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Immunological Disruption of Antiangiogenic Signals by Recruited Allospecific T Cells Leads to Corneal Allograft Rejection

Yaohong Tan,* Fernando Cruz-Guilloy,**† Carlos A. Medina-Mendez,* Nicholas J. Cutrufello,* Rosa E. Martinez,* Maitee Uribeta,* David Wilson,* Yiwen Li,* and Victor L. Perez,*†

Corneal transplantation is the most common solid organ transplantation. The immunologically privileged nature of the cornea results in high success rates. However, T cell-mediated rejection is the most common cause of corneal graft failure. Using antiangiogenesis treatment to prevent corneal neovascularization, which revokes immune privilege, prevents corneal allograft rejection. Endostatin is an antiangiogenic factor that maintains corneal avascularity. In this study, we directly test the role of antiangiogenic and immunological signals in corneal allograft survival, specifically the potential correlation of endostatin production and T cell recruitment. We report that 75% of the corneal allografts of BALB/c mice rejected after postoperative day (POD) 20, whereas all syngeneic grafts survived through POD60. This correlates with endogenous endostatin, which increased and remained high in syngeneic grafts but decreased after POD10 in allografts. Immunostaining demonstrated that early recruitment of allospecific T cells into allografts around POD10 correlated with decreased endostatin production. In Rag2−/− mice, both allogeneic and syngeneic corneal grafts survived; endostatin remained high throughout. However, after T cell transfer, the allografts eventually rejected, and endostatin decreased. Furthermore, exogenous endostatin treatment delayed allograft rejection and promoted survival secondary to angiogenesis inhibition. Our results suggest that endostatin plays an important role in corneal allograft survival by inhibiting neovascularization and that early recruitment of allospecific T cells into the grafts promotes destruction of endostatin-producing cells, resulting in corneal neovascularization, massive infiltration of effector T cells, and ultimately graft rejection. Therefore, combined antiangiogenesis and immune suppression will be more effective in maintaining corneal allograft survival. The Journal of Immunology, 2012, 188: 000–000.

Since the first reported human corneal allograft by Zirm 100 years ago, corneal allotransplantation has become a widely practiced sight-restorative treatment for corneal dysfunction, such as keratoconus, bullous keratopathy, corneal scarring, corneal dystrophy, infection, and failed previous grafts (1, 2). The typically avascular corneas in these conditions afford a high transplant success rate. Even in vascularized corneas, allotransplantation remains the treatment of choice. However, the success rate is dismal secondary to immunological rejection (3, 4). Unfortunately, these patients remain blind because the use of systemic immune-suppression is required to prevent graft rejection, and this is not commonly done or offered.

Neovascularization is the most important predisposing factor for immunologic rejection of a corneal allograft. An increased number of vascularized quadrants of the cornea correlate with lower success rates in both humans and mice (5, 6). Pepose et al. (7) reported that corneal allograft rejection is a T cell-mediated process. CD4+ T cells play a crucial role in corneal allograft rejection, because in vivo depletion of CD4+ T cells through Ab application or by gene deletion results in a sharp reduction in corneal graft rejection (8–11). Following activation, CD4+ T cells produce a wide array of effector molecules, such as IFN-γ, NO, superoxide radicals, and TNF-α, each capable of inflicting extensive injury to the corneal allograft (12). FasL-mediated apoptosis and perforin may also contribute to CD4+ T cell-mediated corneal allograft rejection. However, even in the absence of either of these effector molecules, mice still readily reject corneal allografts (13–17). This suggests that multiple effector mechanisms are involved in corneal allograft rejection. Additionally, the relationship between corneal neovascularization and the previously mentioned immunological mechanisms remains poorly understood. Our laboratory demonstrated that the early recruited allospecific T cells play a critical role in the immunological rejection of high-risk vascularized corneal allografts (18). We hypothesize that the increased tempo and magnitude of T cell recruitment to the site of transplantation are essential for this process and that the presence of blood vessels in the cornea alters the immune response. However, how the emerging immunological and angiogenic responses lead to the rejection of avascular corneal allografts is not totally understood.

