·Basic Research·

Inhibitory effects of regorafenib, a multiple tyrosine kinase inhibitor, on corneal neovascularization

Halil Ibrahim Onder[1], Mesut Erdurmus[2], Yasin Yücel Bucak[3], Hüseyin Simavli[3], Murat Oktay[4], Ahmet Sahap Kukner[2]

[1]Department of Ophthalmology, Duzce University Medical Faculty, Duzce 81160, Turkey
[2]Department of Ophthalmology, Abant Izzet Baysal University Medical School, Bolu 14280, Turkey
[3]Ophthalmology Clinic, Izzet Baysal State Hospital, Bolu 14000, Turkey
[4]Department of Pathology, Duzce University Medical Faculty, Duzce 81160, Turkey
Correspondence to: Mesut Erdurmus. Department of Ophthalmology, Abant Izzet Baysal University Medical School, Golkoy, Bolu 14280, Turkey. merdurmus@yahoo.com
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Abstract

• **AIM: To evaluate the inhibitory effects of regorafenib (BAY 73-4506), a multikinase inhibitor, on corneal neovascularization (NV).**

• **METHODS: Thirty adult male Sprague-Dawley rats weighing 250-300 g, were used. Corneal NV was induced by NaOH in the left eyes of each rat. Following the establishment of alkali burn, the animals were randomized into five groups according to topical treatment. Group 1 (n= 6) received 0.9% NaCl, Group 2 (n = 6) received dimethyl sulfoxide, Group 3 (n = 6) received regorafenib 1 mg/mL, Group 4 (n=6) received bevacizumab 5 mg/mL and Group 5 (n= 6) received 0.1% dexamethasone phosphate. On the 7d, the corneal surface covered with neovascular vessels was measured on photographs as the percentage of the cornea's total area using computer-imaging analysis. The corneas obtained from rats were semiquantitatively evaluated for caspase-3 and vascular endothelial growth factor by immunostaining.**

• **RESULTS: A statistically significant difference in the percent area of corneal NV was found among the groups (P <0.001). Although the Group 5 had the smallest percent area of corneal NV, there was no difference among Groups 3, 4 and 5 (P >0.005). There was a statistically significant difference among the groups in apoptotic cell density (P=0.002). The staining intensity of vascular endothelial growth factor in the epithelial and endothelial layers of cornea was significantly different among the groups (P<0.05). The staining intensity of epithelial and endothelial vascular endothelial growth factor was significantly weaker in Groups 3, 4 and 5 than in Groups 1 and 2.**

• **CONCLUSION: Topical administration of regorafenib 1 mg/mL is partly effective for preventing alkali-induced corneal NV in rats.**

• **KEYWORDS:** corneal neovascularization; regorafenib; tyrosine kinase inhibitor
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INTRODUCTION

Angiogenesis, the formation of functional microvascular networks, is normal during growth and development and wound healing [1]. The process includes protrusion and outgrowth of capillary buds from pre-existing blood vessels. Neovascularization (NV) is a pathologic form of angiogenesis characterized by the proliferation of blood vessels in abnormal locations. Corneal NV is usually associated with inflammatory, infectious, traumatic, toxic, degenerative or immunologic disorders of the ocular surface and cornea [1,2]. It may cause edema, scar formation, or lipid deposition, leading to significant visual impairment and blindness [1]. Therefore, treating this potentially blinding condition is of clinical significance[1-4].

The regulation of neovascular processes and microenvironment influences on NV are not fully understood. Regulation of NV is generally believed to involve a complex balance between angiogenic and antiangiogenic stimulants and factors [2-4]. Among various numbers of proangiogenic factors that stimulate endothelial cells to multiply and form new blood vessels, the vascular endothelial growth factor (VEGF) family plays a major role. VEGF molecules bind to three cell membrane receptors [5]. Increases in VEGF receptors, VEGF messenger ribonucleic acid (mRNA), and protein levels have been demonstrated in experimental animal models[6-8]. VEGFs and their receptors also increase in corneal buttons with corneal NV in humans [9]. Neutralizing antibodies and small-molecule tyrosine kinase inhibitors have been developed to inhibit VEGF activity. Both neutralizing antibodies and tyrosine kinase inhibitors inhibit the angiogenic effect of the VEGF system by affecting the different parts of VEGF-activated angiogenesis.

Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2 (TIE2) is a crucial regulator of angiogenesis that is exclusively or predominantly expressed in endothelial cells. As such, it is indispensable for maturing immature vessels via interactions with the ligands angiopoietin (Ang)1, Ang2, VEGF, and fibroblast growth factor [10]. Regorafenib is a multikinase inhibitor that targets kinases, including VEGF receptors 1, 2, and 3; TIE2; platelet-derived growth factor β; mutant oncogenic kinases; and the fibroblast growth factor receptor, which is involved in NV and oncogenesis [11]. The Food and Drug Administration approved regorafenib in September 2012 to treat patients with metastatic colorectal cancer.

The chemotherapeutic potential of regorafenib for the inhibition of corneal NV is undetermined. Therefore, we established a rat corneal alkali burn model of NV to investigate the effectiveness of topical regorafenib for the inhibition of corneal NV. We also compared the efficacy of regorafenib to that of topical bevacizumab and dexamethasone.

MATERIALS AND METHODS

Materials The animals used in this study were treated under The Association for Research in Vision and Ophthalmology (ARVO) Resolution for the Use of Animals in Ophthalmic and Vision Research. The institutional ethics committee approved the study. Thirty adult male Sprague-Dawley rats weighing 250-300 g were used. The rats were housed under conditions of controlled temperature(23±2)℃ and illumination (12 h light-dark cycle) and food and water were provided *ad libitum*.

Drugs The drugs used were regorafenib (BAY 73-4506; Bayer Schering Pharma AG, Berlin, Germany), bevacizumab (Altuzan; Roche, Istanbul, Turkey), dimethyl sulfoxide (DMSO; Sigma- Aldrich, St. Louis, MO, USA), a commercially available form of preservative-free dexamethasone sodium phosphate 0.1% (Dexa-Sine SE; Alcon Couvreur, Puurs, Belgium) and 0.9% NaCl solution.

Methods

Experimental procedures Before all procedures, the rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (25 mg/kg body weight) and with xylazine hydrochloride (5 mg/kg body weight). All eyes were examined under a binocular microscope to exclude corneal scaring, opacity, and NV before the study. Topical 0.5% proparacaine hydrochloride eye drops were applied to the left eye of each rat and allowed to dry. Corneal NV was induced by application of sodium hydroxide (NaOH). Pieces of Whatman #3 filter paper (3 mm diameter) were dipped in 2 mol/L NaOH for 20s and then placed in the center of each rat's left eye for 60s. Then the burn area and conjunctival sac were then irrigated with 50 mL saline for 1min. To increase the reproducibility of the injuries, a single investigator (M.E.) cauterized all animals. For each eye, the burn stimulus response was scored as 0 (no blister, not raised above corneal surface), +1 (small blister, slightly raised above the surface), +2 (medium blister, moderately raised above the surface), and +3 (large blister). Only the corneas with a burn stimulus score of +1 or higher were included in the calculation of the mean burn stimulus and NV scores in each group[12].

Following the establishment of alkali burn, the animals were randomized into five groups according to topical treatment. Group 1 (n=6) received 0.9% NaCl, Group 2 (n=6) received DMSO, Group 3 (n=6) received 1 mg/mL regorafenib, Group 4 (n=6) received 5 mg/mL bevacizumab, and Group 5 (n=6) received preservative-free dexamethasone phosphate 0.1% solution. DMSO is the solvent of regorafenib; therefore we included a group using only DMSO to clarify the efficacy. All groups received the topical drops twice daily at equal intervals for 7d. On 7d, the control eyes were fully vascularized; thus, the endpoint of the study was achieved because the NV in control eyes has a tendency to gradually regress after 7d [13]. The rats were reanesthetized using the above-described method. Photographs of the left cornea of each rat were taken with an SL-D7 slit-lamp with an attached DC-3 camera (Topcon, Tokyo, Japan). The apex of the cornea was placed in the same direction as the slit lamp to take standardized photos and ensure that the whole cornea was visible in the photograph.

