·Basic Research·

Comparison of subconjunctivally injected bevacizumab, ranibizumab, and pegaptanib for inhibition of corneal neovascularization in a rat model

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Abstract

• AIM: To compare the efficacies of subconjunctival bevacizumab, ranibizumab, and pegaptanib sodium injections for the inhibition of corneal neovascularization in an experimental rat model.

• METHODS: Sixteen corneas of 16 rats were chemically cauterized and randomized into four groups: bevacizumab group that treated with 0.05mL/1.25mg bevacizumab, ranibizumab group that treated with 0.05mL/0.5mg ranibizumab, pegaptanib group that treated with 0.05mL/0.15mg pegaptanib sodium, and control group that treated with 0.05mL saline solution. Digital photographs of the corneas were taken and analyzed using an image analysis software program. All corneas were excised and examined histologically on the 15th day.

• RESULTS: Each treatment group had significantly less neovascularized corneal areas and fewer blood vessels than the control group (all P <0.05). In addition, bevacizumab group had significantly less neovascularized corneal areas and fewer blood vessels than ranibizumab and pegaptanib groups (both P <0.05). However, there was no significant difference between the ranibizumab and pegaptanib groups regarding percentage of neovascularized corneal areas and number of blood vessels (both P >0.05).

• CONCLUSION: Subconjunctival bevacizumab, ranibizumab, and pegaptanib sodium were effective with no corneal epitheliopathy for inhibiting corneal neovascularization after corneal burn in rats . Bevacizumab was more effective than ranibizumab and pegaptanib sodium.

• **KEYWORDS:** corneal neovascularization; bevacizumab; ranibizumab; pegaptanib; subconjunctival injection
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INTRODUCTION

Corneal neovascularization is one of the major causes of visual impairment worldwide [1,2]. It may be seen secondary to infectious, inflammatory, traumatic and metabolic diseases of the cornea [2,3]. Although the pathogenesis has not been fully understood, vascular endothelial growth factor (VEGF) has been demonstrated to have a main role in corneal neovascularization[4,5]. It has been shown that VEGF stimulates angiogenesis in both non-inflammatory and inflammatory models of corneal neovascularization in the mouse and rat corneas[4,6].

Recently, there has been growing interest in the use of anti-VEGF agents for the treatment of corneal neovascularization. Anti-VEGF agents, pegaptanib sodium (Macugen; OSI/Eyetech, Inc., USA) and ranibizumab (Lucentis; Genentech, Inc., USA) have been approved by Food and Drug Administration (FDA) for the treatment of choroidal neovascularization secondary to age-related macular degeneration (AMD). Another anti-VEGF agent, bevacizumab (Avastin; Genentech, Inc., CA), has been used off-label for neovascular AMD and other exudative and ischemic retinal diseases. Among these agents, efficacy of bevacizumab for the treatment of corneal neovascularization has been revealed in many studies [7-11]. However, there are very few studies investigating the efficacy of ranibizumab and pegabtanib sodium for the inhibition of corneal neovascularization[12,13].

Therefore, in this study we aimed to compare the efficacies of subconjunctival bevacizumab, ranibizumab, and pegaptanib sodium injections for the inhibition of corneal neovascularization in an experimental rat model.

MATERIALS AND METHODS

Animal model of corneal vascularization Sixteen corneas of 16 male Sprague-Dawley rats weighing 300-450g have been used in this study. Approval of the experimental protocol was obtained from the Başkent University Medical School Research Committee. The animals were treated and maintained in accordance with the tenets of the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research. Corneal neovascularization was induced with silver nitrate cauterization [14] under general anesthesia (by intraperitoneally administered 50mg/kg body weight ketamine hydrochloride and xylazine combination) which supplemented with topical anesthesia (0.5% proparacaine hydrochloride). Central area of each cornea was cauterized by the same investigator (EE) *via* pressing an applicator stick (diameter 1.8mm, coated with 75% silver nitrate and 25% potassium nitrate) for 10 seconds under the operating microscope. Excess silver nitrate was removed by washing the eyes with 5mL of balanced salt solution.

The extent of burn stimulus response was scored by the same investigator for each cornea as 0 (no blister, not raised above the corneal surface), +1 (small blister, raised slightly above the surface), +2 (medium blister, raised moderately above the surface), and +3 (large blister)[15].

