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Exacerbated corneal inflammation and neovascularization in the HO-2 null mice is ameliorated by biliverdin

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ABSTRACT

Heme oxygenase (HO-1 and HO-2) represents an intrinsic cytoprotective and anti-inflammatory system based on its ability to modulate leukocyte migration and to inhibit expression of inflammatory cytokines and proteins. HO-2 deletion leads to unresolved corneal inflammation and chronic inflammatory complications including ulceration, perforation and neovascularization. We examined the consequences of HO-2 deletion on hemangiogenesis and lymphangiogenesis in the model of suture-induced inflammatory neovascularization. An 8.0 silk suture was placed at the corneal apex of wild type and HO-2 null mice. Neovascularization was assessed by vital microscopy and quantified by image analysis. Hemangiogenesis and lymphangiogenesis were determined by immunofluorescence staining using anti-CD31 and anti-LYVE-1 antibodies, respectively. Inflammation was quantified by histology and myeloperoxidase activity. The levels of HO-1 expression and inflammatory cytokines were determined by real time PCR and ELISA, respectively. Corneal sutures produced a consistent inflammatory response and a time-dependent neovascularization. The response in HO-2 null mice was associated with a greater increase compared to the wild type in the number of leukocytes ($827{,}600 \pm 129{,}000$ vs. $294{,}500 \pm 57{,}510$; $p < 0.05$), neovessels measured by vital microscopy (21.91 ± 1.05 vs. 12.77 ± 1.55 mm; $p < 0.001$) 4 days after suture placement. Hemangiogenesis but not lymphangiogenesis was more pronounced in HO-2 null mice compared to wild type mice. Induction of HO-1 in sutured corneas was greatly attenuated in HO-2 null corneas and treatment with biliverdin diminished the exaggerated inflammatory and neovascular response in HO-2 null mice. The demonstration that the inflammatory responses, including expression of proinflammatory proteins, inflammatory cell influx and hemangiogenesis are exaggerated in HO-2 knockout mice strongly supports the notion that the HO system is critical for controlling the inflammatory and neovascular response in the cornea. Hence, pharmacological amplification of this system may constitute a novel therapeutic strategy for the treatment of corneal disorders associated with excessive inflammation and neovascularization.

1. Introduction

Neovascularization is regarded as a component of the inflammatory reparative response of a given tissue to injury. While neovascularization has some positive effects such as facilitating healing by enabling transport of immunologic humoral and cellular factors for repairing tissues, persistence of blood vessels within the cornea is detrimental. Neovascularization of the normally avascular cornea is seen in many pathological conditions, which include infection, mechanical and chemical injury, chronic exposure to hypoxia and following corneal transplantation. Corneal neovascularization leads to decreased vision, graft rejection and incompetent barrier function, thus presenting a serious clinical problem for which treatment is often lacking.

The molecular mechanisms that control corneal neovascularization are not fully understood. Since inflammation is closely linked with neovascularization, many studies have concentrated on the role of humoral and corneal-derived inflammatory mediators in the regulation of this process and on angiogenic factors that drive the neovascularization process. Less attention has been focused on the identification of intrinsic corneal anti-inflammatory mediators and cellular pathways. In recent years the

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heme oxygenase (HO) system has emerged as a key cytoprotective signal. HO is the rate-limiting enzyme in heme catabolism. It cleaves heme into iron, sequestered by ferritin, carbon monoxide (CO), and biliverdin which is reduced to bilirubin by biliverdin reductase (Abraham and Kappas, 2008). Two isoforms, HO-1 and HO-2, are the major source of HO activity in most tissues. HO-1 is an inducible enzyme, while HO-2 displays a constitutive expression in all cell types. HO-1 and HO-2 are alike in terms of mechanisms of heme oxidation, cofactor and substrate specificity, and susceptibility to inhibition by porphyrins (Abraham and Kappas, 2008; Maines, 1998). They differ in their postulated function; whereas, HO-2 is believed to function as the constitutive HO activity contributing to cell homeostasis, HO-1 expression is relatively low in most normal tissues. Following injury, however, HO-1 expression is greatly enhanced to play a significant role in cytoprotection (Abraham and Kappas, 2008).

