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Inhibition of VEGF Expression and Corneal Neovascularization by siRNA Targeting Cytochrome P450 4B1

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Abstract

Injury to the cornea leads to formation of mediators that initiate and amplify inflammatory responses and neovascularization. Among these are lipid mediators generated by a cytochrome P450 (CYP) enzyme identified as CYP4B1. Increased corneal CYP4B1 expression increases limbal angiogenic activity through the production of 12-hydroxyeicosatrienoic acid (12-HETrE), a potent inflammatory and angiogenic eicosanoid. We used siRNA duplexes targeting CYP4B1 to substantiate the link between CYP4B1 expression, 12-HETrE production and angiogenesis in a model of suture-induced corneal neovascularization. Intrastromal sutures induced a time-dependent neovascular response which was significantly attenuated by CYP4B1-specific siRNAs but not by nonspecific siRNA. CYP4B1 mRNA was reduced by 60% and 12-HETrE's levels were barely detected in corneal homogenates from eyes treated with the CYP4B1-specific siRNA. The decreased neovascular response in CYP4B1 siRNA-treated eyes was associated with a 75% reduction in corneal VEGF mRNA levels. Transfection of rabbit corneal epithelial cells with CYP4B1 cDNA induced VEGF expression. Conversely, treatment with CYP4B1 siRNA or addition of a CYP4B1 inhibitor significantly decreased VEGF mRNA levels; addition of 12-HETrE potently increased them. The results strongly implicate the corneal CYP4B1 as a component of the inflammatory and neovascular cascade initiated by injury and further suggest that CYP4B1-12-HETrE is a proximal regulator of VEGF expression.

INTRODUCTION

Neovascularization of the normally avascular cornea is seen in many pathological conditions which include infection, mechanical and chemical injury, chronic exposure to hypoxia and following corneal transplantation. Corneal neovascularization, whatever the cause, leads to decreased vision, recurrent corneal erosion and incompetent barrier function, thus presenting a serious clinical problem for which treatment is poor. The molecular mechanisms that control corneal neovascularization are not fully understood. Since inflammation frequently precedes corneal neovascularization, many studies have concentrated on the role of humoral and corneal-derived inflammatory mediators in the regulation of this process. Among these mediators are arachidonic acid-derived eicosanoids of the cytochrome P450 monooxygenase (CYP) pathways.

Previous studies have identified a CYP-dependent arachidonic acid metabolism in the corneal epithelium and established it as a primary corneal epithelial inflammatory pathway in

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experimental models of ocular surface inflammation.¹ This corneal epithelial CYP metabolizes arachidonic acid to the potent inflammatory and angiogenic 12-hydroxyeicosatrienoic acid (12-HETrE). 12-HETrE is a critical corneal-derived mediator of ocular surface inflammation based on studies demonstrating that: 1) its synthesis in the corneal epithelium and primarily that of the bioactive R enantiomer increases following hypoxic or chemical injury *in vitro* and *in vivo*; 2-4 2) its levels in the cornea positively correlate with the *in situ* inflammatory and neovascular response;^{5,6} and 3) its biological activities, in particular those of 12(R)-HETrE, *in vitro* and *in vivo*, are characteristic of potent inflammatory mediators (including vasodilation,⁷ neutrophil chemotaxis,⁸ and angiogenesis⁹). The importance of 12-HETrE to human pathophysiology is underscored by its presence in human tears, and more significantly, by the fact that its levels are much higher in tears from subjects with ocular inflammation.¹⁰