Evaluation of corneal NV and photographic analysis The objective of the semiautomatic processing of the photographs taken during the experiments was to calculate the percentage of the whole cornea containing newly formed vessels. In the first step of processing, commercially available software (Adobe Photoshop 7.0; Adobe Systems, Mountain View, CA, USA) was used to cut the corneas out of the photographs. The next step involved manual determination of the cornea's border regions with and without vessel formation. For task, a graphically powerful and high-resolution computer monitor was used. A semiautomatic program was developed in MATLAB 7 (MathWorks, Natick, MA, USA) to calculate the area with NV. The algorithm included selection of a threshold level by which it was possible to convert the color image into a black-and-white image and calculation of the number of pixels in the region with no vessel formation. The quantification of the NV was performed in a blinded fashion. The total corneal area was outlined, with the innermost vessel of the limbal arcade used as the border. The total area of NV was normalized to the total corneal area, and the percentage of the cornea covered by new vessels was calculated.

Tissue preparation and immunostaining Rats were sacrificed and their globes were excised and fixed in 10% buffered formalin for 24h. The tissues were dehydrated in graded ethanol and set in paraffin. Immunohistochemically, the primary antibodies were anti-VEGF antibody (Abcam, Cambridge, UK), anti-active Caspase-3 antibody (Thermo Scientific, Fremont, CA, USA) and anti-CD31 antibody (Labvision, 1:100). The paraffin-embedded tissues were cut

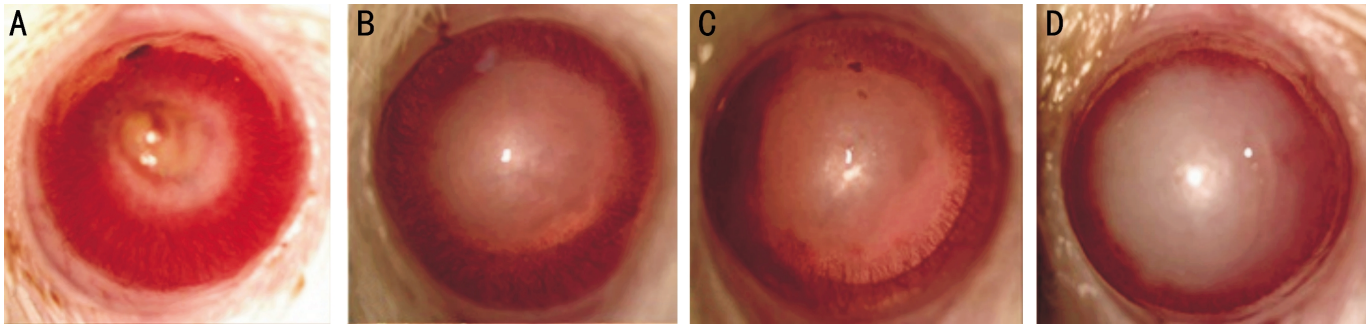

Figure 1 Digitally enhanced slit-lamp photographs of the rat corneas 7d after alkali burn showing the new vessels invading the cornea A: Isotonic saline, B: Bevacizumab 5 mg/mL, C: Regorafenib 1 mg/mL, and D: Dexamethasone phosphate 0.1% group. The degree of reduction in neovascular growth was significantly less in dexamethasone group compared to bevacizumab and regorafenib groups. The percent area of corneal NV also less pronounced in bevacizumab and regorafenib groups than the control group.

into slices 5 μm thick, mounted on polylysine-coated slides, deparaffinized with xylene, and rehydrated through graded concentrations of ethanol. For antigen retrieval, the sections were immersed in 10 mmol/L sodium citrate buffer for 5min (pH 6.0) and heated for 20min in a microwave oven. Endogenous peroxidases were quenched in 0.3% (vol/vol) hydrogen peroxide for 5min. After washing in phosphate-buffered saline, the slides were incubated with blocking reagent for 10min. Sections were incubated for 30min at 37℃ with primary antibodies. The sections were further incubated with anti-polyvalent biotinylated antibody (ScyTek Laboratories, Logan, UT, USA). The immune complexes were detected using an aminoEthyl carbazole chromogen (ScyTek Laboratories) according to the manufacturer's instructions[14].

