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Promotion of Graft Survival by Vascular Endothelial Growth Factor A Neutralization After High-Risk Corneal Transplantation

Bjoern O. Bachmann, MD; Felix Bock, MSc; Stanley J. Wiegand, PhD; Kazuichi Maruyama, MD; M. Reza Dana, MD, MSc; Friedrich E. Kruse, MD; Elke Luetjen-Drecoll, MD; Claus Cursiefen, MD

Objective: To evaluate whether hemangiogenesis, lymphangiogenesis, and concomitant invasion of mononuclear phagocytes occurring after high-risk corneal transplantation in already vascularized high-risk recipient corneal beds increase the risk for subsequent immune rejection.

Methods: Three intrastromal sutures were left in place for 6 weeks in the corneas of BALB/c mice, causing neovascularization. Three weeks after suture removal, keratoplasty was performed (donors C57BL/6 mice). The treatment group received a vascular endothelial growth factor A (VEGF-A)–neutralizing cytokine trap at 0, 4, 7, and 14 days postoperatively (Fc protein was used as the control treatment). Morphometry was performed in corneal flat mounts using lymphatic endothelial hyaluronan receptor-1 (a specific lymphatic endothelial marker), CD31 (a panendothelial marker), and F4/80 (a marker for mononuclear phagocytes).

Results: After corneal transplantation, significant additional hemangiogenesis (mean area covered by vessels [SD], 68% [18%] postoperatively vs 40% [18%] preoperatively; \( P = .03 \)) and lymphangiogenesis (12% [1.3%] postoperatively vs 9% [2.8%] preoperatively; \( P = .03 \)) were observed. Postoperative neutralization of VEGF-A inhibited operation-induced hemangiogenesis (35% [8%]; \( P = .007 \)) and lymphangiogenesis (6% [1.6%]; \( P = .03 \)) and decreased the recruitment of mononuclear phagocytes into the graft (mean [SD], 501 cells/mm² [152] in treated mice vs 684 cells/mm² [35] in Fc controls; \( P = .03 \)). After 8 weeks, 23% of the treated corneas were not rejected, whereas all control corneas were rejected after 21 days (\( P = .007 \)).

Conclusions: Neutralization of VEGF-A after high-risk corneal transplantation effectively inhibits postoperative hemangiogenesis, lymphangiogenesis, and recruitment of antigen-presenting cells and improves corneal graft survival.

Clinical Relevance: Blocking of VEGF-A after high-risk corneal transplantation may be a novel approach to improve graft survival.

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Although orthotopic corneal transplantation is the oldest and most commonly performed solid-tissue transplantation, \(^1\) the nonophthalmic transplant community has paid it remarkably little attention. This community perhaps sees little chance of translating the high rate of success achieved with corneal transplants into other tissue compartments, because that success relies on site-specific characteristics, such as corneal immune privilege and corneal avascularity. \(^2\) Although a very low 2-year rejection rate of about 10% is seen in transplantations performed in avascular, immune-privileged, so-called low-risk recipients, \(^3\) grafting into pathologically prevascularized corneal beds, so-called high-risk corneal transplantations, leads to more than a 50% immune rejection rate. \(^4,5\)

It has been known for many decades that the presence of preexisting blood vessels \(^6\) in high-risk eyes is a strong risk factor for subsequent immune rejection. Recently, clinically undetectable preexisting lymphatic vessels have also been identified as risk factors. \(^3,5-7\) More recently, researchers showed that postoperative growth of blood and lymphatic vessels into the preoperatively avascular recipient bed is also a strong promotor of subsequent immune rejection in the mouse model of low-risk corneal transplantation. \(^5\) However, no studies have examined the role of neovascularization occurring after transplantation into already vascularized high-risk corneal beds. Because immune rejections are very common in high-risk eyes and because postoperative hemangiogenesis and lymphangiogenesis promote immune rejection even in low-risk eyes, we hypothesized that postoperative induction of angiogenesis, and particularly the formation or reformation of lymphatic vessels, together with the recruitment of mono-
nuclear phagocytes, might also promote graft rejection in prevascularized high-risk eyes. Recently, researchers have shown that macrophages, which can be recruited through vascular endothelial growth factor receptor 1 (VEGFR-1) by vascular endothelial growth factor A (VEGF-A), are essential for inflammation-induced hemangiogenesis and lymphangiogenesis.

