Role of Inflammatory Cytokine-Induced Cyclooxygenase 2 in the Ocular Immunopathologic Disease Herpetic Stromal Keratitis

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Ocular infection with herpes simplex virus (HSV) results in a blinding immunoinflammatory stromal keratitis (SK) lesion. Early preclinical events include polymorphonuclear neutrophil (PMN) infiltration and neovascularization in the corneal stroma. We demonstrate here that HSV infection of the cornea results in the upregulation of the cyclooxygenase 2 (COX-2) enzyme. Early after infection, COX-2 was produced from uninfected stromal fibroblasts as an indirect effect of virus infection. Subsequently, COX-2 may also be produced from other inflammatory cells that infiltrate the cornea. The induction of COX-2 is a critical event, since inhibition of COX-2 with a selective inhibitor was shown to reduce corneal angiogenesis and SK severity. The administration of a COX-2 inhibitor resulted in compromised PMN infiltration into the cornea, as well as diminished corneal vascular endothelial growth factor levels, likely accounting for the reduced angiogenic response. COX-2 stimulation by HSV infection represents a critical early event accessible for therapy and the control of SK severity.

Herpes stromal keratitis (HSK) is a chronic immunoinflammatory reaction in the corneal stroma caused by ocular infection with herpes simplex virus (HSV) (35). The pathogenesis is complex with the key lesion-producing event being the influx of inflammatory T cells that orchestrate the stromal keratitis (SK) lesions (26, 29). However, multiple events occur early after infection which, if inhibited, can control the SK lesion severity (11). These events include the production of proinflammatory mediators, prominent invasion by polymorphonuclear neutrophil (PMN) and corneal angiogenesis (11). However, the factors responsible for initiating the inflammatory and angiogenic events in the corneal stroma after virus infection are not still well understood. Thus, multiple cytokines, chemokines, and angiogenic factors have been identified as early participants, but their cellular source and mechanism of induction by virus infection are not clear. Studies in other models of inflammation, including ocular inflammation, have implicated cyclooxygenase 2 (COX-2)-induced prostanooids as key factors in controlling the production of early inflammatory mediators (30, 32, 36). In addition, COX-2-induced production of prostanooids is often implicated in chronic inflammatory diseases, characterized by edema, production of chemotactic factors, and infiltration of inflammatory cells (1, 37). Unlike COX-1, which is a constitutive enzyme and is essential for various physiological functions, COX-2 is an inducible enzyme that is induced in a variety of cell types by diverse stimuli (41). At present, it is not known whether a COX-2-mediated inflammatory cascade represents a key event in the pathogenesis of SK.

The present study was undertaken with two major objectives. First, to find out whether HSV infection causes upregulation of COX-2 expression in the cornea and, second, to investigate the role of COX-2 in SK. Our results indicate that COX-2 is expressed promptly after virus infection. Blocking COX-2, as could be achieved by a selective COX-2 inhibitor, resulted in diminished corneal angiogenesis and SK severity in COX-2 inhibitor treated mice compared to vehicle control animals. This difference in disease phenotype was an indirect event and was shown to be the consequence of a compromised early inflammatory response. Taken together, our results demonstrate that COX-2-mediated prostanooid production is critical in SK pathogenesis and that the use of drugs to inhibit COX-2 represents a valuable approach for disease control.

MATERIALS AND METHODS

Mice. Wild-type, female, 6- to 8-week-old, BALB/c and C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were sex and age matched for all experiments. Mice transgenic (Tg) for interleukin-1 (IL-1) ra protein (T14 hemizygous line) were kindly provided by David Hirsh (Department of Biochemistry and Molecular Biophysics, College of Physician and Surgeons, Columbia University, New York, NY). All manipulations involving the immunocompromised mice were performed in a laminar flow hood. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Virus. HSV type 1 (HSV-1) RE (obtained from Robert Hendricks Laboratory, University of Pittsburgh School of Medicine, PA) was used in the present study. HSV-1 RE-pgC-green fluorescent protein (GFP) was derived in the RE strain background of HSV-1, detailed by the Hendricks group (23), and is a null mutant at the gC locus. For construction, a cloned PstI-EcoRI fragment (sequences from positions 95811 to 96789 in the wild-type HSV-1 genome) containing the gC promoter and first part of the gC open reading frame (ORF) in the corresponding sites of puc8, was first collapsed by digestion, blunt end generation, and religation to remove PstI, HindIII, and sites in between. An NheI-XbaI fragment derived from pEGFP-N1 (Clontech, Palo Alto, CA) was then inserted into the unique Nhel site located at the sequence encoding residue 6 of gC, resulting in an in-frame placement of the enhanced GFP (EGFP) gene immediately after the glycophorin C first six residues and gC promoter. This plasmid was linearized, cotransfected with purified HSV-1 RE DNA into Vero cells by calcium