We have isolated a 1.82 kb CYP cDNA from hypoxic rabbit corneal epithelium with a 98% sequence homology to the lung CYP4B1.^{11,12} The expression of the CYP4B1 in rabbit corneal organ cultures was induced by hypoxia and its increased expression was associated with increased production of 12-HETrE.¹¹ Further studies demonstrating that antibodies against CYP4B1 inhibited hypoxia-induced 12-HETrE synthesis¹¹ provided substantial evidence that in the corneal epithelium CYP4B1 is involved in the hypoxia-induced 12-HETrE synthesis and ocular surface inflammation. Indeed, increased expression of CYP4B1 mRNA in the corneal epithelium during hypoxic injury *in vivo* corresponded well with the progression of the anterior surface inflammatory response, including corneal thickness and inflammatory score, as well as with the synthesis of 12-HETrE.¹³ In mice experiencing exaggerated and unresolved inflammation, corneal CYP4B1 expression is highly elevated and so are the corneal levels of 12-HETrE.¹⁴ In a recent study, we provided substantial evidence that the CYP4B1 is the enzymatic source for the production of 12-HETrE in the cornea. Moreover, transfection of CYP4B1 into the cornea yielded an inflammatory response in the ocular surface and stimulated angiogenic activity of limbal explants that was mediated by increased production of 12-HETrE.¹⁵

The current study was undertaken to further substantiate the cause and effect relationship between CYP4B1 expression, 12-HETrE production and neovascularization in the cornea. We used small interfering RNAs (siRNA) targeting CYP4B1 in a well-established model of inflammatory corneal neovascularization.¹⁶⁻¹⁹ Herein, we demonstrate that siRNA targeting the corneal CYP4B1 inhibits corneal neovascularization further supporting the role of CYP4B1 as an angiogenic pathway in the cornea.

MATERIALS AND METHODS

Design of gene targets and SiRNA

SiRNA duplexes were derived from the coding sequence of the rabbit CYP4B1 cDNA (GenBank AF176914).¹⁵ Synthesis of siRNAs was done by GeneLink (Hawthorne, NY). Sequences identified were BLASTed against the GenBank database. All siRNAs were 21-nucleotide long double stranded RNA oligos with a two-nucleotide (TT) overhang at the 3' end. The target sequence (5' to 3') and the siRNA duplexes, designated as 4B1a, 4B1b and 4B1c, are shown in Table 1, with the position of the sequence in the rabbit CYP4B1 cDNA shown in parentheses; a nonspecific siRNA duplex was used as control. SiRNA solutions were made in RNase free water at concentration of 200 μ M.

In vitro efficacy of siRNA

Immortalized rabbit corneal epithelial (RCE) cells were a generous gift from Dr. K. Araki, Department of Ophthalmology, University of Osaka, Japan.²⁰ Cells were grown to 80% confluence in DMEM/F-12's 1:1 Ham's nutrient mixture (Hyclone, Logan, UT)) supplemented

with 15% heat-inactivated fetal bovine serum and 1% antibiotics and antimycotic solution. Rabbit CYP4B1 cDNA (1.82Kb; GenBank #AF176914) was cloned into the multiple cloning site of pIRES-EGFP vector (Clontech, Mountain View, CA) at AccI and XmaI restriction sites¹⁵. The pIRES-EGFP plasmid was used as control. One day before transfection, the medium was substituted with serum-free medium. For the transfection, cells were incubated in DMEM (500 ml/well) containing 1 µg of plasmid, either pIRES2-EGFP or pIRES2-EGFP-4B1, and 4 µl of the LipofectAMINETM (Invitrogen, Carlsbad, CA) for 6 h at 37°C. After incubation, the medium was replaced with serum-containing medium and incubation continued overnight. Cells were then placed in serum- and antibiotics-deprived DMEM (500 ml/well) containing 40nM siRNA and 6 µl of LipofectAMINETM for 6 hour at 37°C. The medium was replaced with DMEM/F12 supplemented with 15% (v/v) FBS and cells were left overnight at 37°C. Expression of pIRES-EGFP and pIRES-EGFP-CYP4B1 was monitored with a Nikon Eclipse TE2000-U immunofluorescent microscope and recorded with a CCD digital camera attached to the microscope. To assess CYP4B1 activity, cells were washed with PBS twice and processed for measurement of arachidonic acid metabolism as previously described.³ Briefly, cells were incubated with ¹⁴C-arachidonic acid (1 µCi) and NADPH (1 mM) for 60 min at 37°C. The incubation medium was extracted with ethyl acetate and radiolabeled metabolites were separated by HPLC and identified by co-elution with authentic standards. The levels of 12-HETrE were further quantified by negative chemical ionization-gas chromatography-mass spectrometry as previously described.²¹

