Overexpression of Human Copper/Zinc Superoxide Dismutase (SOD1) Suppresses Ischemia–Reperfusion Injury and Subsequent Development of Graft Coronary Artery Disease in Murine Cardiac Grafts

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Background—Ischemia–reperfusion injury is an important risk factor for graft coronary artery disease (GCAD). We hypothesized that overexpression of SOD1 in donor hearts would suppress ischemia—reperfusion injury and thereby reduce GCAD.

Methods and Results—In one series, donor hearts of C57BL/6 (H-2b) transgenic mice overexpressing human SOD1 or C57BL/6 wild-type mice were heterotopically transplanted into C57BL/6 recipients and procured after 4 hours of reperfusion (n=6 each). Superoxide, TNF-α, and MCP-1/CCL2 production were significantly reduced in the SOD1 transgenic donor heart recipients, and graft injury determined by serum CPK-MB levels was significantly decreased. Cardiomyocyte apoptosis and caspase-3 and caspase-9 activities were significantly decreased in these recipients; caspase-8 activity was unchanged. Fas ligand but not Fas expression was also reduced. In a second series, transgenic and wild-type hearts were transplanted into C-H-2bm12 KhEg (H-2bm12) recipients, and then procured on day 56 (n=7 each). Cardiac graft beating was significantly better in the SOD1 transgenic donor heart recipients on days 28, 42, and 56 (but not day 14). Significant reduction in luminal narrowing, the intima/media ratio, and the percentage of diseased vessels was seen in the SOD1 transgenic donor heart recipients, and MCP-1/CCL2, ICAM-1, and VCAM-1 production were significantly reduced.

Conclusions—Overexpression of SOD1 attenuates both apoptosis and the inflammatory response during ischemia—reperfusion injury and therefore mitigates against the subsequent development of GCAD. (Circulation. 2004;110[suppl II]:II-200–II-206.)

Key Words: apoptosis • arteriosclerosis • ischemia • reperfusion • transplantation
common mediators of apoptosis; oxygen free radicals are directly implicated in the initiation of apoptosis.7

Mice that overexpress SOD1 provide a unique tool for assessing the ability of increased intracellular SOD activity to modulate both the inflammatory and microvascular responses normally elicited by ischemia–reperfusion injury and hence the development of GCAD. We used transgenic mice bearing extra copies of cloned human SOD1 cDNA to achieve high-level expression of the transgene in the heart and investigated whether overexpression of human SOD1 in donor hearts would reduce GCAD by suppressing ischemia–reperfusion injury.

Methods

Animals

Inbred male mice of several strains, 6 to 10 weeks old, were used. Mice of the various strains were purchased from The Jackson Laboratories (Bar Harbor, Me). A breeding pair of SOD1 transgenic mice [C57BL/6-TgSOD110Cje, H-2^b] was obtained, prepared originally by Epstein et al.,8 who used a previously characterized plasmid, phGSOD-Svneo, encoding human SOD1. Transgenic founder mice were identified by Southern blotting of tail DNA with hSOD1 cDNA used as a probe. Lines were established from 4 founders. The genotypes of the SOD1 transgenic mice were identified by polymerase chain reaction (PCR) as described.8 The transgenic mice were heterozygous for the human SOD1 gene, and littermates without the gene were used as wild-type controls. Both SOD1 transgenic mice and wild-type littermates were used as donors. C57BL/6 (H-2^b) mice were used as recipients for the study of ischemia–reperfusion injury (syngeneic transplant), and C-H-2^bm12 KhEg (H-2^bm12 ) mice were used as recipients for the study of GCAD (major histocompatibility complex class II mismatch chronic rejection model). All mice were housed in the animal care facility at Stanford University Medical Center (Stanford, Calif), kept under standard temperature, humidity, and timed lighting conditions, and were provided mouse chow and water ad libitum. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals9 prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996).

