Alkali-Induced Corneal Stromal Melting Prevention by a Novel Platelet-Activating Factor Receptor Antagonist

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Objective: To evaluate the effect of LAU0901 (2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid ester), a novel platelet-activating factor (PAF) receptor antagonist, on a rabbit model of severe corneal alkali injury.

Methods: Adult New Zealand albino rabbits were anesthetized and the right eyes were injured with 2N sodium hydroxide for 60 seconds using a 12-mm plastic well, then rinsed. After the injury, 10 rabbits were treated topically with LAU0901 every 2 hours 4 times per day and received a subconjunctival injection of 200 µL of LAU0901 once per week and 10 rabbits were treated with vehicle the same way. Over the course of 4 weeks, the corneas were examined daily by slitlamp microscopy and corneal ulcers were graded with a clinical scoring system. Ten additional rabbits were treated as described but 1 rabbit from each group was killed at 1, 3, 7, 14, or 21 days after injury. The corneas were processed for histopathologic and immunofluorescence examination.

Results: Persistent epithelial defects were present in both groups from day 5 postinjury, but from day 9 through day 25, the average clinical scores of both epithelial defects and stromal ulcerations in the vehicle-treated eyes were significantly higher than those in the LAU0901-treated eyes (P<.01). By day 28, 90% of the eyes in the vehicle-treated group perforated, while only 20% of the eyes in the LAU0901-treated group developed deep ulceration and none were perforated. Histologic examination showed that the corneas treated with LAU0901 for 4 weeks were completely reepithelialized, with fewer inflammatory polymorphonuclear leukocytes and more repair fibroblasts (myofibroblasts) in the stroma as compared with those treated with vehicle.

Conclusions: LAU0901 inhibits corneal ulceration and perforation in a severe alkali-burn model in the rabbit. In the cornea, PAF is a strong inflammatory mediator, a chemotactic to inflammatory polymorphonuclear leukocytes, and an inducer of several proteases that degrade the extracellular matrix.

Clinical Relevance: The inhibition of PAF action by LAU0901 could be important in the immediate and intermediate treatment of chemical injuries to preserve the integrity of the cornea.

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augments the inflammatory process by inducing the expression of cyclooxygenase-2 and increasing the production of prostaglandins. At the transcriptional level, PAF selectively stimulates the expression of enzymes that degrade components of the extracellular matrix and are involved in tissue remodeling, such as MMPs MMP-1 and MMP-9 and urokinase plasminogen activator, as well as tissue inhibitors of metalloproteinase (TIMPs) TIMP-1 and TIMP-2. Platelet-activating factor delays corneal epithelial wound healing by inhibiting adhesion of epithelial cells, increasing apoptosis of stromal cells, and inducing an imbalance in favor of MMP-9 activation. All these activities exerted by PAF are receptor mediated and can be halted by selective PAF receptor antagonists. Corneal epithelial cells, kerocytes, and endothelial cells express the PAF receptor, and in corneal epithelial cells, injury upregulates PAF receptor gene expression. Although the expression of the receptor has not been found in corneal fibroblasts, it was detected in myofibroblasts, and in recent studies, we found that PAF up-regulates expression of endoproteinase furin, MT1-MMP, MMP-9, and TIMP-2 and induces apoptosis in these cells.

LAU0901 (2,4,6-trimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid ester) is a novel and potent PAF receptor antagonist. In a rabbit model of diffuse lamellar keratitis, an inflammatory condition induced by laser in situ keratomileusis surgery, LAU0901 significantly suppressed the inflammatory response and reduced kerocyte apoptosis. These results suggest that LAU0901 could be used in a therapeutic approach to prevent diffuse lamellar keratitis. The current study was undertaken to investigate the effect of LAU0901 treatment on a model of severe alkali burn in rabbit corneas that progresses to irreversible corneal perforation.

