[Frontiers in Bioscience 12, 4932-4945, September 1, 2007]

Heme oxygenase-1 in organ transplantation

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1. ABSTRACT

Cells have a plethora of defense mechanisms that are activated upon exposure to oxidative stress. These aim at limiting the deleterious effects of oxidative stress and re-establishing homeostasis. In the particular context of organ transplantation, these defense mechanisms contribute to sustain graft survival via at least two interrelated mechanisms. First, cytoprotection *per se* should support survival and function of cells within a transplanted organ. Second, cytoprotection could reduce immunogenicity of the graft and modulate the activation of the recipient's immune system to promote regulatory (suppressive) responses that sustain graft survival. Others and we have gathered evidence, to suggest that the stress-responsive enzyme Heme Oxygenase-1 (HO-1 encoded by the gene *Hmox1*) acts in such a manner. Upon organ transplantation, HO-1 is ubiquitously expressed in a transplanted organ, becoming the rate-limiting enzyme in the catabolism of heme into carbon monoxide (CO), iron (Fe) and biliverdin (1). There is accumulating evidence to support the notion that HO-1 expression in a graft and in the recipient can prevent rejection and promote immune tolerance. We will argue that these effects are mediated to a large extent by limiting the deleterious effects of free heme as well as by the inherent cytoprotective and/or anti-inflammatory effects of the end-products generated via heme catabolism.

2. PROTECTIVE RESPONSES IN A TRANSPLANTED ORGAN: ACCOMMODATION

The notion that when expressed in a transplanted organ a given gene or set of genes may be important in sustaining its survival was based on findings of several investigators in the 1980s including Slapak, Alexandre and Bannett, who successfully transplanted kidneys between ABO blood group incompatible individuals, an approach that ordinarily led to hyperacute rejection of those kidneys (2, 3). As hyperacute rejection was known to originate from the deposition of anti-blood group antibodies and complement activation on the vascular endothelium of the transplanted organ, these investigators thought to deplete those circulating antibodies (and complement) from the recipient, using multiple plasmaphereses. The ABO blood group incompatible kidney grafts were then transplanted under immunosuppression and repetition of the plasmaphereses for a few days after transplantation (2, 3). This insured that the levels of circulating antibodies and complement remained at low levels for those very few days that followed transplantation. These investigators noticed however, that when anti-blood group antibodies returned to normal levels days after transplantation, there was no rejection (2, 3).

The conceptual model to explain this phenomenon was provided by one of us (FHB) who

suggested that these ABO incompatible kidney grafts might survive because endothelial cells (EC) in the graft, instead of being "activated" and promoting a pro-inflammatory response leading to hyperacute rejection, up-regulated the expression of one or several "protective genes" during the first days after transplantation (4, 5). Presumably these protective genes prevented the pro-inflammatory response associated with EC activation, thus suppressing hyperacute rejection (4, 5). The survival of a transplanted organ in the presence of anti-EC antibodies and complement, that would otherwise lead to the rejection of a naïve graft, was referred to as "accommodation" (4) (*reviewed in* (6)) and several groups of investigators, including ourselves, became interested in explaining the cellular and molecular basis of this phenomenon.

That accommodation could be established experimentally was first suggested using porcine to primate kidney xenografts and thereafter using a more "easy to handle" hamster to rat cardiac xenograft model (7, 8). We adopted the second experimental model and used it extensively to confirm initially that xenografts did accommodate (9) and 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 putative protective genes, including heme oxygenase-1 (HO-1 encoded by *Hmox1*), the zinc finger protein A20 and bcl-X (11). Thereafter, low levels of anti-EC antibodies were shown to induce the expression of some of these protective genes (12, 13), thus establishing a casual link between the kinetics of return of anti-graft antibodies to the circulation and the expression of protective genes in accommodated xenografts (*reviewed in* (6)).

3. A MOLECULAR BASIS FOR ACCOMMODATION: EXPRESSION OF HEME OXYGENASE-1

While useful to raise the notion that "protective genes" might counter the rejection of a transplanted organ, the technical inability to manipulate the graft (hamster) genome made it impossible at the time to assess which, if any, of these putative "protective genes" was involved in sustaining graft survival. To test this hypothesis experimentally, an alternative experimental model was set-up by Nozomi Koyamada and Tsukasa Myatake working with us. Briefly, these investigators found that in a manner similar to hamster to rat transplants, mouse to rat cardiac xenografts also accommodated when transplanted into cobra venom factor (CVF) plus cyclosporine A (CsA) treated rats (14). This experimental system, i.e. using the mouse as a graft donor, had a significant advantage in that it allowed for genetic deletion of putative protective genes in the graft (mouse), enabling us to assess their role in the establishment of accommodation (11, 14, 15). We were aware at this time of the seminal work from Rex Tyrrel (16, 17), Karl Nath (18), György and József Balla (19) as well as that of Dean Willis (20) and colleagues, suggesting that HO-1 acted in a protective manner in a variety of situations. We decided, based largely on these data, to test the putative role of HO-1 in graft accommodation, by making use of a mouse strain generated by Kenneth Poss in which the *Hmox1* gene had been deleted by homologous recombination (21, 22). Yuan Lin, a surgeon and transplant immunobiologist who joined our laboratory at that time, compared the ability of cardiac transplants harvested from wild type ($Hmox1^{+/+}$) *versus Hmox1* deficient ($Hmox1^{-/-}$) mice to accommodate when transplanted into CVF plus CyA treated rats. Contrary to grafts from $Hmox1^{+/+}$ mice that accommodated, those from $Hmox1^{-/-}$ did not (15). This simple observation demonstrated unequivocally that expression of a single protective gene in a transplanted organ, namely HO-1, could be absolutely critical to its survival (6)(*reviewed in* 23). These observations also brought back something first suggested by George Snell more than 50 years ago that never gained traction in the clinic: that manipulation of the donor organ could be used to promote graft survival. Our studies focused the attention on one single gene, i.e. *Hmox1* that could be of absolutely critical importance in organ transplantation. The next question become why was this one gene so critical to sustain graft survival?