The rats were then randomized into four groups (each group included 4 corneas): bevacizumab group that treated with a single subconjunctival injection of 0.05mL/1.25mg bevacizumab, ranibizumab group that treated with a single subconjunctival injection of 0.05mL/0.5mg ranibizumab, pegaptanib group that treated with a single subconjunctival injection of 0.05mL/0.15mg pegaptanib sodium, and control group that treated with a single injection of subconjunctival 0.05mL salin. Each subconjunctival injection was performed one day after the cauterization, by using a 30-gauge needle, 1mm away from the limbus.

Analysis of corneal neovascularization All rats were anesthetized as mentioned above and each cornea was evaluated by slit-lamp biomicroscopic examination on the 15th day by the same investigator who was blinded to the groups. Corneal photographs were taken using a digital camera attached to the slit-lamp microscope (×25 magnification). The neovascularization area was measured in terms of pixels, and its ratio to the entire corneal area was determined as the percentage by using an image analysis software program (Pixcavator Image Analyzer; Intelligent Perception, Huntington, W. Va., USA).

Analysis of neovascularization by histological examination After the analysis of corneal neovascularization, the animals were euthanized and enucleations were performed. Then, the globes were penetrated with a 27-gauge needle 1.0mm from the limbus at the 3 and 9 o'clock meridians to allow the fixative to pass into the eyes rapidly. 10% formaldehyde was used to prepare the eyes for histological examinations. After fixation for 24 hours, they were removed from the fixative and corneas were dehydrated and sectioned. Tissue sections of 8-μm thickness were created and stained with hematoxylin and eosin for light microscopy. The number of visible blood vessels was counted for each viewed area by using a light microscope with ×200 magnification. To reduce the inaccuracy, the average number of blood vessels from three different views was recorded for each specimen. Finally, the average number of blood vessels was calculated for each group.

Table 1 Percentage of neovascularization area and the number of blood vessels in different treatment groups compared with the control group $\bar{x} \pm s$

Groups	Number of vessels (n)	Vascularized corneal area (%)
Bevacizumab	1.75±0.50[a]	16.19±1.72[a]
Ranibizumab	15.50±9.67[c, e]	28.81±7.29[c, e]
Pegabptanib	13.50±6.87[c, e]	25.82±7.18[c, e]
Control	19.50±5.80	40.38±12.74

[a]P<0.05, bevacizumab group *vs* each of the other groups; [c]P>0.05, ranibizumab group *vs* pegabtanib group; [e]P<0.05, ranibizumab and pegaptanib groups *vs* the control group.

Statistical Analysis All data are expressed as means±SD. Kruskal-Wallis test was used for comparisons among the groups. After finding differences among the groups, Mann-Whitney U test was used for pair wise comparisons. Statistical significance was set at P<0.05.

RESULTS

All corneas had burn stimulus scores of ⩾+2 and the mean burn stimulus scores were not statistically different among the groups (P >0.05). The mean percentages of neovascularized corneal areas and the average numbers of blood vessels are given in Table 1. The groups were statistically different concerning mean percentages of neovascularized corneal areas (P>0.05). Dual comparisons of the groups showed that each treatment group had significantly less neovascularized corneal areas than the control group (all P<0.05). In addition, bevacizumab group had significantly less neovascularized corneal areas than ranibizumab and pegaptanib groups(both P<0.05). However, there was no significant difference between the ranibizumab and pegaptanib groups (P>0.05). Figure 1 displays the demonstrative images of corneas on the 15th day of the experiment in each group. The treatments caused no corneal epitheliopathy.

Histological examination displayed that the groups were statistically different regarding the average number of blood vessels (P<0.05, Table 1). Each treatment group had significantly fewer blood vessels than the control group (all P<0.05). Furthermore, bevacizumab group had significantly

