Donor Treatment With Carbon Monoxide Can Yield Islet Allograft Survival and Tolerance

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Treatment of animals or certain cells with carbon monoxide (CO), a product of heme degradation by heme oxygenase-1 (HO-1), has potent anti-inflammatory and antiapoptotic effects that contribute to the survival of transplanted organs. We report here that inducing HO-1 in, or administering CO to, only the donor can be used in a therapeutic manner to sustain the survival of transplanted allogeneic islets. Similar treatments of only the islets or only the recipient are also salutary. Administering CO only to the donor frequently leads to long-term survival of those islets in untreated allogeneic recipients, which are then antigen-specifically tolerant. Several proinflammatory and proapoptotic genes that are strongly induced in islets after transplantation in the untreated situation were significantly suppressed after administering CO to the donor without further treatment. These included tumor necrosis factor-α, inducible nitric oxide synthase, monocyte chemoattractant protein-1, granzyme B, and Fas/Fas ligand, all of which contribute to the pathogenesis of the rejection of transplanted islets. This correlated with a lesser infiltration of recipient macrophages into the transplanted islets. Our present findings show that induction of HO-1 in, or administration of CO to, only the donor, islets, or the recipient or combinations of such treatments improve allogeneic islet survival. *Diabetes* 54:1400–1406, 2005

Transplantation of islets is an important approach to the treatment of diabetes. Although recent changes in immunosuppression and other facets of the transplant procedure have improved results (1,2), cells in the islets after transplantation frequently undergo apoptosis, and islet function is compromised. Thus, more islets are needed for each transplant, leading to a greater shortage of islets.

Data from others and ourselves show that the induced expression of heme oxygenase-1 (HO-1) in recipients, which leads to the expression of HO-1 in islets as well as elsewhere, has salutary effects in terms of sustaining graft survival (3,4). These findings have been interpreted to suggest that the expression of HO-1 protects the islet cells from apoptosis as well as suppresses the proinflammatory response of the recipient (5,6).

HO-1 degrades heme into three products: carbon monoxide (CO), biliverdin, and Fe^{++}. Biliverdin is converted to bilirubin by biliverdin reductase, and Fe^{++} stimulates an iron pump that removes free iron from the cells as well as leads to the upregulation of ferritin, an iron-binding protein. Each of these products has protective (antiapoptotic and/or anti-inflammatory) properties, and one or more of them likely accounts for the protection afforded by HO-1 in any given situation (7–9).

There is accumulating evidence that in the majority of the disease models in which HO-1 is beneficial, administration of exogenous CO can substitute for HO-1 action (10–13). That is, if HO-1 is absent or its action blocked, the administered CO will have the "same" effects as if HO-1 were expressed. Based on these findings, we tested the effects on islet graft survival of CO administration to the donor and the recipient, or to either one alone. Unexpectedly, treatment of only the donor with CO led to prolonged islet graft survival in the untreated recipient and tolerance in those untreated recipients carrying long-term (>100 day) surviving islets. Furthermore, the proinflammatory response normally seen in the islets after transplantation was markedly suppressed. This novel finding, which quite likely contributes in a significant manner to the prolongation of survival and recipient tolerance, was unexpected. Because CO has a very short half-life, the only effect can be on the islets themselves. Thus, the lack of a proinflammatory response mounted by the recipient must somehow relate to changes that CO effects in the transplanted islets.

RESEARCH DESIGN AND METHODS

Male DBA/2 (H-2^d) and B6AF1 (H-$2^{b,k/d}$) mice 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Recipients were rendered diabetic by a single intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) at a dose of 225 mg/kg. At 5 days after streptozotocin administration, animals with two consecutive (daily) blood glucose levels >350 mg/dl were used as islet recipients. All animals were raised under standard conditions; the protocol was approved by the animal care committee of the Beth Israel Deaconess Medical Center.

Islet isolation and transplantation. Islets were isolated from DBA/2 donors by collagenase V (Sigma) digestion and purification on density gradients, as

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CoPP, cobalt protoporphyrin-IX; HO-1, heme oxygenase-1; IL, interleukin; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; MST, mean survival time; TNF-α, tumor necrosis factor-α.

described elsewhere (14). Islets (400–500 equivalents) for the experiments in which HO-1 was induced and 350–400 islet equivalents for the experiments with CO were transplanted under the left kidney capsule of the diabetic recipients. Glucose levels were measured twice weekly after transplantation using an Accu-Chek Glucometer (Roche, Indianapolis, IN). Glucose levels of <200 mg/dl were considered normoglycemic. Grafts are deemed rejected when two consecutive daily nonfasting glucose levels were >300 mg/dl after a period of primary graft function.

Cobalt protoporphyrin-IX and CO treatment. Cobalt protoporphyrin-IX (CoPP; MP Biomedicals, Irvine, CA) was used to induce HO-1 expression in the donors and the recipients. CoPP was dissolved in 0.1 N sodium hydroxide, and the pH was adjusted to 7.4 with hydrochloride acid. Treatment of the donors involved a single dose of CoPP at 20 mg/kg given 24 h before harvesting the islets, whereas treatment of the recipients involved injections of CoPP at 20 mg/kg every 48 h from day −1 to day +7 (4). Administration of CO was performed in a chamber containing 250 parts per million (ppm) CO. Treatments were for 20 h in the donors and continuously from day −1 till day 13 in the recipients. Isolated islets were also cultured in CO-saturated medium for 24 h after their isolation and before transplantation. "Saturation" of the medium was achieved by bubbling CO into the medium for 10 min before the islets were placed into the medium and then culturing in a 1% CO and 5% CO_2 atmosphere. For studies involving HO-1 induction and cytokine expression, the islets were transplanted immediately after isolation. In those cases in which either donors or recipients were treated with CO, the islets were cultured for 24 h in CMRL medium with 10% serum in 5% CO_2 at 37°C after isolation and before transplantation.

