Metallothionein Protects Islets from Hypoxia and Extends Islet Graft Survival by Scavenging Most Kinds of Reactive Oxygen Species

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Islet transplantation is a promising therapy for Type 1 diabetes, but many attempts have failed due to early graft hypoxia or immune rejection, which generate reactive oxygen species (ROS). In the current study, we determined that transgenic overexpression of the antioxidant metallothionein (MT) in pancreatic beta cells provided broad resistance to oxidative stress by scavenging most kinds of ROS including H₂O₂, peroxynitrite radical released from streptozotocin, 3-morpholinosydnonimine (SIN-1), and superoxide radical produced by xanthine/xanthine oxidase. MT also reduced nitric oxide-induced beta cell death. A direct test of hypoxia/reperfusion sensitivity was made by exposing FVB and MT islets to hypoxia (1% O₂). MT markedly reduced ROS production and improved islet cell survival. Because MT protected beta cells from a broad spectrum of ROS and from hypoxia, we considered it to be an ideal candidate for improving islet transplantation. We first tested syngeneic transplantation by implanting islets under the kidney capsule of the same strain, FVB mice, thereby eliminating the immune rejection component. Under these conditions, MT islets maintained much greater insulin content than control islets. Allotransplantation was then tested. MT transgenic and normal FVB islets were implanted under the kidney capsule of BALB/c mice that were previously treated with streptozotocin to induce diabetes. We found that MT islets extended the duration of euglycemia 2-fold longer than nontransgenic islets. The benefit of MT was due to protection from ROS since nitrotyrosine staining, an indicator of free radical damage, was much lower in MT grafts than in FVB grafts. The time course of protection suggested that the major mode of MT action may have been protection from hypoxia or hypoxia/reperfusion. These data demonstrate that treatment with a broad spectrum antioxidant protects islets from ROS damage such as that produced during the early phase of islet transplantation.

Transplantation of pancreatic islets is considered to be one of the most effective treatments for Type 1 diabetes (1). Recently, islet transplantation using the Edmonton protocol (2) achieved insulin independence in 12 out of 15 diabetic patients for 1 year. However, widespread application of transplantation therapy is still limited by the need for more than one donor pancreas per recipient and difficulties in maintaining long term euglycemia (1). One obstacle has been that many islets are lost during the initial stages of transplantation (3, 4). Shortly after implantation, islet grafts function poorly, and many transplanted beta cells undergo apoptosis prior to stable engraftment. This increases the mass of islets needed to achieve euglycemia (5). Unfortunately, there is an extreme shortage of human pancreatic islet donors. Therefore, instead of increasing the number of islets implanted, a more desirable strategy is to improve islet graft survival during the early stages of transplantation. However, to date, no impressive regimen has been devised to prevent early graft damage.

Reactive oxygen species (ROS) are involved in both early islet graft loss and longer term immune rejection. Shortly after implantation, islet grafts are exposed to nonspecific inflammatory events (3) that generate proinflammatory cytokines, nitric oxide, and ROS. These local, nonspecific inflammatory mediators attack implanted islets. In the rat islet transplant model, grafts are destroyed by high levels of nitric oxide released from allogenic (6) or syngenic (7) macrophages. In addition, the graft suffers from an initial period of hypoxic ischemia after transplantation. Oxygen tension measured within the islet graft is initially very low (8). In fact, newly transplanted islets are essentially avascular, leaving them with insufficient oxygen and nutrients until the process of revascularization is completed. This ischemic microenvironment, followed by reperfusion as a consequence of revascularization, produces conditions known to induce detrimental ROS in transplanted organs (9–11).

