Inhibition of Alkali-Induced Corneal Ulceration and Perforation by a Thiol Peptide

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Corneal ulceration and perforation following a severe alkali burn occur as a consequence of collagen destruction by locally released enzymes. A thiol peptide, which recently was shown to be a potent inhibitor of corneal collagenase in vitro, was tested in alkali-burned rabbit corneas to determine its effectiveness in inhibiting corneal ulceration. Following a standard alkali burn to one eye of each rabbit, ten animals were treated topically six times daily and subconjunctivally one time daily with a 1 mM solution of the peptide for a period of 3 weeks. A control group of ten rabbits was administered vehicle only using the same regimen as the experimental group. Corneal ulceration occurred in ten out of ten of the control eyes and seven out of ten progressed to perforation. The experimental group demonstrated ulcerations in four out of nine animals, only one of which was deep (one of nine), and no perforations. There was no significant difference when comparing the onset of ulceration between the two groups, but the difference was significant when comparing the total number of ulcerations (0.02 < P < 0.05), deep ulcers (0.01 < P < 0.02) and perforations (0.001 < P < 0.01) between the two groups. Histologic examination of the corneas after 3 weeks of treatment revealed that the experimental, thiol-treated corneas that did not ulcerate contained relatively few PMNs, whereas the control corneas demonstrated a marked inflammatory infiltrate in the form of PMNs, most notably at sites of corneal ulceration. These findings demonstrate that a synthetic thiol peptide inhibits alkali-induced corneal ulceration and perforation in vivo. Invest Ophthalmol Vis Sci 31:107-114, 1990

The alkali-burned eye presents a major therapeutic challenge to the ophthalmologist.1 Depending on the clinical condition of the cornea post-burn, numerous therapeutic approaches are used in treating the alkali-injured eye, including steroids, heparin, collagenase inhibitors, contact lenses, fibronectin, conjunctival flaps and corneal transplantation.1,2 Recently, several experimental therapeutic approaches, including sodium citrate and sodium ascorbate, have been advocated.3-5 Numerous studies in the past have demonstrated that the corneal damage following an alkali burn is due to the destruction of corneal collagen by locally released enzymes.6-8 Corneal collagenase has been implicated as one of the principle enzymes involved in the destruction of the cornea.9,10 Therefore, a well justified approach to therapy has been to inhibit collagenase, thus preventing this destruction.

Inhibitors of collagenase have been used for about 20 years in the treatment of alkali-induced corneal ulceration. Many compounds have been tested for their efficacy in preventing corneal ulceration and perforation in this setting but, in general, they have been found to be relatively ineffective in vivo.1 Compounds that have been used as collagenase inhibitors include acetylcysteine,11 cysteine,11,12 Na2EDTA13 and penicillamine.14 Acetylcysteine (Mucamyst) is approved for use as a mucolytic agent only, but is used topically as a collagenase inhibitor in human corneal alkali burns.1,2 Tetracycline compounds have been recently shown to inhibit alkali-induced corneal ulceration, presumably by inhibiting collagenase.15

Recently we have shown that a newly developed β-mercaptomethyl tripeptide (Fig. 1) is highly active in inhibiting purified collagenase from alkali-burned rabbit corneas, in vitro.16 In comparison to other compounds used in the treatment of alkali-induced corneal ulceration, the thiol compound was demonstrated to be far greater in potency in inhibiting corneal collagenase.

In view of our in vitro findings, the present study was undertaken to evaluate the efficacy of the thiol...
HSCH$_2$CH$_2$CO-NHCH$_2$CONHCH$_2$CONH$_2$

CH$_2$

CH

(CH$_3$)$_2$

HSCH$_2$Leu-Phe-Ala-NH$_2$ (Thiol Peptide)

Fig. 1. Structure of the thiol peptide used in this study. The compound is a $\beta$-mercaptomethyl tripeptide developed specifically as an inhibitor of collagenase. The thiol peptide has previously been shown to be a potent inhibitor of corneal and synovial collagenase and has been demonstrated to be far more potent than its carboxyalkyl analogue.

peptide in preventing alkali-induced corneal ulceration and perforation in rabbits.

