Selective Activation of the Prostaglandin E₂ Circuit in Chronic Injury-Induced Pathologic Angiogenesis

Elvira L. Liclican, Van Nguyen, Aaron B. Sullivan, and Karsten Gronert

PURPOSE. Cyclooxygenase (COX)-derived prostaglandin E₂ (PGE₂) is a prevalent and established mediator of inflammation and pain in numerous tissues and diseases. Distribution and expression of the four PGE₂ receptors (EP1-EP4) can dictate whether PGE₂ exerts an anti-inflammatory or a proinflammatory and/or a proangiogenic effect. The role and mechanism of endogenous PGE₂ in the cornea, and the regulation of EP expression during a dynamic and complex inflammatory/repairative response remain to be clearly defined.

METHODS. Chronic or acute self-resolving inflammation was induced in mice by corneal suture or epithelial abrasion, respectively. Reepithelialization was monitored by fluorescein staining and neovascularization quantified by CD31/PECAM-1 immunofluorescence. PGE₂ formation was analyzed by lipidomics and polymorphonuclear leukocyte (PMN) infiltration quantified by myeloperoxidase activity. Expression of EPs and inflammatory/angiogenic mediators was assessed by real-time PCR and immunohistochemistry. Mice eyes were treated with PGE₂ (100 ng topically, three times a day) for up to 7 days.

RESULTS. COX-2, EP-2, and EP-4 expression was upregulated with chronic inflammation that correlated with increased corneal PGE₂ formation and marked neovascularization. In contrast, acute abrasion injury did not alter PGE₂ or EP levels. PGE₂ treatment amplified PMN infiltration and the angiogenic response to chronic inflammation but did not affect wound healing or PMN infiltration after epithelial abrasion. Exacerbated inflammatory neovascularization with PGE₂ treatment was independent of the VEGF circuit but was associated with a significant induction of the eotaxin-CCR3 axis.

CONCLUSIONS. These findings place the corneal PGE₂ circuit as an endogenous mediator of inflammatory neovascularization rather than general inflammation and demonstrate that chronic inflammation selectively regulates this circuit at the level of biosynthetic enzyme and receptor expression. (*Invest Ophthalmol Vis Sci.* 2010;51:6311–6320) DOI:10.1167/iov.10-5455

The successful execution of an acute inflammatory response is nowhere as critical and evolved as in the delicate visual axis because uncontrolled ocular inflammation impairs vision

and leads to blindness. In particular, the transparent, avascular, and immune-privileged cornea has a tightly regulated and remarkable inflammatory/repairative response that is highly dependent on the generation and release of specific and temporally defined arrays of mediators.^{1–4} In this regard, lipid autacoids such as arachidonic acid (AA)-derived cytochrome P450 metabolites, lipoxins, and hydroxyicosatetraenoic acids (HETEs) have emerged as key mediators of corneal inflammation and pathologic angiogenesis and the resolution of these responses.^{3,5–7}

Lipid autacoids are formed in most tissues and are essential regulators of inflammatory and immune responses.^{1,8–11} Recent findings have uncovered a critical role for resident protective lipid circuits, namely 12/15-lipoxygenase (12/15-LOX; Alox15), in the cornea. 12/15 LOX is highly expressed in mouse and human corneas^{12,13} and generates AA- and ω -3 polyunsaturated fatty acid-derived mediators (i.e., lipoxins, resolvins, and protectins) that inhibit inflammation and pathologic angiogenesis and promote wound healing.^{7,13–15} This emerging field underscores the importance of resident endogenous lipid autacoid circuits in the eye to maintain and execute essential acute inflammatory responses.

Cyclooxygenase (COX)-derived prostaglandins (PG) are of primary interest because they are critical and early response mediators that initiate or amplify inflammation. In particular, PGE₂ has traditionally been identified as a prevalent inflammatory mediator in many tissues and inflammatory diseases.^{10,16} More recently, it has been implicated in tumor-driven angiogenesis.¹⁷ Not surprisingly, it is a primary target of several important classes of clinically used drugs (i.e., nonsteroidal anti-inflammatory drugs [NSAIDs] and corticosteroids) for inflammation, pain, and colon cancer. PGE₂ is produced by many cells of the body, including fibroblasts, macrophages/monocytes, dendritic cells, and some types of malignant cells, and it exerts its effects through binding to G-protein-coupled receptors (EP). Four EP receptor subtypes have been identified—EP-1, EP-2, EP-3, and EP-4—which are coupled to different intracellular signal transduction pathways.^{18,19} EP-2 and EP-4 receptors are coupled to G_s and mediate increases in cyclic adenosine monophosphate (cAMP) concentrations, whereas activation of EP-3 inhibits adenylate cyclase through G_i; EP-1 stimulates intracellular Ca²⁺ mobilization through G_q. Hence, the distribution and relative expression of these four receptor subtypes provide an elegant system that can account for the ability of PGE₂ to evoke pleiotropic and sometimes opposing bioactions that are tissue- and cell type-specific.^{18–20} Although PGE₂ induces reactions that give rise to the initial cardinal signs of inflammation (i.e., edema),²¹ it can also inhibit inflammation by downregulating host responses. PGE₂ is a negative regulator of neutrophil, monocyte, and lymphocyte function.^{10,11,18} Specifically, PGE₂ has been shown to negatively regulate allergic reactions, including contact hypersensitivity, conjunctivitis, and asthma, through EP-3 in several murine experimental models, and, through EP-4, to

From the Vision Science Program, School of Optometry, University of California at Berkeley, Berkeley, California.

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Corresponding author: Karsten Gronert, Vision Science Program, School of Optometry, University of California at Berkeley, 594 Minor Hall, MC 2020, Berkeley, CA 94720-2020; kgronert@berkeley.edu.

maintain intestinal homeostasis by downregulation of the immune response.^{22–25} Moreover, PGE₂ decreases macrophage production of proinflammatory cytokines such as TNF- α ²⁶ and stimulates anti-inflammatory cytokine production,²⁷ mechanisms that both contribute to the resolution of inflammation. PGE₂ can also promote the resolution of inflammation through the induction of key enzymes, namely 12/15-LOX, in polymorphonuclear leukocytes (PMNs), and the formation of the anti-inflammatory and pro-resolving lipoxin A₄.²⁸ Hence, the role of PGE₂ in inflammatory and immune responses is complex; it is temporally defined and largely dependent on EP receptor expression.

The capacity of ocular tissues to synthesize PGE₂ was initially determined on the basis of ¹⁴C-AA conversion *in vitro* and *in vivo*.^{29–31} Subsequently, studies that used NSAIDs, PGE₂, and PGE₂ analogs, as well as EP receptor knockout mice, have demonstrated that in the eye, this prostanoid causes miosis, vasodilation, disrupts the integrity of the blood-aqueous barrier, and, through regulating the dynamics of aqueous humor flow, can increase or reduce intraocular pressure.^{32–35} Importantly, NSAIDs are a primary treatment option for ocular indications including the inhibition of intraoperative miosis and the management of postoperative inflammation and pain. However, in contrast to other tissues and despite reports of COX-2 upregulation and PG formation in models of ocular inflammation,³ the endogenous role of this prominent prostanoid and primary therapeutic target in the corneal inflammatory/repairative response, surprisingly, remains to be fully defined. To this end, we assessed the role of the PGE₂ circuit in mediating inflammatory responses in the cornea.