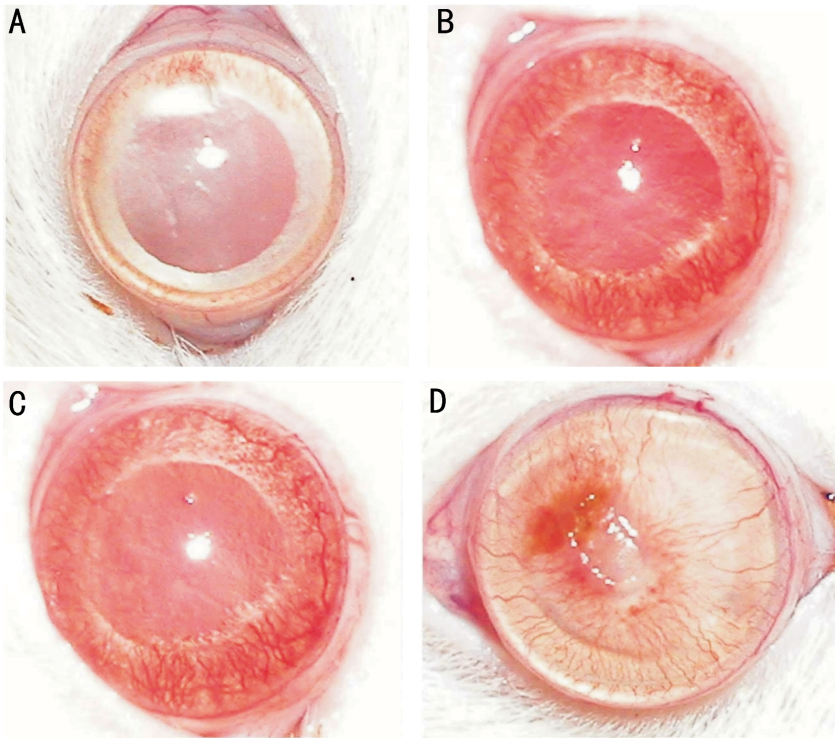

Figure 1 Microscopic examination (×25 magnifications) of cornea on the 15th day of treatment Bevacizumab group (A) showed more significant reduction of neovascularization area than ranibizumab (B) and pegaptanib (C) groups. All treatment groups (A, B, C) had less neovascularized corneal areas compared with the control group (D). Ranibizumab (B) and pegaptanib (C) groups had similar neovascularized corneal areas.

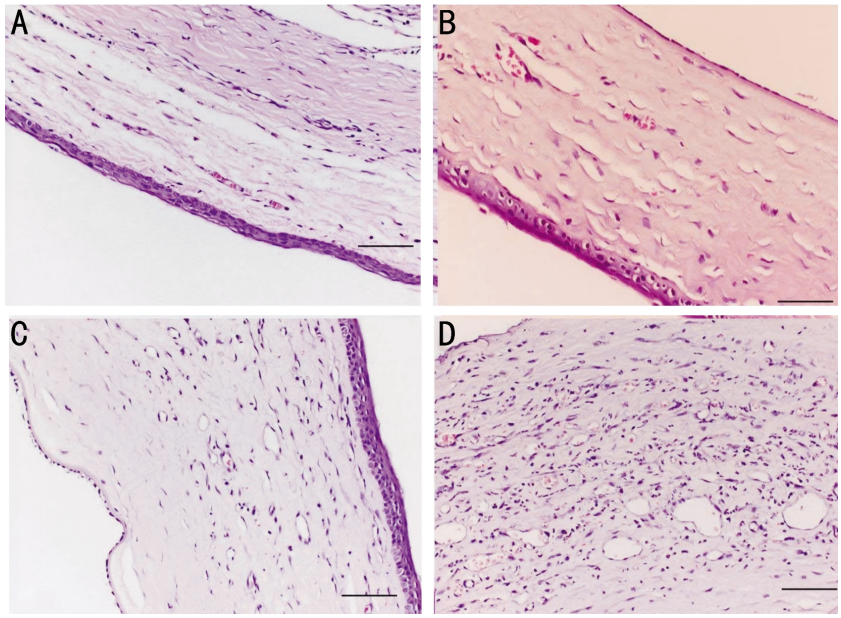

Figure 2 Histopathological sections (×200 magnification) of corneal specimens stained with hematoxylin and eosin There were fewer blood vessels in the bevacizumab group (A), than ranibizumab (B) and pegaptanib (C) groups. All treatment groups (A, B ,C) had fewer blood vessels compared with the control group (D). Ranibizumab (B) and pegaptanib (C) groups had similar numbers of blood vessels (scale bar: 100μm).

fewer blood vessels than the other treatment groups (both P <0.05). On the other hand, there was no significant difference between the ranibizumab and pegaptanib groups (P >0.05). Figure 2 shows the demonstrative histological images of the corneas for each group.

DISCUSSION

This study demonstrated that subconjunctival injection of bevacizumab, ranibizumab, and pegabtanib sodium significantly inhibited corneal neovascularization in experimental rat model. In addition, bevacizumab was more effective than ranibizumab and pegaptanib sodium. On the other hand, there was no significant difference between ranibizumab and pegaptanib sodium. Quantitative evaluation of the corneal neovascularization was consistent with the

clinical results. Epitheliopathy was not detected on the cornea in any treatment group during the experiment.

Photodynamic treatment with verteporfin, laser photocoagulation, and some medical therapies including steroids, cyclosporin A, and thymoquinone have been used for the treatment of corneal neovascularization[16,17]. However, an established therapeutic method is not currently available.