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phosphate coprecipitation, and progeny virus with EGF driven by the native gC promoter were identified by fluorescence and plaque purified, and the insert was confirmed by Southern blot analysis. The virus was propagated and titrated on a monolayer of Vero cells (American Type Culture Collection [Manassas, VA] catalog no. CCL81) by standard protocols (34). Infected Vero cells were harvested, titrated, and stored in aliquots at −80°C until used.

E. coli LPS and endotoxin were used as positive controls of COX-1 and COX-2 and have been used previously (18, 24). BALB/c mice were administered 500 μg of anti-Gr-1 antibody (clone RB6-8C5; BD Pharmingen) and isotype controls for 30 min. Neutrophil depletion was performed before the corneal micropocket assay was performed (4). For detection of COX-2, goat anti-Cox-2 (M-19) (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:100 and incubated for 1 h at room temperature. Samples were then treated with rabbit anti-goat IgG biotinylated antibody (1:200; Vector Laboratories, Burlingame, CA), followed by horseradish peroxidase-conjugated streptavidin for 45 min (1:10,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) and 3,3′-diaminobenzidine substrate (Biogenex, San Ramon, CA) and counterstained with hematoxylin (Richard Allen Scientific, Kalamazoo, MI). Irrelevant biotinylated antibody was used as a negative control.

Histopathology. For histopathologic analysis, eyes from BALB/c mice were extirpated at day 20 p.i. and fixed in 10% buffered neutral formalin. Staining was performed with hematoxylin and eosin (Richard Allen Scientific, Kalamazoo, MI).

Immunohistochemistry and immunofluorescence staining. For immunohistochemistry, eyes from BALB/c mice were enucleated at the indicated time points and snap-frozen in OCT compound (Miles, Elkart, IN). Six-micron-thick sections were cut, air dried, and fixed in acetone-methanol (1:1) at −20°C for 10 min. Endogenous peroxidase activity was blocked with a 50% alcohol solution containing 0.3% hydrogen peroxide for 15 min, and sections were blocked with 3% bovine serum albumin—phosphate-buffered saline. For detection of COX-2, goat anti-Cox-2 (M-19) (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:100 and incubated for 1 h at room temperature. Sections were then treated with rabbit anti-goat IgG biotinylated antibody (1:200; Vector Laboratories, Burlingame, CA), followed by streptavidin Alexa Fluor 546 (Molecular Probes) for 25 min. The cells were thoroughly washed and spun down on microscopic glass slide by using a cytopsin and mounted with Vectashield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA) and visualized under a confocal microscope (Leica, Wetzlar, Germany).

ELISA of corneal lysate. For preparation of corneal lysates, six corneas per time point were pooled and processed as described previously (2). Lysates were analyzed by a standard sandwich enzyme-linked immunosorbent assay (ELISA) protocol. Anti-IL-6 capture and biotinylated detection antibodies were from BD Pharmingen (clone MP5-20F3), and standard recombinant murine IL-6 (r-mIL-6) was from R&D Systems (Minneapolis, MN). Anti-IL-1α, anti-IL-1β, anti-IL-18, anti-VEGF, anti-IL-18, and anti-TNF-α capture, biotinylated detection antibodies and recombinant standards were from R&D Systems. The color reaction was developed by using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (Sigma-Aldrich) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Prostaglandin E2 extraction and quantification was according to the manufacturer’s protocol. Briefly, one to five corneas per time point were homogenized in a microtome, dipped briefly in 100 μM indomethacin in saline to stop prostanstadin synthesis and wash off excess blood, and placed in a homogenizer tube containing garnet beads and 0.5 ml of ethanol. The corneas were homogenized by using a tissue homogenizer (PRO Scientific, Inc., Monroe, CT). Protein precipitate was pelleted in a microcentrifuge, and the ethanol layer was removed to a clean tube. The ethanol was evaporated by vacuum centrifugation, the residue was redissolved in enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI), and samples were analyzed for prosta glandin E2 (PGE2) and leukotriene B4 (LTB4) by using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Flow cytometry. Single cell suspensions were prepared from four corneas (BALB/c) at 48 h.p.i., as described previously (12). The Fc receptors on the cells were blocked with unconjugated anti-CD16/32 (BD Pharmingen) for 30 min. Samples were incubated with fluorescein isothiocyanate-labeled anti-Gr-1 antibody (clone RB6-8C5; BD Pharmingen) and isotype controls for 30 min. All samples were collected on a FACScan (BD Biosciences, San Diego, CA), and data were analyzed by using CellQuest 3.1 software (BD Biosciences).

