The Roles of Urokinase-Type Plasminogen Activator in Leukocyte Infiltration and Inflammatory Responses in Mice Corneas Treated With Lipopolysaccharide

Koji Sugioka, Aya Kodama, Koji Yoshida, Kiyotaka Okada, Hiroshi Mishima, Keiichi Aomatsu, Osamu Matsuo, and Yoshikazu Shimomura

1Department of Ophthalmology, Kinki University Faculty of Medicine, Osaka, Japan
2Department of Biochemistry, Kinki University Faculty of Medicine, Osaka, Japan
3Department of Physiology and Regenerative Medicine, Kinki University Faculty of Medicine, Osaka, Japan

Cornea

Mice Corneas Treated With Lipopolysaccharide

The urokinase-type plasminogen activator (u-PA) plays an important role in corneal wound healing, yet its role in corneal inflammation remains poorly understood. We investigated the role of u-PA in a murine model of lipopolysaccharide (LPS)-induced corneal inflammation.

METHODS. The corneal epithelium was scraped and LPS was applied to u-PA wild-type (u-PA+/+) and u-PA-deficient (u-PA−/−) mice. Corneal re-epithelialization and opacity were measured by stereomicroscopy. Fibrin zymography was performed to detect plasminogen activators in corneas from u-PA+/+ and u-PA−/− mice. Neutrophil, macrophage, and u-PA receptor (u-PAR) expression were determined by immunohistochemistry. Gene expression of corneal macrophage chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 was assessed with reverse transcription-polymerase chain reaction. The in vitro effects of endogenous u-PA on MCP-1, MIP-2, matrix metalloproteinase (MMP)-2, and MMP-9 expression, and macrophage migration activity in mouse ocular fibroblasts stimulated by LPS, were examined.

RESULTS. The u-PA+/+ mice showed enhanced corneal inflammation as compared with u-PA−/− mice. The u-PA expression was increased by LPS stimulation. Immunohistochemical analyses indicated that more neutrophils and macrophages were present in corneas from u-PA+/+ mice than u-PA−/− mice. The u-PAR expression was detected in inflammatory cells and in the leading edges of the epithelial migrating cells. Enhanced mRNA expression of MCP-1 and MIP-2 was observed in corneas from u-PA+/+ mice compared to u-PA−/− mice. Macrophage chemoattractant protein-1, MIP-2, and MPP-9, but not MMP-2, significantly increased in corneal fibroblasts from u-PA+/+ mice compared with u-PA−/− mice.

CONCLUSIONS. These data indicate that u-PA promotes LPS-induced leukocyte infiltration in cornea and that u-PA is an important component in LPS-induced corneal inflammatory responses.

Keywords: u-PA, LPS, wound healing, leukocyte, cornea

Microbes or microbial endotoxins such as lipopolysaccharide (LPS) cause severe corneal inflammation. When the cornea is inflamed, many inflammatory cells infiltrate into the cornea through the limbal blood vessels and the tear fluid. Infiltrating inflammatory cells, which are first to arrive at the inflammatory site, are mainly polymorphonuclear neutrophils (PMNs), followed by macrophages. Inflammatory cells play an important role in removal of bacteria and restoration of corneal transparency, while persistence of inflammatory cells in the cornea contributes to the development of corneal stromal dysfunction and eventually induces severe stromal damage.

During the corneal inflammatory process, corneal resident cells or inflammatory cells release physiological substances, such as cytokines and chemokines. Studies have reported that various proinflammatory mediators influence the infiltration of leukocytes into the inflamed cornea. For example, macrophage inflammatory protein (MIP)-2 is known as a potent chemoattractant and activator of PMN. Intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 are known to be upregulated by inflammatory cytokines. Macrophage chemoattractant protein (MCP)-1 has been implicated in the process of macrophage adhesion and migration.

These studies have indicated the importance of cytokines or chemokines for attracting leukocytes in the inflamed cornea. However, proteases and their inhibitors released from resident and infiltrating cells are also critically involved in the regulation of leukocyte infiltration and tissue inflammation.

