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Knockdown of Heme Oxygenase-2 Impairs Corneal Epithelial Cell Wound Healing

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Abstract

Heme oxygenase (HO) represents an intrinsic cytoprotective system based on its anti-oxidative and anti-inflammatory properties mediated via its products biliverdin/bilirubin and carbon monoxide (CO). We showed that deletion of HO-2 results in impaired corneal wound healing with associated chronic inflammatory complications. This study was undertaken to examine the role of HO activity and the contribution of HO-1 and HO-2 to corneal wound healing in an in vitro epithelial scratch injury model. A scratch wound model was established using human corneal epithelial (HCE) cells. These cells expressed both HO-1 and HO-2 proteins. Injury elicited a rapid and transient increase in HO-1 and HO activity; HO-2 expression was unchanged. Treatment with biliverdin or CORM-A1, a CO donor, accelerated wound closure by 10% at 24 h. Inhibition of HO activity impaired wound closure by more than 50%. However, addition of biliverdin or CORM-A1 reversed the effect of HO inhibition on wound healing. Moreover, knockdown of HO-2 expression, but not HO-1, significantly impaired wound healing. These results indicate that HO activity is required for corneal epithelial cell migration. Inhibition of HO activity impairs wound healing while amplification of its activity restores and accelerates healing. Importantly, HO-2, which is highly expressed in the corneal epithelium, appears to be critical for the wound healing process in the cornea. The mechanisms by which it contributes to cell migration in response to injury may reside in the cytoprotective properties of CO and biliverdin.

Keywords

Heme Oxygenase (HO-1 and HO-2); Biliverdin; Carbon Monoxide; Wound healing; Cornea

INTRODUCTION

A rapid healing of corneal epithelial wounds is important not only for the maintenance of corneal transparency but also to protect the underlying stroma from further damage and to avoid infection. Repair of corneal surface wounds initiates with migration of existing epithelial cells to cover the wound followed with proliferation of epithelial cells to restore the normal thickness of the cornea and a return to homeostasis. This process is regulated by a multitude of growth factors, cytokines and structural proteins that orchestrate the necessary steps for completion of healing including migration, adhesion, proliferation, differentiation as well as apoptosis (Imanishi et al., 2000; Lu et al., 2001). We have shown that a deficiency in heme oxygenase (HO) activity impairs corneal wound healing whereas amplification of

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HO activity accelerates epithelial repair suggesting that the HO system is an integral component of the corneal repair system (Patil et al., 2008; Seta et al., 2006).

HO is the rate-limiting enzyme in heme metabolism cleaving heme, a potent oxidant, into three products: CO, biliverdin and iron. Biliverdin is further reduced by biliverdin reductase to bilirubin. The significance of the HO (HO-1 and HO-2) system extends far beyond its original role in the catabolism of heme in senescent heme proteins (Abraham and Kappas, 2008). It is clear that the HO system has potent cytoprotective and anti-inflammatory properties that are critical in many organs including the eye (Bellner et al., 2008; Seta et al., 2006) and are attributed to its products biliverdin/bilirubin and carbon monoxide (CO). At physiologic concentrations bilirubin (and to a lesser extent biliverdin) is a potent and efficient scavenger of reactive oxygen species (Dore et al., 1999; Stocker et al., 1987). Biliverdin/bilirubin decreases LPS-induced pro-inflammatory transcription factors (NF- κ B) and cytokines (IL-6, TNF α) (Foresti et al., 2004; Gibbs and Maines, 2007; Soares et al., 2004) and this may be mediated via its ROS scavenging mechanisms as the expression of many pro-inflammatory genes is redox-sensitive (Chen and Kunsch, 2004). CO, as well as biliverdin, contributes substantially to the anti-inflammatory properties of HO by suppressing pro-inflammatory cytokines and chemokines while increasing expression of the anti-inflammatory mediator interleukin-10 (Kim et al., 2006; Morse et al., 2003; Ollinger et al., 2007; Song et al., 2003; Wegiel et al., 2009). Iron derived from heme via HO is effectively controlled by ferritin, which is readily upregulated by HO, *via* sequestration and ferroxidase activity (Balla et al., 2007). HO-1 and HO-2 are expressed in most tissues. HO-1 is induced by numerous factors that increase cellular stress while HO-2, in general, is constitutively expressed and developmentally regulated and is altered in many human pathological conditions (Zenclussen et al., 2003). In terms of mechanisms of heme oxidation, substrate and cofactor requirements and susceptibility to porphyrins, they are alike. But they differ in function; while HO-2 is thought to serve as the constitutive HO activity maintaining cell homeostasis, HO-1 expression is relatively low in most tissues and its expression rises rapidly in response to cellular stress (Abraham and Kappas, 2008). Induction of HO-1 has been shown to attenuate ocular surface inflammation (Connors et al., 1995; Laniado Schwartzman et al., 1997), accelerate corneal wound healing (Patil et al., 2008), attenuate retinal detachment-induced photoreceptor apoptosis (Shyong et al., 2008), protect photoreceptors from light damage (Sun et al., 2007) and prevent retinal ganglion cell death (Hegazy et al., 2000).