Heterotopic Cardiac Transplantation

Heterotopic abdominal cardiac transplantation was performed according to the method of Corry et al.,9 with some modifications. Anesthesia was induced with 5% inhaled isoflurane (Halocarbon Laboratories, River Edge, NJ). During surgery, the animals were maintained on 2.5% inhaled isoflurane. Donor animals were systemically heparinized (50 mg/kg) before heart procurement. The donor heart was rapidly excised after retrograde coronary perfusion with ice-cold saline. The procured hearts were kept in ice-cold saline for 20 minutes. Because standard graft implantation averages ~30 minutes, the total ischemic time was 50 minutes. The donor aorta and pulmonary artery were anastomosed to the recipient aorta and vena cava respectively with 10-0 nylon sutures.

Experimental Groups

In the first of this 2-part study, indicators of ischemia–reperfusion injury were analyzed in native hearts (4 transgenic hearts, 4 wild-type hearts) and in 4-hour reperfused grafts (6 transgenic donor hearts, 6 wild-type donor hearts). In the second part, GCAD was measured in grafts explanted at 56 days (7 transgenic hearts, 7 wild-type hearts).

In Situ Oligo Ligation Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Analysis

The grafts were sectioned transversely, frozen in optimal cutting temperature compound (Sakura Finetek USA Inc), and stored at −80°C. In situ staining of DNA strand breaks in serial sections of each specimen was detected by means of the ApopTag in situ oligo ligation kit with oligo A as directed (Intergen, Purchase, NY) with some modifications. The endogenous biotin was blocked with the use of an avidin/biotin blocking kit (BioGenex). TACS Blue Label was used as a peroxidase substrate, and methyl green was used as a counterstain of nuclei. Oligo A was synthesized as described by Didenko et al.10 Because conventional TUNEL assay can detect nonspecific DNA fragmentation caused by necrosis, a more specific in situ ligation assay for identification of apoptotic nuclei was used with hairpin oligonucleotide probes. The number of TUNEL-positive cells in each cardiac allograft was counted manually by 2 investigators (G.K.M., R.D.T.) blinded to the experimental conditions. Cells were counted in 6 animals (4 fields each) at ×200 magnification. The percentage of TUNEL stained cells was recorded, ie, the number of labeled nuclei divided by total number of nuclei.

Caspase-3, Caspase-8, and Caspase-9 Activity Assays

Caspase activities were determined with the use of the caspase-3 activity assay kit (Clontech), and the caspase-8 and caspase-9 activity assay kit (R&D Systems) as directed. Results are expressed as the amount of absorbance of the p-nitroanilide cleaved by caspase 405A standardized to the total protein in micrograms per hour.

Superoxide Production

Superoxide levels were measured in excised tissue by the spin trap method after 4 hours of reperfusion. Superoxide accumulation was measured by using conditioned medium supplemented with the spin trapping agent 4-aminoo-2,2,6,6-tetramethylpiperidine-1-oxyl (tempamine; Sigma-Aldrich) as previously described.11 Electron paramagnetic resonance (EPR) spectra were recorded at room temperature with a spectrometer (Model 8400; Resonance Instruments). The EPR signal intensity was quantified by comparing the double integration of recorded first derivative EPR peaks of each sample with that of a standard tempamine spin solution. When tempamine reacts with other radical species such as superoxide, it loses its EPR signal. Thus, the reduction in peak height is directly proportional to the amount of superoxide produced. All measurements were normalized to the protein concentration of each sample as determined by the bicinchorinic acid method (Pierce Chemical).

Myeloperoxidase Assay

To determine the extent of neutrophil infiltration into the reperfused cardiac grafts, myeloperoxidase (MPO) activity was assessed as previously described.12 The amount of MPO per milliliter of sample was calculated by dividing the activity of the sample by the volume (0.035 ml). MPO activity per minute was calculated from a standard curve using peroxidase (Sigma) as the standard enzyme. MPO values were standardized to the protein concentration of each sample as determined by the bicinchorinic acid method. Data are expressed as units per milligram of total protein.

Protein Isolation and Enzyme-Linked Immunosorbent Assay

Snap-frozen myocardial tissue specimens were homogenized in phosphate-buffered saline and centrifuged at 12 000g for 20 minutes at 4°C. The protein concentration of the supernatant was determined by the bicinchorinic acid method, and aliquots were stored at −80°C. Intragraft tumor necrosis factor-α (TNF-α), monocyte/macrophage chemotactant protein-1 (MCP-1/CCL2) (BioSource International, Camarillo, Calif), Fas, Fas ligand (FasL), intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (R&D Systems) levels were determined by enzyme-linked immunosorbent assay as directed.