We initially asked whether the mechanism(s) underlying the protective effects of HO-1 would involve the end-products generated via heme catabolism, i.e. CO, biliverdin (which is rapidly converted to bilirubin) and Fe (which up-regulates ferritin). We decided to test CO, based on unpublished preliminary data made available to us at the time by Leo Otterbein and Augustine Choi, demonstrating that this gas exerted potent anti-inflammatory effects in monocyte/macrophages (24). By that time, these investigators had also shown that CO inhalation prevented the lethal effect of lung hyperoxia in mice (25). It became apparent from their data that it was technically feasible to apply CO *in vivo* and eventually test its putative role in preventing the rejection of transplanted organs and/or promoting accommodation. Koichiro Sato a post-doctoral fellow with us did these experiments and demonstrated that CO inhalation could act elsewhere than in the lung (a gas exchange surface) to afford potent protective effects, i.e. replace HO-1 and sustain the accommodation of mouse to rat cardiac transplants (26). While critical in establishing that CO generated by HO-1 could sustain graft survival, we felt that there were at least two main questions that should be addressed. First, could these findings be applied to experimental settings that mimicked more closely what occurs clinically when organs are transplanted and second, why was the enzymatic reaction catalyzed by HO-1 and in particular the gas CO so efficient in preventing the rejection of transplanted organs?

4. EXPRESSION OF HO-1 PREVENTS ISCHEMIA-REPERFUSION INJURY, THE INITIAL PHASE OF GRAFT REJECTION

There is now ample evidence that induced expression of HO-1 will ameliorate the damage done by ischemia-reperfusion (IRI), an event inherent to all transplanted organs to varying extents and critically involved in their survival (*reviewed in* (27, 28)). The great majority of the experimental work to support this notion has been done in rodents (29-32) (*reviewed in* (28, 33)),

with the original observation being provided by the group of Jerzy W. Kupiec-Weglinski showing that over-expression of HO-1 in the liver prevented IRI associated with prolonged ischemia (24h, 4^0C), as measured by survival rate after transplantation into syngeneic recipients (34). Presumably IRI in this experimental model occurs independently of the recipient´s adaptive immune response, given that the graft does not express mismatched histocompatibility antigens that can be recognized by the recipient's T and B cell antigen receptors. Therefore, the finding that HO-1 overexpression could override IRI suggested that it prevented tissue injury that occurs independently of B and/or T cells. This finding also supported our original observation that HO-1 prevented xenograft rejection under conditions where CsA suppressed T cell activation (15).

It is now well-established that innate immune responses are critically involved in the pathogenesis of IRI. This notion emerged from the finding that a given number of germline encoded innate receptors, i.e. Toll like receptors (TLR) are required to trigger IRI in transplanted organs (35, 36). As HO-1 suppresses IRI, one possibility would be that HO-1 would interfere with the ability of TLR to trigger IRI. The observation that pharmacological inhibition of HO activity increased TLR expression in organs subjected to IRI and that in the absence of one single TLR, i.e. TLR4, HO-1 was no longer required to prevent IRI (37) supported this notion. The molecular basis for this phenomenon has been recently provided by a set of observations made by the group of Augustine Choi. These investigators have shown that over-expression of HO-1 in a mouse monocytic cell line (RAW264.7) interferes with TLR cellular localization into lipid rafts, an event shown to be required to support TLR signaling (38). This phenomenon was demonstrated for several TLR, including TLR2 and TLR4 (38), the two TLR shown to be functionally involved in the pathogenesis of IRI (35, 36). As discussed in more detail below, this effect relies on the ability of HO-1 to dampen the generation of reactive oxygen species (ROS).

Ischemia involves more than just keeping the organ in low concentrations of O_2, it also involves temperature changes and other factors. After removal from the donor, the organ to be transplanted 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 and is reperfused in the recipient when blood flow is re-established. This leads to the abrupt delivery of O_2 to the graft. Because these organs have been previously exposed to ischemia, O_2 cannot readily be used by the mitochondrial electron transport chain, becoming available to oxidative enzymes, e.g. NADPH oxidase, that can promote the generation of ROS, i.e. superoxide ($O^{\cdot}_2$), hydrogen peroxide (H_2O_2) and hydroxyl ions ($OH^{\cdot}$) (39). These ROS can trigger lipid peroxidation, oxidative modifications of proteins as well as protein unfolding and DNA damage, all of which are highly cytotoxic events (40).