Reverse transcriptase-PCR. Total RNA from BALB/c corneas were extracted at reverse transcriptase-PCR. Total RNA from BALB/c corneas were extracted at different days p.i. by using Tri-reagent (Molecular Biology, Cincinnati, OH). Total RNA (1 μg) was reverse transcribed by using murine leukemia virus reverse transcriptase (Life Technologies, Bethesda, MD) with oligo(dT) as the primer (Invitrogen, San Diego, CA). All cDNA samples were divided into aliquots and stored at −20°C until further use. PCR was performed in PTC-200 programmable thermal controller (MJ Research, Cambridge, MA) using Hot Start PCR master mix (Promega, Madison, WI). The primers used were murine GAPDH forward (TACCTGTCACACACAAGCTTGG) and reverse (GCC TGCTTCACGTTTCTCGATG). murine IL-1β forward (CAACCAAAAGTGATAT) and reverse (GATTCCAGATTCCAGTGTCGA), and murine
RESULTS

Mice treated with COX-2 inhibitor demonstrated diminished SK severity and corneal angiogenesis after ocular HSV-1 infection. Mice (BALB/c and C57BL/6) were ocularly infected with HSV-1 RE and at different days p.i., and corneal levels of COX-1, COX-2 mRNA, and PGE2 protein were measured by real-time PCR and ELISA, respectively. As demonstrated in Fig. 1C to F, infected animals showed higher COX-2 mRNA and PGE2 protein levels at different days p.i. in comparison to naive cornea. The levels of COX-2 mRNA and PGE2 protein were below the limit of detection in naive corneas. However, COX-1 mRNA was found to be constitutively expressed in naive corneas, and after HSV infection no significant \( P < 0.05 \) increase in the expression level was observed (Fig. 1A and B). Although COX-1 and COX-2 mRNA were readily demonstrable in scratch control, PGE2 protein levels were below the detection sensitivity level (Fig. 1A, B, C, D, E, and F). Taken together, these data indicate that corneal inflammation during the preclinical phase is associated with a prompt increase in the COX-2 expression, an enzyme essential for the synthesis of various proinflammatory prostanooids such as PGE2.

To elucidate the essential role of COX-2 in HSK pathogenesis, three groups of BALB/c (5 × 10^6 PFU) and C57BL/6 (5 × 10^6 PFU) mice were treated (till day 10 p.i.) with COX-2 inhibitor (SC-236), COX-1 inhibitor (SC-560) or vehicle, respectively. These mice were evaluated clinically for the development of SK following ocular HSV-1RE infection over a 20 days test period. As shown in Fig. 2A and B (BALB/c) and Fig. 3A and B (C57BL/6), mice receiving COX-2 inhibitor demonstrated significantly \( P < 0.05 \) reduced SK severity compared to COX-1 inhibitor-treated mice and vehicle control. In addition, whereas 87.5% (BALB/c) and 62.5% (C57BL/6) eyes demonstrated clinically evident lesions (score of 3 or more), only 25% (BALB/c) and 12.5% (C57BL/6) COX-2 inhibitor-treated eyes developed such lesions (Fig. 2C and 3C). Attempts to infect mice with a higher virus dose were lethal, but still a few developed an SK score of ≥3 in COX-2-inhibitor treated mice (data not shown). Histopathologic analysis of representative eyes of vehicle and COX-1 inhibitor treated BALB/c mice revealed severe inflammatory changes and cellular infiltration in the corneal stroma at day 20 p.i. (Fig. 2D). However, in mice treated with COX-2 inhibitor protein, only mild, inflammatory changes and cellular infiltrations were evident (Fig. 2D).

