Gene Transfer of Manganese Superoxide Dismutase Extends Islet Graft Function in a Mouse Model of Autoimmune Diabetes

Suzanne Bertera,[1] Megan L. Crawford,[1] Angela M. Alexander,[1] Glenn D. Papworth,[2] Simon C. Watkins,[2] Paul D. Robbins,[3] and Massimo Trucco[1]

Islet transplantation is a promising cure for diabetes. However, inflammation, allorejection, and recurrent autoimmune damage all may contribute to early graft loss. Pancreatic islets express lower levels of antioxidant genes than most other tissues of the body, and β-cells in particular are sensitive to oxidative damage. Therefore, damage from oxidative stress may pose a major obstacle to islet replacement therapy in that both the islet isolation and transplantation processes generate oxygen radicals. To determine whether antioxidant gene overexpression in isolated pancreatic islets can prevent oxidative damage and prolong islet function after transplantation, we used the NOD mouse model to study oxidative stress encountered during both transplantation and autoimmune attack. We transferred an antioxidant gene, manganese superoxide dismutase (MnSOD), by adenoviral infection into isolated islets that were transplanted into streptozotocin-treated NOD*scid* recipient mice. Functioning islet grafts were subsequently exposed to diabetogenic spleen cells and monitored until graft failure. The results show that islet grafts overexpressing MnSOD functioned ~50% longer than control grafts. This significant prolongation of graft function suggests that the antioxidant activity of MnSOD is beneficial to transplanted islet survival and may be used in combination with other strategies aimed at islet graft protection. *Diabetes* 52:387–393, 2003

Islet transplantation for young patients with type 1 diabetes is not currently considered as an option to replace the traditional therapy of daily insulin injections. The risks associated with chronic diabetes (managed with insulin therapy) are certainly less damaging for the patient than the risks associated with the chronic use of the immunosuppressive drugs necessary to prevent transplant rejection. Even with the success of the nonsteroidal antirejection drug regimen of the Edmonton protocol (1), islet transplantation is still considered only for adult patients who have a history of complications such as end stage renal failure; those who already have or are receiving another transplanted organ; or those who, despite strict adherence to insulin therapy, cannot adequately regulate their blood glucose levels (2).

If a way can be found to circumvent the need for a chronic immunosuppressive treatment while preserving donor islet function, then the relatively low risk and generally uncomplicated surgical procedure required to transplant allogeneic islets could become the therapy of choice for young patients with newly diagnosed diabetes. Islet replacement may also help curtail the development of long-term complications associated with the disease (3).

Tissue-specific gene therapy may provide a way to protect islet grafts from the host environment without systemic immunosuppression. Genes may be transferred ex vivo to the donor islets before transplantation to provide protection from the host's immune response in the microenvironment surrounding the graft (4).

Antioxidant genes are not highly expressed in pancreatic islets compared with other tissues of the body (5,6). For this reason, islets may be particularly susceptible to cytotoxic superoxide radicals generated during the inflammatory immune response that promotes type 1 diabetes onset or occurs to some degree with any transplanted tissue. Isolated islets should be protected from oxidative stress during and after transplantation. This could lessen the early graft loss as a result of ischemia/reperfusion injury and nonspecific inflammation (7,8). MnSOD (or SOD2) is a nuclear encoded mitochondrial antioxidant enzyme that is expressed in response to the inflammatory cytokines interleukin (IL)-1β (9,10), γ-interferon (IFN-γ) (11,12), and tumor necrosis factor-α (TNF-α) (13). Previous studies have shown that overexpression of MnSOD protects insulinoma and other tumor cell types from IL-1β damage in vitro (14,15).

This study was designed to determine whether protecting islets from oxidative stress can prolong islet function in vivo after isolation and transplantation in an autoimmune animal model without the added complication of allorejection. The MnSOD antioxidant enzyme was overexpressed in islets isolated from young, prediabetic NOD mouse donors. These MnSOD overexpressing or control islets were transplanted into a syngeneic but immunocom-

From the [1]Division of Immunogenetics, Department of Pediatrics, University of Pittsburgh School of Medicine, Rangos Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania; [2]Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania; and [3]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Suzanne Bertera, PhD, Children's Hospital of Pittsburgh, Rangos Research Center, 3460 Fifth Ave., Pittsburgh, PA 15213. E-mail: subst5@pitt.edu.

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FACS, fluorescence-activated cell sorter; IFN-γ, γ-interferon; IL, interleukin; KRB, Krebs-Ringer's Buffer; MnSOD, manganese superoxide dismutase; NO, nitric oxide; PI, propidium iodide; SNAP, S-nitroso-N-acetylpenicillamine; STZ, streptozotocin; TNF-α, tumor necrosis factor-α

promised mouse model (NOD*scid*). Before transplantation, NOD*scid* mice were rendered diabetic with streptozotocin (STZ). After transplantation and return of euglycemia, donor islets were challenged with spleen cells from spontaneously diabetic NOD mice. These challenged grafts were then followed until hyperglycemia returned. Our results show that the mice that received islet transplants overexpressing MnSOD were euglycemic an average of 50% longer than those that received either untreated islets or islets carrying the LacZ marker gene.

