Treatment of Alkali-Injured Rabbit Corneas With a Synthetic Inhibitor of Matrix Metalloproteinases

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Alkali injuries of the cornea remain a challenging clinical problem. A number of therapeutic strategies have been proposed to reduce stromal ulceration and stimulate epithelial regeneration, and these have been reviewed thoroughly.1−3 Different treatments attempt to interfere with specific phases of corneal ulceration. Glued-on contact lenses attempt to block the physical infiltration of inflammatory cells,4 ascorbate attempts to replace depleted levels of vitamin C in the aqueous humor,5 citrate attempts to block polymorphonuclear leukocyte activation,6 fibronecin attempts to replace a destroyed extracellular matrix component important for cell attachment and migration,7 and immunosuppressive drugs deplete ocular inflammatory cells.8 Several agents have been tested which can inhibit collagenase activity. Some collagenase inhibitors appear to act by nonspecifically chelating the zinc cation present at the active site of matrix metalloproteinases. These agents include sodium edetate (EDTA),9 tetracycline,10 cysteine, and acetylcysteine.11 Recently, thiol-containing synthetic inhibitors of collagenase have been developed that are significantly more potent than the previous generation of collagenase inhibitors.12−14

Although these treatments interfere with different phases of the environment of an alkali injury, a major objective of most regimens is to limit the amount of active enzymes in the cornea which are responsible for degrading the extracellular matrix. These enzymes include the mammalian matrix metalloproteinases (MMP)—collagenase, gelatinase, stromelysin, polymorphonuclear leukocyte collagenase, elastase, and macrophage elastase—and the serine proteases (such as plasmin and tissue plasminogen activator).15,16

Recently, a new synthetic inhibitor of MMP (GM6001) was developed.17 This inhibitor is an extremely potent inhibitor of fibroblast collagenase (inhibition constant [K_i] = 0.4 nmol/l),17 gelatinase (K_i = 3 nmol/l; RE Galardy, unpublished data), and stromal collagenase (K_i = 0.07 nmol/l; RE Galardy, unpublished data). It is also a potent inhibitor of matrix metalloproteinase-related activities such as gelatinase (K_i = 0.07 nmol/l; RE Galardy, unpublished data). The effectiveness of GM6001 in preventing corneal ulceration after severe alkali injury and in promoting stable epithelial regeneration after moderate alkali injury has been demonstrated in rabbit corneas.17

Healing of corneal alkali injuries remains a severe clinical challenge. The authors evaluated the effect of a new synthetic inhibitor of matrix metalloproteinases (GM6001 or N-[2(R)-2-(hydroxamido carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) on preventing ulceration of rabbit corneas after alkali injury. Topical treatment of corneas with severe alkali injuries with 400 μg/ml or 40 μg/ml GM6001 alone prevented ulceration for 28 days, although 8 of 10 corneas treated with vehicle perforated. Corneas treated with 4 μg/ml GM6001 had midstromal depth ulcers. Corneas treated with 400 μg/ml of GM6001 contained very few inflammatory cells and had significantly reduced vessel ingrowth compared with vehicle-treated corneas. Epithelial regeneration after moderate alkali injuries was also investigated. Persistent epithelial defects developed 4 days after moderate alkali injury in rabbit corneas treated with vehicle and progressively increased to an average of 20% of the original 6 mm diameter wound by 27 days after moderate alkali injury. By contrast, epithelial regeneration was complete and persisted for 21 days for corneas treated with a formulation containing GM6001 (400 μg/ml), epidermal growth factor (10 μg/ml), fibronectin (500 μg/ml), and aprotinin (400 μg/ml). Sporadic punctate staining developed in 20% of the corneas treated with the combination of agents between days 21−28 after moderate alkali injury. These results demonstrate that topical application of GM6001 prevented corneal ulceration after severe alkali injury and that a combination containing GM6001, epidermal growth factor, fibronectin, and aprotinin promoted stable regeneration of corneal epithelium after moderate alkali injury. Invest Ophthalmol Vis Sci 33:3325–3331, 1992
melysin (K, = 40 nmol/l; RE Galardy, unpublished data). We evaluated the effect of topical treatment of GM6001 to reduce corneal ulceration of rabbit corneas after severe alkali injuries. In addition, we evaluated a formulation consisting of a combination of GM6001, epidermal growth factor (EGF), fibronectin, and aprotinin on epithelial regeneration of rabbit corneas after a moderate alkali injury.