Here, we report that endogenous PGE₂ in the cornea is not a general inflammatory mediator. Increased PGE₂ formation and upregulation of EP-2 and EP-4 receptor expression were key features of a severe and chronic injury that was associated with inflammatory neovascularization but not a feature of acute self-resolving inflammation. Moreover, topical PGE₂ treatment selectively amplified leukocyte recruitment and pathologic angiogenesis in response to chronic inflammation but did not affect wound healing or leukocyte recruitment during acute epithelial injury, providing evidence for a selective role of the PGE₂ circuit in mediating corneal inflammatory responses.

MATERIALS AND METHODS

Animals

Female C57Bl/6J stock 000664 mice (6–10 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hour day/12-hour night cycle and fed a standard diet *ad libitum* (Rat/Mouse diet LM-485; Harlan Tekland, Madison, WI).

Models of Corneal Injury and Treatment

All animal studies were approved by the University of California at Berkeley in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and in strict accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with ketamine (Ketaset, 50 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (20 mg/kg) by intraperitoneal injection, and 1 drop of tetracaine-HCl 0.5% was applied to the eye to deliver local corneal anesthesia before subjecting animals to corneal injury. To induce a mild and self-resolving injury, the corneal epithelium up to the corneal/limbal border was removed by mechanical abrasion with a 0.5 mm-corneal rust ring remover (Algerbrush II; Alger Equipment, Lago Vista, TX) while leaving the underlying stroma intact, using a dissecting stereomicroscope (Carl Zeiss, Jena, Germany) as previously described.^{13,14} To induce a more severe and chronic injury, a single sterile 8-0 silk suture was placed intrastromally

extending over the corneal apex, without disrupting the iris.¹⁵ Mice were treated topically three times a day with PGE₂ (100 ng; Cayman Chemical, Ann Arbor, MI) or sterile saline alone (HBSS, pH 7.4) for up to 7 days. Ethanol from the PGE₂ solution was rapidly removed under a gentle stream of nitrogen, and PGE₂ was immediately resuspended in sterile HBSS and applied to the eye (5 μ L drop). Eyes were enucleated at the respective time points under a stereomicroscope, and corneas were carefully dissected on ice to remove the limbus area and all noncorneal tissue. Isolated corneas were either snap frozen for RNA or lipidomic analyses or immediately processed for immunohistochemistry.

Assessment of Wound Healing, Neovascularization, and Inflammation

Reepithelialization (wound healing) was assessed by fluorescein staining and digital image analyses 48 and 96 hours after abrasion.^{13,14} For quantification of neovascularization, isolated corneas were rinsed in PBS, fixed in acetone (100%) for 30 minutes, blocked in a 2% bovine serum albumin/PBS solution, and incubated in PBS containing fluorescein isothiocyanate-conjugated CD31/PECAM-1 monoclonal antibody overnight (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100).¹⁵ Flat-mounts were prepared by sectioning the cornea and fixing them to slides. Mosaic images were taken with a microscope (Axioplan 2; Zeiss) equipped with a camera (AxioCam MR; Zeiss) and compiled using specialized software (MosaicX and AxioVision 4.5; Zeiss). Neovascularization was quantified by manually tracing the length of all vessels (Image Pro-Express software; Cyber Media, New Delhi, India) and was expressed as total pixels.

Assessment of Inflammation

Myeloperoxidase (MPO) activity, a quantitative index of tissue leukocyte infiltration, was measured 48 hours after initiating injury, as previously described.^{13–15} In brief, corneas (1 cornea/data point) were homogenized with a hand-held tissue grinder in 450 μ L of 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0). This was followed by sonication, three cycles of freeze-thaw, and a second sonication. After centrifugation, MPO activity in the supernatant was measured by spectrophotometry using *o*-dianisidine dihydrochloride oxidation as a colorimetric indicator. Calibration curves for conversion of MPO activity to PMN number were established with PMN collected from zymosan A-induced peritoneal exudates in mice.

Gene and Protein Expression

RNA from mouse corneas was isolated (RNeasy Mini Kit; Qiagen Sciences, Germantown, MD). RNA integrity was verified using agarose gel electrophoresis, quantified by spectrophotometry, and reverse transcribed (High-Capacity Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Nucleotide primer sequences are listed in Table 1 and were selected from the Harvard Primer Bank (pga.mgh.harvard.edu/primerbank/) and verified by the NIH GenBank database. Real-time PCR was performed (Fast SYBR Green Master Mix; Applied Biosystems) with a quantitative PCR system (StepOnePlus; Applied Biosystems). Amplifications were run in duplicate, and efficiencies for each primer pair were established. Comparative quantification of gene expression was performed (StepOne software; Applied Biosystems) using the $\Delta\Delta_{CT}$ method. Expression of all genes is referenced to a positive mRNA control that was generated by pooling mRNA from C57Bl/6J mouse kidney and spleen.

For immunohistochemical analysis of EP receptor expression, whole eyes were embedded in OCT and sectioned at 5- μ m thickness. Sections were washed in PBS, followed by incubation at room temperature with 10% normal goat serum for 1 hour to block nonspecific antigenic binding sites. After overnight incubation at 4°C with the primary antibody (EP-2 or EP-4, 1:40 in 1% goat serum; Alpha Diagnostic International Inc., San Antonio, TX), the sections

TABLE 1. Primer Sequences

Primer Set	Sense Primer, 5'→3'	Antisense Primer, 5'→3'
COX-1	AGTGC GG TCCAACCTTATCC	GCAGAA TGGCAGTATAGTAGCTC
COX-2	TGAGCA ACTATTCCAAAC CAGC	GCACGTAGTCTTCGATCACTATC
EP-1	GGGCTTAACCTGAGCCTAGC	GTGATGTGCCATTATCGCCTG
EP-2	TCCCTAAAGGAAAAGTGGGACC	GAGCGCATTAAACCTCAGGACC
EP-3	CCGGAGCACTCTGTGAAG	CCCCACTAAGTCGGTGAGC
EP-4	ACCATTCTCTAGATCGAACCGT	CACCACCCGGAAGATGAACAT
VEGF-A	TCACCAAAGCCAGCACATAGGAGA	TTCGTTTAACTCAAGCTGCCTCGC
VEGF-C	GAGGTCAAGGCTTTTGAAGGC	CTGTCCTGGTATTGAGGGTGG
Soluble VEGF-R1 (sFLT-1)	AGGTGAGCACTGCGGCA	ATGAGTCCTTTAATGTTTGAC
VEGF-R2 (FLK-1)	TTTGGCAAATACAACCCCTTCAGA	GCAGAAGATACTGTCACCACC
VEGF-R3 (FLT-4)	CTGGCAAATGGTTACTCCATGA	ACAACCCGTGTGTCTTCACTG
CCR3	TCAACTTGGCAATTTCTGACCT	CAGCATGGACGATAGCCAGG
Eotaxin-1	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
Eotaxin-2	GGTTCAGAGGCACATACAAAAC	AAACCTCGGTGCTATTGCCAC
Actin	ACGGCCAGGTCATCACTATTG	AGGGGCCGGACTCATCGTA

were washed in PBS and incubated for 3 hours at room temperature with an aminomethyl coumarin acetic acid-labeled goat-anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Positive staining was detected by immunofluorescence with a microscope (Axioplan 2; Zeiss).

