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Dysregulated Heme Oxygenase-Ferritin System in Pterygium Pathogenesis

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Abstract

Purpose—Cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 monooxygenases (CYP) derived eicosanoids have been implicated in ocular surface inflammation and neovascularization. These eicosanoids are subjected to regulation by enzymes such as heme oxygenases (HO) and ferritin.

Methods—Quantitative PCR and LC-MS/MS based lipidomics were performed on pterygia from patients undergoing surgical pterygium excision. Control tissues consisted of donor corneas. In addition, LC-MS/MS-based lipidomics was performed on tears collected from patients prior to surgery.

Results—mRNA-expression of HO-2, the constitutive HO isoform, was upregulated by 40 % in pterygia as compared to control tissue, while the mRNA level of the inducible form, HO-1, was downregulated by more than 50 %. Levels of CYP4B1 mRNA showed an approximate 2-fold increase in pterygia compared to control. Lipidomics analysis of tissues indicated a doubling in PGE₂ and TxB₂ levels in pterygia as compared to control. Among LOX-derived metabolites, the anti-inflammatory 15-HETE levels were significantly reduced in pterygia (79.3±48.11 pg/mg protein) as compared to control (586.2±213.5 pg/mg protein), whereas pro-inflammatory LOX- and CYP4B1-derived 12-HETE levels were 10-fold higher in pterygia (2768±832.3 pg/mg protein) compared to control (231.4±87.35 pg/mg protein). PGE₂ and the HETEs were also present in tears from patients with a pterygium, but were not detected in tears from healthy volunteers. Ferritin light and heavy chain mRNA expression levels were 60 % and 30 % lower in pterygia as compared to control.

Conclusion—We believe a dysfunctional HO-Ferritin system leads to increased levels of proinflammatory mediators, thus contributing to the inflammation characteristic of pterygia.

Keywords

Pterygium; eicosanoids; heme oxygenase; inflammation; ferritin

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INTRODUCTION

The pathogenesis of the pterygium continues to be poorly understood, in part, because of the absence of an animal model. Pterygia are prone to become inflamed; controlling inflammation is especially important during and after surgical excision¹. If inflammation is not controlled, recurrence rates of up to 80% have been reported². Current conjunctival graft techniques have greatly reduced recurrence rate as compared to classical techniques without conjunctival grafting³.

Heme oxygenases (HO) catalyze the rate limiting step in heme metabolism, rendering equimolar amounts of carbon monoxide, iron and biliverdin⁴. Biliverdin is subsequently converted by biliverdin reductase to bilirubin, a potent endogenous antioxidant. Iron has to be rapidly sequestered by ferritin in order to avoid oxidative damage to proteins, lipids and DNA⁵⁻⁷. In humans, there are two isoforms of HO, named HO-1 and HO-2⁴: whereas HO-1 is an inducible form of the enzyme and is readily upregulated in response to injurious insults, including ultraviolet irradiation^{4,8}, HO-2 is constitutively expressed and, unlike HO-1, has two additional binding sites for heme, thereby making HO-2 important in the regulation of intracellular heme levels⁹. Despite their differences, both HO-1 and HO-2 are major players in cellular homeostasis due to their cytoprotective, anti-inflammatory, and immunomodulatory properties.

The HO system has been studied extensively in mouse and rabbit corneal epithelium and it has become clear from these studies, and studies performed on human corneal epithelial cells, that HO is critical to the maintenance of orderly wound healing and control of inflammation following cornea surface injury¹⁰⁻¹⁸. HO activity in human corneal epithelium is up to 20 % of that of liver¹⁹. HO has been shown to regulate the levels of various arachidonic acid-derived eicosanoids of the cyclooxygenase, lipoxygenase and cytochrome P450 monooxygenase (CYP) pathways^{4,20,21}. These lipid mediators participate in the initiation, progression and resolution of inflammation as well as in the regulation of the neovascular response of the chronically inflamed cornea. Upregulation of HO-1 and increased HO activity suppress cyclooxygenase (COX)-2-derived inflammatory eicosanoid such as prostaglandin E2 (PGE₂)²² and induce arachidonate 15-lipoxygenase (ALOX15)-derived anti-inflammatory and protective lipid signals such as 15-hydroxyeicosatetraenoic acid (HETE), lipoxins and resolvins.

