Effect of a Metalloproteinase Inhibitor on Established Corneal Ulcers After an Alkali Burn

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Proteinase inhibitors have been shown to prevent corneal ulceration and perforation when used immediately after an experimental alkali burn injury. To evaluate the clinical efficacy of a synthetic metalloproteinase inhibitor, HSCH₂CH(CH₂CH(CH₃)₂)CO-Phe-Ala-NH₂ (SIMP), treatment with inhibitor was withheld until corneal ulceration ensued after a standard alkali injury to the rabbit eye. When topical therapy with a 1 mmol/l solution of SIMP was initiated after corneal ulceration had progressed to a mid-stromal level (clinical score of 2), there was no significant difference in the progression of corneal ulceration between the treated vs. control group after 6 d of therapy. In the second study in which treatment was initiated earlier at the onset of superficial ulceration (clinical score of 1), there was a significant difference in clinical scores between the two groups after 1 d of treatment until termination of the experiment at 21 d (P < 0.005). In the inhibitor-treated group, 88.9% of the corneas showed a reversal or cessation of progression of the ulceration process. Eighty-seven-and-a-half percent of the control corneas progressed to descemetocele formation or perforation by day 14 of treatment. This study suggests that SIMP may be used for effective treatment of corneal ulcers resulting from an alkali burn injury in the human eye. It also shows that early and aggressive initiation of therapy is critical.

Collagenase, a zinc metalloproteinase, is released by the cornea after a chemical injury such as an alkali burn. It is thought to be the principal enzyme involved in the destruction of the cornea.1-3 Inhibitors of collagenase have been a recent approach to the treatment of experimental corneal alkali burns. Several nonspecific collagenase inhibitors have been evaluated in the past and were found to be relatively ineffective in vivo.4-6 We have shown that a newly developed synthetic metalloproteinase inhibitor (SIMP) is a particularly potent inhibitor of rabbit corneal collagenase when tested in vitro against other compounds.7 This β-mercaptopryethyl tripeptide also was shown to prevent alkali-injured rabbit eyes from undergoing corneal ulceration and perforation in vivo when treatment was started immediately after the injury.8

In our previous rabbit experiments,8 topical treatment with SIMP was begun immediately post alkali burn and continued until termination of the study at 21 d. From a clinical standpoint, it is important to determine whether SIMP would be effective if treatment were delayed until the corneal ulcer was established. The use of ascorbate, for example, has had favorable results in the alkali burned eye when applied at onset, although its efficacy depended on the severity of the burn and the route of administration.9-12 When treatment was withheld until corneal ulceration ensued, ascorbate had no significant effect.13 It also has been shown that citrate protects the rabbit eye from ulceration and perforation in the severe alkali injury when used immediately14,15 or after establishment of anterior corneal ulcers.16

We conducted the present studies to determine whether established corneal ulcers would respond to topical application of SIMP. In the first study, we allowed the corneal ulcers to progress to a moderate or mid stromal ulcer before treatment was begun (average, day 11 post burn). In a second study, we initiated inhibitor therapy earlier in the ulceration process with treatment beginning at the appearance of superficial or anterior stromal ulcers (average, day 7 post burn).

Materials and Methods

Alkali Burn Procedure

Twenty New Zealand Dutch strain albino rabbits of both sexes weighing between 2.0 and 2.5 kg were used for each study. Rabbits were anesthetized by intramus-
cular injection of 10 mg/kg xylazine and 37.5 mg/kg ketamine HCl. Rabbit eyes also were anesthetized topically with two drops of proparacaine hydrochloride (Alcon Laboratories, Ft. Worth, TX). One eye of each animal received a sharply defined 12.7 mm corneal burn by pipetting 0.5 ml of 2 N NaOH into a plastic well held firmly against the cornea for 60 sec. The interior of the well and the eye surface then were irrigated with saline for 5 sec. Thorough irrigation was continued for 5 sec after the well was removed. Animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Criteria for Entry of Animals Into the Study
External examinations of each rabbit cornea were performed twice daily throughout the entire study. Detailed, double-masked slit-lamp examinations of the alkali burns were performed every morning at 8:00 AM. Corneas were examined for the presence of corneal defects, ulceration, perforation, vascularization, or infection. Each cornea was assigned a clinical score according to the severity of corneal ulceration using our previous system. Briefly, ulcers were classified into the following groups: (1) no ulceration, score = 0; (2) superficial ulceration (ulcers limited to the anterior one-third of the cornea), score = 1; (3) moderate ulceration (ulcers extending to the middle one-third of the cornea), score = 2; (4) deep ulceration (ulcers extending to the posterior one-third of the cornea), score = 3; (5) descemetocele formation, score = 4; (6) perforation, score = 5. To qualify for treatment in the first study, rabbit corneas had to have reached a clinical score of 2 for two consecutive examinations. Animals with a score of 3 or greater were admitted into the study on the day observed. This criterion ensured that the burned corneas had a moderate or midstromal ulceration upon initiation of treatment.