To prevent the deleterious effects of ROS, cells can up-regulate the expression of several anti-oxidant genes (41, 42). However, the net anti-oxidant capacity afforded by 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 and pass a threshold level beyond which cytotoxicity becomes irreversible, causing cells to undergo necrosis or apoptosis, one of the main features associated with IRI (43). So, the question becomes: how can HO-1 counter these events?

It is likely that both experimental and clinical IRI are associated with a certain level of hemolysis, leading to hemoglobin (Hb) release into the extra-cellular space. In the presence of ROS, ferrous (Fe^{++}) Hb is rapidly oxidized into methemoglobin (MetHb) (Fe^{+++}) that in contrast with ferrous Hb releases heme (*reviewed in* (44)), a hydrophobic molecule that can intercalate in cytoplasmic membranes. Seminal work by Balla and colleagues has demonstrated that free heme derived from Hb does act in a deleterious manner in EC (19, 45). Because the graft endothelium is the physical interface between the recipient's blood and the parenchyma of the graft itself, it is reasonable to assume that the cytotoxic effects of free heme may be exerted primarily on the graft endothelium. While the exact mechanism via which this occurs is not entirely clear, the deleterious effects of free heme are most probably due to the capacity of Fe in its inner core to act as a Fenton reactor and in this manner promote the generation of ROS (*reviewed in* (44)) (46) (47). Based on the well-established role of ROS in the pathogenesis of IRI, it is also likely that mechanisms that would prevent free heme from accumulating should have salutary effects in preventing EC injury and therefore in suppressing IRI. As it turns out, HO-1 does exactly that, i.e. it degrades heme (1). It thus seems reasonable that heme degradation would be one of the mechanisms via which HO-1 prevents IRI. However, this effect is probably not sufficient by itself to afford protection against IRI.

In the process of heme degradation, HO-1 generates potentially deleterious free Fe, which promotes the generation of ROS via the Fenton reaction (47). Others have referred to this as the HO-1 "Trojan Horse" effect (48). However, one needs to consider that the Fe released from heme degradation induces post-transcriptionally (thus very rapidly) the expression of heavy chain (H) ferritin (49), which forms a multimeric protein complex with light chain ferritin that "scavenges" free cellular Fe and preclude it from promoting the generation of ROS (50). This process, originally shown by Balla and colleagues to afford cytoprotection in EC (19) should in principle mitigate IRI.

Pascal Berberat in our laboratory collaborating with Masamichi Katori from Jerzy W. Kupiec-Weglinski´s laboratory tested this hypothesis and confirmed that indeed H-ferritin is anti-apoptotic in EC, showing in addition that it prevents IRI when transduced into rat livers transplanted into syngeneic recipients after a prolonged (24 hours) period of cold ischemia (51). Presumably, this protective effect occurs via the ability of H-ferritin to negate the

participation of free Fe in the generation of ROS, an event that promotes apoptosis via a complex mechanism involving sustained c-Jun NH (2)-terminal kinase (JNK) activation (52-54). HO-1 and H-ferritin may have additional effects that could contribute to suppress IRI as well.

Based on the recent demonstration that HO-1 controls TLR-driven signaling via inhibition of ROS generation (38), it is likely that H-ferritin, which suppresses ROS by limiting the participation of free Fe in the Fenton reaction (52), would also contribute to this effect. While this remains to be tested experimentally, such an effect would be consistent with the ability of HO-1 and/or H-ferritin to suppress IRI via inhibition of TLR signaling.

In addition to their inherent cytotoxicity, ROS can promote the expression of pro-inflammatory genes associated with EC activation. 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 chemotactic 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)) (*reviewed in (*55)). Expression of these pro-inflammatory 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 (*55)).

Expression of most of the pro-inflammatory genes associated with EC activation is under the control of nuclear factor kappa B (NF-κB), a family of transcription factors that belong to the metazoa Rel/Dorsal family and share among other features a common Rel homology domain (RHD) (reviewed in (56)). There are five NF-κB family members in humans and mice, i.e. RelA, c-Rel, RelB, p50 and p52. The predominant member in EC is most probably RelA, a protein of molecular mass ranging from 60-65KDa depending on several possible post-translational modifications that include phosphorylation at serines 276 (57), 311 (58) and 529 (59) as well as acetylation at lysines 122, 123, 218 , 221 and 310 (60-63).

NF-κB mediated gene transcription is triggered upon degradation of the cytoplasmic NF-κB inhibitor molecules, i.e. IκB (reviewed in (56)). This allows for rapid nuclear translocation and NF-κB binding to DNA κB binding motifs GGGRNNYYCC (where R is a purine (A or G), Y is a pyrimidine (C or T), and N is any base) in the promoter region of NF-κB dependent genes. For many years IκB degradation was thought to be the main regulatory step in NF-κB activation. However, several additional mechanisms have now been shown to be essential in regulating NF-κB activity (*reviewed in (*64)). These include, but are not restricted to the phosphorylation of Ser205 (65), Ser276 (66-68), Ser281 (65) and Ser311 in the N-terminal domain of RelA (58). Phosphorylation of these phosphoacceptors is under the control of several protein kinases including protein kinase A, i.e. Ser276 (67), mitogen and stress activated kinase-1 (MSK1), i.e. Ser276 (67) and protein kinase C zeta (PKCζ), i.e. Ser276 (66) and Ser311 (58). As recently shown by Josef Anrather and colleagues the net phosphorylation status of these serines control not only the overall transactivation activity of RelA but also its specificity for different subsets of target genes (65).

