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Forum Review

Therapeutic Applications of Bilirubin and Biliverdin in Transplantation

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ABSTRACT

Bilirubin is the end product of heme catabolism by heme oxygenases. The inducible form of these enzymes is heme oxygenase-1 (HO-1), which is the rate-limiting enzyme that can degrade heme into equimolar quantities of carbon monoxide (CO), biliverdin, and free iron. Biliverdin is very rapidly converted to bilirubin by the enzyme biliverdin reductase, and free iron upregulates the expression of ferritin. HO-1 is a ubiquitous stress protein and is induced in many cell types by various stimuli. Induced HO-1 exerts antiinflammatory effects and modulates apoptosis. Expression of HO-1 *in vivo* suppresses the inflammatory responses in endotoxic shock, hyperoxia, acute pleurisy, and organ transplantation, as well as ischemia–reperfusion injury, and thereby provides salutary effects in these conditions. Accumulating evidence indicates that biliverdin/bilirubin can mediate the protective effects of HO-1 in many disease models, such as IRI and organ transplantation, *via* its antiinflammatory, antiapoptotic, antiproliferative, and antioxidant properties, as well as its effects on the immune response. This review attempts to summarize these protective roles as well as the molecular mechanisms by which biliverdin/bilirubin benefit IRI and solid-organ transplantation, including chronic rejection, and islet transplantation. *Antioxid. Redox Signal.* 9, 2175–2185.

INTRODUCTION

Transplantation of solid organs has become clinically routine as a successful treatment for end-stage organ failure. However, life-long immunosuppression is required for patients bearing allografts, resulting in increased morbidity due to infections and cancer (12, 25). Further, the immunosuppressants used do little if anything to suppress ischemia–reperfusion injury (IRI) or chronic rejection (33, 51). Thus, less toxic treatment regimens and strategies to induce tolerance to allografts are needed to improve quality of life and survival in transplant recipients.

HO-1 is a key player in IRI, allograft rejection, and tolerance induction (6, 14, 55, 64). Induction/overexpression of HO-1 improves outcome after experimental transplantation in several models (4, 19, 37, 52, 56, 63, 69, 71, 73, 84). Evidence indicates that each of the three products of heme degradation has protective properties when expressed in a given tissue or when administered to an animal. We have focused our attention on biliverdin and bilirubin. Several experimental studies, *in vitro* as well as *in vivo*, demonstrated that the bile pigment bilirubin (and its precursor biliverdin), at least in part account for the protective effects of HO-1 (23, 34, 42, 50, 53, 54, 79).

Bilirubin is a lipophilic linear tetrapyrrole, abundant in human blood plasma with "normal" concentrations from 0.3 to 1.2 mg/dl (7). It is the final product of heme catabolism, as heme oxygenases cleave the heme ring to form the water-soluble biliverdin, which is reduced by biliverdin reductase (BVR) to bilirubin (68) (Fig. 1). Because bilirubin is insoluble, it must be glucuronidated before being excreted into the bile (7). Excessive elevations of bilirubin (>20 mg/dl) lead to substantial deposits in the brain, with the resultant kernicterus causing ma-

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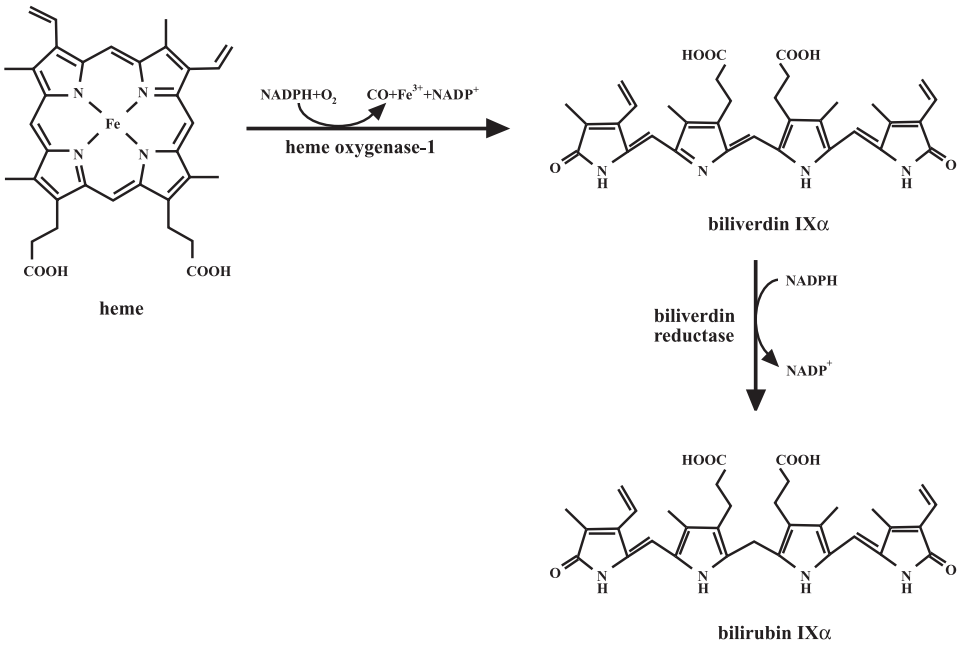

FIG. 1. Two steps of heme degradation. The first reaction is cleavage of the heme ring by the heme oxygenases. In the second reaction, biliverdin reductase reduces the central methene bridge of biliverdin, producing bilirubin.

jor brain damage in newborns (45, 81). However, accumulating evidence now suggests a beneficial role for the bile pigment. First, bilirubin is a potent antioxidant (65). Second, many recent clinical studies have shown an inverse correlation between normal plasma bilirubin levels and various diseases. Individuals with above-normal bilirubin concentrations are also protected from atherosclerosis (60, 76). Although this is only an association, the fact that bilirubin acts as a beneficial therapeutic in experimental atherosclerosis implicates bilirubin as the molecule accounting for the reduction in atherosclerosis. Third, accumulating experimental data *in vitro* as well as *in vivo* show beneficial effects of the bile pigment(s) in various disease models.