In the second study, treatment was started after the corneas had maintained a clinical score of 1 for two consecutive days. Animals with a score of greater than 1 were entered on that day of examination. Thus, treatment was initiated at the onset of an active superficial or anterior stromal ulcer.

Statistics were performed using the two-tailed Student's t-test.

Inhibitor Preparation
The thiol peptide inhibitor was generously provided by Drs. Krysztof Darlak and Arno Spatola from the Department of Chemistry, University of Louisville, Louisville, KY. The synthesis and characterization of SIMP has been described previously. A stock solution of peptide was prepared in 95% ethanol containing 1 mmol/l acetic acid. This solution then was diluted in Adsorbotear without EDTA or thimerosal (Alcon Laboratories) to give a final peptide concentration of 1 mmol/l. Fresh solutions of the peptide were made every 24–48 hr. The inhibitor was stable under these conditions as shown by high pressure liquid chromatography analysis. An equal volume of ethanol containing 1 mmol/l acetic acid was added to a second solution of Adsorbotear as the control vehicle. The final ethanol concentration did not exceed 5% by volume. Both solutions were kept on ice when not in use.

Treatment Regimen
Upon entry into the study, animals were randomly assigned into two groups. One group of 10 rabbits received treatment with SIMP topically every hour from 8:00 AM to 8:00 PM daily. The other 10 animals received hourly treatment with vehicle only as controls on the same schedule. Both groups were treated with subconjunctival injections of inhibitor (1 mmol/l) or vehicle at 8:00 PM daily. After intramuscular and topical anesthesia as described in the previous section, injections of 0.5 ml of the solutions were made subconjunctivally at the 12-o'clock position using a sterile tuberculin syringe and a 30 G needle. All animals were noted to have subconjunctival blebs in the superior fornix after injection. Animals received topical gentamicin sulfate ointment (Pharmafair, Inc., Hauppage, NY) once daily after examination for the duration of the experiment. This treatment regimen was continued until corneas reached a clinical score of 5 (perforation) or until termination of the study. Animals were killed by pentobarbital overdose via ear vein injection. After the animals were killed, the treated eye of each was proptosed and the anterior chamber was entered with a scalpel blade. Corneas were excised at the limbal margin with corneal scissors and placed immediately in 10% formalin. The formalin fixed corneas then were embedded in paraffin, thinly sectioned, and stained with hematoxylin-eosin.

Results
The results of the first study are shown in Figure 1. Inhibitor treatment was initiated when the ulcer reached a clinical score of 2. There was no statistical significant difference in the severity of ulceration between the corneas treated with SIMP vs. control. Because the SIMP-treated eyes were clearly unchanged from control even after 6 d of treatment (P > .01), and both groups had progressed to an average clinical score of 4, the study was terminated at day 15 post burn.
We designed our next study to begin treatment earlier in the ulcerative process, at a clinical score of 1. Results shown in Figure 2 reveal a striking difference between the SIMP-treated and control-treated group, beginning after only 2 d of treatment ($0.01 < P < 0.005$). In this study, one animal was terminated immediately after the burning process because of technical difficulties. Two animals did not progress to ulceration and were not entered in the study. (These two rabbits were observed until the end of the study; one rabbit remained at clinical score 0 and one had progressed to a score of 2 only.) In all cases but one, SIMP either reversed the ulceration process or halted significant progression of ulceration. Only one of the SIMP-treated corneas worsened to a deep stromal ulcer. However, it remained at a score of 3 to the end of the study.

**Light Microscopy**

All of the corneas were examined by light microscopy after sectioning and staining with hematoxylin-eosin. In the first experiment, there was no difference in the extent of inflammation or the overall appearance of the corneas between the SIMP-treated and control groups.

**Fig. 1.** Results from study one. The graph demonstrates the clinical progression of corneal ulceration post alkali burn in the inhibitor treated group vs. control. Examinations were performed daily. The X-axis corresponds to the number of treatment days, with day 0 corresponding to the day when treatment was initiated (after the cornea had maintained a clinical score of 2 for the first two consecutive examinations.) All 10 animals in each group had reached a score of 2, and received at least 2 full days of treatment by termination of the study. The Y-axis corresponds to the average clinical score of the two groups. Average scores were calculated in each group of animals based on the following clinical criterion: no ulceration, score = 0; superficial ulcer (depth to the anterior one-third), score = 1; moderate ulcer (depth to the middle one-third), score = 2; deep ulcer (depth to the posterior one-third), score = 3; descemetocoele, score = 4; perforation, score = 5. There was no significant difference in the progression and severity of ulceration in the two groups after statistical analysis using the two-tailed Student's $t$-test.

**Fig. 2.** Results from study two. The progression of clinical changes after treatment of established ulcers post alkali burn at a score of 1 (superficial ulceration). The X-axis corresponds to the average clinical score after daily examinations. Statistical analysis was performed using the two-tailed Student's $t$-test, with a significant difference in the severity and progression of ulceration between the two groups noted after 1 day of treatment: $*P < 0.01$; $**P < 0.005$. 

