

Gene Therapy With Extracellular Superoxide Dismutase Attenuates Myocardial Stunning in Conscious Rabbits

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Background—Administration of Cu/Zn superoxide dismutase (SOD) without catalase fails to alleviate myocardial stunning, but extracellular SOD (Ec-SOD) may be more effective because it binds to heparan sulfate proteoglycans on the cellular glycocalyx. We therefore used in vivo gene transfer to increase systemic levels of Ec-SOD and determined whether this gene therapy protects against myocardial stunning.

Methods and Results—The cDNA for human Ec-SOD was cloned behind the cytomegalovirus (CMV) promoter and incorporated into a replication-deficient adenovirus (Ad5/CMV/Ec-SOD). Injection of this virus (2×10^8 pfu/kg IV) produced high levels of Ec-SOD in the liver, which could be redistributed to the heart and other organs by injection of heparin. Conscious rabbits underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles for 3 consecutive days starting 3 days after intravenous injection of Ad5/CMV/Ec-SOD or Ad5/CMV/nls/LacZ (negative control). Both groups were given heparin (2000 U/kg IV) 2 hours before the first sequence of occlusions. The severity of myocardial stunning was measured as the total deficit of LV wall thickening after the last reperfusion. On day 1, the total deficit of wall thickening was markedly decreased in Ad5/CMV/Ec-SOD rabbits versus controls and similar to that seen on days 2 and 3 in controls.

Conclusions—The results demonstrate that in vivo gene transfer of the cDNA encoding Ec-SOD provides the heart with substantial protection against myocardial stunning without the need for concomitant administration of catalase. The present observations provide the basis for controlling gene therapy at the posttranslational level and for simultaneously protecting multiple organs from oxidant stress. (*Circulation*. 1998;98:1438-1448.)

Key Words: genes ■ superoxide dismutase ■ free radicals ■ stunning, myocardial ■ ischemia ■ reperfusion

Considerable evidence indicates that reactive oxygen species, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), contribute importantly to myocardial ischemia/reperfusion injury (reviewed in Reference 1). Numerous animal studies have shown that intravenous (IV) administration of antioxidant enzymes (superoxide dismutase [SOD] and catalase) can protect the heart against reversible postischemic dysfunction (myocardial stunning).¹ The combination of these enzymes consistently alleviates myocardial stunning; however, the administration of the cytosolic form of SOD alone has been found to be ineffective.^{1,2} We hypothesized that a potential reason for the lack of efficacy of SOD alone is its inability to detoxify intracellular $\cdot O_2^-$, because this enzyme is largely restricted to the extracellular space after IV administration. We postulated that the extracellular isoform of SOD (Ec-SOD) that is anchored to the glycocalyx of cardiomyocytes might provide better cardioprotection than the freely soluble enzyme.

In addition to the need for combined administration of catalase with SOD, another limitation in using antioxidant enzymes against myocardial ischemia/reperfusion injury is

that they need to be given parenterally and have short plasma half-lives. These limitations could potentially be overcome by use of gene therapy to create an endogenous source of enzyme to provide sustained antioxidant protection. Although numerous studies have used antioxidant enzymes prepared by recombinant techniques,²⁻⁷ none of them have used in vivo gene transfer to protect intact animals from ischemia/reperfusion injury.

The goal of this study was to construct a recombinant adenovirus (Ad5) that overexpresses Ec-SOD and to compare the protection against myocardial stunning afforded by this vector with that afforded by the late phase of ischemic preconditioning. Particular care was taken to select a dose of vector that would increase Ec-SOD levels while avoiding an inflammatory response⁸ that might confound the results. The liver was targeted for gene transfer to preclude any possibility of such adverse effects in the heart and to exploit the efficiency with which Ad5 transfects hepatocytes after simple IV injection. A conscious rabbit model of myocardial stunning⁹⁻¹¹ was used to obviate the confounding effects of factors associated with

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open-chest preparations, which could interfere with myocardial stunning^{12,13} or ischemic preconditioning.^{14,15}

Methods

Adenoviral Vectors

The nuclear-localized LacZ reporter virus Ad5/CMV/nls-LacZ has previously been reported.¹⁶ The plasmid shuttle vector used to generate Ad5/CMV/Ec-SOD was constructed by subcloning the 1396-bp *EcoRI* fragment bearing the cDNA for human Ec-SOD from plasmid pPS3¹⁷ into the corresponding site of an expression vector (p Δ E1sp1B/CMV/BGH) previously constructed by inserting the 1276-bp *BgIII-PvuII* fragment (containing the cytomegalovirus [CMV] immediate early [IE] promoter, polylinker, and bovine growth hormone polyadenylation signal) from pcDNA3 (Invitrogen Corp) into the polylinker of an Ad5 shuttle vector (p Δ E1sp1B) kindly provided by Bett et al.¹⁸ The resulting plasmid shuttle/expression vector (p Δ E1sp1B/CMV/Ec-SOD) was cotransfected with pJM17 into the permissive 293 host cell line¹⁹ to generate Ad5/CMV/Ec-SOD. Viral isolates were collected from 293 monolayers showing evidence of cytopathic effect. These isolates were plaque-purified, verified by restriction analysis, and evaluated for their potential to overexpress SOD activity in 293 cells. Purified viral clones were propagated in 293 cells, isolated, concentrated, and titered by plaque assay according to Graham and Prevec.¹⁹

Pilot Studies

Pilot studies were conducted to assess the pharmacokinetics of liver-directed gene therapy with Ad5/CMV/Ec-SOD. Plasma samples were collected from the ear artery of 5 rabbits on day 0 before administration of increasing doses of Ad5/CMV/Ec-SOD (8.5×10^7 , 8.5×10^8 , and 4.3×10^9 pfu/kg IV). At days 1, 3, 5, and 7 after gene transfer, plasma samples were again collected before and 10 minutes after the IV injection of 0.8 mg/kg dextran sulfate (5000 MW, Sigma Chemical Co) to displace recombinant Ec-SOD from its extracellular binding sites in the liver.

Enzymatic Assay for SOD Activity

An enzymatic assay for SOD activity (Calbiochem International) was used to determine the amount of Ec-SOD activity produced by gene therapy relative to ambient levels of Cu/Zn-SOD and Ec-SOD activity.²⁰ All samples for SOD analysis were extracted with ice-cold $\text{CHCl}_3/\text{EtOH}$ (1:2) to remove hemoglobin and inactivate Mn-SOD.²⁰ Thus, the assay measured total non-Mn-SOD activity and did not distinguish between recombinant and endogenous SOD activity or between the cytoplasmic and extracellular forms of SOD (Cu/Zn-SOD and Ec-SOD, respectively). Protein content was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories), and each SOD assay was run in duplicate. The SOD assay was conducted at 37°C with an Ultra-Spec 3000 spectrophotometer (Pharmacia Biotech), and kinetic analysis was performed with the software package supplied with the instrument. Samples that exceeded linear range were diluted appropriately and assayed again. SOD activity was normalized to cytochrome *c* units by use of a calibration curve generated from a Cu/Zn-SOD standard (Sigma) with a specific activity of 3500 U/mg as determined by the method of McCord and Fridovich.²¹

Surgical Preparation

The conscious rabbit model of myocardial stunning has been described previously.⁹⁻¹¹ Briefly, New Zealand White male rabbits (weight, 2.1 ± 0.2 kg; age, 3 to 4 months) were instrumented under sterile conditions with a balloon occluder¹⁴ around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal²² in the center of the region to be rendered ischemic, and bipolar ECG leads on the chest wall. Gentamicin was administered before surgery and on days 1 and 2 after surgery ($0.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ IM). Rabbits were allowed to recover for a minimum of 14 days after surgery.