Materials and Methods

General Considerations

Twenty New Zealand Dutch strain albino rabbits of both sexes weighing between 2.0 and 2.5 kg were anesthetized by intramuscular injection of 10 mg/kg xylazine and 37.5 mg/kg ketamine HCl. One eye of each animal received topical tetracaine and was proposed for alkali burning. A sharply defined 12.7 mm corneal burn was produced by pipetting 0.5 ml of 2N sodium hydroxide into a plastic well held firmly against the cornea for 60 sec. The interior of the well and the surface of the eye were then thoroughly irrigated with saline for 5 sec. Irrigation was then continued for 5 sec after removal of the well. Erythromycin ophthalmic ointment (Pharmafair, Inc., Hauppage, NY; 0.5%) was immediately applied to the eye and once daily thereafter. Rabbits were randomly assigned into two groups, one group being treated with the peptide and the other being treated with vehicle only. The methods of this study were in accord with the ARVO Resolution on the Use of Animals in Research.

Solution Preparation

Preparation of the thiol peptide, shown in Figure 1, has been described previously. The peptide was dissolved in 95% ethanol containing 1 mM acetic acid. The solution was then dissolved in Adsorbotear without EDTA or thimerosol (Alcon, Inc., Fort Worth, TX) to give a final peptide concentration of 1 mM. This solution was then stored at 4°C until ready for use. Ethanol concentration varied between 2 and 5% in the final solution depending upon the concentration of the stock solution of the peptide. Fresh solutions of the peptide were made every 24 to 48 hr. Concentration of the peptide in Adsorbotear was confirmed by HPLC monitoring of the solution for representative peaks. Equal volumes of ethanol containing 1 mM acetic acid were added to a second solution of Adsorbotear for use as the control vehicle which was also kept at 4°C.

Peptide preparations used for subconjunctival injections were made immediately prior to the time of treatment. The thiol peptide was dissolved in 95% ethanol containing 1 mM acetic acid. The solution was added to normal saline to form a 1 mM concentration of peptide in a total volume of 0.5 ml. Ethanol concentration varied between 2 and 5% in the final solution, depending upon the concentration of the stock solution of the peptide. Equal volumes of 95% ethanol containing 1 mM acetic acid were dissolved in normal saline for use as the control vehicle. Solutions were used immediately after preparation.

Treatment Regimen

Animals were treated topically with one drop of either the peptide or the vehicle only in the alkali-burned eye six times daily, every 2 hr from 8 AM to 6 PM. Animals were returned to their cages immediately after treatment. At 8 PM daily, rabbits were treated with subconjunctival administration of peptide or the vehicle only. Following topical anesthesia with tetracaine, injections of 0.5 ml of the solutions were made subconjunctivally at the twelve o'clock position of each alkali-burned eye using a sterile tuberculin syringe and a 30-guage needle. All animals were noted to have subconjunctival blebs in the superior fornix following injection. At 9 PM daily, topical erythromycin ointment was applied to the alkali-burned eye of each rabbit. This treatment regimen was continued for 3 weeks following the alkali burn.

Clinical Observations and Tissue Analysis

External examinations of each eye were done once daily during the entire study. Detailed, double-masked, slit-lamp examinations of each rabbit were performed every other day initially for the first 10 days and then every day following the onset of corneal ulceration. 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. 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 by classification 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 pos-
terior one-third of the cornea): score = 3; (5) descemetocele: score = 4; (6) perforation: score = 5. Corneal features were documented by macrophotography using an Olympus Zuiko Medical Macro camera. Photography was done twice weekly initially for the first 10 days and then as needed to document any significant change in pathology. Topical and subconjunctival treatments were continued until the animals were sacrificed by pentobarbital overdose via ear vein injection on day 21 of the experiment. Animals which developed corneal perforations were sacrificed at the time the perforation was discovered.

After sacrifice, the anterior chamber of the alkali-burned eye was entered with a scalpel blade, and the entire cornea was excised from the eye with corneal scissors. Corneas were immediately placed in 10% formalin. Following formalin fixation, corneas were embedded in paraffin and stained with hematoxylin and eosin in preparation for routine histologic examination. One animal in the experimental group was sacrificed on the fourth day of the experiment after suffering a spinal injury; the data were excluded from the final analysis of the results.