RESEARCH DESIGN AND METHODS

Experimental animals. NOD/LtJ female mice 3–4 weeks of age and NOD*scid* female mice 8–12 weeks of age were obtained from Jackson Laboratories (Bar Harbor, MA). They were kept in our American Association for Laboratory Animal Science–certified animal facility of the Rangos Research Center and housed in microisolator caging within National Institutes of Health guidelines for animal care in a specific pathogen-free environment. Animal protocols for this experiment were approved by the Children's Hospital of Pittsburgh Animal Research and Care Committee.

Islet isolation and culture. Mouse islets were isolated from the exocrine pancreas by collagenase digestion under sterile conditions. The procedure follows the classical previously published method (16) with slight modifications. After separation on a Ficoll gradient, the islets were further purified by hand picking to eliminate any remaining exocrine tissue. Whole islets were maintained in culture medium consisting of RPMI 1640 medium supplemented with 10% FCS, 20 mmol/l HEPES, 1% L-glutamine, 1% penicillin/streptomycin, and 50 μmol/l β-mercaptoethanol (2-ME) at 37°C in an atmosphere of 95% air, 5% CO_2. All culture reagents were purchased from Life Technologies (Grand Island, NY). Collagenase Type V, Ficoll type 400, and 2-ME were purchased from Sigma (St. Louis, MO).

Adenoviral vectors. An E1- and E3-deleted, first-generation recombinant adenovirus (replication-deficient) was used as a vector in these experiments. Human MnSOD cDNA was cloned and sequenced previously (17), and its function was described by Wong et al. (18). The transgene was excised from the pRK5-MnSOD plasmid by digestion with *Eco*RI and *Pvu*I (19). The resulting 700-bp fragment was then cloned into pAD.CMV Link that contains the CMV immediate early promotor/enhancer and an SV40 poly A site. Recombinant adenovirus was generated by co-transfection of *Nhe*I cut pAD plasmid with *Cla*I cut Ad5.sub360 (E1/E3-deleted) viral DNA (19) to insert the MnSOD cassette into the E1 region of the adenovirus. The adenovectors encoding the LacZ and GFP marker genes have been described previously (20).

Gene transfer to islets. Islets were incubated with the Ad-MnSOD, Ad-LacZ, or Ad-GFP vectors in serum-free medium at a multiplicity of infection of 100 at room temperature for ~1 h with agitation every 15 min. A multiplicity of infection of 100 was chosen based on dose-response experiments showing an infection rate of 70–90% of the surface area of the islets without cytotoxic effects (21,22). After infection, islets were placed back in complete medium and incubated for 2–3 days before further analysis to allow for transgene expression.

Cell preparation and fluorescence-activated cell sorter analysis. Two days after Ad-GFP infection, islets were dissociated to single cells with enzyme-free dissociation buffer (Gibco-BRL), washed, and placed in fluorescence-activated cell sorter (FACS) medium (Hanks' balanced salt solution with 0.1% sodium azide and 0.5% BSA). They were then incubated with a Glut-2 mAb (Chromaprobe) for 20 min and then washed and fixed with 1% paraformaldehyde. Flow cytometry was done on a FACSCalibur flow cytometer (Becton Dickinson). Analysis was performed on groups of 200 islets isolated and infected on three separate occasions.

Antibody staining of MnSOD. Whole islets infected with Ad-MnSOD were fixed in a 2% paraformaldehyde and permeabilized with 0.1% Triton-X solution. After blocking with goat serum, the primary rabbit polyclonal IgG MnSOD antibody (Upstate Biotechnology, Lake Placid, NY) was added for 2 h. A fluorophore-labeled secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), was added for a 1-h incubation protected from light. Hoechst 33342 (Molecular Probes) was used as a counter stain and applied for 20 min.

Multiphoton microscopy. Islets were imaged using a multiphoton laser scanning confocal microscope comprising a titanium-sapphire ultrafast tunable laser system (Coherent Mira Model 900-F), Olympus Fluoview confocal scanning electronics, and an Olympus IX70 inverted microscope with custom-built power attenuation and external PMT detection systems. The three-dimensional multiphoton data stack acquisition used two-photon excitation at 800 nm (Hoechst 33342) and 870 nm (Alexa Fluor 488), with fluorescence emission detected respectively using D450/50 mol/l and HG510/50 steep bandpass emission filters (Chroma, Brattleboro, VT).

