Recombinant Tissue Inhibitor of Metalloproteinases Type 1 Suppresses Alkali-Burn-Induced Corneal Ulceration in Rabbits


Purpose. To test if recombinant tissue inhibitor of metalloproteinases (TIMP-1) was effective in reducing corneal ulceration after alkali injury to the rabbit cornea. The effect of TIMP-1 was compared with that of a proven synthetic metalloproteinase inhibitor.

Methods. After a defined alkali injury to the rabbit cornea, a topical treatment regimen was followed for 24 days; one group was treated with vehicle only, a second group with recombinant TIMP-1, and a third group with the synthetic metalloproteinase inhibitor. Corneas were scored for ulceration during the 24-day period and the scores for the three groups were compared.

Results. The incidence and progression of ulceration and perforation, in the alkali-burned corneas receiving treatment with recombinant TIMP-1 or the synthetic inhibitor, were significantly less than in corneas receiving vehicle treatment alone.

Conclusion. Recombinant TIMP-1 is as effective as a proven synthetic inhibitor in ameliorating corneal ulceration and perforation after an alkali injury. Invest Ophthalmol Vis Sci. 1994;35:677-684

Corneal ulceration after alkali injury is largely the result of proteolytic enzymes that degrade the extracellular matrix of the cornea.1-3 The source of these enzymes can be the cellular components of the cornea itself or inflammatory cells that rapidly invade the corneal tissue after chemical injury. The matrix metalloproteinases are a family of zinc-dependent enzymes capable of catalyzing the degradation of a variety of extracellular components; these enzymes include stromelysins, gelatinase, and collagenase.4 The expression of several metalloproteinases by tissue-cultured corneal tissue was demonstrated recently.5,6 Metalloproteinases are also produced by numerous cell types (see review by Brown et al9), including macrophages and polymorphonuclear leukocytes (PMNs) that invade the injured cornea.7 The serine proteases may also play a role in corneal ulceration.8 One approach to treating the alkali-injured cornea has been the use of compounds that inhibit the enzymes responsible for tissue destruction. Nonspecific inhibitors such as acetylcysteine,9 cysteine,10 sodium ethylenediaminetetraacetic acid,11 and tetracycline12 have all been demonstrated to exert a beneficial effect on alkali-burn-induced corneal ulceration; most of these compounds chelate zinc at the active site of the metalloproteinases. More recently, specific inhibitors of metalloproteinases have been synthesized; some of these compounds are very effective at reducing corneal ulceration and preventing perforation.13-16

The matrix metalloproteinases are endogenously regulated by natural inhibitors.3 There are two principal types of matrix metalloproteinase inhibitors: α2 macroglobulin and the tissue inhibitors of matrix metalloproteinases referred to as TIMPs (tissue inhibitor of metalloproteinases). The two forms, TIMP-1 and TIMP-2, are synthesized and secreted by many cell types; they have been isolated and characterized biochemically.3,17 Purified recombinant TIMP-1 has potent inhibitory activity against collagenase, stromelysin, and gelatinase.17 This study was therefore de-
signed to test if the recombinant TIMP-1 was effective in reducing corneal ulceration and perforation after an alkali injury to the rabbit cornea. The effect of TIMP-1 was compared directly with that of a synthetic inhibitor of metalloproteinases previously shown to reduce ulceration and perforation after alkali injury.14,15

**MATERIALS AND METHODS**

**Preparation of TIMP-1**

Human TIMP-1 expression from a human cytomegalovirus promoter was amplified using glutamine synthetase as a selectable marker in a Chinese hamster ovary cell line (GS 19.6) that was subsequently adapted for growth in suspension.18 Growth of the cell line in a 200 l air lift fermenter generated conditioned media containing approximately 22 g TIMP-1. After concentration and filtration through a 0.2 µm cartridge, the TIMP-1 was purified using an anti-TIMP-1 immunoglobulin G (MAC 15) Sepharose column followed by gel permeation chromatography on Sephacryl S200 (Pharmacia, Uppsala, Sweden), essentially as described by Williamson et al.19 Precautions taken to avoid pyrogen contamination included the rinsing of equipment in 0.1 M NaOH, the use of pyrogen-free water in all buffers and additional sterile filtration steps. The purified TIMP-1 in phosphate-buffered saline at pH 7.5 was stored as 1 mg aliquots at 4°C before use. Densitometry analysis of a Coomassie blue stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis reduced gel demonstrated that the TIMP-1 was >92% pure and its concentration using an A1%/280 (ε1cm = 10 (calculated from a total amino acid analysis and a molecular mass of 28.5 KDa) was 0.2 mg/ml. It was shown to be fully active by titration against human stromelysin-20 using the fluorescent substrate dinitrophenyl-4-aminobenzilic acid substrate (SIMP).21 The material used in the current study passed a rabbit pyrogen test22 at a concentration of 0.1 mg/kg and was estimated to contain 2.79 endotoxin units/mg by limulus amebocyte lysate assay23 using a kit supplied by Whittaker Bioproducts (Walkersville, MD).

