Effects of Topical and Subconjunctival Bevacizumab in High-Risk Corneal Transplant Survival

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PURPOSE. To investigate whether corneal graft survival could be improved by topical or subconjunctival bevacizumab in a murine model of vascularized high-risk corneal transplantation.

METHODS. Before corneal transplantation, intrastralum sutures were placed for 2 weeks in the corneas of BALB/c mice, inducing intense angiogenesis. Allogeneic corneal transplantation was performed using C57BL/6 donor mice. Topical bevacizumab (2.5%) was delivered 3 times a day for 3 weeks in one treatment group, and 0.02 ml (0.5 mg) bevacizumab was injected subconjunctivally at days 0, 4, 8, and 15 after transplantation in the other treatment group. The control group received no treatment. Grafts were examined twice a week for 8 weeks by slit-lamp microscopy and were photographed once a week by slit-lamp digital camera and scored for opacity. For assessment of corneal neovascularization (NV), a quantitative method was used to measure three primary metrics including neovascular area, vessel caliber, and neovessel invasion area.

RESULTS. Both topical and subconjunctival bevacizumab treatment reduced neovascular area and vessel caliber; however, the regression of corneal NV was more profound when treated subconjunctivally. The mean percentage reduction of neovascular area was 55% (P < 0.05) by week 8 in the subconjunctival treatment group and 33% (P = 0.15) in the topical group. Only subconjunctival bevacizumab treatment resulted in significant regression of neovessel invasion area (P < 0.05). All corneal transplants in both the control and the topical groups were rejected by 4 weeks after transplantation. However, in the subconjunctival treatment group, 33% of corneal grafts survived (P < 0.01).

CONCLUSIONS. Subconjunctival bevacizumab may offer an adjunctive measure to conventional therapies in preventing graft rejection in high-risk corneal transplantation. (Invest Ophthal-mol Vis Sci. 2010;51:2411–2417) DOI:10.1167/iovs.09-3745

Alograft rejection is a leading cause of corneal graft failure and thus a leading indication for repeat penetrating keratoplasty.1 Indeed, repeat grafting as a result of previous failure has become the second leading indication for corneal transplantation, as reported.2 It has been known for many decades that the presence of preexisting blood vessels is a strong risk factor for corneal graft immune rejection.3–5 Grafting into vascularized corneal beds, or so-called high-risk corneal transplantations, leads to a rate of immune rejection of greater than 50%, even with a strict regimen of topical and systemic immunosuppressive drugs.6 In fact, stratification of risk factors for immunologic rejection in penetrating keratoplasty has identified recipient vascularization as a critical proximal cause for earlier and more fulminant rejection episodes.4,5,7,8

Targeting angiogenesis to modulate immune responses after corneal transplantation has been the core area of interest for many investigators.5–12 Why the relative immunologic quiescence of the eye, which is a central facet of its immunoprivileged state, is disturbed in patients with corneal neovascularization (NV) is not fully understood.13 However, experimental evidence strongly suggests that molecular factors such as the local immunosuppressive cytokine milieu (transforming growth factor-β, α-melanocyte-stimulating hormone) and functional attributes (anterior chamber-associated immune deviation), which play a critical role in maintaining the physiologic quiescence in anterior segment, are subverted in the presence of corneal NV.14 In addition to blood vessels in vascularized high-risk corneas, lymphatic neovessels can ingrow in parallel with hemangiogenesis, facilitating effective access of donor and host antigen-presenting cells and antigenic material to regional lymph nodes, where accelerated sensitization to graft antigens occurs.15,16 Thus, treatment of corneal NV after corneal transplantation can potentially limit both the afferent (sensitization) and efferent (rejection) arms of allograft immunity and, hence, reduce the propensity for immunoinflammatory reactions that can jeopardize graft survival.14

Vascular endothelial growth factor (VEGF) is thought to be a key mechanistic mediator of NV.17 The prominent role of VEGF in the pathophysiology of corneal NV has been demonstrated in experimental models of corneal angiogenesis.18 It has been shown that VEGF is upregulated in inflamed and vascularized corneas in humans and in animal models.19 VEGF inhibitors, including pegaptanib sodium, ranibizumab, and bevacizumab, are used for the treatment of neovascular age-related macular degeneration.20 Recently, there has been growing interest in using topical and subconjunctival anti-VEGF for the treatment of corneal NV.21–26 Our data in a prospective clinical study have demonstrated a significant reduction in the severity of corneal NV in response to topical bevacizumab
therapy in patients with stable corneal NV. In an animal model of high-risk corneal transplantation, it has also been shown that intraperitoneal (systemic) injection of a VEGF-neutralizing cytokine trap can improve corneal graft survival. These reports suggest that treatment with topical or locally injected anti-VEGF could offer an adjunctive measure to conventional therapies (e.g., corticosteroids) to curb the inciting factors of graft rejection in the setting of vascularized high-risk corneal transplantation. Therefore, we sought to evaluate whether corneal graft survival in vascularized high-risk corneal transplantation can be improved by initiating local (topical or subconjunctival) bevacizumab treatment in a murine model.

