Plasminogen Kringle 5 Inhibits Alkali-Burn–Induced Corneal Neovascularization

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PURPOSE. Plasminogen kringle 5 (K5) is a potent angiogenic inhibitor. The purpose of the present study was to evaluate the therapeutic effect of K5 on alkali-burn-induced corneal neovascularization (NV) and to investigate its mechanism of action.

METHODS. Corneal NV was induced in rabbits by NaOH. The rabbits received eye drops containing K5 or vehicle alone, four times per day. Corneal NV and inflammation were monitored every other day with a slit lamp microscope, and the length of the vessels in the cornea and the area of NV were measured. Vascular endothelial growth factor (VEGF) was determined by immunohistochemical and Western blot analyses. The TUNEL assay was used to assess the apoptosis of endothelial cells. The effects of K5 on primary bovine aortic endothelial cells (BAECs) were determined by MTT assay, flow cytometry, transmission electron microscopy, and DNA fragmentation assay.

RESULTS. Alkali-burn–induced progressive corneal NV and inflammation in the cornea. K5 delayed the onset of corneal NV (P < 0.05) and decreased NV areas (P < 0.05) in a dose-dependent manner. K5 treatment, after the formation of corneal NV, induced regression of newly formatted vessels in the cornea. K5 decreased the inflammatory index in the corneas at different time points after the alkali burn. Corneal VEGF levels were reduced by K5 treatment. K5 inhibits proliferation and induces apoptosis in BAECs.

CONCLUSIONS. Topical application of K5 may have therapeutic potential for the chemical burn-induced corneal NV and inflammation. The inhibitory effect of K5 on corneal NV may be by downregulation of VEGF expression. (Invest Ophthalmol Vis Sci. 2005;46:4062–4071) DOI: 10.1167/iovs.04-1330

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Corneal neovascularization (NV), abnormal formation of blood vessels in the cornea, is a common and serious complication of many corneal diseases. The condition is associated with severe visual impairment and is a high risk factor for graft rejection after allograft corneal transplantation.1–7 Histopathologically, corneal NV is found in 20% of corneas, necessitating keratoplasty, with graft rejection or insufficiency accounting for one third of corneal buttons with NV.8 Corneal NV is a major cause of blindness that affects millions of people.8–10 Several groups have shown some mechanisms responsible for corneal neovascularization.9,10 Angiogenesis is tightly controlled by two counterbalancing systems: angiogenic stimulators such as vascular endothelial growth factor (VEGF), and angiogenic inhibitors such as angiostatin.11–13 Endogenous angiogenic inhibitors are essential for keeping the cornea avascular.14 In some pathologic conditions, such as corneal transplantation or chemical burn, some regions in the cornea increase the production of angiogenic stimulators and decrease the production of angiogenic inhibitors, disturbing the balance between the positive and negative regulators of angiogenesis. As a result, capillary endothelial cells overproliferate, leading to NV.11

Several angiogenic and antiangiogenic factors have been shown to be involved in ocular angiogenesis.1–7 VEGF, a potent and highly selective vascular endothelial mitogen and a modulator of vascular permeability (vascular permeability factor), has been shown to play a key role in corneal NV.15,16 It has been demonstrated that VEGF may be essential for the inflammatory neovascularization of the rat cornea.16

Plasminogen kringle 5 (K5), a proteolytic fragment of plasminogen, is a potent inhibitor of angiogenesis.17–19 Plasminogen contains 5 kringle domains, each consisting of 80 amino acids.19 Among them, K5 displays the most potent inhibitory activity toward endothelial cell proliferation.17,18 Because of its high efficacy, cell-type selectivity, and short amino acid sequence, K5 has considerable potential in the treatment of neovascular diseases.17,20 Recently, it has been shown that intravitreal injection of K5 prevents the development and stops the progression of ischemia-induced retinal NV in rats.21 However, its application in the treatment of corneal NV has not been explored, and the mechanism underlying the antiangiogenic activity of K5 has not been established.