The use of HO-2 null mice has brought new insights into HO research. One such insight is the association of HO-2 deletion with impaired HO-1 expression indicating that HO-2 is critical for HO-1 induction (Seta et al., 2006). The result of this is a mouse deficient in HO activity. A recent study described the altered course of acute inflammation and the reparative response in these HO-2 null mice following mechanical de-epithelialization of their corneas. Wild type (WT) mice heal the epithelial defect in seven days, the acute inflammatory response and the associated inflammatory cytokines and infiltrating neutrophils resolve in a few days and there is no significant neovascularization. In stark contrast to the WT mice, the HO-2 null mice corneas experience an unresolved acute inflammation from the injury, epithelial regeneration is greatly impaired, massive revascularization is evident and, by day seven, all corneas suffer perforation (Seta et al., 2006). Attempts to rescue the HO-2 phenotype in HO-2 null mice have been made using biliverdin. Application of this HO bioactive product rescued the acute inflammatory and reparative response in HO-2 null mice with a two-fold increase in the rate of re-epithelialization reaching that of the WT mice (Seta et al., 2006). In the suture-induced model of corneal inflammatory neovascularization, HO-2 null mice displayed an exaggerated inflammatory and neovascular response that is greatly attenuated with topical application of biliverdin (Bellner et al., 2008).

The present study further explores the role of HO in and the contribution of each isoform (HO-1 and HO-2) to corneal epithelial wound healing using cultured human corneal epithelial cells in a model of epithelial injury. The results indicate that injury activates the HO system and that HO activity is important for re-epithelialization. Moreover, HO-2 and not HO-1 appears to be the critical component of the HO system for epithelial wound healing.

MATERIALS AND METHODS

Materials

Biliverdin, heme and metalloporphyrines were from Frontier Scientific Inc. (Logan, UT). Carbon Monoxide releasing molecule (CORM-A1) (Motterlini et al., 2005) was obtained from Dr. John R. Falck (University of Texas Southwestern Medical Center, Dallas, TX). Inactive CORM (iCORM) was prepared by placing CORM-A1 in PBS buffer for 24 h at room temperature. Hydroxyurea was from MP Biochemical (Solon, OH). Silencing inhibitory RNAs (siRNAs) against the human HO-1 and HO-2 as well as non-specific and GFP-conjugated siRNAs were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies against HO-1 and HO-2 were from Assay designs (Ann Arbor, MI) and β -actin from Sigma Aldrich (St Louis, MO).