In concurrence with lesion incidence and severity, marked differences were apparent in the average extent of angiogenesis in mice treated with COX-2 inhibitor and vehicle only after ocular infection with HSV-1 RE (5 × 10^6 PFU) for BALB/c and 5 × 10^6 PFU for C57BL/6) at day 20 p.i. (Fig. 4A and B). At day 20 p.i., 10 of 16 eyes (BALB/c) and 8 of 16 eyes (C57BL/6) of vehicle-treated mice had developed an angiogenesis score of >10 (Fig. 3A and C). In marked contrast, no eyes of COX-2 inhibitor treated BALB/c and C57BL/6 mice developed equivalent scores (Fig. 4A and B). When the overall angiogenesis was compared in the groups of vehicle treated and COX-2 inhibitor-treated mice (BALB/c and C57BL/6), vehicle control mice showed three to four times (day 20 p.i.) more angiogenesis than COX-2 inhibitor-treated mice (Fig. 4A and B).

II-1β induces COX-2 expression in murine cornea after ocular HSV-1 infection. To evaluate the cellular source of COX-2 at an early stage of virus infection, BALB/c corneas were infected with HSV-1-pgC-GFP. After 24 h p.i., corneas were isolated, and GFP\(^+\) (infected) and GFP\(^-\) (uninfected) corneal cells were sorted by fluorescence-activated cell sorting (FACS) and analyzed for COX-2 mRNA expression by real-time PCR analysis. Interestingly, uninfected corneal cells expressed significantly \( P < 0.05 \) higher COX-2 mRNA in comparison to infected cells (Fig. 5A). When stained for COX-2 protein, uninfected corneal cells (GFP\(^+\)) were stained positive for COX-2 at 24 h p.i. (Fig. 5B). PCR of GFP\(^-\) (uninfected) corneal cells revealed 3,000-4,000-fold less viral mRNA (gB, tk) compared to GFP\(^+\) (infected) at this time point (Fig. 5A). Erroroneous sorting could possibly miss some infected cells harboring few infectious viral particles at their early replicative cycle, thus having feebel GFP expression and escaping the segregation process, resulting in tk and gB expression in a GFP-ve (uninfected) population. In addition, we were able to demonstrate immunohistochemically that corneal stromal fibroblasts and a few corneal epithelial cells expressed COX-2 at 24 h p.i. (Fig. 5C). To rule out the possibility of neutrophils as a possible source of COX-2 at this time point, Gr-1-depleted BALB/c mice and control mice were ocularly infected with HSV-1 RE, and the COX-2 transcript level in the cornea was measured by using real-time PCR analysis. No significant difference in COX-2 mRNA level was observed between these two groups at 24 h.
In addition, we could find COX-2 expression in Gr-1-depleted corneal stroma after HSV infection, indicating the role of uninfected corneal resident cells as a major source of COX-2 at 24 h p.i. (Fig. 5D). Additional experiments were carried out to detect the factors responsible for inducing COX-2 expression in corneal stromal fibroblasts after viral infection. Based on previous findings, potential candidates for inducing COX-2 were proinflammatory cytokines. Thus, a murine stromal fibroblast cell line was stimulated in vitro by various doses of proinflammatory cytokines known to be upregulated in murine corneas after HSV infection (IL-1α, IL-1β, TNF-α, IL-6, IL-18, gamma interferon, IL-2, and VEGF). Interestingly, only IL-1β (in all of the doses used [Fig. 6A]) and TNF-α (only at the highest dose used [data not shown]) induced COX-2 mRNA expression at 24 h p.i. as revealed by reverse transcription-PCR. Semiquantitatively, real-time PCR analysis revealed a significant ($P < 0.05$) and a dose-dependent increase in COX-2 mRNA expression in IL-1β-treated stromal fibroblast cells but not in corneal epithelial cells (Fig. 6A). Hence, IL-1β produced as a consequence of corneal HSV infection (Fig. 6B) may serve as a potential molecule to induce COX-2 and drive the early inflammatory process. Supporting this notion, we were able to demonstrate that administration of IL-1β in the murine cornea by micropocket assay resulted in a significant ($P < 0.05$) and dose-dependent increase in COX-2 mRNA level (Fig. 6C). Blocking of IL-1β activity using IL-1 receptor antagonist transgenic mice resulted in a significantly ($P < 0.05$) diminished COX-2 mRNA and PGE$_2$ levels in murine cornea after ocular HSV-1 infection (Fig. 6D and E).
Diminished inflammatory response in mice treated with COX-2 inhibitor during the preclinical phase of HSK. Normally, after corneal HSV-1 infection, PMN infiltrate the corneal stroma at 48 h p.i. (38). However, in mice treated with COX-2 inhibitor and eventually showing a reduction in HSK severity, the influx of PMN was severely compromised (Fig. 7). As revealed by FACS analysis at 48 h p.i., there was an approximately five- to sixfold reduction in the number of PMN in COX-2-treated corneas in comparison to vehicle controls (Fig. 7).