BILIRUBIN/BILIVERDIN IN SOLID ORGAN TRANSPLANTATION

Transplantation of allogeneic organs requires suppression of the host's immune response to prevent rejection. Current clinical immunosuppressive protocols involving "induction therapy" (*e.g.*, alemtuzumab, anti-thymocyte globulin, IL-2 antagonists), calcineurin inhibitors, and antiproliferative agents are successful in preventing acute, T cell–mediated allograft rejections. However, toxicity of these regimens is a big issue because of the increased incidence of tumors (*e.g.*, lymphomas) and the fact that patients are prone to viral, fungal, and bacterial infections (12, 25). Further, no exhilarating improvements have been made in the last decades with respect to long-term outcomes after solid-organ transplantation, hampered by a process called "chronic rejection." Kidney allograft survival is not better than 50% at 10 years after transplantation (85), and chronic allograft arteriopathy is present in 50% of heart allografts 5 years after transplantation (21). The holy grail of organ transplantation, tolerance of the recipient's immune system to the allogeneic organ, has not yet been achieved in the clinic.

In 1980, Sima and colleagues (62) were the first to attribute "immunosuppressive" effects to bilirubin, demonstrating direct effects on lymphocytes and granulocytes *in vitro* as well as a decrease in "antibody-forming cells" in the spleens of bilirubin-treated mice that had been immunized (46, 62, 75). In 1996, Haga and colleagues (28, 29) were able to show that bilirubin at a concentration of 100–200 μM inhibits cytotoxic T-lymphocyte activity *in vitro.* The same group demonstrated that bilirubin impairs phytohaemagglutinin A–induced T-cell proliferation (27). In a rat model of autoimmune encephalitis, bilirubin interfered with the invasion of inflammatory cells by protecting the blood–brain barrier from free radical–induced permeability changes (42).

However, until the discovery of the immunosuppressive/tolerizing effects of HO-1, no attention was paid to the use of bilirubin as a potential treatment to prevent acute or chronic allograft/islet rejection. Induction of HO-1 counteracts both acute rejection episodes and chronic changes after solid-organ transplantation (14, 16, 37, 52, 64, 71). We recently showed that two protocols that induce tolerance to cardiac allografts in wild-type mice fail to do so in mice lacking HO-1 (84). This finding is a dramatic example of the importance of HO-1 in at least some tolerance protocols (we suspect those involving generation of Tregs). Presumably, the products of heme degradation account for the immunomodulating effects of HO-1. In a mouse model of heart transplantation, biliverdin inhibits proliferation of primary T cells after stimulation with anti-CD3 and anti-CD28 mAbs by interfering with IL-2 synthesis by inhibition of NFAT/NF-κB activation (Fig. 2). *In vivo* administration of biliverdin at 35mg/kg once before transplantation and then daily for 13 days after transplantation significantly prolonged sur-

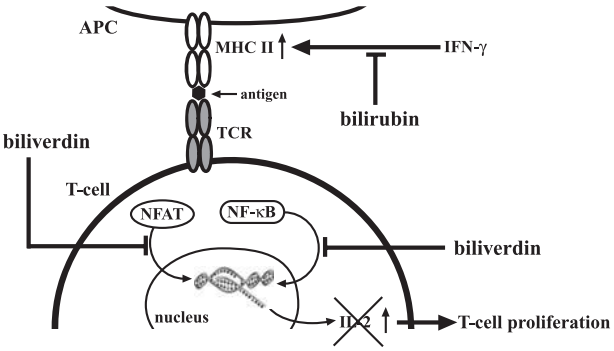

FIG. 2. Mechanisms of bilirubin/biliverdin action on acute rejection. Bilirubin inhibits MHC class II expression. Biliverdin inhibits nuclear translocation of NFAT and NF-κB that induce transcription of IL-2 resulting in impaired T-cell proliferation.

vival of B6AF1 (H-$2^{k/d,\ b}$) allografts in DBA/2 (H-2^{d}) recipients (with a median survival of 20.5 *vs*. 11.5 days in the control), whereas two or three daily injections at the same dose for the same period led to >200 days of graft survival in 66% of the animals tested (Fig. 3A). We challenged those "long-term survivors" with a second heart (transplanted heterotopically to the neck after the first allograft had been placed in the abdomen) from FVB (H-2^{q}) "third-party" mice. These were promptly rejected, whereas second DBA/2 allografts were accepted indefinitely without any further treatment, suggesting tolerance specifically to the donor antigens (Fig. 3B). Biliverdin treatment resulted in a diminished infiltrate of immunocompetent cells in the grafts and suppressed the proliferative activity of recipient splenocytes, as assessed by mixed leukocyte cultures (83). In an *in vitro* study using 2F2B endothelial cells, Wu *et al*. (82) demonstrated that bilirubin (but not CO) administration dose-dependently inhibited IFN-γ–induced MHC class II expression that is presumably required for T-cell activation during acute allograft rejection (see Fig. 2). Studies are needed further to understand the mechanisms of the effects of the bile pigments on the acute rejection of solid allografts.

BILIRUBIN/BILIVERDIN IN PANCREATIC ISLET TRANSPLANTATION

Type 1 diabetes is caused by immune system–mediated destruction of pancreatic β cells in the islets. As a consequence, patients require insulin to prevent hyperglycemic ketoacidosis, as well as long-term consequences of chronic hyperglycemia (*e.g*., microangiopathy, polyneuropathy, and renal insufficiency). In patients with end-stage diabetic glomerulopathy that require a kidney transplant (and who will thus be immunosuppressed anyway), simultaneous transplantation of a pancreatic allograft has become clinically routine (66). However, transplantation of the whole organ is a demanding procedure prone to complications of surgery and is hampered by the lack of suitable organs. For decades, an alternative approach has been tried: transplanting isolated allogeneic pancreatic islets to the recipient. Significant progress has been made during recent years (8, 61); however, islet transplantation has not yet been accepted as a first-line treatment of patients with type 1 diabetes. Difficulties might be explained in part by the need for islets from more than one organ to have a sufficient islet mass so that a patient does not require insulin.