Cell culture

Primary cultures of human corneal epithelial cells (passage 2–5; ScienCell, Carlsbad, CA) and a human corneal epithelial cell line (HCE; passages 29–45) obtained from Dr. Haydee Bazan (LSU Health Science Center) (Sharma et al., 2005), were used in these experiments. Cells were grown in fibronectin-coated T-75 flasks and maintained in serum-free keratinocyte growth medium (KGM, Lonza, USA) supplemented with appropriate growth factors and antibiotics. For all experiments, cells (6–12-well plates) were grown to 60–70% confluency in KGM and then the medium was changed to a growth factor- and supplement-free keratinocyte basal medium (KBM) and cells were incubated for additional 24 h. Scratch injury was performed with a sterile 20 μ l pipet tip to remove cells in two perpendicular linear scraps and generate a wound approximately 0.1 cm in width. The medium was discarded and cells were cultured in fresh KBM medium in the presence and absence of hydroxyurea (0.5mM), chromium mesoporphyrin (CrMP, 10 μ M), CORM-A1 (100–400 μ M) and Biliverdin (5–20 μ M). Phase-contrast images of the injured area near the crossing point were acquired at the time of injury and at the indicated time points thereafter with an inverted microscope equipped with a charge-coupled device camera (Axiovert; Carl Zeiss, Thornwood, NY). The wound area (mm^2) was determined by computerized planimetry using Axiovision 4.6 software (Zeiss). The extent of healing over time was defined as percent of the denuded area at the time of injury. Cell proliferation and viability was measured using the methyl thiazolyl tetrazolium (MTT) colorimetric assay.

siRNA transfection of HCE cells

HCE cells were grown in 12-well plates to 60–70% confluency. Transfection of siRNA was performed according to the manufacturers' instructions (Santa Cruz). Briefly, cells were incubated with 500 μ l of transfection solutions containing a mixture of siRNA (100 nM) and siRNA transfection reagent (5 μ l) (Santa Cruz) for 6 h. Afterwards, the transfection solutions were replaced with fresh KGM-2 growth medium for an additional 24 h. Medium was changed to the growth factor- and supplement-free KBM medium and injury performed 24 h later. Cells were also transfected with control siRNA (100 nM) in parallel to ensure specific gene silencing. At the end of the experiment (24 h after injury), cells were collected for Western blot analysis and real time PCR. Transfection efficiency was assessed using GFP-conjugated non-specific siRNA (Santa Cruz).

HO Activity/CO Measurement

HCE cells were collected by trypsinization 4 h after injury. Cells were washed with phosphate buffered saline, pH 7.4 and resuspended in oxygenated Krebs Buffer. HCE cells were subsequently incubated for 1 h in the dark at 37°C with 2 mMhemin in the presence and absence of CrMP (10 μ M). CO released in the headspace gas was analyzed by gas chromatography-mass spectrometry (GC-MS, HP5989A interfaced to HP5890; Hewlett Packard, Palo Alto, CA). The amount of CrMP-sensitive CO was calculated from standard curves constructed with an abundance of ions m/z 28 and m/z 29 or m/z 31, as previously described (Bellner et al., 2009).

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated using RNeasy Protect Mini Kit (QIAGEN, Carlsbad, CA) and RNA was quantified by Nano drop. Reverse transcription reaction of total RNA was performed using the qScript cDNA synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantitative real-time PCR was performed using PerfeCTa SYBR Green QPCR FastMix (Quanta Bioscience) and the Mx3000 real-time PCR system (Stratagene, La Jolla, CA). Specific primers were designed based on published sequences (GenBank) and were as follows: HO-1 sense, 5'-TGCTCAACATCCAGCTCTTT-3' and anti-sense, 5'-GCAGAATCTTGACTTTTGTT-3'; HO-2 sense, 5'-ATGTCAGCGGAAGTGGAAAC-3' and anti-sense, 5'-CGAGAGGTCAGCCATTCTCA-3'; 18S sense, 5'-GATGGGCGGCGGAAAATAG-3' and anti-sense, 5'-GCGTGGATTCTGCATAATGG-3'. PCR efficiency for each primer pair was determined by quantitating amplification with increasing concentrations of template cDNA, and specific amplification was verified by subsequent analysis of melt curve profiles for each amplification. A nontemplate control served as negative control to exclude the formation of primer dimers or any other nonspecific PCR products. RNA expression of target genes was calculated based on the real-time PCR efficiency (E) and the threshold crossing point (CP) and is expressed in comparison to the reference gene 18S as described (Bellner et al., 2009).

Western blot

HCE cells were homogenized in T-PER tissue protein extraction reagent containing Halt protease inhibitor cocktail (Pierce Biotechnology, Inc., Rockford, IL). Proteins were separated by gel electrophoresis, and immunoblotting was performed using the following primary antibodies: rabbit anti-human HO-1 (1:2,500), rabbit anti-human HO-2 (1:2,500) and β -actin mouse monoclonal antibody (1:10,000). Detection and densitometry analysis was performed as previously described (Bellner et al., 2009).

