Chemical Injury-Induced Corneal Opacity and Neovascularization Reduced by Rapamycin via TGF-β1/ERK Pathways Regulation

Young Joo Shin,1 Joon Young Hyon,2,3 Won Seok Choi,1 Kayoung Yi,1 Eui-Sang Chung,4 Tae-Young Chung,3 and Won Ryang Wee2

1Department of Ophthalmology, Hallym University College of Medicine, Seoul, Republic of Korea
2Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Republic of Korea
3Department of Ophthalmology, Seoul National University Bundang Hospital, Seongnam, Gyeonggi, Korea
4Department of Ophthalmology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea

Purpose. To investigate the protective effect of rapamycin against alkali burn-induced corneal damage in mice.

Methods. BALB/c mice were treated with 0.1 N NaOH to the cornea for 30 seconds. Corneal neovascularization and opacity were clinically evaluated at 1, 2, and 4 weeks after chemical burn injury. Rapamycin was delivered topically to right eyes (1 mg/mL) and injected intraperitoneally (0.2 mg/kg) once a day. Concentrations of interleukin-6 (IL-6) and transforming growth factor-beta1 (TGF-β1) in the cornea were measured by enzyme-linked immunosorbent assay (ELISA). In vitro-cultured human corneal stromal cells were treated with 0 to 500 nM rapamycin for 3 days and then assessed by immunofluorescence staining of vimentin and alpha-smooth muscle actin (α-SMA). Western blotting assays for α-SMA, phosphorylated extracellular signal-regulated kinase (p-ERK 1/2), and total ERK 1/2 were also performed.

Results. Corneal neovascularization and corneal opacity scores measured 4 weeks after the chemical burn corneal injury were lower in the rapamycin group than in the control group. Two weeks after the chemical burn injury, a significant elevation in the corneal IL-6 levels of the positive control group was observed, compared to the levels in the negative control group or the rapamycin group (P < 0.05). Corneal TGF-β1 levels were lower in the rapamycin-treated group than in the control group at 4 weeks after chemical burn injury (P < 0.05). Moreover, rapamycin inhibited TGF-β1-induced α-SMA expression and augmented ERK 1/2 phosphorylation.

Conclusions. Rapamycin treatment reduced corneal opacity and corneal neovascularization in BALB/c mice. Rapamycin protected the cornea from chemical damage via reduction of IL-6 and TGF-β1 expression. Rapamycin reduced α-SMA expression through the ERK 1/2 pathway.

Keywords: rapamycin, alpha-smooth muscle actin, extracellular signal-regulated kinase, corneal stromal cell, chemical burn, interleukin-6, transforming growth factor-beta1

Corneal chemical injuries may produce extensive tissue damage resulting in permanent visual impairment.1 Although it is essential to control inflammation in the acute phase, the development of corneal opacity and neovascularization (NV) in the later phase has been reported to be the main cause of permanent visual loss. The effectiveness of several drugs for the treatment of chemical burn-induced corneal NV and opacity has been investigated.2–4 Nevertheless, the clinically prescribed drugs have not been effective as prophylactic treatments to prevent corneal NV and opacity development. The role of vascular endothelial growth factors (VEGF) in corneal chemical burn has been investigated in previous studies.5,6 In this study, we focused on the effect of rapamycin on other mechanisms of corneal chemical burn. Chemical burn has been reported to cause a chronic inflammatory state.9 Inflammation of the cornea results in corneal NV.3 Thus, suppression of the inflammatory cytokine can reduce corneal NV. Interleukin-6 (IL-6) has been reported to induce the inflammation7 and angiogenesis.8 Furthermore, NV often causes fibrosis,10 which results in corneal opacity.11 Transforming growth factors-beta1 (TGF-β1), which can be modulated by IL-6,12 has been reported to be involved in the initiation of the fibrotic response in vivo.13 Thus, the drugs that suppress inflammation and reduce fibrosis can be the most effective in treatment of corneal chemical burn.