In this study, we investigated the antiangiogenic effects of K5 on corneal NV induced by alkali burn in rabbits. The molecular mechanism was examined in primary endothelial cells.

MATERIALS AND METHODS

Alkali-Induced Corneal NV

New Zealand albino rabbits of either sex (2.0–2.5 kg), were used in the study. Care, use, and treatment of all animals in this study were in strict agreement with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.
Induction of corneal NV was performed as described by Ormerod et al.22 with some modifications. Briefly, a 5.5-mm-diameter circular filter disc was incubated with 20 μL 1 N NaOH for 60 seconds. The filter disc was placed on the corneal surface for 30 seconds in anesthetized rabbits. The ocular surface was then irrigated with 20 mL physiological saline. The rabbits were randomly divided into four groups of 10 rabbits per group.

Recombinant human K5 was expressed, purified, and analyzed, as described previously.21 The right eye of each rabbit was treated with different concentrations of K5 eye drops: 5 mg/L in group 1; 10 mg/L in group 2; 20 mg/L in group 3, and vehicle only in group 4 (control). The left eye was given a drop of PBS as the control. Ten microliters of eye drops were administered four times daily for 4 weeks.

Evaluation of NV and Inflammation

NV was examined by slit lamp every other day after the alkali burn. Measurements of NV were made with a slit lamp by a single masked observer who was an ophthalmologist. Vessel growth onto the clear cornea was noted in millimeters at each time point. NV was quantified by calculating the wedge-shaped area of vessel growth with the formula: \[ A = C/12 \times 3.1416 \left( r^2 - (r - \delta)^2 \right) \], where \( A \) is the area, \( C \) is time (in hours), \( r \) is the radius from the center to the border of vessel growth, and \( \delta \) is the radius of the cornea (7 mm).

Inflammatory response was evaluated by slit lamp. Serial photog- graphs of the cornea were taken. The inflammatory index was analyzed as previously described.24 Briefly, the inflammatory index was analyzed, based on the following parameters: ciliary hyperemia (absent, 0; present but less than 1 mm, 1; present between 1 and 2 mm, 2; present and more than 2 mm, 3); central corneal edema (absent, 0; present with visible iris details, 1+; present without visible iris details, 2; present without visible pupil, 3); and peripheral corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present with no visible iris, 3). The final inflammatory index result was obtained by summing the crosses of the different parameters divided by a factor of 9.24

The rabbits were killed on postoperative day (PD) 28. The eyeballs were removed and fixed in formalin. The corneal specimens were embedded in paraffin, cross-sectioned, and stained with hematoxylin and cosin.

Measure of Regression of Alkali-Burn–Induced Corneal NV

The same alkali-burn–induced corneal NV model was used. At 14 days after the injury, the rabbits were randomly divided into the K5 treatment group and control group, five rabbits per group. The right eye of each rabbit in the K5 treatment group was treated with 20 mg/L K5 eye drops for 14 days. The control group received the PBS eye drops. The extent of corneal NV was examined weekly, and the NV area was measured. At the end of the treatment, the corneas were harvested and paraffin embedded for endothelial cell counting and TUNEL assay. The corneal lysates were used for Western blot assay of VEGF.

Assessment of VEGF Expression

Immunohistochemical analysis for VEGF was performed on 4-μm paraffin-embedded sections. A monoclonal mouse anti-rabbit VEGF antibody (clone C-1; Santa Cruz Biotechnology, Santa Cruz, CA) was used according to the manufacturer’s instructions. Negative control experiments without primary antibody and negative tissue control experiments were performed, as well as positive tissue control experiments, including breast cancer and human tonsil (Dako, Glostrup, Denmark). Sections were then photographed by microscope (Axioskop microscope; Carl Zeiss Meditec; Oberkochen, Germany) using color reversal films (Ektachrome 41 T; Eastman Kodak, Stuttgart, Germany).

The corneal VEGF level was analyzed by Western blot analysis using anti-VEGF antibody (1:300; Santa Cruz Biotechnology, Inc.).