Occlusion/Reperfusion Protocol

Throughout the protocol, rabbits were kept in a quiet, dimly lit room. Left ventricular (LV) systolic wall thickness (WTh), range gate depth, and the ECG were continuously recorded on a thermal array chart recorder (Gould TA6000). No sedatives or antiarrhythmic agents were given at any time. The experimental protocol consisted of 3 consecutive days of coronary artery occlusions (days 1, 2, and 3). On each day, the rabbits underwent a sequence of six 4-minute coronary occlusions interspersed with 4 minutes of reperfusion. Successful coronary occlusions were verified by ST-segment elevation and changes in the QRS complex on the ECG and by the appearance of paradoxical systolic wall thinning on the ultrasonic crystal recordings.

Measurement of Regional Myocardial Function

Regional myocardial function was assessed as systolic thickening fraction by the pulsed Doppler probe, as previously described.²² Percent systolic thickening fraction was calculated as the ratio of net systolic thickening to end-diastolic WTh multiplied by 100.²² The total deficit of systolic WTh after reperfusion (an integrative assessment of the overall severity of myocardial stunning after the sixth reperfusion) was calculated by measuring the area between the systolic WTh-versus-time line and the baseline (100% line) during the 5-hour recovery phase after the sixth reperfusion.^{9-12,15,23,24} In all animals, measurements from ≥ 10 beats were averaged at baseline and from ≥ 5 beats at subsequent time points.

Dose-Ranging Studies

Two empirical dose-ranging studies were undertaken to develop a gene therapy protocol that was effective against myocardial stunning. In the first, 2 instrumented rabbits were injected IV with Ad5/CMV/Ec-SOD at 2 different doses (2.0×10^8 and 4.3×10^9 pfu/kg) and were subjected 3 days later to the 3-day occlusion/reperfusion (O/R) protocol described above. These 2 doses of Ad5/CMV/Ec-SOD had no effect on myocardial stunning, suggesting that cardioprotective levels of Ec-SOD had not been achieved.

A second dose-ranging study was undertaken to determine whether cardioprotective levels of Ec-SOD could be obtained by displacing recombinant Ec-SOD from the liver into the bloodstream for systemic distribution. Although dextran sulfate had been used in pilot studies, heparin gives comparable results²⁵ and was chosen for these experiments because the effects of IV heparin on the tissue distribution of Ec-SOD are better characterized.²⁶ The first rabbit in this series was injected IV with 2.0×10^8 pfu/kg of Ad5/CMV/Ec-SOD 3 days before the first day of the 3-day O/R protocol. The rabbit was treated with heparin (2000 U/kg IV) 2 hours before the first occlusion, and protamine (10 mg/kg IV) was injected before the first occlusion to reverse the effects of heparin. The severity of myocardial stunning (total deficit of WTh) on day 1 in this rabbit was 45% lower than anticipated, indicating a marked cardioprotective effect. A second rabbit was injected with a higher dose of Ad5/CMV/Ec-SOD (8.5×10^8 pfu/kg). Three days after Ad5 injection, the rabbit was similarly treated with heparin and protamine on day 1 just before the O/R protocol. In contrast to the previous rabbit, the total deficit of WTh on day 1 in this rabbit was similar to that of controls. The results of the limited dose-ranging studies thus indicated that a gene therapy protocol including the injection of heparin and protamine on day 1 might be effective at a dose of 2.0×10^8 pfu/kg, whereas a 4-fold higher dose of Ad5 might exceed the therapeutic window for cardioprotection by SOD.⁵

Histology and X-gal Histochemistry

Livers from 8 rabbits were examined 3 days after IV injection with 2×10^8 pfu/kg of recombinant Ad5 to assess histopathology and the extent of gene transfer to the liver. The liver samples were frozen at -28°C in a cryostat and embedded in tissue freezing medium (Triangle Biomedical Sciences). Frozen sections ($5 \mu\text{m}$ thick) were collected on glass slides, fixed for 5 minutes at 4°C in 1.25% glutaraldehyde/PBS, and stained for 16 hours in X-gal chromagen.¹⁶ Sections were then rinsed in PBS and lightly counterstained with

hematoxylin and eosin. The percentage of X-gal-stained hepatocyte nuclei was determined by counting stained and unstained nuclei at high magnification ($\times 40$) in random fields selected in the portal and central zones of the hepatic lobule. Nuclear staining was evaluated in 50 to 100 high power fields in 2 X-gal-stained sections from each animal. Each $\times 40$ field contained 200 to 300 hepatocytes. Adjacent sections were directly stained with hematoxylin and eosin for evaluation of inflammatory changes or other degenerative changes.

Experimental Protocol

A protocol for evaluating the cardioprotective effects of Ec-SOD was developed on the basis of the dose-ranging studies reported above. Conscious rabbits were assigned to 2 groups: group 1 (Ad5/CMV/nls-LacZ-treated) and group 2 (Ad5/CMV/Ec-SOD-treated). In stage 1, both groups were subjected to 3 consecutive days of O/R to establish a baseline for comparison. After the stage 1 analysis, rabbits were allowed to recover for at least 14 days before the next occlusion to preclude any effect of late preconditioning on stage 2.²³ Three days before the stage 2 O/R protocol, rabbits were randomly assigned to group 1 or group 2 and then treated with 2×10^8 pfu/kg of recombinant adenovirus by ear vein injection. The third day after gene transfer was selected as day 1 of the 3-day stage 2 O/R protocol because pilot studies had indicated that maximal levels of Ec-SOD would be available 3 days after gene transfer. On day 1 of the stage 2 O/R protocol, rabbits in both groups received an IV injection of heparin (2000 U/kg) 2 hours before the first sequence of occlusions. Protamine (10 mg/kg) was administered over the 8 minutes preceding the first occlusion on day 1 to reverse the effects of heparin and promote interstitial binding of Ec-SOD. On days 2 and 3, these rabbits underwent the same coronary O/R sequence without the prior administration of heparin or protamine.

Statistical Analysis

Data are reported as mean \pm SEM. For intragroup comparisons, hemodynamic variables and WTh were analyzed by a 2-way repeated-measures ANOVA (time and day) to determine whether there was a main effect of time, a main effect of day, or a day-by-time interaction. If the global tests showed a significant main effect or interaction, post hoc contrasts between different time points on the same day or between different days at the same time point were performed with Student's *t* tests for paired data, and the resulting *P* values were adjusted according to the Bonferroni correction. For intergroup comparisons, continuous variables were analyzed by either a 1-way or a 2-way repeated-measures (time and group) ANOVA, as appropriate, followed by unpaired Student's *t* tests with the Bonferroni correction. All statistical analyses were performed with the SAS software system.²⁷ Two-way ANOVA was performed with the GLM (General Linear Models) procedure.²⁷

Results

Pilot Studies With Ad5/CMV/Ec-SOD

Pilot studies were undertaken to assess the time course of Ec-SOD expression after IV injection of Ad5/CMV/Ec-SOD and to determine whether supraphysiological levels of SOD activity could be achieved in the plasma of intact rabbits. Increasing doses of Ad5/CMV/Ec-SOD were administered IV to rabbits, and plasma samples were drawn at regular intervals before and after dextran sulfate was injected to displace Ec-SOD from the liver. Plasma SOD activity reached half-maximal levels 1 day after gene transfer, maximal levels 3 days after gene transfer, and steadily declined to lower levels at later time points. Dose-dependent increases in plasma SOD activity were observed, but the highest dose (4.3×10^9 pfu/kg) raised plasma SOD levels by $<60\%$ (Figure 1A).