**Synthetic Inhibitor of Metalloproteinases (SIMP)**

The synthetic inhibitor is a β-mercaptomethyl tripeptide developed by Darlak et al.24 This compound has been shown to inhibit metalloproteinases in vitro and to reduce corneal ulceration and perforation after alkali burns.14,15 For the current study, the peptide was purchased from Peptides International (Louisville, KY).

For topical application, the peptide was dissolved in 95% ethanol containing 1 mM acetic acid to generate a 30 mM solution. The 30 mM stock solution was diluted daily with argon-purged, phosphate-buffered saline to 1 mM before use. Argon-purged, phosphate-buffered saline was used to minimize oxidative inactivation of the inhibitor.

**Alkali Burn Procedure**

Thirty New Zealand Dutch strain albino rabbits of both sexes, weighing between 2 and 2.5 kg, were used in this study. Rabbits were housed in the University of Louisville Research Resource Center for the duration of the study and treated in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was induced by an intramuscular injection of 10 mg xylazine/kg body weight and 37.5 mg ketamine HCl/kg body weight. Immediately before the alkali burn procedure, two drops of proparacaine hydrochloride (Alcaine, Alcon Laboratories, Fort Worth, TX) were applied to the cornea.

After administration of topical anesthesia, one eye of each animal was gently proptosed and a sharply defined 12.7 mm diameter circular area alkali burn was made to the cornea. The alkali burn technique is well established in this laboratory.14,15 In brief, approximately 0.5 ml of 2 N sodium hydroxide is placed by pipette into a plastic well held firmly against the cornea for 60 seconds. The well is positioned such that the alkali injury does not extend beyond the limbal region. After 60 seconds, the sodium hydroxide is aspirated from the well and the interior of the well is irrigated with saline. The well is then removed and the external eye thoroughly irrigated with saline for at least 20 seconds. Animals were randomly assigned to three groups of ten: one group for TIMP-1 treatment, another group for treatment with the synthetic peptide inhibitor, and a third group that would receive vehicle treatment only.

**Treatment Regimen**

Starting within 2 hours of the alkali burn, the alkali-injured eyes received one 50 µl drop of the appropriate solution: TIMP, synthetic peptide, or phosphate-buffered saline. Topical treatment was repeated every 2 hours, daily, from 8 AM to 6 PM. At 8 PM daily, all treated eyes were injected subconjunctivally. Rabbits were anesthetized as described earlier; 0.5 ml of the appropriate treatment solution was injected subconjunctivally at the 12 o'clock position using a tuberculin syringe and a sterile 30-gauge needle. After subconjunctival injection, the eyes were treated with topical gentamycin sulfate ointment (Gentrasul; Bausch & Lomb, Tampa, FL) prophylactically. This treatment regimen was continued for 24 days or until the cornea perforated, at which point the rabbit was killed. At 25 days, all animals remaining in the study were killed by...
an overdose of sodium pentobarbital delivered via a marginal ear vein.

After death, all the treated eyes were enucleated and immediately placed in 10% formalin for histology. Formalin-fixed eyes were embedded in paraffin and thin sections were stained with hematoxylin and eosin.

Assessment of Corneal Ulceration and Perforation

External examination of all treated eyes was performed daily with a handheld slit lamp, in a double-masked fashion, while the animal was under anesthesia before delivery of subconjunctival injection at 8 PM. Corneas were examined for the presence of defects, ulceration, perforation, vascularization, and infection. Photographic record was obtained at selected intervals.

Each treated cornea was assigned a score based on the severity of corneal ulceration. Ulcers were classified as follows: 0, no defect or ulceration; 1, ulceration limited to the anterior one-third of the cornea; 2, ulceration extending to the middle third of the cornea; 3, ulceration extending into the posterior third of the cornea; 4, descemetocele formation; and 5, perforation. The scoring system, based on a study by Pfister et al., has been used extensively in our previous studies.14,15

Statistical Analysis of Data

Mean daily ulcer scores for each treatment group were calculated together with 95% confidence intervals. Comparison of mean ulcer scores between groups was made using resampling-based multiple testing.26 The analysis was performed with PROC MULTTEST (SAS Institute, Cary, NC).27 Resampling-based multiple testing is a powerful test that does not make any assumptions about the distribution of P values. The method performs simultaneous t tests and adjusts the bootstrapped P values for multiplicity.