We found that HO-1 expression in EC down-regulates the expression of pro-inflammatory genes associated with EC activation (69). Subsequently, Mark Seldon and Gabriela Silva and one of us (MS) showed that this effect results from inhibition of NF-κB (69). In collaboration with Josef Anrather we found that inhibition of NF-κB occurs through the down modulation of labile Fe, an effect that targets specifically RelA Ser276 (*Seldon et al, submitted for publication*). Inhibition of RelA phopshorylation at Ser276 down regulates RelA transcriptional activity (65, 66) thus inhibiting the transcription/expression of pro-inflammatory genes associated with EC activation. Given that there are several NF-κB dependent pro-inflammatory genes that contribute to the pathogenesis of IRI, it is reasonable to assume that inhibition of their expression via the mechanism described above should counter the pathogenesis of IRI.

We have argued that the control of NF-κB activation, such as afforded by the expression of HO-1 in EC, aims at a functional compromise in that it inhibits the expression of pro-inflammatory genes associated with EC activation while maintaining the expression of several NF-κB dependent genes, required to support the cytoprotective effect of HO-1 (70). It is worth noticing that H-ferritin expression can be controlled at the transcriptional level via activation of NF-κB (71), an effect that is strictly required to prevent TNF mediated apoptosis (52). Therefore it is likely that H-ferritin would be one of the NF-κB dependent genes "spared" by HO-1, an effect that would explain the requirement of NF-κB activation to promote the cytoprotective effect of HO-1 in EC (70). This notion is also supported by the additive effect of CO and H-ferritin in preventing EC from undergoing TNF-mediated apoptosis (51).

In addition to H-ferritin, other end-products of heme degradation by HO-1, i.e. CO and biliverdin are also protective in IRI. The first demonstration that CO affords protection against IRI was probably provided by Tomoyuki Fujita in the laboratory of David Pinsky (72), showing that both HO-1 and inhaled CO were highly protective against lung IRI (72).

Yorihiro Akamatsu, a post-doctoral fellow in our laboratory followed this line of research and demonstrated that the protective effects of HO-1 in cardiac IRI in rats could also be mediated via CO (32). Similar effects were also observed in other studies using different experimental rodent models of IRI (73-77) and more recently porcine (78) IRI models. Interestingly, the original studies of Yorihiro Akamatsu demonstrated unequivocally that pre-

treatment of only the donor or the organ itself with CO afforded beneficial effects (32). In each case, there was better survival of the organ after transplantation than without CO treatment (32). 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. If this is the case, then CO may act in the organ to suppress this type of damage, via a mechanism that remains to be established in organs but may be known, at least in part, for pancreatic islet transplantation.

Our collaborator in Boston, Hongjun Wang, has shown that treatment of only the donor of islets with CO (or bilirubin – see below) results in suppression of inflammation in those islets after transplantation (79)). Without treatment, there is strong expression in the transplanted islets of several pro-inflammatory genes, including TNF (79). With only treatment of the donor of the islets, the expression of these pro-inflammatory genes is very significantly suppressed (79). Based on the concept that inflammation promotes the activation of adaptive immune responses, these findings suggest that the adaptive immune response leading to rejection of those islets may be blunted due to the suppression of inflammation.

Based on the work of Sophie Brouard in our laboratory in Boston we would argue that the protective effect of CO during IRI is mediated to a large extent via its cytoprotective effects (80). This notion is supported by the original finding of Sophie Brouard that HO-1 derived CO acts via the p38 mitogen activated protein kinase (MAPK) signal transduction pathway to prevent EC from undergoing apoptosis (80). While cytoprotective *per se*, such an effect may also limit the overall inflammation/immunogenicity of a transplanted organ, based o the ability of endogenous TLR ligands to promote inflammation (see next section).

The molecular mechanism(s) via which CO affords cytoprotection is still not clear. The data obtained recently by Gabriela Silva at the Instituto Gulbenkian de Ciência would suggest that CO inhibits specifically the expression of the pro-apoptotic p38α isoform, thus directing signals emanating from upstream kinases towards the cytoprotective p38β isoform (81). That p38β sustains the anti-apoptotic effect of HO-1 is demonstrated by the observation that the cytoprotective effect of HO-1 are ablated once p38β expression is suppressed using siRNA (81) or when p38β is deleted by homologous recombination (82). Taken together these data would suggest that the anti-apoptotic effect of CO requires not only the expression of p38β (82) but also the inhibition of p38α expression (81). That this effect acts *in vivo* to prevent IRI is likely to be the case as the ability of CO to prevent liver IRI was shown to involve the activation of the p38 MAPK signal transduction pathway (29).