Lipid Autacoid Analysis

Eicosanoids were identified and quantified by LC/MS/MS-based lipidomics. Endogenous lipid autacoid analysis was performed as previously described.¹⁵ In brief, corneas were immediately homogenized with a hand-held tissue grinder in 66% methanol (4°C); PGE₂-d₄, 15(S)-HETE-d₈, and leukotriene B₄-d₄ (400 pg/each) were added as internal standards to calculate the recovery of prostanoids or monohydroxy- and dihydroxy-containing fatty acids. Lipid autacoids were extracted by solid phase using solid-phase extraction cartridges (Samplic Q C18; Agilent Technologies, Santa Clara, CA)³⁶ and were analyzed by a triple quadrupole linear ion trap LC/MS/MS system (MDS SCIEX 3200 QTRAP) equipped with a LUNA C18-2 mini-bore column using a mobile phase (methanol/water/acetate, 65:35:0.02, vol/vol/vol) with a 0.50 mL flow rate. MS/MS analyses were conducted in negative ion mode, and eicosanoids were quantified by multiple reaction monitoring (MRM mode) using specific and established transition ions for PGE₂ (351→271, 351→189 *m/z*), thromboxane B₂ (369→169 *m/z*), 5(S)-HETE (319→115 *m/z*), 12(S)-HETE (319→179 *m/z*), and 15(S)-HETE (319→175 *m/z*).¹⁵ Calibration curves (0.5–1000 pg) and LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI), and structures were confirmed for selected autacoids by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1.

Statistical Analysis

All data are expressed as mean ± SEM unless otherwise indicated. Student's *t*-test was used to evaluate the significance of differences between two groups, and multiple comparisons were performed by regression analysis and one-way analysis of variance. *P* < 0.05 was considered significant.

RESULTS

Selective Formation of PGE₂ during Chronic Injury

In a previous study, LC/MS/MS-based lipidomic analyses demonstrated distinct and temporally defined formation of

select COX- and LOX-derived autacoids in corneal pathologic angiogenesis.¹⁵ Importantly, PGE₂ was the most prominent eicosanoid formed in the inflamed and vascularized cornea. Hence, to further define the role of endogenous PGE₂ in the cornea during inflammation, we used two well-established and distinct injury models. Acute self-resolving inflammation was induced by epithelial abrasion, which creates a mild, reproducible, uniform and quantifiable epithelial injury without stromal injury and is characterized by transient PMN recruitment, temporally defined wound healing, and epithelial regeneration without neovascularization.^{13,14,37} After epithelial debridement, corneas were stained with fluorescein for direct quantification of the epithelial defect (Fig. 1A). Chronic inflammation and neovascularization were induced using the corneal suture model.^{15,38,39} In contrast to epithelial abrasion, placement of the suture penetrates and induces partial injury to the stroma and evokes a robust and nonresolving inflammatory response as a consequence of chronic injury and irritation. Neovascularization can be observed by day 2 and becomes pronounced by day 7 (Fig. 1A), and it persists as long as the sutures remain in the cornea.^{38,39} Consistent with our previous report,¹⁵ lipidomic analyses revealed select formation of LOX- and COX-derived autacoids (i.e., 5(S)-, 12(S)- and 15(S)-HETE, and PGE₂ and thromboxane B₂, respectively) after 4 days of suture injury (Fig. 1B). Corneal levels of endogenous PGE₂ formation significantly increased 4.3-fold and 6.6-fold after 2 and 4 days of suture-induced chronic inflammation, respectively (774 ± 24 and 1123 ± 279 pg/cornea on day 2 and 4, respectively, vs. 147 ± 11 pg/cornea in uninjured corneas), and remained elevated through day 7 (825 ± 125 pg/cornea; Fig. 1C). Importantly, the increase in endogenous PGE₂ formation correlated with pronounced neovascularization. In contrast, basal corneal levels of PGE₂ demonstrated no significant increase throughout the course of injury and wound healing during an acute, self-resolving epithelial abrasion (Fig. 1C). To rule out strain-specific induction of PGE₂ circuits as a consequence of chronic inflammation, we also assessed PGE₂ formation in the Balb/c strain of mice, which exhibits a distinct phenotype of inflammation. The immune response in Balb/c mice is predominantly a humoral (T_H2) response, whereas C57Bl/6J mice have a predominant cell-mediated (T_H1) response.⁴⁰ As was seen in C57Bl/6J mice, PGE₂ formation selectively increased in chronic inflammation that induced neovascularization (Supplementary Fig. S1, <http://www.iovs.org/content/51/12/6311/suppl/DC1>).

FIGURE 1. PGE₂ formation is a key feature of chronic inflammation and neovascularization. Endogenous lipid autacoids were quantified in uninjured corneas and corneas collected after 2, 4, and 7 days of acute (epithelial abrasion) or chronic (suture) injury ($n = 4-7$; $*P < 0.05$ vs. uninjured) from female C57Bl/6J mice by MS/MS-based lipidomic analyses using a triple quadrupole linear ion trap LC/MS/MS system and multiple reaction monitoring (MRM) for specific transition ions. (A) Representative images of epithelial abrasion and suture injury models of ocular inflammation. (B) Representative MRM lipidomic profile of prominent eicosanoids (PGE₂, thromboxane B₂ [TXB₂], 12-HETE, 5-HETE, 15-HETE) in uninjured corneas and corneas collected after 4 days of suture injury. (C) Quantification of endogenous PGE₂ formation in corneas with epithelial abrasion or suture injury.

PGE₂ Amplifies Inflammatory Angiogenesis

Although PGE₂ is an established and prominent inflammatory mediator in many tissues, our findings in Figure 1 indicate that an increase in endogenous PGE₂ formation is a feature of a more severe and chronic injury that is associated with inflammatory neovascularization rather than general inflammation of the cornea, suggesting an involvement of PGE₂ in mediating the angiogenic response. Thus, to directly examine the role of PGE₂ in neovascularization, mice were topically treated with PGE₂ (100 ng, three times a day) for 7 days after suture placement. Neovascularization, characterized by the development of new microvasculature arising from the limbus, was detected by vital microscopy and immunofluorescence using CD31/PECAM-1, a specific and established marker for vascular endothelial cells.^{15,41} Compared with saline-treated control mice, treatment with PGE₂ for 7 days markedly amplified pathologic neovascularization, as evidenced by a 54% increase in total blood vessels (Fig. 2B; PGE₂ = 49,636 ± 3048 pixels vs. saline = 32,257 ± 1814 pixels). In contrast, topical treatment with PGE₂ did not affect the rate of epithelial wound healing in a model of acute, self-resolving injury that directly depends on the extent of inflammation (Fig. 2A).^{13,37} Two days after epithelial debridement, vehicle- and PGE₂-treated mice exhibited

68% ± 3% and 71% ± 2% wound healing, respectively, and complete wound healing by 4 days. These findings demonstrate that PGE₂ specifically exacerbates inflammatory neovascularization, which correlates with its increased endogenous formation triggered by severe and chronic injury.