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. In normal corneas, angiogenesis is controlled by two counterbalancing systems: proangiogenic factors (vascular endothelial growth factor [VEGF], basic fibroblast
growth factor, matrix metalloproteinases, integrins, and cytokines) and antiangiogenic factors (pigment epithelium-derived factor, VEGFR3, soluble VEGFR-1, angiostatin, and endostatin) (19, 20). Antiangiogenic therapy may prove a promising direction to reduce graft failure (21). Various chemical compounds and drugs, such as steroids (22), methotrexate (23), heparin (24), and thalidomide (25), are reported to inhibit corneal neovascularization. Recently, anti-VEGF therapy targeting corneal angiogenesis showed successful results in animal experiments and clinical trials (26). However, anti-VEGF therapy has not produced the predicted increased graft survival. These observations suggest that using endogenous mechanisms to prevent corneal angiogenesis could be more efficient in maintaining avascularity and graft survival.

Endostatin is a 20-kDa fragment from the C-terminal non-collagenous domain 1 of collagen XVIII (27). Collagen XVIII is found in almost all ocular structures (i.e., the basement membranes [BMs] of the corneal and conjunctival epithelia, Descemet’s membrane, the anterior border layer and posterior pigmented epithelium of the iris, the BMs of the pigmented and nonpigmented ciliary epithelia, the internal wall of Schlemm’s canal and trabeculae, the ciliary and iris muscle cells, the BMs of the pigmented epithelium of the retina, and the internal limiting membrane). Endostatin shows a corresponding pattern, but with additional expression in corneal and conjunctival epithelial cells (28, 29). The constitutive presence of endostatin in corneas suggests that this molecule may play an important role in maintaining corneal avascularity and transparency. The current hypothesis surrounding endogenous endostatin activity is that, during endothelial activation, the production of proteolytic enzymes from BMs leads to a release of antiangiogenic fragments to serve as local inhibitors of angiogenesis (30). We hypothesize that endostatin is the “endogenous factor” that links angiogenesis to the immunological rejection of corneal allografts. In this study, we sought to understand how the immune system plays a critical role in the disruption of the antiangiogenic pathway of endostatin after corneal transplantation, leading to poor corneal allograft survival. Our data demonstrated that, after transplantation, the cornea attempts to maintain its avascular status by producing endostatin; however, the early recruitment of allospecific T cells into the site of transplantation leads to the destruction of endostatin-producing cells, thus leading to neovascularization of the corneal allotransplant and massive infiltration of effector CD4+ T cells, which result in graft rejection. We propose that the dual regulation of endostatin production and allospecific T cell recruitment is critical to increase graft survival and improve visual outcomes.

Materials and Methods

Animals

Female BALB/c (H-2b), C57BL/6 (H-2b), and Rag knockout (−/−) BALB/c mice between 6 and 8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for corneal transplantation. All of the mice between 6 and 8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for corneal transplantation. All of the mice were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), and the procedures were approved by the University of Miami Animal Care and Use Committee.

Orthotopic corneal transplantation and graft survival evaluation

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. before the surgical procedure. Orthotopic corneal transplantsations were performed with wild-type (WT) or Rag−/− BALB/c mice as the recipient, using C57BL/6 mice as donors for allografts and BALB/c mice as donors for syngeneic grafts, as previously described (18, 31). Initially, a central 2-mm full-thickness trephination of the recipient cornea was performed, followed by excision with corneal scissors. Corneas from donor mice were followed by excision with corneal scissors. Corneas from donor mice were then prepared in a similar fashion and secured to the recipient bed using eight interrupted 11-0 nylon sutures (Sharpoint, Reading, PA). Erythro-mycin ointment was applied, and transplants were examined 72 h after surgery. Corneal grafts with flat anterior chamber, ulceration, or other complications related to surgical difficulties were excluded. All corneal sutures were removed at postoperative day (POD) 7. After suture removal, corneal grafts were evaluated twice a week using slit lamp biomicroscopy and clinical scoring of clarity. A standard scoring system of 0–4 was used for corneal opacification: 0 = clear, 1 = slight haze, 2 = increased haze but anterior chamber structures are visible, 3 = advanced haze with difficult view of anterior chamber structures, and 4 = opaque cornea without view of anterior chamber structures. Grafts that received two consecutive scores ≥ 3 without resolution were considered rejected (18).