Effect of K5 on Corneal Neovascularization

Cell Isolation, Culture, and Characterization

For isolation of bovine aortic endothelial cells (BAECs), fresh bovine aorta was treated with PBS with penicillin (100 IU/mL), streptomycin (100 μg/mL), and amphotericin B (2.5 μg/mL). The aortas were washed with Hanks’ balanced salt solution (EuroClone Ltd., Cramlington, UK) and opened longitudinally. They were placed with the lumi- nal surface down in a 0.03% solution of collagenase type II (Sigma-Aldrich, St. Louis, MO). After incubation at 37°C for 15 minutes, the cells were removed with a cell scraper. BAECs were characterized by positive immunofluorescent staining for factor VIII-related antigen (Dako), and by their cobblestone appearance. BAECs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; EuroClone Ltd.) supplemented with 20% fetal calf serum (FCS) and antibiotics and were grown in an environment of 5% CO2 at 37°C. Culture medium was changed every third day. Bovine vascular smooth muscle cells (BVSMCs) were isolated as described previously.25 BVSMCs were identified by positive staining with a monoclonal antibody for α-actin (Sigma-Aldrich). Bovine aortic fibroblast cells (BAFCs) were isolated as described previously.26 Fibroblasts were cultured in RPMI 1640 culture medium with 10% inactivated calf serum and placed in 5% CO2 at 37°C for generational culture at 100% relative humidity. Fibroblasts were identified by positive staining with a monoclonal antibody to vimentin (Sigma-Aldrich). The cells were cultured in DMEM supplemented with 10% FCS and antibiotics.

Treatment of Cells with K5 and an Assay of Cell Proliferation

Recombinant human K5 was expressed, purified, and analyzed, as described previously.21 BAECs were seeded in 96-well plates at a density of 5 × 103 cells/well in 200 μL DMEM with 10% FBS. After 24 hours, the medium was replaced with 200 μL DMEM and 1% FBS for 24 hours, and K5 was added to the wells to different concentrations (0, 20, 40, 80, 160, 320, and 640 nM) in triplicate. After 30 minutes of incubation, various concentrations of VEGF and/or bFGF (Sigma-Aldrich) were added to each well. An MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenylenetrazolium bromide; AMRESCO Inc., Solon, OH) assay was performed 72 hours after the treatment, as described elsewhere.27 BVSMCs and BAFCs were assayed as described for BAECs.

Flow Cytometric Cell-Cycle Analysis

BAECs were grown in 35-mm dishes until confluent, under the conditions just described. K5 was added to the medium to 80 nM. Incubation was continued for 72 hours at 37°C. Cells were removed using 0.05% trypsin in PBS buffer, pelleted (300g for 5 minutes), washed twice in 1% BSA in PBS, resuspended in 1% BSA in PBS, and fixed in 70% cold ethanol. A flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NJ) was used to acquire all data.

Transmission Electron Microscopy

For transmission electron microscopy, BAECs cultured with or without K5 (80 nM) in 24-well tissue culture plates were fixed for 1 hour with 2% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.4), postfixed with 2% osmium tetroxide, dehydrated with a graded ethanol series, and finally embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead nitrate and examined with an electron microscope (H600; Hitachi, Tokyo, Japan).

DNA Fragmentation Assay

In vitro, BAEC cellular DNA extraction and DNA fragmentation assays were performed as previously reported.28 After electrophoresis on 1.8% agarose, gels were examined under UV light and either photographed or digitally recorded using an analysis system (Biocom, Les Ulis, France).

For the in situ detection of apoptotic cells, a DNA fragmentation-detecting terminal deoxynucleotidyl transferase deoxyUTR-nick end labeling (TUNEL) assay was performed with a kit (Chemicon, Te-
mecula, CA). Corneas were harvested at PD28 after 20 mg/L K5 or PBS treatment for 14 days. A peroxidase apoptosis detection kit (Apop Tag In Situ) was used according to the recommendations of the manufacturer (Serologicals, Norcross, GA). Images were obtained with a microscope (Carl Zeiss Meditec).