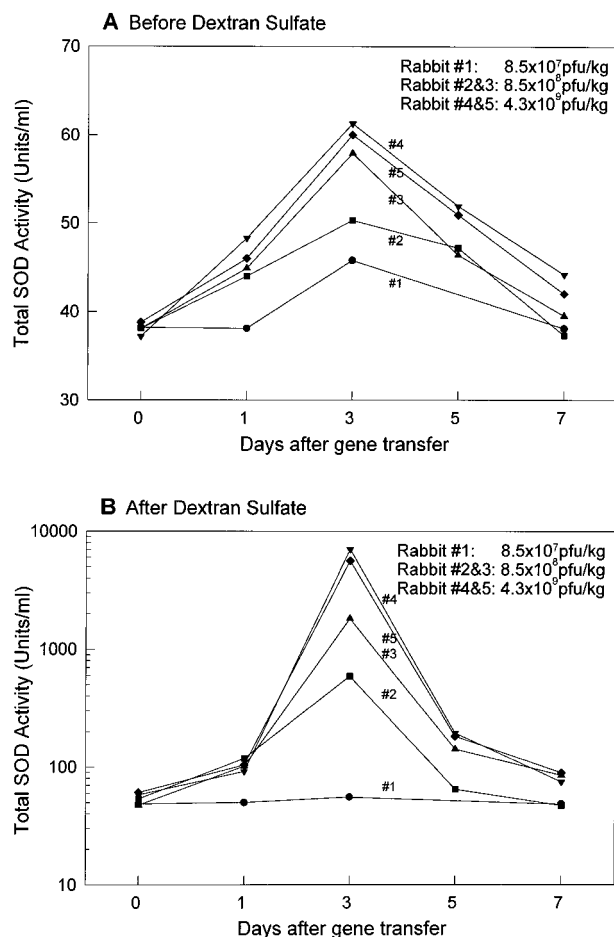


Figure 1. Time course of recombinant Ec-SOD expression in vivo. Three doses of Ad5/CMV/Ec-SOD were studied in 5 rabbits (1 through 5), as indicated in figure. Two plasma samples were drawn from each rabbit on each day, 1 before and 1 after injection of dextran sulfate. Plasma samples were assayed for total Cu/Zn-SOD activity as described in Methods, and results were expressed as total U/mL plasma. A, Levels of SOD activity in plasma samples taken before injection of dextran sulfate on days 0, 1, 3, 5, and 7. B, Levels of SOD activity in samples drawn 10 minutes after injection of dextran sulfate (0.8 mg/kg IV). Note that y axis of A is plotted on a linear scale but high levels of SOD activity necessitated a log scale in B.

In contrast, much higher elevations in SOD activity were measured in plasma samples drawn 10 minutes after the injection of dextran sulfate (Figure 1B). Ec-SOD is a secreted, extracellular enzyme with high affinity for the liver,²⁶ but it can be released into the circulation if it is displaced from its extracellular binding sites with an IV injection of heparin or dextran sulfate.²⁵ The dramatic increase in plasma SOD after the injection of dextran sulfate can be attributed both to the hepatic production of recombinant protein resulting from in vivo gene transfer²⁸ and to the natural affinity of Ec-SOD for the liver.²⁶

Extent of Hepatic Gene Transfer

A recombinant Ad5 vector expressing a nuclear-localized LacZ reporter gene was used to assess the extent of hepatic gene transfer mediated by the 2.0×10^8 pfu/kg dose of recombinant Ad5. Four rabbits were injected IV with 2×10^8

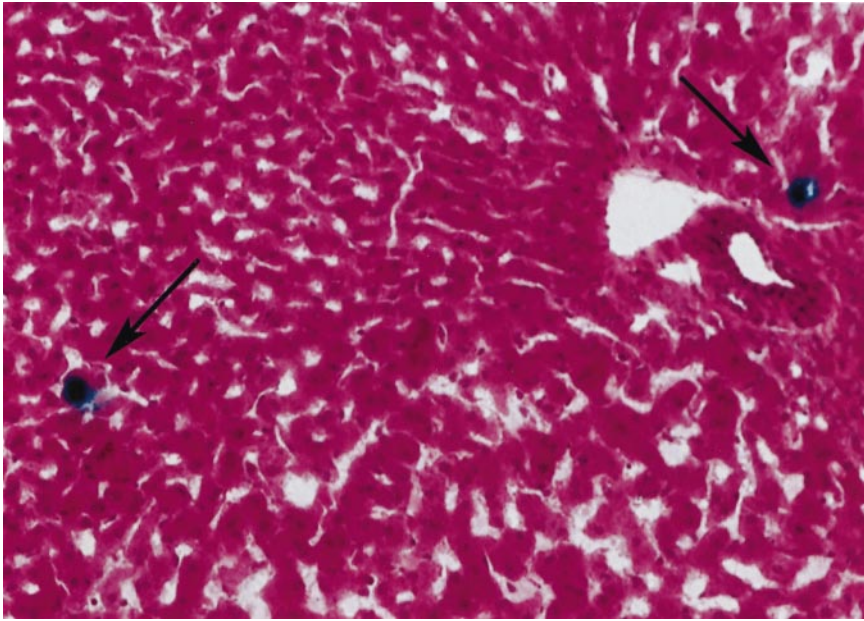


Figure 2. Histochemical assessment of Ad5-mediated *in vivo* gene transfer to liver. Frequency and distribution of gene expression in rabbit liver 3 days after IV injection of 2×10^8 pfu/kg of Ad5/CMV/nls-LacZ. Arrows indicate hepatocytes expressing nuclear-localized LacZ reporter gene. Quantitative image analysis of 8 hepatic cross sections obtained from 4 different rabbits indicated that $0.09 \pm 0.02\%$ (mean \pm SEM) of hepatocytes were transfected under these conditions.

pfu/kg of Ad5/CMV/nls-LacZ, and liver samples were removed 3 days later for X-gal histochemistry and histopathological examination. Another 4 rabbits were injected with 2×10^8 pfu/kg of Ad5/CMV/Ec-SOD for histopathological examination and to serve as negative controls for the X-gal histochemistry. Figure 2 is a representative histological section from the liver of a rabbit injected with the nls-LacZ reporter virus, which was first stained with X-gal to identify hepatocytes expressing the reporter gene, then lightly counterstained with hematoxylin and eosin. The frequency of gene transfer at this dose was low, but the distribution of X-gal-stained nuclei was fairly uniform throughout the liver. The fraction of hepatocytes expressing the nls-LacZ gene averaged $0.09 \pm 0.02\%$, indicating that this dose of Ad5/CMV/nls-LacZ was effective in transfecting a small but reproducible percentage of hepatocytes. Liver sections from rabbits injected with Ad5/CMV/Ec-SOD showed no evidence of X-gal staining (data not shown). Additional sections from each of the 8 rabbits were fully stained with hematoxylin and eosin for examination under light microscopy. Focal, mild, periportal lymphocytic infiltrates were noted in 1 of the 8 rabbits, but there was no evidence of active hepatitis or ongoing hepatocyte necrosis in any of the specimens examined.

Regional Myocardial Function

The preliminary dose-ranging studies indicated that injecting Ad5/CMV/Ec-SOD at a dose of 2×10^8 pfu/kg IV followed 3 days later by heparin and protamine injections before the first coronary occlusion might be effective in attenuating myocardial stunning. Furthermore, this dose of Ad5 transfected a relatively small fraction of hepatocytes without any histological evidence of liver damage (Figure 2). On the basis of these results, an experimental protocol (summarized in Figure 3A) was devised to evaluate the cardioprotective effects of Ec-SOD gene therapy on myocardial stunning. Of the 18 rabbits instrumented for this study, 9 were assigned to the control group (group 1) and 9 to the Ec-SOD group (group 2). Of the

9 rabbits assigned to group 1, 4 were excluded because of malfunction of the WTh probe after stage 1. Of the 9 rabbits assigned to group 2, 1 failed to complete stage 2 because of balloon occluder failure, and 2 were excluded because of malfunction of the WTh probe after stage 1.