Measurement of endostatin and VEGF in corneal transplant extracts

Harvested whole corneas (including host corneas and grafts) were dissected into quadrants, and protein extracts were prepared by sonication with a Sonic Dismembrator (Fisher Scientific). Extracts were then centrifuged, and supernatants were stored at −80°C until assayed. For endostatin measurement, an anti-mouse endostatin Ab (1 μg/ml in PBS) (Sigma, St. Louis, MO) was used as the coating Ab, and a biotinylated anti-mouse endostatin Ab (R&D Systems, Minneapolis, MN) at 1:200 dilution was used as the detection Ab. Recombinant mouse endostatin (R&D Systems) was used as the standard protein. ELISA was performed per the manufacturer’s instructions. VEGF was detected with a mouse VEGF Quantikine Immunoassay (R&D Systems), per the manufacturer’s instructions.

In vivo endostatin therapy

To directly test the role of endostatin in corneal allograft survival, 2 μg endostatin (1 μg/ml; Sigma) or 2 μg PBS (pH = 7.4) was injected in the subconjunctival region of eyes that received cornea transplants. Injections were performed every 3 d from either POD0 to POD30 or POD20 to POD50, when the allografts were vascularized.

Adoptive transfer of T cells

CD3+ T cells from spleen and lymph nodes from BALB/c mice were isolated using a mouse CD3+ T-Lymphocyte Enrichment Set (BD Biosciences, San Jose, CA). The purification of CD3+ T cells was 89%, as verified by flow cytometry (data not shown). A total of 1 × 106 CD3+ T cells in 100 μl PBS was injected into BALB/c Rag−/− mice through the tail vein at POD20 after corneal transplantation.

Corneal blood vessel labeling and visualization with 1,1′-dioctadecyl-3,3′,3′′-tetramethylethlenediamineperchorlate dye

Mice were euthanized with CO2. The thoracic cavity was opened, and a blood collection set (23G 3/4) connected to a three-way stopcock luer valve, was inserted through the left ventricle into the ascending aorta, as described (32). Ten milliliters of PBS, followed by 10 ml of 1,1′-dioctadecyl-3,3′,3′′-tetramethylethlenediamineperchorlate (DiI) mixture (Sigma) and then 10 ml 4% paraformaldehyde in PBS were perfused. Whole corneas were excised and flat mounted, and the vessels were viewed under a Leica SP5 laser-confocal microscope.

Real-time PCR

Corneas were excised, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), as per the manufacturer’s protocol. The cDNA was synthesized using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The freely available PrimerBank (http://www.pga.mgh.harvard.edu/primerbank/index.html) was used to design primers. Details about primers used are given in Table 1. Real-time PCR was carried out using an iCycler IQ system (Bio-Rad). Relative mRNA expression was determined by the comparative Ct (ΔΔCt) method, whereby the relative amount of each target gene in the corneal samples is compared with the relative amount of each target gene in normal corneas (calibrator) and the relative amount of the endogenous control PGK-1 gene (33).

Immunohistochemical staining

Eyes were enucleated, snap frozen, and cut at 8-μm thickness. For T cell staining, rat anti-mouse CD4 and CD8 Abs (BD Biosciences) were used per the following protocol. Slides were fixed in 4% paraformaldehyde for 25 min and then blocked in 3% BSA and 0.05% Tween-20 in PBS for 1 h at room temperature. Staining was performed by incubating slides for 2 h at room temperature with CD4 or CD8 Ab (1:50), followed by a 1-h incubation with Alexa Fluor 594-labeled anti-rat IgG Ab (Molecular Probes,}

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Carlshad, CA) at 1:1000 dilution. For endostatin staining, slides were incubated with a goat anti-mouse endostatin (Sigma) at a concentration of 0.1 μg/ml in PBS containing 0.25 mg/ml saponin (Calbiochem, La Jolla, CA), followed by incubation with Alexa Fluor 594 anti-goat IgG Ab (Molecular Probes) at 1:1000 dilution. Images were taken with a Leica TCP SP5 laser-confocal microscope. Image Pro Plus (Media Cybernetics, Silver Spring, MD) was used to quantify the number of T cells recruited into the allografts.

**Statistical analysis**

The Student t test was used to test for differences of infiltrated CD4 and CD8 T cells and CD31 + cells; the relative mRNA copy numbers of CD4, CD8, and VEGF2; endostatin production; and blood vessels in the corneas. Corneal transplant survival data were plotted with a Kaplan–Meier curve, and differences in survival rate were compared with the log-rank test using PRISM software (GraphPad Software, San Diego, CA).

