·Basic Research·

Topically administered bevacizumab had longer standing anti-angiogenic effect than subconjunctivally injected bevacizumab in rat corneal neovacularization

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Abstract

• **AIM: To compare the effect of topically administered and subconjunctivally injected bevacizumab on experimental corneal neovascularization in rats for two weeks after treatment.**

• **METHODS: Twenty-eight Sprague-Dawley rats were divided into four groups of 7 animals. Each corneal center of right eye was cauterized with silver/potassium nitrate for 8s. After corneal burning, bevacizumab (12.5mg/mL) was topically administered three times per day (TB group) for two weeks or subconjunctivally injected on days 2 and 4 after cauterization (0.02mL; SB group). As negative controls, rats received 0.9% saline topically three times per day (TS group) or subconjunctivally on days 2 and 4 (0.02mL; SS group). Digital photographs of the cornea were taken 1 and 2 weeks after treatment and analyzed to determine the area of cornea covered by neovascularization as the percentage of corneal neovascularization.**

• **RESULTS: One week after treatment, the percentage of corneal neovascularization was significantly lower in the TB and SB groups than in the TS and SS groups (all $P<0.05$). Two weeks after treatment, the percentage of corneal neovascularization was significantly lower in the TB group than in the TS group ($P<0.05$). In all groups, the percentage of neovascularization was decreasing as time passed (all $P<0.05$)**

• **CONCLUSION: Topically administered bevacizumab has longer standing anti-angiogenic effect than subconjunctivally injected bevacizumab in corneal neovascularization following chemical injury in rats.**

• **KEYWORDS:** corneal neovascularization; bevacizumab; topical and subconjunctival administration

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INTRODUCTION

The cornea maintains an avascular state through the balanced operation of angiogenic and antiangiogenic factors [1-3]. However, inflammatory, infectious, degenerative, and traumatic diseases that afflict the organ can cause corneal neovascularization with significant effects on eyesight [4]. If activated, vascular endothelial growth factor (VEGF), an angiogenic factor in the cornea, triggers corneal neovascularization, whereas the control of VEGF expression restrains formation of new vascular tracts [5,6]. Bevacizumab (Avastin®; Roche, Basel, Switzerland) is a full-length humanized antibody directed against VEGF and, in combination with anticancer drugs, is used for the treatment of metastatic colon cancer [7]. Intravitreal bevacizumab injection is used as a treatment for choroidal neovascularization, macular eodema, and other diseases that are accompanied by age-related macular degeneration [8-12]. There has been growing interest in the treatment of corneal neovascularization using bevacizumab and many studies have shown that topical administration or subconjunctival injection of the drug is effective for control of anterior segment neovascularization [13-22]. Three of them simultaneously compared the anti-angiogenic effect of topically administered and subconjunctivally injected bevacizumab for equal or less than 7d after treatment [20-22]. Their results showed that there was no significant difference between two groups. On the other hand, Dastjerdi *et al*[23] showed single topically administered bevacizumab (1%) with denuded epithelia and subconjucnvally injected bevacizumab (0.5mg) still remained in the corneal stroma up to 14d. To our knowledge, there was a paucity of study that compared the anti-angiogenic effect of topically administered and subconjunctivally injected bevacizumab for more than 7d after treatment. The present study compares the effects of subconjunctivally injected and topical bevacizumab in

inhibiting neovascularization after experimental corneal ablation in rats until two weeks after treatment.

MATERIALS AND METHODS

Chemical Cauterization Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-eight 8-week-old Sprague-Dawley (SD) rats, without corneal lesions, and weighing 225-275g, were used. Rats were anesthetized with 10mg/kg zolazepam (Zoletil; Yuhan, Corp. Seoul, Korea) and 10 mg/kg xylazine hydrochloride (Rompun; Bayer, Inc., Frankfurt, Germany), injected into the abdominal cavity, and 0.5% (w/v) proparacaine hydrochloride, topically applied to the right eyes. After drying with gauze, the corneal centers of right eyes were cauterized with silver/potassium nitrate [75% (w/v) silver nitrate/25% (w/v) potassium nitrate; Arzol Chemical, Keen, NH] for 8s, creating 3mm-diameter light gray chemical burns. Each cornea was washed with 10mL balanced salt solution (BSS®, Alcon Laboratories, Inc., Fort Worth, TX, USA) immediately after the burn. To create corneal chemical burns that were uniform in size, a single experimenter cauterized all rats for equal periods of time.

On day 2 after corneal injury, the extent of burn stimulus response was scored on a scale of 0 to +3 in accordance with the experimental model devised by Mahoney and Waterbury to test blister responses [24]. Rats with scores higher than +2 were included in experiments and animals that developed keratitis, endophthalmitis, or corneal perforation, were excluded.

Grouping The 28 rats included in experiments were randomly divided into four groups of 7 animals. From day 2 after cauterization, bevacizumab (12.5mg/mL) was administered topically three times a day (TB group). On days 2 and 4, 0.02mL bevacizumab (12.5mg/mL) was subconjunctivally injected using a 30G insulin syringe (Ultra-Fine™; Becton Dickinson, Franklin Lakes, NJ; SB group). In the negative control groups, saline was administered topically three times a day to animals commencing on day 2 after corneal burning (TS group), or 0.02mL saline was injected subconjunctivally using 30G insulin syringe (Ultra-Fine™; Becton Dickinson) on days 2 and 4 (SS group).