Baseline systolic thickening fraction in group 2 (gene therapy) was $37 \pm 2\%$, $38 \pm 2\%$, and $38 \pm 1\%$ on days 1, 2, and 3, respectively, in stage 1 and $40 \pm 3\%$, $38 \pm 5\%$, and $40 \pm 5\%$, respectively, in stage 2. There were no significant differences in the baseline systolic thickening fraction between the 2 stages on the same day or among different days within the same stage. The fact that thickening fraction on day 1 was no different at baseline during stages 1 and 2 indicates that the injection of Ad5/CMV/Ec-SOD, heparin, and protamine had no significant effect on regional myocardial function in the nonischemic heart.

Figure 4A reports the serial measurements of thickening fraction during the 6 O/R cycles and during the 5-hour recovery phase, expressed as a percentage of preocclusion measurements, in group 1 during stages 1 and 2. Similarly, Figure 4B reports the serial measurements of thickening fraction during the 6 O/R cycles and during the 5-hour recovery phase in group 2 during stages 1 and 2. On day 1 of stages 1 and 2 for both groups 1 and 2, the extent of paradoxical systolic thinning during ischemia did not change significantly with subsequent occlusions, so that during the sixth occlusion it was similar to that measured during the first occlusion (Figure 4A). There were no significant differences between the 2 stages in the extent of systolic thinning during the 6 occlusions in either group.

Group 1 (Control Group)

On day 1 of both stages 1 and 2, contractile function remained significantly depressed for 4 hours after the sixth reperfusion (Figure 4A), with the total deficit of WTh being similar between the 2 stages (Figure 5A). Thus, both on day 1 of stage 1 and on day 1 of stage 2, the sequence of six 4-minute

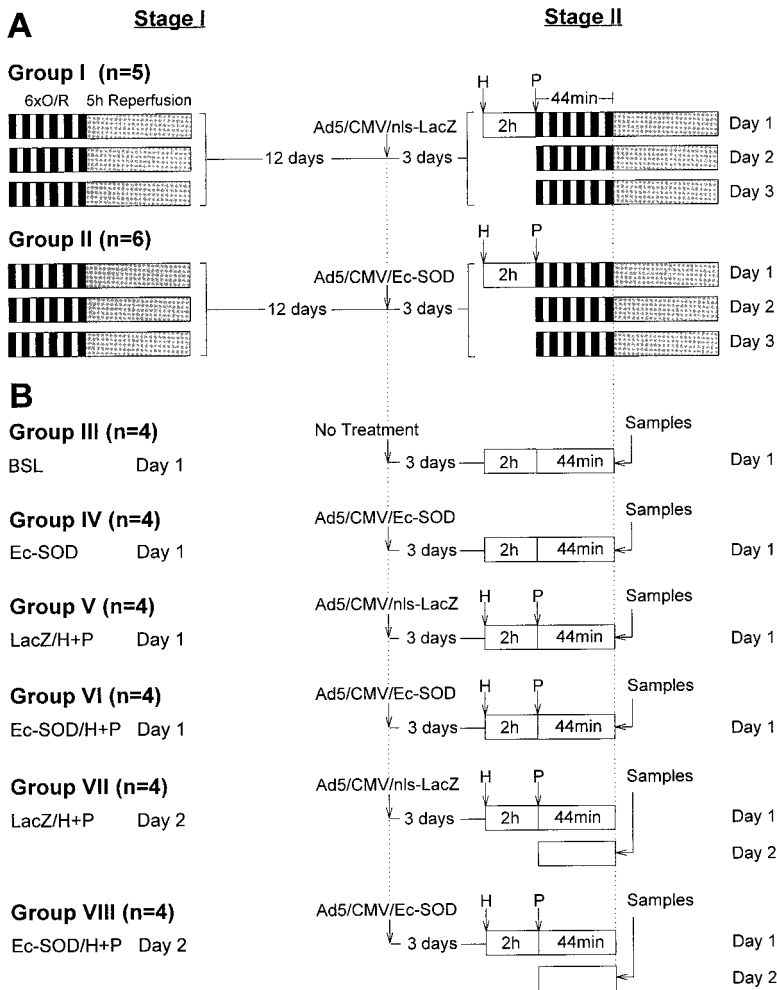


Figure 3. Experimental protocol and corresponding tissue samples. A, Experimental protocol for studying effects of gene therapy on myocardial stunning. Two groups of rabbits were studied (groups 1 and 2). Stage 1 consisted of a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles (6xO/R) performed on 3 consecutive days (days 1, 2, and 3). Recovery of contractile function over each 5-hour recovery period was expressed as percentage of preocclusion thickening fraction (Figure 4). At completion of stage 1, a 12-day period was allowed to prevent late preconditioning from interfering with stage 2. Group 1 was then injected with 2×10^8 pfu/kg of Ad5/CMV/nls-LacZ, while group 2 was injected with equivalent dose of Ad5/CMV/Ec-SOD. Stage 2 O/R protocol was initiated 3 days later with injection of heparin (H) followed 2 hours later by injection of protamine (P). Immediately thereafter, the same O/R protocol performed during stage 1 (6xO/R) was repeated again for 3 consecutive days. B, Experimental protocol in A related to tissue SOD levels reported in Figure 7. Group 3 (BSL) serves as control. Group 4 (Ec-SOD) represents group 2 rabbits before injection of heparin (H) and protamine (P) on day 1 of stage 2; group 5 (LacZ/H+P), group 1 rabbits after injection of H and P on day 1 of stage 2; group 6 (Ec-SOD/H+P), group 2 rabbits after injection of H and P on day 1 of stage 2; group 7 (LacZ/H+P), group 1 rabbits on day 2 of stage 2; and group 8 (Ec-SOD/H+P), group 2 rabbits on day 2 of stage 2.

occlusions resulted in severe myocardial stunning that lasted an average of 4 hours. There were no significant differences in the total deficit of WTh between the 2 stages on day 1 (Figure 5A), indicating that the experimental manipulations undertaken during stage 2 (which included the injection of recombinant Ad5, heparin, and protamine) did not alter myocardial stunning.

On day 2 of stages 1 and 2, the recovery of WTh after the six 4-minute occlusions was markedly improved compared with day 1 (Figure 4A). In stage 1, the thickening fractions on day 2 (expressed as a percentage of preocclusion values) were significantly greater ($P < 0.05$) than those on day 1 at 30 minutes and 1, 2, and 3 hours of reperfusion. Whereas it took 4 hours for thickening fraction to return to 91% of baseline values on day 1, on day 2 thickening fraction reached 98% of baseline after just 3 hours of reperfusion. The total deficit of WTh after the sixth reperfusion was 58% less on day 2 than on day 1 ($P < 0.01$) (Figure 5A). On day 3, the recovery of WTh after the six 4-minute occlusion/4-minute reperfusion cycles was again enhanced compared with day 1 and similar to that observed on day 2 (Figure 5A). The results from stage 2 were very similar to stage 1, and although the 2 curves diverged at the 30-minute time point (Figure 4A), there were no significant differences in the total deficit of WTh between the 2 stages on day 2 or 3 (Figure 5A). Thus, myocardial

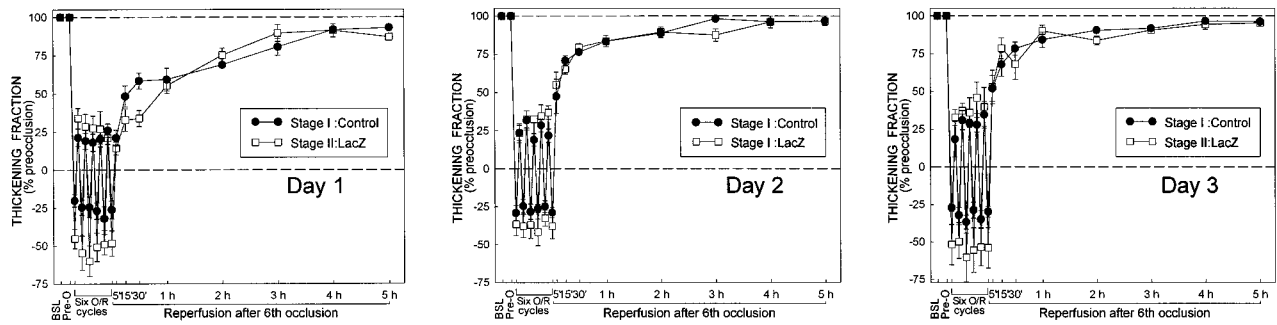
stunning during both stages was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1, indicating late preconditioning against myocardial stunning in both stages.

