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Heme Oxygenase-1 (HO-1) in Transplantation

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ABBREVIATIONS USED:

CO, carbon monoxide; **HO-1**, heme oxygenase-1; **iNOS**, inducible nitric oxide synthase; **M ϕ** , Monocyte/macrophages; **MHC**, major histocompatibility complex; **MAPK**, mitogen activated protein kinase; **NF- κ B**, nuclear factor kappa B; **TNF- α** , tumor necrosis factor- α .

1. SUMMARY

Heme oxygenase-1 (HO-1) is a stress responsive enzyme that acts during inflammatory reactions as the rate-limiting step in the catabolism of heme, yielding equimolar amounts of iron (Fe), biliverdin and the gas carbon monoxide (CO) (1). Expression of HO-1 regulates inflammatory and immune responses, such as those involved in the rejection of transplanted organs. We will discuss here accumulating evidence supporting that HO-1 expression in a transplanted organ can prevent its rejection. We will argue that the protective effects exerted by HO-1 are mediated to a large extent by the end products that are generated via the catabolism of heme.

2-PROTECTIVE RESPONSES IN A TRANSPLANTED ORGAN: ACCOMODATION.

The idea that a “protective gene” such as HO-1 may be important in transplantation came from findings of several investigators in the 1980s, who successfully transplanted kidneys against ABO barriers that ordinarily led to hyperacute rejection of the kidney (2, 3). These investigators depleted the antibodies (and complement) in the recipient by multiple plasmaphereses and only transplanted the kidney (under immunosuppression) after the antibodies were at low levels (2, 3). The repeated plasmaphereses were continued for a very few days and then the antibodies were allowed to return to normal levels. Yet, given this procedure, even in the presence of antibodies and complement levels that would have led to hyperacute rejection if the kidney had been transplanted directly without the plasmaphereses, there was no rejection (2, 3).

At the time, we hypothesized that the kidneys survived because the endothelial cells (EC), instead of reacting to a stimulus such as the antibodies and complement by promoting a pro-inflammatory response, reacted by up-regulation “protective genes” that acted to prevent the pro-inflammatory response and thus prevent rejection (4, 5). We referred to the survival of an organ in the presence of anti-EC antibodies and complement as “accommodation” (4)(*reviewed in* (6)). That accommodation could be established experimentally was first suggested using porcine to primate kidney xenografts (2) and thereafter using hamster to rat cardiac xenografts (7, 8). We have used the second experimental model to show that these xenografts did accommodate (9), that the mechanism leading to accommodation was associated with i) slow return of anti-graft antibodies to the circulation after an initial period of depletion, (10) and ii) expression in the graft

of protective genes, including HO-1 (11). More recently low levels of anti-EC antibodies have been shown to induce the expression of protective genes (12, 13), thus establishing a casual link between the return of anti-EC antibodies to the circulation and the expression of protective genes in accommodated xenografts (*reviewed in* (6)). We were later able to test the hypothesis that the expression of the protective gene HO-1 was critical to establish graft accommodation (*reviewed in* (6)).

Among the earliest investigations of a possible role for HO-1 in transplantation, we studied a xenotransplantation model. Mouse hearts transplanted to rats were rejected in a matter of a very few days (14). If the rats were treated with cobra venom factor (CVF) to block complement and cyclosporin A (CsA) to suppress the T cell response, the mouse hearts survived indefinitely (14). The grafts expressed high levels of HO-1 in the vasculature, as well as throughout the interstitial tissues and cardiomyocytes (11, 14, 15). If the mouse heart was taken from a HO-1 deficient strain that was congenic with the wild-type mouse donor strain, the hearts were rejected by CVF + CsA treated rats as rapidly as untreated rats rejected the wild-type mouse hearts (15). We subsequently demonstrated that the protective effect of HO-1 was mimicked by exogenous CO, suggesting that CO contributes in a critical manner to the protective effect of HO-1 expression in organ transplantation (16)