Therefore, we sought to evaluate whether corneal graft survival could be improved in a murine model of high-risk corneal transplantation by pharmacologically neutralizing VEGF-A, thereby inhibiting postoperative hemangiogenesis, lymphangiogenesis, and mononuclear phagocyte recruitment.

In this study, we demonstrate not only that additional ingrowth of new blood and lymphatic vessels occurs after high-risk corneal transplantation in already prevascularized corneas owing to the operation itself, but also that the process depends on VEGF-A and thus can be inhibited by using VEGF-A–specific cytokine traps. Reducing VEGF-A through the use of such traps also results in a less robust invasion of macrophages into the corneal allograft. Finally, we show that temporarily inhibiting postoperative hemangiogenesis and lymphangiogenesis promotes graft survival, even after high-risk transplantation.

**METHODS**

**MICE AND ANESTHESIA**

Six- to 8-week-old female C57BL/6 mice were used as graft donors and female BALB/c mice of the same age were used as recipients. All animals were treated in accordance with the Association for Research and Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. For surgical procedures, mice were anesthetized using a mixture of ketamine hydrochloride and xylazine (120 mg and 20 mg per kilogram of body weight, respectively).

**MODIFIED MOUSE MODEL OF HIGH-RISK CORNEAL TRANSPLANTATION**

To be able to visualize changes in both hemangiogenesis and lymphangiogenesis after corneal transplantation and to more closely mimic the situation of human high-risk corneal transplantation (where transplantation is usually not performed in the acute inflammatory period), a modified model of high-risk corneal transplantation was developed. In contrast to the established model, a less intense degree of both hemangiogenesis and lymphangiogenesis was induced prior to corneal transplantation, thus enabling measurement of additional hemangiogenesis and lymphangiogenesis. As in the clinical setting, grafting was delayed relative to the time of the initial corneal injury, allowing for the resolution of the acute inflammatory response. Threestromal interrupted 11-0 sutures were inserted in the corneas of BALB/c recipients and left in place for 6 weeks. At this time, the corneas were intensely vascularized and the sutures were removed. Three weeks later, penetrating corneal transplantation was performed using age-matched C57BL/6 donors. Briefly, donor corneas were excised by trephination using a 2.0-mm bore and were cut with curved Vannas scissors. Until grafting, corneal tissue was placed in chilled phosphate-buffered saline. Recipients were anesthetized and the graft bed was prepared by trephining a 1.5-mm central avascular zone of the graft.

**IMMUNOHISTOCHEMISTRY AND MORPHOMETRY OF VASCULARIZATION AND INVASION OF ANTIGEN-PRESENTING CELLS**

Morphometric analysis of both hemangiogenesis and lymphangiogenesis in corneal flat mounts was performed as described previously. Briefly, corneal flat mounts were rinsed in phosphate-buffered saline, fixed in acetone, rinsed in phosphate-buffered saline, blocked in bovine serum albumin, 2%, stained with fluorescein isothiocyanate–conjugated CD31/endothelial cell adhesion molecule-1 antibody overnight (1:100; Santa Cruz Biotechnology, Santa Cruz, California); then washed, blocked, and stained with antibodies against lymphatic endothelial hyaluronan receptor-1 (LYVE-1 [1:500], a lymphatic endothelium-specific hyaluronic acid receptor)6,14; washed, blocked, and stained with Cy3 (1:100; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania); then analyzed by microscope. To illustrate the amount of mononuclear phagocytes invading the corneal allograft concomitant with vascularization, flat mounts were stained with antibodies against F4/80 instead of LYVE-1 at day 7 postoperatively.

Digital photographs of the flat mounts were taken with an image-analysis system. Then, the area that was covered by blood vessels and lymphatic vessels was measured morphometrically on the flat mounts with National Institutes of Health Image software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/nih-image). The total corneal area was outlined, with the innermost vessel of the limbal arcade serving as the border. The total area of blood vs lymphatic neovascularization was then normalized to the total host corneal area and the percentage of the cornea covered by each vessel type was calculated. At day 7 after corneal transplantation, when (in some corneas) vascularization was established within the allograft, the total area of blood vs lymphatic neovascularization was normalized to the total corneal area. F4/80+ cells were counted within a square of 145,000 µm² of the central avascular zone of the graft.
STATISTICAL ANALYSIS

The statistical difference in graft rejection rates between treatment and control groups was analyzed using the Mann-Whitney test and Kaplan-Meier survival curves. The day of the onset of rejection was used for this statistical analysis and to calculate the median survival time. In some animals treated with VEGF Trap, rejection did not occur before the experiment was terminated (8 weeks after corneal transplantation). In these cases, we assigned the day after study termination as the day of rejection for statistical purposes.