Statistics

The results were analyzed for significance using the Chi-square test with Yate's correction and the student t-test. The clinical severity of ulcers was compared for significance using the Mann-Whitney U test.

Results

The severe alkali burns employed in this study resulted in corneal stromal opacities extending to the limbus of each burned eye. None of the eyes became infected and none of the corneas became significantly vascularized. Clinical examination of the subconjunctival injection sites of both the experimental and control eyes showed no signs of necrosis or tissue damage and there was no difference in clinical appearance of the injection sites between the control and inhibitor groups.

Table 1. Analysis of clinical observations for corneal alkali burns at end of experiment

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<tr>
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<th>Experiments (n = 9)</th>
<th>Controls (n = 10)</th>
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<tbody>
<tr>
<td>Nonulcerated corneas</td>
<td>5</td>
<td>0</td>
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<tr>
<td>Total vascularizations</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Superficial stromal ulcers</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Moderate stromal ulcers</td>
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<td>Deep stromal ulcers</td>
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<td>Descemetocoeles</td>
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<td>Perforations</td>
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A striking difference was noted between the experimental and control groups in the development of corneal ulcerations and perforations. The control group demonstrated ulcerations in ten (100%) of ten corneas compared with four (44%) of nine corneas in the experimental group (0.02 < P < 0.05). A total of eight (80%) of the ten control corneas progressed to deep ulceration or descemetocele formation followed by seven (70%) of ten progressing to perforation. The experimental group showed one (11%) of nine progressing to deep ulceration (0.01 < P < 0.02) and none progressing to descemetocele formation or perforation (0.001 < P < 0.01). Table 1 summarizes the overall results from the two groups and Table 2 illustrates the difference between the two groups in terms of incidence of overall ulceration, deep ulceration and perforation.

The mean length of time for appearance of corneal ulceration was 14 ± 4 days (range 8–19) in the control group and 16 ± 1 days (range 15–17) in the experimental group. There was no significant difference when comparing the time of onset of ulceration. The tendency was for the control group to develop ulcers sooner and, once established, to progress rapidly to deep ulcers and descemetoceles. The mean length of time for the development of corneal perforation was 17 ± 2 days (range 15–20) in the control group. As mentioned previously, the experimental group demonstrated no perforations.

Figure 2 illustrates data obtained from clinical observations during the course of the study. Exam 0 represents the day of the alkali burn. The final examination (exam 12) occurred on day 20 post-burn. The number of control eyes decreased as they were removed secondary to perforation. The depth of ulcers was represented by a numerical score, as explained in Methods, above. The depth of ulceration progressively worsened in the control group from exam 5 through exam 12, while the thiol treatment significantly reduced the progression of ulceration. The two groups were significantly different in average clinical
Light Microscopy

All of the corneas were examined by light microscopy following the clinical aspect of the study. It was noted that none of the corneas in either group showed significant reepithelialization. The degree of vascularization varied between corneas, but it was limited to the periphery, and there was no notable difference in vascularization between the two groups.

The anterior stroma and mid-stroma surrounding the ulcerations and perforations, in general, were infiltrated with inflammatory cells. In comparing ulcerating and nonulcerating corneas from the two groups, it was of interest that corneas in the experimental group which were not ulcerating (five of nine) were essentially devoid of polymorphonuclear leukocytes (PMNs) in the central area of the cornea. These experimental corneas also showed a total loss of keratocytes but essentially no loss of stromal substance. The other corneas in the experimental group had varying degrees of PMN infiltration which seemed to correlate with the depth of ulceration present. A representative nonulcerating cornea from the experimental group which demonstrates a lack of PMN infiltration is seen in Figure 3A.

The control corneas demonstrated a marked amount of PMN infiltration throughout six of the ten corneas. It was noted that all four control corneas that did not have generalized PMN infiltration progressed to perforation, and three of the four perforated by day 15 and the fourth on day 17 of the study. The most striking infiltration in these corneas took place at sites of corneal ulceration. A representative area of PMN infiltration in a nonperforated control cornea is shown in Figure 3B. It also was noted that the three eyes in the control group that did not perforate had PMN infiltration throughout the cornea, varying from mild in one to marked in two.