**METHODS**

**LAU0901**

LAU0901 was developed by Bazan and associates. The drug was dissolved at a concentration of 30 µg/µL in 2-hydroxypropyl-β-cyclodextrin (Sigma, St Louis, Mo). The solution was stored in the dark at 4°C until use.

**ANIMALS**

New Zealand albino rabbits of both sexes weighing between 2.5 and 3.5 kg were used in all the experiments. The rabbits were housed in the Neuroscience Center at the Louisiana State University Health Sciences Center (New Orleans) and treated in compliance with the guidelines of the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research, and the experimental protocol was approved by the Institutional Animal Care and Use Committee, Louisiana State University Health Sciences Center.

**CORNEAL ALKALI-BURN MODEL**

Rabbits were anesthetized with intramuscular xylazine hydrochloride (10 mg/kg of Rompun; Mobay, Shawnee, Kan) and ketamine hydrochloride (37.5 mg/kg of Ketaset; Parke Davis, Morris Plains, NJ) and topical tetracaine hydrochloride anesthetic. The right eye of each rabbit was gently proptosed, and a sharply defined 12-mm burn was made on the cornea by pipetting 0.5 mL of 2N sodium hydroxide into a circular plastic well held firmly against the cornea for 60 seconds. The sodium hydroxide was aspirated from the well, and the interior of the well and the ocular surface were thoroughly irrigated with 0.9% sodium chloride (Baxter Healthcare Corp, Deerfield, Ill).

**TREATMENT REGIMEN**

After the injury, the rabbits were randomly divided into 2 groups. Ten rabbits were treated topically with LAU0901 (30 mg/mL) every 2 hours 4 times per day and received a subconjunctival injection of 200 µL of LAU0901 once a week, and 10 rabbits were treated with vehicle the same way. Animals of both groups received topical 0.3% gentamicin sulfate ointment (Gentax; Agepharma, Vienna, Austria) once daily for the duration of the experiment. This treatment regimen was continued until corneas reached a clinical score of 5 (perforation) or until the termination of the study. No analgesics were given to exclude any possible interference with the treatment.

**CLINICAL EVALUATION**

External examinations of each eye were done once daily over the entire course of the study. Detailed slitlamp examinations of each rabbit were performed (in a double-masked manner) every other day initially for the first week and then every day following the onset of corneal ulceration. Eyes were examined for the presence of corneal epithelial defects, ulceration, perforation, vascularization, or infection. The size of the corneal epithelial wound was measured beginning 24 hours after the alkali burn, then once every other day by using 0.3% solution of methylene blue dye to delineate the wound area. Two linear dimensions, the longest line parallel and the longest one perpendicular to it within the confines of the epithelial defect, were measured with the aid of a standard slitlamp (Haag-Streit 900; Haag-Streit International, Bern, Switzerland) at ×10 magnification. The area of the equivalent rectangle was calculated by multiplying the 2 measured perpendicular linear dimensions as described by Mukerji and colleagues. This technique produces results similar to those of a computer-assisted image analyzer. Corneal ulceration was graded according to the clinical scoring system described by Burns and colleagues as follows: 0, no ulcer; 1, superficial ulceration (anterior third of the cornea); 2, moderate ulceration (middle third of the cornea); 3, deep ulceration (posterior third of the cornea); 4, descemetocele; and 5, perforation. Data were analyzed statistically using a 2-tailed t test.