The damaging effects of ROS on pancreatic islets have been widely investigated in diabetes (12, 13) as well as in islet transplantation (14–16). Exposure of isolated human islets (17), rodent islets (18, 19), or beta cell lines (20) to ROS markedly inhibits beta cell function and results in beta cell death. When compared with other cell types, pancreatic beta cells are particularly susceptible to destruction caused by ROS (21). This is probably because islet cells contain very low levels and activities of several ROS detoxifying systems (22). Recent studies reported that early islet graft loss could be ameliorated by various antioxidant combinations such as α-tocopherol (23) and other vitamins (24). Other reports have investigated transgenic overexpression of a single, specific antioxidant protein. Protection from the mitochondrial superoxide radical by adenoviral-mediated expression of Mn-superoxide dismutase (25) was suf-

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1 The abbreviations used are: ROS, reactive oxygen species; MT, metallothionein; STZ, streptozotocin; SIN-1, streptozotocin, 3-morpholinosydnonimine; SNAP, S-nitro-N-acetylpenicillamine; CM-H₂DCFDA, 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; ANOVA, analysis of variance.
efficient to extend islet graft function by 50%. However, in our laboratory, we found that overexpression of the specific, hydrogen peroxide detoxifying protein catalase failed to prevent insulin loss in syngeneic islet grafts (18). To test whether beta cell protection could be improved by protecting against multiple species of ROS, several laboratories have expressed more than one antioxidant enzyme. In insulin-secreting RINm5F cells, combined expression of CuZn-superoxide dismutase plus catalase or CuZn-superoxide dismutase plus glutathione peroxidase provided more protection against hydrogen peroxide, superoxide radical, and nitric oxide than expression of either transgene alone (26, 27). Co-administration of superoxide dismutase and catalase in cultured rat islets more effectively protected against alloxan-induced destruction than either antioxidant alone (28). These results indicated that enhanced protection was possible by scavenging more than one species of ROS. Therefore, we hypothesized that a significant improvement of islet graft survival could be achieved if the donor islets were protected by a potent antioxidant protein with a broad spectrum of ROS scavenging activity, such as metallothionein (MT).

MT is a low molecular weight, cysteine-rich, and highly inducible protein that binds heavy metal with high affinity. MT appears to play an important role in metal metabolism and detoxification. Due to its many cysteine residues, MT also functions as a potent antioxidant. Elevated expression of MT in pancreatic beta cells, produced either by zinc induction (29, 30) or by transgenic techniques (31), has been shown to protect from streptozotocin (STZ)-induced beta cell damage and diabetes. Studies in cell-free systems have demonstrated that MT is able to scavenge a wide range of ROS including superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide at higher efficiencies than other antioxidants such as GSH (32–34). However, it is not certain that MT will provide such a broad spectrum of antioxidant function in vivo.

The present study was designed to test whether overexpression of a single protein with broad spectrum antioxidant activity improved beta cell protection. MT is first shown to be a broad spectrum antioxidant in islets, capable of scavenging many different types of ROS. We then show that MT islets exposed to hypoxia produce fewer ROS and suffer reduced cell death. In syngeneic transplants, MT markedly improved the preservation of graft insulin content, and in allogeneic transplants, MT extended the period of euglycemia 2-fold.

MATERIALS AND METHODS

Animals—MT transgenic mice were established in our laboratory on the FVB strain with pancreatic beta cell overexpression of the human MT II gene, as described previously (31). The HMT-1 transgenic line was used in this study since this line has the highest expression of MT. Recipient BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in ventilated cages at the University of Louisville Research Resources Center with free access to water and standard mouse diet. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

Chemicals—Streptozotocin, 3-morpholinosydnonimine (SIN-1), N-nitro-N-acetyl-penicillamine (SNAP), hypoxanthine, xanthine oxidase, collagenase (type V), Ficoll, and trypsin were obtained from Sigma. Hanks’ balanced salt solution, RPMI 1640 medium, and fetal bovine serum were supplied by Invitrogen. Rat insulin standard was bought from Linco (St. Charles, MO). Rabbit antiserum to guinea pig insulin was purchased from BioGenex (San Ramon, CA). Monoclonal anti-nitrotyrosine antibody was supplied by Cayman (Ann Arbor, MI). 5-(6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) was purchased from Molecular Probes (Eugene, OR). Alamar Blue was purchased from BIOSOURCE International (Camarillo, CA).