We reported that the induced expression of HO-1 in islet transplantation can provide salutary effects (56, 69). Inducing HO-1 in the islet donor or recipient or both results in a significant benefit in terms of prolongation of islet survival in allogeneic recipients, in many cases leading to long-term survival (>100 days) and antigen-specific tolerance. Further studies indicated that administration of biliverdin/bilirubin to the donor, the islets, and/or the recipient manifested similar beneficial effects as inducing HO-1 (79). DBA/2 (H-2^{d}) islets transplanted into B6AF1 (H-$2^{b,\ k/d}$) recipients are rejected within 24.9 ± 5.3 days (n = 11). Administering bilirubin at 8.5 μmol/kg to the islet donor 1 h before islet transplantation led to a significant

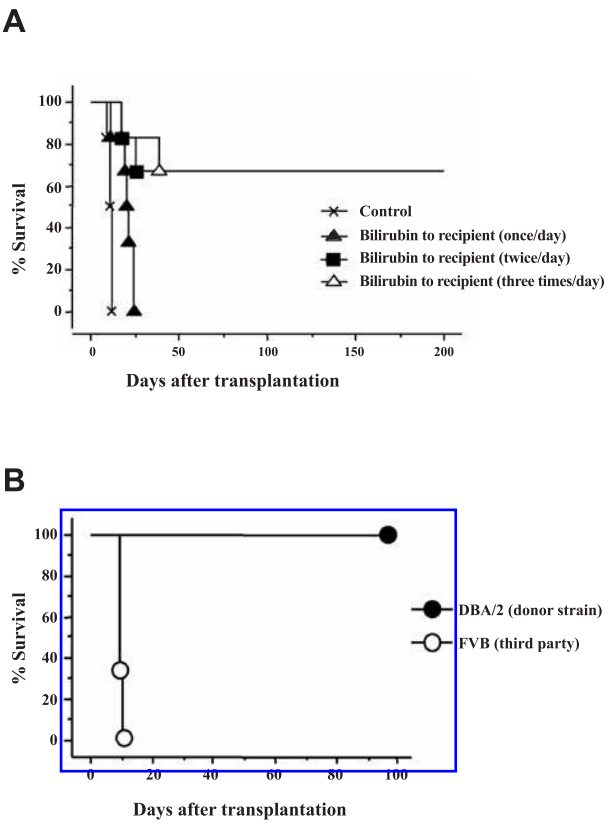

FIG. 3. Exogenous biliverdin administration induces donor-specific tolerance to cardiac allografts. (**A**) Kaplan-Meier plotting of the DBA/2 cardiac allograft survival in B6AF1 mice. Donor and recipient animals received non-treatment (✖) or biliverdin (at 50 μmol/kg, i.p.) once (▲), twice (■), or three times (△) daily (n = 6 per each group). All treatments were terminated at 2 weeks after transplantation. $p < 0.01$, control vs. biliverdin (once per day), and $p < 0.001$, control vs. biliverdin (both twice and three times per day). (**B**) Kaplan-Meier plotting of the second cardiac allograft survival. Long-term heart graft accepting B6AF1 recipients by biliverdin treatments (twice or three times per day) accepted second heart grafts from theDBA/2 (●) but not from FVB (○) mice (n = 3 per each group). $p < 0.05$, DBA/2 *vs*. FVB.

percentage (41.7%; $n = 12$) of long-term surviving islets. In groups in which only the recipient was treated, a single daily dose of bilirubin at 17 μmol/kg/day from day –1 until day 13 led to 50% long-term survival ($n = 6$), whereas giving two doses (every 12 h at 8.5 μmol/kg) led to 72.7% ($n = 11$) of islet grafts surviving long-term (Fig. 4A). Just incubating islets in bilirubin-containing medium (at 100 μM) for 1 h after isolation and before transplantation led to 40% long-term survival of islets in the recipient ($n = 5$; $p = 0.0004$ *vs*. control; Fig. 4A). The protective effect of administering bilirubin to the islet donors was confirmed in a donor–recipient combination differing by a stronger immunogenetic disparity: islets from BALB/c (H-2^d) mice were transplanted to C57BL/6 (H-2^b) mice. A very significant prolongation of islet graft survival was observed when bilirubin was given only to the donor (8.5 μmol/kg, 1 h before islet isolation; $n = 6$) compared with the control, in which the donors received vehicle only ($n = 6$; $p = 0.0074$ *vs*. control; Fig. 4B). The same study indicates that bilirubin pretreatment to the donor reduces the number of macrophages infiltrating into the islet grafts and reduces the expression of proinflammatory and proapoptotic genes that contribute to the destruction of transplanted islets. Bilirubin treatment to the islet donor significantly suppresses expression of TNF, inducible nitric oxide synthase (iNOS), chemokines CCL1 and CXCL10, Fas, caspase-3, -8, and -9, and bcl-2–interacting domain in transplanted grafts at various days after transplantation. At the same time, upregulated expression of protective genes was seen, including HO-1 and bcl-2. Bilirubin protected βTC3 cells (the insulinoma cell line) from lipid peroxidation induced by hydroxyl radicals in an *in vitro* culture (Fig. 5).

BILIRUBIN/BILIVERDIN IN CHRONIC REJECTION

As mentioned earlier, long-term survival of allografts is hampered by a process called "chronic rejection" or "chronic allograft dysfunction (CAD)." The pathologic mechanisms of CAD are not completely understood; however, the severity of CAD correlates with the damage resulting from IRI (innate immunity) as well as alloimmunologic factors (adaptive immunity) (38). The vessels of a chronically rejecting transplanted organ show neointimal hyperplasia, based on vascular smooth muscle cell (VSMC) proliferation. This results in chronic allograft arteriosclerosis, leading to irreversible organ damage and finally to allograft loss (58). The cells in the neointima are thought to migrate from the media of the vessel, after the endothelium is activated/destroyed by the recipient's immune system (74). Accumulating evidence suggests that a significant portion of those neointimal vascular smooth muscle–like cells originates from pluripotent recipient cells (5). Nonetheless, inhibition of proliferation of the neointimal layer is an effective approach to prevent/treat chronic allograft arteriosclerosis experimentally (57). Overexpression/induction of HO-1 is effective in reducing VSMC proliferation and neointima formation in various animal models (2, 22, 39, 41, 71, 72). Likewise, bilirubin/biliverdin induce apoptosis in rat VSMCs stimulated with 2% serum (41). We found that bilirubin/biliverdin (at 10% FCS) do not cause apoptosis in rat and mouse VSMCs but inhibit cell-cycle progression in the G_0/G_1 phase (53). This was mediated *via* suppression of p38 MAPK activation and hypophosphorylation of the retinoblastoma tumor–suppressor protein, presumably dependent on p53 (53, 54) (Fig. 6). Strikingly,