Materials and Methods
Synthetic Inhibitor of MMP
GM6001 is a dipeptide analogue with the structure of N-[2(R)-2-(hydroxamido carbonylmethyl)-4-methylpentanoyl]-L-tryptophanc methylamide (Fig. 1). It was synthesized as described (Grobclny D, Poncz L, Galardy RE, unpublished data) and was approximately 95% pure as determined by reverse-phase high-performance liquid chromatography. Topical solutions of GM6001 were prepared at concentrations of 4 μg/ml, 40 μg/ml, and 400 μg/ml in vehicle consisting of 50 mmol/l N-2-hydroxyethylpipcrazine-N'-2-cthane sulfonic acid (HEPES) buffer, pH 7.4, with antimicrobial drugs (ie, penicillin 100 U/ml, streptomycin 100 μg/ml, and amphotericin B 0.25 μg/ml), and 0.1% dimethyl sulfoxide. The aprotinin, HEPES, dimethyl sulfoxide, and antimicrobials were obtained from Sigma (St. Louis, MO). The EGF and fibronectin were supplied by Chiron Intraophthalmics (Irvine, CA). One solution of GM6001 was prepared at the start of each experiment and was used for the entire experiment. Solutions were sterilized by filtration through a 0.22-μm pore filter and were stored at 4°C for the duration of the experiment. The stability of GM6001 in solution was measured by reverse-phase high-performance liquid chromatography, and a degradation rate of approximately 2% per month was detected during storage at 4°C.

Severe and Moderate Alkali Injuries
Rabbits were used under protocols approved by the Animal Care and Use Committee of the University of Florida and in compliance with the ARVO Resolution on the Use of Animals in Research. Male New Zealand white rabbits weighing 4–6 lb, which were free of systemic or ocular diseases, were anesthetized with ketamine and xylazine. A lid speculum was inserted and the cornea was anesthetized with proparacaine.

To create a severe alkali injury for studying corneal ulceration, a plastic block with a 12.5 mm internal diameter well was pressed onto the cornea, and the well was filled with 1 ml of 2 N sodium hydroxide. After 60 sec of exposure, the sodium hydroxide was rapidly removed by vacuum aspiration, and the well was continuously flooded for 1 min with a total of 50 ml of phosphate-buffered saline while the well was simultaneously aspirated with a vacuum line.

To create a moderate alkali injury for studying epithelial regeneration, the 6.6 mm diameter (34.2 mm²) vacuum collar of a 7 mm Hessburg-Barron vacuum trephine was attached to the center of the cornea. The well was filled with 1 N sodium hydroxide. After 60 sec of exposure, the sodium hydroxide was removed by vacuum aspiration, and the well was continuously flooded for 1 min with a total of 50 ml of phosphate-buffered saline while the well was simultaneously aspirated with a vacuum line. Phenyl red was included in the phosphate-buffered saline to confirm that the pH of the wash solution had returned to normal before the well was removed from the surface of the eye.

Treatment of Severe Alkali Injuries with GM6001
After severe alkali injuries were made to the right eye of each of 20 rabbits, 10 rabbits were randomly assigned to treatment with vehicle, and 10 rabbits were assigned to treatment with vehicle containing 400 μg/ml of GM6001. The animals were housed individually and received food and tap water ad libitum on a 12-hr light–dark schedule. The rabbits were treated with two drops every 2 hr between 8 AM and 6 PM, and 500 μl of GM6001 solution or vehicle were injected subconjunctivally at 6 PM.