Graft Survival and Allograft Function Analysis

Mice used in the second part of the study were monitored daily. Graft viability was assessed by direct abdominal palpation of the hetero-
Topically transplanted hearts. Cardiac graft function was expressed as a beating score and assessed by the Stanford cardiac surgery laboratory graft scoring system (0, no contraction; 1, contraction barely palpable; 2, obvious decrease in contraction strength, but still contracting in a coordinated manner; rhythm disturbance; 3, strong, coordinated beat but noticeable decrease in strength or rate and distention/stiffness; 4, strong contraction of both ventricles, regular rate, no enlargement or stiffness).

Morphometric Analysis of GCAD
At 56 days after transplantation, the cardiac grafts were harvested and embedded in paraffin. Elastica von Gieson staining was performed for morphometric analysis of arterial intimal proliferation, which was performed as described by Armstrong et al. Briefly, the neointima, media, and lumen were measured with the use of SPOT Advanced Version 3.4.2 software (Diagnostic Instruments, Inc). The neointima was defined as the area bound by the internal elastic lamina and the lumen, the media as the region between the internal and external elastic membranes, and the lumen as the clear region in the vessel. Diseased vessels were identified as those with >10% luminal narrowing. Multiple sections from the middle of the heart were used for analysis. Middle-sized coronary arteries were analyzed (>8 arteries for each graft).

Statistical Analysis
Values are expressed as mean±SD. All comparisons shown are between SOD1 transgenic donor heart recipients and wild-type littermate donor heart recipients. Differences in values were analyzed statistically by the unpaired Student t test and the differences in cardiac graft beating score were analyzed by a 2-way repeated-measures ANOVA (StatView 5.0; SAS Institute). Significance was accepted at P<0.05.

Results
Overexpression of SOD1 Suppresses Superoxide Production, Cardiomyocyte Apoptosis, and Inflammation Caused by Ischemia–Reperfusion Injury in Cardiac Grafts
Myocardial injury after ischemia–reperfusion is mediated by oxygen-derived free radicals such as superoxide anion; significant quantity of this radical is generated during ischemia–reperfusion. We observed significantly reduced superoxide radical generation in SOD1 transgenic donor hearts after 4 hours of reperfusion (Figure 1). Ischemia–reperfusion injury causes cardiomyocyte apoptosis and superoxide is directly implicated in this context. At 4 hours of reperfusion, in situ oligo ligation TUNEL-positive apoptotic cardiomyocytes were significantly less in SOD1 transgenic cardiac grafts compared with wild-type littermate donor hearts (Figure 2A). Correlating with the decreased number of apoptotic cardiomyocytes, caspase-3 and caspase-9 activities in lysates from the SOD1 transgenic donor hearts were also reduced when compared with those from the wild-type donor hearts (Figure 2B and 2D). However, there was no significant difference in caspase-8 activity between these 2 groups (Figure 2C). FasL expression was significantly decreased in the cardiac grafts of SOD1 transgenic donor heart group (Figure 2E), whereas Fas expression did not differ between these 2 groups (Figure 2F). These results suggest that SOD1 overexpression in cardiac grafts leads to inhibition of cardiomyocyte apoptosis mediated by caspase-3 and caspase-9–dependent pathways.

Ischemia–reperfusion injury produces a pro-inflammatory environment, which includes an influx of injurious cytokines and chemokines. We therefore determined whether SOD1 overexpression in the cardiac graft reduces the inflammatory response after transplantation. We examined neutrophil-produced MPO, because neutrophils are known to be predominant effector cells in the local inflammatory response. We also determined the levels of the pro-inflammatory cytokines and chemokines, TNF-α, and MCP-1/CCL2. The levels of MPO and the tested pro-inflammatory cytokines were significantly lower in the cardiac grafts of the SOD1 transgenic donor heart group compared with the wild-type donor heart group at 4 hours of reperfusion (Figure 3). Moreover, the serum levels of CPK-MB were significantly lower in the SOD1 transgenic donor heart recipients compared with the wild-type donor heart recipients 4 hours after reperfusion, indicating decreased cardiac graft damage (Figure 3D). Taken together, these results suggest that reduced superoxide generation by SOD1 overexpression in cardiac grafts inhibits cardiomyocyte apoptosis mediated by caspases and limits inflammation in the early phase after ischemia–reperfusion injury to cardiac grafts. Thus, SOD1 overexpression may promote cardiomyocyte survival by efficient detoxification of free radicals and render the heart resistant to ischemia–reperfusion injury.

