



**Heme Oxygenase-2 as a novel target to treat
inflammation and chronic neuropathic pain
associated with corneal injury and surgery**

Doctorate Thesis

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UNIVERSITY OF CATANIA

NEW YORK MEDICAL COLLEGE

International Ph.D. program in Neuropharmacology

XXIV cycle



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List of abbreviations

AA	<i>arachidonic acid</i>
ATP	<i>adenosine-5'-triphosphate</i>
BrdU	<i>bromodeoxyuridine (5-bromo-2'-deoxyuridine)</i>
c-TEN	<i>Customised, TransEpithelial, No-touch surgery</i>
CO	<i>carbon monoxide</i>
COX-2	<i>cyclooxygenase-2</i>
DHA	<i>docosahexaenoic acid</i>
DLK	<i>diffuse lamellar keratitis</i>
EGF	<i>epidermal growth factor</i>
HGF	<i>hepatocyte growth factor</i>
HO	<i>heme oxygenase</i>
LASEK	<i>Laser Epithelial Keratomileusis</i>
LASIK	<i>Laser-assisted in situ Keratomileusis</i>
MMP-1	<i>matrix metalloproteinase-1</i>
MMP-9	<i>matrix metalloproteinase-9</i>
MT1-MMP	<i>membrane type 1 metalloprotease</i>
NGF	<i>nerve growth factor</i>
NSAIDs	<i>nonsteroidal anti-inflammatory drugs</i>

O/E-1	<i>Olf-1/early B-cell factor</i>
ORNs	<i>olfactory receptor neurons</i>
PAF	<i>platelet-activating factor</i>
PCNA	<i>Proliferating Cell Nuclear Antigen</i>
PGE2	<i>Prostaglandin E2</i>
PMNs	<i>polymorphonuclear neutrophils</i>
PRK	<i>photorefractive keratectomy</i>
ROS	<i>reactive oxygen species</i>
ShRNA	<i>short hairpin RNA</i>
siRNA	<i>small interfering RNA</i>
TUNEL	<i>Terminal deoxynucleotidyl transferase dUTP nick end labeling</i>
VEGF	<i>Vascular endothelial growth factor</i>
12(S)-HETE	<i>12-(S)-Hydroxyeicosatetraenoic acid</i>
15(S)-HETE	<i>15-(S)-Hydroxyeicosatetraenoic acid</i>

Preface

Corneal refractive surgery aims at correcting alteration of the shape of the cornea correlated with myopia, hyperopia and astigmatism. More than 12 million patients have undergone refractive surgery since it was approved (see <http://www.laser-eye-surgery-statistics.com/>). Laser-assisted in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK) are the most used techniques to perform experimental corneal surgery.

Several studies demonstrated that after epithelial removal (first step of refractive surgery) an inflammatory response arises and corneal subbasal nerve density does not recover for up to five years. Furthermore, the number of stromal nerves decreases by nearly 90% after LASIK leading to possible complications. One of the most notable adverse effects of refractive surgery correlated with the trans-section of basal nerve, is pain, which typically occurs within the first 72 h after surgery.

Topical ocular nonsteroidal anti-inflammatory drugs (NSAIDs) has demonstrated efficacy in controlling pain after surgery and they are commonly used during the postoperative period. However, some studies reported delayed epithelial wound healing as most notable side effect following topic administration of this class of drugs.

During the inflammatory response the epithelial cells activate a series of endogenous protective mechanisms in the attempt to reduce and/or limitate the propagation of the inflammatory response. Among these mechanisms, the heme oxygenases systems seem to play a major role.

In the recent years the heme oxygenase system (HO-1 and HO-2) has emerged as a fundamental endogenous cytoprotective and anti-inflammatory system in many tissues. It is readily upregulated in response to injury and its activity (heme degradation to bilirubin and carbon monoxide) attenuates tissue damage with significant reductions in inflammatory events including leukocyte adhesion and migration, and production of inflammatory cytokines.

Furthermore HO has been shown to provide neuroprotection and participates to neuronal development in a number of models. We evaluated the role of HO-1 and HO-2 in the corneal inflammatory and reparative response to injury and we assessed the putative mechanisms underlying the cytoprotective/anti-inflammatory function of the HO system in the cornea. Specifically we determined the spatial and temporal changes in HO-1 and HO-2 expression and HO activity in response to injury and correlate these changes to cell infiltrate and wound closure. Furthermore, we determined whether supplementation of HO by-products (CO and/or biliverdin) “rescue” the cornea from the aberrant inflammatory and reparative response in a model where the HO system is impaired.

INTRODUCCION

1- CORNEA

The human cornea, like those of other primates, has five layers (from the anterior to posterior layer): corneal epithelium, bowman's layer, corneal stroma, descemet's membrane and corneal endothelium (Gronert *et al.* 2005). Apart from being an important component of the refractive system of the eye, the cornea protects the most delicate structures of the anterior segment of the eye from injury; it also represents the initial barrier to the external environment and it is in intimate and continuous contact with microorganism and toxins, thus, constantly threatened by processes and agents leading to tissue injury and inflammation (Bellner *et al.* 2009).

Despite this challenge, this tissue is avascular, transparent and shows an extraordinary capacity for epithelial regeneration while maintaining a unique immune-privileged environment. Corneal injury provokes a vital inflammatory response that is characterized by the activation of resident corneal cells and recruitment of leukocytes to produce lipid and protein mediators that initiate and amplify inflammation (Bellner *et al.* 2011a). However aberrant activation of these pathways can lead to tissue destruction, ulceration, perforation and neovascularization, and ultimately to loss of vision.

To maintain the cornea as an optically transparent barrier, a sophisticated self-resolving inflammatory -reparative process must be in place to balance inflammation and immune privilege while promoting wound repair (Bellner *et al.* 2011a). Such a process must include pro- as well as anti-inflammatory circuits that work in concert to initiate, mediate and resolve inflammation in a controlled manner so as to allow

the repair process to proceed towards complete restoration of structure and function, i.e., healing and repair.

2- CORNEAL INNERVATION

The innervation of the cornea and bulbar conjunctiva is provided by a relatively small number of primary sensory neurons located in the ipsilateral trigeminal ganglion (about 1.5% of the total number of neurons of the ganglion, (Felipe *et al.* 1999). Nevertheless, the small size of the cornea and the extensive branching of the peripheral axons of corneal neurons makes this structure the most densely innervated tissue of the body (Rozsa and Beuerman 1982;Felipe *et al.* 1999); (Muller *et al.* 2001).

Corneal neurons can be classified as thin myelinated (A-delta type, 30% in the mouse) or unmyelinated (C type, 70% in the mouse), depending on the size and presence of a myelin sheath in the axon (Belmonte and Giraldez 1981). All peripheral axons of corneal neurons lose the myelin sheath when they enter the corneal stroma, mainly grouped in a variable number of radially oriented nerve bundles. They then branch extensively, forming a subepithelial plexus from which thin branches ascend up traversing the Bowman's layer and enter into the basal layer of the epithelium. There, they run parallel to the corneal surface forming the leashes and terminate in the superficial layers of the corneal epithelium (Fig.1) (ZANDER and WEDDELL 1951;Chan-Ling 1989;Muller *et al.* 2001;Muller *et al.* 2003).

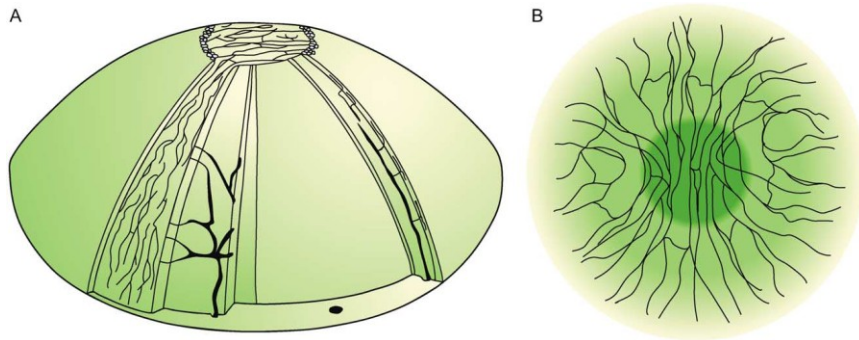


Fig. 1. (A) Schematic distribution of nerves in the stroma and subbasal plexus in human corneas. (B) Adapted scheme on the organization of the subbasal plexus. In the apex the nerve bundles show a preferred orientation in the superior-inferior direction and in the surrounding area they tend to be oriented in the nasal-temporal direction. It is estimated that there are approximately 7000 nociceptors per mm² in the human corneal epithelium.

The mechanisms by which corneal nerve fibres maintain a healthy cornea and promote wound healing after eye injuries is currently under active research in several laboratories. The results obtained in co-culture studies suggest the existence of a possible crosstalk between neurons and corneal epithelial cells through the mutual release of soluble substances. For example, trigeminal neurons release diffusible factors (e.g. neurotransmitters and neuropeptides) that stimulate corneal epithelial cell growth, proliferation, differentiation, and type VII collagen production (Baker *et al.* 1993; Garcia-Hirschfeld *et al.* 1994). Stromal keratocytes also produce neurotrophins (Lambiase *et al.* 2000) (You *et al.* 2001), however, the extent to which these substances exert trophic influences on corneal nerve fibres remains to be determined.

The majority of corneal sensory fibers (about 70%), named polymodal nociceptors, are equally activated by near-noxious mechanical energy but they also respond to heat, to exogenous chemical irritants and to a large variety of endogenous chemical mediators released by damage corneal tissue, by resident inflammatory cells or originating from the

plasma leaking from limbal vessels (protons, potassium ions, ATP, prostaglandins and other arachidonic acid metabolites, amino acids, amines, cytokines, kynins, growth factor) (Belmonte *et al.* 2004). Polymodal nociceptors possibly contribute, together with mechanonociceptors, to the sharp mechanical pain that arises when the cornea is acutely exposed to mechanical force, but they are also the principal source of nerve impulse activity caused by chemical irritation, heat or noxious cold (Belmonte and Giraldez 1981;Beuerman and Rozsa 1985). During inflammation, locally released mediators stimulate polymodal nociceptors, leading to a continuous firing that produces sustained sensations of pain.

Many studies demonstrated that an healthy status of innervations and corneal sensitivity are both essentials for maintaining a healthy ocular surface, mainly because corneal nerves modulate cell proliferation, differentiation and wound healing (Beuerman and Schimmelpfennig 1980;Garcia-Hirschfeld *et al.* 1994). In addition, corneal nerves have been demonstrated to play a role in ion transport, and collagen expression (Jones and Marfurt 1996;Baker *et al.* 1993).

Furthermore, it has been shown that corneal denervation induces apoptosis of resident corneal cells (Cortina *et al.* 2011). However, the mechanisms underlying these effects are not completely understood (Cortina *et al.* 2011).

3- SURGYCAL PROCEDURESES ON CORNEA WITH OR WITHOUT EPITHELIUM REMOVAL

The corneal refractive surgery is based on “the law of thickness” proposed by Barraquer in 1964 (Barraquer JI 1964) “...*changing the*

thickness of the cornea follows the idea that the cornea is a stable lens, removing tissue in the center or adding tissue on the periphery therefore flattens the cornea.” The argon fluoride (193 nm) excimer laser permits the excision of corneal tissue with minimal damage to the adjacent tissues. It employs an high energy ultraviolet radiation to break the covalent bonds between molecules in the corneal stroma without generating high levels of heat (Krauss *et al.* 1986). This procedure has been termed photoablative process and is the principal reason making laser refractive surgery a relative predictable and safer procedure. These are the main differences in the method used to remove the epithelial surface:

PRK and LASIK - were the first surgical procedures, in which laser was used to shape the corneal surface. The most important difference between the two procedures is the way of how the middle layer of the cornea is exposed. During PRK the epithelium is mechanically scraped off using a special instrument and epithelium is not placed back after refractive surgery whereas LASIK, consists in the preparation of a flap and in a replacement of the epithelium back to its position (Lombardo *et al.* 2011).

LASEK - was a modification of PRK method. During LASEK the epithelium layer is loosen from the tissue below with alcohol and is moved aside for the time of the surgery. The replaced epithelium layer will act as a natural contact lense.