Tolerance test. The kidney under which the initial islets were transplanted was removed from a number of animals that had islets surviving long term (>100 days), and then islets syngeneic with the original donor (DBA/2) were transplanted under the other kidney without further treatment. If those second transplanted islets also survived >100 days without further treatment, the recipients were considered tolerant. Antigen-specific tolerance was assessed by transplanting islets of a third-party donor (DBA/1, H-2^q) that does not share either class I or class II antigens with the original donor.

Graft collection for cytokine determination. Islet grafts were collected at 1, 3, 7, 10, 15, and 20 days after islet transplantation. Under anesthesia, the left kidney of the mouse was exposed; grafts can be identified as a whitish area under the kidney capsule. The graft was excised along with the adherent capsule and was snap-frozen in liquid nitrogen for the extraction of total cellular RNA.

RNA isolation and cDNA synthesis. Total cellular RNA was extracted using an RNA kit (Qiagene, Charworth, CA). Then, 1 μg of total RNA was transcribed to cDNA in the presence of murine leukemia virus transcriptase (Applied Biosystems, Foster City, CA). The reaction was performed in a final volume of 100 μl containing 1 × RT buffer containing 5.5 mmol/l $MgCl_2$, 0.5 mmol/l each dNTP, 0.4 units/μl RNase inhibitor, 2.5 μmol/l random hexamers, and 3.125 units/μl reverse transcriptase. Samples were held at 25°C for 10 min and incubated at 48°C for 30 min. Reverse transcriptase was inactivated by heating at 95°C for 5 min, and cDNA samples were stored at −15 to −25°C.

Quantitation of gene expression by real-time RT-PCR. Real-time RT-PCR was performed using the ABI Prism 7700 sequence detection system. Materials used in real-time RT-PCR were purchased from Applied Biosystems unless otherwise stated. RT reactions were set up in a total volume of 25 μl by using 2 × Taqman Universal PCR Master Mix containing 5 mmol/l $MgCl_2$, 200 μmol/l of dNTP, 0.05 units/μl AmpliTaq Gold DNA polymerase, 0.01 units/μl AmpErase UNG, and a passive reference dye, ROX. Each cDNA sample was analyzed in duplicate in the presence of 1 × commercially available assay-on-demand primers together with FAM-labeled fluorogenic probes (Applied Biosystems) specially designed for each target gene. Glyceraldehyde-3-phosphate dehydrogenase expression of each sample was performed in parallel by using the predeveloped primer and VIC-labeled fluorogenic probe to standardize the amount of sample cDNA added to the reaction. Freshly isolated islet mRNA was analyzed each time and used as a calibrator. The PCR ran for 2 min at 50°C, 10 min at 95°C, and for 40 cycles composed of an incubation at 95°C for 15 s plus another at 60°C for 1 min. Relative quantification of all target genes was analyzed based on a comparative C_T method. The amount of target gene, normalized to glyceraldehyde-3-phosphate dehydrogenase and relative to the calibrator, is calculated based on the manufacturer's instructions (available from Applied Biosystems' website for the comparative C_T method at http://www.appliedbiosystems.com/support/apptech/#rt_pcr).

RT-PCR analysis. HO-1 induction in islets was detected by RT-PCR after CoPP injection. Primers for HO-1 were 5′-TGAAGGAGGCCACCAAGGAGGT (forward primer) and 5′-AGGTCACCCAGGTAGCGGGT (reverse primer). β-Actin was used as an internal control. Primers for β-actin were 5′-GCCATCCTGCGTCTGGACCTGG (forward primer) and 5′-TACTCCTGCTT GCTGATCCACA (reverse primer). The amplification condition were as follows: 5 min at 94°C followed by multiple cycles of 94°C, 55°C, and 72°C at 1 min each, 23 cycles for HO-1 and 20 for β-actin to allow cDNA amplification in the linear range.

Western blot. Protein samples (40 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with rabbit anti–HO-1 (StressGen, San Diego, CA) and mouse anti–α-tubulin (Sigma) antibodies, followed by peroxidase-labeled secondary antibodies. Signals were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfont, U.K).

Immunohistochemistry. Islet grafts were immersed in freezing medium (Tris-buffered saline; American Mastertech Scientific, Lodi, CA) together with a portion of the kidney and snap-frozen in precooled 2-methylbutanol in liquid nitrogen. Then 5-μm sections were dried at room temperature for 30 min and fixed in cold acetone for 3 min, followed by immunohistochemistry staining with peroxidase. The antibody F4/80 (Serotec, Oxford, U.K.) was used to detect the presence of macrophages in the graft. The secondary antibody was biotinylated anti-rat antibody (Vector Labs, Burlingame, CA). Avidin-biotin complex–horseradish peroxidase (Dako, Carpinteria, CA) and 3,3′-diaminobenzidine substrates were used to develop the color (Vector). Counterstaining with hematoxylin was preformed after dehydration, and slides were covered with Cytoseal 280 mounting medium (American Mastertech Scientific) for observation.