Group 2 (Gene Therapy Group)

On day 1, the recovery of function was markedly improved in stage 2 over stage 1, resulting in a total deficit of WTh that was 54% less in stage 2 than in stage 1 ($P < 0.01$, Figure 5B). Whereas it took 5 hours for thickening fraction to return to 95% of baseline values in stage 1, in stage 2 thickening fraction reached 91% of baseline after only 2 hours of reperfusion (Figure 4B). These results indicate that gene therapy markedly attenuated myocardial stunning during stage 2 relative to the untreated stage 1. Furthermore, the degree of cardioprotection provided by gene therapy on day 1 of stage 2 was similar to that provided by late preconditioning on day 2 or 3 of stage 1 (Figure 5B).

On day 2, the results in stage 2 were similar to those obtained in the same group in stage 1 or in controls (group 1) in either stage. As observed in controls, in Ad5/CMV/Ec-SOD-treated rabbits, the recovery of WTh on day 2 was significantly improved compared with the same group on day 1 of stage 1 (Figure 4B), so that the total deficit of WTh on day 2 of stage 2 was significantly lower than that observed on

A Group I: Control -- Ad5/CMV/nls-LacZ (n=5)



B Group II: Gene Therapy -- Ad5/CMV/Ec-SOD (n=6)

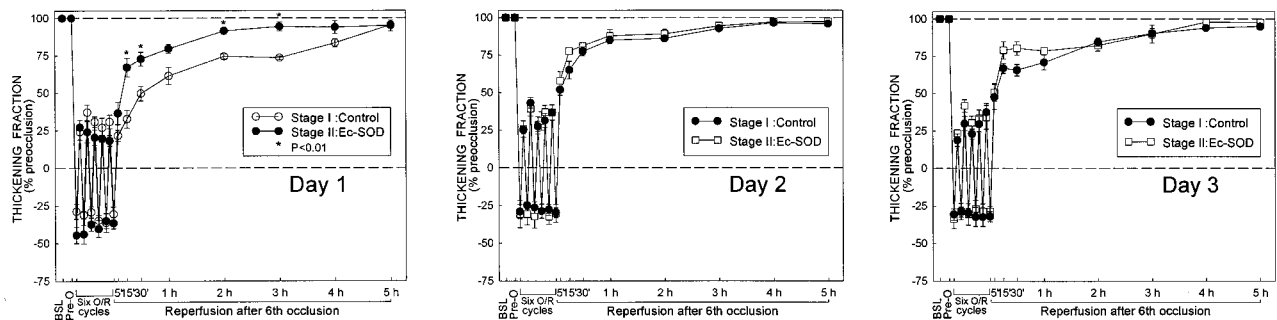


Figure 4. Systolic thickening fraction in rabbits before and after injection of Ad5 vectors. Thickening fraction is expressed as percentage of preocclusion values, and data are mean \pm SEM. This figure illustrates measurements of thickening fraction in ischemic-reperfused region at baseline (BSL), immediately before first occlusion (preocclusion [PreO]), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during 5-hour reperfusion interval after sixth reperfusion. Measurements taken during stage 1 are indicated by continuous lines with solid circles, and measurements taken during stage 2 (after injection of Ad5) by continuous lines with open squares. A, Thickening fractions in control rabbits on day 1 (left), day 2 (middle), and day 3 (right) of stages 1 and 2. B, Thickening fractions in gene therapy rabbits on day 1 (left), day 2 (middle), and day 3 (right) of stages 1 and 2. B, On day 1, marked improvement in recovery of contractile function was noted in rabbits treated with gene therapy (stage 2, Ec-SOD) vs same rabbits before gene therapy (stage 1, control).

day 1 of stage 1 ($P < 0.01$, Figure 5B). The total deficit of WTh on day 2 of stage 2 in group 2 was similar to that of the same rabbits on day 2 of stage 1. Moreover, the total deficit of WTh on day 2 of stage 2 in group 2 was similar to the corresponding value obtained in controls (group 1) on day 2 of either stage (Figure 5). Thus, in the Ad5/CMV/Ec-SOD-treated rabbits, gene therapy provided substantial cardioprotection on day 1 of stage 2 but did not alter contractile function on day 2 of stage 2 relative to controls.

Postmortem Analysis

The size of the occluded-reperfused vascular bed was similar in the 2 groups: $20.9 \pm 1.4\%$ of LV weight in group 1 and $22.9 \pm 3.0\%$ in group 2. Triphenyltetrazolium chloride staining confirmed the absence of infarction in all rabbits included in the final analysis, indicating that the myocardial dysfunction associated with the 3-day O/R protocol was completely reversible.

Plasma SOD Activity on Day 1 of Stage 2

Because the gene therapy protocol used in group 2 provided substantial protection against myocardial stunning on day 1, it

was important to determine the levels of plasma SOD activity that were present during this period. Toward this end, plasma samples were drawn from the 6 rabbits composing group 2 on day 1 of the stage 2 protocol, and the total levels of SOD activity were determined as described under Methods. As illustrated in Figure 6, resting plasma levels were elevated an average of 44% 3 days after the injection of Ad5/CMV/Ec-SOD. On the injection of heparin, Ec-SOD was released from the liver, and the average plasma SOD activity rose to levels 2-fold higher than that observed at baseline on day 0. These levels steadily declined over the next 2 hours to 59% above baseline as Ec-SOD was redistributed across the body mass. Plasma SOD activity continued to decline after protamine (10 mg/mL IV) was injected to reverse the effects of the remaining heparin.

Tissue SOD Activity After Gene Therapy

Because tissue samples could not be obtained during the studies of myocardial stunning, additional rabbits ($n=24$) were used in a parallel study to assess total SOD levels in the liver and heart at various times during the gene therapy protocol. To facilitate comparisons, the experimental design