RESULTS

The incidence and progression of ulceration and perforation in alkali-burned corneas receiving treatment with recombinant TIMP-1 or the synthetic peptide inhibitor were significantly less than that in corneas receiving vehicle treatment only. Table 1 gives the daily score for each eye in the study, together with daily mean values and 95% confidence intervals for each treatment group. These data are also given in Figure 1. Statistical comparison of the groups was made using resampling-based multiple testing as described earlier.

Applying this test to the means shown in Table 1 gave the raw and adjusted P values shown in Table 2. Using a significance level of adjusted P value = 0.05 revealed a difference between control and SIMP-treated corneas beginning at day 15 and difference between control and TIMP-treated corneas beginning at day 10. This statistical treatment revealed no difference between the group treated with SIMP or TIMP.

An unusual feature of the ulceration profile was the transient appearance of superficial ulceration in all groups between days 1 and 8 (Fig. 1); this was not observed in our previous studies, and we have no explanation for it. The subsequent pattern of ulceration and perforation in the vehicle treated group and the synthetic peptide inhibitor treated group was essentially identical to that in our previous studies.14,15 Of the ten corneas in the vehicle-treated eyes, three perforated, four developed descemetoceles, one progressed to score 2, and another two remained at score 1. At the termination of the study, only two of the synthetic-inhibitor–treated corneas had developed ulceration at a score of 2; four corneas had ulceration at score 1, and three had no ulceration. One animal died at day 19 with a corneal score of 0. Alkali-burned corneas treated with TIMP-1 exhibited a pattern of clinical pathology very similar to that of the SIMP–treated corneas. In the TIMP-treated group, mean clinical scores remained below a score of 1 throughout the study; at the termination of the study, only two of the corneas developed a corneal ulcer with a score of 2, five corneas had a score of 1, and three corneas showed no ulceration.

During the course of the experiment, one rabbit in the SIMP–treated group died; postmortem examination did not reveal the cause of death. Ocular infection developed in none of the rabbits, presumably because of the prophylactic use of gentamycin. Neovascularization was minimal in all alkali-burned eyes, extending to only 2 to 3 mm of ingrowth in a few animals in each of the three groups.

Histopathology of vehicle-treated corneas revealed extensive PMN infiltration, stromal destruction, and the absence of a continuous, intact epithelium (Fig. 2A). The anterior chamber of the eye contained exudate and cells. Eyes treated with synthetic peptide inhibitor, in contrast to control eyes, exhibited minimal PMN infiltration, largely intact corneal stroma, and less anterior chamber exudate (Fig. 2B); these findings parallel those of earlier studies with the synthetic inhibitor.14,15 Alkali-burned corneas treated with TIMP-1 in general had intact corneal stroma and limited anterior chamber exudate. PMN infiltration appeared to be reduced from that seen in vehicle-treated eyes but possibly greater than that seen in the SIMP–treated eyes (Fig. 2C); no attempt was made to quantify these differences.
| Rabbit # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| C       | 0.5 | 1.5 | 1.5 | 1 | 1 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2.5 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 |
| O       | 0.5 | 1.5 | 1.5 | 1 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 | 4 | 4 | 5 | 5 | 5 |
| N       | 0.5 | 1.5 | 1.5 | 1 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 | 3 | 3 | 3 | 5 | 4 | 4 | 4 |
| T       | 0.5 | 1.5 | 1.5 | 1 | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 | 3 | 5 | 4 | 4 | 4 | 4 |
| R       | 0.5 | 1.5 | 1.5 | 1 | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 | 3 | 5 | 4 | 4 | 4 | 4 |
| O       | 0.5 | 1.5 | 1.5 | 1 | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 | 3 | 5 | 4 | 4 | 4 | 4 |
| L       | 0.5 | 1.5 | 1.5 | 1 | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 0.5 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 | 3 | 5 | 4 | 4 | 4 | 4 |
| 20      | 0   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
| 210     | 0   | 1   | 1   | 1   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |

**TABLE 1. Individual and Mean Corneal Ulceration Scores**

<table>
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<tr>
<th>Time (Days)</th>
<th>MEAN</th>
<th>95% CI</th>
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<td>0.05</td>
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<td>0.099</td>
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Clinical score: 0 = no ulcer; 1 = superficial (anterior 1/3); 2 = moderate (middle 1/3); 3 = deep (posterior 1/3); 4 = descemetocoele; 5 = perforation. 95% CI = 95% confidence intervals.
FIGURE 1. The progression of corneal ulceration in alkali-injured rabbit corneas. (A) Compares the control vehicle-treated group with that treated with the synthetic inhibitor of metalloproteinases. (B) Compares the vehicle-treated group control with that treated with recombinant TIMP-1. The control data are the same in both figures. The vertical bars represent SEM. The clinical scoring system and the number of rabbits used is described in the text.

