Effect of Inhibition of Inflammatory Mediators on Trauma-Induced Stromal Edema

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PURPOSE. To determine the specific biochemical pathways involved in the initial-phase inflammatory response that causes stromal edema after epithelial debridement of the rabbit cornea.

METHODS. Adult New Zealand White rabbit corneas were treated with 2 mM synthetic inhibitor of metalloproteinase (SIMP)-1, 1 mM DFU (a specific cyclooxygenase [COX]-2 inhibitor) in 50/50 dimethyl sulfoxide (DMSO)/Ringer’s solution, 300 KIU aprotinin (a serine protease inhibitor), 0.05% or 0.10% IL-1 receptor type II solution, 1 mM glitoxitin (a Ras farnesyltransferase inhibitor), or vehicle alone (the control). These were applied topically in vivo in five doses over a 3-hour period except IL-1 receptor type II, which was applied in vitro. After rabbits were killed, the corneas were mounted in perfusion chambers with the endothelium bathed in a modified Ringer’s solution and the epithelium bathed with silicone oil. Corneal thickness was measured with an automatic specular microscope. The corneal thickness typically stabilized 1 hour after mounting. After stabilization, the corneal epithelium was removed with a rotating bristle brush and stromal thickness monitored for 1 hour. Paired control corneas were treated similarly.

RESULTS. Stromal swelling after epithelial debridement was significantly less in most treated corneas, compared with untreated controls: 18.4 ± 5.3 μm vs. 28.6 ± 7.7 μm (n = 6, P = 0.004); SIMP-1, 18.7 ± 10.2 μm vs. 34.3 ± 10.2 μm (n = 7, P = 0.02); DFU, 19.3 ± 10.2 μm vs. 23.5 ± 8.4 μm (n = 6, P = 0.01); and IL-1 receptor type II (0.05%), 26.2 ± 5.6 μm vs. 30.4 ± 5.6 μm (n = 5, P = 0.03) and (0.10%), 26.6 ± 5.6 μm vs. 32.1 ± 7.4 μm (n = 8, P = 0.03). Glitoxitin was not effective (21.5 ± 8.0 μm vs. 21.9 ± 6.2 μm; n = 5, P = 0.94).

CONCLUSIONS. The reduction of stromal edema after topical administration of the inhibitors demonstrates the involvement of the COX-2 enzyme, the matrix metalloproteinase family, plasminogens, and the IL-1 system in the trauma-induced inflammatory response of the rabbit cornea. The inflammatory process in the cornea associated with trauma can proceed along multiple redundant parallel pathways. (Invest Ophtalmol Vis Sci. 2003;44:2507–2511) DOI:10.1167/iovs.02-0926

Although the inflammatory response can be triggered in many ways, an initial response is the formation of edema at the site of injury. The swelling takes place in two phases, and the etiology of the initial phase response (within 1 hour) is poorly understood in the edema models generally used (rat paw, mouse ear, rat lung). However, we recently provided evidence in the cornea that this edema may be related to the increased tissue osmotic effects of proteolysis. Stimula to the corneal epithelium induces a cascade of inflammatory effects that includes keratocyte apoptosis and the initiation of stromal wound healing. In addition, activities of various proinflammatory mediators, such as proteases and interleukins, have been identified within corneal tissues after epithelial trauma. We have found that corneal epithelial debridement, as an initial step in preparation for excimer laser photorefractive keratectomy (PRK), causes rapid stromal edema localized to the area subjacent to the insult. This response can be used as a model to explore the etiology of the acute edema response in inflammation.

Because there are multiple pathways that are candidates for producing this inflammatory response, the present study was undertaken in an in vivo rabbit corneal model of trauma-induced stromal edema. Topical application of biochemical inhibitors was used to demonstrate involvement of specific mediators in this inflammatory pathway.

METHODS

Animals

This study adhered to the standards of the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee (IACUC), which are in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult New Zealand White rabbits of either sex and weighing between 2 and 4 kg were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (5 mg/kg). The rabbits were kept anesthetized throughout drug administration while their eyes were taped shut to prevent corneal drying. The rabbits were subsequently killed with a lethal dose of pentobarbital sodium (100 mg/kg) administered intravenously through the marginal ear vein.

Drug Administration

Five drops (50 μL/drop) of the experimental inhibitor or control vehicle were topically applied during a 3-hour period at 45 minute intervals before rabbits were killed. One eye received a drug and the companion eye served as the control, receiving only vehicle. The water soluble inhibitors 300 KIU aprotinin (Sigma, St. Louis, MO), 2 mM SIMP-1 (Peptides International, Louisville, KY), and 1 mM glitoxitin (Sigma, St. Louis, MO) were dissolved in a modified Ringer’s solution. For the aprotinin series, the endothelial perfusate also contained 300 KIU aprotinin. The non–water-soluble cyclooxygenase (COX)-2 inhibitor, 1 mM DFU (Merck and Co., Inc., Whitehouse Station, NJ), was dissolved in dimethyl sulfoxide (DMSO) and diluted 1:1 with modified Ringer’s fluid. IL-1 receptor type II (Immunex Corp., Seattle, WA) was supplied in a PBS solution and was diluted to 0.05% and 0.10% concentration in PBS. IL-1 receptor type II was administered by immersing the corneal epithelium in either the drug or PBS for 30 minutes after the corneal-mounting procedure.
Corneal Mounting and Perfusion