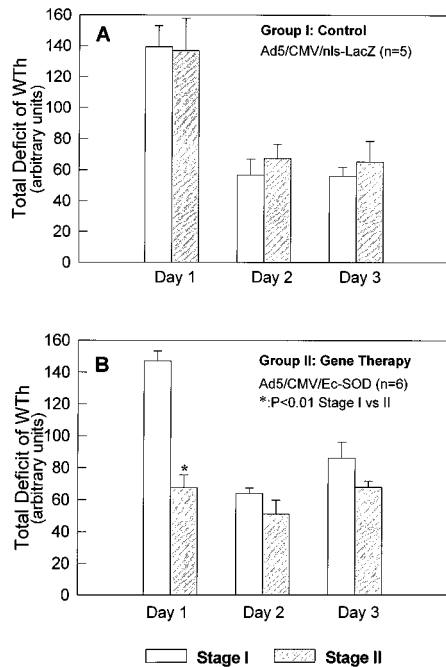


Figure 5. Total deficit of WTh after sixth reperfusion on days 1, 2, and 3 in control (n=5) and gene therapy (n=6) groups (groups 1 and 2, respectively). Total deficit of WTh is an integrated measure of magnitude and duration of postischemic dysfunction, used here to facilitate comparisons of severity of postischemic dysfunction among different days and different animals. A, Total deficit of WTh in group 1 on days 1, 2, and 3 during stage 1 (before injection of Ad5, open bars) and in same rabbits during stage 2 (after injection with Ad5/CMV/nls-LacZ, shaded bars). B, Total deficit of WTh in group 2 on days 1, 2, and 3 during stage 1 (before injection of Ad5, open bars) and in same rabbits during stage 2 (after injection with Ad5/CMV/Ec-SOD, shaded bars). Note that total deficit of WTh in gene therapy rabbits (group 2) on day 1 of stage 2 was markedly reduced vs same rabbits on day 1 of stage 1 (before gene therapy).

of the tissue analysis study is summarized in Figure 3B beneath the corresponding design of the functional study of myocardial stunning (Figure 3A). The baseline (BSL) group (group 3) consisted of normal rabbits and reflects the natural state before gene transfer. Group 4 (Ec-SOD) was designed to assess the myocardial levels of SOD activity that would have existed in gene therapy rabbits (group 2) on day 1 of stage 2 had the heparin and protamine injections been omitted. The negative control group (group 5, LacZ/H+P) represents group 1 on day 1 of stage 2 at the end of the last coronary occlusion. The gene therapy group (group 6, Ec-SOD/H+P) represents group 2 on day 1 of stage 2 at the end of the last coronary occlusion. Two additional groups were used to assess the residual levels of SOD activity that remained in the hearts of group 1 versus group 2 rabbits on day 2 of stage 2: group 7 (LacZ/H+P) represents group 1 on day 2, and group 8 (Ec-SOD/H+P) represents group 2 on day 2.

The results of the analysis of tissue SOD activity are presented in Figure 7, with results from liver samples reported in Figure 7A and results from the LV (taken from the position of the Doppler thickening probe) reported in Figure 7B. In the liver, the baseline level of total cytosolic and extracellular Cu/Zn-SOD activity in group 3 (BSL) rabbits was 66.4 ± 8.4 U/mg protein (Figure 7A). Similar levels of

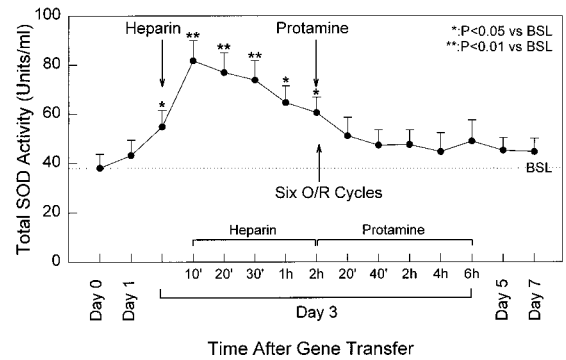


Figure 6. Time course of total plasma SOD activity in gene therapy rabbits. Plasma samples were drawn from rabbits (n=6) at times indicated on x axis, and total plasma SOD activity was determined according to Methods. A single plasma sample was drawn from each rabbit on days 0, 1, 5, and 7 after gene transfer, but 11 samples were drawn on day 3 after gene transfer during analysis of myocardial stunning (on day 1 of stage 2 O/R protocol). After first plasma sample was taken, heparin (2000 U/kg IV) was injected to release recombinant Ec-SOD from liver into bloodstream (vertical arrow). Additional plasma samples were drawn 10, 20, and 30 minutes and 1 and 2 hours after heparin injection (as indicated beneath x axis). Heparin antidote protamine was then injected (10 mg/kg IV) just before first occlusion of stage 2 O/R protocol (6 O/R cycles, vertical arrow). Five more plasma samples were drawn at 20 and 40 minutes and 2, 4, and 6 hours after first occlusion (as indicated beneath x axis). Results of assays for total plasma SOD activity were expressed as U/mL and plotted as mean \pm SEM. Note that resting plasma levels of total SOD activity were elevated by 44% on day 3 after gene transfer but that injection of heparin further increased this activity to 115% over baseline (BSL, dotted line).

activity were found in negative control livers (LacZ/H+P) on day 1 (group 5) and day 2 (group 7). However, 3 days after gene transfer, the livers of rabbits injected with Ad5/CMV/Ec-SOD (group 4; Ec-SOD) contained levels of SOD activity that were 4.4-fold higher than baseline ($P < 0.01$ versus group 3, BSL). Livers from rabbits treated with the complete experimental protocol (Ec-SOD/H+P) contained intermediate levels of activity on day 1 (group 6) and day 2 (group 8). These levels were significantly elevated over group 3 (BSL) because of ongoing production of Ec-SOD but not as high as those found in group 4 (Ec-SOD) on day 1 (because of heparin-mediated release of Ec-SOD from the liver in groups 6 and 8 on day 1). In separate experiments, Northern blot analysis using a riboprobe specific for the 3' end of human Ec-SOD mRNA documented that at 3 days after injection, human Ec-SOD mRNA was present in the livers of rabbits treated with Ad5/CMV/Ec-SOD (group 4) but not in the livers of untreated rabbits (group 3, data not shown).

The results of the analysis of LV myocardial SOD activity are presented in Figure 7B. The baseline level of total cytosolic and extracellular Cu/Zn-SOD activity in group 3 (BSL) rabbit hearts was 3.6 ± 0.6 U/mg protein, $\approx 5.4\%$ of that found in liver at baseline. These levels were not significantly changed by injection of negative control virus (Ad5/CMV/nls-LacZ) or by injection of heparin and protamine either on day 1 (group 5) or day 2 (group 7) of the stage 2 O/R protocol. In contrast, the hearts of rabbits injected with Ad5/CMV/Ec-SOD (group 4, Ec-SOD) contained levels of SOD activity that were 2.1-fold higher than baseline (group 3,

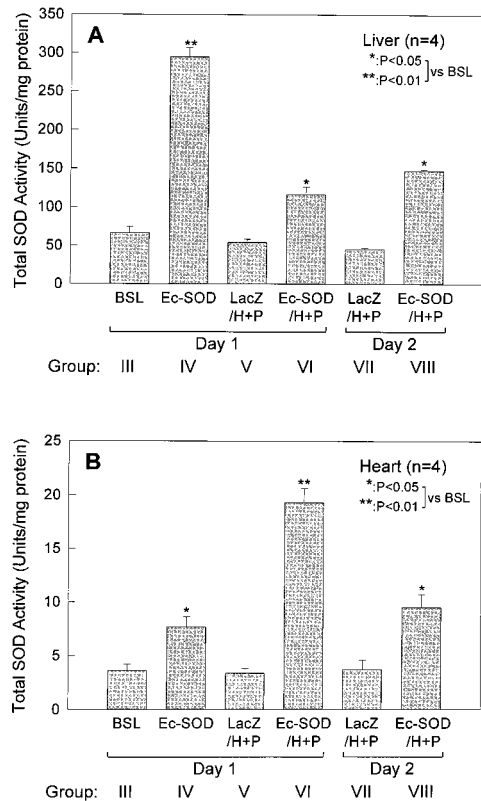


Figure 7. Impact of Ec-SOD gene therapy on total SOD activity in liver and heart. A, Total SOD activities measured in liver samples taken from 6 groups of rabbits defined in Figure 3B ($n=4/\text{group}$). B, Total SOD activities measured in heart samples taken from 6 groups of rabbits defined in Figure 3B ($n=4/\text{group}$). Note that scale used for y axis of A is far wider than that used for B because endogenous baseline (BSL) level of total SOD activity in rabbit liver is much higher than that found in rabbit heart. Days 1 and 2 in this figure refer to days 1 and 2 of O/R protocol initiated 3 days after gene transfer. Manipulations involving heparin (H) and protamine (P) undertaken in group 2, stage 2, day 1 were successful in releasing bulk of recombinant Ec-SOD from liver (A, group 4 vs group 6) and redistributing a significant amount of that SOD activity to heart (B, group 4 vs group 6).