The urokinase-type plasminogen activator (u-PA) is a serine protease that plays a major role in fibrinolytic processes, where it converts plasminogen to plasmin. This protease degrades extracellular matrix (ECM) proteins and mediates pericellular proteolysis. Urokinase-type plasminogen activator also contributes to the regulation of cell recruitment, cell migration, cell adhesion, chemotaxis, and metastasis and...
Role of u-PA in Leukocyte Infiltration in Cornea

PA receptor is normally present in many tissues and has been expressed in cultured human corneal fibroblasts in vitro 21 and Pseudomonas aeruginosa in Ophthalmic and Vision Research.

vivo experiments were approved by the DNA Recombination Committee of Kinki University Faculty of Medicine and were performed using the roles of u-PA in leukocyte infiltration and inflammatory response in the cornea. We developed a murine model of LPS-induced corneal inflammation, using u-PA-deficient (u-PA−/−) mice. In the present study, we showed that u-PA was involved in LPS-induced corneal inflammation. Furthermore, we showed that u-PA-induced corneal leukocyte infiltration was mediated by both proteolytic-dependent and proteolytic-independent mechanisms.

Materials and Methods

Animals

The u-PA+/+ mice were produced by standard gene targeting in embryonic stem cells. Details have been published elsewhere.26 Eight- to 16-week-old mice of both sexes were used. Age-matched male or female C57BL/6 mice, which correspond to u-PA+/+, were used as the controls. In C57BL/6 mice, corneal opacity is not restored after infection with P. aeruginosa, and corneal perforation, shrinkage, or both, eventually occur.27 In vivo experiments were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Kinki University Faculty of Medicine and were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Murine Model of Lipopolysaccharide-Induced Keratitis

Mice were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan), and 0.4% oxybuprocaine (Santen Pharmaceutical, Osaka, Japan) eye drops were topically administered before scraping. A 3-mm-diameter area of the central corneal epithelium of the right eye was scraped with a microsurgery blade (Straight; MANI, Inc., Utsunomiya, Japan) and 10 μg/ml LPS from P. aeruginosa serotype 10 (Sigma-Aldrich Corp., St. Louis, MO, USA) was topically applied onto the scraped cornea.28

Clinical Observations

Corneal opacity was classified under a dissection microscope as follows: grade 0, totally clear, with no opacity seen by any method of slit lamp examination; grade 1, haze of minimal density seen with difficulty; grade 2, mild haze, easily visible; grade 3, moderately dense opacity that partially obscured the iris details; and grade 4, severely dense opacity that completely obscured the details of intraocular structures.29 In this study, n = 8 mice in each group.

Histology and Immunohistochemistry

The u-PA+/+ and u-PA−/− mice were killed at 1 day, 3 days, and 7 days after LPS exposure. The eyes were enucleated and fixed in Super Fix (Kurabo Industries, Osaka, Japan), embedded in paraffin, and sectioned (4 μm). The sections were stained with hematoxylin-eosin. For immunohistochemical analysis, the sections were deparaffinized and incubated for 20 minutes at 120°C with Target Retrieval Solution (×10; DAKO, Glostrup, Denmark). Then the samples were cooled for 30 minutes to room temperature (RT), washed three times with TNT wash buffer (0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween-20) and incubated for 30 minutes with a mixture of methanol (200 ml) and H2O2 (1 ml) at RT. After washing three times with TNT wash buffer, the sections were exposed to TMB blocking buffer (0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.5% Blocking Reagent) (Perkin Elmer, Waltham, MA, USA) for 1 hour at RT, then incubated with anti–NIMP-R14 (1:600 dilution in TNB; Serotec, Oxford, UK), anti-F4/80 (1:600 dilution in TNB; Serotec), and anti–u-PAR (1:500 dilution in 10% normal rabbit serum) (Perkin Elmer) according to the manufacturer’s instructions. Slides were mounted in Vectashield containing 4',6-diamidino-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), and the number of neutrophils and macrophages in each section was determined by direct counting under a fluorescence microscope (Zeiss Axioshot; Carl Zeiss, Oberko-chen, Germany). All experiments were performed in duplicate.