RT-PCR. For RT-PCR, total RNA was extracted from whole islets using TRIzol reagent according to the manufacturer's directions (Life Technologies). First-strand cDNA was synthesized from total RNA by RT reaction using the SuperScript Preamplification System (Life Technologies), and the reaction was run on a Perkin-Elmer GeneAmp PCR System 9600 thermocycler (Perkin-Elmer). Oligonucleotide primers were synthesized in house using an Applied Biosystems synthesizer, model 394-08. MnSOD primers are 25 mer each in length covering nucleotides 128–152 for the upper primer and 388–412 for the lower primer with sequence as follows: 5′ primer-GCTGGCTCCGGCTTTGGGGTATCTG; 3′ primer-GCTGAGGTTTGTCCAGAAAATGCTA. These primers amplify a section of the MnSOD gene that is specific for the human sequence and yields a PCR product of 284 bp. Primer sequences were obtained from Epperly et al. (19). As an internal control for islet RNA, the murine β-actin gene was amplified with the primer sequences 5′ primer-ATCCGTAAAGACCTCTATGC (nucleotides 945–964) and 3′ primer-AACGCAGCTCAGTAACAGTC (nucleotides 1,212–1,231), yielding a 287 bp PCR product (23). The same PCR conditions were used for both MnSOD and β-actin amplifications: one cycle of 94° for 3 min; 35 cycles of 94° for 30 s, 59° for 50 s, 72° for 90 s; and one cycle of 72° for 5 min. Each 25-μl reaction mixture contained 2.5 μl of 10× buffer, 1.5 μl of 25 mmol/l $MgCl_2$, 0.5 μl of 10 mmol/l dNTPs, 0.5 μl of 10 pmol upper primer, 0.5 μl of 10 pmol lower primer, 0.25 μl *Taq* polymerase, 1 μl of cDNA, and 18.25 μl of distilled H_2O. Amplification products were visualized by electrophoresis on a 1% agarose gel. Fragment size was confirmed using a 100-bp DNA ladder (Boehringer Mannheim, Mannheim, Germany).

Assessment of islet cell viability. The assay that we chose to determine cellular damage of whole islets was based on a method by Liu et al. (24) in which viability was determined by a semiquantitative test using propidium iodide (PI), a vital dye that can permeate only cells with damaged membranes. The percentage of dead cells in the islet is then approximated by visual enumeration. PI was added to whole islets for 4–5 min before examination under a fluorescent microscope with a rhodamine (TRITC) filter. The number of dead or damaged (PI-positive) cells was counted by two independent observers blinded to islet group identity. In some instances, Hoechst 33342 was added before PI for 20 min as a counterstain. Because an average-size murine islet (~150 μm in diameter) consists of ~1,000 cells, an islet was considered nonviable when ≥500 cells (>50%) stained positive for PI. When an islet had <500 cells staining positive for PI, it was considered viable. An average of 35 islets were counted for each islet group in each independent set of experiments. Each set of experiments was performed in triplicate.

Glucose stimulation assay. Whole islets were cultured in first low (3 mmol/l) and then high (20 mmol/l) concentrations of glucose in Krebs-Ringer's Buffer (KRB). KRB consisted of 115 mmol/l NaCl, 24 mmol/l $NaHCO_3$, 5 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 25 mmol/l HEPES, 0.1% BSA, and either 3 or 20 mmol/l glucose. The static incubation assay was performed in a 96-well flat-bottomed culture plate with 2–3 islets/well and 3–6 duplicate wells for each islet group. After a 30-min preincubation in 3 mmol/l KRB, supernatant from each well was collected after each 30-min incubation, and the concentration was measured using a mouse insulin enzyme-linked immunosorbent assay (ALPCO Diagnostics). This kit has a measurable range of 250–7,500 ng/ml. β-cell function was measured as the fold increase in insulin secretion when going from a low to high glucose concentration (21).

Cytotoxic exposure in vitro. Islets from uninfected, Ad-LacZ–infected, and Ad-MnSOD–infected groups (30–40 islets per group) were exposed to three different agents in vitro: oxygen free radicals, nitric oxide (NO), and inflammatory cytokines. Each agent was tested in triplicate on independently infected islet groups for a total of ~100 islets of each type tested per agent. For the first set of experiments, alloxan was used as an oxygen free radical generator (25,26). Alloxan was dissolved in a cold 0.01% HCl solution and used immediately after preparation. Islets were exposed to 4 mmol/l alloxan for 5 min at room temperature. The islets were then washed, placed into fresh complete medium, and incubated at 37°C for an additional 2 h before being examined for cellular damage. Second, the three islet groups were exposed to a generator of NO radicals, S-nitroso-N-acetylpenicillamine (SNAP). SNAP produces NO and disulfides as it degrades. Similar compounds such as SIN-1 and SNP were not used because they release products other than NO that also mediate cell death. SNAP was dissolved in DMSO to stabilize the compound before it was added to islet cultures. Islets were incubated with medium containing 5 mmol/l SNAP for 18 h at 37°C and then examined. The third test exposed the islet groups to inflammatory cytokines that have been reported to initiate events in vivo that may lead to β-cell death. (27) On the basis of previous studies, islets were cultured with 50 units/ml recombinant mouse IL-1β (Genzyme, Cambridge, MA), 10^3 units/ml TNF-α, and 10^3 units/ml IFN-γ

(R & D, Minneapolis, MN) for 48 h to induce β-cell damage before being examined (24,28).