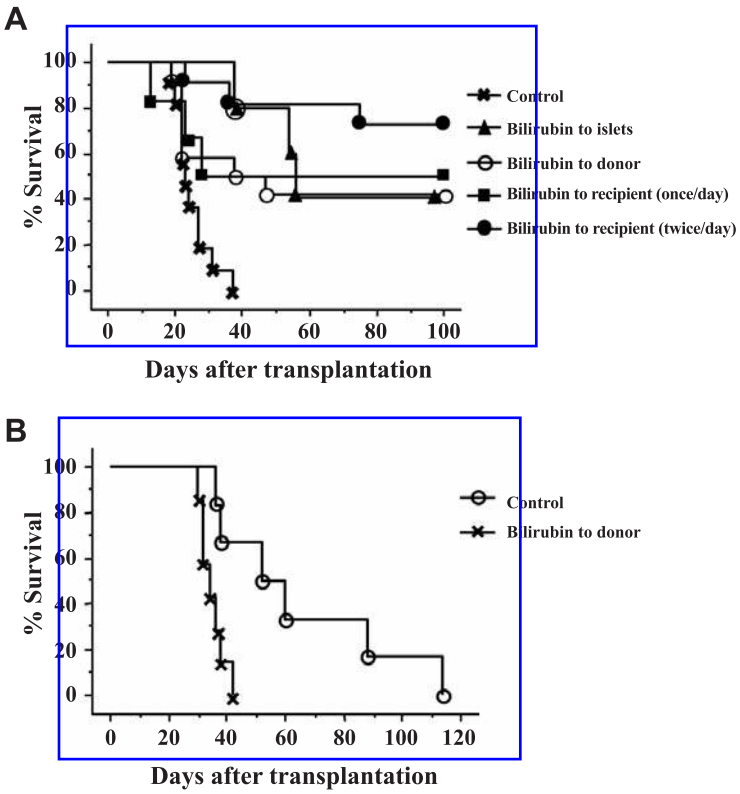

FIG. 4. Bilirubin induces long-term survival to allogeneic islet allografts. (**A**) Kaplan-Meier plotting of the DBA/2 islet allograft survival in B6AF1 mice. Bilirubin was administered to the donor at 8.5 μmol/kg 1 h before islet isolation (○), ex vivo to the islets (▲) or the recipient at either 8.5μM/kg every 12 h (■) or at 17 μM/kg every 24 h (●) from day 1 until day 13. Control mice (✖) received no treatment. (**B**) Kaplan-Meier plotting of the BALB/c islet allograft survival in C57BL/6 mice. Donors received either 8.5 μmol/kg bilirubin (✖) or vehicle control (○) 1 h before islet isolation.