**Statistical Analysis**

Analysis of the significance of differences between two groups was performed by using the paired or unpaired Student’s t-test (SPSS 10.0 statistical software; SPSS, Chicago, IL). Pearson’s analysis was used to
The effect of K5 on corneal neovascularization was evaluated by comparing the length of new vessels and the area between the K5-treatment group and the PBS control. (C) The area of corneal NV was measured and is expressed as the mean ± SD (n = 5). The K5-treated group showed significantly decreased NV area, compared with the PBS control (*P < 0.05 at PD21 and PD28). (D) The corneas were sectioned at PD28 and endothelial cells (ECs) in the corneal section were counted (mean ± SD, n = 5). The K5 treatment group showed significantly fewer ECs than did the control (*P < 0.05).

RESULTS

Alkali-Burn–Induced Corneal NV Model

After removal of the alkali-immersed disc from the eye, the injured central corneal stroma appeared opaque, with a distinct margin (Fig. 1A). Opaque tissue appeared in the central cornea with ingrowth of new vessels toward the central cornea by PD14 (Fig. 1D); vessels reached the center of the cornea by PD21. By PD28, the pannus was elevated above the corneal surface (Fig. 1E). Under light microscopy, a large number of vessels and inflammatory cells were observed in the cornea after the alkali burn (Fig. 1F). These results show that the alkali-burn–induced corneal NV is a simple and useful animal model for corneal inflammation and NV.

There were variations between individual animals in the progression of NV, despite the almost identical early appearance of the wounds. NV was also nonuniform around the circular injury in the cornea. Therefore, two measurements were devised to evaluate the severity of corneal NV at different time points after alkali burn. The length of the longest vessels in the cornea was measured by a slit lamp and the total area of corneal NV was calculated. The results showed that both the length of vessels and the NV area increased rapidly in the first 2 weeks after the burn and then slowed (Figs. 2A, 2B). The total NV area started to decline, whereas the length of the vessels grew slowly after the PD18 (Fig. 2B).

Inhibitory Effect of K5 Eye Drops on Corneal NV

The effect of K5 on corneal NV was evaluated by comparing the length of new vessels and the NV area between the K5-treated and control groups at different time points. The results showed that the onset and progression of corneal NV were delayed in the groups treated with 10 and 20 mg/L K5. The length of the longest corneal NV of these K5-treated groups was significantly shorter than in the control group (Fig. 2A). Similarly, the K5-treated groups also had smaller corneal NV areas than did the control (Fig. 2B). No significant differences were found between the control group and the group treated with 5 mg/L K5, suggesting a K5 dose-dependent inhibitory effect.

The effect was evaluated by slit lamp. Representative slit-lamp photographs of corneal NV of each treatment group showed the inhibitory effect of K5 on corneal NV at PD28 (Fig. 2C).

Effect of K5 on Regression of Corneal NV

In our model, corneal NV was formed at 2 weeks after the injury. At this point, K5 treatment resulted in an obvious regression of corneal NV (Fig. 3). Quantification of the NV area confirmed a significantly smaller in NV area in the K5-treated eye than in the PBS control (*P < 0.05 after K5 treatment for 7 and 14 days; Fig. 3). The average number of vascular endothelial cells was significantly fewer in K5-treated corneas than in the control after K5 treatment for 14 days (P < 0.05), suggesting that K5 induces regression of newly formed vessels in the cornea (Fig. 3).

Anti-inflammatory Effect of K5 Eye Drops

To determine the effect of K5 on corneal inflammation, the inflammatory index was studied at four different time points: 24 and 72 hours and 5 and 7 days after the K5 eye-drop treatment. As shown in Table 1, corneas treated with 5 mg/L K5 (group 1) showed a significant decrease in inflammatory index after 24 hours of the K5 treatment (Wilcoxon test, P < 0.001), but not at other time points. At concentrations of 10 and 20 mg/L (groups 2 and 3), K5 eye drops significantly decreased the inflammatory index in the cornea at all time points.
points of the study, compared with the vehicle control (group 4; Wilcoxon test, \( P < 0.001 \)), suggesting an anti-inflammatory effect of K5.