We will argue that most, if not all the signal transduction exerted by CO is mediated via its binding to divalent metals such as Fe^{++} in the heme prosthetic groups of heme proteins (83). One of such heme proteins is $gp91^{phox}$, a major component of the multimeric NADPH oxidase complex, involved in the generation of ROS by activated monocyte/macrophages and neutrophils. Both HO-1 and CO have been shown to down modulate NADPH expression/activity (38, 84), an effect that as discussed above should contribute to suppress IRI.

In addition to CO, other end products of HO-1 activity, i.e. biliverdin/bilirubin, have salutary effects including the suppression of IRI. Several studies have been done both with testing of a heart on a Langendorff after the ischemic period (85) and with transplantation of the organ. Livers, the small intestine, kidney, and hearts have all been shown to be beneficially affected by pre-treatment with either biliverdin or bilirubin (86, 87). It is presumed, but not proven, that a large part of the salutary effects of such treatments relates to the anti-oxidant effects of bilirubin (88). There is also preliminary evidence that biliverdin/bilirubin and CO can act in an additive manner in terms of overcoming IRI (74, 89).

While CO and biliverdin have beneficial effects, we found that these molecules act by modulating different aspects of the pathogenesis of IRI. Using the small bowel to study IRI, CO and biliverdin both suppressed injury (18). However, the two products acted on different pathological processes. For instance, while CO improved blood flow and biliverdin did not, biliverdin suppressed the expression of adhesion molecules associated with EC activation and reduced cellular infiltration while CO did not (18)(69). We shall return to a discussion of these different functions of CO and biliverdin later.

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

There is now considerable evidence to suggest that tissues exert control over effector responses generated by adaptive immunity (90, 91). The overall view is that cytotoxicity is a highly pro-inflammatory event that promotes the activation of antigen presenting cells, namely dendritic cells (92). Key to understanding this phenomenon is the notion that dendritic cells can "sense" cytotoxicity via recognition of intracellular hydrophobic molecules released during necrosis (93). Such molecules have been shown to include among others, uric acid (94) and high mobility group box 1 (HMGB1) (95).

Recognition of hydrophobic domains in these molecules is thought to be mediated primarily via TLR (93) and probably other innate recognition receptors such as recently shown for the Nod-like protein 3 (NALP3), which recognizes uric acid (96) as well as the receptor for advanced glycation end products (RAGE) that recognizes to at least some extent HMGB1 (97). These germline encoded innate receptors can trigger DC activation, a critical event in the process by which these professional antigen presenting cells become immunogenic, i.e. can trigger the activation of naïve $CD4^+$ T helper (T_H) cells (reviewed in (98)). Given the above, the cytoprotective effects exerted in a graft via the expression of HO-1 are likely to down-modulate DC activation, in a manner that would inhibit the subsequent activation of T_H leading to graft rejection. This hypothesis remains however, to be tested experimentally.

Our work relating to acute graft rejection has focused initially on testing whether exogenous CO and/or biliverdin would impact this type of rejection. We found that CO did not do so, although we hesitate to draw a final conclusion until more doses and schedules have been tested.

That HO-1 modulates T_H cell activation and/or proliferation was suggested initially by the observation that HO-1-deficient mice (*Hmox1*$^{-/-}$) have higher numbers of circulating activated peripheral T_H cells, as compared to wild type (*Hmox1*$^{+/+}$) mice (21, 22). With Santiago Zelenay and colleagues at the Instituto Gulbenkian de Ciência we have confirmed this observation in BALB/c and C57BL/6 *Hmox1*$^{-/-}$ mice. However, we have so far failed to detect any significant changes in T_H cell proliferation whether T_H cells are isolated from *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ mice (99). This would suggest that when expressed physiologically in T_H cells, HO-1 does not regulate TCR signal transduction leading to proliferation (99). Others, however, have shown that T_H cell activation/proliferation is exacerbated in *Hmox1*$^{-/-}$ versus *Hmox1*$^{+/+}$ mice (100). The reasons for this discrepancy are not clear but could be related to differences in the genetic background of the mouse strains used.

HO-1 over-expression in T_H cells inhibits T_H cell proliferation (101). While this observation may have therapeutic value, it does not provide evidence that HO-1 is a physiologic regulator of T_H cell activation/proliferation. Our data suggesting that proliferation of T_H cells from *Hmox1*$^{-/-}$ mice is similar to that of T_H cells from *Hmox1*$^{+/+}$ mice would argue that expression of HO-1 in T_H cells is not part of a physiologic response that regulates T_H cell activation/proliferation (99). A caveat to this conclusion is that expression of HO-1 might control T_H cell activation/proliferation only under conditions of oxidative stress, which has so far not been tested.

When applied exogenously, CO (101, 102) as well as biliverdin (103) down-modulate T_H cell proliferation. This would suggest that these end-products of HO-1 activity limit T_H cell activation and/or proliferation, a notion at first difficult to reconcile with the observation that HO-1 expression in T_H cells does not seem to modulate T_H cell proliferation (99). However, these effects may be explained by the recent observation that pharmacological induction of HO-1 in DC, using cobalt protoporphyrin IX, can suppress T_H cell proliferation in response to those DC, suggesting that HO-1 might regulate T_H cell activation/proliferation via DC cells (104). Angelo Chora at the Instituto Gulbenkian de Ciência has confirmed these results and shown that induction of HO-1 in DC suppresses the activation of self-reactive T_H cells (105).