Epi-LASIK (Epi-K) - is the newest method for removing epithelium. Epithelium layer is separated from below layers by using a special microkeratome. As the remaining epithelium cells are not damaged by

alcohol or mechanical scraping instrument, the recovering of the eye after Epi-K method is faster and the chance of arising haze is smaller than with using PRK or LASEK method (Reilly *et al.* 2010).

c-TEN - The cTen system was introduced by an Italian company called iVis Technologies. It includes a 1,000-Hertz excimer laser (iRES) and a software planning system called CIPTA. cTen is an acronym for Customised, TransEpithelial, No-touch surgery. It is indicated for complex cases with irregularity of the cornea (e.g irregular astigmatism). They use the excimer laser to remove the epithelial skin layer and use topography information from a Scheimpflug camera scanner to correct imperfections across the cornea stroma (Thomann and Schipper 2010).

4-PRK AND LASIK COMPLICATIONS

More than 12 million patients have undergone LASIK and PRK since they were approved (see <http://www.laser-eye-surgery-statistics.com/>).

These are the most popular and used techniques to perform experimental corneal surgery. Many studies demonstrated that corneal subbasal nerve density does not recover for up to five years (Erie *et al.* 2005), and the number of stromal nerves decreases by nearly 90% after LASIK surgery (Erie *et al.* 2005) then complications, although not frequent, do occur. Complications may arise intraoperatively or during the postoperative period (Filatov *et al.* 1997;Ghadhfan *et al.* 2007;Melki and Azar 2001;Stein 2000). Some patients may develop glare, halos, monocular diplopia, changes in contrast sensitivity, and dry eye (Hersh *et al.* 1997;Hersh *et al.* 1990;Seiler and McDonnell 1995). Postoperative complications after LASIK include flap striae and folds, dislodging of the flap, interface debris, epithelial ingrowth, diffuse lamellar keratitis, and

corneal infections (Davis *et al.* 2000; Gimbel *et al.* 1998; Melki and Azar 2001). Instead, postoperative complications after PRK, are related to epithelial debridement and include chronic pain, delayed epithelial healing, infection, and corneal scarring/haze (Alio *et al.* 2008).

4a- Chronic neurophatic corneal pain, inflammation and impaired wound healing.

The cornea is the most powerful pain generator in the human body. The density of corneal pain receptors has been estimated to be 40 times higher than dental pulp. It is estimated that there are approximately 7000 nociceptors per mm² in the human corneal epithelium.

Rosenthal *et al.*, suggested that after surgery the damaged corneal nerve fibers (i.e. polymodal nociceptor) could be the cause of all patient symptoms, such as glare, halos, monocular diplopia, sustained pain, impaired wound healing, changes in contrast sensitivity, whether or not the initial disease is severe dry eye or corneal neuropathy. Patients undergoing to this surgery report severe, unremitting, burning pain and photophobia. They also demonstrated using *in vivo* confocal microscopy that all these patients presented nerve abnormalities and this alteration in corneal nerves (Cruzat *et al.* 2010) are similar to those reported in skin biopsies of neuropathic pain conditions (Lauria and Devigili 2007; Sommer 2008).

Recently, Cortina *et al.* (Cortina *et al.* 2010) confirmed by immunohistochemistry that epithelial removal due to surgical procedures lead to the transection of afferent sensory nerve fibres (Figure 2) and the aberrant regenerated corneal nerves are likely to be among the most important factors associated with impaired wound healing and exaggerated inflammatory response into the corneal layer after surgery

(Ambrosio, Jr. *et al.* 2008). They also demonstrated that the epithelial removal and the impairment of the sub-basal nerve plexus correspond to a lack of sensitivity measured by Coche-Bonnet esthesiometer (Cortina *et al.* 2010).

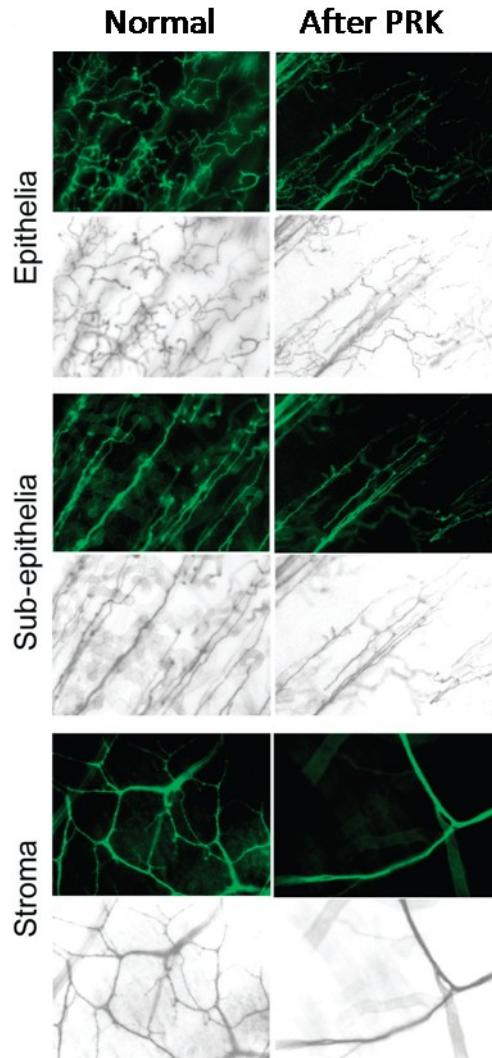


Fig.2 Immunohistochemistry of epithelia, subepithelia, and stroma of rabbit corneal whole mounts stained with anti- β III tubulin antibody 8 weeks after surgery.

Some studies (Tuunanen *et al.* 1997;Tuisku *et al.* 2007) suggest that the improvement of corneal wound healing, showed a direct relation

with the recover of the corneal subbasal nerve plexus to maintain corneal healing and transparency.

4b- Chronic neurophatic corneal pain treatment and side- effects on epithelial regrowth.

As mentioned above one of the most notable adverse effects of PRK is pain, which typically occurs within the first 72 h after surgery (Assouline *et al.* 1998;McCarty *et al.* 1996).

Topical ocular nonsteroidal anti-inflammatory drugs (NSAIDs) have demonstrated efficacy in controlling pain after PRK surgery (Sher *et al.* 1993;Arshinoff *et al.* 1996) and they are commonly used during the postoperative period. The best choice of NSAID, however, is yet to be determined.

Topical NSAIDs have been shown to reduce pain after PRK in numerous clinical trials. Some studies, however, report delayed epithelial wound healing, (Assouline *et al.* 1998;Rajpal and Cooperman 1999) and others have shown no adverse effects on corneal reepithelialization when topical NSAIDs were used (Sher *et al.* 1993). These conflicting results highlight the need for comparative studies among agents. Durrie *et al.* (2007) evaluated the efficacy of 3 approved ophthalmic NSAIDs nepafenac 0.1%, ketorolac 0.4%, and bromfenac 0.09% to determine their effects on corneal reepithelialization and postoperative pain control in patients undergoing PRK surgery (Durrie *et al.* 2007). No difference in time to corneal reepithelialization was noted between nepafenac 0.1% and ketorolac 0.4%. Average time to complete healing was approximately 5.5 d with both regimens; however, patients treated with bromfenac 0.09% demonstrated a delay in corneal healing of approximately 1.5 d (mean healing time, 7 d). In a recent study

comparing nepafenac 0.1% with ketorolac 0.4%, the average time to healing was approximately 4 d in both treatment groups (Donnenfeld *et al.* 2007). It has been postulated that the effects of NSAIDs on the arachidonic pathway, similar to corticosteroids, may play a role in corneal healing (Hersh *et al.* 1990).

Mechanistically, NSAIDs have varying effects on the cyclooxygenase and lipoxygenase pathways. This makes the comparative studies in this kind of field important in assessing differences between agents. In addition, specific preservatives (eg, thimerosal) have been implicated in delayed healing (Assouline *et al.* 1998).

The dose and duration of use may play an important role because higher doses and longer duration of use seem to have a greater association with delayed healing.

4c- Biochemical pathway involved in corneal inflammatory response after injury.

Cytokines and growth factors released after injury are the soluble factors mediating the signals and interaction between different cells and components to restore corneal functionality (Bazan 2005).

Inflammation is the first response of corneal tissue to an insult, and during the first hours, the cells of the corneal layers respond by releasing arachidonic acid (AA) (Eakins *et al.* 1972) from membrane phospholipids and converting this fatty acid into eicosanoids and platelet-activating factor (PAF) (Bazan *et al.* 1993).

When inflammation is more severe and persists, cells that infiltrate the cornea, mainly neutrophils, amplify the response and contribute to the increase in these lipids. Some of these mediators increase pain, delay wound healing, and promote neovascularization through an induction of

overexpression of MMP-1, MMP-9, COX-2 and VEGF in the epithelial cell (Bellner *et al.* 2011a). PAF also increases keratocyte apoptosis and stimulates myofibroblasts to synthesize furin, a convertase that activates growth factors and receptors (Bazan 2005). Blocking the action of PAF, with PAF-antagonist (LAU-0901), prevents both diffuse lamellar keratitis (DLK) after LASIK surgery and stromal melting after severe alkali burn. On the other hand, two lipoxygenase metabolites, 12(S)-HETE and 15(S)-HETE, are activated by growth factors and are involved in proliferation and wound repair (Figure 3) (Gronert *et al.* 2005).

Growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF), released in response to corneal injury, utilize signal mechanisms involving lipoxygenase activation to exert their proliferative effects on epithelial cells and contribute to the repair phase. Bazan *et al.* (Bazan 2005) demonstrated that the omega-3 fatty acid docosahexaenoic acid (DHA) potentiates the corneal nerve regenerative action by nerve growth factor (NGF) after PRK and also stimulation of cyclooxygenase-2 (COX-2) produces a different pattern of prostaglandin synthesis in epithelial and endothelial cells, suggesting different functions; e.g. PGE₂ released from epithelial cells has been implicated in pain. In conclusion, lipids play an important role in the complex inflammatory responses that occur after corneal injury (Cortina *et al.* 2011).

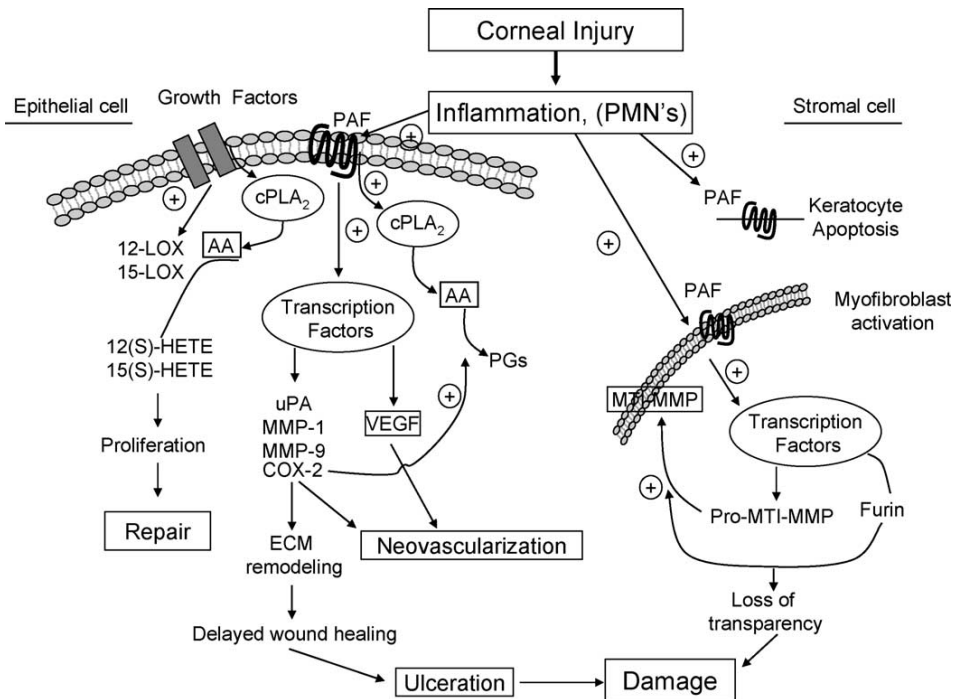


Fig.3 Schematic representation of current knowledge on the actions of lipid mediators in corneal damage and repair (Bazan 2005).

5- HEME OXYGENASE AND ITS ANTI-INFLAMMATORY, ANTIOXIDANT AND NEUROPROTECTIVE PROPERTIES.

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 system extends far beyond its original role in the catabolism of heme in senescent heme proteins (Abraham and Kappas, 2008). In the recent years the heme oxygenase system (HO-1 and HO-2) has emerged as a fundamental endogenous cytoprotective and anti-inflammatory system in many tissues producing compounds that uniquely combine vasodilatory (CO), anti-oxidant (CO and bilirubin) and anti-apoptotic (CO, bilirubin) properties (Abraham and Kappas, 2008).