Regulation of Leukocyte Recruitment by PGE₂ Is Injury Specific

The normally avascular cornea in humans and mice is generally devoid of leukocytes. Suture-induced inflammatory neovascularization is a well-established model in which the underlying driving force of the neovascular response is inflammation.^{38,39} In this regard, leukocyte infiltration is an essential feature of suture-induced neovascularization. Thus, we assessed the effect of PGE₂, a prominent inflammatory mediator, on leukocyte recruitment. The predominant leukocyte type in suture-induced chronic injury, even 7 days after suture placement, is the PMN.⁴¹ PMN infiltration was quantified by measuring MPO activity as a specific marker for PMN.¹³⁻¹⁵ Uninjured corneas had no detectable MPO activity (Fig. 3); after 48 hours, both epithelial abrasion and suture injury significantly induced PMN infiltration. More importantly, topical PGE₂ treatment increased PMN infiltration 4.5-fold (saline = 45,223 ± 15,141 PMNs/cornea vs.

FIGURE 2. PGE₂ treatment exacerbates inflammatory neovascularization but does not affect epithelial wound healing. Mouse eyes were treated topically with PGE₂ (100 ng, three times a day) for up to 7 days after epithelial abrasion or suture injury. **(A)** The corneal epithelium was removed by mechanical abrasion with an Algerbrush. Epithelial wound healing was assessed by fluorescein staining and digital image analyses 48 and 96 hours after epithelial removal with or without topical PGE₂ treatment ($n = 9-12$). **(B)** An 8-0 silk suture was placed intrastromally, extending over the corneal apex without disrupting the iris. Hemangiogenesis was assessed by immunohistochemistry using CD31 as a specific endothelial antigen. Corneas were collected after 7 days of suture injury with or without PGE₂ treatment and were incubated in PBS containing FITC-conjugated CD31/PECAM-1 monoclonal antibody and analyzed under a microscope ($n = 4$; $*P < 0.05$ vs. saline treatment). Images show representative whole corneal flat mounts. CD31⁺ vessels in the entire cornea were traced manually and are expressed as total pixels.

PGE₂ = $246,818 \pm 44,917$ PMNs/cornea) in response to suture injury but did not affect PMN recruitment after epithelial abrasion, indicating that PGE₂ regulates PMN infiltration in an injury-specific manner.

PGE₂ Circuit Is Regulated by Inflammatory Neovascularization

COX-1 and COX-2 have traditionally been defined as the constitutive and inducible isoforms, respectively. However, basal expression of COX-2 has been detected in several tissues, including the corneal epithelium and endothelium,⁴² and the role of COX-1 in mediating inflammatory responses is now appreciated.⁴³ In this regard, it is important to define the expression and role of COX-1 and COX-2 in corneal inflamma-

tory neovascularization. The bioactions of PGE₂ are mediated by binding to EP receptors, of which there are four known subtypes (EP-1, EP-2, EP-3, and EP-4). Importantly, EP receptor expression has been documented in "normal" human corneal tissue^{44,45} and mouse ocular tissue by immunohistochemical analysis.⁴⁵ However, the regulation of EP receptor expression during a highly dynamic and complex inflammatory/repairative response is completely unknown in both human and mouse cornea. Thus, for direct detection of the PGE₂ circuit in the cornea, we used fluorescence-based kinetic (real-time) PCR. Quantitative real time-PCR analysis established the basal expression of the two COX isoforms and four EP receptor subtypes in uninjured corneas (Figs. 4A, 4B). Although COX-1 expression was induced with suture injury (0.19 ± 0.01 vs. 0.30 ± 0.02 relative quantity (RQ) in uninjured and suture injury, respectively; $P < 0.05$), gene expression of COX-2 was upregulated 54-fold (0.21 ± 0.04 vs. 11.46 ± 2.8 RQ in uninjured and suture injury, respectively; $P < 0.05$), suggesting that the increased endogenous PGE₂ formation seen with neovascularization can likely be attributed to the induction of COX-2. More importantly, expression of the EP receptors was regulated by chronic inflammation. After 2 days of suture injury, the gene expression of EP-1 was unchanged, whereas expression of EP-2, EP-3, and EP-4 all increased, with EP-2 and EP-4 exhibiting 14-fold and 8-fold increases, respectively ($P < 0.05$). Based on reports that EP-3 signaling upregulates tumor-associated angiogenesis and tumor growth,⁴⁶ we treated mice with sulprostone, a selective EP-3 agonist, to assess its role in corneal inflammatory neovascularization. Sulprostone did not affect pathologic angiogenesis during chronic injury (Supplementary Fig. S2, <http://www.iovs.org/content/51/12/6311/suppl/DC1>). These results are in agreement with our gene expression data and suggest that EP-3 does not play a major role in regulating corneal inflammatory neovascularization. Immunohistochemical analysis demonstrated the distinct expression of both EP-2 and EP-4 receptors in uninjured corneal epithelium and endothelium, with a weak expression in the stroma (Fig. 4C). In correlation with their upregulated gene expression, immunohistochemical detection of EP-2 and EP-4 receptors was markedly enhanced in the stroma and was ex-

FIGURE 3. PGE₂ regulation of neutrophil infiltration is injury specific. Corneas were collected from uninjured eyes and after 48 hours of epithelial abrasion or suture injury with or without PGE₂ treatment (100 ng, three times a day). PMN content in corneas was quantified by measuring MPO activity as a specific marker for PMN ($n = 4-10$; $*P < 0.05$ vs. saline treatment). An MPO calibration curve was established with inflammatory exudate peritoneal PMNs and was used to calculate relative tissue PMN numbers.

FIGURE 4. The PGE₂ circuit is expressed in the cornea and selectively regulated by inflammatory neovascularization. (A, B) mRNA expression of COX isoforms and EP receptor subtypes in mouse corneas collected from uninjured eyes and after 2 days of suture injury was quantified by real-time PCR and normalized to β -actin ($n = 3-5$; * $P < 0.05$ vs. uninjured). mRNA expression is expressed as relative quantity based on a mouse positive RNA control using the $\Delta\Delta C_T$ method. (C) Mouse eyes were collected, fixed, and sectioned, and 5- μ m sections were probed with antibodies for EP-2 or EP-4 receptors ($n = 3-4$). Shown are representative immunohistochemical images of corneas from uninjured eyes, 4 days after epithelial abrasion or 7 days after suture injury.

pressed in infiltrating leukocytes after 7 days of suture injury compared with uninjured corneas. In contrast, no changes in EP-2 or EP-4 receptor expression were detected 4 days after initiating abrasion injury. Taken together, these data demonstrate that the PGE₂ circuit is expressed in the uninjured cornea and, moreover, is differentially regulated by corneal inflammatory responses. A more severe and chronic injury that was associated with inflammatory neovascularization, but not an acute self-resolving epithelial injury, upregulated the PGE₂ circuit.