STZ treatment. NOD*scid* recipients were rendered diabetic with a single high dose (200 mg/kg) of STZ delivered intraperitoneally in a 0.9% saline solution (29). The animals were monitored by blood glucose readings from the tail vein until hyperglycemia was established. An animal was considered diabetic after two consecutive readings of 300 mg/dl or higher.

Islet transplantation and graft recovery. A total of 400 islets were transplanted per mouse. Under sterile conditions, the recipient animal was anesthetized with a 2.5% solution of avertin, injected intraperitoneally at a dose of 0.015–0.017 ml/g body wt (30). The kidney was externalized through a small incision in the flank. The islets were aspirated into PE50 tubing (Harvard Apparatus, Holliston, MA), then inserted into the space between the capsule and the kidney. The kidney was replaced in the abdominal cavity, and the incision was closed with small wound clips. Uninfected, Ad-LacZ–infected, or Ad-MnSOD–infected islets isolated from 3- to 4-week-old NOD mice were transplanted into a total of 40 STZ-treated NOD*scid* animals. Of these animals, 29 were challenged with spleen cells from diabetic NOD mice, 10 were monitored for long-term function (≥90 days), and 1 was killed after 14 days for histological examination of the graft. For graft recovery, the graft-bearing kidney was removed. When hyperglycemia was evident in 2–5 days after graft removal, the animal was considered graft-dependent.

Autoimmune challenge in vivo. Spleens were removed from spontaneously diabetic NOD female mice (blood glucose >300 mg/dl). A single-cell suspension was prepared by manual disruption of the spleen and passage through a 40-μm nylon mesh. The cells were washed, counted, and resuspended at a concentration of 66.7×10^6 cells/ml. NOD*scid* recipients to be challenged received an intraperitoneal injection of $\sim 20 \times 10^6$ spleen cells (31).

Histologic analysis. Paraffin sections (4 mm) were cut from graft-bearing kidneys of treated animals. Tissue sections were stained with either an insulin (diluted 1:150; DAKO, Carpinteria, CA) or an MnSOD (diluted 1:300; Upstate Biotechnology, Lake Placid, NY) primary antibody followed by biotinylated secondary antibody (30 min) and labeled streptavidin (30 min). Chromogen substrate 3,3′-diaminobenzidine (Sigma, St. Louis, MO) was added for 10 min for enzymatic stain development. Mayer's hematoxylin was applied as a counterstain.

Statistical analysis. A two-sample test of binomial proportions with a 95% CI was used to determine significant differences between groups for the in vitro results. A two-sample *t* test for independent samples with unequal variances was used to test for statistical significance of the mean time of graft function for the experimental animal group in comparison with the control animal groups.

RESULTS

Adenoviral gene transfer to islets. Human MnSOD expression was demonstrated by immunohistochemistry performed on islets transfected with the adenoviral vector. Figure 1 is a confocal microscopic montage of a typical islet infected by Ad-MnSOD. This figure depicts serial cross-sections from different focal planes throughout the islet. Although in this example only the layer of cells surrounding the surface of the islet seem to be infected, our study demonstrates that these cells provided enough antioxidant expression to protect significantly the islet as a whole from exogenously encountered oxygen radicals.

FACS analysis. The percentage of cells expressing the transgene after Ad-GFP infection was found to be 18–32% of the total number of islet cells, varying slightly with the group of islets tested. The number of cells expressing Glut-2 alone was 59–76%, and those expressing both GFP and Glut-2 was 4–8% of the total cells of the islet (data not shown).

Detection of MnSOD transgene. RT-PCR amplification was used to detect the presence of human MnSOD mRNA in the Ad-MnSOD–infected islets. The gel picture in Fig. 2 shows that MnSOD mRNA was detected in the three independently infected Ad-MnSOD islet preparations (*lanes 1–3*) but not in uninfected or Ad-LacZ–infected islet preparations (*lanes 4* and *5*, respectively). The primers used were specific for the human gene as they did not amplify MnSOD in murine kidney (*lane 6*), which is known to express abundant levels of endogenous MnSOD (5).