VEGF is one of the major factors involved in the pathogenesis of corneal neovascularization. VEGF-A, VEGF-B, VEGF-C, and VEGF-D are the members of the human VEGF family [18]. VEGF-A is believed to be the most important member of this family, especially relating to pathologic hemangiogenesis. As a result of variations in mRNA splicing there are four different forms with various numbers of amino acids: VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206} [19]. VEGF-A_{165} is the dominant proangiogenic isoform. Both bevacizumab and ranibizumab use the same mechanisms and inhibit all of the VEGF-A isoforms nonspecifically [20]. Nevertheless, differently from bevacizumab and ranibizumab, pegaptanib does not inhibit all of the VEGF isoforms; it specifically binds to VEGF-A_{165}.

Although there are many studies showing the efficacy of bevacizumab for inhibiting corneal neovascularization [7-11], to our best knowledge, there is only two studies [12,13] investigating the efficacies of ranibizumab and pegaptanib sodium in comparison with bevacizumab. Sener *et al*[12] assessed the efficacies of subconjunctival injections of 1.25mg/0.05mL bevacizumab, 0.5mg/0.05mL ranibizumab, and 0.3mg/0.1mL pegaptanib sodium for inhibiting corneal neovascularization in an experimental rat model. Dursun *et al*[13] evaluated the effects of subconjunctival 2.5mg bevacizumab and 1mg ranibizumab injections. In accordance with our study, in these two previous studies all the treatment groups had less corneal neovascularization than the control groups and bevacizumab was more effective than other agents.

We have found bevacizumab to be more effective than pegaptanib sodium for inhibiting of corneal neovascularization. As mentioned above, differently from bevacizumab and ranibizumab, pegaptanib sodium inhibits only VEGF-A_{165}. This specifity might cause the less efficacy of pegaptanib sodium and we can speculate that some VEGF-A isoforms other than VEGF-A_{165} might play role in corneal neovascularization. On the other hand, although both bevacizumab and ranibizumab similarly inhibit all of the VEGF-A isoforms, we have demonstrated that bevacizumab was interestingly more effective than ranibizumab for preventing corneal neovascularization. We considered two reasons for this interesting finding. Firstly, this might be caused by the pharmacokinetics of subconjunctival ranibizumab application. The half-life of bevacizumab (1.25mg) after intravitreal injection is 4.9 days whereas the half-life of ranibizumab (0.5mg) ranges between 2.8-3.2 days [12]. Therefore, ranibizumab might be rapidly eliminated after subconjunctival injection and did not maintain for enough time and at enough concentration. Secondly, the dose or concentration of ranibizumab might be insufficient. Due to subconjunctival dose and concentration of ranibizumab for inhibition of corneal neovascularization have not been determined yet, we have used the intravitreal dose and concentration (0.5mg/0.05mL).

In our study, none of three anti-VEGF agents that we used could completely prevent corneal neovascularization. In this regard, several reasons may be considered. Firstly, absorption from conjunctiva, the dose and concentration, and treatment frequency of agents might be insufficient; more repetitive subconjunctival injections with higher doses or concentrations might be necessary. Secondly, the time of starting the treatment (one day after the chemical cauterization in our study) might affect the efficacy; beginning the treatment just after the chemical cauterization might cause less corneal neovascularization. Thirdly, some other members of VEGF family other than VEGF-A (such as VEGF-B, VEGF-C, and VEGF-D) and several angiogenic factors other than VEGF (*i.e.* bFGF, PDGF, TGF-α, and TGF-β) might induce corneal neovascularization. Finally, the affinity of anti-VEGF agents for VEGF in rats may be lower than in human [21]. In this regard, although anti VEGF-A agents have been found to be effective for treatment of choroidal neovascularization in humans [22], it has been revealed that these agents were not effective for treating choroidal neovascularization in a rat model[23].

The limitation of the present study was the lack of a steroid treatment group. The inhibitor effect of steroids on corneal neovascularization has been shown previously [16,24]. Further studies could compare the effect of anti-VEGF agents with steroids for the treatment of corneal neovascularization.

In conclusion, we have revealed that subconjunctival bevacizumab, ranibizumab, and pegaptanib sodium were effective with no epitheliopathy in controlling corneal neovascularization after corneal burn in rats. In addition, we found that bevacizumab was more effective than ranibizumab and pegaptanib sodium. Ranibizumab and pegaptanib sodium had similar efficacy. To improve the effectiveness of treatments, combination therapy with other antiangiogenic agents, and/or repeated subconjunctival injections with higher doses and concentrations of a longer duration may be valid options. Further studies on the doses and concentrations, administration routes, durations, and frequencies are warranted.

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