demonstrated that a single intravenous injection of an AAV9 vector directing the expression of EcSOD from the cTnT promoter protects the mouse heart against both MI and subsequent LV remodeling.

The minimal cTnT promoter used here is 411 bp in length and confers a high degree of cardiac selectivity, with 40% of the strength available from the ubiquitously active and strong CMV promoter (Figure 1). As shown in Figure 1B, the heart: liver ratio of luciferase activity from the CMV promoter was 7.3 for AAV6 and 1.4 for AAV9, suggesting that the AAV capsid is more selective for cardiomyocytes than AAV9. This notwithstanding, the transduction efficiency of AAV9 after systemic injection is ≈8-fold higher than that of AAV6 (Figure 2B). By combining the cTnT promoter with the AAV9 capsid, we were able to harness the superior transduction efficiency of the AAV9 capsid while retaining a >400-fold specificity for the heart over liver, as measured here using an in vitro luciferase assay performed at 42 days after vector administration.

The prolonged lag phase and low level of gene expression provided by AAV2 in the present study (Figure 2 and Figure II in the online-only Data Supplement) are consistent with previous reports.5,10 In contrast, AAV9 (closely followed by AAV8) provides robust and early-onset gene expression in the heart as compared with the other serotypes tested (Figure 2). The early onset of gene expression provided by the newer AAV serotypes has been attributed to a more efficient uncoating of viral DNA that facilitates the rapid annealing of single-stranded AAV genomes.14

The transduction efficiency with AAV9 was 84% at a dose of only 3.15x10¹¹ vg/mouse. At the same dose, the ratio of vector genomes: host genomic DNA in the heart was 2.3x10⁹ copies per 1 µg of genomic DNA. AAV9 provided a uniform distribution of eGFP gene expression throughout the myocardium. These results, obtained after the injection of 5-week-old mice, are in close agreement with previous results obtained after the injection of neonatal mice.4 Furthermore, the intensity of eGFP fluorescence on a cardiomyocyte-by-cardiomyocyte basis reflects a gene-dose effect and can be modeled as a Poisson distribution, as shown previously.4 Collectively, these results show that among the serotypes tested, AAV9 is the capsid of choice for gene delivery to the heart, particularly when deployed in combination with a cardiac-specific promoter (eg, cTnT).

Elevated cardiac EcSOD levels have previously been shown to reduce infarct size in animal models of IR injury.15,16 However, no gene delivery method has previously been shown to program therapeutic levels of gene expression in the heart after systemic administration. Furthermore, no previous study has demonstrated that cardiac overexpression of EcSOD can inhibit post-MI LV remodeling independently of its pronounced effect on infarct size. Finally, this is the first study in which CMR has been used to quantitatively assess LV remodeling after the systemic injection of AAV.

The use of late gadolinium-enhanced CMR was critical in proving that 60 minutes of coronary occlusion was sufficient
to equalize infarct size between groups, particularly considering the ability of EcSOD to reduce the size of infarcts resulting from shorter occlusion times. Infarct size after a 30-minute occlusion in the acute study was \( \approx 13\% \) of LV mass in the EcSOD-treated group and \( \approx 24\% \) in the control group by triphenyltetrazolium chloride staining compared with \( \approx 39\% \) across all groups after a 60-minute occlusion in the LV remodeling study by late gadolinium-enhanced MRI. Thus we used AA V9 and the cTnT promoter to program cardiac-selective overexpression of EcSOD and then evaluated therapeutic efficacy in separate models of acute MI and post-MI LV remodeling to clarify the role of EcSOD in ischemia/reperfusion injury. Systemic administration of the AAV9 vector carrying AcTnTEcSOD provided sustained elevations of EcSOD activity in the mouse heart (Figure 4). Further, the level of EcSOD expression from the cTnT promoter was sufficient to decrease infarct size as percentage area at risk by 40\% compared with control (Figure 5) and to significantly reduce LV remodeling as compared with control groups (Figure 6). Nevertheless, the results should be appreciated in the context of lack of significant improvement in LVEF, absence of 4-week infarct size data, and the fact that most histological studies were performed only at early time points postinjury.

Many of the beneficial effects of nitric oxide are abrogated by its diffusion-limited reaction with superoxide, and the product of this reaction (peroxynitrite) can inactivate or destroy many essential molecules within a cell.\(^\text{17}\) Nitric oxide is also known to mediate angiogenesis in vivo, as shown by Ziche et al.\(^\text{18}\) Indeed, the release of nitric oxide by growth factors, such as vascular endothelial growth factor, basic fibroblast growth factor, and transforming growth factor-\(\beta\), plays a critical role in their angiogenic actions,\(^\text{19}\) actions that are abrogated in the presence of superoxide.