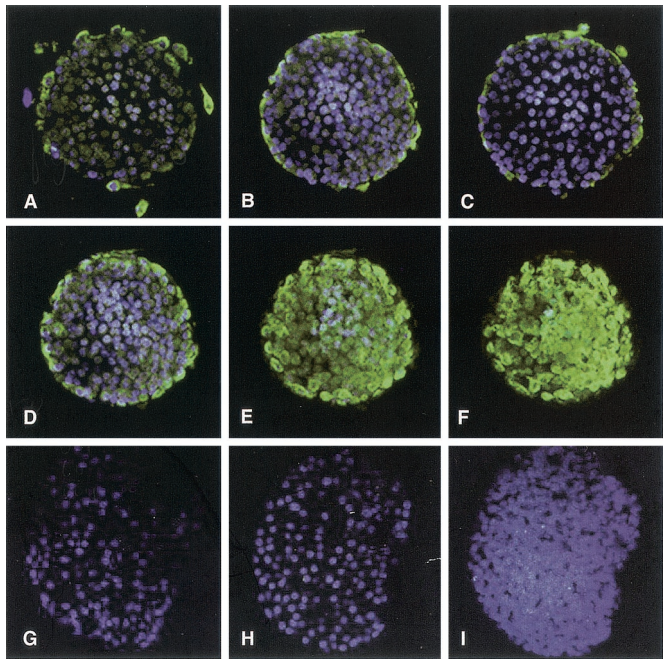

FIG. 1. Cell infection pattern of islet exposed to Ad-MnSOD. *A–F*: Confocal montage of a single islet infected with Ad-MnSOD viral vector (100 MOI). *G–I*: Control islet infected with Ad-LacZ (100 MOI). Both islets were stained with an MnSOD antibody and FITC secondary. FITC (green) indicates the presence of MnSOD protein. Cell nuclei are counterstained in blue with Hoechst 33342. Original magnification 20×.

Islet viability after cytotoxic exposure. Whole islets were counted as viable or nonviable after exposure to one of three different agents known to be toxic to islet cells: alloxan, SNAP, and inflammatory cytokines. Figure 3 shows examples of the method used to quantify the results. Figure 3*IA* shows viable islets characterized by <500 (<50%) dead cells per islet, as indicated by only a few PI-positive (red) cells. Nonviable islets are shown in Fig. 3*IB* and are characterized by >500 (>50%) dead cells (PI-positive) in each islet. This method was used to determine the number of viable islets remaining after exposure to each agent in vitro. Figure 3*II* shows representative islets from each treatment group. Figure 3*IIA–C* shows islets after alloxan exposure. The Ad-MnSOD islets have the least amount of dead cells in this group of cells, suggesting that they were protected from alloxan damage.

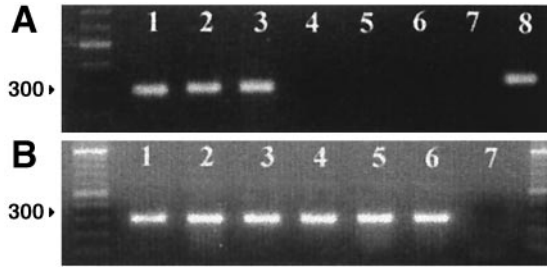

FIG. 2. RT-PCR products of modified and unmodified islets amplified with primers specific for human MnSOD. *Lanes 1–3*: Three different groups of Ad-MnSOD–infected mouse islets. *Lane 4*: Unmodified controls. *Lane 5*: Ad-LacZ–infected islets. *Lane 6*: Mouse kidney. *Lanes 7 and 8*: Negative and positive controls. Panel *B* shows β-actin amplification of samples in panel *A*.