PGE₂ Regulates Expression of Inflammatory and Angiogenic Mediators

Angiogenic growth factors, including those of the vascular endothelial growth factor (VEGF) family, have been implicated in mediating neovascularization in the normally avascular cornea.^{39,47,48} Traditionally, hemangiogenesis has been attributed to the actions of VEGF-A^{39,47}; however, VEGF-C is now recognized as another important mediator of inflammatory hemangiogenesis.^{49,50} VEGF-mediated inflammatory hemangiogenesis is governed through binding to specific tyrosine kinase receptors, of which three isoforms—VEGFR-1/FLT-1, VEGFR-2/FLK-1, VEGFR-3/FLT-4—have been identified. In addition, a soluble form of VEGFR-1, sFLT-1, exists in the cornea and acts to trap VEGF-A. Thus, to further characterize the angiogenic response to suture injury, we examined the gene expression of select key mediators of inflammation and angiogenesis in uninjured corneas and, after 2 days of suture injury, with and

without PGE₂ treatment. Consistent with the model of inflammatory neovascularization and our previous findings,¹⁵ key members of the VEGF circuit were markedly upregulated with suture injury, including VEGF-A and VEGF-C, as well as VEGFR-3/FLT-4, the receptor for VEGF-C (Fig. 5). Interestingly, topical treatment with PGE₂ during chronic inflammation did not alter the expression of VEGF-A or other key mediators of the VEGF circuit. In fact, PGE₂ treatment tended to reduce the expression of these mediators, indicating that PGE₂ exacerbation of inflammatory neovascularization is independent of the VEGF circuit.

Given that PGE₂ exacerbation of inflammatory neovascularization was shown to be independent of the VEGF circuit, we analyzed the possible involvement of the eotaxin-CCR3 axis because this circuit has recently been shown to contribute to choroidal neovascularization characteristic of age-related macular degeneration.⁵¹ CCR3 gene expression was markedly increased after 2 days of suture injury and remained elevated at 7 days compared with corneas from uninjured eyes (Fig. 6). More importantly, topical PGE₂ treatment for 7 days significantly increased CCR3 expression 1.2-fold compared with suture alone (0.18 ± 0.04 vs. 0.08 ± 0.01 RQ, respectively). We then examined the gene expression of eotaxin-1 and eotaxin-2 (CCL11 and CCL24, respectively), the principal mouse ligands for CCR3, after 7 days of suture injury. Although eotaxin-1 gene expression was unaffected by PGE₂ treatment, the gene expression of eotaxin-2 was increased 3.4-fold with PGE₂ treatment com-

FIGURE 5. PGE₂ regulation of inflammatory neovascularization is independent of the VEGF circuit. mRNA expression of key mediators of the VEGF circuit, VEGF-A, and VEGF-C, soluble VEGF receptor-1 (sFLT-1), and VEGF receptor-3 (FLT4) was quantified by real-time PCR in corneas collected from uninjured eyes and after 48 hours of suture injury or after 7 days of suture injury for VEGF receptor-2 (FLK) ($n = 5-9$; $*P < 0.05$ vs. uninjured, $\#P < 0.05$ vs. suture). mRNA expression is expressed as relative quantity based on a mouse positive RNA control using the $\Delta\Delta C_T$ method.

pared with suture alone (0.61 ± 0.14 vs. 0.14 ± 0.03 RQ, respectively; $P < 0.05$). Collectively, these data suggest that PGE₂ exacerbation of inflammatory neovascularization involves regulation of the eotaxin-CCR3 circuit.

DISCUSSION

Lipid autacoids, such as COX-derived prostaglandins, mediate a broad range of physiological processes and, more importantly, are some of the earliest signals triggered by injury and stress.

FIGURE 6. Topical PGE₂ upregulates the expression of a novel regulator of ocular angiogenesis. mRNA expression of the eotaxin-CCR3 circuit was quantified by real-time PCR in corneas collected from uninjured eyes and after 2 or 7 days of suture injury ($n = 3-11$; $*P < 0.05$ vs. uninjured; $\#P < 0.05$ vs. suture). mRNA expression is expressed as relative quantity based on a mouse positive RNA control using the $\Delta\Delta C_T$ method.

Among the five classical prostanoids derived from COX-dependent metabolism of AA, PGE₂ is the major product in most tissues. Indeed, it is one of the most prominent inflammatory mediators released in response to injury. Although it has long been detected in animal models of ocular inflammation,⁵² the role of the PGE₂ circuit in mediating inflammatory responses in the cornea remains poorly defined. Using two well-established and distinct injury models, the present study demonstrates for the first time that endogenous PGE₂ in the cornea is not a general inflammatory mediator. Lipidomic analyses revealed that endogenous levels of PGE₂ do not significantly increase after initiating an acute epithelial abrasion injury (Fig. 1). In sharp contrast, PGE₂ formation markedly increased with a more severe and chronic corneal injury that correlated with the development of pathologic angiogenesis and induction of EP-2 and EP-4 expression in the stroma (Fig. 4). Hence, our findings indicate an injury-specific and selective role for the PGE₂ circuit in mediating corneal inflammatory responses, whose induction likely depends on the extent and duration of tissue injury. In this regard, it is important to note that in a model of hypoxia-induced corneal inflammation, the induction of COX-2 expression was not associated with a parallel increase in PGE₂ formation.⁵³ In accordance with the induction of PGE₂ formation as a selective response to chronic injury, amplification of the PGE₂ circuit by topical treatment with PGE₂ amplified inflammatory neovascularization (Fig. 2). In sharp contrast, treatment with PGE₂ did not affect the wound healing response induced by acute epithelial abrasion.

Inflammation and angiogenesis are intimately linked and fundamental responses to injury. Indeed, a body of evidence indicates that many of the cell types involved in inflammatory processes also release several factors that act directly or indirectly on endothelial cells to promote an angiogenic response.⁵⁴ Moreover, suture-induced inflammatory angiogenesis, with both inflammatory and angiogenic components, is a dynamic response. In this regard, our data show that PGE₂ treatment amplified both pathologic angiogenesis and PMN recruitment in response to chronic suture injury (Figs. 2, 3). Indeed, the ability of PGE₂ to induce an angiogenic response and to modulate inflammatory and immune responses is well documented.^{18,34,55,56} Hence, we cannot discern whether

PGE₂ exacerbation of inflammatory neovascularization is the result of direct amplification of angiogenesis or enhancement of the inflammatory response; further studies are needed to dissect the contribution of these two components to PGE₂ regulation of corneal inflammatory neovascularization.