We reasoned that the scarce cellular influx in the treated group was a result of a block in the induction of proinflammatory cytokines and chemokines, as a consequence of impaired COX-2 activity. As demonstrated in Fig. 8, indeed there was a significant reduction (P < 0.05) in IL-6, IL-1α, MIP-2, and PGE2 levels during the preclinical phase. However, no significant difference was observed in IL-1β and TNF-α levels at these time points (Fig. 8). Although the LTB4 level was undetectable at day 1 p.i., there was no significant (P < 0.05) reduction at 3 and 7 days p.i. (Fig. 8).

Compromised angiogenic response after ocular HSV infection in COX-2 inhibitor-treated mice. As stated above, groups of mice treated with COX-2 inhibitor showed diminished angiogenic responses in comparison to vehicle-treated controls. These mice when tested for corneal VEGF level during the preclinical phase, demonstrated significantly (P < 0.05) reduced levels of VEGF at 3 and 7 days p.i. compared to vehicle controls (Fig. 9A).

Previous studies in tumor systems demonstrated that IL-1β can induce VEGF production through up-regulation of COX-2 and prostanoids (20). To demonstrate the angiogenic activity of COX-2 induced prostanoids in murine corneas, different doses of rmIL-1β were administered by corneal micropocket assay, and the extent of angiogenesis was measured at day 3 postimplantation. Interestingly, IL-1β induced a dose-dependent angiogenic response (Fig. 9B). In such eyes, COX-2 protein was readily detectable by immunohistochemistry around the pellet containing recombinant IL-1β protein (200 ng) in corneal stroma at day 4 postimplantation (Fig. 9C). In addition, IL-1β (200 ng)-implanted eyes demonstrated a significant (P < 0.05) increase in the corneal PGE2 and VEGF protein levels compared to pellet only at this time point (Fig. 9E). Administration of COX-2 inhibitor in those mice resulted in a significant (P < 0.05) decrease in the angiogenic response (Fig. 9D). This decrease in angiogenic
FIG. 3. C57BL/6 mice receiving COX-2 inhibitors show reduced HSK severity. (A) Mean lesion HSK score at day 20 p.i. of C57BL/6 mice infected with $5 \times 10^6$ PFU of HSV-1 RE. Each dot represents the HSK score from one eye. Horizontal bars and figures in the parentheses indicate the mean for each group. *, Statistically significant difference ($P < 0.05$) compared to vehicle control. (B) Kinetic study of HSK score at different days p.i. infected with $5 \times 10^6$ PFU (C57BL/6) HSV-1 RE. *, Statistically significant differences ($P < 0.05$) compared to vehicle control. (C) Bar diagram demonstrates the percent severity of each group of C57BL/6 mice infected with $5 \times 10^6$ PFU at day 20 p.i. *, Statistically significant difference ($P < 0.05$) compared to vehicle control.