BSL) 3 days after gene transfer ($P<0.05$ versus BSL). Moreover, the hearts of rabbits treated with the complete protocol contained statistically elevated levels of SOD activity on both day 1 (group 6) and day 2 (group 8) of the stage 2 O/R protocol. On day 1 of stage 2 (at the time when cardioprotection against stunning was manifest in Figures 4 and 5), the total SOD activity in group 6 hearts (Ec-SOD/H+P) was 5.4-fold higher than baseline (group 3, BSL), whereas on day 2 of stage 2 (at the time when late preconditioning might also account for the cardioprotective effect), the total SOD activity in group 8 hearts (Ec-SOD/H+P) was 2.6-fold higher than baseline.

Discussion

The use of in vivo gene transfer to increase systemic levels of therapeutic protein has considerable potential, but such an approach has not previously been attempted with antioxidant enzymes. The present study demonstrates that a systemic elevation in Ec-SOD attenuates myocardial stunning in con-

scious rabbits, indicating that gene transfer can enhance endogenous antioxidant defenses against ischemia/reperfusion injury in vivo. Although numerous studies have demonstrated that antioxidant enzymes can provide substantial protection against myocardial stunning (reviewed in Reference 1), to the best of our knowledge this is the first demonstration that antioxidant gene therapy can effect a physiological and therapeutic change in the intact animal. This is also the first in vivo study to show that Ec-SOD attenuates myocardial stunning without the need for concomitant administration of catalase, although purified Ec-SOD enzyme has previously been shown to reduce myocardial damage in intact pigs³ and rats,⁴ and overexpression of Ec-SOD has been shown to preserve postischemic myocardial function in hearts isolated from transgenic mice.⁶ Finally, the present study suggests general methods for controlling gene therapy at the posttranslational level and for simultaneously protecting multiple tissues from the consequences of ischemia/reperfusion injury.

Methodological Considerations

The rabbit model used in this study is characterized by stable baseline systolic wall thickening for several weeks after surgical instrumentation, reproducible degrees of myocardial stunning, and consistent development of late preconditioning against stunning.⁹⁻¹¹ The rationale for use of a conscious preparation was to avoid a number of factors that could interfere with the assessment of postischemic myocardial dysfunction: anesthesia, surgical trauma, fluctuations in body temperature, abnormal hemodynamic conditions, elevated catecholamine levels, cytokine release, etc.^{12,13} Most importantly, the use of conscious animals obviates the exaggerated oxyradical formation observed in open-chest animals,¹² which would interfere with the assessment of Ec-SOD.

The choice of the antioxidant enzyme was also a critical methodological consideration in the present study. SOD should provide antioxidant protection by (1) inactivating $\cdot\text{O}_2^-$, (2) sparing nitric oxide from destruction, and (3) preventing $\cdot\text{O}_2^-$ from forming more destructive reactive oxygen species, such as peroxyxynitrite and its reaction products, including hydroxyl radical ($\cdot\text{OH}$). The vast majority of animal studies examining the role of antioxidant enzymes in protecting the myocardium from ischemia/reperfusion injury have used SOD, either alone or in combination with other antioxidants or antioxidant enzymes.¹ Many of these studies relied on continuous IV infusion of recombinant Cu/Zn-SOD or Mn-SOD protein and thus examined the function of intracellular enzymes while they were being delivered to the extracellular space. However, careful examination of the distribution kinetics of Cu/Zn-SOD indicates that the interstitial levels of this enzyme (rather than the plasma levels) are primarily responsible for protection against myocardial ischemia/reperfusion injury.⁷ This being the case, it was reasonable to consider an isoform of SOD that has natural affinity for the interstitial space. The selection of Ec-SOD for these studies was also influenced by the fact that it is the only known isoform of SOD that is secreted from cells and is uniquely suited for hepatic production and systemic distribution.

The possibility of an inflammatory response of the host against the first-generation Ad5 vector was another important consideration in the design of this study and was a major factor in the decision to target gene transfer to another tissue besides the heart. Unlike the heart, the liver has a profound regenerative capacity, and remarkably high frequencies of Ad5-mediated transfection (>90%) can easily be obtained without compromising hepatic function.²⁸ Furthermore, recombinant Ad5 is extremely effective at mediating efficient and selective gene transfer to the liver after simple IV injection,²⁸ whereas surgical²⁹ or interventional³⁰ methods are required to target recombinant gene expression to the heart. The liver was chosen as the source of recombinant Ec-SOD not only because it is a far more opportune target for Ad5-mediated gene therapy but also because it served to alleviate concerns regarding the possibility of inflammation in the heart and its potential impact on myocardial stunning and preconditioning.

Previous Studies of the Cardioprotective Effects of Ec-SOD

A wealth of studies have been published concerning cardioprotection by Cu/Zn-SOD and Mn-SOD (reviewed in Reference 1). However, comparatively few investigations have examined the effect of Ec-SOD on myocardial ischemia/reperfusion injury, and no previous study has examined the effect of Ec-SOD on myocardial stunning in intact animals. Of the studies that have examined the effect of Ec-SOD on myocardial damage, only a few have been conducted in intact animals. Wahlund et al⁴ reported that Ec-SOD reduced creatine kinase release in rats subjected to 10 minutes of coronary occlusion and 24 hours of reperfusion. Hatori et al³ reported that retroinfusion of purified, recombinant Ec-SOD protein into the great cardiac vein decreased the size of myocardial infarctions in open-chest pigs subjected to 45 minutes of coronary occlusion followed by 4 hours of reperfusion and that the cardioprotection provided by the combination of catalase with Ec-SOD was no different from that provided by Ec-SOD alone. This is congruent with our present finding that Ec-SOD without catalase was effective in alleviating myocardial stunning.

Chen et al⁶ recently used transgenic mice overexpressing human Ec-SOD to demonstrate preserved posts ischemic myocardial function in isolated murine hearts. This cardioprotective effect is consistent with the present results; however, in vivo gene transfer has potential for clinical application. Furthermore, results obtained from isolated, buffer-perfused, globally ischemic hearts may not be as clinically relevant as results from intact, blood-perfused, regionally ischemic hearts in conscious animals.