To make a thorough and comprehensive assessment of corneal NV, a quantitative method was implemented to measure three primary metrics—neovascular area, vessel caliber, and neovessel invasion area. Our results indicate that while both topical and subconjunctival bevacizumab therapy inhibit corneal NV after high-risk transplantation, only the subconjunctival route is significantly effective in improving graft survival.

**METHODS**

**Animals and Anesthesia**

Eight- to 12-week-old C57BL/6 and BALB/c male mice were obtained from Taconic Farms (Germantown, NY). Mice were housed in a specific pathogen-free environment at the Schepens Eye Research Institute animal facility. All animals were treated according to guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Public Health Policy on Humane Care and Use of Laboratory Animals (US Public Health Review), and all procedures were approved by the Institutional Animal Care and Use Committee. Anesthesia was administered intraperitoneally by ketamine/xylazine solution at a dose of 120 mg/kg body weight and 20 mg/kg body weight, respectively.

**High-Risk Graft Bed Preparation**

Aspects of this procedure were adapted and modified from Cursiefen et al. to enhance corneal NV. First a 1.5-mm trephine was used to mark the central corneas of BALB/c mice. A figure-of-eight suture knot was then placed with two intrastromal incursions approximately 120° apart, and each incursion extended apically from slightly above the limbus to the trephine demarcation (Fig. 1A). Three interrupted figure-of-eight suture knots were placed using 11-0 nylon sutures (Sharpoint; Vanguard, Houston, TX) for 14 days, after which graft beds exhibited extensive neovascularization. At this time, the sutures were removed, and penetrating corneal transplantation was performed using age-matched C57BL/6 donors.

**Corneal Transplantation**

This procedure has been detailed elsewhere. Briefly, the central cornea (2-mm diameter) was excised from a donor mouse using scissors (Vannas; Storz Instruments, San Dimas, CA) and was placed on ice in corneal preservation media (Optisol-GS; Bausch and Lomb, Rochester, NY). The graft bed was prepared by excising a 1.5-mm site in the central cornea of a recipient mouse. A donor button was then placed onto the recipient bed and was secured with eight interrupted 11-0 nylon sutures (Sharpoint, Reading, PA) (Fig. 1B). Graft survival was evaluated regularly twice a week using a slit-lamp biomicroscope over the course of 8 weeks. We used a standardized opacity-grading scheme (range, 0–5+) to identify rejection, defined as a score of ≥5+ for two consecutive examinations.

**Bevacizumab Treatment**

Topical bevacizumab 2.5% (25 mg/mL; Avastin; Genentech, South San Francisco, CA) was applied three times a day for 3 weeks in one treatment group (n = 10). For the subconjunctival group (n = 10), a volume of 0.02 mL bevacizumab 2.5% (0.5 mg) was injected at days 0, 4, 8, and 15 after transplantation. The control group (n = 10) received no treatment.

**Quantification of Corneal Neovascularization**

We developed a method to objectively quantify corneal NV for application of this study. This method consists of first capturing a series of digital slit-lamp images of corneas and then, using graphics editing software (Photoshop CS2; Adobe Systems Inc., Mountain View, CA) to digitally trace the blood vessels in a corneal image to
remove the background of nonvessel areas (Fig. 2A). This method yields a very clear picture with which to qualitatively evaluate corneal NV levels in vivo (Fig. 3A) and, importantly, allows each individual mouse to be followed at various time points throughout the study (Fig. 3B). In this regard, this method is different from other quantitative image analyses that have been designed for immunohistologic assessment of corneal lymphangiogenesis and hemangiogenesis. Three primary metrics for corneal NV were considered (Fig. 2B). The first, referred to as the neovascular area (NA), involves measuring the area of the corneal vessels themselves when projected onto the plane of a photograph. The second metric, referred to as vessel caliber (VC), involves determining an approximate mean diameter of the corneal vessels. The third metric, referred to as invasion area (IA), measures the fraction of corneal area in which vessels are present.

### Statistical Analysis

Error bars displayed in the figures were calculated from the SEM. Statistical analyses, including Student’s t-tests, were performed throughout the study, as indicated in the respective figure captions. Kaplan-Meier analysis constructed survival curves and respective log rank tests compared with rates of corneal graft survival. P < 0.05 was considered significant.

### RESULTS

**Comparison of Topical versus Subconjunctival Bevacizumab Treatment on Corneal NV**

We first compared the effects of topical versus subconjunctival bevacizumab treatment in suppressing corneal NV after high-risk corneal transplantation. We found that though both topical and subconjunctival delivery of bevacizumab inhibited corneal NV after high-risk transplantation, subconjunctival treatment was clearly more effective to this end (Fig. 3B). This was the case for early time points (e.g., week 1) and late time points (e.g., weeks 4 and 8) after transplantation (Fig. 3B).