Immunofluorescence assay

HCE cells were grown in 16-well chamber slides at 37°C to 100% confluence. Scratch injury at different time points was performed with a sterile 0.1–10 μ l pipet tip to remove cells in a single linear scrape. Briefly, slides were fixed in 4% paraformaldehyde for 10 minutes at room temperature, washed with PBS and then blocked with 5% goat serum in PBS whereupon the slides were incubated with rabbit anti-human HO-1 antibody (1:400) or with rabbit anti-human HO-2 antibody (1:50) overnight at 4°C, then washed and further incubated with a secondary Cy3 conjugated goat anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. To further verify cellular entity, the slides were washed and counterstained for nuclei with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 15 minutes in dark at room temperature. Pictures were taken adjacent to the wound edge and distal to wound edge ($\geq 300 \mu$ m) using a Zeiss Axioplan-2 fluorescent microscope. The total fluorescence intensity (normalized to cell number) was measured using AxioVision 2 multi channel image processing software (Zeiss,

Göttingen, Germany) and the intensity at the wound edge was compared to the intensity obtained distal to wound edge.

Statistical analysis

Results are the mean $\pm$ SEM. Significance of difference in mean values was determined using one-way ANOVA followed by Newman-Keuls post-hoc test for multiple comparisons. $P<0.05$ was considered to be significant.

RESULTS

HO-1 and HO-2 expression in Human corneal epithelial cells

Primary cultures of human corneal epithelial cells express HO-1 and HO-2. In response to injury, HO-1 protein levels increased by 1.8- and 2.1-fold at 2 and 4 h after injury (Figure 1A). HO-2 expression was not affected by injury (Figure 1B). The HCE cell line has been extensively used to elucidate cellular mechanisms of corneal epithelial wound healing. These cells express HO-1 and HO-2 and oxidative injury elicited a rapid activation of the HO system (Abraham and Kappas, 2008). Similar to primary cultures, HCE cells responded to injury with significant increases in HO-1 expression at 2 and 4 h after injury with no change in HO-2 expression (Figure 1C and D). The increase in HO-1 expression was accompanied by a 50% increase in HO activity, as measured by the amount of CrMP-sensitive CO (Figure 1E).

The effect of injury on HO-1 and HO-2 expression was also assessed by immunofluorescence imaging of cells at the edge of the wound and at a distance of 1–2 μ m from the wound. As seen in Figure 2, HO-1 protein expression 1 h after injury increased by 2.5- and 1.7-fold at the wound edge and distal to the wound. HO-1 protein levels remained elevated for 4 h and returned to control (pre-injury) 8 h after injury. In contrast, HO-2 expression either at the wound edge or far from the wound was not altered after injury (Figure 2).

Human Corneal epithelial cells healing enhanced by biliverdin and CORM-A1

Scratch wounds in HCE cells were closed by $50.72\pm 3.59\%$ and $78.24\pm 2.67\%$ at 12 and 24 h, respectively (Figure 3). Addition of 10 μ M of biliverdin enhanced wound healing by 11% ($61.54\pm 2.71\%$ closure) and 10% ($89.77\pm 2.49\%$ closure) at 12 and 24 h, respectively. The effect of biliverdin was dose-dependent (Figure 4A). Addition of CORM-A1 which releases CO in aqueous solutions (Motterlini et al., 2005) was less efficacious than biliverdin; at 100 μ M CORM-A1 enhances wound healing by 5% ($55.59\pm 2.95\%$ closure) and 10% ($87.68\pm 1.53\%$ closure) at 12 and 24 h, respectively (Figure 3). CORM-A1 effect was also dose-dependent (Figure 4B). The inactive CORM (iCORM) at concentrations of 100–400 μ M had no significant effect on wound closure (data not shown).