Discussion

Ocular alkali burns create a complex therapeutic challenge for the ophthalmologist. Alkali injuries can progress to corneal ulceration and perforation, thus causing loss of vision and permanent visual disability. The control and prevention of this pathologic progression have been the focus of numerous studies. The treatment approaches that have been used in treating alkali injuries have been thoroughly reviewed. Treatment modalities that have been successful in reducing the incidence of corneal ulceration and perforation in the alkali-burned cornea fall into five categories: (1) agents which inhibit the locomotion...
Fig. 3. Representative histologic sections from alkali-burned corneas. (A) Nonulcerated cornea from the experimental group (rabbit #776) following 3 weeks of topical and subconjunctival treatment with 1 mM thiol peptide. The cornea is essentially devoid of cells and there are no signs of ulceration (hematoxylin-eosin, original magnification ×100). (B) Ulcerated cornea from the control group (rabbit #772) following 3 weeks of topical and subconjunctival treatment with vehicle only. The anterior and mid-stroma are infiltrated with PMNs and other inflammatory cells at the site of an ulcer (hematoxylin-eosin, original magnification ×100).

Collagenase has been identified as a major contributor to the breakdown of the cornea following such an injury. Compounds that have been used previously as collagenase inhibitors in treating alkali burns in humans have been relatively ineffective in vivo and, thus, work has continued to produce more effective treatment approaches. Our findings in the present study indicate that a recently developed peptide derivative designed to inhibit collagenase has a significant impact upon reducing alkali-induced corneal ulceration and perforation at markedly lower concentrations than previous compounds. This effect of the peptide almost certainly can be ascribed to its ability to interfere with the collagenase-mediated destruction of the corneal stroma.

Synthetic inhibitors of collagenase have been designed specifically to bind with collagenase and, thus,
block the enzyme’s collagen-cleaving action.\textsuperscript{17,24,25} The mechanism by which the peptide presumably functions is by binding to the substrate recognition site of the enzyme and coordinating with catalytically essential Zn\textsuperscript{2+} at the active site.\textsuperscript{26} Development of these peptides is based upon strategies used to develop potent inhibitors of angiotensin-converting enzyme.\textsuperscript{26} Previous studies have shown that these compounds are highly potent inhibitors of collagenases from several sources including: (1) pig synovial collagenase\textsuperscript{17,25}; (2) rabbit V-2 tumor collagenase\textsuperscript{17,25}; and, most recently, (3) purified corneal collagenase from alkali-burned rabbit corneas.\textsuperscript{16} Thiol peptides, such as the one used in this study (Fig. 1), have been demonstrated to be far greater in potency than their N-carboxyalkyl counterparts.\textsuperscript{16,25} In comparison to other compounds that have been demonstrated to reduce the incidence of alkali-induced corneal ulceration, the thiol peptide was shown to be far greater in potency in inhibiting corneal collagenase in vitro. This included comparison to acetylcysteine, cysteine, sodium citrate, sodium ascorbate and tetracycline compounds.\textsuperscript{16} For this reason, the thiol peptide was evaluated in vivo for its efficacy in reducing alkali-induced corneal ulceration.

In the present study we have shown one particular thiol peptide to be a potent inhibitor of corneal ulceration and perforation following a severe alkali burn in rabbits. The burn was very severe; 70% of the control eyes perforated within 3 weeks. The area of the burn extended to the limbus, which prevented significant vascularization or reepithelialization. Collagen destruction occurred rapidly once ulceration appeared. In the experimental (thiol-treated) group there was a significant reduction in the progression of ulceration and perforation. Overall, the majority of corneas in the experimental group did not ulcerate and only one progressed to deep ulceration; this was a marked difference in comparison to the extreme pathology noted in the control group. This observation strongly supports the contention that corneal ulceration following the release of collagenases is effectively inhibited by a synthetic collagenase inhibitor. Qualitative examination of the histologic sections of the ulcerated eyes showed a marked PMN infiltration moving from the limbal region inward (Fig. 3B). Infiltration of the cornea with acute and chronic inflammatory cells is a well described phenomenon occurring during corneal ulceration, with the PMN being the predominant cell type following alkali burns.\textsuperscript{20,27,28} It was of some interest to note that examination of nonulcerated experimental eyes revealed relatively few inflammatory cells in the cornea following treatment with the thiol peptide (Fig. 3A).