Rapamycin (Sigma-Aldrich, St. Louis, MO) is an immunosuppressant used in several cancer treatments14 and in allograft rejection treatment.15 Rapamycin acts through inhibition of mammalian target of rapamycin (mTOR).16 Multiple upstream signals, including those triggered by growth factors and nutrients, are integrated into the mTOR signaling pathway. Inhibition of mTOR leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression, and autophagy induc-
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METHODS

Animals

All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week-old male BALB/c mice were obtained (NARA Biotech, Seoul, Korea) and maintained in the colony room of our institution for 1 to 3 days before the experiments. The temperature in the colony room was maintained at 25°C, and the 12-hour day–night cycle was 8 AM to 8 PM and 8 PM to 8 AM.

Corneal Chemical Burn Injury

Anesthesia was achieved by intraperitoneal injection of tiletamine and zolazepam (30 mg/kg; Zoletil 50; Virbac, Carros, France) and xylazine hydrochloride (5 mg/kg). Alkali burn injury was inflicted by pressing an application stick soaked in 0.05 M NaOH onto the cornea of the right eye for 30 seconds. The corneal surface was then carefully rinsed with 10 mL physiological saline solution for 2 minutes.

Drug Preparation and Treatment Protocol

Rapamycin was delivered into right eyes topically (1 mg/mL) and injected intraperitoneally (0.2 mg/kg) once per day. The animals were separated into three groups: the negative control group (n = 5) with no chemical burn injury, the positive control group (n = 5) that received a PBS injection after the corneal chemical injury, and the rapamycin group (n = 5) that received the chemical burn injury followed by topical and intraperitoneal rapamycin treatment. The mice were clinically evaluated and then sacrificed at 1, 2, and 4 weeks after corneal chemical injury.

Assessment of Corneal Neovascularization and Increased Opacity

Corneal NV and opacity were clinically evaluated at various intervals (1, 2, and 4 weeks) after the chemical burn injury. The areas of NV and epithelial defects were measured, and their percentages of the entire cornea were calculated. Corneal opacity was scored using a system of scaling from 0 to 4, where 0 = opacity, completely clear cornea; 1 = slightly hazy, iris and lens visible; 2 = moderately opaque, iris and lens still detectable; 3 = severely opaque, iris and lens hardly visible; and 4 = completely opaque, with no view of iris and lens. Haze and NV grade values (means ± SD, n = 5) were calculated at each postoperative follow-up time for the eyes.

Enzyme-Linked Immunosorbent Assay (ELISA)

The tissues were homogenized and then sonicated in 1 mL antiprotease-supplemented Nonidet-P40 (NP-40) buffer. Specimens were centrifuged at 12,000g at room temperature for 10 minutes and frozen at −70°C for future cytokine measurement. A double-sandwich ELISA was performed to determine mice IL-6 and TGF-β1 concentrations. All experiments were performed with commercially available IL-6 and TGF-β1 kits (R&D Systems, Minneapolis, MN) and following the manufacturer’s protocols.

The color reagent 3,3′,5,5′-tetramethylbenzidine (TMB; 100 μL) was then applied for 20 minutes to develop a blue color, and the reaction was stopped by addition of 50 μL of 1 M H₂SO₄. The absorbance was measured at a wavelength of 450 nm, using an automatic plate reader (SpectraMax 384; Molecular Devices, Sunnyvale, CA) with a reference wavelength of 570 nm. The cytokine concentrations were adjusted on the basis of the total protein concentrations measured by using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL).

Isolation and Culture of Human Corneal Stromal Cells

This study was performed according to the tenets of the Declaration of Helsinki and was reviewed and approved by the institutional review board/ethics committee of Hallym University Medical Center. The human stromal cells were obtained from discarded corneal-scleral rings after penetrating keratoplasty. These tissues were stored in OptisolGS (Bausch and Lomb, Inc., Rochester, NY) at 4°C until they were processed for culture.

The cells were cultured in accordance with previously published methods. The corneal stromal cells (obtained from remnant donor tissue after corneal transplantation) were harvested at or before 7 days after death. Descemet's membrane and epithelium were removed using forceps and an ophthalmic knife. The stroma was minced under laminar flow. Midstroma and posterior stroma explants were then suspended in culture medium and cultured in 24-well plates. The corneal stroma was sliced into quarters and digested overnight with 2 mg/mL collagenase (Roche, Basel, Switzerland) in Dulbecco's modified Eagle medium (DMEM) at 37°C. Isolated cells were washed in DMEM and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (PBS; Gibco-Invitrogen, Grand Island, NY).