Overall, HO has a well-deserved reputation of a potent endogenous anti-oxidant cell defense system. So far, HO-1, transcriptionally upregulated in response to oxidative stress, was considered the only HO isoform that provided cytoprotection. However, it is becoming clear that, in the brain and in many other tissues, HO-2, rather than HO-1, participates in a multitude of housekeeping functions directed at maintaining tissue homeostasis and protecting against oxidative stress under constantly changing conditions (Chen *et al.* 2003).

The ability of HO-2 to be post-transcriptionally activated provides immediate actions including blocking oxidant-producing systems, scavenging reactive oxygen species (ROS), and, therefore, preventing and eliminating further damage caused by ROS (Parfenova and Leffler 2008). For example, the cerebroprotective functions of HO-2 include CO-mediated increases in cerebral blood flow in response to seizures, hypoxia, hypotension, and glutamate aimed at providing neurons with nutrients and oxygen (Chen *et al.* 2003). In addition, CO, via its ability to strongly bind to heme, inhibits heme-containing ROS-generating systems, NADPH oxidase and the mitochondrial respiratory chain, thus reducing oxidative stress (Parfenova and Leffler 2008).

Furthermore, bilirubin, a potent ROS scavenger, eliminates preformed oxidant radicals thus strengthening the anti-oxidant effects of CO. Therefore, HO-2 builds a strong cerebroprotective system in the brain and cerebral circulation that, in contrast to HO-1, provides immediate responses to cerebrovascular stress and prevents potential damage to neurons, astrocytes, and cerebral vascular endothelium (Parfenova and Leffler 2008). Chen *et al.* (Chen *et al.* 2003) investigated the roles of HO-1 and HO-2 in the homeostasis of a neuronal population using the

olfactory system as a model and knock out mice for both HO isoforms. The olfactory system is an important neuronal and developmental model (Graziadei and Monti Graziadei 1980; Calof and Chikaraishi 1989; Hansel *et al.* 2001), and may be used to delineate the functions of HO in neurons. The olfactory epithelium contains one of the few neuronal populations capable of robust neurogenesis throughout life, and is the only mammalian neuronal population known to be able to replace itself functionally (Graziadei and Monti Graziadei 1980). They provided evidence that HO-1 and HO-2 are both expressed in neurons and in embryonic and adult olfactory epithelium, where they are positioned to mediate autocrine/paracrine functions (Chen *et al.* 2003).

They demonstrated that loss of HO-1 or HO-2 affects the homeostasis of the neuronal population. Olf-1/early B-cell factor (O/E-1) was used as a marker to identify post-mitotic cells of the olfactory neuronal lineage. O/E-1 labeling was significantly reduced in both HO-1 and HO-2 animals. The numbers of cells labeled with BrdU were also decreased in HO-1 and HO-2 null mice. In contrast, the number of TUNEL-positive cells was only increased in the HO-2 null mouse, indicating that only HO-2 mediated survival cues. The proliferative and survival functions of HO could be mediated by any of the products of HO action then further studies need to be made to evaluate this hypothesis. Thus Chen *et al.* (2003) were able to show that HO-2 supports neuronal survival even under baseline condition.

Aim of the study

The aim of my research has been an extensive characterization of the HO system status in the cornea correlated to the inflammatory/repairative response to injury elicited by corneal epithelial removal. For this purpose, I first used an *in vitro* model of human corneal epithelial cells and successively I employed an *in vivo* model in which the corneal epithelial removal was used to mimic the first step of the surgical procedure. Both models were used to correlate spatial/temporal changes of HO expression and activity with inflammatory and reparative response following epithelial removal. Leukocyte infiltration, pro-inflammatory mediators production, and wound closure were evaluated under various experimental conditions in which HO activity is differently upregulated or inhibited.

Chapter 1

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

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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.

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 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). 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 2-3; 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 (mm²) 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 mM hemin 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 integrity was verified by agarose gel electrophoresis and quantitated 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'-GCAGAATCTTGACTTTGTT-3'; HO-2 sense, 5'-ATGTCAGCGGAAGTGGAAC-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

Western blot analysis, 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 either the Mann-Whitney U-test or 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 within 1 h and returned to control levels 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 a transient increase in HO-1 expression 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 μm 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 $10 \mu\text{M}$ 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 $100 \mu\text{M}$ 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 inactivated CORM (iCORM) at concentrations of 100-400 μM had no significant effect on wound closure (data not shown).

Inhibition of HO activity impairs wound healing

CrMP at the concentration used ($10 \mu\text{M}$) 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 29.74 and 63.14%, 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

than the control untreated or cell treated with non-specific siRNA at all time points (Figure 7). That the siRNAs specifically knockdown their corresponding proteins is seen in Figure 8. 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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FIGURES

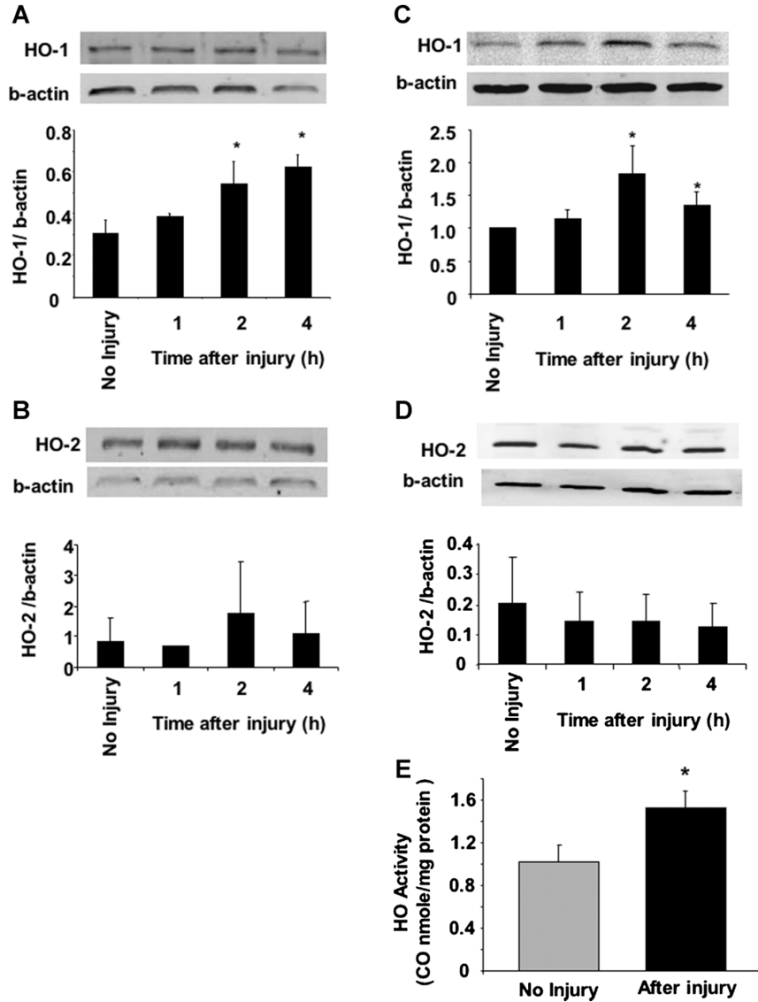


Figure 1: HO expression and activity in human corneal epithelial cells. (A) A representative Western blot of HO-1 and HO-2 proteins and (B) densitometry analysis (n=3) in primary cultures of human corneal epithelial cells. (C) A representative Western blot of HO-1 and HO-2 proteins and (D) densitometry analysis (n=3) 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)

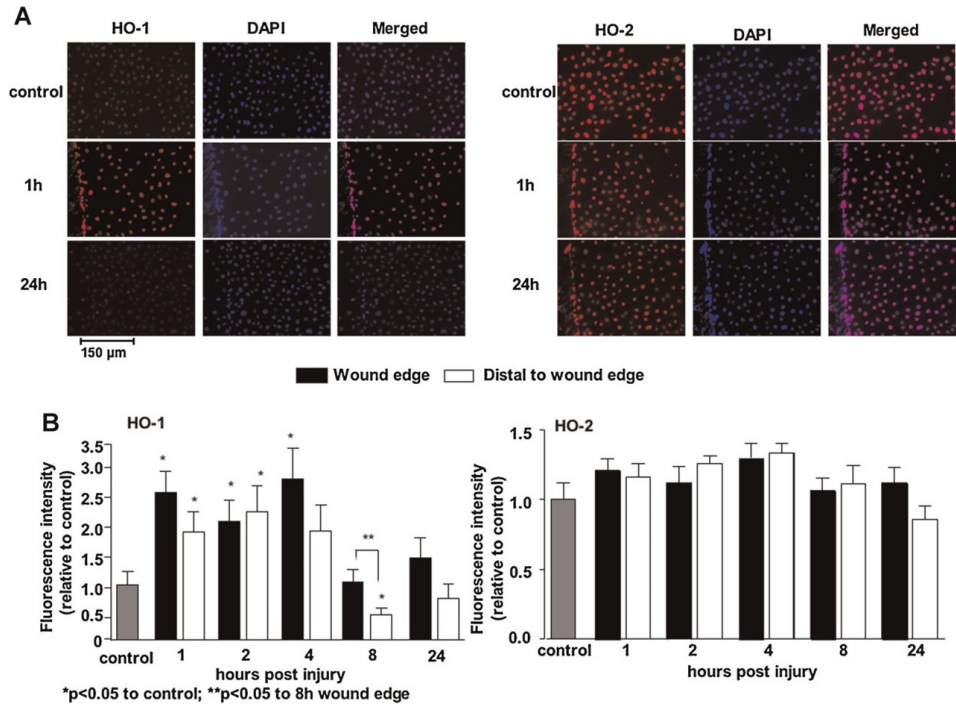


Figure 2: HO-1 and HO-2 expression in HCE cells. (A) Immunofluorescence assay for HO-1 (left panel) and HO-2 (right panel). Scratch injury was performed and expression of HO-1 and HO-2 determined 1, 2, 4, 8, 24 h after injury with 20X magnification of Zeiss Axioplan-2 fluorescent microscope. (B) Quantitative 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).

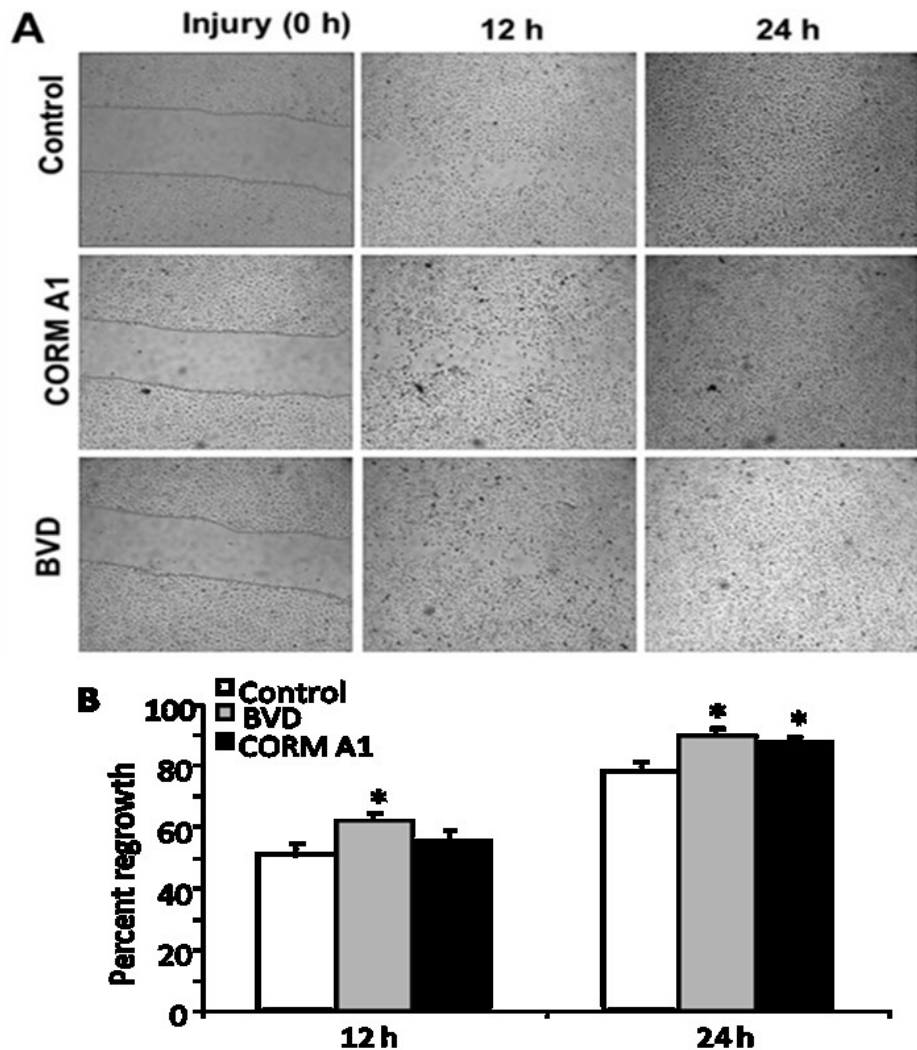


Figure 3: Effect of biliverdin and CO on HCE wound healing. Scratch injury was performed in the absence and presence of biliverdin (BVD, 10 μ M) or CORM-A1 (100 μ M) and percent healing was determined at 12 and 24 hr after injury. A) Representative images of wounded HCE cells. B) Quantitative analysis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