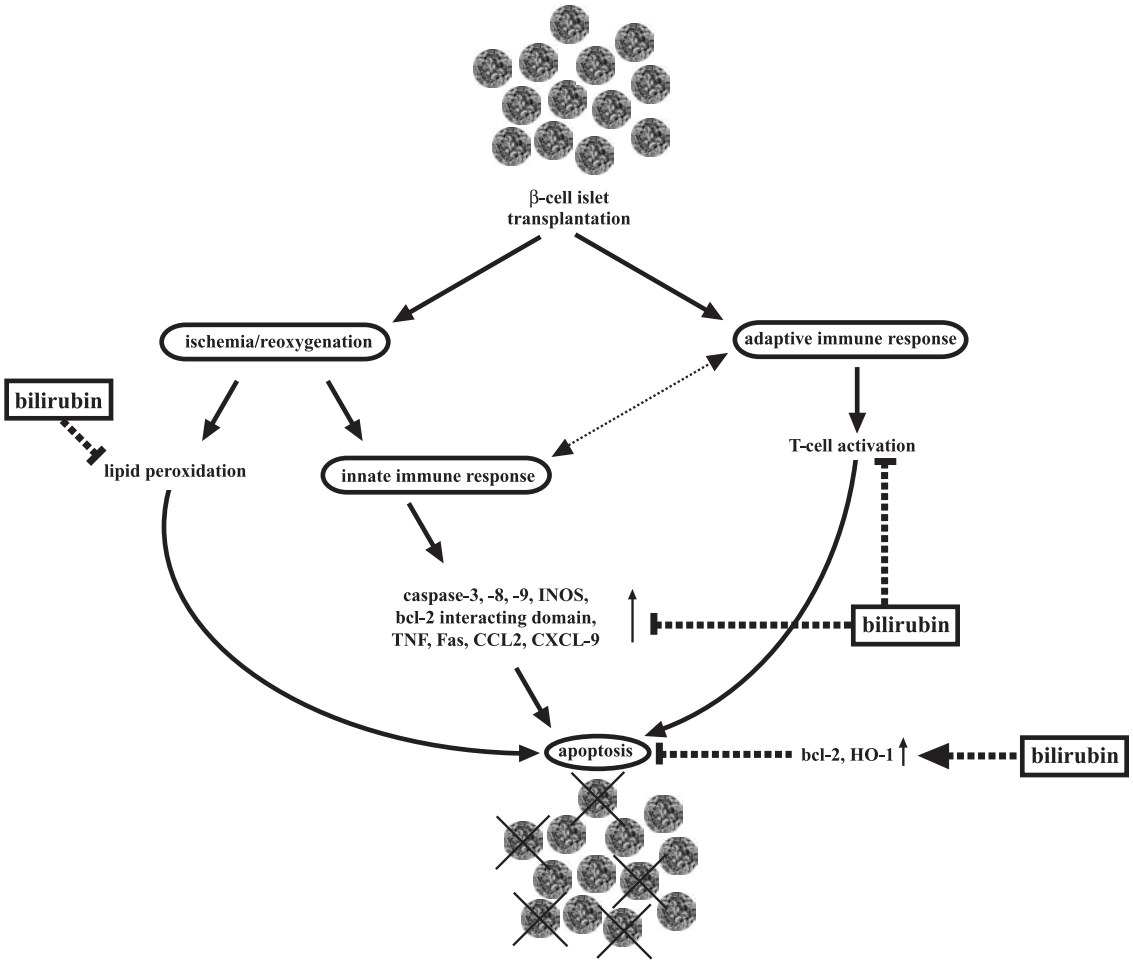

FIG. 5. Potential mechanism of the beneficial effect of bilirubin on allogeneic transplanted β-cell islets. Bilirubin suppresses the expression of pro-apoptotic and pro-inflammatory genes and induces expression of anti-apoptotic HO-1 and bcl-2. Further, bilirubin reduces oxidative stress and T-cell mediated immune responses.

in transgenic rats congenitally having high bilirubin levels (12.0 mg/dl), VSMC-derived neointima formation that is seen in WT rats (serum bilirubin, 0.9 mg/dl) after balloon injury was nearly absent (Fig. 7). Similarly, pretreatment of the arteries with biliverdin was effective in reducing neointima formation after injury. In concert with these findings, we have preliminary data in a mouse model of chronic allograft arteriosclerosis that bilirubin treatment of the recipient ameliorates neointimal lesions when compared with the vector-treated control (unpublished observations).

BILIRUBIN/BILIVERDIN IN IRI

Ischemia (hypoxia) is a consequence of the interruption of blood supply during solid-organ (islet) transplantation. Subsequently, damage to metabolically active tissue occurs as a consequence of hypoxia and the lack of nutrients. Restoration of blood flow is necessary to salvage the tissue from death; however, on reperfusion, a cascade of events occurs that leads to additional cell injury. The consequences of interruption and restoration of blood supply are generally referred to as IRI, although this term is not applied to islet transplantation (10). The reperfusing blood flow leads to a well-orchestrated series of interactions between vascular endothelium and the innate immune

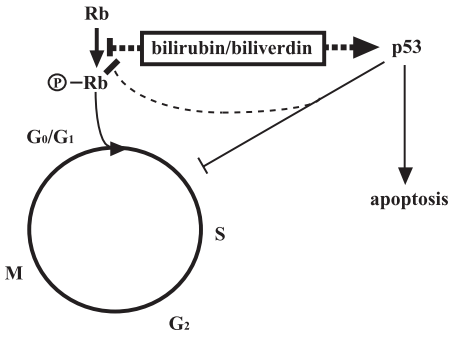

FIG. 6. Bilirubin/biliverdin inhibits proliferation of VSMCs. Bilirubin/biliverdin inhibit Rb-phosphorylation and cell cycle progression, presumably via p53 and induce apoptosis to (starved) VSMCs.

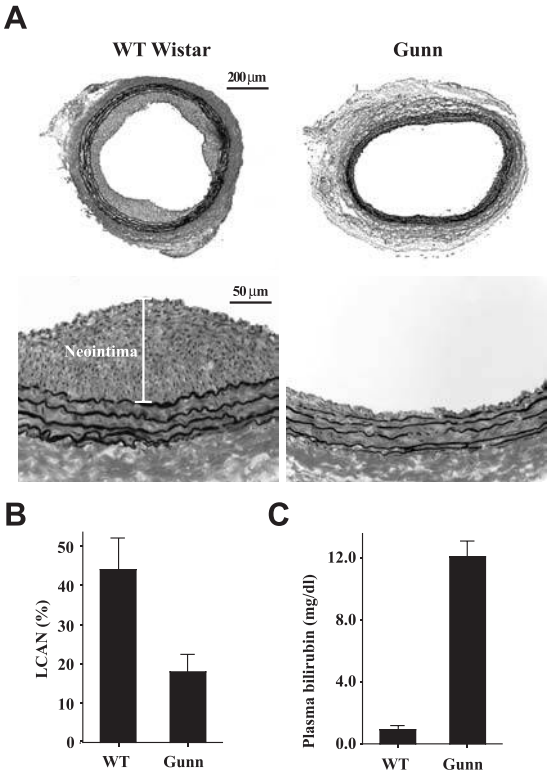

FIG. 7. Bilirubin suppresses neointimal hyperplasia associated with balloon injury. (**A**) Elastica van Giesson stain of wild type Wistar and Gunn rat carotid arteries 2 weeks following balloon injury. (**B**) Luminal cross-sectional area narrowing (LCAN) of wild type Wistar and Gunn rat carotid arteries at 2 weeks after balloon injury. (**C**) Plasma bilirubin levels measured in wild type Wistar and Gunn rats prior to balloon injury ($n = 6$; values are expressed as mean ± SD).

system (involving neutrophils, monocytes, eosinophils), resulting in an inflammatory burst (10). Cell-adhesion molecules are rapidly expressed on the surface of the endothelium (*e.g.*, P-selectin) that enable rolling of leukocytes (bearing L-selectin, the counterligand of P-selectin that is constitutively expressed on neutrophils), along the endothelial surface. Subsequently, neutrophils that express β_2-integrins (*e.g.*, CD11/CD18) adhere to members of the immunoglobulin superfamily (*e.g.*, ICAM-1, VCAM-1) and are finally firmly attached to the vessel wall, a process that is triggered by endothelial chemoattractants (leukotrienes, chemokines, PAF) and by leukocyte expression of IL-1 and TNF. Tissue damage occurs because of the release of proteases, collagenases, lipoxygenases, phospholipases, and myeloperoxidase by neutrophils. Consequently, vascular endothelial cells in the early phase (1–2 h) and organ-specific cells (*e.g.*, cardiac myocytes, kidney tubular cells) in the later phase (4–6 h) undergo apoptosis, leading to deterioration of organ function (3). Induction of HO-1 has been shown to ameliorate tissue damage associated with essentially all models of IRI (9, 19, 20, 31, 40, 73, 77, 86–88). Bilirubin/biliverdin at least in part account for the protective effects of HO-1. The bile pigments ameliorate organ function in various models of IRI (Fig. 8) by promoting antiinflammatory and antiapoptotic as well as possibly other effects (Fig. 9).