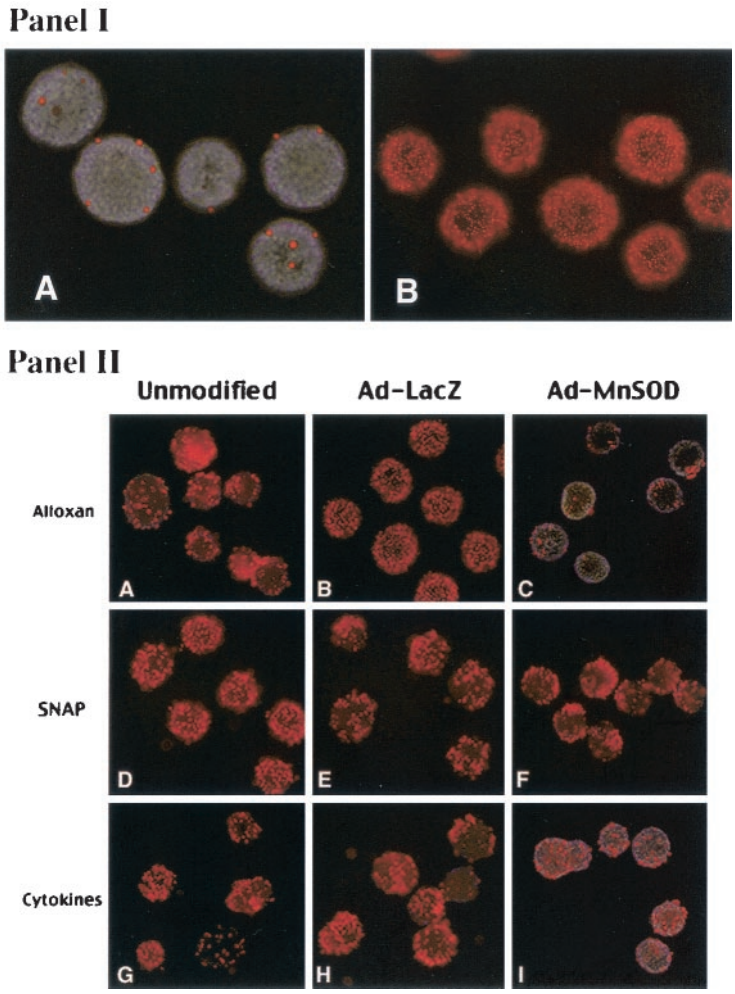

FIG. 3. Panel I: Method of vital dye staining of viable and nonviable mouse islets. Fluorescent images of islets stained with Hoechst 33342 nuclear dye (blue) and propidium iodide (PI) in red. Only cells without membrane integrity (i.e., either dead or damaged) cannot exclude PI. *A*: Normal viable mouse islets. *B*: Heavily damaged, nonviable islets. Panel II: Examples of unmodified Ad-LacZ and Ad-MnSOD–infected islets after exposure to cytotoxic agents in vitro. *A–C*: Islets exposed to 4 mmol/l alloxan for 5 min and examined after 2 h. *D–F*: Islets exposed of 5 mmol/l SNAP for 18 h. *G–I*: Islets exposed to 50 units/ml IL-1β, 10^3 units/ml TNF-α, and 10^3 units/ml IFN-γ for 48 h. Original magnification 20×.

Figure 3*IID–F* are islets that were exposed to SNAP. Approximately 80% of the islets were nonviable at the end of this exposure period regardless of adenovirus modification. These data indicate that the MnSOD enzyme confers minimal protection to the islets from exposure to SNAP. Figure 3*IIG–I* shows islets that were cultured with a combination of IL-1β, TNF-α, and INF-γ for 48 h. Ad-MnSOD–treated islets exhibited significantly less cell death than either the untreated or the Ad-LacZ–treated islets, suggesting some protection against exposure to proinflammatory cytokines.

A summary of these in vitro results is shown in Table 1. Ad-MnSOD–modified islets show a statistically significant increase in the number of viable islets over controls with a 95% CI for both the alloxan- and the cytokine-treated islets. In contrast, islets exposed to NO show minimal survival advantage as a result of MnSOD overexpression.

TABLE 1
Summary of islet viability after in vitro challenges

	Percentage of viable islets*		
Treatment	Unmodified	Ad-LacZ	Ad-MnSOD
Alloxan	10% (n = 112)	4% (n = 102)	95% (n = 110)
SNAP	15% (n = 105)	20% (n = 100)	22% (n = 90)
Cytokines	20% (n = 98)	29% (n = 104)	66% (n = 96)

*Percentage of islets was determined by dividing the number of viable islets remaining after treatment (those with <500 dead cells) divided by the total number of islets tested per treatment (n). Results are from three separate experiments of the three different islet groups.

Assessment of islet function. Insulin secretion for unmodified, Ad-LacZ infected, and Ad-MnSOD infected islets was measured in response to low (3 mmol/l) and high (20 mmol/l) concentrations of glucose. The data shown in Table 2 are average values from three separate experiments obtained for each treated and nontreated control group. The ability of all of the nontreated islet groups to increase insulin production six- to sevenfold when exposed to increased levels of glucose indicates that the adenoviral vector and the MnSOD and LacZ transgenes did not impair β-cell responsiveness. The same assay was then run after islet groups were exposed to alloxan, SNAP, and inflammatory cytokines. When looking at the fold increase in insulin secretion, the islets overexpressing MnSOD are most protected against alloxan exposure. The increase after SNAP exposure of less than onefold probably reflects that the insulin was released as the β-cells were dying. The increase in secretion after cytokine exposure probably also reflects dying β-cells even though the MnSOD islets show a slightly more elevated response than do control islets.

Long-term function and graft dependence. Of the 40 animals that received a transplant, 11 of the graft recipients were followed as unchallenged controls. One animal

TABLE 2
Glucose-stimulated insulin release of control and adenovirus-treated islets with and without exposure to cytotoxic agents

Treatment	Glucose Concentration	Unmodified	Ad-LacZ*	Ad-MnSOD*
No Treatment	3 mmol/l	511 ± 95	622 ± 113	490 ± 82
	20 mmol/l	3,490 ± 130	3,933 ± 93	3,450 ± 179
	Fold increase	6.8	6.3	7.0
Alloxan	3 mmol/l	1,462 ± 186	592 ± 254	587 ± 135
	20 mmol/l	3,643 ± 260	1,498 ± 367	4,123 ± 119
	Fold increase	2.5	2.5	7.0
SNAP	3 mmol/l	1,245 ± 49	640 ± 42	1,195 ± 35
	20 mmol/l	715 ± 162	495 ± 261	980 ± 113
	Fold increase	0.6	0.8	0.8
Cytokines	3 mmol/l	1,145 ± 132	390 ± 41	2,128 ± 220
	20 mmol/l	1,381 ± 241	504 ± 26	4,266 ± 367
	Fold increase	1.2	1.3	2.0

Values shown are ng/ml insulin after 30 min of static incubation. Table shows means ± SD of duplicate samples from three to six experiments. Fold increase was calculated by dividing the mean value of the high glucose concentration (20 mmol/l) by the mean value of the low concentration (3 mmol/l). *Islets were infected with either Ad-LacZ or Ad-MnSOD at 100 multiplicity of infection.

was killed after 14 days for histological examination of the graft (Fig. 4*B*) and the other 10 were monitored for ≥90 days for long-term function (data not shown). All of these animals, three with uninfected, three with Ad-LacZ–infected, and four with Ad-MnSOD–infected islet grafts, were euglycemic for >90 days. After removal of the graft-bearing kidney, hyperglycemia returned in each animal with blood glucose levels >300 mg/dl by day 4 after graft removal.