Overexpression of SOD1 in the Donor Heart Improves Cardiac Allograft Function and Reduces GCAD and Local Cytokine Production
We next determined whether the reduction in ischemia–reperfusion injury early after transplantation results in im-
proved cardiac allograft function and limits the development of GCAD over time. We therefore determined cardiac allograft function, GCAD indices, and cytokine production in the chronic phase. All animals survived for the 56-day study duration. Graft beating scores were significantly better in the SOD1 transgenic donor heart group at 28, 42, and 56 days after transplantation (Figure 4). Marked fibrointimal thickening and luminal narrowing, morphologically resembling typical human GCAD, were observed in wild-type donor hearts (Figure 5A [a]). In contrast, less intimal thickening and preserved vessel lumen were observed in SOD1 transgenic donor hearts (Figure 5A [b]). GCAD, assessed by the mean percentage of luminal narrowing, the intima-to-media ratio, and the percentage of diseased vessels, was significantly less in the SOD1 transgenic donor hearts compared with the wild-type donor hearts (Figure 5B). In contrast, intimal thickening and preserved vessel lumen were observed in SOD1 transgenic donor hearts (Figure 5A [b]). GCAD, assessed by the mean percentage of luminal narrowing, the intima-to-media ratio, and the percentage of diseased vessels, was significantly less in the SOD1 transgenic donor hearts compared with the wild-type donor hearts (Figure 5B). In contrast, intimal thickening and preserved vessel lumen were observed in SOD1 transgenic donor hearts (Figure 5A [b]).

**Discussion**

Numerous reports describe the efficacy of antioxidants and free radical scavengers in minimizing ischemia–reperfusion injury. Although transgenic mice overexpressing SOD1 have been used to understand the protective role of SOD1 against ischemia–reperfusion injury in myocardial infarction, to our knowledge, ours is the first study of the preventive effect of SOD1 on both apoptosis and inflammation during ischemia–reperfusion injury as well as the development of GCAD in cardiac grafts obtained from transgenic mice. Controversy remains, however, as to the degree to which antioxidants protect the heart during ischemia–reperfusion injury. A previous study has shown that exogenous SOD1 expression inhibited reperfusion injury in rodent renal allografts. The location of the SOD1 overexpression near the mitochondrion may have prevented injury from a variety of oxidant species as well as subcellular organelle dysfunction caused by membrane lipid peroxidation. Transgenic mice, which overexpress an expression vector containing SOD cDNA were found

### Figure 2. Effect of SOD1 overexpression on indices of cardiomyocyte apoptosis during ischemia–reperfusion injury. A, In situ oligoligation TUNEL-positive cell counts. B, Caspase-3 activity. C, Caspase-8 activity. D, Caspase-9 activity. E, FasL expression. F, Fas expression. Values are mean±SD. WT indicates wild-type donor heart; SOD1, SOD1 transgenic donor heart; Native, native heart of each group (n=4, each group); Transplanted, transplanted cardiac graft of each group (n=6, each group); NS, not significant.

### Figure 3. Effect of SOD1 overexpression on inflammatory response during ischemia–reperfusion injury. A, MPO activity. B, TNF-α production. C, MCP-1/CCL2 production. D, Recipient serum CPK-MB level. Values are mean±SD. WT, wild-type donor heart group; SOD1, SOD1 transgenic donor heart group; Native, native heart of each group (n=4, each group); Transplanted, transplanted cardiac graft of each group (n=6, each group); NS, not significant.
leads to caspase-8 and then caspase-3 activation.19 Activated and TNF receptor-mediated death receptor pathway, which subsequent caspase-9 and caspase-3 activation, and the Fas chrome c from the mitochondria into the cytosol and mediated stress pathway, which involves release of cyto-

cortic activity.17 Two major signal transduction

to delay apoptotic cell death considerably.7 Injection of antisense SOD expression vector into neurons decreased the amount of SOD, simultaneously delaying apoptosis, whereas SOD injected after the development of oxidative stress had no effect on apoptosis.7