Previous work exploring the presence of inflammatory and angiogenic molecules in pterygium has focused primarily on bioactive proteins and has ignored the contributions of lipid mediators. Although eicosanoids released from infiltrating cells can amplify the inflammatory response, their ability to be produced endogenously from the injured tissue renders them able to initiate the inflammatory response by altering vascular permeability and stimulating leukocyte chemotaxis. Prominent proinflammatory and angiogenic eicosanoids include the COX-derived prostaglandins (PGE₂ and TxB₂), the lipoxygenase (LOX)-derived leukotrienes, and the CYP-derived 12-(R)-hydroxyeicosatrienoic acid (12-HETE)²³⁻²⁵. Among the anti-inflammatory eicosanoids are the LOX-derived lipoxins²⁶ and the CYP-derived epoxyeicosatrienoic acids (EETs)²⁷. The role of eicosanoids in the pathogenesis of pterygium is largely unknown. Here, we present a limited genomic and lipidomic analysis in pterygia to explore a possible linkage between the HO system, eicosanoids and pterygia.

MATERIALS AND METHODS

Specimen

Thirty-one pterygia were obtained from a total of 30 patients undergoing pterygium excision. Medical history, age, and gender could be obtained for 28 of 30 patients. The mean age $\pm$ SD of those 28 patients (14 females, 14 males) was 54 ± 14 . Five patients were being treated for diabetes and/or hypertension at the time of pterygium excision. Twenty-seven of the 31 collected pterygia were primary and the remaining four recurrent. Control tissues consist of sclera, conjunctiva, cornea and limbus from 17 donor corneo-scleral grafts (mean age $\pm$ SD: 40 ± 21). Collected tissues were kept on ice for less than an hour for pickup and transport to the laboratory and then snap frozen in liquid nitrogen and stored at -80°C . Tears were collected from patients with pterygia at the time of pre-surgical examination and from healthy volunteers (mean age $\pm$ SD: 35 ± 17). Tears were immediately transferred to 100% methanol and stored at -80°C . Tear and tissue samples were routinely recovered according to the guidelines set down by the Declaration of Helsinki and the institutional review board.

Lipidomics

Extraction—For a detailed protocol please see supplement. Briefly, tissues were homogenized in ice-cold distilled HPLC-grade water/100 to which was added 1 ml ice-cold methanol and internal standards. Samples were then placed at -80°C for at least 30 minutes where after they were centrifuged at 10,000g for 10 minutes at 4°C and supernatants collected. The pellet was saved and used for protein determination using the Bradford Protein Assay. Solid-Phase lipid extraction was performed using C18-ODS AccuBond II 500-mg columns (Agilent Technologies, Santa Clara, CA) as described¹⁷, or as described in the supplemental Materials and Methods.

Analysis—For a detailed protocol please see supplement. Briefly, eicosanoid identification and quantification was carried out using a Q-trap 3200 linear ion trap quadrupole liquid chromatography-tandem mass spectrometry (LC/MS/MS) equipped with a Turbo V ion source operated in negative electrospray mode (AB Sciex, Framingham, MA) as described in the supplemental Materials and Methods. Synthetic standards (Cayman Chemical) were used to obtain standard curves (5–500 pg) for each eicosanoid (linear regression R^2 values > 0.99) and internal standard. The given amounts in each sample were corrected for loss during extraction and normalized to protein content (tissue samples) or given as total amount (tears). MRM ion pairs for standards and internal standard are summarized in Supplemental Table 1.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from pterygia and control tissues using the RNeasy® Plus Micro Kit (Qiagen, Germantown, MD) and RNA was quantitated using a Take3 plate and a Synergy HT spectrophotometer (Biotek, Winooski, VT). Reverse transcription reaction of total RNA was performed using the qScript™ cDNA Synthesis Kit (Quanta BioSciences, Inc., Gaithersburg, MD) according to the manufacturer's instruction. Quantitative real-time PCR was performed using PerfeCTa™ SYBR® Green FastMix™ Kit (Quanta BioSciences, Inc.) and the Mx3000 real-time PCR system (Stratagene, La Jolla, CA). Specific primers, purchased from Gene Link™ (Hawthorne, NY), were designed using Primerbank (<http://pga.mgh.harvard.edu/primerbank>) based on published sequences (GenBank) and were as shown in Supplemental Table 2. Quantitative analysis was performed as previously described^{11, 18}.