How two of the end products of HO-1 activity, i.e. CO and biliverdin, inhibit the rejection of transplanted organs also remains to be elucidated (103, 106-108). James McDaid in our laboratory in Boston showed that pharmacological induction of HO-1 by cobalt protoporphyrin IX can trigger alloreactive T_H cells to undergo activation induced cell death (AICD) (109), a regulatory mechanism that controls the extent of T_H cell responses and appears to be involved in some mechanisms by which tolerance can be induced (110-112). The observation that CO promotes Fas/CD95 mediated AICD, suggests that it can mediate the pro-apoptotic effect of HO-1 in T_H cells (102).

It is at first intriguing that CO would act as an anti-apoptotic molecule in EC (15, 80) while promoting activated T_H cells to undergo apoptosis (102). However, these effects may synergize to prevent the rejection of transplanted organs (*reviewed in* (27)). As discussed above it is likely that by protecting EC from undergoing apoptosis, HO-1 would limit irreversible graft injury associated with IRI while on the other hand, by promoting T_H cells to undergo AICD, probably limiting additional injury to the graft. This may help to explain how the expression of HO-1 in a graft recipient affords protective effects in terms of preventing acute graft rejection in rodents (108, 113).

Kenichiro Yamashita in our laboratory found that biliverdin has a profound impact on acute rejection (103), probably explaining how expression of HO-1 achieves this effect. 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 (103). 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 as well as H-2I. The biliverdin was given twice or three times a day because of the very short half-life of circulating bilirubin (approximately 3 hours) that is generated very rapidly after biliverdin administration (103). 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 (103). This finding, demonstrates that biliverdin can induce donor specific immune tolerance.

In an attempt to understand further the cellular basis of this phenomenon, Kenichiro Yamashita and James McDaid studied the effects of biliverdin on T cells *in vitro*. Biliverdin exerted potent anti-proliferative effects, impairing signaling originated via the TCR and leading to *IL*-2 transcription, required for alloreactive T_H cell activation and proliferation (103). Biliverdin blocks nuclear translocation of transcription factors required for *Il*-2 transcription, i.e. nuclear factor kappa B (NF-κB) and nuclear factor of activated T cells (NF-AT) (103). Such an effect is likely to suppress alloreactive T cell activation involved in acute as well as chronic graft rejection.

Based on the immunoregulatory effects of HO-1, we reasoned that when generated endogenously, CO and/or biliverdin might promote donor specific tolerance of a transplanted organ. To test this hypothesis Kenichiro Yamashita, James McDaid and Robert Öllinger asked whether HO-1 expression was required for the induction of tolerance when hearts from C57BL/6 (H-2^b) mice are transplanted into BALB/c (H-2^d) recipients treated with the

tolerance-inducing regimen of anti-CD40L antibody (MR-1) plus donor specific transfusion (DST). We reasoned that if HO-1 was required for tolerance induction, then *Hmox1* deletion should lead to graft rejection. We confirmed initially that grafts from *Hmox1*$^{+/+}$ C57BL/6 donors could be tolerized when transplanted into *Hmox1*$^{+/+}$ BALB/c recipients receiving DST 7 days before transplantation plus anti-CD40L. However, this was no longer the case when these grafts were transplanted under the same regimen into *Hmox1*$^{-/-}$ recipients (114). Similarly, tolerance was also ablated when HO activity was blocked pharmacologically using zinc protoporphyrin IX (114). These observations suggest that expression of HO-1 in the recipient can be critical to establish tolerance.

These co-workers followed up this line of investigation by asking whether expression of HO-1 in the graft was also required for the establishment of tolerance. They found that grafts from *Hmox1*$^{-/-}$ BALB/c donors were tolerized when transplanted under the same regimen into *Hmox1*$^{+/+}$ recipients, suggesting that expression of HO-1 in a graft is not strictly required to support tolerance induction against that graft (114).

Having established that expression of HO-1 in the graft recipient can be critical for tolerance induction, Kenichiro Yamashita went on to test whether pharmacological modulation of HO-1 could be used therapeutically to promote tolerance induction. As previously established, DST alone (day 0, at the time of transplantation) failed to prolong the survival of DBA/2 (H-2^{d}) hearts transplanted into B6AF1 (H-2$^{b,k/d}$) recipients. However, long-term survival and (dominant peripheral) tolerance were readily induced when DST was combined with pharmacological induction of HO-1 by cobalt protoporphyrin IX (114). The tolerogenic effect of HO-1 plus DST was dependent on the presence of regulatory T_H cells (Tregs), as suggested by adoptively transferring these cells into irradiated recipients under various regimens (114).