VEGF immunostaining was evaluated in the epithelial, stromal, and endothelial layers of the corneas. Staining intensity was determined semiquantitatively with a previously described technique as none (0), weak (1), moderate (2), and intense (3)[15]. The numbers of new corneal vessels were determined on slides immunostained with anti-CD31 antibodies, as described previously, by an examiner with no knowledge of the experimental procedures [14]. Most sections were obtained from the central region of the cornea. The number of new corneal vessels was evaluated in at least two sections of each eye, from one limbus to another. Apoptosis was evaluated in the epithelium of the cornea on the anti-active caspase-3 antibody-stained slices. Five microphotos were captured from each slice of every sample at 200 magnification. Anti-active caspase-3-positive cells were counted in the photos. All evaluations were performed by an examiner with no knowledge of the experimental procedures. NIS Elements 3.1 software and an Eclipse 80i microscope (Nikon Instruments, Tokyo, Japan) were used.

Statistical Analysis Statistical calculations were performed using the SPSS 15 statistical package (SPSS, Chicago, IL, USA). A P value of <0.05 was considered statistically significant. All data are presented as medians (minimum-maximum). The Kruskal-Wallis test was used to compare continuous variables. The Bonferroni-adjusted Mann-Whitney U-test was used for multiple comparisons and P values of <0.005 were considered statistically significant. The Chi-square (Fisher's exact) test was used for categorical variables and the data are presented as the frequency and percent (%).

RESULTS

The burn stimulus intensity was similar between the groups, and median stimulus scores were 2 or higher (P>0.05). The mean percent areas of corneal NV were 68.5 (64.1-75.2) in Group 1, 58.0 (54.7-68.9) in Group 2, 39.9 (36.4-48.8) in Group 3, 42.1 (36.7-48.7) in Group 4, and 21.9 (14.0-39.6) in Group 5 (Figure 1). The differences in the percent areas of corneal NV among the groups were statistically significant (P<0.001). Although Group 5 had the smallest percent area of corneal NV, there were no differences among the Groups 3, 4, and 5 (P>0.005). Group 1 had the highest mean area and statistically significant differences were found between Group 1 and other groups excluding Group 2.

The mean number of new corneal vessels was 20.0 (16.3-21.7) in Group 1, 16.0 (13.7-18.7) in Group 2, 10.0 (7.0-11.7) in Group 3, 9.8 (7.7-15.7) in Group 4 and 8.5 (7.3-10.0) in Group 5. The differences in the number of new corneal vessels among the groups were statistically significant (P<0.001). There were no differences among Groups 3, 4, and 5 in the number of new corneal vessels. Groups 1 and 2 had the highest numbers of new corneal vessels.

The mean number of active caspase-3-positive cells was 3.7 (3.0-4.0) in Group 1, 3.3 (1.7-3.7) in Group 2, 1.3 (0.0-3.0) in Group 3, 2.3 (2.0-4.0) in Group 4, and 1.3 (0.0-2.3) in Group 5. The differences among the groups were statistically significant (P=0.002). Statistically significant differences were observed between Groups 1 and 3 (P=0.002) and Groups 1 and 5 (P=0.002) in the number of active caspase-3-positive cells. Table 1 shows the percent areas of corneal NV, the number of new corneal vessels, and the number of active caspase-3-positive cells and significance levels among the groups. Figure 2 shows the corneal sections in each group in terms of the distribution of active caspase-3-positive cells.