Corneal neovascularization and in vivo delivery of siRNA

All animal studies adhered to the ARVO statement for the Use of Animals in Ophthalmic and Vision research. Male New Zealand rabbits (2.2–2.5 kg) were anesthetized with Ketamine (50 mg/kg) and Xylazine (20 mg/kg) intramuscularly. Following a local administration of 0.5% proparacaine hydrochloride to minimize discomfort, a single 7.0 silk suture was inserted at midstromal depth approximately 2 mm from the limbus. Immediately after suture placement, siRNA duplexes (20 µl) were administered by subconjunctival injection adjacent to the suture location using a 32-gauge Hamilton syringe. The left eye received a subconjunctival injection of 20 µl solution of CYP4B1 siRNA (200 µM); the right eye received 20 µl of nonspecific siRNA (200 µM). Injections of siRNA were repeated at day 2 and 4 after suture placement. In some experiments, eyes were treated with a daily drop (10 µl, 30 µM) of the selective CYP4B1 inhibitor 17-octadecynoic acid (17-ODYA)^{4,15}. Corneal neovascularization was evaluated at days 2, 4 and 7 after suture placement by slit lamp microscopy attached to a video camera (Q Imaging, Canada). Neovascularization was quantified in digitalized images by comparing total vessel length. Vessel length was determined by tracing all and each vessel from the limbus to the suture. Total length is the sum of these vessels in mm and was determined using Image Pro-Express Software (Cyber Media) as previously described.¹⁵ Rabbits were sacrificed 7 days after suture insertion and corneal wedges containing the sutures were dissected and processed for measurements of CYP4B1 mRNA expression. Functional expression of CYP4B1 was further evaluated by measuring the levels of 12-HETrE, its arachidonic acid metabolite. Briefly, corneal wedges were homogenized and lipids were extracted with ethyl acetate as previously described. Extracts were constituted with methanol and subjected to reverse phase HPLC. 12-HETrE was detected by absorbance at 205 nm and identified by its co-elution with chemically synthesized 12-HETrE standard²². In addition, microsomes prepared from corneal wedges were incubated with arachidonic acid (7 nmol) and NADPH (1 mM) for 1 h at 37°C. 12-HETrE synthesis was measured by gas chromatography/mass spectrometry as previously described.¹⁰

Real time PCR

Corneas were dissected from eyes using sterile instruments and were cleaned in sterile PBS (4°C) under a dissecting microscope to remove all non-corneal tissue. Total RNA was isolated

using TRIzol reagent (Invitrogen, Carlsbad, CA) and RNA integrity was verified by agarose gel electrophoresis and quantitated by spectrophotometry using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). RT reaction of total RNA (5 µg) was performed using SuperscriptTM III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Quantitative Real-Time PCR was performed using Brilliant[®] SYBR Green QPCR Master Mix and the Mx3000pTM Real-Time PCR System (Stratagene, La Jolla, CA). Specific primers were designed based on published sequences (Genebank) and were as follows: CYP4B1 sense 5'-TCTCTGGGTTGGACAGTTCATTG-3' and antisense 5'-TGTCTCCTTTGCCAAACGTACAC-3' (359 bp); VEGF A sense 5'-GGAGTACCCTGATGAGATCGA-3' and antisense 5'-CTTTGGTCTGCATTACATTTGT-3' (165 bp); 28S sense 5'-AAACTCTGGTGGAGGTCCGT-3' and antisense 5'-CTTACCAAAGTGGCCCACTA-3' (305 bp). PCR efficiency for each primer pair was determined by quantitating amplification with increasing concentrations of template cDNA (15.6, 31.2, 62.5, 125.0, 250.0, and 500.0 ng) and specific amplification was verified by subsequent analysis of melt curve profiles for each amplification. A non-template control served as the negative control to exclude the formation of primer dimers or any other non-specific 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 28S RNA as described.²³