Histologic analysis of serial sections revealed that groups 2 and 3 had fewer inflammatory cell infiltrates in the cornea than did groups 1 and 4 (Fig. 4). Retrocorneal membrane (RCM), an inflammatory membrane, was observed in groups 1 and 4 but not in groups 2 and 3 (Fig. 4). This histology further confirmed the anti-inflammatory effect of K5 in this model.

**Downregulation of VEGF Expression in the Cornea**

To determine whether K5’s effect on corneal NV is through downregulation of VEGF, a major mediator of corneal NV and inflammation, corneal VEGF levels were measured by immunohistochemistry and Western blot analysis. Immunohistochemistry with an anti-VEGF antibody demonstrated intensive VEGF signal in the corneas after the alkali burn (Figs. 5A, 5B). The stromal and intravascular inflammatory cells of group 4 stained strongly positive, as did some capillary vascular endothelial cells, basal corneal epithelial cells, and certain cells of the capillary wall (e.g., pericytes and extravasated monocytes). In contrast, rabbits treated with high doses of K5 displayed weaker VEGF signals in the same areas under the same exposure intensity as those in group 4 (Fig. 5).

The effect of K5 on VEGF expression was semiquantified by Western blot analysis using the anti-VEGF antibody (Fig. 5E). Consistent with the result of immunohistochemistry, treatment of 20 mg/L K5 eye drops resulted in a fivefold decrease in corneal VEGF levels (\( P < 0.01 \); Fig. 5E) when compared with the control corneas treated with PBS, suggesting that K5 down-regulates VEGF expression in the cornea with alkali burn.

**Specific Inhibition of the Growth of BAECs by K5**

At first, we performed cell viability assays in the presence of VEGF or bFGF. VEGF stimulated proliferation of BAECs in a dose-dependent manner (1–25 ng/mL), with a maximum response at 10 ng/mL VEGF (data not shown). At a concentration of 2 ng/mL, VEGF induced a 50% increase in BAEC proliferation. K5 was added to the culture medium of BAECs, BVSMCs, and BAFCs, which were preincubated with 2 ng/mL VEGF for 24 hours. K5 decreased viable cell numbers in BAECs in a dose- and time-dependent manner (Fig. 6), with an apparent ED\(_{50}\) of 80 nM (Fig. 6). Under the same conditions, K5 did not inhibit the proliferation of either BVSMCs or BAFCs, suggesting that the inhibitory effect of

<table>
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<tr>
<th>Group</th>
<th>Inflammatory Index</th>
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<tr>
<td></td>
<td>24 h</td>
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<tr>
<td>Group 1 (5 mg/L K5)</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>Group 2 (10 mg/L K5)</td>
<td>0.64 ± 0.07</td>
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<tr>
<td>Group 3 (20 mg/L K5)</td>
<td>0.61 ± 0.06</td>
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<tr>
<td>Group 4 (vehicle control)</td>
<td>0.83 ± 0.05</td>
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*Data are the mean ± SD; \( n = 10 \) for each group.

**Figure 4.** Corneal histopathology at PD28 in the K5-treated eye and the control. The rabbits were treated with K5 and vehicle for 28 days. The corneas were sectioned and stained with hematoxylin-eosin. (A) Group 1 treated with 5 mg/L K5, showing some inflammatory cell infiltration; (B) group 2 treated with 10 mg/L K5, and (C) group 3 treated with 20 mg/L K5, showing corneas devoid of vessels and with only minor inflammatory cell infiltration and NV. (D) Group 4 (vehicle control) showing increased inflammatory cells and NV in the stroma. A significant retrocorneal membrane (arrows) was observed under the corneal endothelial cell layer in both groups 1 (A) and 4 (D). Magnification, ×400.
K5 is endothelial cell specific (Fig. 6). The inhibition of BAECs was also dependent on the incubation time with K5 (Fig. 6). Similarly, K5 inhibited BAECs growth induced by bFGF (data not shown).