The VEGF staining intensity of the corneal epithelial layer

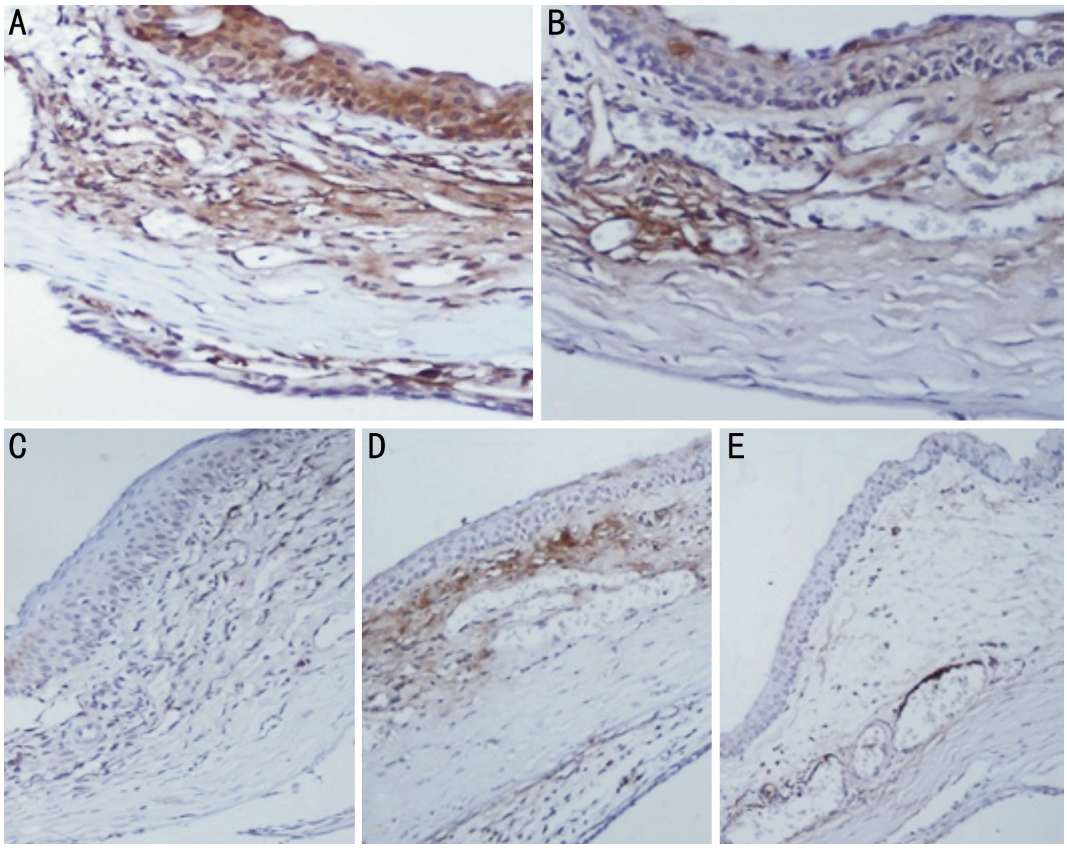

Figure 2 Representative sections of corneas after treated with A: Isotonic saline; B: Dimethyl sulfoxide; C: Regorafenib 1 mg/mL; D: Bevacizumab 5 mg/mL, and E: Dexamethasone phosphate 0.1% groups in terms of active caspase-3 positivity. The higher density of active caspase-3 positive cells in isotonic saline and dimethyl sulfoxide groups compared to other treatment groups. Regorafenib and dexamethasone groups had the lowest density of apoptotic cells (40×).

Table 1 The percent areas of corneal NV, the number of new corneal vessels and active caspase-3-positive cells in the groups

Parameters	Group 1 NaCl (*n*=6)	Group 2 DMSO (*n*=6)	Group 3 regorafenib (*n*=6)	Group 4 bevacizumab (*n*=6)	Group 5 dexamethasone (*n*=6)
The percent areas of corneal NV	68.5 (64.1-75.2)	58.0 (54.7-68.9)	39.9 (36.4-48.8)	42.1 (36.7-48.7)	21.9 (14.0-39.6)
The number of new corneal vessels	20.0 (16.3-21.7)	16.0 (13.7-18.7)	10.0 (7.0-11.7)	9.8 (7.7-15.7)	8.5 (7.3-10.0)
The number of active caspase-3-positive cells	3.7 (3.0-4.0)	3.3 (1.7-3.7)	1.3 (0.0-3.0)	2.3(2.0-4.0)	1.3 (0.0-2.3)