**HISTOLOGIC EXAMINATION AND IMMUNOFUORESCENCE STAINING**

Ten additional rabbits used for histologic analysis were also assigned to 2 groups and treated according to the same regimen described earlier but 1 rabbit chosen at random from each group was killed by pentobarbital sodium overdose via ear-vein injection at 1, 3, 7, 14, or 21 days after injury. The isolated corneas were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 2 hours at room temperature or overnight at 4°C. After gradient dehydration in 10%, 15%, and 20% sucrose in 0.1M phosphate-buffered saline (PBS) each for 4 hours, the cornea was cut into 2 halves and embedded in OCT (optional cutting temperature) compound (Sakura Finetek USA, Inc, Torrance, Calif). Serial 6-µm cryostat sections were cut, air-dried, and stored at −80°C until use. These sections were used for routine hematoxylin-eosin staining or for immuno-
the Mann-Whitney vehicle-treated group. Clinical scores were compared for significance using a mounting medium (Aqua-Mount; Lerner Laboratories, Pittsburgh, Pa), viewed, and photographed with a fluorescence microscope (Nikon Eclipse TE200; Nikon UK Ltd, London, England) equipped with a Nikon digital camera (DXM1200; Nikon UK Ltd) using the MetaVue imaging software (Nikon USA, Melville, NY). In all assays, negative controls were prepared using normal mouse IgG (Santa Cruz, sc-2025) or 0.1M PBS for the primary antibody to exclude nonspecific staining.

TRANSFERASE-MEDIATED URIDIN-5'-TRIPHOSPHATE NICK END LABELING ASSAY

The sections were also stained for terminal deoxynucleotidyl transferase mediated uridine-5'-triphosphate nick end labeling (TUNEL) by using a fluorescein apoptosis-detection system (Promega, San Luis Obispo, Calif), according to the manufacturer’s instructions. Positive and negative control slides were included in each assay. We used DAPI for nuclear counterstaining. Photographs were obtained as described earlier.

RESULTS

CLINICAL EVALUATION

Examination of corneas immediately after alkali injury showed a sharply defined circular stromal opacity limited within the burned area and a narrow band of clear cornea of about 0.5 mm remaining in the limbal area. During the course of the experiment, none of the eyes became infected. For the first 3 days after the injury, obvious conjunctival edema and eyelid swelling were observed and the kinetic rate of epithelial wound closure was linear for both groups (Figure 1A). By day 4, most of the eyes were covered with the regenerating epithelium but small epithelial defects were detected. From day 5 on, persistent corneal epithelial defects were present in both groups, and from day 7 to day 21, the average areas of epithelial defects in vehicle-treated eyes were significantly larger than those in the LAU0901-treated eyes. Corneal ulceration occurred at day 5, and there was no difference between the 2 groups with regard to the time of onset. However, from day 9 through day 23, the average clinical scores in the vehicle-treated eyes were significantly higher than those in the LAU0901-treated eyes (Figure 1B). When an ulcer was established in the vehicle-treated eyes, it progressed rapidly to deep ulceration and descemetocele. Figure 2A shows the clinical appearances of all the corneas treated with LAU0901 and vehicle at day 18 after the injury. Almost all the eyes in the vehicle group developed deep corneal ulcers and descemetoceles, while no deep ulcers were observed in the LAU0901 group. One eye (V5) in the vehicle-treated group had perforated at day 16 after alkali injury. At the end of observation, 9 of 10 eyes in the vehicle-treated group perforated, but only 2 of 10 eyes in the LAU0901-treated group developed deep ulceration and none were perforated (Figure 2B).

HISTOLOGIC ANALYSIS

Immediately after injury, all the cellular components within the burned area were destroyed in this model of
severe corneal alkali burn. A layer of regenerating epithelium grew to cover the epithelial defects from day 1 to day 3. The central stroma remained acellular, but at the peripheral area, the anterior stroma was intensely infiltrated with inflammatory cells as shown by myeloperoxidase staining (Figure 3). Compared with the vehicle-treated eyes, the LAU0901-treated eyes displayed intense α-SMA positive staining, indicating the presence of myofibroblasts, and fewer PMNs in the limbus. At day 7, the central areas of LAU0901-treated corneas were covered by 2 to 3 layers of epithelial cells and the anterior stroma was infiltrated with fewer PMNs and more myofibroblasts than those in the vehicle-treated eyes (Figure 4). The endothelial layer in both treated and untreated eyes was replaced by a retrocorneal membrane that also stained positive with α-SMA (Figure 4). At day 14, vehicle-treated eyes developed deep ulceration with numerous PMNs infiltrating into the ulcerating area, while the LAU0901-treated eyes showed intact epithelia and the stroma filled with myofibroblasts (Figure 4). Figure 5 shows the pathologic changes of specimens taken from the 2 eyes shown in Figure 2B at day 28 after the injury. In the LAU0901-treated eyes, the cornea was covered with 4 to 5 layers of epithelial cells; the stroma was populated with reparative fibroblasts and had few PMNs and macrophages, as stained with antitymeyloperoxidase and anti-CD14. The endothelia were replaced by the retrocorneal membranes made up of α-SMA–positive cells in both samples. Sections from the vehicle-treated eye reveal that there were numerous inflammatory cells, mainly PMNs, present in the ulcerating area and new vessels at the ulcerating edge.
STROMAL CELL APOPTOSIS AFTER ALKALI INJURY

