INTRODUCTION

Corneal neovascularization occurs secondary to inflammatory, infectious, traumatic, ischemic, and degenerative conditions and loss of the limbal stem cell barrier. It can be observed clinically in patients with infectious keratitis, trauma, alkali burns, or contact lens wear. Neovascularization can help corneal wound healing, but it can also lead to a reduction in corneal transparency and, consequently, significant visual impairment. In addition, it can worsen the prognosis of corneal transplantation because of the loss of immune privilege in the transplanted cornea. Inhibition of corneal neovascularization has been reported in several studies through medications like steroids, NSAIDs, and cyclosporine A; laser therapies like thermal argon laser photocoagulation, and surgeries like limbal tissue transplantation and amniotic membrane transplantation. However, there is currently no definitive treatment because of side effects and considerations of clinical safety.

Vascular endothelial growth factor (VEGF) is a neurotrophin that plays a key role in the angiogenic process. Some studies have shown that its secretion and receptor expression are increased in corneal neovascularization. VEGF expression has been shown to be associated with the activation of nuclear factor-kappaB (NF-kappaB).
Curcumin is a phenolic pigment extracted from the turmeric plant. In several studies, it has been shown to possess potent anti-oxidative, anti-inflammatory, anti-angiogenic, and anti-carcinogenic effects, but not cytotoxicity.20–22 Although the exact pharmacologic mechanism and target molecule have not been determined, curcumin has been reported to inhibit several signal transduction pathways, including the pathway leading to NF-kappaB activation.23–25 Therefore, we hypothesized that curcumin therapy would inhibit corneal neovascularization through the reduction of NF-kappaB activation and VEGF expression. The object of the current study was to investigate the anti-angiogenic effects of topical curcumin in an \textit{in vivo} rabbit model of corneal neovascularization.

**MATERIALS AND METHODS**

**Animals**

We studied 26 eyes in 13 New Zealand white albino rabbits (weight range, approximately 2.0–2.5 kg). All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**In Vivo Rabbit Model of Corneal Neovascularization**

Rabbits were deeply anesthetized by intramuscular injection of 50 mg/kg tiletamine plus zolazepam (Zoletil; Virbac, Carros, France). Topical proparacaine hydrochloride 0.5% (Alcaine®, Alcon, Forth Worth, Texas, USA) was instilled in 24 eyes in 12 rabbits, then 7-0 black silk suture was placed at midstromal depth at the 12 o'clock position in the corneas of both eyes, while avoiding anterior chamber perforation. The sutures were placed parallel to the limbus, without burying knots. Levofloxacin ophthalmic solution (Oculevo®, Samil, Seoul, Korea) was instilled two times a day. Seven days postoperatively, the sutures were removed under anesthesia after examination of corneal neovascularization. In two eyes of one test rabbit as negative control, sutures were not placed, and nothing was applied to represent the normal state.

**Curcumin Application and Experiment Design**

Stock solution of curcumin (Sigma, St. Louis, Missouri, USA) was made at 10 mmol/L concentration in dimethyl sulfoxide (DMSO) (Sigma), and diluted to 40, 80, and 160 μmol/L with balanced salt solution (BSS). The final concentration of DMSO for all treatments was less than 0.1%.

After suture removal, the animals were randomly divided into four groups (six eyes from three rabbits in each group). Different concentrations of curcumin were applied topically, two times daily for seven days: BSS in group A (control), 40 μmol/L in group B, 80 μmol/L in group C, and 160 μmol/L in group D.

**Analysis of Corneal Neovascularization by Biomicroscopic Examination**

All treatment and control animals were photographed at a standard magnification before treatment, as well as three and seven days after treatment. After anesthesia administration, images of the corneal neovascularization were captured using a camera (Contax D-7, Stuttgart, Germany) attached to a biomicroscope (S21, Carl Zeiss, Jena, Germany). The images were analyzed using the Axiovision digital imaging software (AxioVision AC Rel 4.5, Carl Zeiss, Hallbergmoos, Germany). Quantification of neovascularization was performed in a blinded fashion. To control for individual variations in the area of neovascularization induced by suture placement, the areas measured before topical application of curcumin and BSS were set at 1, and other area values were recorded as the ratio of the remaining neovascularized area.