Inhibition of HO activity impairs wound healing

CrMP at the concentration used (10 μ M) inhibited HO activity measured as CO production by 82 ± 2 and $92\pm 3\%$ in control and injured cells, respectively (mean $\pm$ SE, $n=3$). CrMP had no effect on cell viability at all time points, however, it significantly inhibited the healing of scratch wounds in HCE cells. As seen in Figure 5A–C, in cells treated with CrMP, healing was inhibited by 65%; only $18.21\pm 1.75\%$ and $28.13\pm 3.22\%$ of the wounds were closed at 12 and 24 h, respectively. The inhibitory action of CrMP was partially negated at 12 h by adding back biliverdin ($34\pm 1.97\%$) or CORM-A1 ($36.08\pm 2.33\%$) (Figure 5). Wound closure at 24 h after injury of cells treated with CrMP and supplemented with either biliverdin or CORM-A1 was not different than that of the control untreated cells, suggesting that HO activity (via production of biliverdin and/or CO) is required for epithelial cell

healing. Addition of CrMP, biliverdin or CORM-A1 did not significantly alter the rate of proliferation after injury (Figure 5D).

To differentiate the contribution of HO to cell proliferation and migration in wound closure, the cell cycle blocker hydroxyurea (0.5 mM) was added in the scratch wound model. Assessment of cell viability indicated that this concentration was sufficient to inhibit HCE proliferation without significantly affecting cell viability. As seen in Figure 6, in the presence of hydroxyurea, healing of the wounds at 12 and 24 h after injury amounted to of 30% and 63%, indicating attenuation of healing by 39% and 20%, respectively. Addition of the HO inhibitor CrMP to cells treated with hydroxyurea further attenuated migration-driven healing at 24 h by 23% when compared to cells treated with hydroxyurea. Addition of biliverdin or CORM-A1, respectively, significantly enhanced migration-driven wound healing by 27% and 42% at 12 h, and 17% and 20% at 24 h (Figure 6).

HO-2 knockdown impairs healing of wounded HCE cells

Knockdown of HO-1 and HO-2 expression was carried out with the use of specific siRNA. Transfection with GFP-conjugated siRNA demonstrated a transfection efficiency of $65 \pm 10\%$ ($n=4$). Scratch injury was performed in cell transfected with HO-1, HO-2 and non-specific siRNAs. As seen in Figure 7, wound closure in cells treated with HO-2 siRNA was significantly impaired; only 50% of the scratch wound closed at 6 and 12 h after injury and 70% at 24 h after injury. In contrast, wound closure in cells treated with HO-1 siRNA was not significantly different from the control untreated or cell treated with non-specific siRNA at all time points (Figure 7). Real time PCR of HO-1 and HO-2 mRNA as well as Western blot analysis of HO-1 and HO-2 protein clearly indicated a specific knockdown of HO-1 and HO-2 gene expression by their corresponding siRNA preparations (Figure 8). Densitometry analysis further demonstrated that suppression of HO-1 and HO-2 by their corresponding siRNA was about the same (viz., 72 ± 9 and $84 \pm 6\%$, respectively, when compared to control; 73 ± 13 and $78 \pm 12\%$ when compared to control siRNA).

DISCUSSION

This *in vitro* study provides further evidence for a critical role for HO in corneal epithelial wound healing. Using an *in vitro* model of epithelial scratch injury in HCE cells, we showed that inhibition of HO activity attenuates wound closure while amplification of HO activity by supplementation of its catalytic products, biliverdin or CO, accelerates wound closure. The beneficial effect of the HO system was further assigned to HO-2, the constitutive isoform of the HO system, based on findings that HO-2-specific siRNA impaired wound closure whereas HO-1-specific siRNA had no significant effect. The fact that HO-2 is highly expressed in the corneal epithelium (Bellner et al., 2008) together with a previous study demonstrating that its deficiency results in aberrant inflammatory and repair response to epithelial injury typified by perforation, ulceration and neovascularization (Seta et al., 2006) suggest that HO-2 is a critical component for corneal epithelial homeostasis. Several findings in the current study provide additional evidence to support an important role for HO-2 expression and HO activity in corneal epithelial homeostasis.