Staining with TUNEL showed that stromal cell apoptosis was present in all the alkali-burned corneas regardless of treatment (Figure 6). At day 1 and day 3, abundant TUNEL-positive cells were present in the limbal area of alkali-burned corneas, but there was less dense staining in the LAU0901-treated eyes than in the vehicle-treated group. In the samples of day 14 and day 28, sparse TUNEL-positive cells were found in the central stroma of LAU0901-treated eyes while densely stained cells were seen in the ulcerative region of vehicle-treated eyes.

COMMENT

Earlier studies in our laboratory demonstrated that alkali burn induces a rapid increase in the levels of PAF in the cornea, and we found that the production of PAF after alkali burn is related to the intensity and time of the injury.23 The more severe the injury is, the higher the levels of PAF, suggesting an important role for this lipid mediator in the pathogenesis of alkali-injured cornea.23 The present study investigated the effect of a PAF receptor antagonist, LAU0901, in the treatment of a severely alkali-burned rabbit cornea. Our results showed that LAU0901 significantly decreased the incidences of corneal ulcerations and perforations. By the termination of the experiments (day 28), only 20% of the treated eyes developed deep ulceration but none were perforated; in the nontreated group, 90% of the eyes were perforated. Corneas treated with LAU0901 for 4 weeks were completely reepithelialized, with fewer PMNs and more repair fibroblasts (myofibroblasts) in the stroma, as compared with those treated with vehicle. Other treatments used in similar models of severe alkali burn, such as topical application of synthetic MMP inhibitor, also prevent corneal perforation but they do not promote a stable, regenerative epithelium.16,17,39 Our findings are in good agreement with our previous...
report, in which LAU0901 treatment prevented laser in situ keratomileusis–induced diffuse lamellar keratitis.36

To further investigate the mechanisms by which LAU0901 treatment might prevent corneal ulceration, we examined qualitatively by immunofluorescence the specimens taken at different times after alkali injury. Although the results were derived from 1 animal for each point, the differences correlate with the clinical findings. A striking difference between the 2 groups, from the early to the late stage, was that the corneas treated with LAU0901 showed much less inflammatory-cell infiltration than did those treated with vehicle. Corneal ulceration is known to be associated with extensive inflammatory-cell infiltration and PMNs are the predominant inflammatory cells after alkali burn.1-3 The PMNs undergo a respiratory burst to fuel their phagocytic and degranulation activities, releasing important enzymes such as type 1 collagenase (MMP-1) and plasminogen activator.40,41 Macrophages, another type of inflammatory cell found in alkali-injured tissue, also produce serine proteases and several MMPs, including MMP-1, MMP-2, MMP-3, and MMP-9.42 The effect of PAF on the recruitment and activation of inflammatory cells is well documented.43,44 Platelet-activating factor also enhances the synthesis and release of proinflammatory cytokines including IL-1, IL-6, IL-8, and tumor necrosis factor α in neutrophils and monocytes.55,46 These cytokines all have been proposed to play an important role in the inflammatory response after alkali injury.6,47,48 Moreover, PAF activates phospholipases, resulting in additional PAF synthesis, along with that of prostaglandins.25,45 LAU0901 reduced the inflammation by blocking the action of PAF in the injured tissue at the top of the cascade of inflammation, thereby lowering the levels of proteases and cytokines released by the inflammatory cells. Therefore, our results suggest that the decreased inflammation resulting from LAU0901 treatment is due to the blocking of PAF-receptor activities by PAF, which thus prevents corneal perforation in this acute model.