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ance of ulceration or vascularization between the treated vs. control groups. In the second study, distinct differences were noted between the control- and inhibitor-treated corneas. First, about half of the control corneas had notable vascularization, but there was no vascularization of the SIMP-treated group. Second, there were more polymorphonuclear leukocytes (PMN) in the control corneas (Fig. 3). PMN were noted in both groups when peripheral reepithelialization was present, but the SIMP-treated corneas had few PMN centrally in all cases.

**Discussion**

The results of the present experiments clearly demonstrate the efficacy of SIMP in the treatment of established corneal ulceration resulting from experimental alkali burns. However, treatment with the inhibitor must be initiated early in the ulceration process before significant corneal melting occurs.

Noninfectious corneal melting caused by severe alkali burn injury can be among the most difficult and challenging of the corneal diseases for the ophthalmol-
ogist to treat effectively. Alkali rapidly penetrates ocular tissues, resulting in saponification of plasma membranes, ischemia, and destruction of collagen. In the case of mild burns, fibroblasts infiltrate and secrete newly synthesized collagen. In a severe burn, however, the epithelial surface and underlying fibroblasts are destroyed, which greatly increases the risk of corneal ulceration, perforation, and infection.

As yet, there is no mainstay of therapy for the ophthalmologist to manage an alkali burned eye and prevent the potentially devastating complications. Initial therapy of alkali injuries involves copious irrigation of the ocular surface with sterile water or normal saline. Subsequent treatment modalities vary from the use of soft contact lenses and epidermal growth factor, which promotes restoration of the corneal epithelium, to the use of agents that inhibit epithelial regrowth and infiltration of PMN into the cornea, such as glued-on contact lenses and cyanoacrylate tissue adhesives. Other treatments have been advocated through animal studies, such as the use of sodium citrate and sodium ascorbate. More recently, however, attention has been focused on the effectiveness of metalloproteinase inhibitors.

Early studies by Brown suggested that nonspecific collagenase inhibitors reduced the incidence of corneal ulceration in experimental corneas after alkali injury. Agents that inhibit collagenase include acetylcysteine, cysteine, NaEDTA, and penicillamine. However, these agents have been shown to have limited efficacy in vivo. Another compound that inhibits collagenase in vitro and is presumed to inhibit collagenolytic activity in vivo is tetracycline. We have demonstrated the efficacy of a synthetic inhibitor of mammalian collagenase (SIMP) in treating the experimental alkali burned cornea. This inhibitor was designed to bind to collagenase, a Zn2+-dependent metalloproteinase, and block collagen degradation by interacting with Zn2+ at the active site. It effectively prevented corneal ulceration when therapy was initiated immediately after the alkali burn.

The present study was designed to test the effect of SIMP on established corneal ulcers, because this situation may be more applicable clinically. SIMP had no effect on the progression of established corneal ulceration compared to control eyes when treatment was begun at a clinical score of 2. The established criterion was that the corneas must maintain a score of 2 for two subsequent examination days before entered into the study. This meant that most of the corneas had anterior stromal ulcers present for several days. Similarly, Pfister et al found that the therapeutic effect of topical citrate diminished as the depth and severity of the established ulcer increased. In the second experiment, we began treatment at the onset of ulceration or at a clinical score of 1 (superficial ulcer) after two consecutive examination days. SIMP was found to have a favorable effect on these established ulcers.

We speculate is that in the first study, when treatment was withheld until mid stromal ulceration, SIMP was ineffective because a larger number of PMN had already entered the cornea to release their destructive enzymes or that a different spectrum of enzymes was present at the time of therapy. In support of this, there appeared to be no difference in the number of PMN present in the SIMP vs. control group upon histologic examination. Histologic examinations of tissue from the second study, which began treatment at the start of ulceration, confirmed the initial studies in our laboratory by Burns et al. The SIMP-treated corneas contained few PMN centrally, but in control corneas, PMN infiltrated throughout the stroma (Fig. 3). The peripheral cornea appeared to have the greatest accumulation of PMN, and their density appeared to correlate with peripheral reepithelialization.

Paterson et al have quantitated the time course of PMN infiltration into the cornea post alkali burn and found it to be a biphasic phenomenon with an early transient peak at 12–24 hr and a second peak around 21 d beginning at days 12–14. In our present experiments, corneal ulcers had progressed to anterior stromal level (score of 1) by days 5–8 post alkali injury and to mid stromal level (score of 2) by days 9–14. An effect of SIMP inhibitor upon PMN chemotaxis might be suspected. However, we have found no direct effect of SIMP upon PMN locomotion using the agarose method to measure chemotaxis (unpublished data). It has been shown that the supernatant from alkali burned commercial collagens is chemotactic for PMN. SIMP may have an indirect effect on PMN influx by inhibiting collagen destruction and the accumulation of degradative products that are chemotactic.

Key words: corneal ulcer, alkali burns, proteinase inhibitor, collagenase, metalloproteinase

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References