First is the demonstration that addition of the HO products, biliverdin and CO, significantly accelerated closure of the wound made by scratching HCE monolayers, consistent with previous findings that upregulation of the HO system attenuates the inflammatory response while accelerating the repair of the cornea following injury (Connors et al., 1995; Patil et al., 2008). It is well documented that biliverdin and CO act as endogenous cytoprotective molecules, each with its specific mode of action. Biliverdin, the final product of HO catalytic activity, is further reduced to bilirubin by biliverdin reductase (BVR) (Abraham and Kappas, 2008). Bilirubin, and to a lesser extent biliverdin, is a powerful endogenous/

physiological antioxidant (Stocker et al., 1987). Studies have found that bilirubin at 10 nM was capable of protecting cells from a 10,000 fold increase in oxidative stress generated by hydrogen peroxide and identified an amplification process for bilirubin bioactions whereby bilirubin acting as an antioxidant, is itself oxidized to biliverdin and then recycled by BVR back to bilirubin a process that could readily afford 10,000 fold amplification (Baranano et al., 2002). BVR is widely expressed in most tissues including the cornea (Bellner et al., *IOVS*, 2008 49, E-Abstract 2378). Hence, administration of water-soluble biliverdin is accompanied with increased tissue/cell levels of bilirubin. The primary mechanism for bilirubin-mediated cytoprotection in various types of stress appears to be due to its potent antioxidant activity. Given that injury by itself evokes in any given tissue or cell a stress response which frequently is accompanied by increased reactive oxygen radicals, i.e., oxidative stress, it is reasonable to assume that the increased healing of wounded HCE in the presence of biliverdin is the consequence of decreasing this stress response. Indeed, application of antioxidants has been shown to increase corneal wound healing in diabetic rats (Hallberg et al., 1996). Moreover, increased reactive oxygen species in high glucose-treated HCE cells is believed to underlie inhibition of the EGFR–phosphatidylinositol 3-kinase/Akt pathway and consequently impairment of wound healing (Xu et al., 2009). Interestingly, BVR/biliverdin has been shown to activate the phosphatidylinositol 3-kinase/Akt pathway and to provide protection against ischemic injury (Pachori et al., 2007). Along with potent antioxidant properties, bilirubin also exerts anti-inflammatory effects including inhibition of NF- κ B activation and release of inflammatory cytokines such as IL-6 and TNF α (Foresti et al., 2004; Nakao et al., 2004; Sarady-Andrews et al., 2005). This may well underlie its beneficial effect in reducing inflammation and increasing healing of the cornea in vivo (Bellner et al., 2008; Seta et al., 2006). It may also contribute to its effect on HCE wound closure since tight coordination and regulation of cytokine production is required for an ordered healing process and for maintaining corneal health (Lu et al., 2001). The other product of the HO catalytic activity, CO, also increased the rate of healing in HCE cells. CO was administered in the form of the CO donor CORM-A1 which has been shown to effectively release CO in aqueous solutions (Motterlini et al., 2005). HO-derived CO has been identified as playing a role in many processes related to tissue viability (Kim et al., 2006). It is anti-inflammatory and cytoprotective and it does so in part through inhibition of pro-apoptotic factors including caspases (Morse et al., 2009) and pro-inflammatory cytokines via activation of the MKK3/p38 MAP kinase pathway (Otterbein et al., 2003). It is interesting to note that p38 kinase has been shown to play an important role in the regulation of cell migration and proliferation in healing corneal epithelium (Saika et al., 2004). Whether this confers the ability of CO to increase the rate of healing is yet to be established.

The second important finding is the observation that inhibition of HO activity greatly impairs wound closure indicating that HO activity is a critical factor in maintaining corneal epithelial renewal. This was further substantiated by the demonstration that adding back the catalytic products of HO, biliverdin or CO, negated the effect of HO inhibition. Epithelial wound healing involves migration (sliding) of surviving epithelium to cover the defect and proliferation of these cells to restore the original number of cells. The in vitro scratch injury used in this study provides a means to separate the relative contributions of HO to these two processes in wound closure. Thus, the use of serum-free medium during the injury and the application of hydroxyurea to inhibit cell proliferation indicate that adequate HO activity is important for epithelial cell migration, the first stage in corneal epithelial wound closure. This was evidenced by the demonstration that treatment with the HO inhibitor altered the rate of wound closure in the presence of hydroxyurea and that migration-driven healing was significantly enhanced by the addition of biliverdin or CORM-A1. Moreover, neither inhibition of HO activity nor addition of the HO catalytic products had a significant effect on the rate of cell proliferation after injury, further suggesting that migration rather than proliferation is the process affected by HO activity. HO activity has been implicated in the

regulation of vascular cell migration and proliferation (Abraham and Kappas, 2008). A recent study by Grochot-Przeczek et al (Grochot-Przeczek et al., 2009) demonstrated that inhibition of HO activity impaired cutaneous wound closure further supporting a role for the HO system in wound healing process.