Statistical analyses. Kaplan-Meier survival curves were performed by using the Statview software, and the statistical difference was assessed by the log-rank test. Values of $P < 0.05$ were considered as significant. Survival data are expressed as mean survival time (MST) ± SE. Differences between cytokines expression were compared for statistical significance by Student's t test.

RESULTS

Induction of HO-1 and islet graft survival. Our initial experiments involved induction of HO-1 expression with injections of CoPP. HO-1, which is constitutively expressed in the spleen (data not shown), was further induced with CoPP at both the mRNA (Fig. 1*A* and *B*) and protein levels (Fig. 1*C*). In islets, HO-1 mRNA was detected within 12 h (the first time point measured) of a single injection of CoPP (20 mg/kg) in the mouse, as seen in Fig. 1*A* and *B*. Islets from CoPP-treated mice manifested HO-1 levels that were approximately half as strong as those seen in spleens from CoPP-treated mice (Fig. 1*B*). HO-1 protein levels were maintained in islets for up to 6 days postexposure in mice receiving a single dose of CoPP (Fig. 1*C*). HO-1 was strongly induced in cells surrounding the islet and marginally induced in some β-cells (data not shown). Administration of CO to a donor mouse (250 ppm, 20 h) did not result in HO-1 expression.

Islet grafts transplanted from DBA/2 to B6AF1 mice (a class I [H-2K] plus a class II [H-2I] incompatibility) were normally rejected in 18.6 ± 8.7 days (MST ± SD) without treatment ($n = 13$) (Fig. 2). None of the control islet grafts in this study led to long-term euglycemia in the recipients. Induced expression of HO-1 in both donor and recipient led to 83.3% (five of six grafts) of DBA/2 islets functioning long term (>100 days) in B6AF1 recipients ($P < 0.0001$ vs. control). The one other islet graft, i.e., that which did not survive long-term, survived for 51 days. Treatment of the recipients only led to 37.5% (three of eight grafts) long-term survival ($P = 0.0002$ vs. control). The MST of the remaining five grafts (35.2 ± 10.9 days) was also significantly longer than controls ($P = 0.007$ vs. control). Most surprisingly, treatment of donors only led to 4 of 10 long-term surviving grafts ($P = 0.0008$ vs. control). The MST of the other six grafts was 29.5 ± 11.8 days ($P = 0.068$ vs. control) (Fig. 2).

Tolerance in recipients of long-term surviving islet grafts. To test whether recipients of long-term surviving

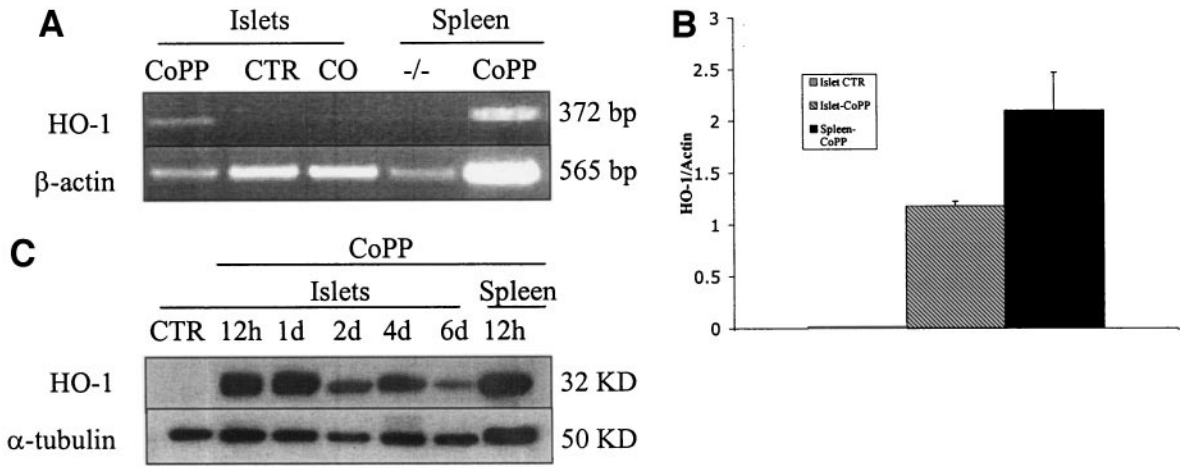

FIG. 1. HO-1 can be induced at the mRNA and the protein level in DBA/2 mice by a single 20-mg/kg injection of CoPP. *A*: RT-PCR. Samples were islets from CoPP-treated, nontreated (CTR), and CO-administered donors, and spleens from HO-1 knockout (negative control) and CoPP-injected (positive control) mice. *B*: The HO-1 expression in islets at the mRNA level was ~50% as strong as that seen in spleen 12 h postinjection. *C*: HO-1 protein expression in islets was detected as early as 12 h after a single injection of CoPP and maintained above the control level for at least 6 days. Results are representative of three independent experiments. d, day.

grafts were antigen-specifically tolerant, second islet grafts were implanted under the same contralateral kidney as that used for the first transplant in each animal. Second transplants that were syngeneic with the original donor survived long-term without any further treatment ($n = 5$), whereas the third-party islets did not show prolonged survival ($n = 3$) (Table 1).