Values are expressed as median (minimum-maximum). DMSO, dimethyl sulfoxide.

was 5 (83.3%) in Group 1, 4 (66.7%) in Group 2, 0 (0%) in Groups 3 and 4, and 1 (16.7%) in Group 5. The stromal VEGF staining intensity was 5 (83.3%), 2 (33.3%), 2 (33.3%), 4 (66.7%), and 3 (50.0%), respectively. The endothelial VEGF staining intensity was 5 (83.3%), 4 (66.7%), 0 (0.0%), 2 (33.3%), and 1 (16.7%), respectively. The VEGF staining intensity of the corneal epithelial and endothelial layers was significantly different among the groups (P<0.05). Figure 3 shows the representative corneal sections of the groups in terms of VEGF staining properties of the epithelial, stromal and endothelial layers. There were no significant differences among the groups in terms of side effects of the topical medications based on macroscopic and histopathologic measurements.

DISCUSSION

Inhibition of vision-threatening corneal NV is a major challenge following corneal chemical insult or inflammation, and in many clinical situations corneal anti-angiogenic treatment would help. Topical corticosteroids remain the first choice for treating corneal NV secondary to inflammatory etiologies [16,17]. However, steroids are not ideal for treating corneal NV because of increased replication of pathogens and delayed wound healing [18,19]. Besides, steroid treatment may cause glaucoma and cataract formation. These side effects limit the application of steroids in corneal NV. Various compounds such as nonsteroidal anti-inflammatory drugs, heparin, caffeic acid phenethyl ester, thymoquinone, cyclosporin A, methotrexate, and therapies such as argon laser treatment and photodynamic therapy have been used in experimental and clinical studies for the treatment and prevention of corneal NV [12,20-25]. None of these treatment options used in corneal NV are ideal because of side effects such as thermal injury to adjacent areas, ocular hypertension, cataract formation, and vessel recanalization[22]. For all of the above reasons, more effective, easier and safer corneal NV treatment is required.