FIG. 4. Mice receiving COX-2 inhibitor show diminished angiogenic response after HSV-1 infection at day 20 p.i. (A) Angiogenesis scores for individual eyes of different groups of BALB/c mice infected with $5 \times 10^5$ PFU HSV-1 RE at day 20 p.i. Horizontal bars and figures show the mean for each group. (B) Angiogenesis scores for individual eyes of different groups of C57BL/6 mice infected with $5 \times 10^6$ PFU HSV-1 RE at day 20 p.i. Horizontal bars and figures show the mean for each group.
FIG. 5. Uninfected stromal fibroblasts are the major producers of COX-2 after ocular HSV-1 infection. (A) Mice (BALB/c) were infected with \(5 \times 10^5\) PFU HSV-1-RE-pgC-GFP. At 24 h p.i., corneas, GFP\(^+\) cells (infected) were sorted out from GFP\(^-\) cells (uninfected), and total RNA was extracted. Real-time PCR analysis was conducted to detect the COX-2, tk, and gB mRNA expression from GFP\(^+\) and GFP\(^-\) cell types as described in Materials and Methods. The results are shown as mean ± the SD of three separate experiments. *Statistically significant difference (\(P < 0.05\)) compared to GFP\(^+\). The number in the upper right-hand corner of dot plot represents percentage of GFP\(^+\) cells at 24 h p.i. (B) Single-cell suspensions (BALB/c mice infected with HSV-1 RE gc-GFP) were prepared at 24 h p.i. Cells were stained for COX-2 as described in Materials and Methods. The cells were spun down on microscopic slides and visualized under a confocal laser scanning microscope (Leica, Wetzlar, Germany). Bar, 80 \(\mu\)m. The arrow indicates the COX-2-positive cells. (C) Immunohistochemistry for COX-2 expression in corneas of BALB/c mice. COX-2 expression (arrows) were detected in corneal stroma of BALB/c mice infected with \(5 \times 10^5\) PFU HSV-1 RE at day 1 p.i. Diaminobenzidine was used as substrate, and sections were counterstained with hematoxylin. Magnification, \(\times 200\). (D) Gr-1-depleted and control mice were ocularly infected with HSV-1 RE. At 24 h p.i., four corneas/group were processed for the extraction of cellular mRNA. Real-time PCR analysis was conducted to detect the COX-2 mRNA expression in corneas of HSV-1-infected mice as described in Materials and Methods. The results are shown as the mean ± the SD of three separate experiments. Immunohistochemistry for COX-2 expression in corneas of Gr-1-depleted BALB/c mice (\(5 \times 10^5\) PFU HSV-1 RE) (arrows) at 24 h p.i. Diaminobenzidine was used as a substrate, and sections were counterstained with hematoxylin. Magnification, \(\times 200\).
FIG. 6. IL-1β-induced COX-2 expression in murine stromal fibroblast cell line. (A) Murine stromal fibroblast cells and corneal epithelial cells were stimulated with different doses of rmIL-1β for 24 h. At 24 h poststimulation, the cells were collected, and the total RNA was extracted. Real-time PCR analysis was conducted to detect the COX-2 mRNA expression as described in Materials and Methods. The results are shown as mean ± the SD of three separate experiments. *, Statistically significant differences (P < 0.05) in comparison to untreated control. (B) Mice (BALB/c) were infected with 5 \times 10^5 PFU HSV-1-RE. At different days p.i. four corneas/time point were taken, and total RNA was extracted. Reverse transcription-PCR was conducted to detect the IL-1β mRNA expression in corneas of mice infected with HSV-1 as described in Materials and Methods. (C) Different concentrations of rIL-1β (50 and 200 ng) were administered in BALB/c corneas (n = 8) by corneal micropocket assay. Real-time PCR analysis was conducted at day 1 p.i. to detect the COX-2 mRNA expression in corneas of mice infected HSV-1 as described in Materials and Methods. The results expressed as mean ± the SD. *, Statistically significant differences (P < 0.05) compared to pellet alone. (D) Control C57BL/6 mice and IL-1 ra Tg mice were infected with 5 \times 10^6 PFU HSV-1 RE. At 1, 2, 5, and 7 days p.i., four corneas/group were
response was associated with a reduction in corneal PGE$_2$ and VEGF protein levels in those corneas (Fig. 9E).

DISCUSSION

This study focuses on the early events in the pathogenesis of the blinding immunoinflammatory lesion SK caused by ocular infection with HSV. We show that corneal infection with HSV results in the upregulation of COX-2, an enzyme that contributes to the inflammatory process in the corneal stroma, via upregulation of prostanoids. The likely source of COX-2, at least initially after infection, was corneal stromal fibroblasts stimulated by proinflammatory cytokines, such as IL-1, induced by HSV replication. The present study demonstrates, for the first time, a critical role of COX-2 for herpetic SK lesion development. Accordingly, selective inhibition of COX-2, but not COX-1, resulted in significantly milder disease and reduced corneal angiogenesis compared to control mice. This difference in disease phenotype was an indirect event and was shown to be the consequence of a compromised inflammatory and angiogenic response. Thus, our results imply that COX-2 plays an important role in the early inflammatory phase of HSV infection.