**Semiquantitative RT-PCR Examination of VEGF Gene Expression**

VEGF mRNA expression was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). For total mRNA extraction, the half area of corneal neovascularization in each eye was homogenized in reagent (Trizol; Gibco BRL, Grand Island, New York, USA). Single-strand cDNA was synthesized by a first-strand synthesis system for RT-PCR (Superscript III; Invitrogen, Carlsbad, California, USA) and a random primer, and used as a template for PCR.

PCR experiments were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. PCR amplification was performed with a primer (sense, 5'-GAGCGGAGAAAGCATTTGTTT-3'; antisense, 5'-TGCACCGACTCTGTTTGT-3') for VEGF mRNA. The conditions were 5-min hot start at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C. Amplified products were separated by electrophoresis on a 1.0% agarose gel and visualized by ethidium bromide staining. To investigate the relative expression of VEGF, band densities were measured.
with densitometric analysis (Image Master VDS 2.0; Pharmacia Biotech Inc., San Francisco, California, USA).

Immunofluorescence Staining

One rabbit without corneal neovascularization (normal) and four rabbits from each group were used for immunofluorescence staining. The half areas of corneal neovascularization were embedded in optical cutting temperature compound (Sakura Finetek, Torrance, California, USA), frozen, and cut into cryosections (6 μm). Frozen sections were thawed and fixed in cold 4% paraformaldehyde at -4°C for 10 min. Sections were incubated with blocking solution (normal goat serum; Zymed, South San Francisco, California, USA) for 30 min. Primary antibodies against phospho-NF-kappaB p65 (Santa Cruz Biotechnology, Santa Cruz, California, USA) were used at a dilution of 1:200, and samples were incubated for 2 hr at room temperature. Secondary antibodies (Alexa-Fluor 555 [red] conjugated goat anti-rabbit IgG [1:5000]; Molecular Probes, Eugene, Oregon, USA) were then applied and incubated in a dark chamber for 1 hr, followed by counterstaining with Hoechst (4′,6-diamidino-2-phenylindole, dihydrochloride; 1:1000) for 5 min. After washing with PBS, anti-fade mounting medium (Gel Mount; Biomedica Corp., Foster City, California, USA) and a coverslip were applied. Staining was evaluated under a fluorescence microscope (IX71-F22FL; Olympus, Tokyo, Japan) and photographed with a digital camera (DP70-Set2; Olympus).

Statistical Analysis

Data were compared among groups and were expressed as means ± standard error of mean. Statistical analysis was carried out using the Mann-Whitney U test, with significance set at \( P < 0.05 \).

RESULTS

Biomicroscopic Analysis of Corneal Neovascularization

On biomicroscopic examination of the neovascularized cornea, neovascularized areas were less regressed in the control and 40 μmol/L groups, but neovascularized areas in the 80 and 160 μmol/L groups were significantly reduced at three days after treatment. At seven days after treatment, the neovascularized areas of control group subjects were slightly regressed. The neovascularized areas in all treated groups were significantly reduced compared with their states before treatment (Figure 1).

Figure 2 shows the mean ratio between neovascularized areas before treatment and three days (A) and seven days (B) after treatment. At three days after treatment, the mean ratios were 0.82 ± 0.04 in the control group, 0.70 ± 0.03 in the 40 μmol/L curcumin group, 0.61 ± 0.03 in the 80 μmol/L curcumin group, and 0.35 ± 0.05 in the 160 μmol/L curcumin group. At seven days, the mean ratios were 0.69 ± 0.01 in the control group, 0.50 ± 0.07 in the 40 μmol/L curcumin group, 0.43 ± 0.08 in the 80 μmol/L curcumin group, and 0.29 ± 0.08 in the 40 μmol/L curcumin group. The ratios in the 80 and 160 μmol/L curcumin groups were significantly reduced at three days, and the ratios in all treatment groups were significantly reduced at seven days compared with the control group (\( P < 0.05 \)). No other complications, such as infectious keratitis, were seen in the rabbit eyes until seven days after treatment.