The realization that HO-1 is strongly induced by oxidative stress and other injurious stimuli in conjunction with the robust ability of HO-1 to guard against oxidative insult (Abraham and Kappas, 2008; Foresti et al., 2004; Otterbein and Choi, 2000) and the demonstrations of its protective role in models of inflammation-mediated injury including xenograft rejection (Song et al., 2003), endotoxin challenge (Ohta et al., 2003; Otterbein et al., 2000; Zampetaki et al., 2003), ischemia-reperfusion injury (Arai-Gaun et al., 2004; Coito et al., 2002; Hegazy et al., 2000; Minamino et al., 2001; Yet et al., 2001), and contact lens-induced hypoxic injury (Conners et al., 1995; Laniado Schwartzman et al., 1997), together with the fact that HO-1 deficiency is associated with a chronically inflamed state and increased leukocyte recruitment as reported in a human (Yachie et al., 1999) and in mice null for the HO-1 gene (Kapturczak et al., 2004; Poss and Tonegawa, 1997), have all led to an examination of the cytoprotective and anti-inflammatory nature of the HO system. Although the mechanisms involved in this cytoprotection are largely unknown, the elimination of excess cellular heme as well as the enzymatic products of the HO system, carbon monoxide and bilirubin, have been shown to protect against tissue damage by exerting antioxidant and anti-inflammatory effects (Stocker et al., 1987; Otterbein, 2002; Ryter et al., 2004). Upregulation of HO-1 as well as administration of biliverdin/bilirubin or CO have been shown to downregulate the inflammatory response by either attenuating the expression of adhesion molecules and, thus, inhibiting leukocyte recruitment (Wagener et al., 1999, 2003), by repressing the induction of cytokines and chemokines (Minamino et al., 2001; Morse et al., 2003; Otterbein et al., 2000; Sarady-Andrews et al., 2005; Song et al., 2003), or by inhibiting proinflammatory hemoproteins such as cyclooxygenase-2 and cytochrome P450 4B1 (Conners et al., 1995; Haider et al., 2002; Laniado Schwartzman et al., 1997; Li Volti et al., 2003). Though many of the postulated mechanisms underlying this protection are assigned to HO enzymatic activity and its products, CO and bilirubin, the contribution of HO-2, the primary source of bilirubin and CO in uninjured tissues, to the regulation of inflammation and reparative response has been largely unexplored.

We have previously documented the presence of HO activity and expression in human and rabbit corneas as well as HO-1 inducibility following oxidative stress in vitro and hypoxic injury in vivo (Abraham et al., 1987, 1995; Bonazzi et al., 2000; Conners et al., 1995; Laniado Schwartzman et al., 1997; Neil et al., 1995). In a recent study, we showed that HO-2 displays a prominent constitutive expression in the cornea and that deletion of HO-2 gene markedly impairs the inflammatory and reparative response of the cornea to epithelial injury (Seta et al., 2006). Hence, HO-2 deficiency led to unresolved corneal inflammation and chronic inflammatory complications including ulceration, perforation and neovascularization. In the present study, we examined the consequences of HO-2 deletion on the corneal inflammatory response including hemangiogenesis and lymphangiogenesis in the model of suture-induced inflammatory neovascularization and examined whether supplementation of the HO-derived product, biliverdin, corrects for HO-2 deficiency.

2. Materials and methods

2.1. Animals

All animal experiments were performed following an institutionally approved protocol in accordance with the National Institute's of Health Guide for the Care and Use of Laboratory Animals. The HO-2 null mice are direct descendants of the HO-2 mutants produced by Poss and colleagues (Poss et al., 1995). These well-characterized HO-2 null mice have a C57BL/6x129/Sv genetic background (Rogers et al., 2003), which was used on age- and gender-matched controls (Jackson Laboratory, Bar Harbor, ME).

2.2. Suture-induced inflammatory corneal neovascularization

Mice were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (20 mg/kg). A drop of tetracaine–HCl 0.5% was applied to the eye to deliver local corneal anesthesia before subjecting animals to injury. One 8.0 silk suture was placed intrastromally with two stromal incursions extending over the corneal apex, the points of the suture placement being about 1.5 mm from the limbus, in order to obtain standardized angiogenic responses. In some experiments, eyes were treated with biliverdin (100 μM, 10 μl eye drop, t.i.d.). The corneal angiogenic response was documented on days 2, 4 and 7 after suture placement using a Zeiss dissecting microscope coupled to an Axiocam Hrc digital camera and Axiovision 4.5 software (Zeiss). Digital images were analyzed by image processing software (ImagePro, Media Cybernetics, Inc., Silver Spring, MD). Corneal vascularization was measured as the total length (mm) of vessels in the cornea. Mice were euthanized after 2, 4, and 7 days and the corneas were dissected and subjected to selected analyses.

2.3. Corneal whole mounts and morphologic determination of hem- and lymphangiogenesis

The corneas were excised, rinsed in phosphate buffered saline (PBS 0.01 M, pH 7.4) and fixed in acetone for 20–30 min, as described previously (Cursiefen et al., 2004). After three additional washing steps in PBS and blocking with 3% BSA in PBS for 3 h, the corneas were stained overnight at 4 °C with FITC-conjugated rat anti-mouse CD31 (PECAM-1) (1:300, eBioscience, San Diego, CA). Thereafter the tissue was washed, blocked and stained with rabbit anti-mouse LYVE-1 (1:500, Abcam Inc., Cambridge, MA) antibody overnight at 4 °C. After a last washing and blocking step, LYVE-1 was detected with a Cy3-conjugated goat anti-rabbit antibody (1:500, Jackson Immunoresearch, West Grove, PA). Corneal hemangiogenesis and lymphangiogenesis were quantified using the digital image analysis software Axiovision (Zeiss) as described (Samolov et al., 2005). The total corneal area was outlined using the innermost vessel of the limbal arcade as the border, and the ratio between the area of the vascularized ($CD31^+$ for hemangiogenesis and $LYVE\text{-}1^+$ for lymphangiogenesis) cornea and the total corneal area was calculated.

2.4. Histology and immunostaining

Dissected corneas were washed twice with PBS and fixed in 4% paraformaldehyde–PBS for 1 h at 4 °C. Corneas were washed five times with PBS, placed in 30% sucrose for 24 h and embedded in OCT compound (Sakura Finetek, Torrence, CA). Croystat sections