RESULTS

INDUCTION OF HEMANGIOGENESIS AND LYMPHANGIOGENESIS AFTER CORNEAL TRANSPLANTATION IN PREVASCULARIZED, HIGH-RISK CORNEAS

To evaluate the existence and amount of additional blood and lymphatic vessel growth after high-risk corneal transplantation, we compared the density of vascularization that was present immediately prior to transplantation with that observed 3 days after allografting (n=8). Preoperatively, vital microscopy revealed a moderate level of corneal vascularity. Immunohistochemical staining, using LYVE-1 as a lymphatic endothelium-specific marker, confirmed that blood vessels (CD31+/LYVE-1−) were present in relatively large numbers, with fewer (mostly) segmented lymphatic vessels (CD31+/LYVE-1−) distributed throughout the corneal circumference (Figure 1). Three days after corneal transplantation, we observed not only an increase in blood vessel density but also the apparent reformation of existing segmented lymphatic vessels as well as concomitant ingrowth of new, continuous lymphatic vessels from the physiologically vascularized limbal arcade (Figure 1 and Figure 2). Quantitative analyses revealed that immediately preoperatively, a mean 40% (SD, 18%) of the corneal surface was covered by pathologic blood vessels, whereas lymphatic vessels covered a mean 9% (SD, 2.8%) of the cornea. Three days after corneal transplantation, there was a significant increase in both blood (hemangiogenesis; \( P < .05 \)) and lymphatic (lymphangiogenesis; \( P < .05 \)) vessel density, covering a mean 68% (SD, 18%) and 12% (SD, 1.3%) of the cornea, respectively.

INHIBITION OF ADDITIONAL HEMANGIOGENESIS AND LYMPHANGIOGENESIS AFTER HIGH-RISK CORNEAL TRANSPLANTATION BY VEGF TRAP

We had previously demonstrated that selective, pharmacological neutralization of VEGF-A by administering VEGF Trap effectively blocked lymphangiogenesis as well as hemangiogenesis following corneal injury or normal-
risk corneal transplantation in mice. Therefore, we elected to use this same agent to evaluate the extent to which combined hemangiogenesis and lymphangiogenesis, occurring after high-risk corneal transplantation, also depends on VEGF-A. Three days postoperatively, vital microscopy revealed that a single injection of VEGF Trap on the day of the operation effectively suppressed additional corneal blood vessel ingrowths, compared with mice treated with Fc protein. Quantitative immunohistochemical and morphometric analyses revealed that 3 days following high-risk corneal transplantation, the density of blood and lymphatic vessels in the corneas of VEGF Trap–treated mice was significantly reduced relative to animals that received Fc protein, respectively (mean [SD], hemangiogenesis-vascularized areas, 35% [8%] vs 68% [18%], P < .01; lymphangiogenesis-vascularized areas, 6% [1.6%] vs 12% [1.3%], P < .001) (Figure 3). Seven days postoperatively, this tendency was even more evident (mean [SD], hemangiogenesis, 37% [19%] in VEGF Trap–treated vs 82% [6%] in Fc-treated mice; P = .004). Notably, in VEGF Trap–treated mice, the corneal area covered by blood and lymphatic vessels 3 days postoperatively was comparable with that seen immediately prior to the operation (mean [SD], 40% [18%] vs 9% [2.8%], respectively) (Figure 1).

SUPPRESSION OF INVASION OF MONONUCLEAR PHAGOCYTES FOLLOWING HIGH-RISK CORNEAL TRANSPLANTATION BY VEGF TRAP

After F4/80+ mononuclear phagocytes are recruited to inflammatory sites, they react differently depending on whether they differentiate into macrophages or dendritic cells. Macrophages are essential for the immune amplification leading to inflammatory neovascularization. In addition, activated inflammatory F4/80+ mononuclear phagocytes can also differentiate into dendritic cells and gain access to the draining lymph nodes through the lymphatic vessels and, by this, contribute to the initiation of allospecific immune reactions. In the murine model of high-risk corneal transplantation, postoperative treatment with VEGF Trap leads to decreased invasion levels of mononuclear phagocytes into the central avascular zone of the corneal allograft compared with treatment with Fc protein, as can be seen 7 days postoperatively (mean [SD], 501 cells/mm² [152] vs 684 cells/mm² [35], P < .05) (Figure 4).