Inhibition of u-PA Activity

We used u-PA-STOP (American Diagnostica, Stamford, CT, USA), a selective u-PA inhibitor, to determine the influence of u-PA inhibition on LPS-induced keratitis. In u-PA+/+ mice, the LPS-induced keratitis model was produced as stated above. Then, intravenous injection of u-PA-STOP (1 mg/kg), topical eye drops (200 μg/ml, u-PA-STOP), or saline (control, n = 4 mice in each group) was administered every 12 hours. After 3 days, the eyes were photographed and fluorescein staining and corneal opacity were estimated under a dissection microscope. The corneal sections were stained with anti–NIMP-R14 and anti-F4/80, and the number of immunoreactive cells in each section was determined by direct counting under a Zeiss Axioshot fluorescence microscope.

Zymographic Analysis

For zymographic analysis of u-PA, tissue-type plasminogen activator (t-PA), and matrix metalloproteinases (MMPs), using the LPS-induced keratitis model in u-PA wild-type (u-PA+/+) and u-PA−/− mice, as stated above, the mice were killed at 1 day, 3 days, and 7 days after LPS exposure. The eyes were enucleated and the separated corneas were frozen at −80°C. The corneas were pulverized with 200 μl extraction buffer (10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycho-
late, and 0.02% sodium azide) and centrifuged at 10,000g for 5 minutes at 4°C. The corneal extracts were used as samples. In this study, \( n = 5 \) mice in each group.

**Fibrin and Gelatin Zymographic Analysis**

The activities of u-PA and t-PA were determined as described previously. Briefly, protein samples were loaded onto a 10% polyacrylamide gel containing 0.55 mg/mL bovine fibrinogen and 0.056 NIH U/mL thrombin (Sigma-Aldrich Corp.). After electrophoresis, gels were soaked in 2.5% Triton X-100 solution for 60 minutes, and then incubated in reaction buffer (0.5 M glycine-NaOH, pH 8.4) at 37°C for 36 hours. The gels were stained with Coomassie Blue R-250 for 1 hour and destained with destaining solution (30% methanol, 10% acetic acid). Matrix metalloproteinase-2, MMP-3, and MMP-9 activities were detected by gelatin zymography using the Gelatin Zymo Electrophoresis Kit (Life Laboratory Company, Yamagata, Japan) according to the manufacturer’s instructions. The intensity of the bands was measured by the LAS-1000 system (Fuji Film, Tokyo, Japan).

**Extraction of Total RNAs and RT-PCR**

Total RNAs were extracted from corneal samples (\( n = 5 \) mice, in each group) by using Isogene (Nippon Gene, Toyama, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) was done by using the SuperScriptIII one-step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Primer sequences are listed in Table 1. Twenty nanograms of total RNA was subjected to reverse transcription at 45°C for 30 minutes. Thermal cycle conditions included the initial denaturation at 95°C for 10 seconds, followed by 32 cycles of PCR amplification (94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 1 minute). The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Cell Isolation and Culture**

Primary cultures of mouse corneal fibroblasts were established from u-PA\(^{+/+}\) and u-PA\(^{−/−}\) mice as described elsewhere. The eyes were enucleated from postnatal day 1 mice, and small explants from corneas were removed with sterile surgical forceps, under a stereo dissection microscope. For the outgrowth of corneal fibroblasts, the explants were placed onto 35-mm culture dishes in medium containing 10% fetal bovine serum. The cells were grown to confluence in a 35-mm culture dish and were then changed to serum-free medium.

**Real-Time RT-PCR**

Real-time RT-PCR was conducted to examine the effects of endogenous u-PA on MMP-2, MMP-9, MCP-1, and MIP-2 expression in mouse ocular fibroblasts. After 24-hour serum starvation, mouse corneal fibroblasts were incubated in the presence or absence of LPS (5 ng/mL) for 24 hours. Single-strand complementary DNA (cDNA) was prepared from total RNA by using random primers under standard conditions with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). The cDNA was subjected to quantitative real-time PCR by using SYBER Premix Ex Taq (Takara Bio, Shiga, Japan) and the ABI 7900HT Sequence Detection System (Applied Biosystems) in a 96-well plate according to the manufacturer’s instructions. The PCR conditions were 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 50°C for 0.5 minutes, and 72°C for 30 seconds. The primer pairs are listed in Table 1. The amount of each gene expression was calculated by normalizing the cycle threshold (Ct) of each gene to the Ct of the β-actin in the same sample, according to the ΔΔCt method. Three independent experiments were performed.