Stromal repopulation after alkali injury by repair fibroblasts, also called myofibroblasts, is a necessary event because these cells have the unique capacity to remodel the damaged stroma by producing collagen and extracellular matrix mucopolysaccharides. In cases of incomplete destruction of the entire keratocyte population, as in the model used in this study, the repair fibroblasts probably derive from the surviving keratocytes at the peripheral cornea. Earlier studies noted the shortage of repair fibroblasts in the ulcerated stroma and considered the lack of healthy fibroblasts as one of the major causes of corneal ulceration after severe alkali burn.1,3 Although the mechanisms responsible for this phenomenon remain unknown,
there are extensive studies indicating that apoptosis plays an important role in regulating cellularity during corneal wound healing. Previous work in our laboratory has shown that PAF is a strong inducer of apoptosis in corneal keratocytes. More recently we found that PAF also induced apoptosis in corneal myofibroblasts, although these are more resistant than keratocytes. In the present study, the much lower density of myofibroblasts that stained with α-SMA in the vehicle-treated corneas compared with that in the LAU0901-treated eyes implies that PAF-mediated apoptosis may have a role in the decrease of cellularity. In the intermediate phase, PAF-induced apoptosis in the remaining keratocytes results in a lack of keratocyte activation and transformation to myofibroblasts. In the reparative phase, PAF might, alone or in combination with other cytokines, induce apoptosis in myofibroblasts. In fact, we have found that when PAF is combined with tumor necrosis factor α, there is a significant increase of apoptosis in corneal myofibroblasts. Therefore, LAU0901 treatment may contribute to the inhibition of PAF-induced apoptosis in corneal keratocytes and myofibroblasts.

Corneal reepithelialization after alkali injury plays a crucial role in the cessation of ulceration by eliminating the stimulus for collagenase production and activation. Herein, we found that, although persistent epithelial defects were present in both groups, the average area of epithelial defects in LAU0901-treated corneas was significantly smaller than that in vehicle-treated corneas during the 3 weeks of observation, suggesting that LAU0901 treatment promotes a stable epithelial regeneration. Platelet-activating factor has been shown to delay corneal epithelial wound healing even in the presence of growth factors. The mechanisms include a reduction of epithelial-cell adhesion to the extracellular matrix, increase of keratocyte apoptosis, and enhancement of the expression of urokinase plasminogen activator and MMP-1 and MMP-9 in corneal epithelial cells. Elevated levels of urokinase plasminogen activator and plasmin activity can impair epithelial cell migration and attachment by degrading extracellular matrices such as fibronectin and laminin. MMP-9 is also very active in degrading basement membrane components. It is then conceivable that LAU0901 promotes wound healing by blocking the action of PAF on cell migration and attachment.

In summary, LAU0901, a novel PAF receptor antagonist, can significantly decrease stromal ulceration and prevent perforation of rabbit corneas after a severe alkali injury by a combination of mechanisms. The beneficial effect of LAU0901 treatment shown in this study also suggests that this agent can be used in combination with other therapies to block the initial inflammatory reaction; to increase epithelial cell migration and adhesion, which would be helpful to promote a stable epithelial resurfacing; and to reduce apoptosis of keratocytes and myofibroblasts, which are important in the synthesis of the extracellular matrix. In addition, this could be an important advance in the treatment of other intractable corneal dis-
orders with similar pathologic features of persistent epithelial defects and sterile stromal ulceration, such as ocular cicatricial pemphigoid, Mooren ulcer, and rheumatoid arthritis.

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