These findings demonstrated unequivocally that the ability of a transplanted organ to express HO-1 can be absolutely critical to its survival (*reviewed in* (6, 17)). In addition it suggests that the gas CO, generated by these organs is a key component of this protective effect (16). We learned another lesson: it was the expression of HO-1 and generation of CO in the donor that made all the difference. These two findings brought something quite new to transplantation. Manipulation of the donor organ has been a goal for decades, dating back to Snell's concept of passenger leukocytes that might be removed from the donor organ, however, no approach has been discovered that really contributes to the survival of transplanted organs to the extent that it has been adopted clinically. Our studies focused our attention on one gene that could be of absolutely critical importance in organ transplantation.

These experiments led us to investigate the role of HO-1, and its products, in models of allotransplantation as well (*reviewed in* (17)). HO-1 has been extensively reviewed in many places in this book, thus no details are needed here except to list the three products of HO-1 action on heme: carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and

free iron (which stimulates the up-regulation of ferritin). Others and we have primarily studied induced expression of HO-1 or the administration of CO or biliverdin/bilirubin. Since to date, it appears that biliverdin accomplishes everything that bilirubin does (with the exception of one study (18)), we shall in this chapter refer only to biliverdin, asking the reader to consider that bilirubin can likely accomplish the same as biliverdin. We have focused primarily on the use of several different organs to test the effects of HO-1 and its products on ischemia-reperfusion injury (following syngeneic transplantation) as well as on allotransplantation of the heart. More recently we have tested HO-1 and its products in a model of islet transplantation in mice.

The rejection of a graft, or damage to that graft, can be artificially divided into three phases. Firstly, there is the damage to the organ or cells, i.e. β -cells form pancreas islets, that occurs during the procedure of taking out the organ, storing it (if, as in most cases, it is not immediately transplanted) and then transplanting it into the recipient, where it is re-perfused. This phase is referred to as ischemia-reperfusion injury. Important to our thinking about ischemia-reperfusion injury are the findings that an organ already shows signs of inflammation in a brain dead donor, before the organ is removed (19, 20). Secondly, there is the well-known acute rejection that is mediated by T lymphocytes (*reviewed in* (21, 22)). It is in this area that the field of transplantation has made the greatest progress through the discovery of immunosuppressive drugs, although there are still toxicities associated with those drugs. Even with the more specifically T cell directed agents, an immunosuppressed individual is more susceptible to infection and to the development of malignancies than is a healthy individual. Thirdly, and the greatest current cause of organ loss is chronic rejection. Chronic rejection, also referred to as transplant-associated arteriosclerosis, is in some ways quite like atherosclerosis including the proliferation of vascular smooth muscle cells with intimal proliferation. There are likely multiple etiological factors leading to chronic rejection; as with most “immunological situations”, damage by both T cells and antibodies has been invoked, although general inflammation from other causes is also on the list of potential precipitating and aggravating agents.

Others and we have examined the role of HO-1 and its products in each of these situations. While the effects of Fe^{++} and ferritin have received much less attention than biliverdin and CO, it appears that HO-1, CO and biliverdin are each beneficial when expressed or administered in at least one phase of rejection. Likewise, ferritin is anti-inflammatory.