The apoptotic process involves a complex series of signals and cell activation steps.13 Two major signal transduction pathways that induce apoptosis, the mitochondria disruption-mediated stress pathway and the Fas and TNF receptor-mediated death receptor pathway, have been investigated in relation to various diseases. Narula et al showed that human cardiomyopathy is associated with the activation of caspase-3, indicating that the effector caspase-3 plays a role in cardiomyocyte apoptosis.18 Caspase-3 activation is regulated by at least 2 pathways, the mitochondria disruption-mediated stress pathway, which involves release of cytochrome c from the mitochondria into the cytosol and subsequent caspase-9 and caspase-3 activation, and the Fas and TNF receptor-mediated death receptor pathway, which leads to caspase-8 and then caspase-3 activation.19 Activated caspase-3 then cleaves a substrate such as poly-(ADP-ribose) polymerase, leading to DNA fragmentation and apoptosis.

In this study, caspase-9 and caspase-3 activities were significantly reduced, whereas no significant reduction in caspase-8 activity was observed in the SOD1 transgenic donor heart recipients at 4 hours of reperfusion. In addition, FasL, but not Fas expression, was reduced in this group. Thus, apoptosis appears to be reduced mainly by inhibition of the caspase-9-mediated pathway in hearts transplanted from SOD1 transgenic donors.

GCAD in human heart transplant recipients is progressive, with no effective therapy short of retransplantation. The precise causative factors leading to GCAD remain elusive. Although immunologic disparity between the donor and recipient undoubtedly contributes to the development of GCAD,20 there remains the possibility that antigen-independent factors also contribute to or accelerate the progression of GCAD. In a human case-control study, the contributions of the number of major histocompatibility complex class I mismatches, donor age, recipient cytomegalovirus status, number of rejection episodes in the first year, and histologic degree of ischemia–reperfusion injury to GCAD were compared, and ischemia–reperfusion injury emerged as the strongest predictor for the subsequent development of GCAD.2 In addition, Wang et al21 showed that ischemia–reperfusion injury is by itself sufficient to induce GCAD in murine cardiac syngenic grafts.

We observed a significant reduction of GCAD in the SOD1 transgenic donor heart group at 56 days after transplantation. GCAD was reduced mainly by the reduction of ischemia–reperfusion injury in the early phase, which may have resulted in decreased production of inflammatory cytokines and adhesion molecules in the cardiac allograft. The resulting decrease in tissue injury translates into a reduction in GCAD.

In transplanted coronary arteries, both medial smooth muscle cells and endothelial cells express VCAM-1.22 Up-regulation of this adhesion molecule may be important, because treatment with anti-VCAM-1 antibody induces long-term acceptance of murine cardiac allografts.23 In addition, treatment with anti-ICAM-1 antibody has been shown to inhibit GCAD in rats.24 The diseased graft shows abundant ICAM-1, especially on the endothelial surface.22 These adhesion molecules may facilitate transmigration of inflammatory and immune cells to the graft and contribute to ischemia–reperfusion injury as well as GCAD. Additionally, reduced ICAM-1 and VCAM-1 production in the chronic phase may relate to decreased GCAD.

Griendling et al25 suggested chemotactic signals are released that stimulate the redox-sensitive processes of smooth muscle cell proliferation and migration, important components of the intimal hyperplasia associated with GCAD. The data presented here strongly support an oxidative stress-induced mechanism of alloantigen-independent GCAD development, because grafts overexpressing SOD1 showed decreased levels of superoxide.

Our findings bring to light the important role of SOD1 in suppressing superoxide generation, apoptosis, and the inflammatory response during ischemia–reperfusion injury, properties which ultimately mitigate against the subsequent development of GCAD in murine cardiac grafts. In addition, overexpression of SOD1 reduces apoptosis mainly by inhibition of the caspase-9-mediated pathway in murine cardiac grafts. The dissection of these cell-signaling events should lead us much closer to new preventive and therapeutic
approaches for ischemia–reperfusion injury and GCAD after cardiac transplantation. Gene therapy approaches that target SOD1 may be important for overcoming GCAD in clinical transplantation.

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