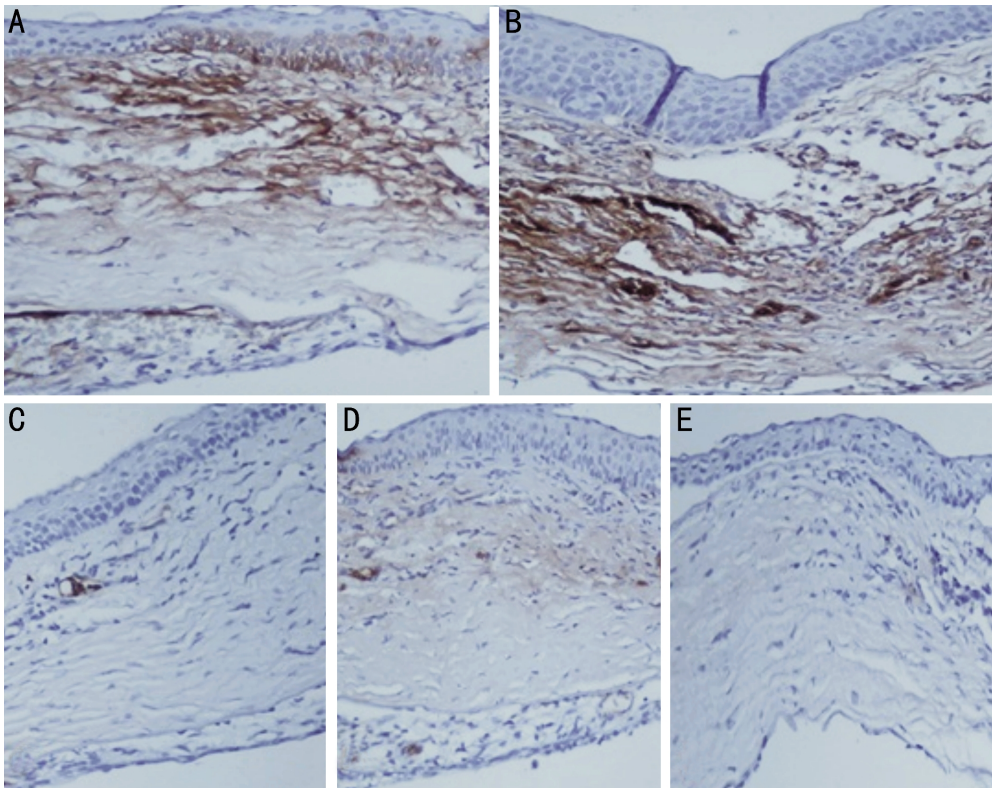

Figure 3 Vascular endothelial growth factor immunostaining in A: Isotonic saline; B: Dimethyl sulfoxide; C: Regorafenib 1 mg/mL; D: Bevacizumab 5mg/mL; and E: Dexamethasone phosphate 0.1% groups. The lower density of vascular endothelial growth factor positive cells in regorafenib, bevacizumab and dexamethasone groups compared to dimethyl sulfoxide and control group (40×).

VEGF acts as the key mediator during inflammation and NV; it regulates the growth of the vascular endothelium and controls the formation of new blood vessels [26]. Binding to its receptors, VEGF triggers a signaling cascade that promotes endothelial cell growth, survival, migration, differentiation, and mobilization of endothelial progenitor cells from the bone marrow into the peripheral circulation [27]. VEGF inhibition for the treatment of NV is a molecular target-based treatment. Molecular target-based treatment may offer fewer side effects and more effective treatment for corneal NV. One way to inhibit VEGF activity is by using humanized monoclonal antibodies such as bevacizumab, which has been widely used in clinical and experimental studies of ocular pathologies[28-30].

Another way to inhibit VEGF is by tyrosine kinase inhibition. Selective inhibition of VEGFR-2 tyrosine kinase with SU5416 has an inhibitory effect on corneal NV [31]. Regorafenib is a novel, potent inhibitor of the VEGF receptor and other angiogenic receptor tyrosine kinases [11,32]. In the present study, we found that regorafenib, a multiple tyrosine kinase inhibitor, has inhibitory effects on alkali-induced corneal NV in rats. The inhibitory effects of topical regorafenib were comparable to those of topical dexamethasone and bevacizumab.

Monoclonal antibodies induce their effect by blocking the effect of VEGF prior to its attachment to endothelial receptors. Tyrosine kinase inhibitors block the VEGF activity by inhibiting tyrosine kinase in the intracellular part of the VEGF cell membrane receptor. This may represent a different opportunity for treatment of the neovascular process in ocular pathologies. Use of tyrosine kinase inhibitors with monoclonal antibodies may also provide a more powerful effect in corneal NV. However, further studies are needed to evaluate the effect of different tyrosine kinase inhibitors on corneal NV.

The mutant plasminogen kringle 5 (mK5) induces apoptosis in alkali-induced corneal NV in rats, and also found significantly inhibits corneal NV [33]. Local administration of the adenosine diphosphate ribosylation factor 1 inhibitor after alkali injury enhances intraocular caspase-3 expression [34]. In the present study, regorafenib significantly inhibited the apoptotic cell count compared to the control group. These conflicting results may be due to differences in measurement methods between studies. In our study, there were no differences in the numbers of active caspase-3-positive cells among the bevacizumab-, regorafenib-, and dexamethasone-treated groups.

To the best of our knowledge, this is the first study to test the inhibitory effects of regorafenib on corneal NV. The main limitations of the study are the lack of expression patterns of regorafenib-related receptors and growth factors as well as the absence of an experimental group evaluating the combined use of tyrosine kinase inhibitors with monoclonal antibodies, which may also provide a more powerful effect.

In conclusion, apoptotic cell count, VEGF immunostaining and corneal NV are reduced by topical administration of regorafenib compared to control. Topical administration of 0.1% regorafenib may have some benefit for prevention of

corneal NV. Further studies are needed to determine the optimal dosage of regorafenib for preventing corneal NV and to evaluate the effects of regorafenib on expression of VEGF mRNA.

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