VEGF mRNA expression in RCE cells

RCE cells were grown to 80% confluency in complete medium and then incubated for 24 h in serum-free medium before adding 12-HETrE (0.1–100 nM) or placing the cells under hypoxic condition using a modular tissue culture chamber (Billups-Rothenburg, DelMar, CA.) supplied continuously with 5%CO₂/2% O₂/93% N₂ (hypoxia), bubbled through deionized H₂O into the chamber within a 37°C incubator. Total RNA was extracted with TriZol, quantified by spectrometry and processed for real time PCR analysis as described above. VEGF mRNA levels were also measured in cells transfected with the plasmids pIRES2-EGFP or pIRES2-EGFP-4B1 and treated with and without CYP4B1 siRNA duplexes (40 nM) or 17-ODYA (20 µM).

RESULTS

In vitro efficacy of CYP4B1 siRNA

We have previously shown that RCE cells overexpressing the CYP4B1 readily converted arachidonic acid to 12-HETrE, whereas, native cells exhibited very low levels of CYP4B1 expression and showed little capacity of 12-HETrE production.²⁴ Therefore, RCE cells overexpressing CYP4B1 were used to evaluate the efficacy of siRNA duplexes targeting CYP4B1. Figure 1A depicts GFP fluorescence in cells transfected with the bicistronic control plasmid pIRES-EGFP (upper panel) and the plasmid expressing the CYP4B1, pIRES-EGFP-CYP4B1 (middle panel); both exhibited similar levels of GFP fluorescence. However, they differ in their capacity to produce 12-HETrE the major arachidonic acid metabolite of CYP4B1. As seen in Figure 1B, HPLC profiles of arachidonic acid metabolites in cells transfected with the CYP4B1-containing plasmid clearly showed that 12-HETrE production is 3–4 fold higher than cells transfected with the control plasmid pIRES-EGFP. Treatment of pIRES-EGFP-CYP4B1-transfected cells with CYP4B1-specific siRNA attenuated GFP fluorescence (Figure 1A, lower panel), suggesting inhibition of CYP4B1 expression. The effect of siRNA on CYP4B1 functional expression was further determined by measuring the production of 12-HETrE. Quantitative analysis is given in Figure 1C. 12-HETrE production in CYP4B1-transfected cells treated with two CYP4B1-specific siRNAs (4B1-b and 4B1-c) was markedly inhibited (81 and 85%, respectively) as compared to untreated CYP4B1-transfected cells. 12-HETrE production was unaltered in cells treated with nonspecific siRNA and amounted to 1.4

± 0.3 nmol/h/ 10^6 cells ($n=3$). The CYP4B1 siRNA duplex 4B1-a was less effective in inhibiting 12-HETrE production in CYP4B1-transfected cells. The 4B1-c siRNA duplex was used in all subsequent experiments as the CYP4B1-specific siRNA.

CYP4B1 siRNA inhibits corneal neovascularization

Corneal neovascularization was apparent in eyes with sutures as early as 2 days after its placement. In eyes treated with saline (vehicle control), the neovascular response increased with time encompassing larger area of the cornea by day 7 after suture placement (Figure 2A). Similar neovascular response was observed in eyes treated with control nonspecific siRNA duplexes (Figure 2A). In contrast, corneal neovascularization was markedly reduced in eyes receiving subconjunctival injections of CYP4B1-specific siRNAs as compared to control eyes or eyes receiving the control nonspecific siRNA (Figure 2A). Quantitative analysis showed that the neovascular response to suture placement in eyes receiving the CYP4B1-specific siRNA was inhibited by about 50 and 60% at day 4 and 7, respectively, when compared to either control eyes or eyes treated with the non-specific siRNA (Figure 2B). To further substantiate the notion that the neovascular response is mediated at least in part by CYP4B1 activity via production of 12-HETrE, experiments were conducted using daily topical treatment of 17-ODYA. Eyes treated with 17-ODYA exhibited a 50% reduction of the neovascular response at day 4 after suture placement as compared to eyes treated with the vehicle control, viz., from 14.95 ± 1.21 to 8.29 ± 1.26 mm neovessels ($n=4$, $p<0.05$). These results, combined with the results obtained with the CYP4B1-specific siRNA, lend support to the notion that the CYP4B1-derived 12-HETrE is a key pathway in the development of corneal neovascularization.