Extensive evidence gathered over many years indicates that the role of SOD in protecting the myocardium against ischemia and reperfusion is extremely complex.¹ Although a few studies conducted in open-chest or isolated heart preparations have indicated that Cu/Zn-SOD alone can protect against ischemia/reperfusion injury, other studies have failed to demonstrate protection with Cu/Zn-SOD alone,⁴ and yet others have indicated that a combination of Cu/Zn-SOD and catalase is more effective.^{1,2} We recently demonstrated the

inability of Cu/Zn-SOD to ameliorate myocardial stunning in the same conscious rabbit model as used here.³¹ The discrepancy between the present results and previous results obtained with exogenous Cu/Zn-SOD^{1,2,31} is most likely a result of differences in the molecular properties of Cu/Zn-SOD and Ec-SOD. First, the local tissue levels of SOD achieved in the present study could be higher than those achieved previously by IV administration because of the extended half-life of Ec-SOD. The half-life of Cu/Zn-SOD after IV injection is ≈ 7 minutes, whereas that of Ec-SOD is ≈ 20 hours.³² Second, and perhaps more importantly, the localization of the enzyme is different. Ec-SOD is concentrated on the cellular membrane and in the extracellular matrix, whereas exogenous Cu/Zn-SOD is freely distributed across the extracellular space. The close proximity of Ec-SOD to the intracellular environment could be critical for its ability to dismutate $\cdot\text{O}_2^-$ produced in this compartment. By virtue of its attachment to the sarcolemma, the strategic location of Ec-SOD might also prevent the diffusion of $\cdot\text{O}_2^-$ from one compartment to another. These unique properties of Ec-SOD may serve to widen its effective dose range (relative to Cu/Zn-SOD) and thereby relax the requirement for concomitant addition of catalase.³

From a pathophysiological standpoint, the present results imply that $\cdot\text{O}_2^-$ plays an important role in the genesis of myocardial stunning. This concept is not necessarily in conflict with previous studies that had concluded that the $\cdot\text{OH}$ is the principal culprit in posts ischemic myocardial dysfunction,^{1,31} because $\cdot\text{O}_2^-$ can serve as a precursor of $\cdot\text{OH}$ through the iron-catalyzed Haber-Weiss reaction or by reacting with NO to form peroxynitrite.³³ We propose that $\cdot\text{O}_2^-$ may contribute to myocardial stunning by causing the formation of more destructive reactive oxygen species (such as $\cdot\text{OH}$ and peroxynitrite), which then inflict the damage responsible for contractile dysfunction. This paradigm would explain the fact that myocardial stunning can be attenuated by interventions that intercept $\cdot\text{O}_2^-$ at critical locations (such as Ec-SOD), by interventions that prevent $\cdot\text{OH}$ formation, and by interventions that scavenge $\cdot\text{OH}$ and/or peroxynitrite.³¹

Present Study

In pilot studies, IV injection of Ad5/CMV/Ec-SOD produced modest elevations in total plasma SOD activity that peaked after 3 days and declined by 1 week (Figure 1). This time course of gene expression after IV injection of recombinant Ad5 is generally consistent with previous reports involving the secretion of recombinant protein after Ad5-mediated, in vivo gene transfer to the liver.²⁸ Even high doses of Ad5/CMV/Ec-SOD resulted in relatively modest plasma levels of Ec-SOD activity (<60% increase over baseline, Figure 1A). However, truly supraphysiological levels of plasma Ec-SOD (up to 100-fold over baseline) could be obtained by displacing the recombinant enzyme from its heparan sulfate proteoglycan binding sites with dextran sulfate. Although the pharmacokinetics of this particular form of Ec-SOD administration have not previously been examined, the results in Figure 1 are generally consistent with other studies examining the release of endogenous Ec-SOD by dextran sulfate or heparin²⁵ and with studies examining the fate of radiolabeled Ec-SOD after IV injection.³² However, direct comparison is

not possible because the curves in Figure 1 represent the net effect of several dynamic processes, including the time course of Ad5-mediated gene expression,²⁸ the kinetics of competitive release and redistribution of Ec-SOD,²⁶ and the in vivo degradation of Ec-SOD.³²

Empirical dose-ranging studies were conducted in conscious rabbits, with myocardial stunning as the primary end point. A protocol including the injection of heparin and protamine was adopted to displace recombinant Ec-SOD from the liver for systemic distribution, thus minimizing the viral load necessary to achieve a cardioprotective effect. The viral dose selected from these studies (2×10^8 pfu/kg IV) resulted in a moderately low frequency of liver-directed gene transfer (0.09% of liver hepatocytes, Figure 2). However, the strength of the CMV IE promoter²⁸ in combination with the 20-hour half-life of Ec-SOD³² caused a 4.4-fold elevation in hepatic SOD activity (Figure 7A), an accumulation sufficient to produce a 2-fold elevation in plasma SOD activity after heparin injection (Figure 6). To reverse the effects of heparin, protamine was injected 2 hours later just before the O/R protocol. This precaution was taken to ensure the interstitial binding of Ec-SOD and to alleviate concerns regarding the potential effect of heparin on other extracellular heparin-binding enzymes.³⁴

The data presented in Figure 7 indicate that the manipulations involving heparin and protamine succeeded in releasing the bulk of recombinant Ec-SOD from the liver into the circulation for systemic distribution, yielding a 5.4-fold increase in total myocardial SOD activity over baseline. In light of previous work²⁴ demonstrating oxyradical production during repetitive episodes of myocardial ischemia and reperfusion, the results presented in Figures 4 and 5 indicate that this increase in myocardial SOD activity was sufficient to neutralize the biological threat imposed by excess $\cdot O_2^-$. Twenty-four hours later (on day 2), total myocardial SOD activity had declined to a level 2.6-fold higher than baseline (consistent with the 20-hour half-life of Ec-SOD in the rabbit³²). This elevation in SOD activity may have contributed to the protection against stunning observed on day 2 of stage 2 in group 2 (Figure 5B); alternatively, this protective effect may be the result of late preconditioning against stunning.⁹⁻¹¹

The experimental design for the study of myocardial stunning incorporated 2 stages (untreated and Ad5-treated), which enabled each rabbit to serve as its own control. Furthermore, comparison of rabbits treated with the negative control virus (Figure 5A) with rabbits treated with gene therapy (Figure 5B) enabled us to formally exclude the possibility that the Ad5 vector or the use of heparin or protamine might somehow contribute to the cardioprotective effect. The data presented in Figure 5 indicate that the degree of cardioprotection provided by the Ec-SOD gene therapy protocol was remarkable, being equivalent to that provided by late preconditioning (ie, similar to that observed on day 2 or 3 of either stage 1 or 2).

From a pharmacological standpoint, the results in Figures 6 and 7 suggest a general method for controlling systemic levels of therapeutic proteins produced by liver-directed gene therapy. Although gene therapy has the novel potential of

providing a sustained source of therapeutic protein, this benefit is partly offset by the difficulty of controlling recombinant protein levels after gene therapy has been applied. Molecular methods are being refined to control gene therapy at the transcriptional level,^{35,36} but such control will require hours or days to become fully manifest and cannot possibly deliver a bolus of protein within a matter of minutes. In theory, recombinant techniques could be used to add the secretory and heparin-binding domains of Ec-SOD to almost any therapeutic protein. Liver-directed gene transfer would then lead to modest resting plasma levels (as in Figure 1A) that could be rapidly increased in a medical emergency with the simple IV injection of heparin (as in Figure 1B). Although many issues need to be resolved with respect to this approach,³⁴ the present results demonstrate for the first time that liver-directed gene therapy can be modulated at the posttranslational level (Figure 7), providing a new form of pharmacological control that may prove useful in fine-tuning gene therapy or applying it as a systemic bolus.

Conclusions

Our understanding of the complex functions of antioxidant enzymes in protecting the myocardium against ischemia/reperfusion injury continues to evolve. The present study expands the existing body of knowledge by demonstrating that gene therapy techniques can be used to attenuate myocardial stunning in conscious rabbits with the use of a single antioxidant enzyme (Ec-SOD) without the need for simultaneous administration of catalase. These results support the efficacy of Ec-SOD in protecting the myocardium against ischemia/reperfusion injury,^{3,4,6} which might be attributed to the extended half-life³² and/or the extracellular binding properties of this unique antioxidant enzyme.

This report also demonstrates that the extracellular binding property of Ec-SOD can be used in conjunction with in vivo gene transfer to impose a novel form of posttranslational control to manipulate the levels of recombinant protein present in various tissue compartments. For the purposes of this study, the manipulations involving heparin and protamine provided a novel method to control the levels of Ec-SOD present in the myocardium. In parallel studies, we found similar increases in SOD activity in other tissues, suggesting a method for simultaneously protecting multiple organs from ischemia/reperfusion injury.

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