3. PROTECTIVE EFFECT OF HO-1 IN ORGAN ISCHEMIA-REPERFUSION INJURY

In most experimental models of ischemia-reperfusion injury associated with organ transplantation, the organ that is transplanted is syngeneic to the recipient. This is done so that an immunological rejection response against the “foreignness” of the organ will be absent and the study will focus on the damage done by ischemia and re-perfusion. Ischemia as discussed here involves more than just keeping the organ in low concentrations of oxygen; it also involves temperature changes and other factors. After removal, the organ is stored in a preservation solution that has been developed to maintain function in the organ. Following a number of hours of storage (to mimic the clinical situation in which organs from brain dead donors are used) the organ is transplanted to the recipient. The organ is then re-perfused in the recipient when blood flow is re-established leading to the delivery high O₂ levels to the graft. Because these organs have been previously exposed to ischemia, the O₂ cannot readily be used by the mitochondrial electron transport chain, becoming available to oxidative enzymes, e.g. NADPH oxidase, that promote the generation of reactive oxygen species (ROS), i.e. superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl ions (OH[•]) (23). The main cellular targets of ROS are probably endothelial cells (EC) that line the vasculature of the transplanted organ. Accumulation of ROS in EC can cause lipid peroxidation, oxidative modifications of proteins as well as protein unfolding and DNA damage (24). To prevent these deleterious effects, cells in a transplanted organ can up-regulate the expression of anti-oxidant genes (25, 26). However, the net anti-oxidant capacity afforded by the regulated expression of these genes is probably not sufficient *per se* to counter the exceedingly high levels of ROS generated during ischemia and reperfusion. Therefore ROS do accumulate during IRI and pass a threshold level beyond which EC undergo necrosis or apoptosis, one of the main features associated with IRI (27). ROS also promote the expression of pro-inflammatory genes associated with EC activation (28). These genes encode vasoconstrictors (e.g. endothelin-1), cytokines (e.g. interleukin-1beta (IL-1β) and IL-6), chemokines (e.g. IL-8 and monocyte chemoattractant protein 1 (MCP-1)), adhesion molecules (e.g. E&P-selectins, intracellular adhesion molecule 1 (ICAM-1), vascular cellular adhesion molecule 1 (VCAM-1)) as well as pro-thrombotic molecules (e.g. tissue factor (TF)) (28). Expression of these genes is directly

responsible for the activation and recruitment of circulating leukocytes, e.g. neutrophils and monocyte/macrophages into the graft, a central aspect of IRI (*reviewed in (28)*).

There were at least two questions that we found pertinent with regard to a possible protective role of HO-1 during ischemia-reperfusion injury. Firstly, we asked whether expression of HO-1 or administration of biliverdin or CO would suppress this type of injury? This hypothesis was based on the observation that HO-1 expression protected livers from genetically obese Zucker rats from ischemia-reperfusion injury and extended survival after transplantation of those livers into syngeneic recipients, i.e. from 40% in untreated controls to about 80% in recipients that received livers expressing HO-1 (29). Secondly, we asked whether the expression of HO-1 or administration of biliverdin or CO to the donor organ, the recipient, or, for that matter, during the period that the organ was being stored in the preservation fluid could afford protection against ischemia-reperfusion injury.

There is now ample evidence that induced expression of HO-1 or the administration of CO or biliverdin will ameliorate the damage done by ischemia-reperfusion. The great majority of this work has been done in rodents (32-35)(*reviewed in (30, 31)*). In at least one of the studies involving organ transplantation, induction of HO-1 in the donor was shown to sufficient to prevent not only ischemia reperfusion injury but also chronic graft rejection that develops many weeks after transplantation (34). We have obtained data recently that supports the notion that treatment of the donor with CO can mimic the protective effects of HO-1 in terms of preventing ischemia reperfusion injury (35) (see table 1). Others have also shown similar protective effects of CO in different experimental models of ischemia reperfusion injury in rodents (36-40). In addition, there is one study in pigs in which it was shown that pre-treatment with CO was beneficial in a model of heart by-pass, which is also a problem of ischemia-reperfusion injury (41).