**Results**

*Endogenous endostatin production and corneal graft survival*

Previous work demonstrated that endostatin is produced by most of the ocular structures, especially the cornea and conjunctiva, as a crucial mechanism to maintain the avascular environment of the eye (28, 29). To determine whether endostatin also plays a role in preventing angiogenesis after transplantation and increased graft survival, we first measured endostatin production in protein extracts from syngeneic (BALB/c [H-2d] to BALB/c [H-2d]) and allogeneic (C57BL/6 [H-2b] to BALB/c [H-2d]) corneal grafts at different time points after transplantation using ELISA. Although all syngeneic corneal transplants remained clear and survived, allografts started to be rejected after POD20 and were significantly rejected by POD60 (Fig. 1A). Interestingly, immediately after corneal transplantation, upregulation of endostatin production occurred in both allogeneic and syngeneic transplants (Fig. 1B). However, in allogeneic transplants, endostatin production started to decline after POD10 and was significantly decreased by POD20, correlating with increased graft rejection. In contrast, syngeneic transplants retained high endostatin levels, graft clarity, and 100% graft survival.

To further investigate the relationship between anti- and proangiogenic signals in corneal graft survival, VEGF production was assessed and compared with endostatin. VEGF expression increased in both groups after transplantation, with a higher production in allotransplants (p < 0.05, Fig. 1C). In contrast to the peak endostatin levels seen at POD10, VEGF production began decreasing after POD3, reaching naive cornea levels by POD10. After that, VEGF production increased again and remained higher in allografts compared with syngeneic grafts (p < 0.05, Fig. 1C). DiI is one of the lipophilic carbocyanine dyes that can incorporate directly into the membrane of endothelial cells, resulting in instantaneous labeling of blood vessels (32). With laser-confocal microscopy, the vessels can be seen clearly in flat-mounted corneas. DiI staining identified a significant number of blood vessels in allogeneic grafts, whereas no vessels were detected in syngeneic grafts at POD40 (Fig. 1D). These data indicate that, after corneal transplantation, the syngeneic graft survival correlated with an avascular environment; upregulation of the antiangiogenic endostatin might play a crucial role in maintaining corneal avascularity following transplantation. However, adaptive immune responses in corneal allografts could alter this process, leading to angiogenesis and poor graft survival.

**Recruitment of T cells correlated with decreased production of endostatin in the corneal allotransplants**

Corneal allograft rejection is an immunological process primarily mediated by T cells. We hypothesize that the early recruitment of allospecific T cells to the site of transplantation is responsible for the disruption of endostatin production and the initiation of an angiogenic cascade that leads to graft rejection and failure. Elucidation of this correlation began with immunohistochemical analysis of corneal allotransplant sections to determine the relationship between the kinetics of T cell recruitment and endostatin
production after transplantation. Similarly to the previously shown ELISA results, immunohistochemical analysis confirmed that endostatin production was upregulated and remained elevated in syngeneic grafts and decreased in allogeneic transplants (Fig. 2). Furthermore, in syngeneic grafts, the vast production of endostatin appeared to occur in stromal cells of the corneal transplants. However, in allogeneic grafts, endostatin production occurred in the host cornea near the wound at POD3. Peak endostatin production was observed at POD10 and was more prominent in the distal stroma of the grafts. Concurrently, T cell infiltration into the allograft started around POD10 and increased thereafter, with concomitant graft rejection. As shown in Fig. 2, infiltrating T cells clustered around the endostatin-expressing cells. An inverse-proportion correlation was found between the numbers of infiltrating T cells and endostatin-producing cells.

As the number of T cells increased, the number of endostatin-producing cells decreased.

To directly test whether T cell recruitment into corneal allografts and disruption of endostatin production leads to increased neovascularization and graft rejection, corneal transplants were performed using Rag2/2 mice. Because Rag2/2 mice are deficient in mature T and B cells, immunological rejection of an allotransplant should not occur, and the kinetics of endostatin production and prevention of neovascularization should be similar to those seen in syngeneic transplants (34, 35). Our data demonstrated that Rag2/2 corneal allografts (C57BL/6 [H-2b] to Rag2/2 BALB/c [H-2d]) did not undergo rejection, and the survival rate was identical to that of the syngeneic transplants (BALB/c [H-2d] to Rag2/2 BALB/c [H-2d]), with no evidence of rejection up to POD60 and longer (Fig. 3A).