Treatment with CO. Our results with CO treatment are remarkably similar to those in which HO-1 was induced (Fig. 3). Without treatment, DBA/2 islets transplanted into B6AF1 recipients were rejected in 21.89 ± 7.67 days ($n = 9$). CO treatment of either the donor or the recipient led to 50% long-term survival ($P = 0.0038$ and $P = 0.025$ vs. control, respectively). The percentage of surviving grafts increased to 71.4% when both donor and recipient were exposed to CO ($P = 0.0006$ vs. control). Long-term survival was achieved in 66.7% (four of six grafts) of cases when islets were cultured in a CO-saturated solution for 24 h after their isolation and before transplantation ($P = 0.0008$ vs. control). The MST of the remaining two grafts was 35 ± 4.24 days, which was not significantly different from the control.

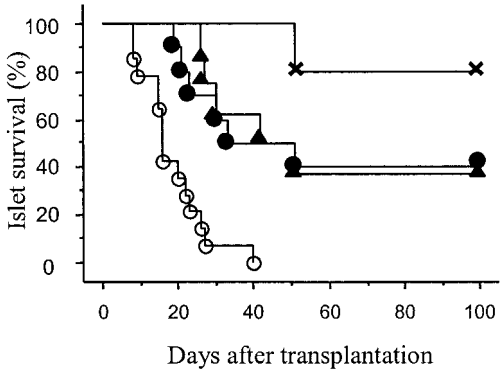

FIG. 2. Induced HO-1 expression in the donor and/or recipient led to long-term islet allograft survival. Crude islets from DBA/2 mice (400–500 islets) were transplanted under the kidney capsule of the B6AF1 recipients. Islet grafts survived significantly longer when HO-1 was induced in the donor (●, $n = 10$), the recipient (▲, $n = 8$), or both (×, $n = 6$) compared with controls (○, $n = 13$).

Tolerance with CO treatment. As with induction of HO-1, treatment with CO led to antigen-specific tolerance in all of the five recipients in which the first transplant of islets survived for >100 days. All three third-party islets were rejected without prolongation (Table 2).

Real-time RT-PCR analysis of cytokine expression. Islets transplanted from untreated donors to untreated recipients upregulated the expression of a number of proinflammatory and proapoptotic genes after transplantation, including tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), granzyme B, Fas, and Fas ligand. In an attempt to understand why donor-only treatment with CO led to graft survival, we analyzed the effect of treating only the donor on the expression profiles of these genes in the graft at 1, 3, 7, 10, 15, and 20 days posttransplantation. In groups in which only the donor was treated, TNF-α, iNOS, granzyme B, Fas, and Fas ligand were suppressed from day 1 until day 10 after transplantation compared with the nontreated controls (Fig. 4*A*–*E*). No difference in the expression levels of cytokines were observed at 15 and 20 days after transplantation, although the expression level for all cytokines was greatly reduced in both control and CO-treated groups compared with cytokine levels immediately after transplantation. In contrast, mRNA expression of the antiapoptotic gene bcl-2

TABLE 1
Tolerance test in the HO-1 induction groups

CoPP treatment	Survival days of first graft before removal	Donor strain of second graft	Second graft survival (days)
Donor + recipient	124	DBA/2	>322
Donor + recipient	105	DBA/2	>242
Donor	112	DBA/2	>171
Donor	105	DBA/2	>241
Recipient	132	DBA/2	>228
Recipient	329	DBA/1	23
Donor	173	DBA/1	29
Donor + recipient	132	DBA/1	28

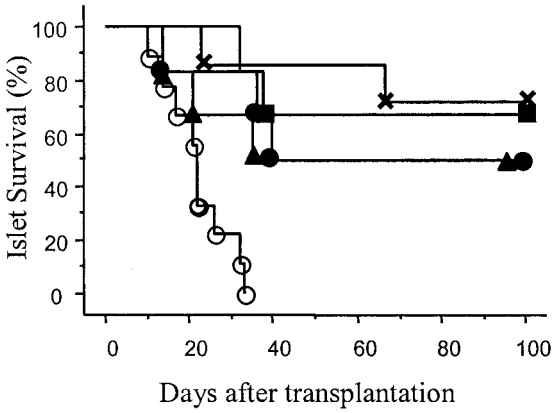

FIG. 3. CO administration only to the donor (●, $n = 6$), the islet (■, $n = 6$), or the recipient (▲, $n = 6$) led to long-term survival of allogeneic islets. A high percentage of grafts survived long-term when both donor and recipient were exposed to CO (×, $n = 7$) compared with the controls (○, $n = 9$). DBA/2 islets (350–400 islets) were transplanted under the kidney capsule of B6AF1 mice. The mean graft survival time in every CO-treated group is significantly longer than the control group ($P < 0.05$ vs. control).

was upregulated in the CO-treated group at 10 days after transplantation compared with the control (Fig. 4*F*).

Macrophage infiltration. We hypothesized that the suppression of proinflammatory cytokine expression after transplantation in the groups in which only the donor was treated was caused by less macrophage infiltration into the graft. To evaluate this, grafts were stained at 1, 7, 10, 15, and 20 days posttransplantation with antibody to the macrophage marker F4/80. Significantly fewer macrophages were found at 1 and 7 days after transplantation in the CO-treated group. There were no significant differences in the prevalence of macrophages at 10 days after transplantation. However, many fewer macrophages were found in grafts at 15 and 20 days posttransplantation in the CO-treated group compared with controls. The morphology of the islets in the CO-treated group looked near normal compared with the control grafts, in which the islets were smaller and the architecture abnormal (Fig. 5). In an attempt to explain the lesser macrophage infiltration in the grafts treated with CO, we analyzed the mRNA, using real-time RT-PCR, for monocyte chemoattractant protein-1 (MCP-1), a chemokine secreted by β-cells that can induce the migration of macrophages to an inflammatory site. Significantly decreased MCP-1 mRNA levels were observed on different days after islet transplantation in the CO-treated group compared with the controls at identical time points (Fig. 6). Thus, CO treatment in donors appears to exert anti-inflammatory effects in part by downregulating MCP-1 message.