Interestingly, pre-treatment with CO has beneficial results no matter whether one treats the donor, the organ *ex vivo* or the recipient (35). In each case, there is better survival of the organ after transplantation than without CO treatment (see table 1). However, treating at all three stages is better than treating at any one stage. One interpretation of these observations is that there is continuing damage from the time the organ is prepared for removal to the reperfusion in the recipient. A caveat for these observations is that one should not necessarily relate all the beneficial effects to CO alone. We have noted in a study of the effects of CO pre-treatment of

animals subsequently exposed to TNF- α + D-galactosamine, that the CO is highly therapeutic in terms of suppressing hepatitis, however that the salutary effect of CO requires other responses (42). CO leads to up-regulation of NF- κ B in hepatocytes followed by inducible nitric oxide (NO) synthase (iNOS) induction with NO production leading to up-regulation of HO-1 (42). Each of these steps is needed in order for CO to mediate its therapeutic effect. It is not clear in how many other situations, CO acts via influencing other genes and their products. Finally, it appears that it is biliverdin, produced by the induced HO-1 that mediates the protective function without the need for the other directions (unpublished findings). More recently CO and biliverdin have been suggested to synergize to afford the protective effect of HO-1 in similar models of liver injury (43, 44)

While both CO and biliverdin have salutary effects on ischemia-reperfusion injury, we found in a model in which both CO and biliverdin were tested that they act by modulating different aspects of the pathogenesis of ischemia-reperfusion injury. Using the small bowel to study ischemia-reperfusion injury, we found that CO and biliverdin both suppressed injury. However, the two products acted on different pathological processes. For instance, while CO improved blood flow and biliverdin did not, biliverdin suppressed adhesion molecules and reduced cellular infiltration while CO did not (18). We shall return to a discussion of these different functions of CO and biliverdin later.

We are only beginning to understand the mechanisms by which CO and biliverdin act. A set of observations studying islet transplantation suggests one effect that may contribute to the beneficial results CO and biliverdin have on ischemia-reperfusion injury as well as other aspects of organ graft rejection. Induction of HO-1 or treatment of islets with either CO or biliverdin while the islets are still in the donor (i.e. donor treatment) leads to a suppression of the pro-inflammatory response normally seen in islets after transplantation to the recipient (Wang, H et al. submitted). Thus, cytokines such as TNF- α , which are normally highly induced in the islets on the early days after transplantation, are suppressed by the donor treatment (figure 1). Lack of inflammation in the islets likely leads to a lesser rejection response. Whether a similar phenomenon will be observed with organs, i.e. donor treatment leading to less inflammation in the organ after transplantation, must still be tested. To the extent that the findings in islets obtain for organ transplantation, for ischemia-reperfusion injury, the less inflammation, the less oxidative stress. The impact of these findings on acute rejection are discussed below.

4. PROTECTIVE EFFECT OF HO-1 ACUTE REJECTION.

Our work relating to acute rejection has focused primarily on testing if CO and/or biliverdin would impact T cell mediated acute rejection. We found that CO did not do so, although we hesitate to draw that as a conclusion until more doses and dosing schedules of CO have been tested. That HO-1 expression can modulate T cell activation and proliferation was first suggested by the observation that HO-1-deficient mice (HO-1^{-/-}) have higher numbers of circulating activated peripheral CD4 T cells, as compared to wild type (HO-1^{+/+}) mice (45, 46). More recently, CD4 T activation was found to be associated with up-regulation of HO-1 expression (47) and over-expression of HO-1 was shown to inhibit CD4 T cell proliferation (47-49). These effects are mimicked by CO (47, 50) as well as by biliverdin (48, 49), suggesting that these end products of HO-1 activity act as negative feed back loop to limit CD4 T cell activation and proliferation. How these end products contribute to the ability of HO-1 to inhibit the rejection of transplanted organs remains to be elucidated (48, 51-53).