CYP4B1 siRNA inhibits CYP4B1 expression and 12-HETrE Synthesis

The effect of siRNA treatment on CYP4B1 mRNA levels was assessed by real time PCR analysis. As seen in Figure 3A, CYP4B1 mRNA levels of corneal limbal wedges indicated a significant inhibition of CYP4B1 mRNA expression in CYP4B1 siRNA-treated eyes. (Figure 3A). Since specific antibody against the rabbit CYP4B1 protein is unavailable, CYP4B1 functional expression was examined by measuring levels of 12-HETrE, the arachidonic acid metabolite of CYP4B1 enzymatic activity. Reverse phase HPLC analysis of homogenates of corneal wedges containing the sutures indicated the presence of 12-HETrE as detected by UV absorbance at 205 nm and co-eluted with synthetic 12-HETrE standard (Figure 3B). 12-HETrE was clearly present in homogenates from corneas treated with the control nonspecific siRNA. In contrast, 12-HETrE was barely detectable in homogenates from corneas treated with CYP4B1-specific siRNA (Figure 3B). Additional experiments were carried out to examine CYP4B1 enzymatic activity in corneal wedges from eyes treated with siRNA duplexes by measuring their capacity to metabolize arachidonic acid to 12-HETrE. Corneal wedges from sutured eyes treated with the CYP4B1 specific siRNA produced 12-HETrE in a significantly lower rate as compared to corneal wedges from nonspecific siRNA-treated sutured eyes, viz., 0.13 ± 0.03 vs 0.18 ± 0.04 pmol12-HETrE/mg protein/h, $n=3$, $p<0.05$.

CYP4B1 siRNA decreases corneal VEGF expression

We have previously shown a relationship between CYP4B1 and VEGF expression in the cornea following hypoxic injury,¹³ an injury that is associated with neovascularization. We also demonstrated that the angiogenic activity of 12-HETrE is mediated at least in part by VEGF; 12-HETrE induces VEGF expression and activity and inhibition of VEGF function diminishes 12-HETrE-induced angiogenesis.²⁵ Therefore, we evaluated the effect of siRNA on VEGF expression and its relation to neovascularization. Real time PCR analysis clearly demonstrated that VEGF expression was markedly reduced by CYP4B1-specific siRNA but was not affected by treatment with nonspecific siRNA (Figure 4).

VEGF expression is regulated by the CYP4B1-12HETrE pathway in RCE cells

To further establish the link between CYP4B1 and VEGF expression, experiments were carried out using RCE cells. As seen in Figure 5A, VEGF mRNA levels were significantly higher in cells transfected with CYP4B1 cDNA than non-transfected cells or cells transfected with the control plasmid pIRES. More importantly, addition of CYP4B1-specific siRNA inhibited CYP4B1-stimulated VEGF induction by 46% (Figure 5A). There was no significant change in VEGF expression levels in CYP4B1-transfected cells treated with the control siRNA (data not shown). Likewise, neither control siRNA nor CYP4B1 siRNA had an effect on VEGF expression in control cells or cells transfected with the control plasmid pIRES. That the effect of CYP4B1 overexpression on VEGF mRNA levels is linked to CYP4B1 catalytic activity is documented in Figure 5B; addition of the CYP4B1 inhibitor 17-ODYA attenuated the effect of CYP4B1 on VEGF mRNA levels by 44%. Since RCE cells transfected with the CYP4B1 cDNA readily produce 12-HETrE from either exogenous or endogenous arachidonic acid,¹⁵ it is likely that 12-HETrE mediates, at least in part, the CYP4B1 effect on VEGF expression levels. To this end, addition of 12-HETrE to RCE cells induces VEGF expression in a concentration-dependent manner (Figure 5C); 12-HETrE at 1 nM induced VEGF to the same extent as hypoxia (2% O₂) attesting to the potency of 12-HETrE as an angiogenic lipid mediator.