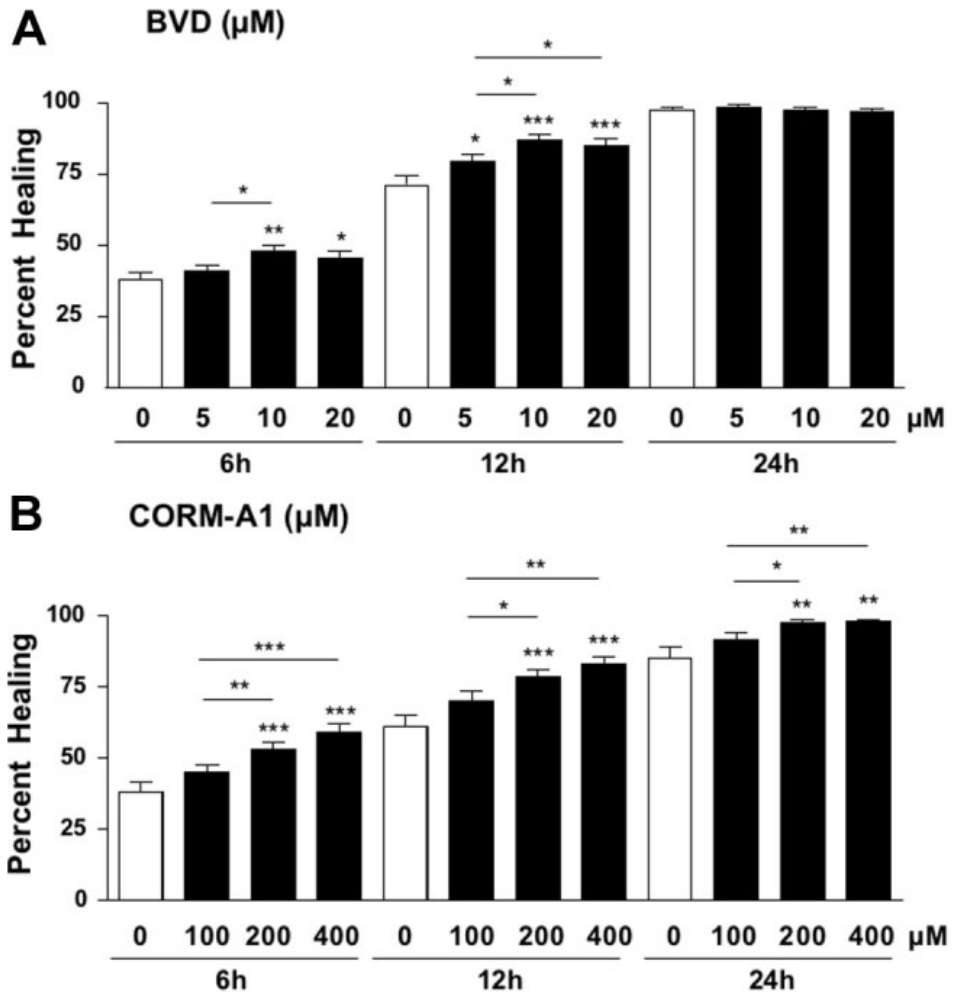


Figure 4: Effect of biliverdin (A) and CORM-A1 (B) concentrations on HCE wound healing 12 and 24 h after injury (n=8-12, *p<0.05, **p<0.01 and ***p<0.001).

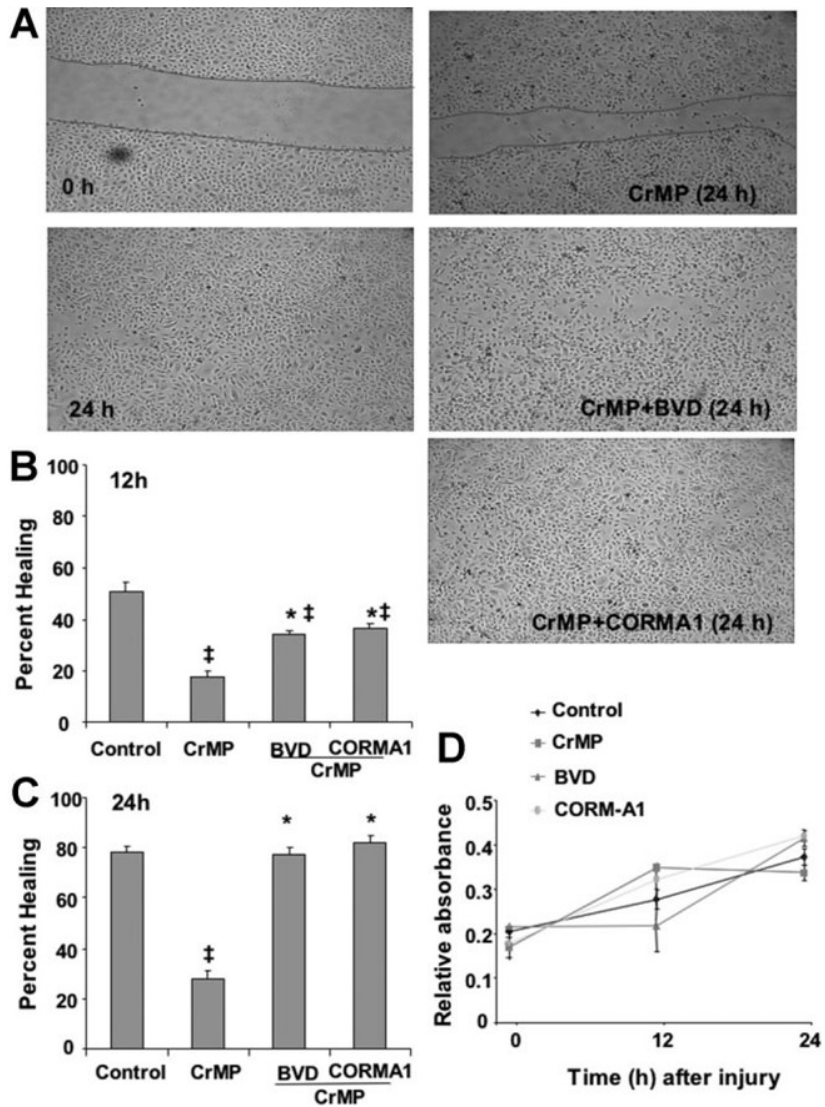


Figure 5: Effect of HO inhibition on HCE wound healing. Scratch injury was performed in the absence and presence of CrMP (10 μ M) with and without addition of biliverdin (BVD, 10 μ M) or CORM-A1 (100 μ M). A) Representative images. Quantitative analysis of percent healing at 12h (B) and 24h (C) after 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).

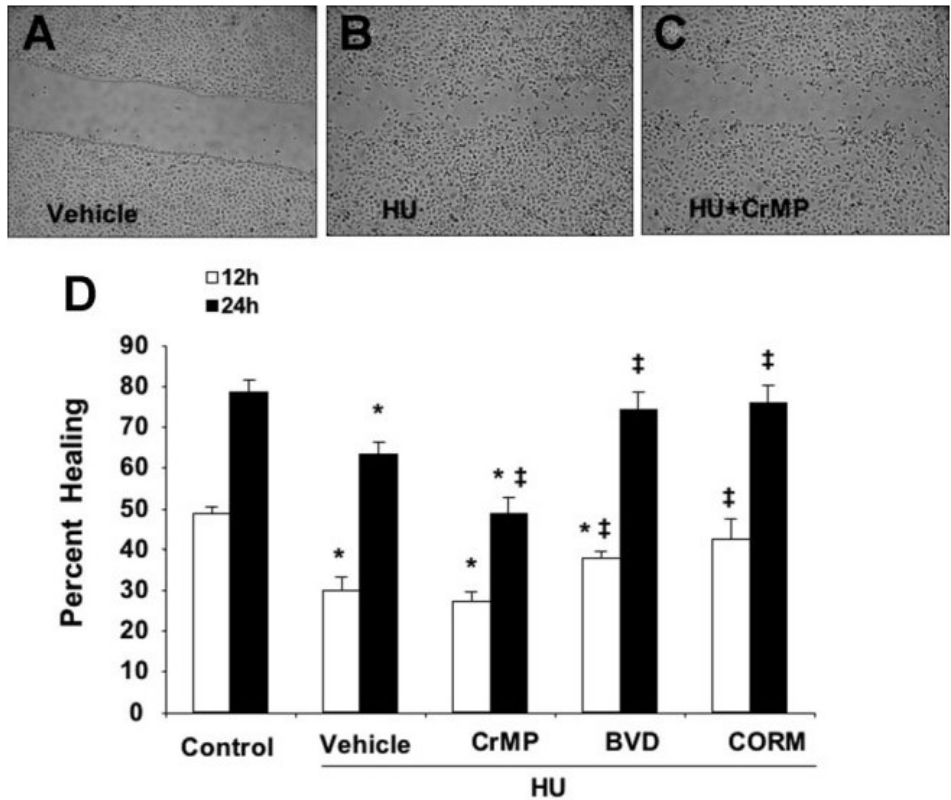


Figure 6: Effect of HO inhibition on migration-driven wound healing. Scratch injury was performed in the absence and presence of hydroxyurea (HU, 0.5 mM) with and without addition of CrMP (10 μ M), biliverdin (BVD, 10 μ M) or CORM-A1 (100 μ M). Representative images of (A) wound at time 0; (B) wound 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).

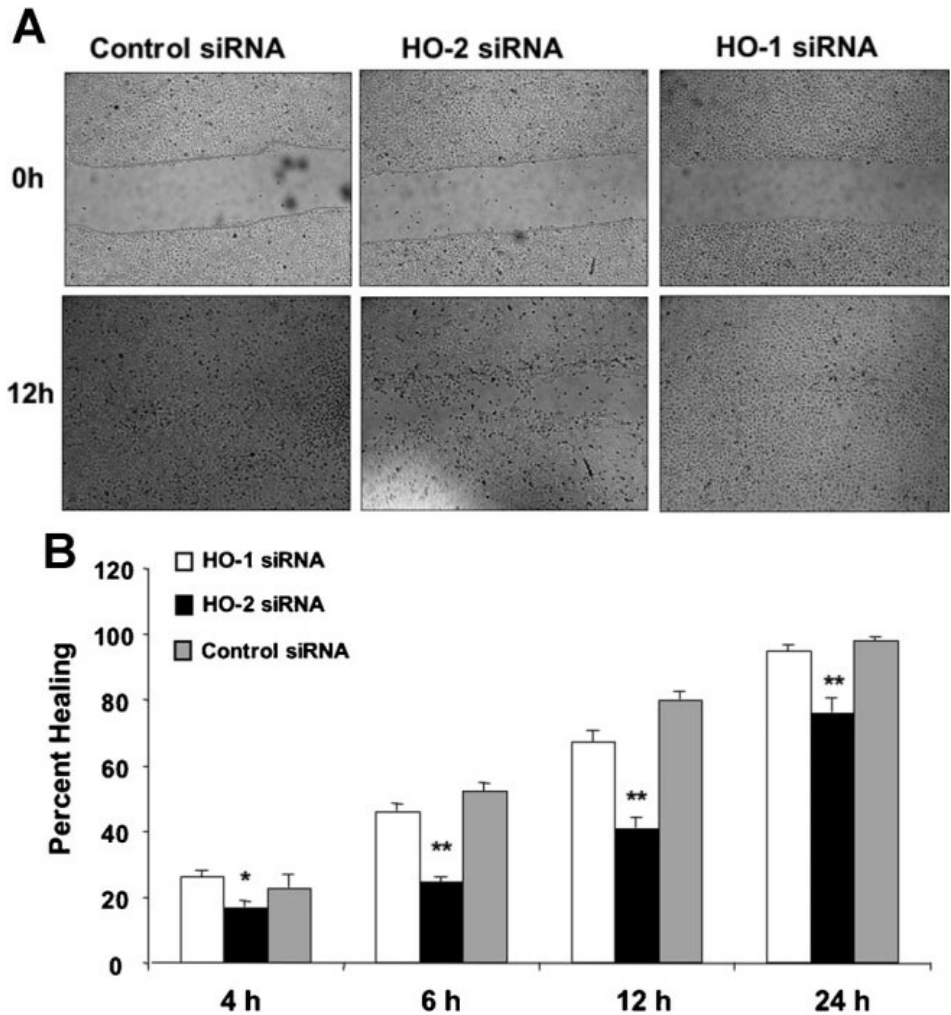


Figure 7: Effect of HO-1 and HO-2 siRNA on HCE wound healing. Cells were transfected with control and gene-specific siRNAs. Scratch injury was performed 48 h later and healing determined at 4, 6, 12 and 24 h after injury (n=8-12, *p<0.05 and **p<0.005 vs corresponding control siRNA-treated cells).