It is the fundamental importance of the balance between nitric oxide and superoxide that motivated the present study of EcSOD in mouse models of MI and LV remodeling. In

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**Figure 5.** Extracellular superoxide dismutase (EcSOD) expression from the cardiac troponin-T (cTnT) promoter protects the heart against myocardial infarction (MI). Mice were injected intravenously with AcTnTeGFP (Crt) or AcTnT EcSOD (EcSOD-treated) packaged in AAV9 (3.15×10\(^{11}\) vg/mouse, n=4–5 per group). A, Four weeks after vector administration, MI was induced by a 30-minute left anterior descending occlusion, and infarct size was determined. Shown are representative midventricular sections of triphenyltetrazolium chloride– and Phthalo blue–stained hearts from control or EcSOD-treated mice. Arrows delineate the infarct borders. B, Bar graph showing area at risk as percentage of LV mass in control and EcSOD-treated mice. C, Bar graph showing infarct size as percent of area at risk in hearts from control and EcSOD-treated mice, \( P=0.035 \) vs control. D, Four weeks after vector administration, fixed-frozen sections from noninfarcted hearts were stained for capillaries using biotinylated GSL I–Iso lectin B4. Representative images from control and EcSOD-treated mice are shown at high-power magnification. Scale bars=10 \( \mu \)m. E, Graph showing quantification of capillaries in hearts from control (n=4) and EcSOD-treated (n=3) groups. F, Low-power magnifications of short-axis cardiac cross-sections from control and EcSOD-treated mice immunostained for neutrophils on day 1 after MI. Scale bars=200 \( \mu \)m. G, Graph showing quantification of neutrophils in infarcted hearts from control (n=2) and EcSOD-treated (n=4) groups harvested 24 hours after MI. Results are expressed as mean±SEM, \( P=0.029 \) vs Ctrl.
Extracellular superoxide dismutase (EcSOD) expression inhibits left ventricular (LV) remodeling after myocardial infarction (MI). AAV9 vectors carrying ActnTeGFP or ActnTeCSCD were administrated intravenously to 5-week-old mice. Four weeks after vector injection, MI was induced by a 60-minute left anterior descending occlusion. Cardiac structure and function were serially assessed by cardiac magnetic resonance (CMR).

Figure 6. Extracellular superoxide dismutase (EcSOD) expression inhibits left ventricular (LV) remodeling after myocardial infarction (MI). AAV9 vectors carrying ActnTeGFP or ActnTeCSCD were administrated intravenously to 5-week-old mice. Four weeks after vector injection, MI was induced by a 60-minute left anterior descending occlusion. Cardiac structure and function were serially assessed by cardiac magnetic resonance (CMR).

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Disclosures
None.

References

**CLINICAL PERSPECTIVE**

In clinical gene therapy applications, it is critical for therapeutic gene expression to be confined to the organ or cell type of interest to prevent any possible deleterious side effects attributable to off-target gene expression. Furthermore, an ideal therapy for ischemic heart disease would provide cardiac-selective gene expression without the complex surgical procedures now required to improve gene delivery to cardiomyocytes using conventional adeno-associated virus (AAV) 2 serotypes. In this study, we show that the AAV9 capsid provides for robust cardiac-restricted gene expression in mouse hearts after systemic administration. Furthermore, although gene therapy with extracellular superoxide dismutase has previously been shown to reduce infarct size in animal models, the combination of the cardiac troponin-T promoter and the AAV9 serotype provided therapeutic levels of extracellular superoxide dismutase gene expression in the heart after systemic administration. Intravenous injection of an AAV9 vector directing the expression of extracellular superoxide dismutase from the cardiac troponin-T promoter protected the mouse heart against both myocardial infarction and subsequent left ventricular remodeling. These studies should expedite future animal experiments aimed at elucidating the function of cardioprotective genes in the mammalian heart, and the beneficial effects of extracellular superoxide dismutase overexpression may suggest potential clinical applications in the setting of ischemic heart disease.
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SUPPLEMENTAL MATERIAL

“AAV9-mediated EcSOD gene delivery in LV remodeling” by Konkalmatt et al.

Supplemental Methods

Determination of AAV vector genome copy number. Genomic copy numbers were determined in mice previously injected with the AcTnTeGFP vector. Total DNA (vector + genomic) was extracted from control and vector-treated hearts and 100 ng was used in real-time PCR reactions using the following primers for amplifying eGFP: 5′-ACATGAAGCAGCACGACTT-3′ (forward) and 5′-GAAGTTCACCTTGATGCCGT-3′ (reverse). Known copy numbers (10^5–10^8) of the plasmid pAcTnTeGFP were used to construct the standard curve for determining vector genome content. The host genome content was based on the 100ng of input DNA.