Statistical Analysis

Student's t-test was used to evaluate the significance of differences between groups. P values less than 0.05 were considered significant. All data are presented as mean $\pm$ SE.

RESULTS

Aberrant expression of the heme oxygenase system in pterygium

Analysis of mRNA levels of the HO system in pterygium and control tissues showed that HO-2 expression was significantly elevated in pterygium tissue (Figure 1B), whereas expression of HO-1 was significantly lowered (Figure 1A). Induction of the heme oxygenase system is normally accompanied by an increase in ferritin levels in order to remove excess of free iron as a result of heme oxygenase activity^{28,29}. Surprisingly, we found that the mRNA levels of both light and heavy chain ferritin were reduced by 60% and 30%, respectively, in pterygium as compared to control tissue (Figure 1C & D). The Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant response pathway is a major cellular defense against the cytotoxic effects of oxidative stress, regulating the gene expression of both HO-1 and Ferritin. Nrf2 mRNA levels were significantly lower in pterygium as compared to control (Figure 1E).

Impaired expression of the HO system correlates with amplification of inflammatory lipid mediators

Induction of HO activity directly impacts functional expression of heme-containing enzymes. In this regard, COX-2 and CYP4B1 are of special interest because these heme-containing enzymes generate well established inflammatory and angiogenic markers of inflammation, especially in the cornea^{30,31}. The activity of both these enzymes is regulated by HO^{22,32}. Analysis of CYP4B1 mRNA, a major enzymatic source for the inflammatory and angiogenic 12-HETrE in the cornea³³, showed a significant increase in pterygium as compared to control (Figure 1F). Analyses of pterygia and control tissue by immunofluorescence showed increased COX-2 protein levels in pterygium as compared to control tissue (data not shown) confirming studies by others^{34,35}. The COX-derived PGE₂ and TxB₂ were both moderately elevated in pterygium as compared to control (Figure 2A & B). The lipid profile also revealed significantly elevated levels of 12-HETE, a prominent proangiogenic lipid mediator and precursor for 12-HETrE, in pterygia as compared to control tissues (Figure 2D). Levels of 5-HETE were unaltered (Figure 2C) while the levels of the anti-inflammatory 15-HETE and EETs^{26,27} were lower in pterygia as compared to control (Figure 2E & F).

Elevated levels of proinflammatory lipid mediators in tears from pterygium patients

In addition to lipids in pterygial tissue, we also analyzed the levels of lipid mediators in tears collected from patients with pterygium and healthy volunteers. The pro-inflammatory lipid mediators 5-, and 12-HETE and PGE₂ were all present in tears from pterygium patients, but were non-detectable in tears collected from healthy individuals (Figure 3A through C). The anti-inflammatory 15-HETE was also present in tears from pterygium patients, but not detectable in tears from healthy volunteers (Figure 3D).

DISCUSSION

The inflammatory response and the process of wound healing in pterygia are clearly disordered. This is evident in the expression of HO-1 and HO-2 mRNA. In normal corneal epithelium, HO-1 mRNA is expressed at very low levels. Oxidative stress, heavy metals and almost any cellular stressor rapidly induces high levels of HO-1 mRNA expression. On the other hand, HO-2 is constitutively expressed and is not induced by factors that induce HO-1

expression; its expression is believed to assist in creating a basal tone of anti-inflammatory signals ^{4, 18}. A study by Synowiec et al., show a correlation between age-related macular degeneration and polymorphisms of the heme oxygenase -1 and -2 genes, clearly indicating that a fully functional heme oxygenase system is crucial for long term eye health ³⁶. We observed in pterygia that expression of HO-1 is significantly reduced compared to the control tissue despite the presence of several known HO-1 inducing cytokines, chemokines, growth factors ^{37, 38} and evidence of damage from oxidative stress ^{39, 40}. HO-1, apparently, is not inducible in pterygia, thus, depriving the pterygium of a critical component of the inflammatory response. It has been postulated that HO-1 serves as a stop signal that initiates the resolution phase of acute inflammation: when this activity is not present, an acute inflammation becomes chronic ^{41, 42}, very similar to the situation seen in pterygia.