Lastly is the finding that despite the fact that HO-1 and HO-2 share the same catalytic activity, knockdown of HO-2 but not HO-1 impaired wound healing. This is a seminal observation given that HO-1 is the stress and injury inducible isoform (Abraham and Kappas, 2008) and its upregulation in many tissues including the cornea (Connors et al., 1995; Laniado Schwartzman et al., 1997; Patil et al., 2008) confers cytoprotection while, HO-2 is constitutively expressed. However, even though HO-1 and HO-2 catalyze the same reaction, notable differences between the two exist (Shibahara, 2003). Human HO-1 and HO-2 share 43% amino acid sequence identity and about 80% to their correspondent rat and mice proteins. HO-2 (316 amino acids; 36 kDa) contains 3 cysteine residues while HO-1 (288 amino acids; 33 kDa) contains no cysteine residues. Unlike HO-1, HO-2 contains additional two heme binding sites which are not involved in heme breakdown reaction and are conserved at equivalent positions of human, mouse and rat HO-2 (McCoubrey et al., 1997). HO-2 expresses at relatively constant levels but its activity is regulated by phosphorylation under the control of different protein kinases (Boehning et al., 2003; Dore et al., 1999). All nucleated cells depend on heme for their survival as heme senses or uses oxygen. Heme functions as a prosthetic moiety of various hemoproteins. Hence, heme must be synthesized and degraded within an individual cell because heme cannot be recycled among different cells. HO is the only enzyme that can degrade heme, maintain cellular heme homeostasis and affect hemeprotein levels (Shibahara, 2003). The distinct properties of HO-2 including constitutive expression, activation by phosphorylation and additional binding sites for heme set it apart from HO-1. Moreover, the finding that HO-2 is cytoprotective without degrading heme (Kim and Dore, 2005; Kim et al., 2005) suggests that HO-2 participates not only in maintaining heme homeostasis but also in cellular defense mechanisms against injury. To this end, the healthy corneal epithelium expresses high levels of HO-2 (Bellner et al., 2008). The absence of HO-2 expression as in the HO-2 null mice leads to an aberrant corneal response to epithelial injury including exaggerated inflammation and lack of healing (Bellner et al., 2008; Seta et al., 2006). Other studies clearly showed that HO-2 deficiency leads to increased oxidative stress and cell apoptotic signaling further supporting a role for HO-2 in cytoprotection (Chang et al., 2003; Goodman et al., 2006; Turkseven et al., 2007).

The demonstration in this study that despite the presence of the two isoforms, HO-1 and HO-2, in HCE cells before and after injury, only a knockdown of HO-2 attenuated wound closure indicates a cytoprotective role for HO-2 not necessarily compensated for by HO-1. Yet, induction of HO-1 increases the rate of re-epithelialization after corneal abrasion injury (Patil et al., 2008) whereas deficiency in HO-1 inducibility is associated with attenuated wound healing response (Biteman et al., 2007; Seta et al., 2006), raising the question of the differential roles of HO-1 and HO-2 in the response of the cornea to injury. In the healthy cornea HO-1 expression is largely absent (Bellner et al., 2008). Injury induces corneal HO-1 expression with most of the expression residing within the inflammatory cell infiltrates (Bellner et al., 2008; Seta et al., 2006). It is well accepted that the wound repair process is closely linked to a complex inflammatory response that must be precisely regulated to ensure proper healing and optimal visual outcome. In fact, early leukocyte migration appears to be important for full corneal re-epithelialization (Li et al., 2006). As the inflammatory and repair responses are intimately linked so is the functional and regulatory relationships between these two isoforms, each provide a distinct contribution to the healing process. The present study together with our previous reports (Bellner et al., 2008; Seta et al., 2006) suggest that HO-2 participates in corneal epithelial homeostasis promoting cell migration

whereas HO-1 is primarily involved in the resolution of inflammation (Willis et al., 2000). In all, it appears that both HO-1 and HO-2 are required for controlled corneal inflammatory and repair responses to injury. Amplification of the HO system through induction of HO-1 or supplementation of the HO catalytic products may be beneficial in conditions such as diabetic keratopathy, basement membrane dystrophies, neuropathies and infections where healing is significantly impaired.