We have recently shown that induction of HO-1 expression triggers alloreactive CD4 T cells to undergo activation induced cell death (AICD) (McDaid et al. FASEB Journal, in press), a regulatory mechanism that controls the extent of CD4 T cell responses and appears to be involved in some mechanisms by which tolerance can be induced (54-56). The observation that CO promotes Fas/CD95 mediated AICD, suggests that CO mediates the pro-apoptotic effect of HO-1 in these cells (50). It is intriguing that CO acts as an anti-apoptotic molecule in EC while promoting activated CD4 T cells to undergo apoptosis. However, these contrasting effects may synergize to prevent the rejection of transplanted organs. By protecting EC from undergoing apoptosis, HO-1 is likely to limit irreversible graft injury associated with IRI or acute graft rejection. On the other hand, by promoting activated CD4 T cells to undergo apoptosis HO-1 probably limits these cells from sustaining additional injury to the graft. This may help to explain how the expression of HO-1 in the recipient affords potent protective effects in terms of preventing acute graft rejection in rodents (49, 53)

We have recently found that biliverdin has a profound impact on acute rejection (48). Treatment of the donor for only 1-2 days and the recipient for 14 days (from day -1 to day +13 with regard to transplantation) with biliverdin (50 µM/kg given twice per day) led to long-term survival (>100 days) in a majority of heart grafts from DBA/2 to B6AF1 mice (48). This

combination differs by one class I antigen (H-2K) and a class II antigen (H-2I), and thus is a weaker combination than some that differ by both H-2K and H-2D and H-2I. The biliverdin was given twice or three times a day because of the very short half-life of the bilirubin (approximately 3 hours) that appeared after biliverdin administration. Only a single dose of biliverdin improved survival but none of the grafts survived long-term (fig 2a; 48). Recipients carrying a long-term surviving first heart graft accepted grafts from the same donor strain without further treatment, while third-party grafts were rejected promptly (fig 2b).

In an attempt to understand the basis of this biliverdin effect, we studied the effects of biliverdin on T cells *in vitro*. Biliverdin exerts potent anti-proliferative effects in T cells, impairing signaling originated via the T cell receptor and leading to the transcription of IL-2, a cytokine required for T cell activation and proliferation (48). Biliverdin blocks nuclear translocation of transcription factors required for IL-2 transcription, i.e. nuclear factor kappa B (NF- κ B) and nuclear factor of activated T cells (NF-AT) (48). Such an effect is likely to contribute to suppress alloreactive T cell activation involved in acute as well as chronic graft rejection (fig. 3).

5. PROTECTIVE EFFECT OF HO-1 IN CHRONIC GRAFT REJECTION.

Induction of HO-1 suppresses chronic graft rejection. This was first suggested by the finding that induction of endogenous HO-1 expression (52) or exogenous HO-1 over-expression (57, 58) can block chronic rejection of cardiac transplants in rodents. Similar protective effects have been observed in experimental models of vascular remodeling driven by either transplantation (59) or wire or balloon injury (60-62). Our work tested whether CO would also suppress chronic rejection. In a model of transplanting a segment of the aorta between allogeneic strains, administration of CO for the full 56 days of the experiment clearly suppressed the development of the arteriosclerosis (60). *In vitro*, CO suppressed the proliferation of vascular smooth muscle cells, almost certainly one of the mechanisms contributing to the *in vivo* action (60). Given that the induction of HO-1 only around the time of transplantation also suppressed chronic rejection, there is need of careful studies to determine how often and for how long CO would have to be administered to effectively suppress the chronic rejection. Recent results suggest that a very short exposure to CO each day can also suppress the signs of chronic rejection such as atherosclerosis.

The cellular and molecular mechanisms by which CO prevents the development of arteriosclerotic lesions remains to be established but there is evidence that CO can act at the level of the vasculature to inhibit leukocyte infiltration/activation as well as smooth muscle cells proliferation (62). Both these effects can suppress neointima formation, an event that plays a critical role in the pathogenesis of chronic graft rejection (62).