In normal corneal epithelium, HO-2 is abundant and is by large constitutively expressed. Studies from our laboratory using animal models provided ample evidence that HO-2 plays a critical role in the execution of an ordered inflammatory and repair processes in response to ocular surface injury. The mechanisms underlying this protective role is believed to be at least in part due to HO-2 ability to degrade heme and produce the anti-inflammatory and anti-oxidative agents, biliverdin/bilirubin and carbon monoxide ⁴. In pterygia, however, HO-2 mRNA levels are 40 % in excess of the corneal/conjunctival control. What emerges from our data on HO expression is a condition marked by high levels of constitutively expressed HO-2 associated with an inability to induce expression of a critical component of the inflammatory response in pterygia, HO-1. In fact, studies have shown that HO-2 induction in response to chronic sunlight-exposure of human dermis ⁴³, or UV exposure of keratinocytes ⁸ prevented HO-1 induction by UV radiation. In contrast, downregulation of HO-2 in numerous human cell lines led to a compensatory upregulation of HO-1, whereas HO-1 up- or downregulation had little to no effect on HO-2 expression ⁴⁴.

UV-A and UV-B exposure is associated with photochemical damage to cells. This damage includes lipid peroxidation, reduced mitochondrial viability, and direct damage of DNA. UV-A radiation leads to an immediate measurable increase in free iron in human skin fibroblast and keratinocyte cytoplasm through proteolysis of ferritin, and at the same time causing a reduction of iron-regulatory protein 1 (IRP-1) binding activity ⁴⁵. Subsequently, released iron is free to catalyze the formation of UV-induced ROS via the Fenton reaction ^{5, 45}. The intracellular free iron concentration is normally tightly regulated and kept at a low level, chiefly by the iron-sequestering action of ferritin. Once thought to be limited to the cytoplasm, ferritin has been found in nuclei of avian corneal epithelium perhaps serving as a defense against UV-induced free radical damage to DNA ^{6, 46}. Ferritin mRNA levels were 3-fold lower in pterygia than in controls suggesting a role in the pro-oxidant and inflammatory status of the pterygium tissue. Many cytoprotective genes, including HO-1, ferritin heavy and light chains, superoxide dismutase, glutathione peroxidase (GPX) and catalase are under the control of the transcription factor NRF2, which is a major regulator of cytoprotection during oxidative stress. Our results showed a downregulation of NRF2 mRNA in pterygium tissue that likely is the reason why HO-1 and ferritin levels are downregulated as well. These data are supported by a study by Balci et al, showing that the enzymatic activity of SOD, catalase and GPX-2 is downregulated in pterygium ⁴⁷. The low level of intracellular ferritin might also provide insight into the formation of the enigmatic Stocker's Line, an iron deposit sometimes seen in the corneal epithelium at the head of a pterygium. The traditional theory is that this iron comes from iron bound to lactoferrin in the tear film which, when released, enters the epithelium. This theory does not identify the source of the iron found in the hundreds of heme containing proteins in every epithelial cell. Due to intense and chronic HO activity and low ferritin levels, cytotoxic iron could accumulate in pterygial cells catalyzing the production of the hydroxyl radical. To avoid

this, iron is extruded to adjacent epithelial cells that have normal levels of ferritin, eventually, forming Stocker's line.