**Enzyme-Linked Immunosorbent Assay**

To detect MCP-1 and MIP-2 in the supernatant of corneal fibroblasts, we used enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (Quantikine; R&D systems, Minneapolis, MN, USA). Corneal fibroblasts from u-PA\(^{+/+}\) and u-PA\(^{−/−}\) mice were grown to subconfluence and then incubated in serum-free Dulbecco’s modified Eagle’s medium for 24 hours, with or without exposure to LPS (5 ng/mL). The supernatant was then harvested for ELISA.

**Macrophage Migration Assay**

Macrophage migration assays were performed by using collagen type-1–coated transmigration inserts (pore size, 3 μm; BD Biosciences, San Jose, CA, USA). The macrophage cell line RAW 264.7 (2 × 10\(^5\)) was seeded onto the upper chambers (collagen type-1–coated inserts). Quadruplicate samples per treatment were tested, and their mean average was obtained. Data were expressed as mean ± standard error of the mean (SEM) of the number of migrated cells/filiter, in three independent experiments.

**Statistical Analyses**

Statistical comparisons between two groups were performed by unpaired Student’s t-test. Values of \( P < 0.05 \) were considered significant. Significant differences between groups are \( *P < 0.05 \), and \( **P < 0.01 \).

**RESULTS**

**Clinical Observations of LPS-Induced Keratitis in u-PA\(^{+/+}\) and u-PA\(^{−/−}\) Mice**

The clinical levels of LPS-induced keratitis in u-PA\(^{+/+}\) and u-PA\(^{−/−}\) mice were examined. As shown in Figure 1A, at 1 day of treatment, corneal opacity was clearly observed in u-PA\(^{+/+}\) mice,
in contrast to u-PA−/− mice, which showed mild opacity. Both types of mice had the same level of corneal epithelial defects. At a later time period, u-PA+/+ mice showed aggravated opacity and recurrent corneal erosion, while u-PA−/− mice showed gradually diminishing opacity, with corneal epithelial defects also reappearing. Neovascularization was observed in u-PA+/+ mice at day 3 and later. Figure 1B shows the ratio of corneal epithelial defects. Corneal epithelial defects were prolonged in u-PA+/+ mice, and at 7 days, complete resurfacing was not observed in any mice. All the u-PA−/− mice showed their defects repaired by 7 days. Error bars represent SEM (n = 8).

**Role of u-PA in Leukocyte Infiltration in Cornea**

The histologic results showed severe damage and persistent inflammatory responses in u-PA+/+ corneas compared with u-PA−/− corneas (Fig. 2). There were numerous inflammatory cells infiltrated into the cornea at 1 day of LPS exposure in both u-PA+/+ and u-PA−/− corneas (Figs. 2A, 2B); however, inflammatory cells infiltrated into u-PA−/− corneas were limited locally (Fig. 2B). Moreover, prolonged corneal epithelial defects and corneal stromal disruption, accompanied with the persistent inflammatory cell infiltration, were observed in u-PA−/− mice gradually decreased, in contrast to the level of opacity of the u-PA+/+ mice, which was greater. At 7 days, the u-PA+/+ mice had four eyes of eight marked grade 4, in contrast to the u-PA−/− mice, which recorded grade 1 in five of eight eyes.

**Leukocyte Infiltration Is Impaired in u-PA−/− Mice**

The histologic results showed severe damage and persistent inflammatory responses in u-PA+/+ corneas compared with u-PA−/− corneas (Fig. 2). There were numerous inflammatory cells infiltrated into the cornea at 1 day of LPS exposure in both u-PA+/+ and u-PA−/− corneas (Figs. 2A, 2B); however, inflammatory cells infiltrated into u-PA−/− corneas were limited locally (Fig. 2B). Moreover, prolonged corneal epithelial defects and corneal stromal disruption, accompanied with the persistent inflammatory cell infiltration, were observed in u-PA−/− mice.