The rabbits were examined daily using slit-lamp microscopy, and qualitative clinical evaluations were made with the slit-lamp microscope. Ulceration was graded by one ophthalmologist who was masked to the identity of the treatment group. A clinical score was assigned based on the depth of ulceration as follows: 0, no ulceration; 1, superficial ulceration limited...
to the anterior one-third of the cornea; 2. moderate ulceration extending to the middle one-third of the cornea; 3. deep ulceration extending to the posterior one-third of the cornea; 4. descemetocele; and 5. perforation. Corneal appearance was documented by photography. Average values for ulceration were calculated for the two groups and were compared for statistical significance using the Mann-Whitney U nonparametric test for two samples.

Vascularization and epithelial defects were also measured clinically by slit-lamp beam lengths. At the time of the animals were killed, their corneas were fixed for 1 hr in 2.5% glutaraldehyde and 2% paraformaldehyde and embedded in JB-4 (Polysciences, Warrington, PA). One-micron thick sections were cut and stained for light microscopy with hematoxylin and cosin.

Dose Response for Inhibition of Ulceration by GM6001

In a separate experiment, a severe alkali injury was made to the right eyes of 20 rabbits. After injury, the rabbits were randomly assigned to one of four treatment groups consisting of five rabbits each. One group of rabbits was treated with the vehicle solution, and the other three groups were treated with vehicle solution containing GM6001 at concentrations of 4 μg/ml, 40 μg/ml, and 400 μg/ml. The rabbits were treated with two drops every 2 hr between 8 AM and 6 PM with no subconjunctival injection. Ulceration was graded every 5 days after injury for 30 days as described, and average scores were calculated and compared for statistical significance using a Mann-Whitney U nonparametric test for two samples.

Effect of a Combination of GM6001, EGF, Fibronectin, and Aprotinin on Epithelial Regeneration After Moderate Alkali Injury

To determine if prolonged treatment with a combination of agents could regenerate a stable corneal epithelium, a moderate alkali injury was made to the right eyes of 10 rabbits, and the rabbits were randomly assigned to one of two groups consisting of five rabbits each. The rabbits were treated with two drops of test solutions every 2 hr between 8 AM and 6 PM with no subconjunctival injection. Test solutions were vehicle and vehicle containing GM6001 (400 μg/ml), human EGF (10 μg/ml), human fibronectin (500 μg/ml), and aprotinin (400 μg/ml).

The animals were evaluated every day beginning the day of injury for epithelial defects using fluorescein staining. The corneas were photographed with a Kowa (Kowa Co., Torrance, CA) camera at a constant magnification of 10× with an ultraviolet flash to reveal fluorescein-stained regions. The surface area of the epithelial defect of each cornea was measured by computerized planimetry of projections of the photograph slides using Sigma Scan software (Jandel Scientific, Corte Madera, CA). The nonepithelialized area of each cornea that stained with fluorescein was expressed as a percent of the initial wound area (34.21 mm²). The average area of epithelial defects was calculated for the two treatment groups, and mean values were compared for statistical significance using a two-tailed Student’s t-test.

Results

Treatment of Severe Alkali Injuries With GM6001

Examination of corneas immediately after alkali injury showed a sharply defined circular stromal opacity extending nearly limbus to limbus with no evidence of conjunctival injury. During the course of the experiment, none of the eyes became infected. Persistent epithelial defects were present in both vehicle- and inhibitor-treated eyes from day 6 postinjury.