INHIBITION OF ADDITIONAL HEMANGIOGENESIS AND LYMPHANGIOGENESIS AND INVASION OF MONONUCLEAR PHAGOCYTES AFTER HIGH-RISK CORNEAL TRANSPLANTATION SIGNIFICANTLY IMPROVES LONG-TERM CORNEAL GRAFT SURVIVAL

Because we demonstrated that additional growth of blood and lymphatic vessels occurs after high-risk corneal transplantation and that this additional ingrowth and the concomitant invasion of mononuclear phagocytes was effectively blocked by postoperative administration of VEGF Trap, we sought to determine whether transient, postoperative neutralization of VEGF-A might also promote corneal graft survival after allogeneic high-risk transplantation. Mice treated with VEGF Trap (n = 13) on the day of the operation and 3, 7, and 14 days thereafter showed significantly prolonged graft survival (P < .05) relative to control mice injected with Fc protein (n = 10). Notably, in the VEGF Trap–treated group, 23% of the grafts survived through the end of the 8-week observation period. In marked contrast, 90% of the grafts were rejected within 2 weeks of transplantation in the Fc control group, with 100% rejection seen by 3 weeks (Figure 5).

COMMENT

Based on the results, one can draw 2 important conclusions: (1) Pharmacologic neutralization of VEGF-A after high-risk corneal transplantation inhibits the induction of operation-induced angiogenesis, lymphangiogenesis, and mononuclear phagocyte recruitment. (2) Novel antiangiogenic treatments to block both postoperative hemangiogenesis and lymphangiogenesis and the concomitant invasion of mononuclear phagocytes can significantly improve the rate of graft survival, opening new treatment approaches for high-risk patients lacking effective therapies today.
Figure 3. A, Flat mount at postoperative day 3 of a cornea treated with Fc protein (control). B, Flat mount at postoperative day 3 of a cornea treated with VEGF (vascular endothelial growth factor) Trap (Regeneron Pharmaceuticals Inc, Tarrytown, New York). Sutures keep the graft in place. Original magnification ×40. C, Morphometry showing hemangiogenesis (P=.007) and lymphangiogenesis (P<.001) 3 days after keratoplasty in Fc protein– and VEGF Trap–treated mice.

Figure 4. A, Flat mount at postoperative day 7 of a mouse cornea treated with Fc protein (control). B, Flat mount at postoperative day 7 of a mouse cornea treated with VEGF (vascular endothelial growth factor) Trap (Regeneron Pharmaceuticals Inc, Tarrytown, New York). Arrowheads indicate inflammatory cells. Original magnification ×100.
When performed in avascular (ie, low-risk) recipient beds, corneal transplantation is the most successful of all organ/tissue transplantation procedures. Its rate of graft rejection, 10% after 2 years, is remarkably lower than that seen in the solid-tissue transplantation of any other organ.5 This success can be attributed, at least in part, to the corneal angiogenic privilege, ie, to the healthy cornea’s lack of the blood and lymphatic vessels that compose the efferent and afferent arms, respectively, of an immune reflex arc.16,17 That immune privilege has long been known to be abolished by preexisting corneal vascularization, a strong risk factor for immune rejection following penetrating corneal transplantation.12,18 In fact, rejection rates of up to 100% have been observed in the mouse model of orthotopic high-risk corneal transplantation in neovascularized high-risk eyes.19 In human transplantations that are defined as high-risk owing to preexisting corneal blood vessels, rejection rates increase to 50% and beyond. These high-risk transplant patients, together with those who elect not to receive or are denied transplantation because of such a poor prognosis, could greatly benefit from improved strategies for preventing immune rejections after high-risk corneal transplantation.

Hemangiogenesis and lymphangiogenesis occurring after low-risk corneal transplantation in preoperatively avascular recipient beds have recently been identified as additional contributors to allograft rejection.3 Our study demonstrates for the first time that the VEGF-A–dependent hemangiogenesis and lymphangiogenesis as well as mononuclear phagocyte recruitment occurring after corneal transplantation in already vascularized high-risk recipient beds might also contribute to allograft rejection.