Islet Preparation—The isolation procedure was based on a modification of the method of Gotot et al. (35) and has been described previously (31). Isolated islets were cultured overnight in RPMI 1640 medium containing 10% bovine serum albumin, 2% penicillin-streptomycin, 50 U/ml insulin, and 200 U/ml recombinant human EPO prior to the transplantation and in vitro studies, which were performed on the second day.

Measurements of ROS Production—To measure ROS production in single islet cells, the overnight cultured islets were first dispersed into individual cells by treatment with trypsin (0.0075%) in Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ solution at 37 °C for 10 min followed with mechanical dispersal by 50 times of repeat pipetting, as described previously (36). A cell membrane-permeable and oxidant-sensitive fluorescent dye CM-H$_2$DCFDA was used to measure ROS. The dispersed islet cells were loaded with 10 μM CM-H$_2$DCFDA for 30 min followed by three washes of fresh culture medium without phenol red. The cells were resuspended in culture medium without phenol red. After the cells were counted, the dispersed islet cells containing CM-H$_2$DCFDA was distributed into a 96-well plate at a concentration of 40,000 cells/well in 200 μl of islet culture medium without phenol red. The exogenous sources of ROS (H$_2$O$_2$, SIN-1 or hypoxanthine/xanthine oxidase) were added quickly to the wells. With the addition of ROS, the increase in fluorescence intensity in each well was measured on a fluorescent microplate reader (Tecan, Durham, NC) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The data were expressed as fluorescent intensity per 40,000 cells.

ROS production in whole islets following hypoxia treatment was measured with a method modified from the procedure of Ye et al. (37) in our laboratory. Briefly, the hypoxia-treated or untreated FVB control or MT transgenic islets were cultured with 5 μM CM-H$_2$DCFDA for 30 min followed by three washes of fresh culture medium. The fluorescence of each islet was activated at an excitation wavelength of 485 nm and recorded at an emission wavelength of 530 nm. ROS was monitored from randomly sampled individual islets using an Olympus IX70 inverted microscope equipped with a digital cooled CCD camera. Images were analyzed with ImagePro software (Media Cybernetics, Silver Spring, MD). More than 100 islets from at least three separate islet isolations were studied for each group. The results were expressed as the mean fluorescence intensity.

Nitric Oxide in Vitro Studies—Isolated FVB and HMT-1 islets were exposed to different concentrations of a nitric oxide donor, SNAP, for 24 h. The apoptotic and necrotic DNA was detected with an anti-biotin/anti-DNA POD ELISA$^{\oplus}$ kit (Roche Applied Science) based on the manufacturer’s instructions. Briefly, 40–50 islets were cultured for 24 h in 500 μl of fresh culture medium in a 1.5-ml microtube with or without SNAP treatment. After treatment, the microtube was centrifuged at 2000 × g for 10 min at 4°C. The supernatant was removed as the necrosis DNA sample. The pellet was lysed with 100 μl of lysis buffer for 30 min at room temperature. The microtube was centrifuged again at 2000 × g for 10 min at 4°C. The supernatant was removed as the apoptotic DNA sample. To quantify the necrotic and apoptotic DNA, both DNA samples were added to the streptavidin-coated microplate contained in the kit. All values were normalized to islet total DNA measured by picoGreen DNA quantification (Molecular Probes). In Vitro Hypoxia Treatment—Isolated FVB and HMT-1 islets were cultured in a 96-well plate placed in a sealed incubator chamber saturated with 1% O$_2$, 5% CO$_2$, and 94% N$_2$ at 37°C. After incubation for 24, 48, or 72 h, the islet cell viability was assessed by measuring islet metabolism as indicated by Alamar Blue absorbance. The data for cell viability were calculated as the percentages of viability of control cells that were cultured normally in 95% air, 5% CO$_2$. ROS production in islets after hypoxia for 7 h was measured with CM-H$_2$DCFDA fluorescence dye, as described above.