The present study is the first to perform a comparison of arachidonic acid lipid metabolites in tears and tissues of patients with pterygia and from healthy donors. This comparison yielded a profile that indicates a shift in favor of pro-inflammatory over anti-inflammatory lipid autacoids in pterygia. The most abundant pro-inflammatory eicosanoid was the LOX-derived 12-HETE a potent proinflammatory and proangiogenic lipid mediator²⁴, whereas the levels of the LOX-derived anti-inflammatory 15-HETE²⁶ were lower in pterygia. We have previously shown that CYP4B1, the major source of the pro-inflammatory and pro-angiogenic 12-HETrE in the eye⁴⁸ is regulated by HO activity²¹. In the pterygium where the HO regulatory system is impaired, the CYP4B1 mRNA levels were 2-fold higher than in control, reflecting the amount of angiogenesis often seen in the diseased tissue. The pro-inflammatory COX-derived metabolites PGE₂ and TxB₂ were elevated in pterygia as compared to control and confirm previous studies showing that COX-2 protein levels are upregulated in pterygium^{34, 35}. Comparing the lipid profile from individual pterygium tissue samples, a pattern emerges: one cluster without and one cluster with pro-inflammatory lipids. We did not distinguish between inflamed and non-inflamed pterygia, but one plausible explanation is the reason for excision, being for cosmetic reasons on one hand, and for inflammatory/irritating reasons on the other. Figure 4 depicts our working hypothesis in which UV exposure leads to an impaired HO-1-ferritin system, which in turn causes upregulation of pro-inflammatory and pro-oxidative pathways and consequently inflammation, neovascularization and neoplastic growth.

Previous studies from this laboratory provide some direction towards controlling inflammation in pterygia which is key to pterygial management especially in the postoperative phase^{12-14, 18}. Removing the corneal epithelium in a wild type mouse creates an inflammation that resolves without complications as the wound heals in a few days. The picture is much different in the HO-2 knockout, a mouse with deficient HO-1 and no HO-2 activity¹⁸. These mice, in the absence of any HO-2 activity, display an exaggerated inflammatory response: wound healing stops, ulcerations form and perforations destroy the cornea¹⁸. Topical application of the HO metabolic product biliverdin, however, restores the HO phenotype mitigating the inflammation, restoring healing and preventing perforation¹³. The HO-2 knockout cornea resembles the pterygium in that wound healing and the inflammatory response are deranged. Other therapeutic possibilities include induction of HO-1 which, besides its anti-inflammatory properties, is also a signal that initiates the resolution of inflammation and the use of agents that promote resolution of inflammation such as the 17-resolvins⁴⁹. Inducing expression of Nrf2 in order to increase expression and activity of anti-inflammatory genes, among them HO-1 and ferritin is another therapy to be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. mRNA expression of select genes in pterygium and control tissue. (A) Heme oxygenase (HO)-1 mRNA, (B) HO-2 mRNA, (C) Ferritin light chain (Ftl) mRNA, (D) Ferritin heavy chain (Fth) mRNA, (E) Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) mRNA, and (F) cytochrome P450 monooxygenase 4B1 (Cyp4B1) mRNA. (n=15–16; *p<0.05; **p<0.01; ***p<0.001 from control tissue).

Figure 2. Lipid profile in pterygium and control tissue. (A) Prostaglandin E₂ (PGE₂), (B) Thromboxane B₂ (TxB₂), (C) 5-hydroxyeicosatetraenoic acid (5-HETE), (D) 12-HETE, (E) 15-HETE, and (F) total epoxyeicosatrienoic acids (EET)s. (n=12–14, *p<0.05; **p<0.01 from control tissue).

Figure 3.

Lipid profile in tears collected from pterygium patients and healthy volunteers. (A) Prostaglandin E₂ (PGE₂), (B) 5-hydroxyeicosatetraenoic acid (5-HETE), (C) 12-HETE, (D) 15-HETE. (n=6–7, *p<0.05 from control).

Figure 4.

Proposed effects of chronic UV-exposure on the heme oxygenase-ferritin cytoprotective system. Cyclooxygenase 2 (COX-2), cytochrome P450 monooxygenase 4B1 (CYP4B1), Heme oxygenase (HO), 12-hydroxyeicosatetraenoic acid (12-HETE), 12-hydroxytrienoic acid (12-HETrE), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), prostaglandin E₂ (PGE₂), thromboxane B₂ (TxB₂).