In vitro, viability of H9C2 rat cardiac myoblasts was assessed at 18 h of hypoxia at defined time points after reoxygenation. Bilirubin, at a concentration of 0.03 mg/dl, (0.5 μM) provided protection against reoxygenation damage (24). These results are similar to those in which HO-1 is induced with hemin, which leads to an increase of bilirubin in the supernatant. In an isolated perfused rat heart model, exogenously administered bilirubin to the perfusate before ischemia increased functional recovery of the myocardium, measured after 30 min of warm ischemia and 60 min of reperfusion, and reduced infarct size from 11.7% in the control to 3.9%. Further, mitochondrial integrity was preserved under bilirubin treatment (18). By adding bilirubin in an isolated perfused rat kidney model, 20 min of warm ischemia and 2-h reperfusion, vascular resistance was reduced, urine output increased dose dependently, creatinine clearance increased, tubular injury minimized, and lipid peroxide levels suppressed compared with the effects in the absence of bilirubin (1). However, by using an *in vivo* model of renal IRI in the rat kidney (by clamping both renal pedicles for 30 min followed by 6 h of reperfusion), the same group published data showing that intravenous administration of bilirubin did not provide complete protection against IRI. Perioperative target doses of 5 and 20 mg/kg bilirubin, respectively, peaking at a median serum bilirubin of 2.9 mg/dl (50 μM) at the end of ischemia, preserved cortical architecture, did not protect the renal medulla, decreased serum creatinine but not blood urea nitrogen (BUN) at 6 h after reperfusion, and did not improve the glomerular filtration rate. The authors thus suggested testing the combination of two of the products of heme catabolism, biliverdin and CO (36). This has also been proposed and tested in rat models of heart or kidney transplantation after a period of cold ischemia of 24 h. Biliverdin was administered intraperitoneally (i.p.) to the donor and the recipient 2 h before surgery and to the recipient immediately after reperfusion. Biliverdin administration alone improved heart isograft survival at 7 days after transplantation to 30% ($p < 0.05$ *vs.* control) and CO treatment alone to 10% ($p < 0.005$ *vs.* control); however, when CO and biliverdin treatment were combined, the survival rate increased to 80% ($p < 0.005$ *vs.* control). Untreated control hearts did not survive. Similarly, kidney isograft survival was prolonged after biliverdin treatment alone, but was again inferior to the dual therapy (*i.e.*, biliverdin in combination with CO) (50).

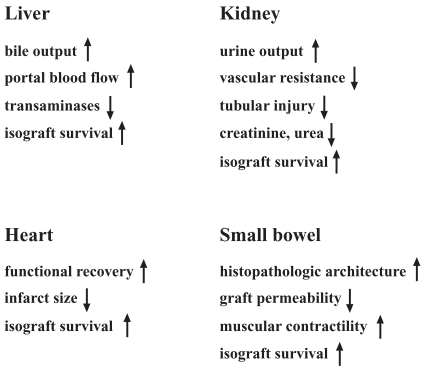

FIG. 8. Effects of bilirubin/biliverdin in various animal models of IRI. In liver, kidney, heart, and small bowel, rodent models of IRI bilirubin/biliverdin substantially improve organ function and parameters associated with IRI.

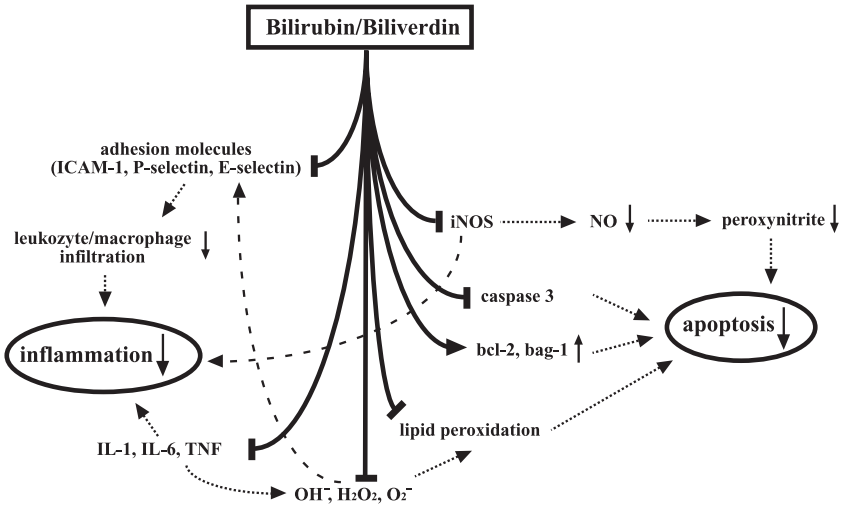

FIG. 9. Potential mechanisms of bilirubin/biliverdin improving organ function during/after IRI. Bilirubin/biliverdin suppress pathological signaling pathways involved in innate and adopted immunity (adhesion and infiltration of immunocompetent cells) as well as oxidative stress and apoptosis and promote anti-apoptotic bcl-2 and bag-1.