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Figure 1. HO expression and activity in human corneal epithelial cells. (A) A representative Western blot of HO-1 and HO-2 proteins and (B) densitometry analysis (n=3–4) in primary cultures of human corneal epithelial cells. (C) A representative Western blot of HO-1 and HO-2 proteins and (D) densitometry analysis (n=6) in HCE cells (E) HO activity measured as CrMP-inhibitable CO production in HCE cells 4 h after scratch injury (n=4, *p<0.05 from control uninjured cells)

Figure 2.

HO-1 and HO-2 expression in HCE cells. (A) Immunofluorescence assay for HO-1 (left panel) and HO-2 (right panel). Scratch injury was performed and expression of HO-1 and HO-2 determined 1, 2, 4, 8, 24 h after injury with 20X magnification of Zeiss Axioplan-2 fluorescent microscope. (B) Quantitative analysis of HO-1 and HO-2 fluorescence intensity 1, 2, 4, 8 and 24 h after injury (HO-1 n=6, *p<0.05 vs control and **p<0.05 vs 8h wound edge; HO-2 n=4).

Figure 3. Effect of biliverdin and CO on HCE wound healing. Scratch injury was performed in the absence and presence of biliverdin (BVD, 10 μ M) or CORM-A1 (100 μ M) and percent healing was determined at 12 and 24 hr after injury. A) Representative images of wounded HCE cells. B) Quantitative analysis of healing (n=8–12, *p<0.05 vs control untreated cells).

Figure 4.

Effect of biliverdin (A) and CORM-A1 (B) concentrations on HCE wound healing 6, 12 and 24 h after injury (n=8–12, *p<0.05, **p<0.01 and ***p<0.001).

Figure 5.

Effect of HO inhibition on HCE wound healing. Scratch injury was performed in the absence and presence of CrMP (10 μ M) with and without addition of biliverdin (BVD, 10 μ M) or CORM-A1 (100 μ M). A) Representative images. Quantitative analysis of percent healing at 12h (B) and 24h (C) after injury. (D) Effect of CrMP, BVD and CORM-A1 at the concentration indicated above on cell proliferation (n=8–12; *p<0.05 vs CrMP, †p<0.05 from control untreated cells).

Figure 6.

Effect of HO inhibition on migration-driven wound healing. Scratch injury was performed in the absence and presence of hydroxyurea (HU, 0.5 mM) with and without addition of CrMP (10 μ M), biliverdin (BVD, 10 μ M) or CORM-A1 (100 μ M). Representative images of (A) wound at time 0; (B) wound in the presence of hydroxyurea at 24 h; and (C) wound in the presence of hydroxyurea and CrMP at 24 h. D) Quantitative analysis of percent healing (n=8–12, *p<0.05 vs corresponding time control, †p<0.05 vs HU alone).

Figure 7. Effect of HO-1 and HO-2 siRNA on HCE wound healing. Cells were transfected with control and gene-specific siRNAs. Scratch injury was performed 48 h later and healing determined at 4, 6, 12 and 24 h after injury (n=8–12, *p<0.05 and **p<0.005 vs corresponding control siRNA-treated cells).

Figure 8. Specificity of gene knockdown by siRNA. HCE cells were transfected with control and gene-specific siRNAs. Cells were collected 24 after scratch injury and analyzed for HO-1 and HO-2 mRNA and protein levels. A) Real time PCR analysis of HO-1 and HO-2 mRNA (n=4–6, *p<0.05 vs control untreated cells or control siRNA-treated cells; [‡]p<0.05 vs HO-1). B) Representative Western blots of HO-1 and HO-2 proteins and densitometry analysis (in parenthesis; n=3).