Alamar Blue Assay—The Alamar Blue assay, which incorporates a redox indicator that changes color and fluorescence in response to cell metabolic activity, is a commonly used method to determine viability and/or proliferation of mammalian cells (38) and microorganisms (39). In our studies, 15 overnight-cultured FVB control islets or HMT-1 transgenic islets were handpicked into 200 μl of fresh culture medium (no phenol red) containing 1:20 diluted Alamar Blue in a 96-well plate. Islets were cultured for 4 h, and the Alamar Blue fluorescence was measured on a fluorescent microplate reader (Tecan, Durham, NC) at the excitation wavelength of 535 nm and the emission wavelength of 595 nm. This measurement provided an absorbance value indicating the pretreatment metabolic activity and was used to normalize the post-treatment metabolic activity. After three washes with fresh culture medium, islets were cultured in 200 μl of culture medium under conditions for 1:5 dilution of hypoxia (1% O$_2$). After 4 h of hypoxia, 1:5 diluted Alamar Blue for a final dilution of 1:20. The color was developed for another 4 h, and the fluorescence was measured again. Islet cell viability was calculated as...
the ratio of fluorescence after treatment to the fluorescence before treatment.

Syngeneic Transplantation—50 FVB and HMT-1 islets were transplanted separately under each kidney capsule in a normal FVB mouse according to a modification of the procedure of Montana et al. (40). Recipient mice were anesthetized via intraperitoneal injection with 10 μl/g of a solution containing 10 mg/ml ketamine and 3.2 mg/ml xylazine. The left side kidney was first externalized through a small incision and kept moist with saline. 50 islets were picked into a gel-loading pipette tip (0.5-mm diameter) mounted on a 1-cc Hamilton syringe (Reno, NV) and allowed to settle. The tip was inserted through an incision beneath the kidney capsule, and the islets were gently forced out of the tip. The body wall and the skin were closed with sutures. Then the transplantation to the right kidney was performed by the same procedure. 6 days later, grafts were recovered by removing a portion of the kidney many fold larger than the visualized graft site. This portion of the kidney was homogenized in acid ethanol (23 ethanol, 2 HCl, 75 H2O, v/v/v) for insulin extraction. To determine the insulin content, we used an anti-insulin antibody-coated tube radioimmunoassay kit (Diagnostic Products, Los Angeles, CA) and rat insulin standards according to the manufacturer’s instructions. Data were calculated from four independent experiments. * and ** indicate that MT and FVB values were different at the same SNAP concentration (p < 0.05 and p < 0.01, respectively, by one-way ANOVA test). Vertical bars indicate S.E.

**FIG. 1.** ROS production in dispersed FVB control and transgenic MT islet cells. ROS was measured with the fluorescent dye CM-H2DCFDA as described under “Materials and Methods.” Islet cells were not treated (A), treated with 100 μM H2O2 (B), treated with 30 μM SIN-1 (C), treated with 1 mM hypoxanthine plus 2 milliunits of xanthine oxidase (D). The solid circles indicate FVB control islet cells. The open circles indicate MT transgenic islet cells. This figure is typical of three independent experiments.

**FIG. 2.** Nitric oxide-induced damage in FVB and MT transgenic islets exposed to SNAP for 24 h. A, representative photomicrographs of FVB and MT islets after SNAP treatment. The concentrations of SNAP are shown on the left. Similar results were obtained in four independent experiments. Magnification was at ×100. B, necrosis and apoptosis in SNAP-treated islets. Cell death was measured as described under “Materials and Methods.” Data were calculated from four independent experiments. * and ** indicate that MT and FVB values were different at the same SNAP concentration (p < 0.05 and p < 0.01, respectively, by one-way ANOVA test). Vertical bars indicate S.E.
islet grafts. The survival time of MT islet grafts was significantly longer than that of FVB grafts ($p < 0.01$ by Mantel-Cox log rank test).