The anti-proliferative effect of CO in smooth muscle cells involves cell cycle arrest at the G1/S phase (60, 63, 64). This is mediated via activation of guanylate cyclase, generation of cGMP, activation of p38 MAPK and expression of the cell cycle inhibitor p21^{Cip1} (62). There is also evidence that CO inhibits cyclin A expression and kinase activity as well as retinoblastoma phosphorylation in smooth muscle cells, an effect that is likely to contribute to the anti-proliferative effect of this gas as well (64). Whether these effects are dependent on the expression of p21^{Cip1} has not been established but seems likely to be the case. It is worth noticing that genetic deletion of p21^{Cip1} inhibits the anti-proliferative effect of CO in cultured smooth muscle cells while it does not impair the ability of CO to inhibit vascular remodeling that occurs following balloon injury in mice (62). This suggests that, in addition from its anti-proliferative effect in smooth muscle cells, CO has additional protective effects that are sufficient *per se* to block vascular remodeling, possibly via the potent anti-inflammatory and cytoprotective that CO exerts in EC and monocyte/macrophages (*reviewed in* (65)).

While we have not tested whether biliverdin would suppress chronic rejection, we have shown that biliverdin suppresses intimal hyperplasia after balloon injury and that biliverdin, *in vitro*, suppresses vascular smooth muscle cell proliferation (öllinger *et al* submitted). It thus seems not unlikely that biliverdin will also suppress chronic rejection. Again, it will be important to test when biliverdin must be given and how often. The effect of these substances on macrophages is a likely additional mechanism that contributes to their *in vivo* actions on arteriosclerosis. Both CO and biliverdin suppress the pro-inflammatory response *in vivo* as well as *in vitro* and to varying extents boost the production of IL-10, the anti-inflammatory cytokine. The pro-inflammatory effects of activated macrophages are thought also to contribute to the development of chronic rejection.

It should be noted that CO and biliverdin suppress smooth muscle cell proliferation by somewhat different mechanisms. Both involve p38 MAPK, a set of signaling molecules that are involved in inflammation and apoptosis. However, while CO induces p38 MAPK to inhibit

smooth muscle cell proliferation, biliverdin does so by suppressing p38 MAPK. Two or the four isoforms of p38 in at least some situations, function in opposing manners. Thus, while p38 α is pro-apoptotic, p38 β is anti-apoptotic. We hypothesize the same to explain the different effects of CO and biliverdin on p38: that one may acts on p38 α and the other on p38 β , elevating one and suppressing the other with the same overall effect.

6. THE PROTECTIVE EFFECTS OF HO-1 IN CLINICAL TRANSPLANTATION.

There is now clear evidence that expression of HO-1 plays a critical role in preventing graft rejection in a clinical setting. Analyses of kidneys transplanted under a standard immunosuppressive regimen reveal that there is a 70 fold up-regulation of HO-1 mRNA expression in grafts undergoing acute rejection, as compared to grafts that do not undergo rejection (66). The question that arises is why these grafts undergo acute rejection despite the expression of HO-1 (66). One possible explanation is that under an aggressive host immune response, expression of HO-1 is not sufficient *per se* to suppress graft rejection (66).

In contrast to grafts undergoing acute rejection, HO-1 expression is almost undetectable in kidney transplants undergoing chronic graft rejection (66). Recent evidence suggests that this can be explained by a guanine-thymine (GT) n length polymorphism in the 5' regulatory region of the human HO-1 gene, which dictates the extent of HO-1 transcriptional inducibility (67). Long (GT) n repeats are associated with low levels of HO-1 expression in response to a given stimulus, while short (GT) n repeats are associated with high expression (*reviewed in* (68)). In two independent studies in which kidney transplant recipients and donors were genotyped for this polymorphism, a significantly better long-term survival was associated with presence of the short (GT) n repeat genotype (high level of HO-1 expression) in the graft (69), as compared to grafts expressing the long (GT) n repeat genotype (low level of HO-1 expression). Recipients of grafts with low HO-1 expression lost their grafts significantly more often due to chronic graft rejection (69, 70). The beneficial effect of the donor short (GT) n repeat genotype was significantly more pronounced in grafts exposed to prolonged ischemia and subjected to a least one episode of acute rejection (69). Together with the data obtained in experimental transplant models, these studies strongly support the theory that grafts can express protective genes that have a major impact in preventing chronic graft rejection.