Flow Cytometry

The effect of K5 on the cell cycle was examined by flow cytometry. BAECs were exposed to DMEM containing 10% FCS alone in the presence or absence of 80 nM K5. After 72 hours, the cells treated with K5 showed a decreased percentage of cells in the S phase (Fig. 7A). The ratio of G2+S/G1 cells was decreased in the K5-treated cells (Fig. 7B), suggesting a decreased proliferation rate of BAECs.

Ultrastructural Characterization of Apoptosis in BAECs Treated with K5

As examined by electron microscopy, the control BAECs cultured in the absence of K5 had a typical endothelial cell morphology with elongated shape and with tight junctions (j) between adjacent endothelial cells (Fig. 8A). The nuclei were ellipsoidal and large, and the cytoplasm contained a few vacuoles (v) and numerous mitochondria (Fig. 8).
Treatment of BAECs with K5 (80 nM) induced marked alterations in endothelial cell morphology. K5-treated cells were mainly characterized by spherical shapes (Fig. 8B) and by some nuclear and cytoplasmic abnormalities. Representative electron micrographs of the K5-treated BAECs are presented in Figures 8B–D. The shapes of the nuclei were frequently disturbed and condensed, a typical feature of apoptotic cells. Condensed chromatin (cc) was observed either inside the nucleus (Fig. 8B) or at the periphery of the nucleus (Fig. 8C). In the K5-treated cells, vacuoles with different morphologies were also observed, some of which may be heterophagic vacuoles, because they frequently contained cell debris that was often located at their peripheries (Fig. 8B). In addition, apoptotic bodies were detected in the K5-treated cells (Fig. 8D), further suggesting that K5 induces apoptosis in endothelial cells.

K5-Induced Apoptosis in Endothelial Cells

Chromosomal DNA was extracted from the control and K5-treated BAECs and analyzed by electrophoresis in 1.8% agarose gels. The cells treated with 80 nM K5 showed typical DNA fragmentation, with 250- to 300-bp multiples that are characteristic of apoptotic cells (Fig. 9A). In contrast, DNA fragmentation was not observed in the control BAECs or in BAECs treated with 20 or 40 nM K5.

For the in situ detection of apoptotic cells, a TUNEL assay was performed on corneas treated with 20 mg/L K5 (Fig. 9C) or PBS (Fig. 9B) for 14 days. The result demonstrated that K5 increased the apoptotic endothelial cells in corneal NV, compared with the PBS control.

DISCUSSION

Corneal NV is a major cause of vision loss in humans. Currently, argon laser photocoagulation and photodynamic therapy (PDT) with different photosensitizers are used to treat corneal NV. Although these therapies generally improve vision, they are not ideal treatments because of vessel recanalization, thermal damage to adjacent tissues, and possible worsening of the lesion. More effective prevention or treatment strategies are therefore desirable. The present study provides the first evidence that natural angiogenic inhibitors have therapeutic potential in the treatment of chemical-burn-induced corneal NV.

Investigators have long sought a satisfactory in vivo model of corneal NV. Studies in the 1980s established standard models of corneal injury using alkali-immersed filter discs. The mechanism by which NV occurs in this model is similar to that of inflammatory corneal disease. Thus, the alkali-induced corneal disease appears to be a suitable animal model for the study of similar corneal conditions in humans. Previous studies have shown corneal inflammation after chemical burn, and our results in rabbits support the findings. Our experiments were performed on 40 2.0- to 2.5-kg New Zealand albino rabbits from the same animal breeder. The experiment revealed the entire inflammation process. This model of alkali-burn-induced corneal NV has many advantages, including convenience and reappearance of the corneal NV. Therefore, it is a useful animal model for inflammatory corneal NV.

From the animal experiments, we found that the topical application of K5 eye drops can inhibit the development of both the alkali-burn-induced corneal NV and inflammation. Corneal NV has a close relationship to inflammation. The development of new blood vessels extending into the area of the burn is associated with increased inflammatory cells within the burn area. Inflammation has the ability to induce tissue...
hypoxia that subsequently causes a series of responses, including increased angiogenic stimulators, decreased angiogenic inhibitors and vascular endothelial cell proliferation, which eventually lead to corneal NV. In addition, K5 induced regression of the newly formed vessels in the cornea caused by alkali burn, which is a more interesting clinical end point. This result is consistent with previous observations that angiostatin inhibits and causes regression of alkali-induced corneal NV.