PGE₂ is produced by two important enzymes, COX-1 and COX-2. Because of the ubiquitous and constitutive expression of COX-1, it has been widely associated with homeostatic functions, whereas COX-2 is mainly an inducible enzyme that has been linked to pathophysiological PG generation.^{8,57} Consistent with the increased levels of PGE₂ in the cornea, we observed a robust induction of COX-2 gene expression with chronic injury (Fig. 4). Expression of COX-1 was also upregulated, suggesting that the increase in endogenous PGE₂ formation and the subsequent neovascularization seen with suture injury may be attributed to COX-1, COX-2, or both. Indeed, early studies in rabbits and rats have shown that topical application of both selective COX-2 and nonselective COX inhibitors reduced corneal neovascularization induced by silver/potassium nitrate cauterization.^{58–60} However, the pronounced induction of COX-2 in our study suggests that it likely is the prominent source of PGE₂ in the cornea, which is consistent with the role of inducible COX-2 activity in many inflammatory responses.

The myriad and specificity of bioactions of PGE₂ can be attributed to the four EP receptor subtypes, whose cellular expression can be regulated by inflammation and PGE₂ itself.^{19,61} It is well established that all four EP receptors contribute to the regulation of cell proliferation, tumor angiogenesis and growth, including mammary epithelial hyperplasia and breast and colon carcinogenesis.^{62–65} However, the role of EP receptors in the cornea is just beginning to unfold. In particular, their regulation during the highly dynamic and complex inflammatory neovascularization has not been determined. We detected basal gene expression of all four EP receptor subtypes in uninjured corneal tissue (Fig. 4), findings that are supported by immunohistochemical data previously reported.⁴⁵ Importantly, though gene expression of EP-3 showed a trend to increase, the expression of EP-2 and EP-4 receptors was markedly upregulated with chronic but not acute injury, correlating with the selective induction of the PGE₂ circuit during inflammatory neovascularization. Indeed, recent evidence implicates a role for EP receptors in ocular angiogenesis. Activation of the EP-4 receptor is reported to contribute to laser-induced choroidal neovascularization and oxygen-induced retinopathy in rat models.⁶⁶ In dogs, corneal neovascularization was a toxic side effect associated with topical administration of a selective EP-4 receptor agonist used to lower intraocular pressure.⁶⁷ In addition, corneal implants of hydroxyethylmethacrylate pellets (Hydron; Interferon Sciences, New Brunswick, NJ) containing an EP-2 or EP-4 agonist induced neovascularization in mice,⁶⁸ and angiogenesis was reduced in EP-2^{−/−} mice in response to basic fibroblast growth factor stimulation by a corneal micro-pocket assay.⁶⁹ EP receptors are expressed on a variety of cell types, including keratocytes, endothelial and epithelial cells, and inflammatory cells (i.e., macrophages and PMNs), and the cellular source of EP-2 and EP-4 during corneal inflammatory neovascularization remains to be determined. Interestingly, both EP-2^{−/−} and EP-4^{−/−} mice exhibit altered ocular inflammatory responses to lipopolysaccharide, implicating the role of these receptors in leukocyte infiltration.³³ Hence, selective upregulation of EP-2 and EP-4 suggests that distinct injury responses, namely, chronic and acute inflammation, induce differential cellular lipid mediator circuits.

Angiogenesis, or the growth of microvessels from existing vessels, is a complex multistep process involving extracellular matrix degradation and the migration, survival, and proliferation of preexisting endothelial cells. This process is regulated

by a variety of mediators, including growth factors and their cell surface receptors, matrix-degrading enzymes, and adhesion receptors. The VEGF family of angiogenic factors and their receptors are key and traditional mediators of ocular pathologic angiogenesis.⁴⁷ Accordingly, downregulation or upregulation of the VEGF circuit results in parallel changes in angiogenesis. Indeed, recent evidence shows that the antiangiogenic effect of LXA₄ can be attributed, at least in part, to the downregulation of key members of the VEGF family.¹⁵ In contrast, PGE₂ upregulation of VEGF has been associated with several experimental models of angiogenesis^{66,70,71} and contributes to the initiation of diabetic retinopathy.⁷² Interestingly, our data demonstrate that exacerbated inflammatory neovascularization with PGE₂ treatment is independent of the VEGF circuit (Fig. 5). This finding illustrates the complexity of the PGE₂ circuit in which the bioactions and signaling of PGE₂ occur in a temporal, tissue-, and cell type-specific manner.

In this regard, our study uncovers a previously unknown association of the PGE₂ circuit with the eotaxin-CCR3 axis (Fig. 6), a newly identified pathway that contributes to ocular pathologic angiogenesis, specifically retinal neovascularization.⁵¹ Consistent with the proposed role of the eotaxin-CCR3 axis in retinal angiogenesis, we observed a significant upregulation of the chemokine receptor CCR3 and the CCR3 ligands eotaxin-1 and eotaxin-2, with chronic injury that induced pathologic corneal angiogenesis. Eotaxin-1 has been reported to directly induce angiogenic responses by human, rat, mouse, and chick endothelial cells.⁷³ Interestingly, PGE₂ treatment further increased the expression of eotaxin-2 but not of eotaxin-1. Further studies are required to establish a direct link between EP receptor signaling and activation of the eotaxin-CCR3 axis and to determine the contribution of eotaxin-1 and eotaxin-2. Moreover, if other signaling pathways mediate PGE₂ modulation of inflammatory neovascularization in the cornea remains to be investigated. In other tissues, evidence has demonstrated PGE₂ to be a potent modulator of angiogenesis *ex vivo* and *in vitro* through the induction of angiogenic regulatory proteins such as basic fibroblast growth factor and amphiregulin, a ligand for epidermal growth factor receptors.^{71,74,75} We also cannot exclude a direct effect to promote angiogenesis because PGE₂/EP has been reported to enhance the survival of endothelial cells and to promote endothelial cell proliferation, migration, and tubulogenesis.^{66,68,69,76}

Pathologic angiogenesis in the cornea and retina affects millions of people worldwide and is a key feature of several forms of ocular disease, including age-related macular degeneration, retinopathy of prematurity, and keratitis (herpetic and bacterial). Thus, elucidation of endogenous pathways that regulate or promote inflammatory neovascularization is of primary interest. PGE₂ is a prevalent inflammatory mediator in many tissues, but the endogenous role of the PGE₂ circuit in mediating inflammatory responses in the cornea remains to be clearly defined. Our data demonstrate that the PGE₂ circuit is present in the cornea and is selectively upregulated in response to a more severe and chronic nonresolving inflammation. Moreover, amplification of this endogenous circuit with topical PGE₂ selectively exacerbated PMN trafficking and angiogenesis in the chronically inflamed cornea but did not alter the sequelae of an acute injury response. PGE₂ exacerbation of inflammatory neovascularization was unexpectedly independent of the VEGF circuit and was associated with induction of the eotaxin-CCR3 axis, a novel regulator of ocular angiogenesis. Together, our data provide strong evidence for an injury-specific and selective role for the PGE₂ circuit in mediating corneal inflammatory and angiogenic responses, which is of interest given the fact that this lipid circuit is a major clinical target for current treatment options for ocular diseases.