DISCUSSION

Corneal neovascularization poses a real threat to vision; even modest numbers of new vessels will reduce visual acuity. In recent years, we have identified in the corneal epithelium the CYP4B1 enzymatic pathway which upon injury is activated and produces a potent angiogenic eicosanoid, namely, 12-HETrE. To date, the link between CYP4B1 expression, 12-HETrE production and neovascularization of the corneal surface was based primarily on studies showing correlation between the progression of the inflammatory and neovascular response to injury, increased CYP4B1 expression and increased production of 12-HETrE. The current study is the first to establish a direct cause and effect relationship between the CYP4B1 pathway and inflammatory neovascularization in the cornea. Moreover, the current study provides additional evidence for a link between the expression of CYP4B1 and VEGF and suggests that CYP4B1 may be a proximal regulator of VEGF expression and bioaction in the cornea.

Gene silencing by siRNA is a useful approach for suppressing gene expression with great specificity and potency and a powerful alternative to other genetic knockdown methods for the analysis of loss-of-function phenotypes; it can be employed locally and at any stage of the experiment. This approach has been successfully used to silence VEGF and VEGF receptor gene expression in several models of corneal neovascularization.^{26–29} Using this approach, we examined whether CYP4B1 gene silencing diminishes the corneal neovascular response in a model of inflammatory neovascularization. CYP4B1 expression is primarily localized to the corneal epithelium. In the intact cornea, CYP4B1 mRNA levels and CYP4B1 catalytic activity as an arachidonic acid metabolic pathway are relatively low; injury, however, rapidly and strongly induces CYP4B1 mRNA followed by an increase in its enzymatic activity, i.e., production of 12-HETrE. To specifically target the localized and induced expression of CYP4B1, siRNA duplexes were injected subconjunctively at the limbus at the time of suture instillation and every other day during the development of neovascularization. The results clearly indicated that, using this experimental approach and siRNA duplexes which shown to exert specificity and potency in vitro, CYP4B1 expression as measured by real time PCR was reduced by 60%. More importantly, treatment with CYP4B1-specific siRNA greatly attenuated the neovascular response suggesting that CYP4B1 is a key participant in the regulation of corneal neovascularization. The corneal CYP4B1 is involved in arachidonic acid metabolism and has been the primary enzymatic source of the angiogenic eicosanoid 12-HETrE.²⁴

Accordingly, treatment with CYP4B1-specific siRNA greatly reduced the corneal levels of 12-HETrE. It is likely that the decrease in CYP4B1 activity and consequently 12-HETrE levels contributed to the reduced neovascular response brought about by the CYP4B1-specific siRNA. It is also likely that suture-induced corneal neovascularization is mediated, at least in part, via the production of 12-HETrE whose levels were barely detected in CYP4B1 siRNA-treated corneas but were predictably prominent in the untreated sutured corneas.

The demonstration that administration of siRNA that specifically targeted CYP4B1 greatly attenuated VEGF mRNA levels is of great significance. We have previously shown that CYP4B1 pattern of expression is parallel to that of VEGF and that a functional interaction links these two angiogenic pathways. This functional interaction is manifested by the demonstration that 12-HETrE's angiogenic activity and, by extension, CYP4B1 angiogenic capacity is mediated, at least in part by VEGF.^{11,13,25} It is also supported in this study by the demonstrations that 1) suppression of CYP4B1 expression *in vivo* reduced VEGF mRNA levels; 2) overexpression of CYP4B1 in corneal epithelial cells or addition of 12-HETrE *in vitro* increased the levels of VEGF mRNA; and 3) inhibition of CYP4B1 enzymatic activity *in vitro* significantly decreased VEGF mRNA levels. Noteworthy, is a report by Chen et al.³⁰ which showed that an inhibitor of the CYP4 enzymes, including CYP4B1, inhibited VEGF-induced angiogenesis in human endothelial cells *in vitro* and corneal neovascularization elicited by VEGF *in vivo*.