In vivo transgene expression. MnSOD expressing islet grafts were histologically examined for long-term function and transgene expression. Tissue sections from graft-bearing kidneys were stained with antibodies to either insulin or MnSOD. The antibodies used here react to both mouse and human protein. Figure 4 shows 4-mm sections of graft-bearing kidneys of transplanted, nonchallenged animals. Figure 4*A* is from an uninfected, syngeneic islet graft that was included as a control. This graft was removed and examined after 96 days. Staining with the MnSOD antibody reveals that the mouse kidney underneath the islet graft is rich in endogenous MnSOD, and staining can be easily seen in the kidney tubules. There is no staining seen in the graft itself, however, suggesting that there is not enough MnSOD in unmodified mouse islets to be detected by this method. Figure 4*B* and *C* are also stained for MnSOD, but these islet grafts had been infected before transplantation with the Ad-MnSOD vector. Both Fig. 4*B*, from a graft harvested at 14 days (short-term), and Fig. 4*C*, harvested at 140 days (long-term), show MnSOD staining throughout the grafted islets. Fig. 4*D* is a serial section to Fig. 4*C* and was stained for insulin. The dark stain here suggests that these islets were still functioning well after 140 days.

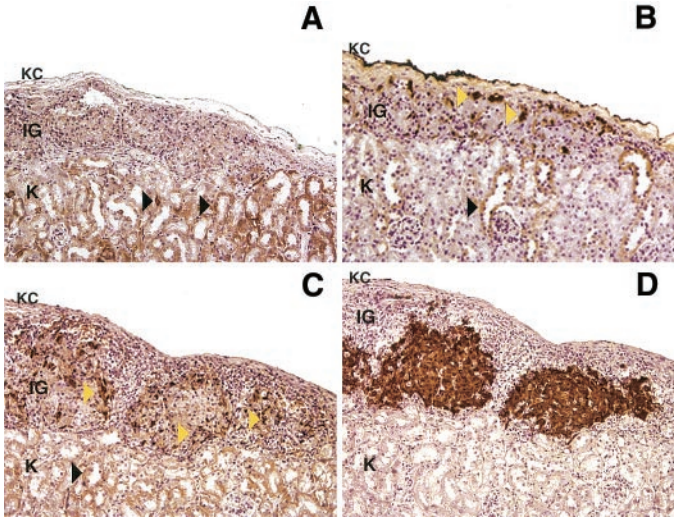

FIG. 4. Transverse sections of islet grafts (IG) transplanted under the capsule (KC) of the kidney (K). Black arrows in the kidney and yellow arrows in the islet grafts point out examples of MnSOD staining. Section *A* is from an uninfected normal islet graft included as a control. This section shows that the tubules of a normal mouse kidney express enough endogenous MsSOD to stain darkly positive for the protein but that the normal mouse islets of the graft do not. Sections *B* and *C* are from Ad-MnSOD–infected islet grafts that have been harvested at two different time points. Section *B* was harvested at 14 days, and section *C* was harvested at 140 days. Both show dark staining for MnSOD in the islets that is not seen in the uninfected control (*A*). Section *D* is stained for insulin expression (dark stain) and is a serial section of the graft in *C*. Sections are H&E counterstained. Original magnification 20×.

Islet function after autoimmune challenge. Of the 40 animals that received a transplant, 29 were challenged with T-cells from NOD mice and were followed until hyperglycemia was evident. In addition to these 29 animals, 22 animals that did not receive a transplant were challenged in parallel as controls for disease transfer. Table 3 summarizes the length of time that these islet-engrafted animals or animals that did not receive a transplant remained disease-free after the introduction of activated T-cells.

The results show that duration of euglycemia after spleen cell transfer for uninfected (mean 30 ± 8) and Ad-LacZ grafts (mean 27 ± 12) did not differ significantly from the controls that did not receive a transplant (mean 31 ± 5). In contrast to the control groups, the islet grafts expressing the MnSOD enzyme showed a significant increase in survival time of 50% over any of the three control groups with a mean value of 45 ± 11 days. This result is statistically significant when compared with each of the control groups ($P = 0.001$).

