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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, intrastromal 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 Ophthalmol Vis Sci. 2010;51:2411–2417) DOI:10.1167/iovs.09-3745

A lllograft rejection is a leading cause of corneal graft failure and thus a leading indication for repeat penetrating keratoplasty. Indeed, repeat grafting as a result of previous failure has become the second leading indication for corneal transplantation, as reported. It has been known for many decades that the presence of preexisting blood vessels is a strong risk factor for corneal graft immune rejection. Graffing 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. 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.

Targeting angiogenesis to modulate immune responses after corneal transplantation has been the core area of interest for many investigators. 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. 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 the anterior segment, are subverted in the presence of corneal NV. 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. Thus, treatment of corneal NV after corneal transplantation can potentially limit both the afferent (sensitization) and efferent (rejection) arms of alloimmunity and, hence, reduce the propensity for immunoinflammatory reactions that can jeopardize graft survival.

Vascular endothelial growth factor (VEGF) is thought to be a key mechanistic mediator of NV. The prominent role of VEGF in the pathophysiology of corneal NV has been demonstrated in experimental models of corneal angiogenesis. It has been shown that VEGF is upregulated in inflamed and vascularized corneas in humans and in animal models. VEGF inhibitors, including pegaptanib sodium, ranibizumab, and bevacizumab, are used for the treatment of neovascular age-related macular degeneration. Recently, there has been growing interest in using topical and subconjunctival anti-VEGF for the treatment of corneal NV. 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, each extending from slightly above the limbus to the circumference of the paracentral cornea, which was marked with a 1.5-mm trephine. Three of these suture knots were placed in BALB/c mice to stimulate robust corneal neovascularization (B1). After 14 days the sutures were removed (B2), and penetrating corneal transplantation was performed using age-matched C57BL/6 donors (B3).

**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 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.

**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

**Figure 1.** Preparation of robust high-risk graft beds for orthotopic corneal transplantation. (A) Schematic depiction of the figure-eight knot used created with two intrastromal incisions, each extending from slightly above the limbus to the circumference of the paracentral cornea, which was marked with a 1.5-mm trephine. Three of these suture knots were placed in BALB/c mice to stimulate robust corneal neovascularization. (B1). After 14 days the sutures were removed (B2), and penetrating corneal transplantation was performed using age-matched C57BL/6 donors (B3).
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. We analyzed all the corneal images quantitatively using a mathematical software program (MatLab; MathWorks Inc., Natick, MA) written specifically to calculate these corneal NV parameters. After the blood vessels were enhanced and traced by using different graphics editing tools and filters, by setting a threshold level, the nonvessel area was erased, and the remaining neovascular area was then pixelized and measured. The calculated blood vessel area was normalized to the whole corneal area to obtain the NA score for each corneal picture. VC was also estimated by using a computational technique to measure the largest diameter circle centered at each pixel inside a blood vessel. The mean value across all pixels within blood vessels was taken as an estimate of the mean VC for a given image. Last, the IA was quantified, and the ends of all vascular sprouts were marked. By connecting all these marks, the contour of the IA was traced and the measured area was again normalized to the whole corneal area (Fig. 2B).

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).
acizumab treatment appeared to be the only effective method to indicate times to 8 weeks after transplantation. Subconjunctival bev-

**Figure 4.** Analysis of topical versus subconjunctival bevacizumab on the corneal NA, VC, and IA. High-risk graft beds in BALB/c mice were transplanted with C57BL/6 cornea, and mice were left untreated ($n = 10$) or were treated topically ($n = 10$) or subconju-

**Figure 5.** Subconjunctival delivery of bevacizumab diminishes opacity of corneal allografts in the high-risk setting. High-risk graft beds in BALB/c mice were transplanted with C57BL/6 cornea, and mice were left untreated ($n = 10$) or were treated topically ($n = 10$) or subconju-

**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 exam-

**Figure 6.** Subconjunctival bevacizumab promotes corneal allograft survival in the high-risk setting. High-risk graft beds in BALB/c mice were transplanted with C57BL/6 cornea, and mice were left untreated ($n = 10$) or were treated topically ($n = 10$) or subconju-

**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 for 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.

Increases in neovessels and blood vessels were calculated and normalized to yield the mean IA at the indicated times to 8 weeks after transplantation. Although topical bevacizumab treatment mildly reduced NA in high-risk corneal transpl-

$P$-test was performed to evaluate statistical significance ($*P < 0.05$, $**P < 0.01$).
DISCUSSION

Corneal NV has long been established as an important risk factor for immune rejection after corneal transplantation, yet an effective treatment has remained elusive. The data from our experiments using a mouse model of high-risk keratoplasty suggest that both topical and subconjunctival bevacizumab treatment can diminish the severity of corneal NV. However, only subconjunctival bevacizumab treatment results in a significant regression of all neovessel metrics, including NA, VC, and IA. Moreover, only subconjunctival bevacizumab treatment significantly promotes graft survival in the high-risk setting. Although topical administration of bevacizumab results in a mild to moderate decrease in the severity of corneal NV, topical bevacizumab shows little impact on graft survival in the experimental model of high-risk transplantation described here. These findings strongly suggest that topical application of bevacizumab may not be able to provide adequate anti-VEGF activity to impact graft survival compared with the subconjunctival route.

It is likely that the limited efficacy of topical bevacizumab results, at least in part, from a lack of adequate penetration through the corneal epithelium. Healthy corneal epithelium contains several layers of cells connected by tight junctions, thus acting as an effective barrier for large molecules. Full-length immunoglobulins, including bevacizumab, with molecular weights of 149 kDa likely have limited capacity to penetrate the intact cornea. Nonetheless, conditions such as inflammation have a significant effect on the corneal epithelium; indeed, it is known that patients with, for example, ocular surface disease have an incompetent barrier function. Moreover, the clinical efficacy of topical bevacizumab in the treatment of corneal NV has been shown by our group and by other investigators, indirectly suggesting that topical bevacizumab may penetrate the epithelial barrier in patients with corneal inflammatory NV. This is also supported by our work in a mouse model of corneal NV that has clearly demonstrated bevacizumab can penetrate the neovascularized cornea after topical application (Sadrai Z, et al. IOVS 2008;49: ARVO E-Abstract 1488). Taken together, however, it is apparent that the level of corneal penetration by topical application of bevacizumab is insufficient in markedly improving corneal NV and significantly promoting allograft survival in high-risk graft beds. In contrast, subconjunctival injection of bevacizumab can bypass the ocular surface epithelial barrier and can produce high corneal levels of drug that lead to a significant suppression of corneal NV, hence promoting graft survival in high-risk settings.

Although our results with subconjunctival bevacizumab were highly significant, the inhibition of corneal NV was far from complete for several possible reasons. First, it is possible that the dosage and duration of treatment were insufficient to effectively antagonize all VEGF activity. Because of concern regarding the potential adverse effects of bevacizumab in treating corneal NV, we sought to limit our treatment of bevacizumab to the first few weeks after transplantation. Similarly, bevacizumab is a humanized monoclonal antibody, and thus its potency in murine models could be significantly impaired. Second, preexisting “stable” or “mature” vessels in the recipient bed may not be as susceptible as developing vessels to anti-VEGF treatment. 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 signal-

References