On the basis of immunohistochemical analyses, as well as neutrophil depletion studies, the early cellular source of COX-2 appeared to be stromal fibroblasts. However, it was not clear how the infection, which occurs in the epithelium (11), caused such a response in the stroma. Conceivably, cytokines released by infected epithelial cells represent the primary stimulus for COX-2 upregulation. The cytokine IL-1β represents the most likely candidate since it is produced early and, as shown by in vitro studies with stromal fibroblasts, may act as a potent stimulator for COX-2 expression. Others, too, have shown that fibroblasts stimulated by IL-1β strongly upregulates COX-2 (13, 27). Moreover, blocking IL-1β by IL-1 receptor antagonist protein in vivo resulted in diminished COX-2 production. Whether virus itself or products derived from virus such as TLR9 stimulating viral nucleic acid also act as stimulants for COX-2 expression requires further investigation. Some reports have associated HSV infection of the trigeminal ganglion with COX-2 transcript expression (17), but it was not clear if this was evident in virus-infected or nearby cells. However, HSV-1 can activate NF-κB (25), and the latter has been shown to induce COX-2 expression in human airway epithelial cells (8). Accordingly, direct effects of HSV infection remains possible and merit further investigation.

An influx of inflammatory cells, primarily PMN, repre-
sents a prominent early event after viral infection of the cornea (38, 39). These responses both serve to control infection and also contribute to corneal neovascularization (38, 39). The influx of PMN was significantly diminished in COX-2 inhibitor-treated animals, supporting previous observations that COX-2 via production of prostanoids plays an important role in PMN recruitment and activation. Such effects were observed in rheumatoid arthritis, dermatitis, periodontitis, and pancreatitis models (5, 28, 33). How COX-2-induced prostanoids in the corneal stroma caused PMN influx remains unclear. Thus, whether the effect is direct or proceeds by the induction of other chemokines, such as MIP-2 (14), that are known to be involved in PMN recruitment requires further evaluation. That the recruitment might be indirect finds support in a radiation-induced central nervous system inflammation model, wherein mice treated with a COX-2 inhibitor had lower levels of several cytokines and chemokines (21). Once recruited to the stroma, PMN and macrophages themselves can serve as an additional source of COX-2-induced prostanoids at the inflammatory site (7, 16). Accordingly, whereas the early source of COX-2 expression may be mainly from noninflammatory cells, the inflammatory cells themselves subsequently become the major source of COX-2. The respective roles of various cell types that produce COX-2 during the course of HSV infection are currently unknown but are under investigation in our laboratory. We anticipate that, early after infection, COX-2 produced from stromal fibroblasts facilitates the influx of PMN, which in turn now acts as an additional source of prostanoids, thus setting the stage for the chronic immunoinflammatory phase.

Early PMN invasion also contributes to corneal pathology by
acting as a major source of angiogenesis factors (22, 31) and perhaps tissue-damaging factors such as nitric oxide (9). Thus, in line with the minimal early PMN response noted in COX-2 inhibitor-treated mice, such animals showed a marked reduction in angiogenesis. One factor derived from PMN, as well as from other cell types involved in neovascularization, is VEGF (31). We demonstrated that VEGF was significantly downregulated in COX-2 inhibitor-treated mice in comparison to control animals. It is not known how COX-2 induces VEGF production, but one mechanism could be via PGE2, a proangiogenic factor (15), that can induce VEGF in some systems (20). However, it is not clear whether this effect is direct or mediated by upregulation of other cytokines such as IL-6, IL-1, and chemokines containing E-L-R motifs. We are currently testing such notions in our HSV model.

Given its role in corneal angiogenesis and SK, COX-2 represents a logical target for therapy. However, caution may be warranted. Previous studies in an endotoxin-induced uveitis model have indicated that disturbance of the arachidonic acid pathway exacerbates this condition in COX-2-deficient mice (40). This was caused by elevated LTB4 and 5-LO metabolized from arachidonic acid via the lipoxygenase pathway. At least in our system we were able to demonstrate that inhibition of COX-2 was not associated with an increase in LTB4, thus justifying our approach in counteracting the corneal immunoinflammatory lesion with COX-2 inhibitors.

Taken together, our results support the hypothesis that the inflammatory milieu and angiogenic stimuli created early after infection play an important role in HSV-induced ocular lesions. An important participant of this environment is COX-2-induced prostanoid synthesis. Blocking the effect of COX-2 by a specific COX-2 inhibitor abrogates the cascade of events involving angiogenesis.
that culminate in SK. This regulation is indirectly mediated by downregulating various signaling molecules previously known to be important in SK pathogenesis and corneal angiogenesis.

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ERRATUM

Role of Inflammatory Cytokine-Induced Cyclooxygenase 2 in the Ocular Immunopathologic Disease Herpetic Stromal Keratitis

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