Kato and colleagues (34) observed significant improvement of organ function in a model of an isolated perfused rat liver after a single 5-min bilirubin rinse. On reperfusion after livers had been exposed to 16 h of cold ischemia, bilirubin caused an increase in bile output when compared with the untreated control. The bilirubin was also able to restore ZnPP-induced repression of bile output, bile salt, and phospholipid excretion (ZnPP blocks HO-1 activity). In a vascularized rat model of liver transplantation (that involves leukocytes, mononuclear cells, and macrophages in contrast to the isolated perfused liver) after 16 h of cold ischemia, rinse of the liver grafts with bilirubin before reperfusion improved survival rate from 67 to 100% and significantly reduced production of acrolein, an end product of lipid peroxidation (34). In concert with these findings, we found that a single bilirubin rinse of cardiac isografts before reperfusion inhibits activation of mitogen-activated protein kinases normally seen on reperfusion (unpublished observations). By using an *ex vivo* model of hepatic IRI after 24 h of cold ischemia and 2 h of reperfusion with whole blood, Fondevilla *et al*. (23) recently demonstrated that biliverdin added to the perfusate improves portal blood flow, increases bile production, and reduces serum glutamic-oxaloacetic transaminase. In an *in vivo* model of rat liver transplantation (24 h of cold ischemia), treatment of the recipient with biliverdin briefly before and 20 h after reperfusion (with or without treatment of the donor) markedly improved survival (as assessed on day 7 after transplantation) and improved liver function measured 6 and 12 h after reperfusion.

Three studies have been published with respect to IRI of the small bowel and bilirubin/biliverdin treatment. In a model of warm IRI of the small bowel by clamping the superior mesenteric artery for 45 min, Ceran *et al*. (15) showed an improvement in the histopathologic architecture in the bilirubin/biliverdin-treated group when compared with the untreated controls. In a rat model of small bowel transplantation (6 h of cold ischemia), biliverdin was administered at 50 mg/kg i.p. to the donor and the recipient 3 h before surgery and to the recipient immediately after reperfusion. Biliverdin, being converted to bilirubin, with plasma bilirubin levels peaking at 1.1 mg/dl at 30 min after administration, which is probably a lower value than the one that is normally seen at 5 or 15 min, caused a decrease in infiltrating polymorphonuclear cells when compared with the control at 24 h after transplantation. Messenger RNA levels of (proinflammatory) IL-6, IL-1, and ICAM-1 and serum IL-6 levels were decreased under biliverdin treatment after IRI as well. Interestingly, NF-κB nuclear translocation was increased in the small bowel after biliverdin treatment of naive rats, which was not observed in the saline-treated controls. Thus, the authors suggest a "preconditioning effect" in addition to the "antioxidant" effects (49).

DISCUSSION

The rejection of an allograft involves a multiplicity of mechanisms ranging from those that are involved in IRI to acute rejection to chronic rejection. Bilirubin or biliverdin or both have potent effects in animal models of transplantation with respect to all three of these phases of allograft dysfunction (Fig. 10). Inflammation, a natural immune response, an elicited and aggressive immune response, and many of the hallmarks of atherosclerosis (inflammation, VSMC proliferation, and others) are the basis of these various phases of rejection. In parallel, heme oxygenase-1 (HO-1) as the gene that degrades heme as well as the products of heme degradation, is similarly multipotent in terms of the therapeutic effects it can mediate. Several of the components of the HO-1 system exert very strong anti-inflammatory effects, including switching the proinflammatory response of cells of natural immunity, such as in the monocyte/macrophage, to an antiinflammatory phenotype. These agents also suppress the elicited T cell–mediated immune response, likely at least in part by favoring the survival and perhaps growth of Tregs, and are effective at suppressing VSMC proliferation and restoring the endothelium after vascular damage.

HO-1 is crucial in preventing pathologic reactions that are a component of many diseases and that are the basis, as well, of allograft rejection. Inflammation, more and more, is being recognized as a critical component of diseases of varying pathogeneses. That is almost certainly true for allograft rejection, as proposed in the danger hypothesis (43). As evidenced by the

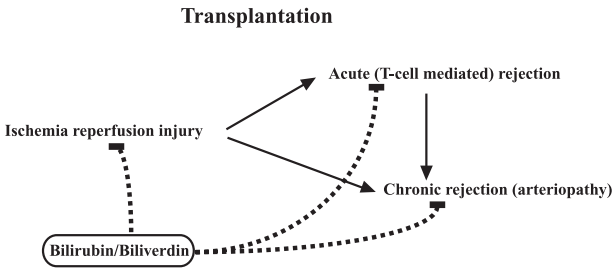

FIG. 10. Bilirubin and biliverdin in transplantation. Outcome in solid organ and islet transplantation is dependent on IRI, acute rejection episodes, and chronic allograft dysfunction. IRI per se can lead to organ loss; further, the severity of IRI is associated with the number and severity of acute rejection episodes. Acute (subclinical) rejections may as well cause allograft loss and promote chronic allograft dysfunction. Bilirubin/biliverdin experimentally counteract IRI, acute rejections, and chronic changes and thus should be considered as potent therapeutics in transplantation.

work with allogeneic islet transplantation reviewed here, treatment of only the donor with induction of HO-1, bilirubin, or CO leads to a very significantly suppressed inflammatory as well as immune response in those islets after transplantation to the recipient (78, 79). The mechanisms for this reduced inflammatory response are only now beginning to be understood at the molecular level. That understanding may well lead to additional potential therapeutic approaches.

Both biliverdin and bilirubin are potent at inhibiting acute T cell–mediated immune responses and thus can contribute to avoiding rejection of organs or islets. In addition, both molecules are potentially antiapoptotic and can suppress the responses involved in chronic allograft dysfunction. These and results from past and ongoing studies in IRI (in which the innate immune system is activated during reperfusion injury) support our goal to apply the "natural" bile pigments clinically in solid-organ transplantation. We are beginning to learn more about the mechanisms underlying the beneficial effects of administering biliverdin or bilirubin. Bilirubin and biliverdin exert potent antioxidant effects (60, 65, 70). In addition, biliverdin interacts with cell-surface biliverdin reductase to suppress proinflammatory responses of macrophages (unpublished observations).