Santiago Zelenay and colleagues at the Instituto Gulbenkian de Ciência explored the possibility that HO-1 expression might be required for Treg development and function, a hypothesis driven initially by the apparent overlap of Treg and HO-1 immuno-regulatory functions in various experimental models including organ transplantation (115). Also supporting this hypothesis were a series of previous studies suggesting that HO-1 expression was required for Treg function (116, 117). However, we found no apparent abnormalities of Treg development and/or function in *Hmox1*$^{-/-}$ versus *Hmox1*$^{+/+}$ mice, indicating that at least under homeostatic conditions Treg development and maintenance are not dependent on HO-1. Further, Treg isolated from *Hmox-1*$^{-/-}$ mice inhibited T_H cell proliferation equally well *in vivo* and *in vitro* as compared to Tregs from wild type Hmox1$^{+/+}$ mice, once more suggesting that Treg development and function are independent of HO-1 expression. These findings are consistent with the observation that *Hmox1* deletion does not result in severe systemic lymphoproliferation early in life, as it occurs in mice in which genes critically involved in Treg development and/or function have been deleted (reviewed in (118)).

It is still possible, however, that HO-1 expression and Treg may interact functionally to exert immunoregulatory functions, including in suppressing the rejection of transplanted organs. One possible scenario would be that HO-1 expression in tissues and/or in DC might affect the activation and expansion of Treg. It is also possible that Tregs may act at least partially via the up-regulation of the expression of protective genes, e.g. HO-1 in tissues and/or DC, thus inducing a tolerogenic effect against transplanted organs (119).

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

It is now well-established that induction of HO-1 suppresses chronic graft rejection, one of the major causes of graft failure in clinical transplantation. This was first suggested by the finding made by Wayne Hancock and colleagues (107), showing that pharmacological induction of HO-1 using cobalt protoporphyrin IX suppressed the development of chronic graft rejection of rodent cardiac transplants (107). In this experimental system, i.e. DST plus CD40L blockade, HO-1 is required to promote tolerance induction (114), and suppress chronic lesions in the graft (107). Interestingly, immunosuppressive protocols that fail to induce tolerance also fail to induce HO-1 expression and possibly for that reason lead to the development of chronic graft lesions (107). In a similar manner, others found that exogenous HO-1 over-expression also suppresses the development of chronic graft rejection (120, 121) and protective effects have also been observed in experimental models of vascular remodeling driven by either transplantation (122) or wire or balloon injury (123-125).

CO at least partially seems to mediate the ability of HO-1 to prevent the development of chronic graft rejection. That work developed in our laboratory in Boston by Manabu Haga, in collaboration with Brian Zuckerbraun and Leo Otterbein, used as an experimental model the transplantation of a segment of aorta between allogeneic rat strains. CO suppressed chronic graft rejection, i.e. administration of CO for the full 56 days of the experiment suppressed the development of arteriosclerosis (123).

More recently, others have suggested that the ability of interleukin (IL)-10 to suppress chronic rejection in the same experimental model is mediated via HO-1 (126), a finding in keeping with the previous observation that HO-1 expression mediates the anti-inflammatory effects of IL-10 (127).

The cellular and molecular mechanisms by which CO prevents the development of arteriosclerotic lesions remain to be established but there is evidence that CO can act at the level of the vasculature to inhibit leukocyte infiltration/activation (24) as well as smooth muscle cell proliferation (125). *In vitro,* CO suppresses the proliferation of vascular smooth muscle cells, almost

certainly one of the mechanisms contributing to the *in vivo* action (123). Both these effects, i.e. inhibition of leukocyte infiltration/activation and smooth muscle cell proliferation, are likely to suppress neointima formation, a hallmark of chronic graft rejection (125).

While we have not tested whether biliverdin would suppress chronic graft rejection, Robert Öllinger in our laboratory has shown that when administered exogenously, biliverdin or bilirubin can suppress intimal hyperplasia that arises after balloon injury and that biliverdin or bilirubin suppresses vascular smooth muscle cell proliferation *in vitro,* (128). Based on the original data obtained by Robert Öllinger, other investigators have reported similar effects (129). Given the above it seems not unlikely that biliverdin/bilirubin will also suppress chronic graft rejection, but this remains to be tested.

Both CO and biliverdin suppress the pro-inflammatory response of monocyte/macrophages *in vivo* as well as *in vitro* and to varying extents boost the production of the anti-inflammatory cytokine IL-10 (130). As discussed above, the anti-inflammatory effects of IL-10 that suppress chronic graft rejection (126) act via a process that is presumably due to the induction of HO-1 (126). Based on these and other data (127), one of us (MPS) suggested that HO-1 may act as an amplification positive feed back loop that enforces regulatory mechanisms limiting tissue injury and inflammation (131), such as involved in the pathogenesis of chronic graft rejection.

It should be noted that while both CO and biliverdin suppress smooth muscle cell proliferation, they do so by somewhat different mechanisms, both involving p38 MAPK. While CO induces p38 MAPK to inhibit smooth muscle cell proliferation (125), biliverdin does so by suppressing p38 MAPK (128). We believe that as demonstrated for the anti-apoptotic effect of CO in EC (81), the anti-proliferative effect of CO in smooth muscle cells may also be exerted via the selective activation of the p38β. This also remains to be tested experimentally.