Mouse model of acute myocardial infarction and histological determination of infarct size. A standard myocardial ischemia-reperfusion protocol was employed. Mice were anesthetized with pentobarbital sodium (100 mg/kg, ip) prior to intubation and artificial respiration was maintained with a rodent ventilator. A thoracotomy was made by cutting the left third and fourth ribs with a cautery pen. A transient occlusion of the left anterior descending coronary artery (LAD) was accomplished by passing a 7-0 silk suture beneath the LAD at the level of the left auricle. The suture was then tied over a length of polyethylene-20 tubing. Reperfusion was achieved by removing the tubing 30 min later. Upon reperfusion, the chest was closed in layers, the endotracheal tube was removed, and the mouse was recovered for a period of 2 h prior to euthanasia by anesthetic overdose. For the analysis of infarct size, 2,3,5-triphenyltetrazolium chloride (TTC) and Phthalo blue staining were used to delineate the infarct region and the area-at-risk, respectively. In brief, the explanted hearts were cannulated via the ascending aorta for perfusion with 2 to 3 ml of 0.9% sodium chloride at 37°C followed by 3 to 4
ml of 1.0\% TTC at 37°C in phosphate buffer (pH 7.4). The LAD was then reoccluded by retying the suture left in the myocardium after reperfusion so that the heart could be perfused with 2 to 3 ml of 10\% Phthalo blue dye (Heubach) to stain the fully perfused regions dark blue. The atria and right ventricle were then trimmed away from the LV, which was frozen and sectioned into 1mm-thick, short-axis slices prior to weighing and digital photography. The risk region, infarct zone, and LV areas were then determined by computer-assisted planimetry and converted to masses according to the weight of each tissue slice. The values for the individual slices were then summed to calculate the infarct size as a percentage of either risk region or LV mass.

**Morphometric analysis of neutrophils and capillaries.** Neutrophil counts were performed using n=2 GFP-treated animals and n=4 animals treated with EcSOD. For each heart, a single mid-ventricular section was used for the counts. Care was taken to select consistent mid-ventricular sections across all animals based on morphological landmarks. Three 10x fields of view encompassing infarct areas in the anterior, lateral and inferior regions of the heart, including the full-thickness of the LV wall from endocardium to epicardium, were imaged in order to sample all areas of tissue injury. Counts were performed with an automated cell counting protocol using the Color Deconvolution and ITCN plug-ins for ImageJ (National Institutes of Health; Bethesda, MD). Because counts were automated, it was not possible to measure interobserver error. However, to determine the effect of the chosen fields of view on counting results, all fields of view were split into two groups containing three fields of view from each section. Counts from the two groups were found to agree strongly (R = 0.92, p<0.01).

Capillary fractional area was performed using n=4 GFP-treated animals and n=3 animals treated with EcSOD. For each heart, a single mid-ventricular section was used for the counts. The short-axis plane was used for all sections as is standard for this metric. Two to three 40x fields of view from the septal region were imaged. Percent fractional area was determined using the threshold tool in ImageJ.
Fig. S1. Cardiac-specific gene expression from AAV serotypes harboring cTnT promoter:

An initial comparison of the AAV vectors harboring CMV and cTnT promoters was made using AAV6 and AAV9 serotypes. Luciferase expression cassettes ACMVLuc or AcTnTLuc were cross-packaged into AAV6 or 9 capsids for administration into 5 week-old mice by iv injection. Luciferase expression was assessed by non-invasive in vivo bioluminescence imaging for 6 weeks, followed by ex vivo bioluminescence imaging of the explanted tissues and quantitative luciferase activity assay. (A) Luciferase expression from the CMV promoter (CMV) was observed throughout the body in both AAV6 and AAV9 injected mice. In contrast, the cTnT promoter (cTnT) restricted luciferase expression to only the left side of the thoracic cavity in both AAV6 and AAV9 vector injected mice. Bioluminescence signals were stronger in AAV9 as compared to AAV6 injected mice, regardless of the promoter used (CMV or cTnT). (B) Ex vivo bioluminescence images of explanted tissues (left to right, top to bottom: heart, liver, thymus, kidney, spleen, intercostal muscle, brain, gastrocnemius muscle) from mice that received ACMVLuc (CMV) or AcTnTLuc (cTnT). Organs were immersed in D-luciferin solution for one minute and bioluminescence was imaged immediately thereafter using the IVIS 100 system. In mice injected with AAV6/CMVLuc, luciferase expression was abundant mainly in heart, skeletal muscles (intercostals and gastrocnemius muscles) and liver. In mice injected with
AAV9/CMVLuc, bioluminescence signals were observed in all the tissues tested, with particularly high levels in heart, skeletal muscle and liver. Much weaker signals were detected in kidney, thymus and brain. In contrast to luciferase expression driven by the CMV promoter, the cTnT promoter expressed luciferase predominantly in the heart, both when delivered by the AAV6 and AAV9 capsids.

Fig. S2. Time course and tissue distribution of cTnT-mediated luciferase expression from five AAV serotypes following IV injection: The AAV reporter vector AcTnTLuc was packaged into the indicated AAV serotype capsids (AAV2, AAV1, AAV6, AAV8 and AAV9). Five-week old mice (n=4 per group) were injected with 1x10^{12} viral genomes by systemic administration. In vivo bioluminescence images of mice were obtained at days 4, 7, 14, 28 and 42 after vector administration.