Transdifferentiation of Corneal Stromal Cells Into Myofibroblasts

The cells were cultured on tissue culture-treated plastic at 4 × 10⁴ cells/cm². After cells reached a confluent state, the corneal stromal cells were maintained in serum-free DMEM/F12 medium containing 50 mM HEPES and 0.1 mM L-ascorbic acid 2-phosphate. The corneal stromal cells were treated with TGF-β1 (8 ng/mL) with 0.1% FBS for the stromal cell transition to myofibroblasts and 0 or 250 or 500 nM rapamycin was added. The treatment continued for 3 days.

Immunofluorescence Staining

Human corneal keratocytes cultured on glass coverslips in 12-well plates were washed with phosphate-buffered saline (PBS) and fixed for 20 minutes in 3.7% formaldehyde solution. The cells were permeabilized with 0.5% Triton X-100 treatment for 10 minutes and then incubated with a blocking buffer (1% bovine serum albumin) for 1 hour at room temperature. After being washed with PBS, the cells were incubated overnight with rabbit polyclonal antibody for vimentin (Sigma-Aldrich) or for α-smooth muscle actin (SMA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C, and then washed with PBS. The cells were incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (IgG) antibody (1:100 dilution) for 1 hour at 37°C in the dark and then counterstained with Hoechst nuclear staining dye (1:2000 dilution; Molecular Probes, Eugene, OR) in accordance with the manufacturer’s recommendations. After extensive washes with PBS, the slides
were coverslipped with mounting medium to reduce photo-bleaching.

**Western Blotting for α-SMA, β-ERK 1/2, Total ERK 1/2, and β-Actin**

Keratocytes were grown under various culture conditions and then extracted in Radioimmunoprecipitation assay (RIPA) buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl [pH, 7.4], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.03 TIU/ml aprotinin), including protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (PhosSTOP; Roche) after the culture medium was removed. The supernatant was collected after centrifugation at 13,000 g for 20 minutes and frozen at −70°C until they were used for the measurement of α-SMA, phosphorylated extracellular signal-regulated kinase 1/2 (β-ERK 1/2), total ERK 1/2, and β-actin levels. Aliquots of cell extracts containing 20 or 30 μg of proteins were subjected to SDS-PAGE. Protein bands were electrophoretically transferred by SDS-PAGE to a membrane (Immobilon-P; Millipore Corp., Bedford, MA). After the membranes were incubated with 5% skim milk in PBS for 1 hour, they were immersed in rabbit antihuman α-SMA antibody (Abcam, Cambridge, MA), rabbit antihuman β-ERK 1/2 antibody (Abcam), rabbit antihuman total ERK 1/2 antibody (Abcam), and rabbit antihuman β-actin antibody (Abcam) at 4°C overnight and alkaline phosphatase conjugated with secondary antibody for 2 hours. The immunoreactive bands were detected using a chromogenic immunodetection kit (WesternBreeze; Invitrogen, Carlsbad, CA) following the manufacturer’s protocols. Data were quantified with a video image analysis system.

**Statistical Analysis**

Data were evaluated using the Kruskal-Wallis test followed by the Mann-Whitney U test. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Corneal Neovascularization and Corneal Opacity Measurement**

The corneal NV area and corneal opacity scores are shown in Figures 1A, 1B. The corneal NV area was smaller in the rapamycin group than in the positive control group at 4 weeks after chemical burn injury (P = 0.017, Mann-Whitney U test) (Fig. 1A). The corneal opacity score was lower in the rapamycin group compared to that of the positive control group at 4 weeks after chemical burn injury (P = 0.009, Mann-Whitney U test) (Fig. 1B). The corneal epithelial defect was not different between rapamycin group and positive control group (Fig. 1C). Toxic effect of topical rapamycin on overlying epithelium was not observed.