References

- Gronert K. Lipid autacoids in inflammation and injury responses: a matter of privilege. *Mol Interv.* 2008;8:28–35.
- McDermott AM, Perez V, Huang AJ, et al. Pathways of corneal and ocular surface inflammation: a perspective from the Cullen symposium. *Ocul Surf.* 2005;3:S131–S138.
- Bazan HE. Cellular and molecular events in corneal wound healing: significance of lipid signalling. *Exp Eye Res.* 2005;80:453–463.
- Streilein JW. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol.* 2003;3:879–889.
- Laniado-Schwartzman M, Dunn MW. Cytochrome P450-derived eicosanoids mediators of ocular surface inflammation. *Adv Exp Med Biol.* 2003;525:47–54.
- Tjebbes GW, van Delft JL, van Haeringen NJ. Production of lipid mediators in experimental keratitis of rabbit eye. *J Lipid Mediators.* 1993;8:87–93.
- Gronert K. Lipoxins in the eye and their role in wound healing. *Prostaglandins Leukot Essent Fatty Acids.* 2005;73:221–229.
- Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science.* 2001;294:1871–1875.
- Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol.* 2008;8:349–361.
- Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. *Trends Immunol.* 2002;23:144–150.
- Rocca B, FitzGerald GA. Cyclooxygenases and prostaglandins: shaping up the immune response. *Int Immunopharmacol.* 2002;2:603–630.
- Chang MS, Schneider C, Roberts RL, et al. Detection and subcellular localization of two 15S-lipoxygenases in human cornea. *Invest Ophthalmol Vis Sci.* 2005;46:849–856.
- Gronert K, Maheshwari N, Khan N, Hassan IR, Dunn M, Laniado-Schwartzman M. A role for the mouse 12/15-lipoxygenase pathway in promoting epithelial wound healing and host defense. *J Biol Chem.* 2005;280:15267–15278.
- Biteman B, Hassan IR, Walker E, et al. Interdependence of lipoxin A₄ and heme-oxygenase in counter-regulating inflammation during corneal wound healing. *FASEB J.* 2007;21:2257–2266.
- Leedom AJ, Sullivan AB, Dong B, Lau D, Gronert K. Endogenous LXA₄ circuits are determinants of pathological angiogenesis in response to chronic injury. *Am J Pathol.* 176:74–84.
- Samuelsson B, Morgenstern R, Jakobsson PJ. Membrane prostaglandin synthase-1: a novel therapeutic target. *Pharmacol Rev.* 2007;59:207–224.
- Wang D, Dubois RN. Prostaglandins and cancer. *Gut.* 2006;55:115–122.
- Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacol Ther.* 2004;103:147–166.
- Sugimoto Y, Narumiya S. Prostaglandin E receptors. *J Biol Chem.* 2007;282:11613–11617.
- Kobayashi T, Narumiya S. Function of prostanoid receptors: studies on knockout mice. *Prostaglandins Other Lipid Mediat.* 2002;68–69:557–573.
- Moncada S, Ferreira SH, Vane JR. Prostaglandins, aspirin-like drugs and the oedema of inflammation. *Nature.* 1973;246:217–219.
- Honda T, Matsuoka T, Ueta M, Kabashima K, Miyachi Y, Narumiya S. Prostaglandin E(2)-EP(3) signaling suppresses skin inflammation in murine contact hypersensitivity. *J Allergy Clin Immunol.* 2009;124:809–818, e802.
- Kabashima K, Saji T, Murata T, et al. The prostaglandin receptor EP₄ suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J Clin Invest.* 2002;109:883–893.
- Kunikata T, Yamane H, Segi E, et al. Suppression of allergic inflammation by the prostaglandin E receptor subtype EP₃. *Nat Immunol.* 2005;6:524–531.
- Ueta M, Matsuoka T, Narumiya S, Kinoshita S. Prostaglandin E receptor subtype EP₃ in conjunctival epithelium regulates late-phase reaction of experimental allergic conjunctivitis. *J Allergy Clin Immunol.* 2009;123:466–471.
- Kunkel SL, Spengler M, May MA, Spengler R, Larrick J, Remick D. Prostaglandin E₂ regulates macrophage-derived tumor necrosis factor gene expression. *J Biol Chem.* 1988;263:5380–5384.
- Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE₂, and PAF. *J Clin Invest.* 1998;101:890–898.
- Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol.* 2001;2:612–619.
- Bazan HE, Birkle DL, Beuerman R, Bazan NG. Cryogenic lesion alters the metabolism of arachidonic acid in rabbit cornea layers. *Invest Ophthalmol Vis Sci.* 1985;26:474–480.
- Preud'homme Y, Demolle D, Boeynaems JM. Metabolism of arachidonic acid in rabbit iris and retina. *Invest Ophthalmol Vis Sci.* 1985;26:1336–1342.
- Kulkarni PS, Fleisher L, Srinivasan BD. The synthesis of cyclooxygenase products in ocular tissues of various species. *Curr Eye Res.* 1984;3:447–452.
- Saeki T, Ota T, Aihara M, Araie M. Effects of prostanoid EP agonists on mouse intraocular pressure. *Invest Ophthalmol Vis Sci.* 2009;50:2201–2208.
- Biswas S, Bhattacharjee P, Paterson CA, Tilley SL, Koller BH. Ocular inflammatory responses in the EP₂ and EP₄ receptor knockout mice. *Ocul Immunol Inflamm.* 2006;14:157–163.
- Kulkarni PS, Srinivasan BD. The effect of intravitreal and topical prostaglandins on intraocular inflammation. *Invest Ophthalmol Vis Sci.* 1982;23:383–392.
- Biswas S, Bhattacharjee P, Paterson CA, Maruyama T, Narumiya S. Modulation of ocular inflammatory responses by EP₁ receptors in mice. *Exp Eye Res.* 2007;84:39–43.
- Gronert K, Clish CB, Romano M, Serhan CN. Transcellular regulation of eicosanoid biosynthesis. *Methods Mol Biol.* 1999;120:119–144.
- Li Z, Burns AR, Smith CW. Two waves of neutrophil emigration in response to corneal epithelial abrasion: distinct adhesion molecule requirements. *Invest Ophthalmol Vis Sci.* 2006;47:1947–1955.
- Cursiefen C, Maruyama K, Jackson DG, Streilein JW, Kruse FE. Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea.* 2006;25:443–447.
- Cursiefen C, Chen L, Borges LP, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest.* 2004;113:1040–1050.
- McClellan SA, Huang X, Barrett RP, van Rooijen N, Hazlett LD. Macrophages restrict *Pseudomonas aeruginosa* growth, regulate polymorphonuclear neutrophil influx, and balance pro- and anti-inflammatory cytokines in BALB/c mice. *J Immunol.* 2003;170:5219–5227.
- Bellner L, Vitto M, Patil KA, Dunn MW, Regan R, Laniado-Schwartzman M. Exacerbated corneal inflammation and neovascularization in the HO-2 null mice is ameliorated by biliverdin. *Exp Eye Res.* 2008;87:268–278.
- Bazan HE, Tao Y, DeCoster MA, Bazan NG. Platelet-activating factor induces cyclooxygenase-2 gene expression in corneal epithelium: requirement of calcium in the signal transduction pathway. *Invest Ophthalmol Vis Sci.* 1997;38:2492–2501.
- Loftin CD, Tian HF, Langenbach R. Phenotypes of the COX-deficient mice indicate physiological and pathophysiological roles for COX-1 and COX-2. *Prostaglandins Other Lipid Mediat.* 2002;68–69:177–185.
- Schlotzer-Schrehardt U, Zenkel M, Nusing RM. Expression and localization of FP and EP prostanoid receptor subtypes in human ocular tissues. *Invest Ophthalmol Vis Sci.* 2002;43:1475–1487.
- Biswas S, Bhattacharjee P, Paterson CA. Prostaglandin E₂ receptor subtypes, EP₁, EP₂, EP₃ and EP₄ in human and mouse ocular tissues—a comparative immunohistochemical study. *Prostaglandins Leukot Essent Fatty Acids.* 2004;71:277–288.
- Katoh H, Hosono K, Ito Y, et al. COX-2 and prostaglandin EP₃/EP₄ signaling regulate the tumor stromal proangiogenic microenvironment via CXCL12-CXCR4 chemokine systems. *Am J Pathol.* 2010;176:1469–1483.