Table 2. Transition of Corneal Opacity Grade of Both Types of Mice

<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>Day 1 u-PA+/+</th>
<th>Day 1 u-PA−/−</th>
<th>Day 3 u-PA+/+</th>
<th>Day 3 u-PA−/−</th>
<th>Day 7 u-PA+/+</th>
<th>Day 7 u-PA−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>5 (cyc)</td>
</tr>
</tbody>
</table>
PA⁺/⁺ corneas at 3 days (Fig. 2C) and 7 days (Fig. 2E) of treatment. In contrast, the number of inflammatory cells was reduced and epithelial defects were healed in u-PA⁻/⁻ corneas at 3 days and 7 days of LPS exposure (Figs. 2D, 2F). To determine the identity of inflammatory cells, immunostaining for neutrophils (NIMP-R14) and macrophages (F4/80) was performed in the corneas at 1 day, 3 days, and 7 days of LPS exposure (Fig. 3). Corneas from u-PA⁺/⁺ mice treated with LPS showed numerous neutrophils and macrophages, compared with u-PA⁻/⁻ mice treated with LPS (Figs. 3A, 3C). The number of neutrophils peaked at 1 day in both u-PA⁺/⁺ and u-PA⁻/⁻ mice (Fig. 3B). In contrast to the distribution pattern for neutrophils, the number of macrophages peaked at 3 days in both u-PA⁺/⁺ mice and u-PA⁻/⁻ mice (Fig. 3D). The number of accumulated neutrophils was approximately 2.4-fold higher at 1 day, 2-fold higher at 3 days, and 16-fold higher at 7 days in u-PA⁺/⁺ mice than in u-PA⁻/⁻ mice (P < 0.01; Fig. 3B).

However, the number of accumulated macrophages was approximately 4-fold higher at 1 day, 1.5-fold higher at 3 days, and 2-fold higher at 7 days in u-PA⁺/⁺ mice than in u-PA⁻/⁻ mice (P < 0.01; Fig. 3D). In summary, these data indicate that u-PA regulates neutrophil and macrophage infiltration into the cornea after LPS exposure.

Effect of u-PA Inhibitor on LPS-Induced Keratitis in u-PA⁺/⁺ Mice

We administered u-PA-STOP via intravenous injections, and by topical eye drops, every 12 hours, to u-PA⁺/⁺ mice. After 3 days, the administration of u-PA-STOP significantly reduced corneal opacity and epithelial defects as compared with the control group (Fig. 4A). Immunofluorescent observations showed that less ingression of NIMP-R14-immunoreactive cells and F4/80 immunoreactive cells was present in the u-PA-STOP-treated group than in the saline-treated group (Figs. 4B, 4C). These results indicated that u-PA contributed to neutrophil and macrophage infiltration into the inflamed cornea after LPS treatment.

Expression of u-PAR in the Cornea After LPS Exposure

Immunohistochemistry showed that u-PAR expression was detected in inflammatory cells and the leading edges of the migrating epithelial cells. Expression of u-PAR in inflammatory cells was more frequently observed in u-PA⁺/⁺ corneas than in u-PA⁻/⁻ corneas (Fig. 5).

Lipopolysaccharide Induces Expression of u-PA and t-PA in the Cornea

The u-PA and t-PA activities from the corneas were measured by fibrin zymography (Fig. 6A). Urokinase-type PA activity was barely detectable in u-PA⁺/⁺ normal corneas. The activity increased in u-PA⁺/⁺ corneas at 1 day, 3 days, and returned to normal at 7 days of LPS exposure. Enzymatic activity for t-PA was also detected, but expression was faint compared with u-PA expression. The faint t-PA expression was seen in both u-PA⁺/⁺ and u-PA⁻/⁻ corneas; however, the t-PA expression pattern appeared to be approximately the same for all time points after LPS exposure.

Activity of MMPs Was Reduced in the Corneas From LPS-Treated u-PA⁻/⁻ Mice

Gelatin zymography results are shown in Figure 6B. Enhanced levels of both latent and active forms of MMP-9 were detected in u-PA⁺/⁺ and u-PA⁻/⁻ corneas at 1 day, 3 days, and 7 days of treatment.
LPS treatment. However, the levels of latent and active forms of MMP-9 were more enhanced in u-PA+/+ than in the u-PA−/− mice, especially at 3 days. Expression of MMP-9 appeared to peak at 3 days in u-PA+/+ mice, whereas the peak was at 1 day in u-PA−/− mice. Zymographic results also showed that u-PA−/− corneas had suppressed MMP-2 activity in LPS-stimulated corneas. In u-PA+/+ mice, the active form of MMP-2 was detected at 1 day and 3 days after LPS stimulation. In addition, MMP-3 was expressed at 3 days and 7 days in u-PA+/+ mice and at 7 days in u-PA−/− mice.