**Corneal IL-6 and TGF-β1 Levels and α-SMA Expression**

Corneal TGF-β1 levels are shown in Figure 2A. The TGF-1 levels of cornea were elevated in the positive control group than in the rapamycin group at 4 weeks after chemical burn injury (P = 0.017, Mann-Whitney U test) (Fig. 2A). However, TGF-β1 levels in the rapamycin group did not differ from those in the negative control group and were lower than those in the positive control group (P = 0.036, Mann-Whitney U test).

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932985/)  
**Figure 1.** Corneal opacity and corneal NV scores. (A) Corneal opacity score was lower in the rapamycin group than that in the positive control group at 4 weeks after chemical burn injury (P = 0.009; Mann-Whitney U test). (B) Corneal NV score was lower in the rapamycin group than in the positive control group at 4 weeks after chemical burn injury (P = 0.017, Mann-Whitney U test). (C) The corneal epithelial defect was not different between rapamycin group and positive control group. Toxic effect of topical rapamycin on overlying epithelium was not observed. *Statistically significant.

As shown in Figure 2B, the IL-6 levels did not differ significantly among the three groups at 1 week. At 2 weeks after chemical burn injury, the corneal IL-6 levels increased in the positive control group compared to the levels in the negative control group or those in the rapamycin group (P = 0.016 and 0.008, respectively, Mann-Whitney U test). However,
**Figure 2.** Corneal TGF-β1 and IL-6 measurement by ELISA and corneal α-SMA expression was determined by Western blotting. (A) Corneal TGF-β1 levels. Corneal TGF-β1 levels increased in the positive control group compared to those in the negative control group at 4 weeks after chemical burn injury infliction ($P = 0.036$, Mann-Whitney $U$ test). However, TGF-β1 levels in the rapamycin group did not differ from those in the negative control group and were lower than those in the positive control ($P = 0.036$). (B) IL-6 levels did not differ significantly among the three groups at 1 week after injury. However, at 2 weeks after infliction of the chemical burn injury, corneal IL-6 levels increased in the positive control, whereas it was not expressed in the negative control and in the rapamycin group. (C) α-SMA of cornea was not expressed in the three groups at 1 week after injury. However, at 2 weeks after chemical burn injury, α-SMA expression increased in the positive control, whereas it was not expressed in the negative control and in the rapamycin group. *Statistically significant.
4 weeks after chemical burn injury, the IL-6 levels in the positive control group were comparable to those in the negative control group. The IL-6 levels were higher in the rapamycin group than those in the negative control group ($P = 0.032$, Mann-Whitney $U$ test).

The $\alpha$-SMA expression of corneal homogenate was not detected 1 week after chemical burn injury. However, 2 weeks after chemical burn injury $\alpha$-SMA expression increased in the positive control and was not detected in the negative control and in the rapamycin group (Fig. 2C).

**Cell Identification**

Cultured corneal stromal cells were identified with immunocytochemistry staining of vimentin (Fig. 3). Vimentin-positive cells were stained green, and the nuclei were stained blue with Hoechst 33,342 dye.

**Immunocytochemistry**

The immunofluorescence staining of $\alpha$-SMA is shown in Figure 4. The control cells showed negligible green fluorescence, and TGF-$\beta$1 (8 ng/mL)-treated cells were visualized as green cells due to $\alpha$-SMA expression. The nuclei were stained with Hoechst 33,342 (blue) dye. Rapamycin treatment reduced $\alpha$-SMA expression in TGF-$\beta$1-treated cells.

**Western Blotting**

Rapamycin inhibited TGF-$\beta$1-induced $\alpha$-SMA expression and augmented ERK 1/2 phosphorylation. Whole-cell lysates were prepared and analyzed for $\alpha$-SMA expression and total/p-ERK 1/2 expression by Western blotting (Fig. 5). The figures show expression of phosphorylation of ERK 1/2 after rapamycin (250 nM and 500 nM) treatment of cells in the absence or presence of 8 ng/mL TGF-$\beta$1. ERK pathway activity was measured by examining the expression of phosphorylated ERK 1/2 (p-ERK 1/2). IL-6 expression was not detected.