For all experimental series except for the IL-1 series, silicone oil was applied topically to both eyes immediately before enucleation. The corneas were dissected and isolated according to the method of Dickstein and Maurice, in thermally controlled perfusion chambers attached to an automated specular tracking microscope. The order of mounting was randomized. The endothelia were continuously perfused with the glutathione-bicarbonate Ringer solution described by Ruberti et al., containing (in mM) 124.7 NaCl, 2.79 glucose, 0.61 MgSO4, 1.21 KH2PO4, 25.4 NaHCO3, 7.26 KCl, 0.65 CaCl2, and 4.75 adenine, and 0.16 glutathione. The epithelia were covered with silicone oil to prevent anterior surface fluid exchange. In the IL-1 series of experiments, the epithelial surface was not covered with silicone oil until the 30-minute drug treatment period was complete.

Epithelial Debridement

Once a stable corneal thickness was achieved, approximately 1 hour after mounting, the perfusion chamber was rotated from beneath the microscope objective and the silicone oil was removed by aspiration to allow for the debridement of the epithelium. The debridement was performed using a Pallikaris debridement brush (courtesy of Autonome Technologies Corp., Orlando, FL) designed specifically for use in refractive surgery, and the epithelial debris and remaining silicone oil were aspirated. The exposed anterior stroma was then covered with fresh silicone oil and rotated back under the objective. The bare stromal thickness was then tracked for 1 hour. Subsequently, this procedure was repeated on the companion eye.

Thickness Measurements

An automatic specular tracking microscope was used to scan the corneal tissue continuously as described previously by Ruberti et al. This system was connected to a computer by a data acquisition board (AT-MIO-16E-10; National Instruments, Austin, TX) with 12-bit resolution and digital signal processing routines developed in commercial software (LabView; National Instruments). The raw data were subjected to a low-pass filter to smooth the signal. A peak detector supplied by the software was used to identify the reflective structures of the cornea.

Statistical Analysis

Statistical analysis was performed on computer (SigmaStat; SPSS Inc., Chicago, IL), by Student’s paired t-tests with a significance level of 0.05. Statistical errors are expressed as standard deviations.

RESULTS

The possibility of proteolysis as a cause of the inflammation led to the use of aprotinin and SIMP-1, both of which can inhibit the breakdown of collagen. The findings for these two drugs were: aprotinin, 18.4 ± 5.3 μm vs. 28.6 ± 7.7 μm (n = 6, P = 0.004); SIMP-1, 18.7 ± 10.2 μm vs. 34.3 ± 10.2 μm (n = 7, P = 0.02). As can be seen in Figure 1, pretreatment with aprotinin resulted in statistically significantly less swelling compared with the control corneas from 20 to 60 minutes after debridement. SIMP-1 pretreatment showed a significant difference only at 60 minutes (Fig. 2); however, there was a trend toward significance at earlier time points. It should be noted that aprotinin was also administered in the endothelial perfusion solution, and its effect on inflammation may be more pronounced because of this.

The knowledge that a nonspecific COX inhibitor, diclofenac sodium (Voltaren Ophthalmic Solution; Ciba Vision Ophthalmics, Duluth, GA), is effective in reducing stromal swelling caused by epithelial trauma was the basis for using DFU, a specific COX-2 inhibitor. It yielded a statistically significant effect at 1 hour after debridement with changes in thickness of 19.3 ± 10.2 μm vs. 23.5 ± 8.4 μm (n = 6, P = 0.01, Fig. 3).

IL-1 was investigated because it is a prominent proinflammatory cytokine that acts upstream of those mediators already tested. The IL-1 receptor type II was used to regulate the biological effects of IL-1. At a concentration of 0.05%, a statistically significant difference was found at 1 hour after removal of the epithelium, with changes in thickness of 26.2 ± 5.6 μm vs. 30.4 ± 5.6 μm (n = 5, P = 0.03, Fig. 4). At an increased concentration of 0.10%, the swelling difference between drug-treated and control corneas was statistically significant from 20 to 60 minutes (at 1 hour, 26.6 ± 5.6 μm vs. 32.1 ± 7.4 μm, n = 8, P = 0.03, Fig. 5).

The last mediator tested was gliotoxin, which inhibits the initiation of the mitogen-activated protein kinase (MAPK) signaling cascade. The edema was not blocked by gliotoxin. No statistically significant differences were observed with this solution, with paired swelling changes of 21.5 ± 8.0 μm vs. 21.9 ± 6.2 μm (n = 5, P = 0.94, Fig. 6).

There were no statistically significant differences in initial stromal thicknesses immediately after debridement between any of the drug-treated corneas and the paired controls. Therefore, there was no reason to believe that the swelling proper-
ties of the stroma were affected by any of the drugs administered in this model. These results help to narrow the possibilities for the most significant pathways involved in stromal swelling after epithelial trauma.