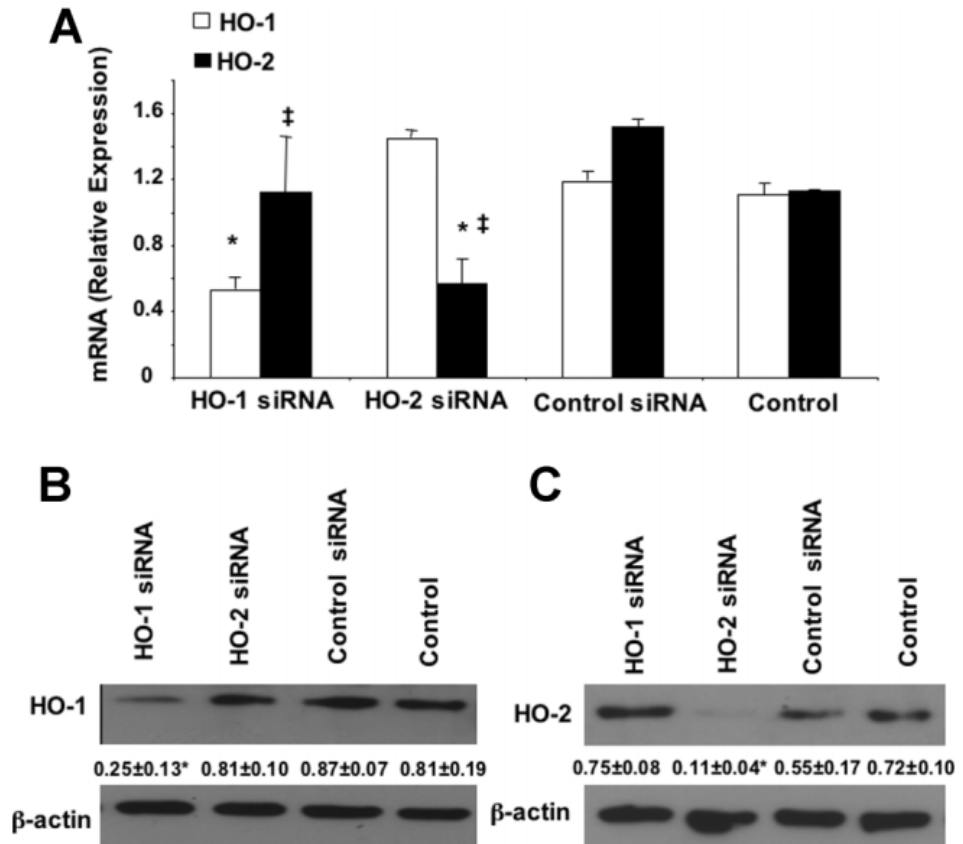


Figure 8: Specificity of gene knockdown by siRNA. HCE cells were transfected with control and gene-specific siRNAs as described in Methods. Cells were collected 24 after scratch injury and analyzed for HO-1 and HO-2 mRNA and protein levels. A) Real time PCR analysis of HO-1 and HO-2 mRNA (n=4-6, *p<0.05 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.

Chapter 2

THE ROLE OF NEUTROPHILS IN CORNEAL WOUND HEALING IN HO-2 NULL MICE^δ

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ABSTRACT

Our studies demonstrated that Heme oxygenase (HO), in particular, the constitutive HO-2, is critical for a self-resolving inflammatory and repair response in the cornea. Epithelial injury in HO-2 null mice leads to impaired wound closure and chronic inflammation in the cornea. This study was undertaken to examine the possible relationship between HO-2 and the recruitment of neutrophils following a corneal surface injury in wild type (WT) and HO-2 knockout (HO-2^{-/-}) mice treated with Gr-1 monoclonal antibody to deplete peripheral neutrophils. Epithelial injury was performed by removing the entire corneal epithelium. Infiltration of inflammatory cell into the cornea in response to injury was higher in HO-2^{-/-} than in WT. However, the rate

of corneal wound closure following neutrophil depletion was markedly inhibited in both WT and HO-2^{-/-} mice by 60% and 85%, respectively. Neutropenia induced HO-1 expression in WT but not in HO-2^{-/-} mice. Moreover, endothelial cells lacking HO-2 expressed higher levels of the Midkine and VE-cadherin and displayed strong adhesion to neutrophils suggesting that perturbation in endothelial cell function caused by HO-2 depletion underlies the increased infiltration of neutrophils into the HO-2^{-/-} cornea. Moreover, the fact that neutropenia worsened epithelial healing of the injured cornea in both WT and HO-2^{-/-} mice suggest that cells other than neutrophils contribute to the exaggerated inflammation and impaired wound healing seen in the HO-2 null cornea.

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INTRODUCTION

In addition to being an important component of the refractive system of the eye, the cornea serves to protect the most delicate structures of the anterior segment of the eye from injury. It represents the initial barrier to the external environment and is in intimate and continuous contact with microorganisms and toxins, thus, constantly threatened by processes and agents which cause tissue injury and

inflammation. Despite this challenge, this tissue is avascular, transparent and shows an extraordinary capacity for epithelial regeneration while maintaining a unique immune-privileged environment. Injury to the corneal surface elicits an inflammatory and repair response that, if is orderly executed, the structure and function of the cornea is restored. The corneal inflammatory response is marked by activation of corneal cells, in particular, stromal keratocytes, and recruitment of leukocytes to produce lipid and protein mediators that initiate, amplify and ultimately resolve the inflammation. The repair response, i.e., re-epithelialization process, proceeds simultaneously with migration and proliferation of epithelial cells. Aberrant activation of these pathways can lead to tissue destruction, ulceration, perforation and neovascularization, and ultimately to loss of vision [1,2].

The recruitment of inflammatory cells into the injured cornea is a critical step in initiation and amplification of not only the inflammatory response (increasing innate immunity to prevent infection) but also the repair and healing process. Neutrophil migration into the corneal stroma following epithelial surface injury is evident within a few hours of injury. These neutrophils infiltrate the corneal stroma through the limbal blood vessels [1,3], and their progression through the stroma to the area of the wound has been suggested to be facilitated by keratocyte apoptosis [4]. Neutrophil infiltration is an immediate response to injury, exerting their phagocytic functions to clear pathogens and cellular debris. However, at the same time neutrophils release into the injured tissue oxidative, hydrolytic and pore-forming molecules capable of damaging otherwise-healthy host cells. As such, an exaggerated neutrophil recruitment in response to injury or inflammatory stimuli contributes to the

immunopathology observed in many diseases [5,6]. The role of the neutrophil in the inflammatory response and healing of the cornea is controversial. Depletion of neutrophils or inactivation of their migration have been shown to both increase and decrease re-epithelialization and healing of the corneal surface [4,7-9].

The heme oxygenase (HO) system has been implicated in the resolution of inflammation [10]. HO is the rate-limiting enzyme in heme catabolism. It cleaves heme to biliverdin, carbon monoxide (CO), and iron; biliverdin is subsequently converted by biliverdin reductase to bilirubin. Two isoforms, HO-1 and HO-2, are expressed in most tissues. HO-1 is an inducible enzyme, whereas HO-2 displays, in general, a constitutive expression that is developmentally regulated [11]. Our previous study showed that a deficiency in HO activity, as in the HO-2 null (HO-2^{-/-}) mice, exacerbates ocular surface inflammation allowing an acute inflammation to become chronic with the stigma of chronic corneal inflammation such as neovascularization, ulceration, and perforation [12-14]. The present study was undertaken to examine the role that neutrophils play in the exaggerated inflammation developed in the HO-2^{-/-} mice. Our results demonstrated that the elevated number of neutrophils recruited to the injured tissues in the HO-2^{-/-} mice are not solely responsible for the delay in healing, on the contrary, the lack of neutrophils, through depletion, impairs significantly the wound healing in both wild type (WT) and HO-2 mice.

MATERIALS AND METHODS

Animals.

All animal experiments were performed following a protocol approved by the Institutional Animal Care & Use Committee (Approval # 40-2-0410.2) and 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 [15]. These well-characterized HO-2-null mice have a C57BL/6x129/Sv genetic background [16], which was used on age- and gender- matched controls (Jackson Laboratory, Bar Harbor, ME). DNA was isolated from the tail and genotyped using HO-2 and Neo specific primers as described [16]. Neutropenia was induced with an intraperitoneal injection of 1.6 mg/Kg of Anti-Mouse Ly-6G (Gr-1), a monoclonal antibody (eBioscience, San Diego, CA) that specifically reacts with Ly-6G, which is present on neutrophils but not with Ly-6C, which is expressed on neutrophils, dendritic cells, and subpopulations of lymphocytes and monocytes [17], 24 hours prior to epithelial injury. Mice were anesthetized with ketamine (50 mg/kg) and xylazine (20 mg/kg) intramuscularly, and a drop of tetracaine-HCl 0.5% was applied to the eye to deliver local corneal anesthesia before animals were subjected to injury. The corneal epithelium was removed up to the corneal/limbal border with a 0.5-mm corneal rust ring remover (Algerbrush II; Alger Equipment, Lago Vista, TX). Wound closure (re-epithelialization) was monitored by fluorescein staining. Images of the anterior surface were taken with a dissecting microscope (Carl Zeiss, Jena, Germany) coupled to a digital camera (AxioCam HRC; Carl Zeiss) and analyzed using Zeiss software (Axiovision 4.5). Mice were sacrificed 2 days after injury, eyes were removed, and corneas free of conjunctival tissue were dissected and processed for selected analyses.

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, Gaithersburg, MD) 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'-GCAGAATCTTGACTTTGTT-3'; HO-2 sense, 5'-ATGTCAGCGGAAGTGGAAAC-3' and anti-sense, 5'-CGAGAGGTCAGCCATTCTCA-3'; Midkine (Mdk) sense, 5'-GAAGAAGGCGCGGTACAATG-3' and anti-sense, 5'-GAGTGGATTCTGCATAATGG-3'; VE-cadherin sense, 5'-CGGCCACGCCACTGTCTTGT-3' and anti-sense, 5'-CCAAGGGCTTGCCCACTCGG-3'; 18S sense, 5'-GATGGGCGGCGGAAAATAG-3' and anti-sense, 5'-GCGTGGATTCTGCATAATGG-3'. PCR efficiency for each primer pair was determined by quantifying amplification with increasing concentrations of template cDNA, and specific amplification was verified by subsequent analysis of melt curve profiles for each amplification. A non-template 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 [13].

Isolation of Neutrophils

Neutrophils were isolated from peripheral blood. Briefly, blood was collected in EDTA-containing tubes. Most of the erythrocytes were removed by sedimentation using Anticoagulant Citrate Dextrose Solution (ACD) and dextran (6% dextran/PBS). The remaining erythrocytes and platelets were removed using ddH₂O and 0.6 M KCl. Neutrophils were then isolated using Histopaque 1077 density centrifugation. Isolated neutrophils were re-suspended in Hank's Balanced Salt Solution. Isolation steps were performed at 4°C. The number of neutrophils purified were counted using hemacytometer (Fisher Scientific, Pittsburgh, PA). The yield was approximately 0.15-0.2x10⁶ neutrophils/ml blood.