**DISCUSSION**

Corneal chemical burn may induce corneal blindness by increasing the corneal opacity and corneal NV. $^{3,9}$ Rapamycin has been reported to inhibit pulmonary and peritoneal fibrosis$^{18,19}$ as well as allograft rejection.$^{16}$ In this study, the corneal opacity and NV scores were lower in the rapamycin group than those in the positive control group at 4 weeks after the chemical injuries. Rapamycin treatment reduced IL-6 levels in the rapamycin group compared to those in the positive control group.
group 2 weeks after the chemical injuries. IL-6 is a proinflammatory cytokine produced in corneal chemical burn tissue and is known to induce angiogenic factors. IL-6 levels in the rapamycin group decreased at 2 weeks and then did not differ at 4 weeks after chemical injury.

The TGF-β1 levels in chemically burned corneas increased 4 weeks after chemical injury infliction. In vivo studies revealed that corneal TGF-β1 levels were higher in the positive control group than in the rapamycin group and the negative control group. TGF-β1 has been reported to stimulate transdifferentiation of corneal stromal cells to myofibroblasts, which then increase the corneal opacity. In vitro studies showed that the expression of α-SMA, a marker of myofibroblast, increased with the TGF-β1 treatment. Furthermore, TGF-β1 treatment stimulated the corneal stromal cells to transdifferentiate into myofibroblasts, whereas rapamycin inhibited TGF-β1-induced myofibroblast transdifferentiation.

Rapamycin treatment also elevated phospho-ERK 1/2 levels. The ERK 1/2 signaling cascade is a central pathway in the regulation of cellular processes such as proliferation, differentiation, and survival. The ERK 1/2 cascade is activated by many extracellular stimuli and internal processes, and the ERKs also phosphorylate a large number of substrates, including Elk1, c-fos, and smad-linker. Crossstalk between ERKs and SMAD signaling pathways has been reported. The activity of SMAD pathway plays a central part in the development of fibrosis. Rapamycin targets the mTOR protein, which is a serine/threonine kinase robustly regulated by a diverse array of upstream signals. ERK has been reported to involve regulation of the mTOR pathway. The mTOR-mediated protein synthesis requires coincident and mutually dependent activity in the PI3K and ERK pathways. ERKs phosphorylate distinct sites on tuberous sclerosis complex 2 (TSC2), leading to greater repression of its GTPase-activating protein (GAP) activity and, consequently, a magnified stimulation of mTOR complex 1 (mTORC1) signaling. mTOR is a major effector of cell growth and protein synthesis via the direct functional control of its downstream targets, ribosomal protein S6 kinase (S6k), and eukaryotic initiation factor 4E-binding protein (4E-BP). IL-6 expression was not detected in an in vitro study, while IL-6 levels of cornea in vivo increased 2 weeks after chemical injuries. In vivo, chemical corneal injuries induce acute and chronic inflammation of ocular surface, where a variety of inflammatory cells are involved. Rapamycin, which is an anti-inflammatory agent, reduced IL-6 level of cornea in vivo study. Even though IL-6 has been described as being secreted by many different cell types including T cells, macrophages and vascular endothelial cells, it is secreted by corneal stromal cells in response to exogenous stimuli including ultraviolet irradiation, infection, and immunological challenge. However, cultured corneal stromal cells may produce extremely low levels of IL-6 if inflammatory stimuli are absent. In an in vitro study, TGF-β1 was used for induction of myofibroblast transdifferentiation, and it did not cause IL-6 secretion as an inflammatory stimulus. TGF-β1 expression in cultured corneal stromal cells was not investigated in the in vitro study because the cells were treated with TGF-β1 exogenously.

The combination of topical and intraperitoneal administration of rapamycin can be the most appropriate way to investigate the effect of rapamycin. Further study is necessary to determine the safest and most effective way to administer rapamycin. In addition, it is not clear whether rapamycin can reduce complete stem cell loss and subsequent conjunctivalization, or whether rapamycin may prevent stromal NV and myofibroblast transdifferentiation only. Further study is necessary to investigate whether rapamycin can prevent conjunctivalization.

CONCLUSIONS

In conclusion, rapamycin reduced chemical burn-induced corneal opacity and NV by inhibiting IL-6 and TGF-β1 expression. Rapamycin reduced TGF-β1-induced myofibroblast transdifferentiation through the ERK 1/2 pathway.

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References

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