Gene Expression of MCP-1 and MIP-2 in LPS-Treated Corneas

To determine whether leukocyte infiltration into the cornea correlated with local inflammatory mediator production, mRNA expression of MCP-1 and MIP-2 was examined before and after LPS stimulation in u-PA+/+ and u-PA−/− mice. Messenger RNA expression of MCP-1 and MIP-2 significantly increased in u-PA+/+ corneas treated with LPS, compared with mRNA of u-PA−/− corneas (Figs. 7A, 7B).

Effects of LPS on u-PA Production in Mouse Corneal Fibroblasts

As shown in Figure 8A, both secreted u-PA and cell-associated u-PA expression were slightly enhanced by LPS treatment, in a dose-dependent manner.

Effects of LPS on the Abundance of MMP-2, MMP-9, MCP-1, and MIP-2 mRNAs in u-PA+/+ and u-PA−/− Corneal Fibroblasts

The effects of LPS on the abundance of MMP-2, MMP-9, MCP-1, and MIP-2 mRNAs in u-PA+/+ and u-PA−/− corneal fibroblasts were assessed by real-time PCR analysis. The effects of LPS on MMP-9, MCP-1, and MIP-2 mRNA levels, but not on MMP-2 mRNA levels, were significantly higher in the u-PA+/+ corneal...
**Figure 4.** Urokinase-type plasminogen activator-STOP (a selective u-PA inhibitor) inhibits LPS-induced neutrophil and macrophage ingress in u-PA<sup>+/+</sup> mice. (A) Representative photographs of corneas in saline-treated groups (a, b) and in u-PA-STOP-treated groups (c, d) 3 days after treatment. (B) Immunofluorescent staining shows infiltration of neutrophils labeled with NIMP-R14 antibody in the saline-treated group (a) and u-PA-STOP-treated group (b). The signal is less in the u-PA-STOP-treated group than in the saline-treated group (a, b). Scale bar: 50 μm. (C) Numbers of NIMP-R14-positive neutrophils in corneal stroma. (D) Immunofluorescent staining shows infiltration of macrophages labeled with F4/80 antibody in the u-PA-STOP-treated group (a) and saline-treated group (b). The signal is less in the u-PA-STOP-treated group than in the saline-treated group (a, b). Scale bar: 50 μm. (E) Numbers of F4/80-positive macrophages in the corneal stroma. Data are representative of four repeated experiments with five corneas per group. Error bars represent SEM. **P < 0.01.

**Figure 5.** Expression of u-PAR in LPS-stimulated u-PA<sup>+/+</sup> and u-PA<sup>-/-</sup> corneas. Immunohistochemistry for u-PAR in the cornea at 1 day and 7 days after LPS exposure in u-PA<sup>+/+</sup> and u-PA<sup>-/-</sup> mice. Paraffin sections were stained with u-PAR antibody. The u-PAR–positive cells were observed in the stroma and the leading edge of the migrating epithelial cells. u-PAR expression of the cells in the corneal stroma was more frequently observed in u-PA<sup>+/+</sup> cornea. Red: u-PAR, blue: DAPI.
fibroblasts than u-PA−/− corneal fibroblasts. The u-PA+/+ corneal fibroblasts had a 4-fold increase in the amount of MMP-9 mRNA, a 2-fold increase in the amount of MCP-1 mRNA, and a 3-fold increase in the amount of MIP-2 mRNA, compared with u-PA−/− corneal fibroblasts (Figs. 8B, 9A).

**Effects of LPS on MCP-1 and MIP-2 Release by u-PA+/+ and u-PA−/− Corneal Fibroblasts**

The u-PA+/+ and u-PA−/− corneal fibroblasts were cultured for 24 hours in the absence or presence of LPS (5 ng/mL), and the amount of MCP-1 and MIP-2 in the culture supernatants were then determined by ELISA. The concentrations of MCP-1 and MIP-2 in the culture supernatants were greatly increased by the presence of LPS. Moreover, in the presence of LPS, both of these cytokines were significantly increased in the supernatants from u-PA+/+ compared with u-PA−/− mice (Fig. 9B).