Although the role of VEGF-A in promoting pathologic hemangiogenesis is well established, we have only recently come to appreciate that VEGF-A can also promote lymphangiogenesis.8,20,21 While VEGF-A might promote lymphangiogenesis in part by acting directly on VEGFR-2 expressed by the lymphatic endothelium,22,23 it also plays a critical role in the initiation of a proinflammatory cascade that locally amplifies cytokine and growth factor signals promoting both hemangiogenesis and lymphangiogenesis.8 After injury, VEGF-A recruits VEGFR-1–expressing leukocytes, especially macrophages, to the cornea. These inflammatory cells appear to contribute to neovascularization both by releasing hemangiogenic and lymphangiogenic growth factors (including VEGF-A, -C, and -D) at the site of injury8 and by actively integrating into the new lymphatic vessels.8 This integration may allow for the rapid reconstitution of partly regressed and disconnected corneal lymphatic vessels to the draining lymphatic vessels at the corneal limbus (and then to the draining regional lymph nodes). In other words, the rapid reorganization of partly regressed and partly dissected lymphatic vessels after corneal transplantation is at least in part VEGF-A dependent and seems to promote graft rejection by allowing for rapid host sensitization through migration of antigenic material and antigen-presenting cells into regional lymph nodes. Such hemangiogenesis and lymphangiogenesis and mononuclear phagocyte recruitment can be successfully inhibited by the pharmacological neutralization of VEGF-A.

It is well established that lymphatic vessels constitute the essential conduits of the afferent arm of the immune response, transporting antigen-presenting cells into the regional lymph nodes, while blood vessels serve as conduits for the efferent arm of the immune response, inducing an alloimmune response that allows effector cells access to the graft.17,24-26 In low-risk keratoplasties in rodents, allosensitization occurs 2 to 4 weeks after corneal transplantation, presumably through lymphatic drainage pathways.3,8 In contrast, after high-risk corneal transplantation in mice, the graft comes into direct contact with blood and lymphatic vessels almost immediately, leading to rapid donor-specific sensitization and a 100% rejection rate within 2 weeks10,12,19 (owing to, at least in part, the direct access to draining lymph nodes afforded to antigens and antigen-presenting cells by preestablished lymphatic vessels. In contrast, after low-risk transplantation, it takes newly generated blood and lymphatic vessels 1 to 2 weeks to reach the corneal graft.5 This delay allows the immune system enough time to initiate tolerogenic mechanisms, such as the eye-specific immune response of anterior chamber–associated immune deviation.27-29 Thus, in the low-risk environment, persistent immune tolerance can be achieved before alloreactive donor-specific T cells can be created.30 In this study, we achieved a similar delay following high-risk corneal transplantation by using VEGF Trap to inhibit the rapid reconstitution and regeneration of lymphatic vessels. Inhibition of this early reconnection seems to delay the onset of sensitization, thereby creating a window of opportunity for tolerogenic mechanisms to set in, at least in a subset of mice. Because most human high-risk transplantations are not performed in the most acute inflammatory period, inhibiting neovascularization in the early postoperative period by trapping VEGF-A should prove beneficial (because the lymphatic vessels of these patients are also partly regressed). Indeed, it was recently shown that lymphatic vessels completely regress after a short period of inflammation in the mouse model of inflammatory hemangiogenesis and lymphangiogenesis.31 In addition, VEGF-A may affect the functional properties of corneal lymphatic vessels and the recruitment.

Figure 5. Kaplan-Meier analysis showing corneal graft survival in mice treated with Fc protein (control) and VEGF (vascular endothelial growth factor) Trap (Regeneron Pharmaceuticals Inc, Tarrytown, New York).

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of mononuclear phagocytes in and out of the cornea. Whereas the primary effect of VEGF Trap on corneal hemangiogenesis and lymphangiogenesis seems to be a direct and local one, it is not possible to rule out indirect systemic effects, such as those on VEGF-recruited hematopoietic progenitor cells.

In summary, neutralization of VEGF-A after high-risk corneal transplantation inhibits postoperative hemangiogenesis and lymphangiogenesis and leads to a decreased number of mononuclear phagocytes within the allograft tissue. Corneal graft survival can be significantly improved by temporally inhibiting this operation-induced neovascularization as well as by interfering with the reconstitution of preexisting, fragmented lymphatic vessels, opening important new avenues of treatment for high-risk patients undergoing corneal transplantation.

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Correspondence: Claus Cursiefen, MD, Department of Ophthalmology, Friedrich-Alexander University Erlangen-Nuremberg, Schwabachanlage 6, 91054 Erlangen, Germany (curcursiefen@yahoo.com).

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