Measurements of endostatin production in protein extracts of the corneal transplants of Rag2/2 BALB/c mice and endostatin kinetics. (A) Rag2/−− corneal allografts (C57BL/6 [H-2b] to Rag2/−− BALB/c [H-2d]) and syngeneic transplants (BALB/c [H-2d] to Rag2/−− BALB/c [H-2d]) showed no evidence of rejection up to POD60 and longer (n = 8). (B) Endostatin production in protein extracts of corneal transplants demonstrated that endostatin levels remained significantly elevated in the Rag2/−− allogeneic and syngeneic groups and were significantly higher in Rag2/−− mice compared with WT-rejected allografts (C57BL/6 [H-2b] to BALB/c [H-2d]) at POD40 (p < 0.01). (C) A total of 1 × 106 CD3+ T cells from WT BALB/c mice was transferred via the tail vein to Rag2/−− mice with corneal grafts at POD20. Forty days after adoptive transfer (POD60), only the allograft group started to be rejected. By 60 d after T cell transfer, three of four allografts were rejected, whereas all of the syngeneic grafts survived (n = 4). (D) ELISA analysis showed that the production of endostatin decreased significantly in rejected allografts, whereas it remained high throughout in syngeneic grafts (up to POD100) of Rag2/−− mice that received T cells at day 20 (p < 0.01, n = 4). *p < 0.01.
corneal transplants also demonstrated that endostatin levels remained significantly elevated in the Rag−/− allogenic and syngeneic group, paralleling the lack of immune rejection for both groups. Endostatin levels were significantly higher in Rag−/− mice compared with WT-rejected allografts (C57BL/6 [H-2b] to BALB/c [H-2d]) at POD40 (p < 0.01) (Fig. 3B). To confirm these observations, the immune system of surviving Rag−/− allogenic and syngeneic transplant recipients was reconstituted by adoptive transfer of 1 × 107 CD3+ T cells from WT BALB/c mice via the tail vein at POD20. Forty days after adoptive transfer (POD60), only the allograft group started rejecting; by 60 d after T cell transfer, three of four allografts were rejected, whereas all of the syngeneic grafts survived (Fig. 3C). Analysis of endostatin production showed that the production of endostatin decreased significantly in rejected allografts, whereas it remained high throughout in syngeneic grafts (p < 0.01, Fig. 3D). These data explain how the adaptive immunological response leads to the disruption of endostatin regulation by the recruitment of allo-specific T cells that destroy endostatin-producing cells, followed by neovascularization and massive infiltration of T cells, leading to subsequent allograft rejection.

Administration of endostatin resulted in increased graft survival rate of corneal allotransplants

To directly test the role of endostatin in maintaining corneal transplant survival, exogenous endostatin was given to the mice with corneal transplants, and the graft survival rate was assessed. The mice were treated with either endostatin or PBS (control) through subconjunctival injections from POD0 to POD30. In the PBS-treated group, the graft rejection started at POD27; by POD40, 6 of 10 were rejected (Fig. 4A). In contrast, all allografts in the endostatin-treated group survived through POD40 (p < 0.01, n = 10, Fig. 4A). After POD40, the allografts from both the PBS- and endostatin-treated groups were eventually rejected (data not shown). Exogenous endostatin did not diminish, but delayed, the allograft rejection. All syngeneic grafts survived in both the endostatin- and PBS-treated groups (Fig. 4B). To see the effect of endostatin on vascularized corneal allografts, the mice received endostatin or PBS treatment, as described above, from POD20, when the allografts were vascularized, to POD50 or until the grafts were rejected. As shown in Fig. 4C, all of the grafts started rejecting around POD30; no difference existed between the endostatin-treated group and the PBS-treated group (p > 0.05, Fig. 4C).

To confirm the effects of endostatin in maintaining an avascular microenvironment in surviving corneal allografts, the expression of VEGF receptor VEGFR2 and the presence of blood vessels were measured. Binding of VEGF-A to VEGFR2 results in the migration and mitogenesis of vascular endothelial cells. Because the abundance of VEGFR2 correlates with neovascularization, we used its expression as an indication of new vessel formation. To determine the abundance of VEGFR2, real-time PCR was performed on corneal RNA from tissue harvested at POD40. The relative mRNA expression of VEGFR2 was greater in the allografts treated with PBS in comparison with those treated with endostatin (p < 0.01, Fig. 5A, Table I). To determine whether regulation of VEGFR2 expression also correlated with blood vessel formation, the number of blood vessels in the corneal allografts was quantified using anti-CD31 Ab staining. Similarly to the observed downregulation in VEGFR2 expression, the number of CD31+ cells concentrated in the corneal stroma was significantly reduced in the endostatin-treated group compared with the PBS-treated group (p < 0.01, Fig. 5B).