VEGF has long been considered as a key angiogenic factor in the cornea;^{31,32} it is induced in response to injury and inhibition of its expression or blockade of its receptors inhibits corneal neovascularization in numerous experimental models.^{27,33,34} VEGF within the injured cornea originates from multiple cell types including resident and recruited inflammatory cells.^{35,36} While the cellular source of 12-HETrE within the injured cornea is primarily the corneal epithelium³, it is also likely that infiltrating cells such as neutrophils contribute to its increased levels as well.³⁷ Notably, 12-HETrE bioactivity includes both chemotactic as well as angiogenic capacity.⁸ Hence, the CYP4B1-12-HETrE pathway may constitute an upstream regulator of VEGF expression and action and an amplification system of neovascularization. Accordingly, injury rapidly induces CYP4B1 activity to produce 12-HETrE; this lipid mediator acting as a chemoattractant, amplifies infiltration of inflammatory cells, a well-established source of VEGF, and activates resident cells including epithelial cells and the adjacent limbal endothelial cells to produce and use VEGF as the mediator of neovascularization.

In summary, this study is the first to demonstrate that inhibition of CYP4B1 expression attenuates corneal neovascularization. The results strongly implicate the corneal CYP4B1 as a component of the inflammatory and neovascular cascade initiated by injury to the ocular surface. Recent interest in inhibiting neovascularization has focused on VEGF.³⁸ Our previous studies showing that VEGF mediates the angiogenic activity of 12-HETrE²⁵ and that injury to the cornea is associated with a concerted induction of CYP4B1 and VEGF,¹³ together with the results presented here, raise the possibility of a new approach to the problem of preventing corneal neovascularization that targets the CYP4B1-12-HETrE system. To this end, we have shown the presence of the cytochrome P450 system in human corneas³⁹ and documented the presence of 12-HETrE in human tears at concentrations that readily induce inflammatory and angiogenic response including VEGF induction.¹⁰ More importantly, ocular surface inflammation of diverse etiologies was associated with increased 12-HETrE tear levels. 12-HETrE's levels were especially high in contact lens-related inflammation in which all patients had slept with their lenses in place and had an acute ocular surface inflammation on waking. Hypoxia, which is believed to be a major factor in contact lens-induced inflammation, has been shown to induce CYP4B1 expression and stimulate 12-HETrE synthesis.^{11,21} Herpes simplex keratitis and corneal foreign body, both injuries to the corneal epithelium, also had marked elevations of tear film 12-HETrE.¹⁰ The significant increase of 12-HETrE in the tear film of

inflamed human eyes supports a positive correlation between 12-HETrE production and ocular surface inflammation that is consistent with the relationship previously established in rabbit models and with its hypothesized role as a *paracrine* mediator of inflammation. The fact that 12-HETrE is produced in other inflamed tissues^{40,41} further extends its clinical relevance.

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Figure 1.

Inhibition of 12-HETrE production by CYP4B1-specific siRNA in RCE cells. **A)** EGFP fluorescence in RCE cells transfected with the pIRES2-EGFP (upper panel) and pIRES2-EGFP-4B1 without (middle panel) and with (lower panel) CYP4B1 specific siRNA (X40). **B)** Representative HPLC elution profiles of arachidonic acid metabolites in RCE cells transfected with the pIRES2-EGFP (upper panel) and pIRES2-EGFP-4B1 (lower panel). The major metabolite co-eluted with 12-HETrE authentic standard; other radioactive peaks correspond to: I, unknown polar metabolites; II, 12,20-diHETE; 12-HETE; and 12-oxo-ETrE as previously reported^{42,43} **C)** Quantitative analysis of 12-HETrE production in RCE cells treated with siRNA duplexes targeting CYP4B1 (4B1-a, 4B1-b, 4B1-c) and non-specific siRNA (control) and incubated with arachidonic acid. Results are mean±SE, n=3, *p<0.05 from control siRNA.