It is evident that there is a delicate balance between angiogenic stimulators and inhibitors and that this balance plays a key role in maintaining the homeostasis of angiogenesis. Under certain hypoxic conditions in the cornea, as found in inflammation and injury, angiogenic stimulators are overproduced, whereas angiogenic inhibitors are decreased. The disruption in the balance between these factors results in NV. VEGF is a major angiogenic stimulator in the cornea, and increased VEGF levels have been shown to be a common pathologic factor in neovascular ocular diseases of humans, as well as in the animal model of alkali-induced corneal disease. In addition to VEGF, other angiogenic stimulators can also contribute to corneal NV. In an effort to block the final pathway of angiogenesis, we should to use endogenous angiogenic inhibitors, such as K5, to counterbalance the overproduction of VEGF in burned corneas. In our study, K5 inhibited not only the expression of VEGF in the cornea, but also VEGF-induced endothelial cell proliferation. Therefore, the K5-induced downregulation of VEGF restores the balance in angiogenesis control and thus, represents a mechanism underlying the inhibitory effect of K5 on corneal NV. The mechanism of the antiangiogenic effect of K5 is elusive. However, previous studies have shown that downregulation of VEGF may be responsible, at least partially, for the antiangiogenic effect of K5. This downregulation may be through inhibition of the activation of HIF-1 and mitogen-activated protein (MAP) kinase, which are known as regulators of VEGF expression. This effect may be mediated by a K5 receptor, although the signaling pathway from the K5 receptor to VEGF regulation has not been revealed.

Several endogenous angiogenic inhibitors (e.g., angiostatin, pigment epithelium-derived factor [PEDF], and endostatin) have been identified in recent years and are widely believed to have therapeutic potential for neovascular diseases. K5 is a potent angiogenic inhibitor and is believed to have therapeutic potential in the treatment of solid tumors. Our results confirm that topical application of K5 eye drops inhibits and reduces corneal NV without detectable immunoresponses or toxicity in the animal model. The data therefore suggest that K5 is promising as a potential therapeutic drug for corneal NV. This study represents the first documented approach to treat injury-induced corneal NV with an endogenous angiogenic inhibitor.

Angiogenesis is a multistep process consisting of endothelial cell proliferation, migration, basement membrane degradation, and formation of lumina. To determine the molecular mechanism of antiangiogenic activity of K5, we applied K5 to primary BAECs, BVSMCs, and BAFCs. In these studies, the inhibitory effect of K5 was specific for endothelial cells. K5 inhibited BAEC proliferation in a dose- and time-dependent manner. In further studies, flow cytometric cell cycle analysis of BAECs showed that K5 decreased the percentage of cells in the S phase and the G2+S/G1 ratio, suggesting an inhibition of BAEC proliferation. Further, both the transmission electron microscopy and DNA fragmentation assays demonstrated that K5 also induced apoptosis in BAECs, providing structural evidence supporting previous observations. By an in situ assay, we also detected more apoptotic endothelial cells in corneal NV treated with K5 than in those treated with PBS. Cell cycle arrest and apoptosis may be closely linked events. Indeed, several agents have been found to affect both events. Glucocorticoids induce G1 arrest and apoptosis in transformed lymphoid cells, and pRb causes G1 arrest but suppresses apoptosis in cultured cells.
The intracellular events that mediate these effects of K5 remain uncertain. However, our immunohistochemical and Western blot studies showed that K5 inhibits VEGF expression in the cornea. As VEGF is essential for endothelial cell proliferation and survival, the K5-induced downregulation of VEGF expression may be responsible, at least in part, for the inhibited proliferation and increased apoptosis in BAECs. It remains to be investigated, however, whether the effect on VEGF expression is mediated by the K5 receptor.37

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References