**Macrophage Migration Assay**

Chemotactic cytokine (MCP-1, MIP-2) analyses showed that LPS-treated u-PA+/+ corneal fibroblasts released more chemotactic cytokine. Such effects might be expected to enhance the migration of macrophages through type-1 coated collagen membranes in vitro. To test this possibility, the macrophage migration assay was performed. The RAW 264.7 macrophage migration was dramatically increased in the medium from LPS-treated u-PA+/+ corneal fibroblasts compared with the medium from LPS-treated u-PA−/− corneal fibroblasts. The LPS-induced macrophage migration was impaired without the existence of corneal fibroblasts, regardless of the genotype (Fig. 10).

**DISCUSSION**

In the current study, we demonstrated that u-PA enhanced LPS-induced corneal inflammation by promoting PMN and macrophage infiltration. Moreover, we showed that LPS enhanced the release of u-PA and MMPs, and the proteolytic inhibition of u-PA inhibited LPS-induced corneal inflammation, suggesting that u-PA was an essential component of LPS-induced corneal inflammation.

Reports on the plasminogen-plasmin system in the cornea suggest that collagenase is activated by plasmin, and consequently dissolves collagen in the corneal stroma during ulceration in alkali-burned corneas. It has also been reported that the tear fluid of normal humans, as well as of a corneal ulcer patient, contains u-PA and t-PA, and that u-PA promotes corneal epithelial cell migration. There is general agreement that the main function of u-PA involves pericellular proteolysis, cell migration, and cell proliferation, whereas t-PA appears to be more responsible for thrombolysis. However, u-PA and t-PA can substitute for each other in the generation of plasmin for fibrinolysis.

In addition to its well-known function in the fibrinolytic system, by regulating leukocyte extravasation to inflamed
tissue, u-PA is increasingly recognized as a critical component of the inflammatory response. During corneal infection, corneal resident cells and inflammatory cells produce and release mediators, resulting in the destruction of the ECM. A number of investigators have characterized the inflammatory response induced by ocular infection. Besides the expression of PAs, there is also the release of several mediators relevant to corneal inflammatory responses, such as the arachidonic acid metabolites, elastase, and IL-1β. Moreover, enzymatic expression of t-PA and u-PA is putatively controlled by several serine protease inhibitors, including plasminogen activator inhibitor (PAI)-1 and PAI-2. Both PAI-1 and PAI-2 are upregulated in the mouse cornea infected with P. aeruginosa.

In the present study, infiltrating cells induced by LPS in the inflamed corneas were mostly neutrophils and macrophages, and u-PAR expression in infiltrating cells was also observed in the inflamed corneas. Previous studies have shown that u-PAR expression is strongly enhanced under inflammatory conditions, particularly in infiltrating leukocytes. Although u-PAR is an important binding partner of u-PA, several studies have demonstrated that u-PAR controls leukocyte responses independently of u-PA. Conversely, it is known that u-PA functional properties are independent of u-PAR. Deindl et al. have demonstrated that u-PA-mediated leukocyte infiltration is not dependent on u-PAR. Reichel et al. have demonstrated that u-PA-mediated intravascular adherence and paracellular transmigration of neutrophils to postischemic tissue via Mac-1 does not require u-PAR.

In this study, we did not determine if u-PA/u-PAR interactions affected leukocyte infiltration. There is the possibility that u-PA enhances leukocyte infiltration through interaction with one of its other binding partners, besides u-PAR.

In the present study, to elucidate the mechanism by which u-PA promoted leukocyte infiltration into the cornea, we used two approaches. In the first approach, the expression of gelatinases was studied both in vivo and in vitro. Urokinase-type plasminogen activator converted plasminogen to plasmin. Plasmin plays an important role during corneal infection and ulceration, and also in activation of MMPs. Once formed, plasmin can degrade fibrin, fibronectin, vitronectin, and laminin, the component of the subepithelial basement membrane. Plasmin also appears to be involved in leukocyte infiltration, by activating MMPs. Among the MMPs, gelatinases have been shown to promote inflammatory cell

Figure 8. Expression of u-PA, MMP-2, and MMP-9 in the ocular fibroblasts derived from postnatal day 1 (P1) wild-type (WT), and u-PA knockout (KO) mice. (A) Fibrin zymographic analysis of u-PA protein expression and u-PA protein secretion in dose-dependent responses to LPS treatment in WT ocular fibroblasts. (B) Adding LPS enhanced expression of MMP-2 and MMP-9 mRNA. Expression of MMP-9 mRNA, but not of MMP-2, was counteracted by lack of u-PA, in ocular fibroblasts. The experiments were performed in triplicate. Error bars represent SEM. **P < 0.01.
The u-PA–activated plasmin is a physiological activator of pro–MMP-9. Moreover, u-PA can directly activate MMP-9.