47. Amano S, Rohan R, Kuroki M, Tolentino M, Adamis AP. Requirement for vascular endothelial growth factor in wound- and inflammation-related corneal neovascularization. *Invest Ophthalmol Vis Sci*. 1998;39:18–22.
48. Cursiefen C, Chen L, Saint-Geniez M, et al. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. *Proc Natl Acad Sci U S A*. 2006;103:11405–11410.
49. Tammela T, Zarkada G, Wallgard E, et al. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature*. 2008;454:656–660.
50. Chung ES, Chauhan SK, Jin Y, et al. Contribution of macrophages to angiogenesis induced by vascular endothelial growth factor receptor-3-specific ligands. *Am J Pathol*. 2009;175:1984–1992.
51. Takeda A, Baffi JZ, Kleinman ME, et al. CCR3 is a target for age-related macular degeneration diagnosis and therapy. *Nature*. 2009;460:225–230.
52. Eakins KE, Whitelocke RA, Perkins ES, Bennett A, Unger WG. Release of prostaglandins in ocular inflammation in the rabbit. *Nat New Biol*. 1972;239:248–249.
53. Schwartzman ML, Bonazzi A, Miesel P, Mezentssev A, Abraham NG, Dunn MW. COX-2 lack of function in hypoxia-induced ocular surface inflammation. *Thromb Res*. 2003;110:293–298.
54. Costa C, Incio J, Soares R. Angiogenesis and chronic inflammation: cause or consequence? *Angiogenesis*. 2007;10:149–166.
55. Form DM, Auerbach R. PGE2 and angiogenesis. *Proc Soc Exp Biol Med*. 1983;172:214–218.
56. BenEzra D. Neovascularogenic ability of prostaglandins, growth factors, and synthetic chemoattractants. *Am J Ophthalmol*. 1978;86:455–461.
57. Smith WL, Langenbach R. Why there are two cyclooxygenase isozymes. *J Clin Invest*. 2001;107:1491–1495.
58. Haynes WL, Proia AD, Klintworth GK. Effect of inhibitors of arachidonic acid metabolism on corneal neovascularization in the rat. *Invest Ophthalmol Vis Sci*. 1989;30:1588–1593.
59. Yamada M, Kawai M, Kawai Y, Mashima Y. The effect of selective cyclooxygenase-2 inhibitor on corneal angiogenesis in the rat. *Curr Eye Res*. 1999;19:300–304.
60. Frucht J, Zauberman H. Topical indomethacin effect on neovascularisation of the cornea and on prostaglandin E2 levels. *Br J Ophthalmol*. 1984;68:656–659.
61. Weinreb M, Machwate M, Shir N, Abramovitz M, Rodan GA, Harada S. Expression of the prostaglandin E(2) (PGE(2)) receptor subtype EP(4) and its regulation by PGE(2) in osteoblastic cell lines and adult rat bone tissue. *Bone*. 2001;28:275–281.
62. Chang SH, Ai Y, Breyer RM, Lane TF, Hla T. The prostaglandin E2 receptor EP2 is required for cyclooxygenase 2-mediated mammary hyperplasia. *Cancer Res*. 2005;65:4496–4499.
63. Chang SH, Liu CH, Conway R, et al. Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. *Proc Natl Acad Sci U S A*. 2004;101:591–596.
64. Amano H, Hayashi I, Endo H, et al. Host prostaglandin E(2)-EP3 signaling regulates tumor-associated angiogenesis and tumor growth. *J Exp Med*. 2003;197:221–232.
65. Kawamori T, Kitamura T, Watanabe K, et al. Prostaglandin E receptor subtype EP(1) deficiency inhibits colon cancer development. *Carcinogenesis*. 2005;26:353–357.
66. Yanni SE, Barnett JM, Clark ML, Penn JS. The role of PGE2 receptor EP4 in pathologic ocular angiogenesis. *Invest Ophthalmol Vis Sci*. 2009;50:5479–5486.
67. Aguirre SA, Huang W, Prasanna G, Jessen B. Corneal neovascularization and ocular irritancy responses in dogs following topical ocular administration of an EP4-prostaglandin E2 agonist. *Toxicol Pathol*. 2009;37:911–920.
68. Kuwano T, Nakao S, Yamamoto H, et al. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB J*. 2004;18:300–310.
69. Kamiyama M, Pozzi A, Yang L, DeBusk LM, Breyer RM, Lin PC. EP2, a receptor for PGE2, regulates tumor angiogenesis through direct effects on endothelial cell motility and survival. *Oncogene*. 2006;25:7019–7028.
70. Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett*. 1995;372:83–87.
71. Cheng T, Cao W, Wen R, Steinberg RH, LaVail MM. Prostaglandin E2 induces vascular endothelial growth factor and basic fibroblast growth factor mRNA expression in cultured rat Muller cells. *Invest Ophthalmol Vis Sci*. 1998;39:581–591.
72. Frank RN. Diabetic retinopathy. *N Engl J Med*. 2004;350:48–58.
73. Salcedo R, Young HA, Ponce ML, et al. Eotaxin (CCL11) induces in vivo angiogenic responses by human CCR3+ endothelial cells. *J Immunol*. 2001;166:7571–7578.
74. Subbaramaiah K, Benezra R, Hudis C, Dannenberg AJ. Cyclooxygenase-2-derived prostaglandin E2 stimulates Id-1 transcription. *J Biol Chem*. 2008;283:33955–33968.
75. Finetti F, Solito R, Morbidelli L, Giachetti A, Ziche M, Donnini S. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1. *J Biol Chem*. 2008;283:2139–2146.
76. Rao R, Redha R, Macias-Perez I, et al. Prostaglandin E2-EP4 receptor promotes endothelial cell migration via ERK activation and angiogenesis in vivo. *J Biol Chem*. 2007;282:16959–16968.