FIGURE 1. Microscopic examination of the neovascularized area of the cornea. Control group (1, 2, 3). Groups treated with 40 μmol/L (4, 5, 6), 80 μmol/L (7, 8, 9), or 160 μmol/L (10, 11, 12) topical curcumin. Initial neovascularization of the cornea was observed (1, 4, 7, 10). Three days after treatment, neovascularized areas had slightly regressed in the control and 40 μmol/L groups (2, 5), but neovascularized areas were significantly reduced in the 80 and 160 μmol/L groups compared with the control group (8, 11). Seven days after treatment, neovascularized areas had slightly regressed in the control group (3); regression was more significant in all treated groups compared with the control group (6, 9, 12).
VEGF mRNA by Semiquantitative RT-PCR

Semiquantitative RT-PCR with normalization to GAPDH showed that the mean levels of gene expression for VEGF in the curcumin treated groups (40, 80, and 160 μmol/L) were 0.75 ± 0.107, 0.06 ± 0.011, and 0.05 ± 0.009, respectively, compared with the control group (BSS) (Figure 3). The corneas of the 80 and 160 μmol/L curcumin treatment groups expressed significantly less VEGF mRNA than those in the control group did (P < 0.05).

NF-kappaB Activation in the Neovascularized Cornea

In the immunofluorescent stain with the phospho-NF-kappaB antibody, NF-kappaB activated cell was stained, but inactivated cell was not stained. Figure 4 showed phosho-NF-kappaB staining in normal, control (BSS), and curcumin treated (40 μmol/L and 80 μmol/L) corneas. The corneal tissue of the

FIGURE 2 The mean ratios between blood vessel areas before and three days (A) and seven days (B) after treatment in each group (★; significantly different, P < 0.05).

FIGURE 3 Mean optical density rate of VEGF mRNA expression in the cornea seven days after treatment. Mean optical densities in the 80 and 160 μmol/L curcumin treatment groups were significantly reduced compared with the control group (BSS).

FIGURE 4 Immunofluorescent stain with phospho-NF-kappaB (p65) antibody in normal, control (BSS), and curcumin-treated (40 μmol/L and 80 μmol/L) corneas. Hoechst was used as a nuclear stain (blue). Original magnification, 400×.
normal group was not stained. And the epithelium and stroma in the control (BSS) and 40 μmol/L curcumin treatment groups were well stained. But the epithelium was stained weakly and few activated cells were examined in the stroma in the 80 μmol/L curcumin treatment group.

**DISCUSSION**

Corneal neovascularization is caused by various corneal diseases. Though it can enhance corneal wound healing and suppress the progression of infection, it also reduces the transparency of the cornea and induces significant visual impairment. Because of the clinical importance of this entity, several treatments, such as medication, laser photocoagulation, and surgery, have been studied.

Corneal neovascularization is caused by an imbalance between angiogenic factors like fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) and antiangiogenic factors like angiotatin, endostatin, or pigment epithelium derived factor (PEDF). VEGF plays an important role in the development and differentiation of normal tissue and the angiogenesis of physiologic and pathologic reactions. In the normal state, it promotes endothelial migration and development and maintains normal vessels by preventing apoptosis of endothelial cells. As the pathologic reaction, its overexpression can cause several vascular diseases and facilitate the growth of the tumor. In ophthalmology, VEGF is related to angiogenesis in diabetic retinopathy, corneal neovascularization, retinopathy of prematurity, and choroidal neovascularization. The treatments of these diseases focus on the reduction of VEGF production or blockage of pre-existing VEGF.

NF-kappaB is the transcription factor that controls the genes involved in cellular proliferation and apoptosis, and it has served as an important target molecule in the treatment of chronic inflammatory diseases and several cancers. Several studies have shown that VEGF expression is associated with the activation of NF-kappaB, and pyrroldine dithiocarbamate, an inhibitor of NF-kappaB, could decrease VEGF mRNA expression.