Beginning on day 12 after alkali injury, the average clinical score of the vehicle-treated eyes steadily increased, indicating progressive ulceration of the corneas (Fig. 2). By contrast, the average clinical score for corneas treated with 400 μg/ml GM6001 remained below a grade of 1 for the 26 days of duration of treatment, indicating the presence of superficial ulceration. Beginning on day 13 after injury and continuing to the end of the experiment 26 days after injury,
average clinical scores were significantly higher for vehicle-treated corneas \((P < 0.05 \text{ on days } 13-16, \ P < 0.001 \text{ on day } 16-26)\). On day 22 after injury, five of the ten corneas treated with vehicle had perforated, and on day 26 after injury, eight of the ten corneas treated with vehicle had perforated. None of the corneas treated with GM6001 perforated during the 26 days after injury.

The appearance of corneas treated with vehicle or GM6001 for 26 days after severe alkali injury is shown in Figure 3. Slit-lamp photomicrographs indicated the severe ulceration that had developed in corneas treated with vehicle (panel A); the corneas treated with GM6001 had only superficial ulceration (panel B). The extent of new blood vessel growth into the cornea also was apparent in the slit-lamp photomicrographs. Measurements of the length of capillary growth into the cornea showed that 50% of the corneas treated with GM6001 had vessels of 2 mm or less in length and 50% had vessels of 3 mm or less in length 26 days after injury. More extensive peripheral neovascularization was found in vehicle-treated corneas. Specifically, all such corneas had peripheral vessels at least 2 mm in length, 50% had vessels 3 mm in length, 40% had vessels 4 mm in length, and 10% had vessels 4 mm or greater in length at the time of perforation or at 26 days after injury. Neither vehicle- or GM6001-treated eyes showed evidence of any reaction at the site of the subconjunctival injections.

Histologic sections of vehicle-treated corneas (panel C) revealed a dense inflammatory cell infiltrate. Polymorphonuclear leukocytes were present throughout the stroma, at the edge of the ulcer, and in a band above Descemet's membrane. By contrast, there were relatively few inflammatory cells present in GM6001-treated corneas, and the inflammatory cells were predominately located in the anterior half of the stroma (panel D). No inflammatory cells were observed along Descemet's membrane. Cells that had the appearance of fibroblasts were observed throughout the stroma. The stromal matrix appeared intact and retained the lamellar organization of collagen fibers characteristic of normal corneas.

**Dose Response for Inhibition of Ulceration by GM6001**

The effect of different concentrations of GM6001 on corneal ulceration after severe alkali injury is shown in Figure 4. As observed in the previous experi-
The dose effect for GM6001 treatment of alkali-injured corneas. A severe alkali injury 12 mm in diameter was made to corneas of rabbits treated with vehicle or GM6001 at the indicated concentrations. Corneal ulcers were graded with a clinical score of 0 = no ulcer, 1 = anterior 1/4 of cornea, 2 = middle 1/4 of cornea, 3 = posterior 1/4 of cornea, 4 = descemetocele, 5 = perforation. Values plotted are the mean and standard error for five rabbits in each treatment group. Statistically significant differences were found between vehicle and all three groups treated with GM6001, between the two highest concentrations of GM6001 and the lowest concentration of GM6001, but not between the two highest concentrations of GM6001, by Mann-Whitney U nonparametric test.

Effect of a Combination of GM6001, EGF, Fibronectin, and Aprotinin on Epithelial Regeneration After Moderate Alkali Injury

Although GM6001 treatment prevented development of significant corneal ulcers after severe alkali injury, all the corneas had large persistent epithelial defects at the end of treatment, with an average size of approximately 5 x 5 mm. Thus, GM6001 alone was not sufficient to promote epithelial regeneration after severe alkali injury. Because the ability of the corneal epithelium to regenerate after a severe alkali injury was severely compromised, we evaluated the effect of a combination of agents on epithelial regeneration of moderate alkali injury. As shown in Figure 5, corneas treated either with vehicle or a combination of GM6001, EGF, fibronectin, and aprotnin rapidly resurfaced the 34.2-mm² injury within the first 3 days after injury. However, the epithelia of corneas treated either with vehicle or the combination of agents was not stable and sloughed off during days 3–5. The epithelium of both treatment groups again regenerated during days 6–9. However, the epithelia of all five eyes treated with vehicle rapidly sloughed off again, and the epithelial defects grew progressively larger until the epithelial defects reached an average of approximately 20% of the original wound area (6.8 mm²) on day 28 after injury. By contrast, the epithelium of eyes treated with the combination of agents remained healed between days 9–20 after injury. During the fourth week after injury, two of the corneas treated with the combination of agents developed sporadic punctate fluorescein staining. All the vehicle-treated corneas developed corneal ulcers that extended into the anterior one-third of the cornea, whereas no ulcers developed in corneas treated with the combination of agents.