Histology and immunostaining

Dissected corneas were washed twice with PBS and embedded in OCT compound (Sakura Finetek, Torrence, CA). Cryostat sections were cut transversely into 7 µm thick sections, stained with Hematoxylin-Eosin and mounted on microscopic slides in Cytosol XYL (Richard-Allan Scientific, Kalamazoo, MI). Immunofluorescence staining was performed using the following antibodies: rat anti-mouse Ly-6G (Gr-1) monoclonal antibody (1:200, eBioscience, San Diego, CA), rat anti-mouse CD68 antibody (1:100, AbD Serotec, Raleigh, NC). Briefly, frozen corneal sections were fixed in 4% paraformaldehyde- PBS for 15 min at room temperature and then blocked in 5% goat serum in PBS-Triton X100 (0.1%) for 1h at room temperature. Then, sections were incubated with either rat anti-mouse Gr-1 or rat anti-mouse CD68 antibody (eBioscience, San Diego, CA) overnight at 4°C, washed and

further incubated with a Cy3- conjugated goat anti-rat antibody (1:500, Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. To further verify cellular entity, sections were washed and counterstained for nuclei with 4',6- diamino-2-phenylindole (DAPI) for 15 minutes. Immunofluorescence was visualized using a Zeiss Axioplan-2 fluorescent microscope. Images were captured and analyzed using AxioVision 2 multi channel image processing software (Zeiss, Gottingum, Germany) [12].

Adhesion assay

Isolation and culture of aortic endothelial cells (mAEC) from WT and HO-2^{-/-} mice were done as previously described [18]. Adhesion of neutrophils to the mAEC monolayer was measured using a CytoSelect Adhesion Assay Kit (Cell Biolabs, San Diego, CA) according to manufacturer's instructions. Endothelial cells were grown in a 48-well plate and treated with TNF- α (50 ng/ml) or its vehicle control for 8 h. Neutrophils were labeled with LeukoTracker, added to the wells, and incubated for 60 min at 37°C in a cell culture incubator. The mAEC were washed to remove the non-adherent neutrophils, lysed and fluorescence at 480 nm/520 nm was measured with a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT). Neutrophil adhesion was expressed as relative fluorescence units (RFU).

Statistical Analysis.

Student's t-test was used to evaluate the significance of differences between groups, and multiple comparisons were performed by regression analysis and one-way analysis of variance. $P < 0.05$ was considered significant. All data are presented as mean \pm SE.

RESULTS

Neutrophil depletion impairs wound healing in both WT and HO-2^{-/-} mice.

The corneal epithelial injury model is a well-established model in which the inflammatory and reparative response has been well characterized [13,19]. Wound healing was assessed in control and neutrophil-depleted WT and HO-2^{-/-} mice. As seen in Figure 1, epithelial injury in WT mice produced a consistent wound in control mice ($6.6 \pm 0.3 \text{ mm}^2$, n=6) that exhibited $44.2\% \pm 5.2\%$ re-epithelialization by day 2 after injury. In contrast, wound closure in neutrophil-depleted WT mice ($7.2 \pm 0.2 \text{ mm}^2$, n=6) was markedly inhibited displaying re-epithelialization of $20.5\% \pm 4.0\%$ at day 2 after injury. Similar results were obtained in the HO-2^{-/-} mice in which wound closure was further impaired following neutrophil depletion. Thus, epithelial injury in HO-2^{-/-} mice produced a consistent wound in control ($7.4 \pm 0.1 \text{ mm}^2$, n=5) and neutrophil-depleted mice ($7.6 \pm 0.2 \text{ mm}^2$, n=5) that was closed by $22.6\% \pm 4.9\%$ and $4.5\% \pm 2.6\%$, respectively, at day 2 after injury (Figure 1 A and B).

Neutrophil depletion reduces the number of infiltrated cells into the injured cornea.

H&E staining of corneal sections from neutrophil-depleted WT or HO-2^{-/-} clearly showed the absence of neutrophils (Figure 2A). The number of neutrophils harvested from control HO-2^{-/-} mice (not injected with Gr-1 antibody) was significantly higher than that in the corresponding WT mice (Figure 2B). However, injection of Gr-1 antibody to either WT or HO-2^{-/-} mice resulted in a comparable

reduction of approximately 70% in the number of systemic neutrophils (Figure 2B). The H&E staining also confirmed previous studies [12,13] showing exaggerated neutrophil infiltration in response to epithelial injury in the HO-2^{-/-} cornea as compared to the WT corneas (Figure 2A). To further substantiate the nature of the cellular infiltrate, immunofluorescence analysis using the anti-mouse Ly-6G (Gr-1) antibody was performed. This antibody preparation is highly specific for neutrophils and have been used extensively to examine the role of neutrophils in innate immunity [17]. As seen in Figure 3A and B, Gr-1 positive staining was seen throughout the cornea; it was greater in the HO-2^{-/-} injured corneas as compared to WT corneas and was nearly absent in corneas of neutrophil-depleted mice of both genotypes. Although, within the time frame of the experiment (2 days post injury), neutrophils clearly constitute the majority (>90%) of the cellular infiltrate [4,20], additional immunofluorescence staining for CD68 (a macrophage-specific marker)-positive cells showed little if any positive signals in the 2-day post injury corneas of WT or HO-2 null mice (Figure 3B).

HO-1 expression in neutrophils

Neutrophils exhibited expression of both HO-1 and HO-2; however, HO-2 expression was absent in neutrophils from HO-2^{-/-} (data not shown). The residual neutrophils in the systemic circulation after injection of Gr-1 antibody displayed a 3-fold higher expression of HO-1 mRNA level in neutrophils purified from WT-depleted mice as compared to neutrophils from control WT mice (Figure 4). Interestingly, while neutrophils from control HO-2^{-/-} mice display similar HO-1 expression, the HO-1 expression failed to increase in the residual neutrophils from

neutrophil-depleted HO-2^{-/-} mice (Figure 4) in agreement with a previous study showing that HO-1 induction in the cornea as well as in stimulated peritoneal neutrophils is impaired in HO-2^{-/-} mice [13].

Neutrophil adhesion to HO-2 null endothelial cells.

Migration of neutrophils through the blood vessel wall is highly regulated by endothelial cells adhesion molecules. The role of HO-2 in neutrophil adhesion was examined using aortic endothelial cells lacking the HO-2 gene [18]. As seen in Figure 5, neutrophils adhere significantly more to HO-2^{-/-} endothelial cells compared to WT mAEC. Moreover, this adhesion was enhanced in the in mAEC treated with TNF- α . Interestingly, mAEC from HO-2^{-/-} mice showed a marked increase in the expression of Mdk, a 13-kDa multifunctional heparin-binding protein that promotes migration of neutrophils, macrophages, and neurons [21] (Figure 6A). The HO-2^{-/-} mAEC also expressed significantly higher levels of VE-cadherin (Figure 6B), a key junctional molecule for transendothelial migration of neutrophils [22].

When examining adherence of neutrophils from HO-2^{-/-} mice to either endothelial cells from WT or HO-2^{-/-} mice, we found no significant difference. Thus, neutrophil adhesion in relative fluorescent units (RFU) to WT and HO-2^{-/-} endothelial cells amounted to 157 \pm 7 and 137 \pm 11, respectively (n=6; p=0.14). The results are interesting and raise the possibility that the interactions between neutrophils and endothelial cells in the HO-2^{-/-} mice are compromised by mechanisms that need to be further explored.

DISCUSSION

This study is a follow-up on previous studies that revealed a functional relationship between expression of HO-2 and neutrophil recruitment after corneal injuries. HO-2 null mice experience an exaggerated inflammatory response after injury marked by a 4-fold increase in neutrophil infiltration in the corneal stroma, which might be fueling the exaggerated inflammation seen in these mice [12,13]. Infiltration of inflammatory cells into injured tissue is the hallmark of wound repair. Polymorphonuclear leukocytes, primarily neutrophils, are the first to migrate into the tissue in response to injury. Numerous studies in different types of ocular surface injury including epithelial abrasion, chemical burns and pathogenic insults have documented the presence of neutrophils within the corneal stroma as early as 6 h after injury [4,8,13,19,23]. These neutrophils transmigrate from the limbal vessels and through the stroma, presumably with the help of keratocytes, into the injured area [24]. The number of neutrophils in the injured cornea peaks 24-48 h after injury and begins decreasing, thereafter, as other cell types, such as macrophages begin to appear. Macrophages act in concert with neutrophils to phagocytose debris and invading pathogenic microorganisms and are a source of chemoattractants and growth factors that promote resolution of inflammation as well as cell migration and proliferation for wound closure and healing [10]. While infiltration of inflammatory cells is crucial for repair, their continuous influx and presence may be detrimental.

Our prior studies have determined that HO-2 is crucial for an ordered execution and, most importantly, resolution of acute inflammation that is essential for wound healing; in the absence of HO-2 activity, an acute inflammation becomes chronic with a continuous influx

of inflammatory cells leading to tissue destruction. We have defined a cytoprotective and anti-inflammatory role for HO in the cornea in a series of publications [12,13,25-27]. This HO function is seen clearly during wound healing after an epithelial injury. Using HO-2 null mice that also express little HO-1, thereby, abrogating all HO activity, a normally self-resolving epithelial injury becomes a massive non-resolving inflammatory defect with attenuated wound healing, ulceration and perforation. These corneas can be rescued by topical application of biliverdin/bilirubin or carbon monoxide CO, both products of HO metabolism of heme [12-14]. Biliverdin/bilirubin and CO serve as stop signals controlling leukocyte migration and activation by attenuating adhesion molecule expression and cytokine and chemokine induction [12,28-32]. Studies using models of epithelial injury [33-35] demonstrated spatial and temporal increases in HO-1 expression that correlated with leukocyte infiltration, keratinocyte proliferation, resolution and wound healing. In fact, HO-1 induction has been implicated as a switch for resolution [10]. The finding in this study together with previous studies demonstrating impaired HO-1 inducibility in the HO-2^{-/-} injured corneas and stimulated neutrophils [13] suggests that such impairment may contribute to increase neutrophil migration and activation, in sum, increasing tissue damage. The increased expression of Mdk, VE-cadherin and increased adherence of neutrophils to HO-2^{-/-} mAEC cells is consistent with increased neutrophil migration and activation.

It is well known that a healthy corneal wound healing requires the timely recruitment of neutrophils. Absence of neutrophil recruitment impairs healing of the cornea, while exaggerated neutrophil infiltration

coupled with impaired resolution leads to delayed and aberrant wound healing [7,13]. Smith and colleagues [4,24], using a model similar to ours, found that the main adhesion molecules involved in neutrophil activity during corneal inflammation are the CD18 integrin and the P and E-selectins. Re-epithelialization was significantly delayed in mice with deletion of these adhesion molecules and in mice made neutropenic. These results are consistent with ours: Neutrophil-depleted WT mice had a substantial delay in wound closure, as did the HO-2 null mice. The most profound delay was seen in HO-2 null mice that were also neutrophil-depleted. On the other hand, Ueno et al., [7] found accelerated wound closure in neutrophil-depleted mice. Their injury model was an alkali burn, which is a different injury from epithelial removal. Histological examination of our WT neutrophil-depleted corneas showed a complete absence of neutrophils as well as substantially delayed wound closure as compared to control WT corneas, which led us to conclude that neutrophil infiltration of the corneal stroma promotes epithelial, wound closure. Neutrophil-depleted WT mice corneas are also denied the benefit of infiltrating neutrophil-derived HO-1 and HO-2. This is further exaggerated in the HO-2^{-/-} mice in which HO-1 inducibility is impaired [13]. In all, this suggests that HO-2 deficiency in corneal cells rather than in inflammatory cells contributes to the exaggerated inflammation and impaired wound healing seen in the HO-2^{-/-} cornea. It also suggests that perturbation in endothelial cell function caused by HO-2 deletion underlies the increased infiltration of neutrophils into the HO-2^{-/-} cornea. Undoubtedly, additional experiments are needed to distinguish the role of neutrophils and corneal cells in this model.