Figure 2. Effect of siRNA treatment on corneal neovascularization. **A)** Representative pictures depicting corneal neovascularization in control eyes (treated with the vehicle saline) and in eyes receiving subconjunctival injections of CYP4B1-specific (4B1-siRNA) or nonspecific siRNAs at days 2, 4 and 7 after suture placement (X16). **B)** Quantitative analysis of corneal neovascularization 4 and 7 days after suture placement. Results are mean $\pm$ SE; n=6; *p<0.05 from control siRNA-treated eyes; ‡p<0.05 from day 4.

Figure 3. Effect of siRNA treatment on corneal CYP4B1 mRNA and 12-HETrE levels 7 days after intrastromal suture placement. **A)** Representative amplification plots for CYP4B1 (*left*) and 28S rRNA mRNA (*right*) by real time PCR analysis in RNA from corneas treated with control (blue line) and CYP4B1-specific (red line) siRNA. **B)** Quantitative analysis of mRNA levels. Results are presented as relative expression and are mean±SE, n=5, *p<0.05 from control siRNA. **C)** HPLC analysis of 12-HETrE levels in corneal homogenates from control and CYP4B1 (4B1) specific siRNA treated eyes; upper panel, elution profile of 12-HETrE standard (st); lower panel, representative analyses (n=3) of corneal homogenates from eyes treated with control (blue line) and CYP4B1-specific (red line) siRNAs.

A

B

Figure 4. Effect of siRNA treatment on corneal VEGF mRNA 7 days after intrastromal suture placement. **A)** Representative amplification plots for VEGF in corneas treated with control siRNA and CYP4B1 (4B1)-specific siRNA. **B)** Quantitative analysis of mRNA levels. Results are presented as relative expression and are mean $\pm$ SE, n=5, *p<0.05 from control siRNA-treated eyes.

Figure 5.

Effect of CYP4B1 expression and 12-HETrE on VEGF mRNA levels in RCE cells. Levels of mRNA were measured by real time PCR. **A)** VEGF mRNA levels in non transfected (control) and cells transfected with the control plasmid pIRES and pIRES plasmid containing the CYP4B1 cDNA (4B1) in the absence and presence of CYP4B1-specific siRNA (4B1+siRNA). Results are expressed as relative expression normalized to 28S levels and are mean $\pm$ SE; n=3; *p<0.05 from control or pIRES transfected cells; †p<0.05 from 4B1 transfected cells. **B)** VEGF mRNA levels in cells transfected with pIRES-GFP-CYP4B1 (4B1) and treated with the CYP4B1 inhibitor 17-ODYA (20 μ M). Results are expressed as relative expression normalized to 28S levels and are mean $\pm$ SE; n=3; *p<0.05 from pIRES transfected cells; †p<0.05 from 4B1

transfected cells. C) VEGF mRNA levels in RCE cells treated with 12-HETrE (0.1–100 nM) or subjected to hypoxia (2% O₂). Results are expressed as relative expression normalized to 28S levels and are mean±SE; n=3; *p<0.05 from control.

TABLE 1

Sequences of siRNA duplexes targeting CYP4B1		
SiRNA duplex	CYP4B1 sequence 5'-3' [cDNA sequence]	SiRNA duplex sequence
4B1a	gcatccacctctacttgaa [1499–1518]	GCAUCCACCUCUACUUGAATT TTCGUAGGUGGAGAUGAACUU
4B1b	gcacctacttgcttcagaa [1745–1757]	GCACCUACUUGCUUCAGAATT TTCGUGGAUGAACGAAGUCUU
4B1c	tgacttgatcggtttat [1914–1932]	UGCACUUGAUCGGCUUUAUTT TTACGUGAACUAGCCGAAUA