DISCUSSION

The results of this study clearly demonstrate that TIMP-1, a natural inhibitor of matrix metalloproteinases, is largely effective in preventing extensive corneal ulceration up to 24 days after an alkali injury. To our knowledge, this represents the first demonstration of a therapeutic effect of TIMP-1 on an ulcerative process in vivo. This initial study shows that TIMP-1 is at least equally effective at ameliorating corneal ulceration up to 24 days after an alkali injury.

TABLE 2. Raw and Adjusted P Values for Differences Between Treatment Groups Generated by Resampling-Based Multiple Testing

<table>
<thead>
<tr>
<th></th>
<th>Control vs SIMP</th>
<th>Control vs TIMP</th>
<th>SIMP vs TIMP</th>
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<tbody>
<tr>
<td></td>
<td>Raw P Values</td>
<td>Adjusted</td>
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<td>Raw P Values</td>
<td>Adjusted</td>
<td>Raw P Values</td>
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</table>

* Significant difference.
Ulcration of the cornea after chemical injury is most likely the result of proteolytic enzymes, largely metalloproteinases, which catalyze the degradation of the extracellular matrix proteins, such as collagen, and proteoglycans of the corneal stroma. Therefore, an obvious strategy to the treatment of the chemically injured cornea has been the exploration of compounds that might inhibit the destructive proteinases. Knowledge of the structure of metalloproteinases has facilitated the development of many different synthetic inhibitors with considerable specificity. At least two of these synthetic compounds have proven to be efficacious in reducing corneal ulceration and perforation after alkali injury. However, relatively little is known about the toxicity of these synthetic compounds, either in their original form or their metabolites; additionally, the stability of these inhibitors is open to question.

The natural tissue inhibitors of metalloproteinases are believed to play a role in regulating the turnover of extracellular matrix proteins. TIMP has been demonstrated by immunolocalization in several tissues. For example, TIMP is present in scar tissue during active remodeling, but it is absent in quiescent tissue. Also in a wound healing model in rabbit colon, TIMP is co-localized with collagenase at the edge of the wound. TIMP has been shown to have a number of significant biologic effects, including the inhibition of tumor cell colonization and the infiltration of bovine vascular endothelial cells into amnion basement membrane. In vivo studies of TIMP are limited, although one study has shown that intraperitoneal injection of TIMP into nude mice prevented tumor cells from metastasizing to the lung. In another study the pathology associated with an animal model of arthritis was reduced by TIMP treatment. The dramatic effect of TIMP-1 on corneal ulceration demonstrated in this study underscores the potential value of metalloproteinase inhibition as a therapeutic approach.

The precise molecular mechanism of TIMP action is still uncertain, but is thought to involve complexing with the metalloproteinase at the zinc-dependent active site. Recent work by Murphy et al has demonstrated that TIMP-1 lacking its C-terminal domain retains inhibitory activity. Thus, there exists the potential to design smaller peptides, based on TIMP structure, for use as therapeutic compounds.

The biologic mechanism of action of TIMP in the alkali-burned cornea presumably is related to the ability of this molecule to inhibit metalloproteinases. One additional aspect of this study was that the cornea treated with TIMP appeared to exhibit better reepithelialization than the eyes treated with either vehicle or synthetic inhibitor. This might be associated with either more effective preservation of an extracellular matrix to support the epithelium, or might be related to the fact that TIMP-1 is reported to possess growth factor activity. Whether such growth factor activity...
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is a direct action of TIMP-1 or an indirect consequence of matrix metalloproteinase inhibitor is currently unknown.

As was observed previously with the synthetic peptide inhibitor, PMN infiltration was reduced in the TIMP-1-treated corneas. Previously, it has been hypothesized that reduced degradation of matrix proteins leads to less production of peptide fragments that are chemotactic for PMNs. Whether TIMP-1 could act directly to inhibit PMN chemotaxis is not known, although, as noted previously, TIMP has been shown to inhibit bovine endothelial cell invasion of basement membrane.

The continued exploration of TIMP and synthetic inhibitors of matrix metalloproteinases would seem a valuable course in the quest to develop better treatment for ulcerative diseases of the cornea. Metalloproteinase inhibitors might also be expected to play a therapeutic role in ulcers associated with bacterial keratitis and dry-eye syndromes, and neurotrophic ulcers. It is recognized, however, that such an approach represents only one aspect of the treatment regimen necessary for complete therapy of the alkali-burned eye. The value of other therapies such as sodium citrate and sodium ascorbate along with anti-inflammatory agents and appropriate emergency treatment cannot be underestimated.

Key Words

recombinant tissue inhibitor of metalloproteinases (TIMP-1), cornea, ulceration, alkali injury, rabbit

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