TABLE 3
Duration of graft function after autoimmune challenge

Transplant type*	Mean†	Median	n	Individuals (days normal)‡
Unmodified	30 ± 8	33	8	19, 20, 26, 33, 33, 35, 35, 42
Ad-LacZ	27 ± 12	30	10	7, 14, 20, 21, 29, 31, 32, 32, 35, 49
Ad-MnSOD	45 ± 11§	44	11	32, 34, 35, 36, 44, 44, 47, 48, 48, 53, 69
Nontransplanted	31 ± 5	31	22	17, 26, 26, 28 (x3), 29, 30, 30, 31 (x3), 32 (x3), 33 (x3), 34, 36, 39, 40

*Groups were unmodified (normal) islet grafts, islets infected with either Ad-LacZ or Ad-MnSOD (100 MOI), and animals that did not receive a transplant to serve as controls for the adoptive transfer of disease. Each graft consisted of 400 islets. †Mean graft survival time in days ± SD. ‡Days of euglycemia after autoimmune challenge for individuals of each transplant type. The day the animal was challenged with 20 million spleen cells from a diabetic NOD was considered day 0. §Mean function length of MnSOD-treated islet grafts is significant when compared with each of the control groups ($P = 0.001$).

DISCUSSION

The present study was designed to test the ability of MnSOD to protect transplanted islets from damage as a result of inflammation generated during transplantation and subsequent autoimmune attack. The presence of human MnSOD mRNA and the mature protein in the adeno-infected murine islets was confirmed by RT-PCR and antibody staining. The MnSOD and control islets had comparable responses to glucose stimulation, indicating no modification in insulin responsiveness after adenoviral infection. Studies have shown that alloxan damages islets both in vitro and in vivo (32,33). Additional studies have shown that cells expressing MnSOD are protected from the oxidant damage mediated by alloxan (14,26). Our study demonstrates that islets expressing MnSOD are protected from alloxan damage. The Ad-MnSOD islets were still functioning and viable, whereas there was extensive damage to the uninfected and Ad-LacZ–infected islets. The MnSOD enzyme actively dismutates superoxide anions to hydrogen peroxide and molecular oxygen, thus neutralizing the oxygen radicals generated by alloxan degradation.

However, MnSOD conferred very little protection against the NO-generating compound SNAP. The mechanism for this may be associated with the formation of excess peroxynitrite ($ONOO^-$). This highly cytotoxic anion, formed by superoxide and NO, takes place at a faster rate than the dismutation reaction (34). Two studies by MacMillan-Crow et al. (35,36) reported that peroxynitrite targets nitration of tyrosine residues (especially Tyr34) in human MnSOD, resulting in enzyme inactivation. They found 3-nitrotyrosine and increased but inactive MnSOD in the tubular epithelium of chronically rejecting human renal allografts. Enzyme inactivation allows increased levels of peroxynitrite that may function to promote apoptosis (37).

The above observations seem to coincide with the results of our cytokine experiments. The inflammatory cytokines TNF-α, IFN-γ, and IL-1β mediate the generation of oxygen free radicals and NO (12,13,27,38). In this context, as NO levels increase, the dismutation activity of MnSOD is not fast enough to prevent some oxygen radicals from reacting with NO to form peroxynitrite that, in turn, deactivates MnSOD. Thus, the MnSOD antioxidant would be able to protect the islets only until peroxynitrite formation reached a level that affected a decrease in enzyme activity. Studies are now under way to determine whether this is the mechanism that eventually leads to graft failure of Ad-MnSOD–modified islets.

The results of the confocal microscopic study showed the adenoviral infection pattern of a typical islet. The peripheral cells of the islet are infected by the adenovirus construct, yet the antioxidant overexpression in the outer layers of cells seems to be sufficient to protect the entire islet, including the non–transgene-bearing cells, from exogenous oxygen radicals. Our in vitro findings after oxygen radical exposure support this interpretation.

Protection was also seen in vivo. The antioxidant-bearing transplanted islets were able to withstand the onslaught of factors involved in this model of islet destruction 50% longer ($P = 0.001$) than uninfected, Ad-LacZ–infected, or endogenous (nontransplanted) islets. Although it can be argued that the method of giving the transplanted islets a chance to engraft before introducing diabetogenic T-cells is artificial, this model is a valid, quantitative test of the potential protective properties of the transgene being studied. If this test had been completely successful (i.e., if the grafts did not eventually fail after autoimmune challenge), then more demanding tests that resemble natural disease onset and clinical transplantation situations more closely would have been attempted.

Because in our model, graft function eventually failed, it is reasonable to believe that overexpression of the MnSOD gene alone will not be enough to protect islets from destruction after transplantation into the diabetic host. However, the MnSOD oxygen radical scavenger has therapeutic value in extending the life of autoimmune challenged islet grafts. A combination of this antioxidant enzyme with another gene (or genes) acting on other pathways of cell death could potentially provide improved protection from the host immune response and disease recurrence. Although the actual mechanism for the destruction of transplanted pancreatic islets remains unknown, it is clear from this and other studies that NO and, consequentially, peroxynitrite generation must be controlled to provide greater graft protection.

When perfected, this type of virtual encapsulation, provided by transgenic overexpression in the outer cell layers, could act as a barrier to protect the interior (mostly β) cells of the islet. This would provide the type of protection for transplanted islets that was theorized as being the most beneficial for young patients, i.e., islet protection without systemic immunosuppression therapy.

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