Curcumin is a polyphenol extracted from the rhizome of the plant *Curcuma longa*. It appears to be nontoxic, and it has anti-oxidative, anti-inflammatory, and anti-carcinogenic properties through the blockade of several signal pathways. Though the exact reaction mechanism and target molecule of curcumin is unknown, the substance has been shown to promote blockage of the signal pathway associated with protein kinase C, phospholipase A2, and arachidonic acid and has been shown to inhibit autophosphorylation of the epidermal growth factor (EGF) receptor. Some studies have shown that curcumin inhibits the expression of VEGF and bFGF, two major angiogenic factors. Other studies have shown its inhibition of VEGF expression and the similar effect with bevacizumab in several tumors. VEGF expression has also been reported to be inhibited by the curcumin-induced reduction in NF-kappaB activity.

In the current study, we investigated whether curcumin could inhibit corneal neovascularization in a rabbit model. Biomicroscopic examination for one week after topical application of curcumin revealed that treated eyes had significantly less neovascular area than control eyes did. There was also a greater reduction in VEGF mRNA expression and NF-kappaB activation in treated eyes compared to control eyes. Therefore, regression of new vessels was promoted prominently after topical application of curcumin.

There are some ophthalmologic studies of curcumin. Arbiser et al. reported that curcumin inhibited basic FGF-mediated corneal neovascularization in the mouse cornea. However, their study only looked at FGF-induced corneal neovascularization that would be different from the neovascularization induced by several clinical diseases. Because we used the suture-induced neovascularization model, our study could assess the clinical corneal neovascularization frequently seen in several corneal diseases. Bian et al. demonstrated that curcumin inhibited activation of NF-kappaB in human umbilical vein endothelial cells and inhibited corneal neovascularization in a corneal alkaline burn rat model. However, our study demonstrated inhibition of NF-kappaB activation and VEGF mRNA expression and regression of corneal neovascularization in the same tissue and, therefore, provided more valuable information.

In our study, VEGF mRNA expression was inhibited in the 80 and 160 μmol/L curcumin treatment groups, but not in the 40 μmol/L treatment group. However, the neovascular area by biomicroscopic examination was reduced with more than 40 μmol/L of curcumin. Neovascular area is related to various factors, such as VEGF, FGF, and TGF-a and b1, but in this study, only VEGF mRNA levels were determined by RT-PCR in neovascular corneas. So, this may affect our results. One study showed the anti-angiogenic effect of curcumin occurred through the inhibition of FGF-2-related signal transduction, not through VEGF.

Being a lipophilic molecule, curcumin interacts with cellular membrane and is subsequently transported inside the cell. In a previous study, quantitative cellular uptake and intracellular localization of curcumin were reported in normal and tumor cells. It revealed that a linear dependency on the uptake was observed with treatment concentration of curcumin, and curcumin...
is located both in the cell membrane and the nucleus. Although it was the experiment with spleen lymphocyte and NIH3T3, not corneal cell, we could analogize the pharmacologic activity of curcumin to the corneal cells. Further study is required to investigate quantitative uptake of curcumin in the corneal cells.

Though our study demonstrated inhibition of NF-kappaB activation and VEGF mRNA expression and reduction of neovascularization in curcumin-treated eyes, it did not reveal the precise anti-angiogenic mechanism by which curcumin exerts this effect. We were able to determine that the anti-angiogenesis effect of curcumin was related to NF-kappaB, VEGF, and neovascularization. Additional studies are needed to determine the anti-angiogenic mechanism and target molecule of curcumin.

In summary, our results indicate that topical curcumin reduces experimental corneal neovascularization related to the inhibition of NF-kappaB and VEGF mRNA expression. These findings might help in the treatment of various corneal diseases causing neovascularization, and our results might indicate the basic dosage required in the clinical application of curcumin. We could not investigate the other effects of topical curcumin on the cornea, and our study had a short follow-up period. Hence, the safety and longevity of the treatment effect remain unknown. Further studies are required to investigate these factors.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