TABLE 2
Tolerance test in the CO treatment groups

CO treatment	Survival days of first graft before removal	Donor strain of second graft	Second graft survival (days)
Donor + recipient	118	DBA/2	>304
Donor + recipient	118	DBA/2	>304
Donor + recipient	110	DBA/2	>320
Donor	112	DBA/2	>320
Recipient	110	DBA/2	>320
Donor + recipient	133	DBA/1	15
Recipient	118	DBA/1	18
Donor	105	DBA/1	15

DISCUSSION

Preservation of islet cells' viability after transplantation is a major concern. In most models used to date, there is significant apoptosis of the islet cells (15,16). This leads to the need for more donor islets, often requiring two or even three transplants to achieve a "cure" (1). Whether directly related to the death of the islets or not, rejection is still a major problem, despite the more intelligent use of immunosuppressive agents.

It has been shown in the past that inducing expression of HO-1 in the recipient leads to prolongation of islet allograft survival (4). We show in this study that CO can "substitute" for HO-1 in this regard: treatment of only the recipient with CO leads to prolongation of islet allograft survival. HO-1 expression or CO may have beneficial effects based on their anti-inflammatory and/or antiapoptotic effects on inflammatory, immune, or other cells. It has previously been shown that expression of HO-1 in, or CO treatment of, monocyte-macrophages, which are often implicated in islet cell destruction, suppresses the proinflammatory response to lipopolysaccharide while at the same time boosting the anti-inflammatory effects (increased expression of interleukin [IL]-10) both in vitro and in vivo (17–18). It has also been demonstrated that HO-1 expression or CO can suppress the T-cell immune response (19,20). We demonstrate here that treatment of only the donor, by either induction of HO-1 or administration of CO, also leads to prolonged survival of islets in the recipient, with tolerance in those recipients carrying transplanted islets for >100 days. There is accumulating evidence that expression of HO-1 in the donor organ can be critical to the survival of a graft after transplantation. In a mouse-to-rat transplantation model, in which the recipient is treated with the immunosuppressive agent cyclosporin A plus briefly with cobra venom factor to block complement action, a mouse heart graft survives indefinitely. However, if the graft is from a mouse deficient in HO-1 (a HO-$1^{-/-}$ mouse), the graft is rejected as though there was no immunosuppression. Because the wild-type donor and the HO-$1^{-/-}$ donor differ by only this one gene, this finding is critical evidence for the importance of HO-1 expression in the donor in at least some transplant situations (21,22). More recently it has been shown that induction of HO-1 in an allogeneic donor organ can facilitate the survival of that organ after transplantation (23,24). These findings, however, do not necessarily indicate that it is the effect of HO-1 on the donor organ that has any role in promoting survival. Expression of HO-1 in the heart after transplantation could lead to the secretion into the blood of the recipient of products of HO-1 action, such as CO and bilirubin, which could have anti-inflammatory or antiapoptotic effects on various cells in the recipient. The same is true of our studies of islet graft survival in which HO-1 was induced in only the donor: we have found HO-1 still being expressed 6 days after a single dose of CoPP.

The most unexpected finding of our present studies was the observation that treatment of only the donor with CO can lead to long-term survival of the islet graft in the