Acute rejection episodes are currently treated/prevented with high levels of immunosuppression, resulting in a fourfold increase in tumor incidence (12) in transplanted patients and a significantly higher rate of bacterial, viral, and fungal infections (25). Despite some single cases of organ-specific tolerance that have been observed in transplanted individuals (13), currently no strategy is available to intentionally induce tolerance in normal organ recipients to the grafts. Experimentally, several strategies have been established to induce donor-specific tolerance in mice by means of a short course (antibody) treatment covering the perioperative period in which no further immunosuppression is needed (35). As shown in mice lacking HO-1, at least some of those tolerance-inducing strategies require the expression of HO-1 (84). We have shown that induction of HO-1 by CoPP can induce tolerance to murine cardiac allografts, on the basis of which we hypothesized that the products of heme catabolism might, at least in part, account for the effects on the immune system observed under HO-1 induction. CO, at the concentrations being tested, did not effectively prolong allograft survival in various strain combinations (83); in contrast, injection of biliverdin twice daily significantly prolonged allograft survival, and in 66% of the recipients, donor-specific tolerance was observed. Adding a third dose for each day did not significantly differ from the results with two doses. The effect of biliverdin/bilirubin on T cell–mediated (acute) rejection likely has at least two bases. We reviewed the findings that biliverdin/bilirubin suppresses T-cell activation, that HO-1 (and maybe biliverdin/bilirubin also) promote activation-induced cell death and the survival of Tregs (44). In addition, however, HO-1 has a profound effect on the stimulation of the immune response by dendritic cells (DCs). High expression of HO-1, and probably bilirubin, in DCs holds those cells in the immature state in which they stimulate Treg. If HO-1 expression in the DCs is low, then the DCs, on stimulation with LPS, mature to cells that stimulate the alloaggressive response. As the authors of the article describing these findings speculated, we also believe that this could be an important part of the effects of HO-1 and biliverdin/bilirubin on tolerance induction (17).

To gain more insight into the mechanism of how HO-1 and the bile pigments suppress immune responses, several studies are being conducted, including studies on IRI. IRI plays an important role during the early phase of the host's immune-system activation. The damage that occurs after reperfusion initially activates the innate immune system, attracting granulocytes and macrophages. In addition to that, apoptosis of the cells as well as expression of endothelial surface antigens triggers the specific immune response that is mediated by T lymphocytes (3, 10). The success of treating IRI with biliverdin/bilirubin probably attests to the antiinflammatory as well as the antiapoptotic effects of these molecules. As with HO-1 induction, biliverdin and bilirubin treatment potently suppresses the nonspecific immune response, as tested in various *ex vivo* and *in vivo* models: biliverdin treatment protects against lethal endotoxic shock in mice that have been challenged with LPS (*via* inhibition of NF-κB activation) (59) and inhibits iNOS expression and NO production in response to endotoxin in rats (80). Little is known about the effects of HO-1 on neutrophil activation and function, but it is clear from our discussion that the overall pathology of IRI involves inflammation and reactive oxygen species (ROS). In this latter regard, biliverdin/bilirubin are among the most potent antioxidants known (65). Thus, one might assume that the antioxidant potential is responsible for the decrease of oxidative damage, as IRI is a result of an oxidative burst that occurs during reperfusion, and the bile pigments might scavenge ROS *via* an antioxidant cycle, as suggested by Sedlak and Snyder (60). However, in addition to these antioxidant effects, preconditioning of the organ by bilirubin/biliverdin, downregulation of adhesion molecules, and antiapoptotic mechanisms might preserve the organs (26). Experiments being conducted currently indicate that bilirubin/biliverdin activate distinct intracellular signaling cascades that might help to protect the organ from damage of the innate immune system (unpublished observations).

The innate immune system initiates the detrimental process leading to islet dysfunction and finally loss (47). Our results provide the basis for a potential approach initiated by biliverdin/

bilirubin that would protect those islets (79). The three aspects of this action are as follows.

1. Bilirubin treatment of the donor leads to a lesser inflammatory response in the islets after transplantation to the recipient as compared with islets from untreated donors. As with IRI in solid allografts, a nonspecific inflammatory response involving mainly macrophages would heighten the alloaggressive immune response.
2. Biliverdin treatment of the donor leads to suppression of iNOS and IL-1β; strong evidence indicates that both NO, the product of iNOS action, and IL-1β can damage islets (32, 48). Thus, the diminution of NO and IL-1β should have salutary effects. Biliverdin/bilirubin may also be able to protect islets by up-regulating protective genes such as HO-1 and bcl-2. The upregulation by biliverdin/bilirubin of HO-1 would lead to an amplification cycle because more biliverdin would be produced by the induced HO-1. In addition to the protective effect of HO-1, upregulation of bcl-2 in β cells can protect the cells from caspase-mediated cell death, thus contributing to the survival of transplanted islets (67). This is consistent with the suppressed level of caspases in freshly isolated islets and islet grafts at various days after transplantation after biliverdin treatment.
3. The antioxidant effects may contribute greatly to their protective actions in islet transplantation, as early interventions aimed at reducing oxidative stress of pancreatic cells and islets have been shown to improve outcome after islet transplantation (11). It would seem that administration of biliverdin or bilirubin might be useful clinically for islet transplantation to treat type I diabetes.

Finally, with regard to chronic rejection, HO-1 and biliverdin/bilirubin appear to have many of the actions that should, and appear to, overcome the pathologic processes involved in chronic rejection. The antiinflammatory effects, the antioxidant effects, and the modulation of apoptosis could all contribute to suppressing chronic rejection. Biliverdin and bilirubin have both been shown to suppress smooth muscle cell proliferation *in vitro*. Certainly, their effects *in vivo* are consistent with those actions (41, 53, 54).

Biliverdin and bilirubin are parts of a natural gene system (HO-1) that arose in evolution a long time ago. It is not unreasonable to think that this gene has served in a protective mode for much of this time. As such, HO-1 itself and the products of heme degradation may protect from a wider range of insults than drugs derived to target a given molecule specifically. Perhaps the testing of these natural, but highly potent, protective molecules in clinical transplantation is reasonable.

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ABBREVIATIONS

BVR, biliverdin reductase; BUN, blood urea nitrogen; CAD, chronic allograft dysfunction; CO, carbon monoxide; DC, dendritic cell; HO-1, heme oxygenase-1; IRI, ischemia–reperfusion injury; LCAN, luminal cross-sectional area narrowing; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

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