Another factor that is likely to modulate HO-1 expression in a manner that could impact graft rejection is the immunosuppressive protocol under which organs are transplanted in a clinical setting. While glucocorticoids have been suggested to down-regulate the expression of HO-1 (71, 72) other immunosuppressive drugs, such as rapamycin can up-regulate HO-1 expression (73), an effect that could contribute to the beneficial effects of these immunosuppressive drugs in terms of preventing chronic graft rejection (74). In keeping with this, immunosuppressive drugs that have been shown to up-regulate the expression of HO-1, such as the allotrap peptide RDP58, can prevent the development of chronic graft rejection in rodents, (75, 76).

7. DISCUSSION.

One of the questions that should rightly be asked after reading the above is “why bother with the individual products of HO-1, at least in terms of using them as therapeutics, when one can just induce HO-1?” There are several reasons. Firstly, and as discussed above, individuals vary in the promoter GT polymorphism for the HO-1 gene. Thus, there is the question whether the inducing agent to up-regulate HO-1 will not be effective enough in those who have the genetically determined lower response. Secondly, we have found examples of conditions that do not appear to respond to CO but do to biliverdin. While it is possible that we used the wrong doses/dosing schedule for CO, our findings raise the possibility that any given condition will respond more or only to one product and not the others. Thirdly, using the individual products such as CO or biliverdin allows one to use relatively higher doses than might be generated by HO-1 action. While toxicity of the product has to be considered, biliverdin, for instance, is thought to have little toxicity in the ranges to which it has been induced in the above-mentioned studies except in the immediate neonatal period. As such, high levels of biliverdin/bilirubin administered for a very short time might be more advantageous than is possible by inducing HO-1. *In vitro* data suggests that HO-1 has a relatively narrow therapeutic window with higher concentrations becoming toxic, most probably due to the high levels of free iron that are generated (77). Our own results suggest that there is only an eight-fold difference in the concentration of HO-1 that led to optimal protective effect, in a model of EC apoptosis, and the dose at which HO-1 became

toxic. Lastly, at least for the moment, inducing HO-1 may involve other responses that are not desirable.

Highly relevant to the present chapter is the association between the HO-1 promoter polymorphism and chronic rejection. As discussed above there are now two studies in which individuals with a higher HO-1 response based on a shorter GT allele have less chronic rejection than individuals with a longer GT allele and thus a poorer HO-1 response (69, 70). While only an association, it does not seem unreasonable to posit that it is the better protection that individuals with a high HO-1 response have that is responsible for the relative absence of arteriosclerosis. While other approaches should of course continue to be tested, we are biased that use of the HO-1 system with its products should be high on any list of approaches to chronic rejection. These are natural products that appear to be involved physiologically in the normal protection against arteriosclerosis.

Very importantly to our minds, the association just mentioned related to the relative response of the donor. The findings suggest that it is protection of the donor organ that is critical for the avoidance of chronic rejection. There was no significant association between the ability to respond by up-regulating HO-1 and graft survival in the recipient. This emphasis of the donor is also consistent with our findings in mouse to rat transplants discussed above. The absence of HO-1 in the mouse heart led to very rapid rejection under conditions in which wild-type hearts would survive indefinitely. It is time for the transplantation community to return to potential therapies, such as expressing HO-1 in or administering an HO-1 product to the donor.

The other very significant problem in transplantation is ischemia-reperfusion injury. It is thought that the injury associated with ischemia-reperfusion injury is in large measure due to oxidative stress. Biliverdin and bilirubin are two of the most potent anti-oxidants known. Further, there is preliminary evidence that biliverdin and CO can act in an additive manner in terms of overcoming ischemia-reperfusion injury (37, 78).