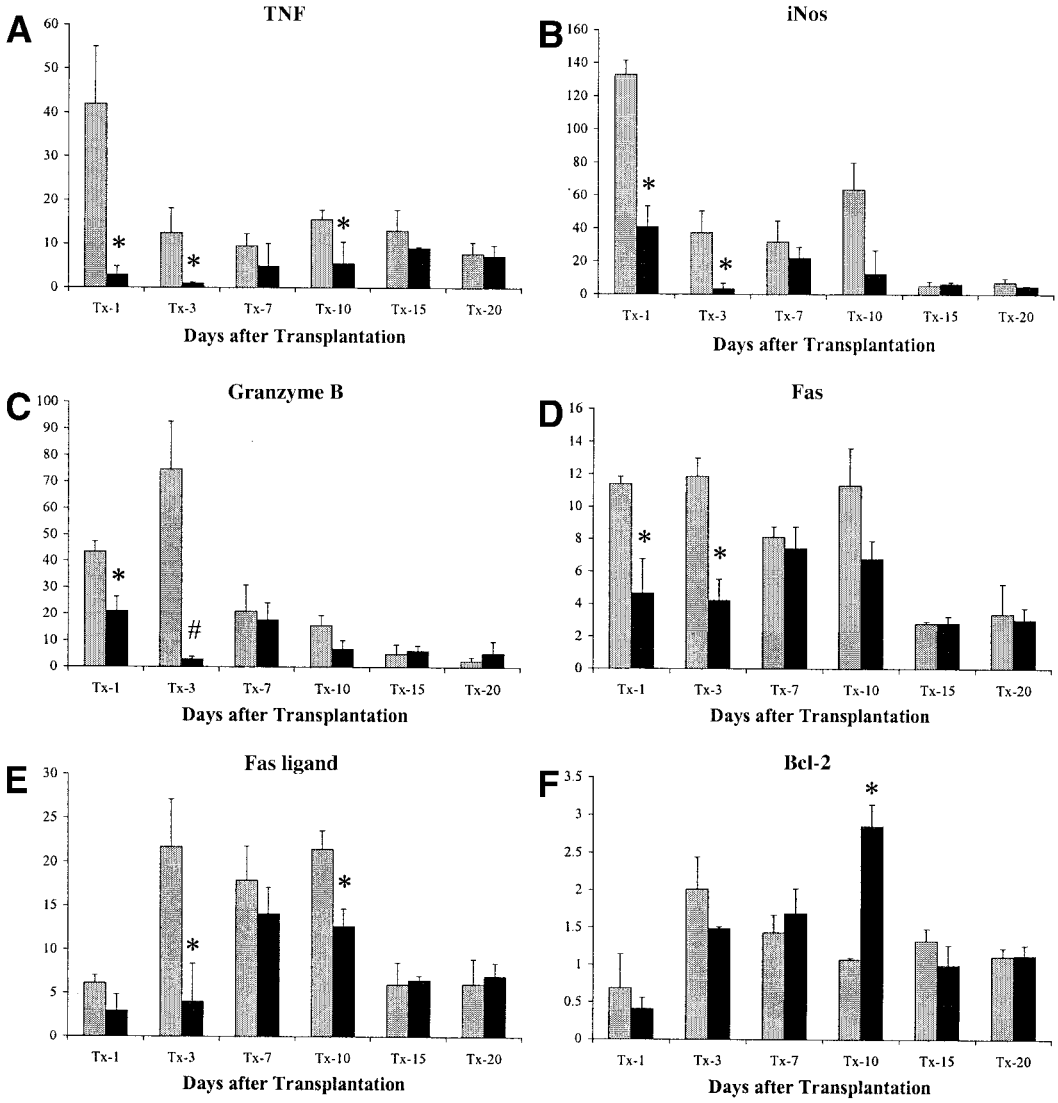

FIG. 4. Effects of donor treatment with CO on the pro-inflammatory response in islets after transplantation. Analysis by real-time RT-PCR at various days after islet transplantation showed CO administration only to the donor led to decreased mRNA expression of TNF-α (*A*), iNOS (*B*), granzyme B (*C*), Fas (*D*), and Fas ligand (*E*) and an increase of bcl-2 (*F*) compared with untreated controls. Results shown are the mean ± SD from three islet grafts at each time point. Values are expression level of genes of interest in the analyzed graft compared with freshly isolated nontreated islets. ▨, islet grafts from untreated donors; ■, grafts from CO-treated donors. *$P < 0.05$, #$P < 0.01$ vs. control.

recipient and tolerance in those recipients in which the islets survive for >100 days. CO treatment of only the donor led to a significant percentage of the islet allografts surviving long term in untreated recipients, suggesting that the CO-induced change in the islets themselves led to survival of those islets. It seems exceedingly unlikely that the CO used for treatment of only the donor would still be able to exert any effects in the recipient, given the very short half-life of CO. We thus focus our discussion on the CO-treated donors.

Our rationale for treatment of only the donor was in part based on studies of the fate of organs or tissues from brain-dead donors that manifested signs of inflammation even during the time in situ in the donor (25,26). Brain death led to macrophage infiltration and significant augmentation of macrophage-associated inflammatory molecules such as IL-1β and -6, TNF-α, and MCP-1 in rat islets (27). It seemed possible that the stress of all the procedures involved in removal of islets from a donor and the manipulations that they undergo before transplantation might also initiate inflammation (28). Thus, to the extent that one might suppress this inflammatory response by treatment of the donor, one might decrease a subsequent rejection response (29).

After transplantation to allogeneic recipients without treatment, islets become a nidus for a strong proinflammatory environment. Activated macrophages become resident in the islets and produce proinflammatory cytokines, including TNF-α, IL-1β, and others. These cytokines can induce perforin/granzyme B and Fas/Fas L expression by the cytotoxic T-cells and lead to β-cell apoptosis. We demonstrate in this study that treatment of the donor with CO suppresses this proinflammatory response in the islets after transplantation. There are several possible reasons why suppression of the proinflammatory response might lead to islet survival.

First, it is known that various cytokines just mentioned and others such as NO can be directly toxic to β-cells. By

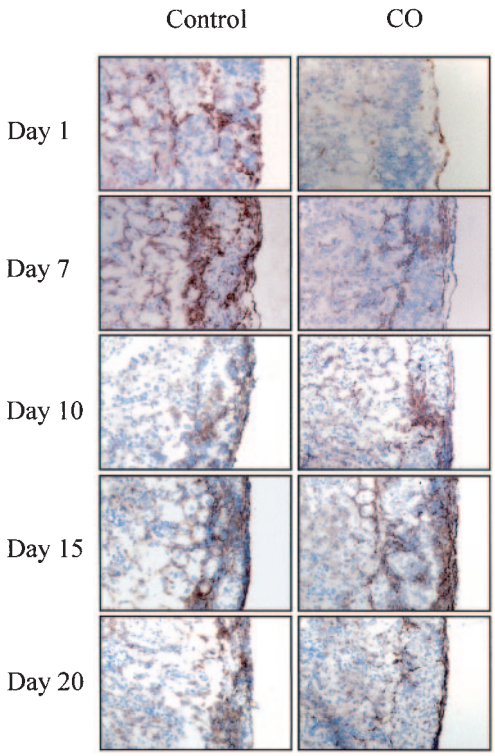

FIG. 5. CO administration only to the donor suppresses macrophage infiltration to the islet grafts in the recipients. Islet grafts from nontreated donors or donors administered CO were harvested at various days after islet transplantation and stained with the macrophage marker F4/80. Magnification is 200×. Samples shown are representative of three animals per group.