In six of the ten control corneas, histological examination revealed PMNs throughout the stroma. The remaining four corneas, all of which perforated, were infiltrated by a large number of PMNs peripherally around the sites of perforation, but PMNs were not seen centrally. It is likely that ulceration and perforation in these corneas took place at the corneal periphery as a result of the accumulation of PMNs and release of their lysosomal enzymes at that site. Perhaps PMN infiltration into the central stroma did not occur because early perforation did not afford the physiological environment or time frame for chemotaxis to take place. It is also possible that the lack of adequate vascularization reduced PMN infiltration, although the finding that six of ten control corneas were diffusely infiltrated even without significant vascularization argues against this theory.

Although the primary impact of the thiol peptide is to directly block the destruction of stromal collagen by collagenase, there also might be an indirect effect upon PMN chemotaxis. Infiltration of the PMNs into a connective tissue matrix may require collagenase and other proteases to cleave stromal collagen fibrils surrounding the PMN, thus allowing more easy penetration and chemotaxis of the PMNs to occur. Thus, inhibition of collagenase might result in reduced PMN infiltration, thereby enhancing the prevention of ulceration and perforation in these corneas. While this speculation is based only upon qualitative histologic observation, the concept that PMN infiltration was reduced in the alkali-burned cornea by treatment with a potent inhibitor of collagenase is interesting; a similar observation was made previously when rabbits with alkali-burned corneas were treated with tetracycline systemically.\textsuperscript{15}

Furthermore, it has been proposed that alkali-burned collagen fragments may be a mediator for PMN influx.\textsuperscript{29} It has been demonstrated that the supernatant from alkali-burned commercial collagens is chemotactic for PMNs and stimulates PMN respiratory burst; it was suggested that following alkali-burning of the cornea, peptide fragments of corneal collagen could diffuse away from the cornea across the surfaces or through the stroma to the limbal area where interaction with serum albumin could facilitate PMN locomotion. After invading the cornea through the damaged collagen matrix, the PMNs might then release their proteolytic enzymes, which would lead to further stromal destruction. Perhaps the thiol peptide has interfered with this feedback mechanism by inhibiting the proteolytic enzymes released by the PMN, thus preventing further generation of chemotactic collagen fragments.

Collagenolytic activity has been found to be pro-
duced by non-PMN sources, such as from epithelial cells and fibroblasts. Initially, lysis of epithelial cells and fibroblasts would cause release of their enzymes and it is likely that the peptide inhibited metalloproteases, which were released by these cells. However, because these cells were destroyed by the alkali burn, it is unlikely that they were a continuous source of proteolytic enzymes following initial release. Thus, the peptide's effect most likely was not due solely to inhibition of enzymes derived from sources other than PMNs. Although the source of collagenase in these corneas is not clear, it is likely that collagenase and proteases released by PMNs were inhibited by the peptide and that the overall protective effect of the peptide was due to this inhibition. The corneas treated with the peptide also showed a total loss of keratocytes but essentially no loss of stromal substance. This finding indicates that stromal collagen was protected from degradation by the thiol peptide.

In summary, this study has shown that a thiol peptide, designed to inhibit collagenase, reduces the incidence of corneal ulceration and perforation in alkali-burned rabbit corneas. The treatment of the alkali-burned cornea should be a combined approach using agents that have different mechanisms of action. Because the PMN plays a major role by releasing destructive enzymes, including collagenase, the inhibition of its locomotion and activation with an agent such as citric acid is a fundamental therapeutic component. Second, ascorbic acid treatment, which replaces the depleted levels of the co-factor necessary for collagen production, is also regarded as a crucial component. Finally, a potent metalloprotease inhibitor should be a component of therapy because of the need to inhibit collagenase and other proteases released into the cornea. The thiol peptide used in this study could be a candidate for this aspect of treatment.

Key words: cornea, alkali burn, corneal ulceration, thiol peptide, collagenase inhibitor

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References