Measurement of Corneal Neovascularization On weeks 1 and 2 after treatment, corneal neovascularization was assessed by photography at 32X magnification (Coolpix 4500™; four megapixels; Nikon Imaging Japan, Inc., Tokyo, Japan) attached to a slit-lamp microscope. Photographs were examined using an image analyzing program (Image J 1.40g written by Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). First, all corneas and neovascularization therein were outlined with reference to the corneal limbus. To determine the percentage of corneal neovascularization, the number of pixels showing neovascularization was expressed as a percentage of the entire corneal pixel number[25].

Statistical Analysis Using the SPSS program (version 16.0; SPSS, Inc., Chicago, IL, USA), the one-way ANOVA was performed for statistical analysis and followed by the Tukey honestly significant difference test between means. The paired Student *t*-test was performed to compare the percentage of corneal neovascularization at one week and two weeks after cauterization. The *P* value below 0.05 was considered statistically significant.

RESULTS

Twenty-eight rats showed scores of +2 or higher in the experimental blister response model. Immediately after cauterization, burn stimulus scores did not statistically differ among the four groups (P=0.74; ANOVA). No animal developed corneal perforation.

At one week after cauterization, the percentage of corneal neovascularization was 34.7±5.9%, 52.5±11.3%, 35.0±7.4%, and 51.7±12.2% in TB group, TS group, SB group, and SS group, respectively. The percentage of corneal neovascularization was significantly lower in the TB and SB groups than in the TS and SS groups (all P<0.05; ANOVA). At two weeks, the percentage of corneal neovascularization was 16.5±5.6%, 30.8±7.3%, 26.4±6.1%, and 25.9±8.7% in TB group, TS group, SB group, and SS group, respectively. The percentage of corneal neovascularization was significantly lower in the TB group than in the TS group (P<0.05; ANOVA). Thus, in each group, the percentage of neovascularization was decreasing as time passed (all P< 0.05; paired Student *t*-test; Figures 1, 2).

DISCUSSION

Many reports have appeared on the effects of bevacizumab inhibiting corneal neovascularization in rats, rabbits, and humans [10,14,16,18,21,26-29]. In most experimental studies of corneal neovascularization, topically adminisered and subconjunctivally injected bevacizumab was applied to prevent early-stage neovascularization. In the present work, bevacizumab was subconjunctivally administered on only days 2 and 4 after corneal burning. Clinically, it is difficult to inject bevacizumab repeatedly because injection-related complications including pain and subconjunctival hemorrhage may develop. In spite of only one week follow-up, the anti-angiogenic effect of topically administered and subconjunctivally injected bevacizumab did not differ at one week after treatment in three previous comparative studies [20-22]. Topical bevacizumab cannot penetrate the unwounded cornea with intact epithelium as it has a molecular weight of 149 kDa which is too large to penetrate the intact corneal epithelium. Dastjerdi *et al*[23] showed single topically administered bevacizumab (1%) with denuded epithelia and subconjunctivally injected

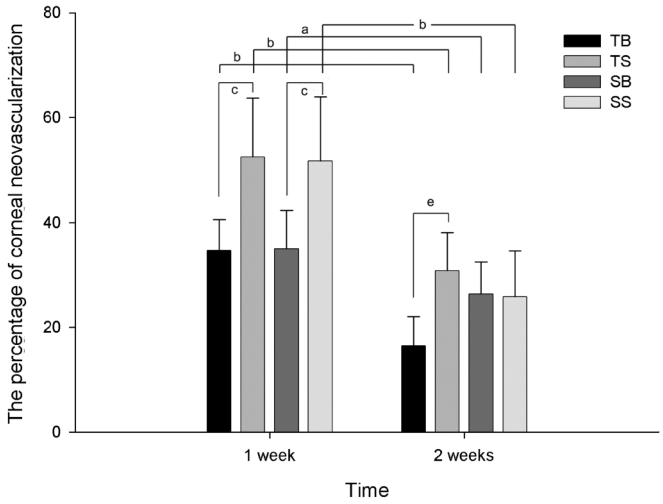

Figure 1 The percentage of corneal neovascularization after chemical burn The percentage of corneal neovascularization after chemical burn. At one week after cauterization, the percentage of corneal neovascularization was significantly lower in the topical bevacizumab (TB) and subconjucntival bevacizumab (SB) groups than in the topical saline (TS) and subconjunctival saline (SS) groups. [a] $P<0.05$, [b] $P<0.001$:Significant differences between 1 week and 2 weeks after cauterization (paired Student t-test); [c] $P<0.05$, [d] $P<0.001$: Significant differences between the two groups at one week after cauterization (ANOVA). [e] $P<0.05$, [f] $P<0.001$: Significant differences between the two groups at two weeks after cauterization (ANOVA, $P<0.05$).