Interestingly, we noticed from our current study and previous reports that in WT the majority of neutrophils were present adjacent to the leading edge of the wound whereas in the HO-2^{-/-} neutrophils are randomly distributed all through the stroma. This raises the possibility that HO-2 deletion impairs guidance of neutrophils to the injured area. It has been suggested that keratocyte network plays a prominent role in neutrophil migration by serving as a source of contact guidance and chemoattraction for migrating neutrophils [24,36,37]. Likewise, injured epithelial cells release signals that attract neutrophils [1]. The fact that neutropenia worsened epithelial healing of the injured cornea in both WT and HO-2 null mice suggest that cells other than neutrophils contribute to the exaggerated inflammation and impaired wound healing seen in the HO-2 null cornea [12-14]. Certainly, the role of HO-2 in epithelial and keratocyte cell function need to be further evaluated.

Endothelial cells lining the blood vessel wall are active participants in the inflammatory response and highly regulated endothelial-leukocyte interactions are in place so as to facilitate leukocyte entrance into the injured tissue. [38] Neutrophils infiltrating the injured tissue are derived from circulating leukocytes in the adjacent limbal vessels that have become transiently leaky in response to signals released by injured cells. Endothelial cell activation initiated at the time of injury is exemplified by increased release of cytokines and chemokines and by increased expression of adhesion molecules including P and E selectins, integrins and ICAM. Activated endothelial cells are “sticky” allowing circulating neutrophils to anchor and roll and ultimately enter the site of inflammation. In a recent study, we showed that vascular endothelial cells derived from HO-2^{-/-} mice are highly

activated; they display increased expression of cytokines and adhesion molecules [18]. Further analysis in the current study revealed that expression of the chemokine Mdk and the adhesion molecule VE-Cadherin are greatly increased in HO-2^{-/-} mAEC, both have been shown to regulate leukocyte-endothelial interactions. Different studies indicate that during an inflammatory process, its expression is increased; the response to the inflammatory stimuli was less severe in midkine knockout (Mdk^{-/-}) mice than in WT mice and the numbers of infiltrating neutrophils and also macrophages were lower in Mdk^{-/-} than in WT mice [39]. VE-Cadherin is a tight-junction protein specific to endothelial cells that is involved in the regulation of endothelium permeability and consequently to leukocyte-endothelial interactions [22,40,41]. It is possible that perturbation in endothelial cell function caused by HO-2 deletion underlies the increased infiltration of neutrophils into the HO-2^{-/-} cornea.

In summary, the HO-2 null mice showed an exaggerated corneal inflammation associated with an increased number of inflammatory cells compared to WT. However, systemic and corneal neutrophil depletion worsened rather than improved the wound healing process in both WT and HO-2^{-/-} mice, suggesting that the absence of HO-2 gene within corneal cells contributed to the impaired corneal healing in the HO-2 null mice. Increasing HO activity may be beneficial in the treatment of corneal inflammatory conditions such as the dry eye syndrome and in the treatment of non-healing corneal defects.

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Author Contributions

Substantial contribution to conception and design analysis and interpretation of data: MLS, GM, LB, AH. Drafting and revisiting the article for important intellectual content: MLS, MWD. Final approval of the version to be published: GLV, FD.

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FIGURES

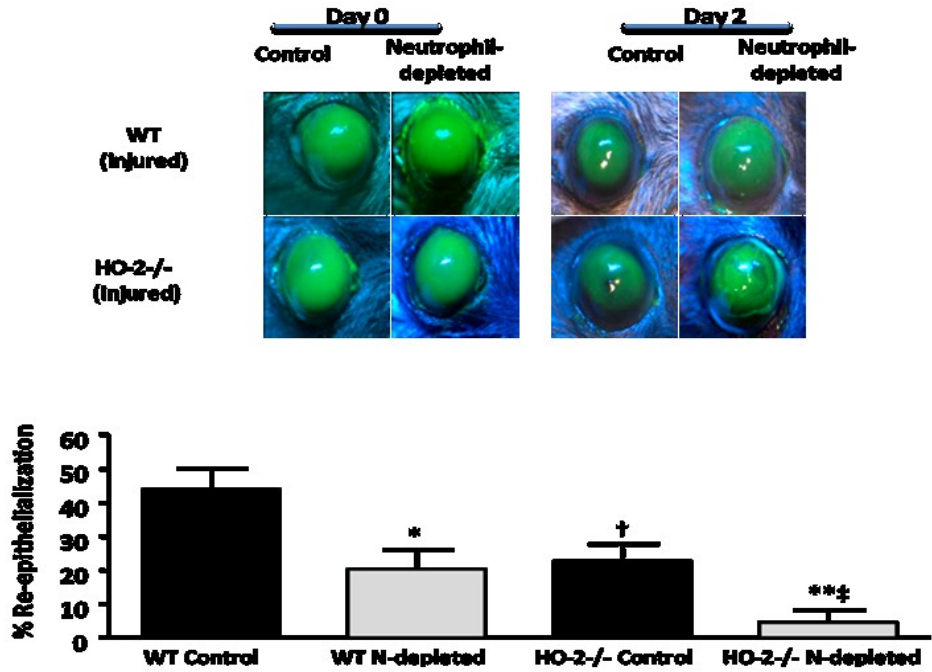


FIGURE 1. Effect of neutrophil depletion on wound healing. (A) Representative images of fluorescein-stained corneas at day 0 and day 2 after injury in control and neutrophil (N)-depleted WT and HO-2-/- mice. (B) Wound closure as percent change from day 0. Results are mean \pm SE; n= 5-7; *p < 0.005 from control WT mice; **p < 0.005 from control HO-2-/- mice; † p < 0.01 from control WT mice; ‡ p < 0.005 from neutrophil-depleted WT mice.

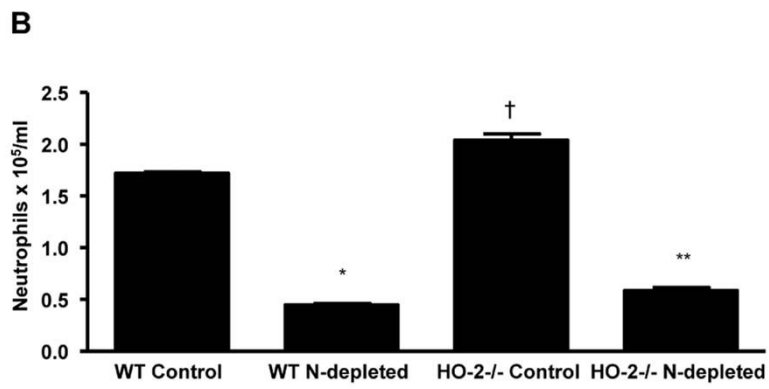
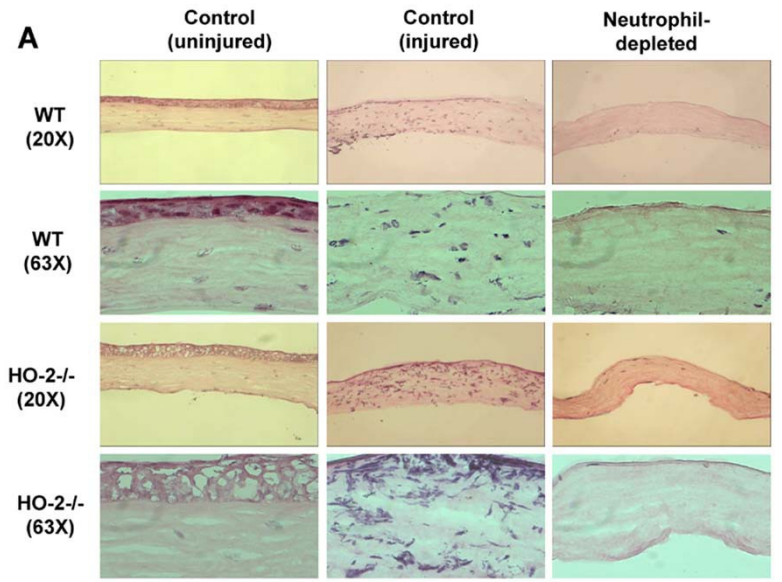


FIGURE 2. Infiltration of Inflammatory cells in the cornea at day 2 after epithelial injury. (A) Representative H&E staining of corneas from uninjured and injured control and neutrophil-depleted WT and HO-2^{-/-} mice 2 days after injury. (B) Number of neutrophils in peripheral blood of control and neutrophil (N)-depleted WT and HO-2^{-/-} mice 2 days after injury. Results are mean ± SE; n=3; *p<0.0001 from control WT; **p<0.0001 from control HO-2^{-/-} mice; † p<0.05 from control WT.

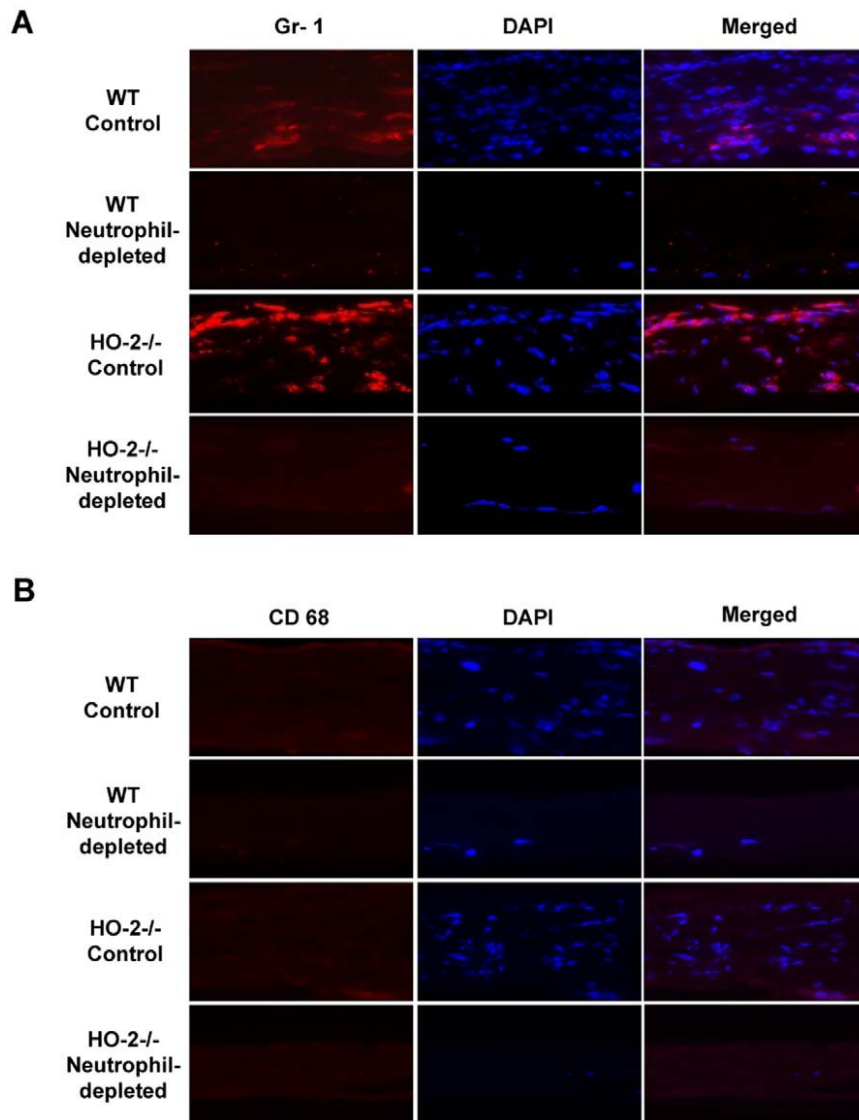


FIGURE 3. Immunofluorescence analysis of (A) Gr-1- and (B) CD68-positive cells in corneas from control and neutrophil-depleted WT and HO-2^{-/-} mice 2 days after injury. Images were taken at 63X magnification and are representative of three separate analyses.