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

Transplanted kidneys undergoing acute rejection under a standard immunosuppressive regimen have 70 fold greater HO-1 mRNA expression, as compared to naïve kidneys (132). There are at least two possible reasons why these grafts undergo acute rejection despite the expression of HO-1 (132). One, that given the aggressive host immune response, physiological expression of HO-1 may not be sufficient per se to suppress graft rejection (132). Alternatively, or in addition, the HO-1 response, which occurs after the stress stimulus, may not be able to overcome an already ongoing rejection response. These two explanations might be summarized as: too little, too late.

Shown to be relevant to chronic graft rejection (133), is 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 (134). Short (GT)n repeats are associated with high expression of HO-1 while long (GT)n repeats are associated with low levels of expression in response to a given stimulus (*reviewed in* (135)). A significantly better long-term survival is associated with the presence of high levels of HO-1 expression in the graft (133), as compared to grafts expressing low levels of HO-1 (133, 136). As the length of ischemia became greater, so did the beneficial effect of a stronger HO-1 response (133). These clinical data, combined with experimental transplant models, support the concept that expression by grafts of protective genes can help prevent chronic graft rejection. 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 deal with chronic rejection. These are natural products that appear to be involved physiologically in the normal protection against arteriosclerosis.

Certain immunosuppressive drugs might interfere with the action of HO-1. Glucocorticoids down-regulate HO-1 expression (137, 138) and more recently, nephrotoxicity associated to Cyclosporin A has been associated with reduced HO-1 expression and to be reverted when HO-1 was induced pharmacologically prior to CsA treatment (139). Other immunosuppressive drugs, however, such as Rapamycin can themselves up-regulate HO-1 expression (140). The latter effect could contribute to the salutary effects of such immunosuppressive drugs in preventing chronic graft rejection (141). The allotrap peptide RDP58, likewise, leads to up-regulation of HO-1 with the same potential benefits (142). Given the above and considering the central role of HO-1 in controlling graft survival, immunosuppressive drugs used in clinical transplantation should be monitored more extensively for their ability to modulate the expression of this gene.

8. SUMMARY AND PERSPECTIVES

Transplantation has served on more than one occasion to allow insights that extend far beyond this one clinically important procedure. Studies in the 60s and 70s used models of transplantation that led to much of what we have learned about cell-mediated immunity in general. The complexity of the processes leading to graft rejection involves several of the same pathological processes that underlie a large number of diseases and disorders. It is of note that 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 major pathological processes: inflammation, oxidative stress, apoptosis, aggressive T cell responses and proliferation of vascular smooth muscle cells. It is absolutely remarkable that a single genetic system would not only itself have a beneficial action (removal of cytotoxic free heme by HO-1) but also lead to the generation of three products that are each protective (the generation of ferritin in the case of Fe and the inclusion of bilirubin generation for biliverdin). We believe that the studies with HO-1 in transplantation may be expanded to a multitude of other inflammatory diseases, a notion supported by emerging data from other as well as our laboratories (105, 143).

There are now multiple threads of evidence that protection of the donor organ is critical for the avoidance of rejection. This is true for early non-function, as especially seen with islets, but also in the studies showing that a stronger HO-1 response in the donor organ leads to a lesser incidence of IRI and subsequently of chronic rejection. We hope that the transplantation community will focus more on potential therapies such as expressing HO-1 in or administering an HO-1 product to the donor.

9. ACKNOWLEDGEMENTS

Miguel P. Soares and Fritz H. Bach worked together for about a decade largely focusing on the issues of transplantation as reviewed here. As of 2003, MPS set up his laboratory at the Instituto Gulbenkian de Ciência in Oeiras, Portugal. We mention in the text the names of collaborators who worked with us in Boston or Oeiras. Individuals that we acknowledge as being major contributors to the data contained herein include (in alphabetical order) Yorihiro Akamatsu Pascal Berberat, Sophie Brouard, Yuan Lin, James McDaid, Tsukasa Myatake, Robert Öllinger, Kenichiro Yamashita, Nozomi Koymada, Koichiro Sato, and Neal Smith among many. This work was partially supported by European Community, 6th Framework Grant LSH-2005-1.2.5-1 (Xenome), Phillip Morris External Research Program, POCTI/SAU-MNO/56066/2004 and POCTI/BIA-BCM/56829/2004 by "Fundaçao para a Ciencia e Tecnologia", Portugal to MPS as well as NIH (HL77721 and HL-58688) and the Julie Henry Fund of the Department of Surgery, Beth Israel Deaconess Medical Center, Boston, MA, USA to FHB.

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Abbreviations: AICD: Activation induced cell death, CO: carbon monoxide; CsA: Cyclosporine A, CVF: cobra venom factor, DST: donor specific transfusion, EC: endothelial cells, Hb: hemoglobin, HO-1: heme oxygenase-1, IRI: Ischemia reperfusion injury, iNOS: inducible nitric oxide synthase, Mø: Monocyte/macrophages, MHC: major histocompatibility complex, MHb: Methemoglobin, MAPK: mitogen activated protein kinase, NF-κB: nuclear factor kappa B, ROS: reactive oxygen species, TCR: T cell receptor, TNF: tumor necrosis factor

Key Words: Heme, Oxygenase-1, Transplantation, cell death, MHC, Major Histocompatibility Complex, Review

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