bevacizumab (0.5mg) still remained in the corneal stroma up to 14d. If the corneal neovascularization and inflammation reduces the integrity of corneal epithelial tight junctions, the bevaciumab can penetrate the tight junction and reach the stroma. Nomoto *et al* [30] demonstrated that after subconjunctival injection of bevacizumab (1.25mg/0.05mL), the half-life of bevacizumab was 1.80 and 2.85 weeks in the iris-ciliary body and retina/choroid, respectively. For these reasons, we initially postulated that the anti-angiogenic effect of topically administered and subconjucntivally injected bevacizumab should not differ. Conversely, in present study it was revealed that the anti-angiogenic effect of topically administered bevacizumab was higher than that of subconjunctivally injected bevacizumab at two weeks after treatment. In the subconjunctivally injected bevacizumab group, the half-life of bevacizumab after subconjunctival injection might not be sufficiently long to demonstrate a continuous effect. In the topically administered bevacizumab group, the regression of corneal neovascularization might be due to the long-standing topical use of bevacuzumab. Kim *et al* [26] demonstrated that topically applied bevacizumab (12.5mg/mL) twice daily decreased corneal neovascularization in 10 eyes of 7 patients within one month. Bock *et al* [31] showed topical bevacizumab (5mg/mL) five times a day for averagely 3.6 months reduced 48% in mean vascular area in 5 eyes of 5 patients with corneal neovascularizaition.

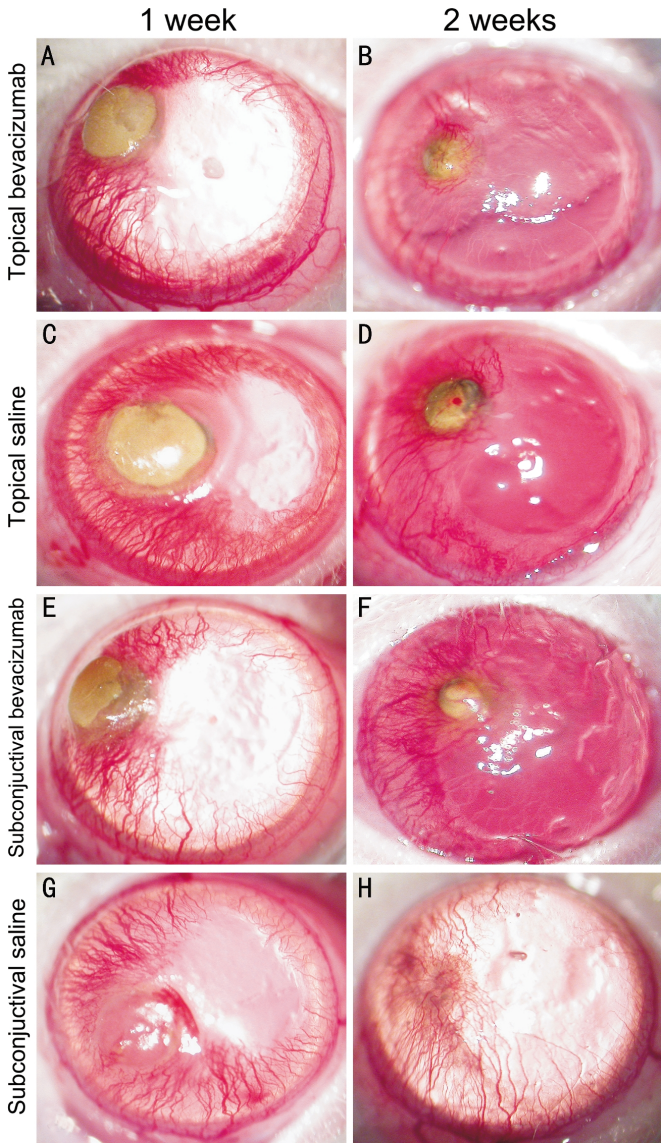

Figure 2 Slit lamp microscopic photographs Slit lamp microscopic photographs to monitor the corneal neovascularization in topical bevacizumab (TB), topical saline (TS), subconjucntival bevacizumab (SB), and subconjunctival saline (SS) groups at one week (A, C, E, and G) and two weeks (B, D, F, and H) after corneal cauterization.

A limitation of our study is that first, we have not compared the effects of different doses of bevacizumab on neovascularization; such work is in progress. Second, a humanized anti-VEGF antibody (bevacizumab) that poorly bound to murine VEGF-A was used in present study. It has been showed that these antibodies bind rat VEGF-A with less specificity and dissociate more easily than human VEGF-A [31]. Third, although corneal neovascularization can be clearly observed in the rat chemical cauterization model, the corneas should have been stained immunohistochemically with anti-CD31 antibody by using whole mounts to get more precise results [32]. Forth, the molecular mechanism of corneal neovascularization such as VEGF expression should be compared among four groups.

In conclusion, we showed that topically administered

bevacizumab was longer-standing than subconjunctivally injected drug in inhibiting corneal neovascularization. Subconjunctival injection of bevacizumab may be feasible in reducing neovascularization for short duration or by repeated manners.

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