limiting the proinflammatory response, these toxic molecules are very significantly neutralized. Second, some of the cytokines/chemokines produced in the islets after transplantation help to recruit host cells into the islets. For example, fewer macrophages were noted in islets obtained from donors treated with CO than in those from controls, consistent with reduced levels of MCP-1, a chemokine that attracts cells such as macrophages to the site of inflammation. Finally, as is inherent in the danger hypothesis (30,31), we speculate that the decreased inflammation in islets after transplantation results in a decreased rejection response. The mechanisms involved in the establishment of tolerance need elucidation. Furthermore, we propose that in the presence of the decreased proinflammatory and rejection responses, the effects of protective genes such as bcl-2, which was present in the islets after transplantation in those cases in which the donor was given CO, shifts the balance from rejection to tolerance.

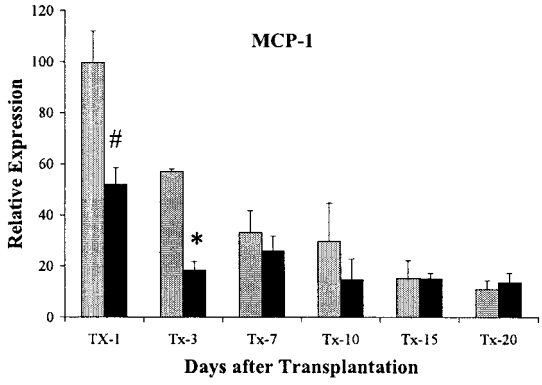

FIG. 6. MCP-1 mRNA expression was suppressed in grafts from donors to which CO was administered compared with the untreated control, as analyzed by real-time RT-PCR. RNA was extracted from islet grafts at various days after transplantation. Results shown are the means ± SD from three islet grafts at each time point. *$P < 0.05$, #$P < 0.01$ vs. control.

But why should CO treatment of the donor decrease the inflammatory response in the islets after transplantation? One possible contributing factor may be the antiapoptotic effects of CO treatment on islets (3). Such an effect would likely still obtain after transplantation, even though the islets were only exposed in the donor. Thus, the debris of dying islets that would ordinarily lead to inflammation would be decreased and the inflammatory response suppressed. Second, treatment of the islets in the donor with CO will suppress a proinflammatory response by resident macrophages to later stresses, including the isolation procedure. Although it is not known how long the antiapoptotic and anti-inflammatory effects of CO persist after treatment, the ability of short-term CO exposure to suppress reactions that normally develop over many hours suggests that such a mechanism might be discovered (32,33). We propose that the suppression of the inflammatory response and of apoptosis are key to allowing survival when only the donor is treated.

The mechanisms underlying the ability of HO-1 or CO to prolong islet graft survival and to induce tolerance deserve further study. Studies of the effects of HO-1 expression or CO administration in antigen-presenting cells or T-cells are only now beginning but may be involved in the results presented herein. Given the above results, it would seem that induction of HO-1 or administration of CO might be useful clinically for islet transplantation. Induction of HO-1, however, has the disadvantage that individuals vary in terms of the strength of their HO-1 induction to a given stimulus dependent on polymorphisms in the promoter of the HO-1 gene (34).

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REFERENCES

1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
2. Inverardi L, Kenyon NS, Ricordi C: Islet transplantation: immunological perspectives. *Curr Opin Immunol* 15:507–511, 2003
3. Gunther L, Berberat PO, Haga M, Brouard S, Smith RN, Soares MP, Bach FH, Tobiasch E: Carbon monoxide protects pancreatic beta-cells from apoptosis and improves islet function/survival after transplantation. *Diabetes* 51:994–999, 2002
4. Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, Inverardi L: Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. *Diabetes* 50:1983–1991, 2001
5. Pileggi A, Cattan P, Molano RD, Berney T, Vizzardelli C, Fraker C, Oliver R, Ricordi C, Pastori R, Bach FH, Inverardi L: Induced heme oxygenase-1 upregulation protects pancreatic beta cells from apoptosis in vitro. *Scientific World J* 1 (1 Suppl. 3):108, 2001
6. Tobiasch E, Gunther L, Bach FH: Heme oxygenase-1 protects pancreatic beta cells from apoptosis caused by various stimuli. *J Investig Med* 49:566–571, 2001