Data Analysis—Data are presented as the means ± S.E. Statistical significance was performed by one-way or two-way ANOVA and Dunnett’s post hoc (2-tailed) test. Kaplan-Meier survival analysis and Mantel-Cox log rank test were used to analyze islet graft survival time. Mann-Whitney rank sum test was used to analyze nitrotyrosine staining in islet grafts. Computations were done using statistical programs from SPSS (version 10.0) and Sigmastat (version 2.03).

Allotransplantation—200 FVB or HMT-1 islets were transplanted under each kidney capsule (400 total) with the same protocol described above. Before transplantation, the recipient BALB/c mice, aged 8–12 weeks, were injected with a single dose of STZ (intraperitoneal 220 mg/kg) to induce diabetes. Only mice with blood glucose ranging from 350 to 500 mg/dl were used as recipients for transplantation surgery. Tail blood glucose levels of the transplanted mice were monitored every other day with a glucose meter (OneTouch Ultra, Life Scan, Milpitas, CA). Graft failure was defined as a return of hyperglycemia (nonfasting blood glucose $> 250$ mg/dl) on two consecutive measurements. Islet graft survival time was calculated as the number of days from transplantation to the first day of hyperglycemia of two consecutive measurements. Grafts from some recipients were recovered 6 days after transplantation and sectioned for hematoxylin/eosin and consecutive measurements. Grafts from some recipients were recovered 6 days after transplantation and sectioned for hematoxylin/eosin and nitrotyrosine staining. In separate experiments in which only one kidney was transplanted, we verified that removal of the graft containing kidney caused a return to glucose levels over 600 mg/dl. 

Immunohistochemistry for Nitrotyrosine—Islet grafts were fixed in 10% formaldehyde in 0.1 mol/liter phosphate buffer (pH 7.2), dehydrated in an ascending graded series of ethanol, and subsequently infiltrated with paraffin. Serial sections were cut at 5 μm, mounted on polylysine-coated slides, and then deparaffinized in xylenes and a descending graded series of ethanol. For nitrotyrosine staining, slides were treated with target retrieval solution (DAKO, Carpinteria, CA) followed by M.O.M. mouse Ig blocking reagent (Vector Laboratories, Burlingame, CA). Nitrotyrosine monoclonal antibody (Cayman) was added to the slides at a concentration of 10 μg/ml and incubated overnight at 4 °C. After three washes in phosphate-buffered saline, slides were incubated with biotinylated anti-mouse IgG reagent, followed by ABC reagent, and developed with DAB as chromagen. Slides without primary antibody treatment were used as negative control. For quantification of nitrotyrosine production, 5 MT graft slides and 5 FVB control graft slides from three independent recipients were scored on a scale from 1 to 5 grades based on the severity of nitrotyrosine staining by two researchers blind to the identity of the section.

Statistical significance was measured by two-way ANOVA. Vertical bars indicate the S.E. ** indicates that MT ROS production in FVB and MT islets exposed to hypoxia for 7 h. The MT transgene reduced hypoxia-induced ROS production in islet cells. ROS production of whole islets was measured as described under “Materials and Methods.” Data are mean values of more than 100 islets/group. a** indicates that hypoxia increased ROS levels in FVB control islets ($p < 0.001$). b** indicates that MT ROS levels were lower than FVB values following 7 h of hypoxia ($p < 0.001$). Statistical significance was measured by two-way ANOVA. Vertical bars indicate the S.E.
RESULTS

Broad Spectrum ROS Scavenging by MT—Our previous study (31) demonstrated that the MT transgene protected against ROS released by STZ. To determine whether MT could protect against many species of ROS, beta cells were exposed to H₂O₂, the superoxide radical produced by hypoxanthine and xanthine oxidase, and the peroxynitrite radical released from SIN-1. Beta cell ROS production measured with CM-H₂DCFDA (Fig. 1) was dramatically reduced by the MT transgene following exposure to all three sources. Islets were also exposed to nitric oxide by incubation with SNAP, a nitric oxide donor. SNAP did not increase CM-H₂DCFDA fluorescence in our assay; consequently, we assessed MT-induced resistance to nitric oxide by observing changes in islet morphology and quantitating islet cell death. As shown in Fig. 2, MT islets were resistant to SNAP-induced morphological damage and cell death as measured by DNA cleavage. These data demonstrate that MT is able to efficiently scavenge all or most forms of free radicals.