Discussion

As pointed out by Kenyon,2 there are no clinical situations more frustrating to the corneal specialist than the relentless ulcerative destruction of the herpe-
glycanase). It is possible that GM6001 may interfere with the chemotactic response of inflammatory cells. Further studies are needed to define more accurately the molecular mechanism by which GM6001 reduces corneal ulceration.

The results of the experiments presented here demonstrate that topical treatment of rabbit corneas after severe alkali injury with GM6001 prevents ulceration; vehicle-treated corneas rapidly progressed to perforation. The mechanism by which GM6001 prevents ulceration has not been fully established, but GM6001 probably acts primarily to inhibit many of the enzymes implicated in corneal ulceration, including collagenase, gelatinase, and stromelysin (proteoglycanase). It is possible that GM6001 may interfere with the recruitment of inflammatory cells into the injured cornea. This could occur by at least two processes. GM6001 could reduce the production of chemotactic fragments generated by destruction of the extracellular matrix. The drug could also reduce the chemotactic response of inflammatory cells. Further studies are needed to define more accurately the molecular mechanism by which GM6001 reduces corneal ulceration.

Extensive toxicologic studies of GM6001 remain to be performed. However, the repopulation of the stroma of severe alkali-burned corneas with fibroblast-like cells and the enhanced regeneration of the epithelium of moderate alkali-burned corneas after prolonged treatment with a formulation containing high concentrations of GM6001 (400 μg/ml) and three other agents suggest that GM6001 has minimal toxic effects on corneal cells.

Prevention of corneal ulceration is a major objective after chemical injury. However, the risk of corneal ulceration remains high until a stable epithelium resurfaces the cornea. Thus, an optimal therapy for treatment of alkali injuries would prevent ulceration and promote regeneration of a stable epithelium of alkali-injured corneas. The agents were selected to correct four different aspects of the cornea which had been reported, or were assumed to be altered after alkali-injury to the cornea: excess MMP activity, low levels of mitogenic growth factors in tears, denatured extracellular matrix proteins, and high levels of plasmin in tears. Our results demonstrated that the combination of four agents (GM6001, EGF, fibronectin, and aprotinin) prevented ulceration and was effective in regenerating a stable epithelium after moderate alkali injury compared with treatment with vehicle. However, the fact that some sporadic epithelial breakdown occurred 21 days after injury suggests that additional (or different) agents may be necessary to prevent long-term epithelial breakdown. Addition of ascorbate to the combination of GM6001, EGF, fibronectin, and aprotinin may further benefit corneal wound healing after alkali injury because ascorbate is severely depressed in aqueous fluid after alkali injury. Immunosuppressive drugs such as acyclovir may further reduce the inflammatory cell infiltration after injury. Because alkali injury damages multiple aspects of normal corneal physiology, it seems logical that successful treatment with multiple agents will be required to return the corneal epithelium to full function.
environment to a state where stable healing and repair can occur. Control of excess MMP activity after alkali injury will surely be necessary, and potent inhibitors such as GM6001 may eventually be very useful as one component of a combination of agents in the clinical management of corneal ulceration.

Key words: aprotinin, collagen, cornea, epidermal growth factor, matrix metalloproteinase inhibitor, libronectin, ulcer

References