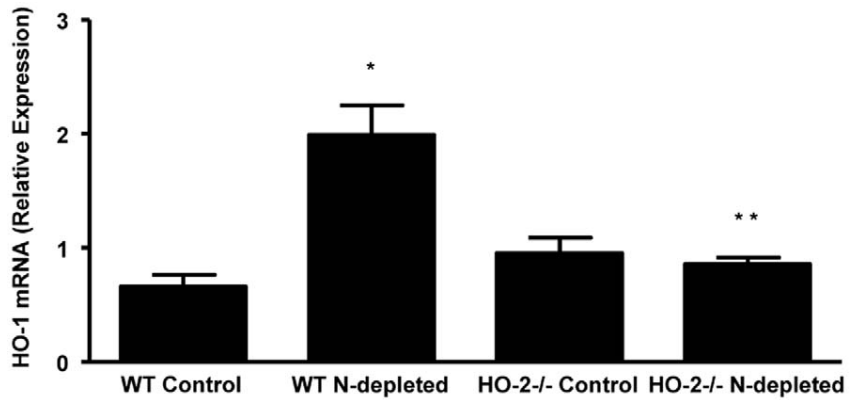


FIGURE 4. Real-time PCR analysis of corneal HO-1 mRNA expression in neutrophils purified from peripheral blood of control and neutrophil (N)-depleted WT and HO-2^{-/-} mice 2 days after injury. Results are the mean \pm SE; n = 3; *p < 0.01 from neutrophils purified from control WT mice; **p < 0.05 from neutrophils purified from neutrophil-depleted HO-2^{-/-} mice.

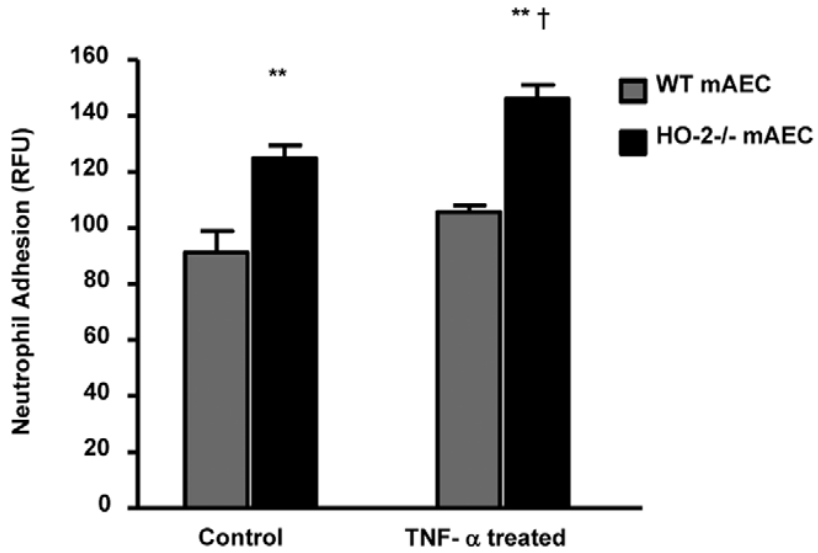
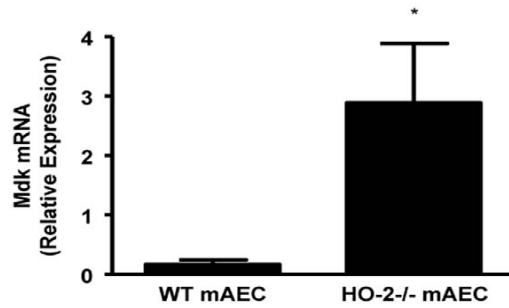


FIGURE 5. Neutrophil adhesion onto untreated (Control) and TNF α -treated WT and HO-2-/- aortic endothelial cells (mAEC). Neutrophil adherence is measured as the fluorescence intensity associated with mAEC and is given in relative fluorescence units (RFU). Results are the mean \pm SE; n = 3; **p < 0.005 from WT mAEC; †p < 0.05 from untreated HO-2-/- mAEC.

A



B

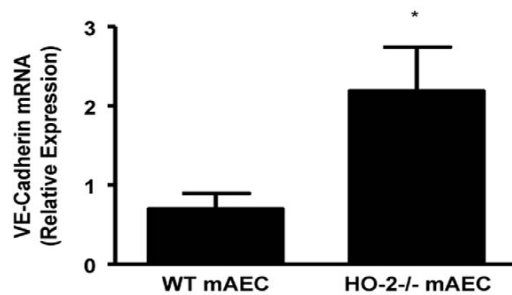


FIGURE 6. Real-time PCR analysis of mRNA expression of (A) midkine (Mdk) and (B) VE-Cadherin in untreated aortic endothelial cells (mAEC) from WT and HO-2-/- mice. Results are the mean \pm SE; n = 4 to 5; *p < 0.005 from WT mAEC.

GENERAL DISCUSSION

Inflammation is a vital physiologic response to injury in living tissues and, when tightly controlled, contributes to normal healing and repair; it enables and crucially drives tissue repair by stimulation of resident cells to migrate and proliferate into the wound site, but severely disturbs wound healing processes when it is prolonged or overly exaggerated.

Hence, an ordered execution and resolution of inflammation is an essential step toward wound healing; it requires balanced and defined formation of pro- as well as anti-inflammatory signals which orchestrate a complex and well controlled biologic and biochemical process involving tissue and immune system cells.

Dr. Schwartzman's group was the first to demonstrate that HO-2 is a key anti-inflammatory and cytoprotective protein in the cornea and showed that its deletion impairs corneal inflammatory and repair response (Seta *et al.* 2006; Halilovic *et al.* 2010; Bellner *et al.* 2011a; Marrazzo *et al.* 2011); HO-2-deficient corneas respond to injury with exaggerated inflammation and lack of resolution leading to impaired wound healing, perforation, ulceration and neovascularization (Seta *et al.* 2006).

My studies were designed to elucidate the specific pathway and the consequences of suppression of HO-2 to the inflammatory and repair response of the cornea after epithelial removal. I used different kind of approaches to evaluate our hypothesis. I assessed the percentage of regrowth after scratch assay using an *in vitro* model of human corneal epithelial cells. Using specific siRNA treatment I was been able to knockdown the HO-2 protein expression (Halilovic *et al.* 2010). These

set of experiments confirmed that a reduction in HO-2 resulted in a significant decrease of the percentage of cell regrowth compared to the control cells.

On the other hand I showed that consistently with previous reports obtained with the HO-2 null mice (Seta *et al.* 2006), in situ suppression of HO-2 in WT mice was associated with attenuation of corneal wound healing. Thus, wound closure was completed by day 7 after injury in corneas treated with nonspecific shRNA, whereas, wound healing was significantly impaired in HO-2 specific shRNA-treated corneas showing a closure of the wound of only about 70% at day 7 after injury. In Dr Schwartzman's *in vivo* model we also found that the impaired healing was associated with increased corneal neovascularization (Seta *et al.* 2006;Bellner *et al.* 2008;Bellner *et al.* 2011a). Take all together, our data suggested that impaired healing and increased neovascularization in response to epithelial injury were similar both when HO-2 expression was inhibited locally (shRNA against HO-2) or depleted globally (knock out mice for HO-2).

It is recognized that leukocytes are not only capable of amplifying the inflammatory response, but are also significantly involved in the repair process. Gan *et al.* (Gan *et al.* 1999) showed in two rabbit models of corneal injury (PRK and a standardized alkali corneal wound) that corneal epithelial healing rate is delayed in the absence of PMNs *in vivo* and PCNA expression, a marker for cell proliferation, increases in the presence of leukocytes. Other studies showed that limiting neutrophil infiltration to the cornea attenuated the inflammatory response and accelerated wound closure (Ma and Martins-Green 2009;Ueno *et al.* 2005), whereas, exaggerated influx is linked to impaired resolution and

repair and chronic inflammation as observed in the HO-2 null mice (Seta *et al.* 2006;Bellner *et al.* 2008;Bellner *et al.* 2011a).

Under our experimental conditions, in accordance with our previous studies, I confirmed that HO-2 null mice experienced an exaggerated inflammatory response after injury characterized by a 4-fold increase in neutrophil infiltration in the corneal stroma, which might be fueling the exaggerated inflammation seen in these mice (Seta *et al.* 2006). My data (Marrazzo *et al.* 2011) suggested that such impairment may contribute to increase neutrophil migration and activation and thus leading to increased tissue damage. The increased expression of Mdk, VE-cadherin and increased adherence of neutrophils to HO-2^{-/-} mouse aortic endothelial cells is consistent with increased neutrophil migration and activation. These data suggest that HO-2 deficiency in corneal cells, rather than in inflammatory cells, contributes to the exaggerated inflammation and impaired wound healing seen in the HO-2^{-/-} cornea. Furthermore, perturbation of endothelial cell function caused by HO-2 deletion underlies the increased infiltration of neutrophils into the HO-2^{-/-} cornea (Marrazzo *et al.* 2011). In summary, the HO-2 null mice showed an exaggerated corneal inflammation associated with an increased number of inflammatory cells compared to WT. However, systemic and corneal neutrophil depletion worsened rather than improved the wound healing process in both WT and HO-2^{-/-} mice, suggesting that the absence of HO-2 gene within corneal cells contributed to the impaired corneal healing in the HO-2 null mice.

We have also previously shown that HO-2 expression is abundantly expressed in the corneal epithelium, which is likely the largest source of HO activity in the uninjured cornea. During injury, however,

inflammatory cells invading the cornea induce HO activity; the majority of this activity appears to be driven by the inducible HO-1 (Willis *et al.* 2000). Seta *et al.* (2006) showed that induction of HO-1 is impaired in the absence of HO-2. The blunted HO-1 induction in the cornea in response to injury in HO-2 null mice was also observed in corneas in which HO-2 was locally suppressed. The nature of the regulatory interactions between these two isoforms remains unknown. However, in view of reports showing that HO-1 expression and activity are maximal during the resolution phase and that induction of HO-1 expression promotes resolution whereas inhibition of HO activity is pro-inflammatory (Patil *et al.* 2008; Bellner *et al.* 2009), it is reasonable to assume that this blunted response may contribute to the diminished defense against injury and consequently to the exaggerated inflammatory response in the HO-2 knock out eyes.

Furthermore, Schwartzman *et al.* found that epithelial injury was also associated with a distinct increase in MMP-2 and MMP-9 expression. The expression of both MMP-2 and MMP-9 has been shown to increase in response to injury and contribute to epithelial wound repair (Matsubara *et al.* 1991; Sivak and Fini 2002). On the other hand, studies with MMP-2 null mice demonstrated decreased corneal neovascularization in response to removal of the corneal epithelium (Azar 2006). Bellner *et al.* (Bellner *et al.* 2011b) showed that lingering and elevated levels of MMP-2 mRNA in response to epithelial debridement were significantly lowered in corneas from HO-2 null mice treated with biliverdin. To further elucidate the mechanisms underlying such response, we performed a full-scale gene microarray comparing WT and HO-2 null corneas; preliminary data further demonstrated a close relationship between HO-2 and MMP-2 expression. In particular,

deletion of the HO-2 gene was associated with a six-fold increase in MMP-2 expression (Bellner, unpublished data). The nature of this relationship with regard to corneal response to injury needs to be further explored.

It has become clear that a functioning HO system is crucial for the anti-inflammatory circuits in the cornea, and that enhancement through treatment with stannous chloride of wild type mice (Patil *et al.* 2008), or partial replenishment in the shape of one of its metabolic products, biliverdin or CO (Bellner *et al.* 2008; Bellner *et al.* 2011b).

In summary these findings showed the key role played by the HO system in corneal wound healing as anti-inflammatory and cytoprotective system. Further, considering the findings by Dr. Chen *et al.* (2003) on olfactory receptor neurons (ORNs), showing that heme oxygenase-1 and heme oxygenase-2 have distinct roles in the proliferation and neuronal development/survival of ORNs mediated by cGMP and bilirubin, respectively. I believe that increasing HO activity may be beneficial in the treatment of corneal inflammatory conditions such as the dry eye syndrome, corneal pain and in the treatment of non-healing corneal defects. Undoubtedly further studies need to be made to investigate the function of the HO system on the regeneration of corneal nerve system.

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Giuseppina Marrazzo's publications during the PhD program:

1) The Role of Neutrophils in Corneal Wound Healing in HO-2 Null Mice.

Giuseppina Marrazzo, Lars Bellner, Adna Halilovic, Giovanni Li Volti, Filippo Drago, Michael W. Dunn, Michal Laniado-Schwartzman

PLoS ONE 6(6): e21180. doi:10.1371/journal.pone.0021180

2) Knockdown of heme oxygenase-2 impairs corneal epithelial cell wound healing.

Halilovic A, Patil KA, Bellner L, **Marrazzo G**, Castellano K, Cullaro G, Dunn MW, Schwartzman ML.

J Cell Physiol. 2011 Jul;226(7):1732-40. doi: 10.1002/jcp.22502.

3) Neuroprotective effect of silibinin in diabetic mice.

Marrazzo G, Bosco P, La Delia F, Scapagnini G, Di Giacomo C, Malaguarnera M, Galvano F, Nicolosi A, Li Volti G.

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