From the results of our zymography assay, considerable damage to the corneal stroma in u-PA+/+ mice was accompanied by upregulation of MMP-2, MMP-3, and MMP-9. However, more enhanced expression of MMP-9 was observed in u-PA+/+ corneas than u-PA−/− corneas, while MMP-2 or MMP-3 expression was only slightly enhanced. These data suggest that MMP-9 was stimulated by u-PA directly or indirectly through the plasminogen/plasmin cascade in inflamed cornea. Additionally, corneal fibroblasts from u-PA+/+ mice showed more MMP-9 expression than u-PA−/− corneal fibroblasts, indicating that increased expression and activity of MMP-9 may be responsible for the u-PA–dependent LPS-induced corneal inflammatory response. Therefore, it is strongly suggested that the u-PA/MMP-9 system is closely involved in leukocyte infiltration during corneal inflammatory processes.

Studies have suggested a role of MMPs in the cornea; however, it remains to be elucidated which MMP is responsible for corneal inflammation. Matrix metalloproteinase-9 plays an important role in corneal epithelial wound healing, whereas MMP-2 may be involved in the process of collagen remodeling in the corneal stroma. Other studies have shown that MMP-9 may play an important role in stromal degradation and remodeling of a new matrix. Macrophage migration requires MMP-9 activation by plasminogen. Additionally, plasmin can activate MMP-3, which can then activate other pro-MMPs, such as pro–MMP-9.

In the present study, we concluded that MMP-9 activation was most important in corneal leukocyte infiltration.

In the second approach, u-PA–induced effects of chemotaxis mediator release were investigated. As MCP-1 and MIP-2 are chemotactic cytokines of macrophages and neutrophils, we focused on these two cytokines. We showed, in vivo, that u-PA+/+ corneas, in comparison to u-PA−/− corneas, had enhanced gene expression of MCP-1 and MIP-2. These results indicated that u-PA contributed to MCP-1 and MIP-2 synthesis in inflamed corneas. From these results, we hypothesized that one potential mechanism underlying the u-PA–mediated leukocyte infiltration might be the release of MCP-1 and MIP-2 from corneal fibroblasts, which promoted the leukocyte infiltration. The results showed that corneal fibroblasts from u-PA−/−, stimulated by LPS, significantly induced more MCP-1 and MIP-2...
production than u-PA−/− corneal fibroblasts, suggesting that u-PA contributed to corneal leukocyte infiltration, possibly through controlling the release of MCP-1 and MIP-2 from corneal fibroblasts. In addition, our in vitro data from macrophage migration assays indicated that the corneal fibroblasts from u-PA+/− mice, stimulated by LPS, significantly promoted chemotaxis of macrophages, presumably through releasing chemotactic factors.

Although further studies are necessary to detect other potential mechanisms, we conclude that at least two different mechanisms for corneal leukocyte infiltration exist: (1) u-PA presumably activates MMP-9 directly and also indirectly through a plasminogen/plasmin cascade in LPS-induced inflamed cornea, in a process that appears to involve leukocyte corneal infiltration; and (2) u-PA-mediated leukocyte infiltration is probably involved in the release of MCP-1 and MIP-2 from corneal fibroblasts, which promote the leukocyte infiltration into the cornea.

Acknowledgments

Supported in part by grants from the Osaka Eye Bank.

Disclosure: K. Sugioka, None; A. Kodama, None; K. Yoshida, None; K. Okada, None; H. Mishima, None; K. Aomatsu, None; O. Matsuo, None; Y. Shimomura, None

References

Role of u-PA in Leukocyte Infiltration in Cornea

Invest Ophthalmol Vis Sci 2014; Vol. 55  No. 8  5349