In Vitro Hypoxia Studies—Hypoxia and reoxygenation are known to induce ROS production (41, 42). To determine whether MT could reduce hypoxia-induced ROS production, we exposed isolated FVB control and MT transgenic islets to 1% O₂. This is close to the microenvironment that transplanted islets are subject to at the graft site (43). ROS production was measured with CM-H₂DCFDA following 7 h of hypoxia and return to normoxic medium. The data in Fig. 3 illustrate the effect of the hypoxia incubation on ROS generation. Both FVB and MT islets produced more ROS following exposure to hypoxia. However, the ROS generation was significantly greater in FVB islets than in MT islets. To determine whether this reduction in ROS generation translated into improved islet survival, we assessed islet viability with the metabolism-sensitive dye Alamar Blue. MT and FVB islets were exposed to 1% O₂ for 24, 48, and 72 h and then assayed with Alamar Blue. As shown in Fig. 4, islet cell metabolism was markedly decreased by hypoxia treatment. Overexpression of MT provided significant resistance to this effect at all time points analyzed.

Transplantation—Ischemia and/or ischemia reperfusion are toxic stressors that grafted islets must contend with during the early phase of transplantation while revascularization progresses. To determine whether the ability of MT to protect against hypoxia and reoxygenation would be beneficial in a real transplantation situation, islets were transplanted into syngeneic, nondiabetic FVB recipients. These grafts are subject to ischemia but not to immune rejection or glucose toxicity. MT transgenic islets were implanted under one kidney capsule,
and control FVB islets were implanted under the other kidney capsule of the same recipient. 6 days after transplantation, the graft was recovered for determination of insulin content. Insulin content is a commonly used indicator of transplanted islet health (40-44). Fig. 5 illustrates that both FVB and MT islet grafts lost part of their insulin content after transplantation. However, FVB islets were more severely affected. MT islets retained about 60% of their initial insulin content, whereas FVB islets retained less than 20% of their initial insulin (p < 0.01).

We also carried out allotransplantation studies using FVB control islets or MT transgenic islets implanted under the renal capsules of STZ-induced diabetic BALB/c mice (Fig. 6). 400 FVB islets were able to maintain near normal blood glucose levels in recipient mice for an average period of 8.36 ± 1.67 days. The same number of MT transgenic islets was more effective. The average euglycemic period for recipients receiving MT islets was almost doubled, to 16.2 ± 2.52 days. Mantel-Cox log rank analysis of islet graft survival time indicated that MT grafts were significantly (p < 0.01) more effective in maintaining euglycemia.

To confirm that the prolonged survival of MT islets was due to protection from ROS, nitrotyrosine staining in islet grafts recovered after 6 days of transplantation was measured. Nitrotyrosine is a marker for the presence of peroxynitrite. As shown in Fig. 7, nitrotyrosine staining in MT grafts was markedly decreased when compared with nitrotyrosine staining in control FVB islet grafts. This result was confirmed by blind ranking of nitrotyrosine intensity on five MT and five FVB graft slides from three different recipients per group (p < 0.02).

**DISCUSSION**

In the present study, we demonstrated that overexpression of MT reduced ROS levels in beta cells exposed to many different prooxidant stimuli. Protection from ROS helped preserve islet viability after treatment with a nitric oxide donor or prolonged exposure to hypoxia. MT transgenic islets maintained almost 3-fold higher insulin content than control islets following syngeneic transplantation. Transplantation with MT islets prolonged euglycemia by 2-fold when compared with transplantation with control islets, and MT reduced the formation of nitrotyrosine in islet grafts into allogeneic, diabetic recipients. The greatest benefit of MT was probably due to protection from hypoxia rather than due to prevention of immune rejection.