An overview of the HO-1 system, which is relevant to transplantation and to other inflammatory conditions, shows that the system functions in physiological protection (reviewed in (65)). Not only is there the evidence recounted above and related to the HO-1 promoter polymorphism but there is also an association between high normal or just above normal levels of bilirubin in normal individuals and a lesser incidence of atherosclerotic-like conditions (79, 80). While this association does not necessarily implicate bilirubin as the molecule that causes

the lesser incidence of atherosclerotic conditions, our recent finding that bilirubin suppresses intimal hyperplasia after balloon injury, inhibits vascular smooth muscle cell proliferation *in vitro* and suppresses the pro-inflammatory response of macrophages provides evidence implicating bilirubin as the molecule of interest in the avoidance of atherosclerotic reactions *in vivo*.

9. An overview.

Inflammation, oxidative stress, apoptosis, T cell responses and proliferation of vascular smooth muscle cells are major pathological processes that lead to rejection of transplanted organs. The expression of HO-1 or the administration of the products of HO-1, including CO and biliverdin, leads to the very actions that contravene these pathologies. It is thus not surprising that HO-1 and its products have shown salutary effects in transplantation.

Why does physiological expression of HO-1 not achieve the same results? There are several potential explanations hinted at above. It may be that the physiological response of HO-1 is simply not strong enough to overcome the complications of transplantation. Alternatively, it may be that the HO-1 response comes too late. Therapeutic use of the HO-1 system can hopefully overcome both of these limitations.

In fact, as mentioned above, individuals who have a strong HO-1 response based on their promoter GT length polymorphism appear to benefit from that response. Those studies suggest that the physiological response is on the borderline of effectiveness.

The doses of CO used in the studies showing the effectiveness of that compound in various phases of transplantation are relatively non-toxic. Further, the extent to which bilirubin was raised by administration of biliverdin, or in a few cases bilirubin itself, would have to be considered non-toxic given our extensive knowledge of bilirubin. If the expression of HO-1 or the administration of CO and/or biliverdin prove to be effective in a large animal model such as the pig, it would seem not unreasonable to test these agents in humans. For the problems of each of the phases of transplantation mentioned above, the products could be used to pre-treat. It is in that situation that the best data has been obtained to show effectiveness.

Table 1**Exposure of the donor and the graft, but not the recipient to CO, protects hearts from transplant associated IRI**

Group	N	Day 1	Day 7	Day 14
Control	6	0% (0/6)	0% (0/6)	0% (0/6)
CO (D)	6	50% (3/6)	33% (2/6)	33% (2/6)
CO (G)	6	50% (3/6)	33% (2/6)	33% (2/6)
CO (D+G)	6	100% (6/6)	83% (5/6)	66% (4/6)
CO (D+R)	5	0% (0/5)	0% (0/5)	0% (0/5)
CO (G+R)	6	33% (2/6)	17% (1/6)	17% (1/6)
CO (D+G+R)	6	100% (6/6)	83% (5/6)	83% (5/6)

Legend: Hearts were harvested from Lewis rats, exposed to ischemia (UW solution, 24 h, 4°C), and transplanted into syngeneic Lewis recipients (R). When indicated, CO was administered to the donor (D), the graft (G), and/or the recipient (R). Treatment of the organ *ex vivo* (G) is done by “saturating” the preservation solution with CO by bubbling CO through the solution for several minutes before the organ is placed in that solution.) Notice the significant increase in the percentage of graft survival under CO administration to the donor and/or the graft vs untreated controls ($P<0.05$). There was no significant difference between the protective effect of CO given to the donor and the graft vs. the donor, the graft, and the recipient ($P>0.05$). There was a significant difference between the protective effect of CO given to the donor and the graft vs. the donor alone ($P<0.05$). Results shown are the percent of graft survival (number surviving grafts/total number of grafts).

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