**DISCUSSION**

Although widely studied because of its potential impact on all tissues in the body, many aspects of inflammation are poorly understood. It has been shown that the cornea responds to trauma, such as epithelial scrape or herpes simplex virus (HSV)-1 infection, with a complex cascade of events.\(^1\)–\(^10\),\(^19\) One of the most significant of these events finds the anterior stromal keratocytes undergoing apoptosis immediately after epithelial trauma. This epithelial-stromal interaction has been implicated in the past, as well as by these experiments, to be part of the inflammatory response after epithelial debridement. Programmed cell death has been linked to various cytokine-mediated pathways that are triggered by epithelial damage.\(^3\)–\(^6\),\(^10\) Many of these same cytokines also have a proinflammatory effect. It is also important to note that apoptosis is not triggered after laser removal of the epithelium. This suggests that the mechanical destruction of the epithelial cells is the trigger for the accompanying keratocyte death. In previous work,\(^1\) we demonstrated that stromal swelling occurs after epithelial debridement and that the use of the nonspecific cyclooxygenase inhibitor, diclofenac sodium, can partially inhibit this edema. Because the inflammatory process comprises a complex interaction of various biochemical mediators with multiple parallel and serial pathways (Fig. 7), we investigated the effects of inhibitors of specific mediators known to be produced by the injured cornea.

Because corneal pretreatment with diclofenac sodium reduced the stromal edema,\(^1\) we differentiated the effect further by showing that the specific COX-2 inhibitor DFU also partially prevented stromal swelling after epithelial debridement. There-
fore, the COX-2 enzyme plays a significant role in the inflammatory reaction in this model. A future study using a specific COX-1 inhibitor would be advisable to determine whether both of these enzymes are involved in the swelling or just the COX-2 enzyme.

It has also been shown that plasmin is upregulated in the traumatized cornea.20 Because this system is not dependent on the cyclooxygenase system, it would be interesting to know whether plasminogen also affect stromal swelling. We tested the effect of the plasmin inhibitor aprotinin on the observed stromal edema. Once again, our results demonstrated a reduction in stromal swelling. This indicates the involvement of plasmin in the acute corneal response to epithelial injury.

It has been suggested that one cause of stromal edema is the breakdown of components in the stromal extracellular matrix resulting from the activation of inflammatory mediators.1 The metalloproteinase family is known to degrade the extracellular matrix and to be upregulated by several inflammatory mediators, such as IL-1 and plasmin.10,11,15,16,21,22 The application of a nonspecific metalloproteinase inhibitor, SIMP-1, clearly demonstrated that these enzymes are upregulated during this form of trauma.

IL-1, one of the key mediators of inflammation, is responsible for increasing the activity of proinflammatory enzymes, such as COX-2 and MMPs, as well as regulating the Fas/Fas ligand system and plasmin levels.6,7,25 The results of the current study demonstrated that the application of soluble IL-1 receptor type II significantly reduced the stromal edema associated with epithelial trauma. This receptor is a natural regulator of IL-1 activity that binds to IL-1 without affecting the actions of the IL-1 receptor antagonist. Although statistically significant, the inhibition of IL-1 did not appear to reduce the edema as much as would be anticipated with a key inflammatory mediator. This may be because the soluble IL-1 receptor type II is relatively large (~50 kDa) and hydrophilic, properties that could hinder the protein’s diffusion into the corneal stroma in sufficient quantities even with the increased contact time with the epithelium. Further investigation involving inhibition of IL-1 may resolve this issue.

It has been found that the Ras protein inhibitor, gliotoxin, can reduce the inflammation in the in vivo cornea.24 Because of this finding, we tested gliotoxin in our model. As the results clearly showed, we did not find any difference between the control and gliotoxin-treated corneas. There are a number of possible explanations for this result. The drug may not have penetrated the epithelium in sufficient quantities, the drug did not remain within the corneal stroma for a sufficient time in our experiment, or this pathway contributes only marginally if at all to the stromal edema created in this model. It is also possible that the Ras protein’s most significant contribution to inflammation is related to the infiltrating macrophages from the surrounding tissue which occurs in vivo and beyond the time frame studied in this in vitro model.

Therefore, the reduced stromal edema seen after topical administration of the inhibitors demonstrates the involvement of the COX-2 enzyme, the matrix metalloproteinase family, plasminogens, and the IL-1 system in the trauma-induced inflammatory response of the rabbit cornea. These results give us a better understanding of the etiology of inflammation within the cornea and could allow for more successful treatments to be developed not only for ophthalmic conditions but also for inflammatory conditions elsewhere in the body.

It is emphasized that this early edema response in the cornea may be an appropriate model to study the vegetative physiologic characteristics of the early inflammatory phase in other tissues. The corneal stroma is normally a dense connective tissue. Proteolytic activity within the connective tissue and swelling may be necessary precursors to the secondary invasive response of the macrophages.

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References