Islets are exposed to many noxious ROS during transplantation and the onset of diabetes. These different ROS can produce additive or synergistic toxicity to beta cells (13, 45). Therefore, it is important that antioxidant treatment protects against as many different types of ROS as possible. However, most enzymatic antioxidant proteins are specific in their substrate and thus limited in the types of ROS that they can inactivate. Catalase and glutathione peroxidase decompose primarily hydrogen peroxide, and superoxide dismutase activity is specific to superoxide. An example of the restricted protection provided by a single antioxidant enzyme was the recent report (25) that elevated Mn-superoxide dismutase expression protected islets from alloxan but provided no protection from nitric oxide. Unlike enzymatic antioxidants, MT has broad antioxidant activity. In cell-free systems, MT has been shown to scavenge nitric oxide radical (46), superoxide radical (47), hydroxyl radical (33), and peroxynitrite (48). We confirmed that MT was effective against all of these ROS when expressed in transgenic pancreatic islets and also showed that MT reduced apoptosis and necrosis produced by nitric oxide exposure. Nitric oxide is generally considered to be an important mediator of beta cell damage produced by many stressors (49). The efficacy of MT against most ROS means that MT overexpression can provide a general test of the role of ROS in beta cell damage produced during transplantation and diabetes.

MT overexpression was able to extend the duration of euglycemia by 2-fold in allogeneic recipient diabetic mice. This amounted to an increase of 8 days. The relatively short period of improved graft function suggests that MT protects most effectively against the damage produced in the early phase of transplantation, prior to immune rejection of the graft. Consistent with this, we found that MT protected graft insulin content in syngeneic recipients, which is in the complete absence of immune rejection. Although there are many factors that contribute to early graft loss, evidence suggests that hypoxia is one important factor. Islet grafts are initially an essentially avascular tissue. Mean P O2 measured in syngeneic transplanted islets (8) is 5–10 mm Hg (about 1% O2). Low levels of oxygen can impair islet cell function and cell survival. It has been shown that there is a strong correlation between reduced oxygen supply and the occurrence of apoptosis in isolated human and rat islets (50). Islet grafts are particularly prone to destruction caused by ischemia followed by reperfusion during the process of revascularization (51). The loss of cell viability produced by *in vitro* hypoxia in the present study is consistent with this sensitivity. MT was very effective in blocking ROS generation and cell death produced by hypoxia. Because our fluorescence detection system was not sealed from air, the generation of ROS may have been due to hypoxia or hypoxia/reperfusion.

Several prior studies have used systemic antioxidants to extend the efficacy of islet transplants (52-55) with varying degrees of success. To improve outcome and focus protection on the islet, Bertera et al. (25) transplanted islets with adenosinergic-mediated overexpression of Mn-superoxide dismutase into an NODscid model of immune rejection. This produced a 50% extension of graft function, which was thought to be due to protection from immune attack. We found that MT produced a similar or modestly greater degree of protection in our transplant system. Since experiments were performed in different models, a direct comparison of the efficacy of the two antioxidants is impossible. Clearly neither transgene provided complete protection. MT is cytoprotective and protected against hypoxia, whereas Mn-superoxide dismutase is mitochondrial and was thought to protect against immune attack. Potentially, the combination of these two differentially localized antioxidants with different modes of action may produce additive or synergistic benefit.

Successful clinical trials using the Edmonton protocol (2) provide encouraging results for islet transplantation. However, the need for multiple donors hampers successful and efficient clinical islet transplantation. The shortage of organ donors is an intractable problem. Therefore, any significant improvement in graft efficiency is worthwhile. MT improved islet function by reducing damage secondary to hypoxia. This demonstrates that powerful ROS scavengers are beneficial for transplanted islet survival and may be useful in combination with other strategies aimed at islet graft protection.

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**REFERENCES**