7. Sass G, Soares MC, Yamashita K, Seyfried S, Zimmermann WH, Eschenhagen T, Kaczmarek E, Ritter T, Volk HD, Tiegs G: Heme oxygenase-1 and its reaction product, carbon monoxide, prevent inflammation-related apoptotic liver damage in mice. *Hepatology* 38:909–918, 2003
8. Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP: Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med* 192:1015–1026, 2000
9. Berberat PO, Katori M, Kaczmarek E, Anselmo D, Lassman C, Ke B, Shen X, Busuttil RW, Yamashita K, Csizmadia E, Tyagi S, Otterbein LE, Brouard S, Tobiasch E, Bach FH, Kupiec-Weglinski JW, Soares MP: Heavy chain ferritin acts as an antiapoptotic gene that protects livers from ischemia reperfusion injury. *FASEB J* 17:1724–1726, 2003
10. Nakao A, Moore BA, Murase N, Liu F, Zuckerbraun BS, Bach FH, Choi AM, Nalesnik MA, Otterbein LE, Bauer A: Immunomodulatory effects of inhaled carbon monoxide on rat syngeneic small bowel graft motility. *Gut* 52: 1278–1285, 2003
11. Katori M, Busuttil RW, Kupiec-Weglinski JW: Heme oxygenase-1 system in organ transplantation. *Transplantation* 74:905–912, 2002
12. Moore BA, Otterbein LE, Turler A, Choi AM, Bauer AJ: Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. *Gastroenterology* 124:377–391, 2003
13. Chauveau C, Bouchet D, Roussel JC, Mathieu P, Braudeau C, Renaudin K, Tesson L, Soulillou JP, Iyer S, Buelow R, Anegon I: Gene transfer of heme oxygenase-1 and carbon monoxide delivery inhibit chronic rejection. *Am J Transplant* 2:581–592, 2002
14. Berney T, Molano RD, Cattan P, Pileggi A, Vizzardelli C, Oliver R, Ricordi C, Inverardi L: Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis. *Transplantation* 71:125–132, 2001
15. Fontaine MJ, Fan W: Islet cell transplantation as a cure for insulin dependent diabetes: current improvements in preserving islet cell mass and function. *Hepatobiliary Pancreat Dis Int* 2:170–179, 2003
16. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC: Vulnerability of islets in the immediate posttransplantation period: dynamic changes in structure and function. *Diabetes* 45:1161–1167, 1996
17. Inoue S, Suzuki M, Nagashima Y, Suzuki S, Hashiba T, Tsuburai T, Ikehara K, Matsuse T, Ishigatsubo Y: Transfer of heme oxygenase 1 cDNA by a replication-deficient adenovirus enhances interleukin 10 production from alveolar macrophages that attenuates lipopolysaccharide-induced acute lung injury in mice. *Human Gene Ther* 12:967–979, 2001
18. Lee TS, Chau LY: Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* 8:240–246, 2002
19. Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol DW, Flavell RA, Billiar TR, Otterbein LE, Choi AM: Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. *J Immunol* 172:1220–1226, 2004
20. Pae HO, Oh GS, Choi BM, Chae SC, Kim YM, Chung KR, Chung HT: Carbon monoxide produced by heme oxygenase-1 suppresses T cell proliferation via inhibition of IL-2 production. *J Immunol* 172:4744–4751, 2004
21. Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH: Expression of heme oxygenase-1 can determine cardiac xenograft survival. *Nat Med* 4:1073–1077, 1998
22. Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP: Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol* 166:4185–4194, 2001
23. Tullius SG, Nieminen-Kelha M, Buelow R, Reutzel-Selke A, Martins PN, Pratschke J, Bachmann U, Lehmann M, Southard D, Iyer S, Schmidbauer G, Sawitzki B, Reinke P, Neuhaus P, Volk HD: Inhibition of ischemia/reperfusion injury and chronic graft deterioration by a single-donor treatment with cobalt-protoporphyrin for the induction of heme oxygenase-1. *Transplantation* 74:591–598, 2002
24. Araujo JA, Meng L, Tward AD, Hancock WW, Zhai Y, Lee A, Ishikawa K, Iyer S, Buelow R, Busuttil RW, Shih DM, Lusis AJ, Kupiec-Weglinski JW: Systemic rather than local heme oxygenase-1 overexpression improves cardiac allograft outcomes in a new transgenic mouse. *J Immunol* 171:1572–1580, 2003
25. Contreras JL, Eckstein C, Smyth CA, Sellers MT, Vilatoba M, Bilbao G, Rahemtulla FG, Young CJ, Thompson JA, Chaudry IH, Eckhoff DE: Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes* 52:2935–2942, 2003
26. Takada M, Nadeau KC, Hancock WW, Mackenzie HS, Shaw GD, Waaga AM, Chandraker A, Sayegh MH, Tilney NL: Effects of explosive brain death on cytokine activation of peripheral organs in the rat. *Transplantation* 65:1533–1542, 1998
27. Toyama H, Takada M, Suzuki Y, Kuroda Y: Activation of macrophage-associated molecules after brain death in islets. *Cell Transplant* 12:27–32, 2003
28. Johansson U, Olsson A, Gabrielsson S, Nilsson B, Korsgren O: Inflammatory mediators expressed in human islets of Langerhans: implications for islet transplantation. *Biochem Biophys Res Commun* 308:474–479, 2003
29. Bottino R, Fernandez LA, Ricordi C, Lehmann R, Tsan MF, Oliver R, Inverardi L: Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation. *Diabetes* 47:316–323, 1998
30. Matzinger P: Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:991–1045, 1994
31. Austyn JM: Death, destruction, danger and dendritic cells. *Nat Med* 5:1232–1233, 1999
32. Morse D, Pischke SE, Zhou Z, Davis RJ, Flavell RA, Loop T, Otterbein SL, Otterbein LE, Choi AM: Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem* 278:36993–36998, 2003
33. Alcaraz MJ, Fernandez P, Guillen MI: Anti-inflammatory actions of the heme oxygenase-1 pathway. *Curr Pharm Des* 9:2541–2551, 2003
34. Exner M, Schillinger M, Minar E, Mlekusch W, Schlerka G, Haumer M, Mannhalter C, Wagner O: Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty. *J Endovasc Ther* 8:433–440, 2001