**Neovascular Area**

At baseline, there was no statistically significant difference in the mean NA among the three study groups. Although NA
values in all three groups decreased, by the week 4 time point it became clear that the subconjunctival route of bevacizumab was considerably more effective than the topical route in reducing NA (Fig. 4A) because the subconjunctival values were significantly and markedly lower than control at weeks 4 ($P < 0.05$), 6 ($P < 0.05$), and 8 ($P < 0.05$). The mean percentage reduction of NA in the topical group was 15% at week 2, 39% at week 4, 13% at week 6, and 33% at week 8, whereas these values in the subconjunctival group were 33% at week 2, 51% at week 4, 50% at week 6, and 55% at week 8. In the control group, the mean percentage reductions of NA were 27%, 26%, 19%, and 13% in weeks 2, 4, 6, and 8, respectively.

**Vessel Caliber**

Estimation of VC using a computational technique showed that values for the three groups were not statistically different at baseline. These values dropped in all three groups to the same level by week 2 and remained relatively stable until week 6. However, control values then rose substantially by week 8, but subconjunctival treatment group remained statistically lower than in the control group ($P = 0.03$). Topical bevacizumab treatment group showed a very marginal statistical significant difference from the control group in reduction of VC at week 8 ($P = 0.05$) (Fig. 4B).
reduce IA. Student’s t-test was performed to evaluate statistical significance (*P < 0.05; **P < 0.01).

Invasion Area
As for the other two metrics, no statistically significant difference in neovessel IA was shown any at baseline between the different study groups. Control and topical groups showed increases rather than significant declines in their IA values (Fig. 4C). The mean percentage reduction values for IA were −8%, −1%, −8%, and −6% in the control group and −2%, 1%, −1%, and −8% in the topical group by weeks 2, 4, 6, and 8, respectively. Interestingly, subconjunctival treatment appeared to be the only effective method to reduce IA (Fig. 4C) because those values remained significantly lower than in the control group at weeks 4 (P < 0.01), 6 (P < 0.01), and 8 (P < 0.025). Mean percentage reductions for IA in the subconjunctival group were 2%, 10%, 9%, and 8% at weeks 2, 4, 6, and 8, respectively.

Increased Efficacy of Subconjunctival versus Topical Bevacizumab on High-Risk Corneal Allograft Survival
To test whether treatment with topical or subconjunctival bevacizumab could promote allograft survival in the high-risk setting, the opacity of transplanted corneal grafts was examined and scored regularly to 8 weeks after transplantation by slit-lamp examination. We found that mice treated topically with bevacizumab did not exhibit a reduced mean opacity score (Fig. 5) or a statistical difference from the mean score of the untreated controls (P > 0.05). In contrast, mice treated subconjunctivally with bevacizumab exhibited a reduced mean opacity score (Fig. 5) that was significantly lower than that of the untreated controls at weeks 2 (P < 0.01), 6 (P < 0.01), and 8 (P < 0.05). Furthermore, Kaplan-Meier survival curve analysis demonstrated that subconjunctival treatment improved survival rates to greater than 30% (P = 0.008), whereas 0% survival was observed among the untreated controls (Fig. 6). Topical bevacizumab treatment also demonstrated 0% survival
proangiogenic factors (including FGF, IL-1, TNF-α, IFN-γ) are also upregulated during wound healing and inflammation. Third, it is clear that other relevant proangiogenic factors (including FGF, IL-1, TNF-α, and IFN-γ) are also upregulated during wound healing and inflammation. Fourth, even in relation to VEGFR2-mediated signaling, it is notable that bevacizumab is a specific antibody for VEGF-A only; other ligands to VEGFR2 (e.g., VEGF-C) are not directly suppressed by bevacizumab.

Corneal NV is almost invariably associated with higher graft rejection rates, and blood vessel levels at the time of transplantation are significantly correlated with graft survival. Khoadadoust reported that endothelial rejection occurred in 3.5% of avascular cases, 13.3% of mildly vascular cases, 28% of moderately vascular cases, and 65% of heavily vascular cases. In the present study, significant and marked regression of all three metrics collectively, which occurred only with subconjunctival treatment, has demonstrated a clear association with improved graft survival rate. Although topical treatment with bevacizumab resulted in a limited decrease in the severity of corneal NV, it could not increase graft survival. These findings could further underscore the significant relationship between size and extent of corneal blood vessels and chance of graft rejection. Corneal blood vessels allow an influx of immune effector cells to the corneal matrix. Therefore, the extent and size of corneal NV, including neovessel area, the caliber of neovessels, and the corneal area invaded by neovessels, particularly the portion of NV that crosses into the graft, may well be crucial for the effector arm of immunity (facilitating delivery of alloreactive T cells), which is detrimental to corneal graft survival.

To conclude, subconjunctival administration of bevacizumab is more effective than topical administration in treating corneal NV and promoting corneal graft survival in a mouse model of high-risk transplantation. Subconjunctival bevacizumab could potentially offer an adjunctive measure to conventional therapies in preventing graft rejection in vascularized high-risk corneal transplantation. More research, however, is needed to define the optimal dosage and frequency of administration to achieve the best clinical outcomes. Moreover, given our previous clinical results with topical bevacizumab in decreasing corneal NV and our data in this study (using a humanized antibody in a mouse model of corneal NV), topical bevacizumab, which clearly is efficacious in suppressing corneal NV, may also (particularly at higher dosing regimens than the one used in this study) be efficacious in preventing graft rejection in high-risk corneal transplantation, warranting further study.

References