![FIGURE 4. Administration of exogenous endostatin promoted corneal allotransplant survival](image-url)

![FIGURE 5. Endostatin suppressed the neovascularization of the corneal allografts.](image-url)
Furthermore, Dil staining of blood vessels at POD40 also confirmed that allografts in the PBS-treated group had significant staining of vessels originating from the limbus in all four quadrants extending to the graft margins and crossing into the grafts. Once vessels grew into the grafts, they exhibited increased branching and proliferated throughout the grafts; although vascularization was inhibited in the endostatin-treated allografts, fewer vessels originating from the limbus extended to the graft margin and did not extend beyond the graft margin into the grafts (Fig. 5C). Together, these data directly confirmed the role of endostatin in corneal graft survival and prevention of rejection by downregulating angiogenic signals and neovascularization.

**T cell infiltration decreased with the administration of endostatin in the corneal allotransplants**

Although there are few absolute principles, it is certain that the immune rejection of corneal allografts is T cell dependent. Real-time PCR data showed increased CD4 and CD8 expression in the PBS-treated group \( (p < 0.01, \text{Fig. 6A, 6B, Table I}) \) compared with the endostatin-treated group. To further address T cell infiltration, frozen sections were obtained from the enucleated eyes with the endostatin-treated group. To further address T cell infiltration, these data directly confirmed the role of endostatin in corneal graft survival and prevention of rejection by downregulating angiogenic signals and neovascularization.

**Discussion**

Conventional dogma indicates that the formation of blood vessels in an avascular cornea alters the afferent and efferent pathways of immune recognition and responses to alloantigens after transplantation. Endostatin is expressed by corneal and conjunctival epithelial cells and is essential in maintaining corneal avascularity and transparency (30). However, there has been no systematic study of endogenous endostatin regulation during immune-mediated graft rejection. Using ELISA, we found basal endostatin production in normal BALB/c murine corneas and a clear upregulation of endostatin upon corneal transplantation, regardless of donor tissue. However, endostatin production began declining at POD10 in allogeneic corneal transplants, reaching the level of normal corneas by POD20 (Fig. 1B). Immunohistochemistry confirmed maximal production of endostatin around POD10, mostly present in the distal stroma of the allografts. Interestingly, early infiltrating allospecific T cells surrounded the endostatin-producing cells by POD10 (Fig. 2). Thereafter, the number of T cells increased, whereas the number of endostatin-producing cells decreased. Importantly, allograft rejection was preceded by a decline in endostatin production to baseline levels. In contrast, early recruitment of T cells was not observed in syngeneic corneal grafts, and endostatin levels remained consistently high throughout, with maintained corneal clarity and survival. We hypothesize that these early infiltrated T cells are allospecific effector T cells that migrate into the allografts to destroy endostatin-producing cells, followed by a decrease in endostatin production and failure to suppress neovascularization, a major contributing factor for graft failure.

Neovascularization can be the result of an imbalance between pro- and antiangiogenic factors. Because VEGF is known to play a major proangiogenic role (as opposed to endostatin), we investigated its expression pattern and found no correlation with rejection in our system; it was expressed at similar levels in allogeneic and syngeneic grafts (slightly higher in allogeneic, Fig. 1C). Therefore, it seems that endostatin upregulation counteracts VEGF in syngeneic, but not allogeneic, recipients; new blood vessels emerge, allowing for a substantial T cell influx into the allograft that precedes graft rejection. We also hypothesize that early inflammatory cells may be the source of VEGF, but further investigation will be needed to corroborate this idea.

**Table I. Primers of VEGFR2 and PGK-1 for real-time PCR**

<table>
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<th>Target Gene</th>
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<th>Reverse Primer (5’–3’)</th>
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<td>PGK-1</td>
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<tr>
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<tr>
<td>CD8</td>
<td>GACGAGCTACGTGGTGTGA</td>
<td>GACGGCTGAAGGTTGGA</td>
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</tbody>
</table>

**FIGURE 6. T cell infiltration decreased in endostatin-treated corneal allografts at POD40.** (A and B) Real-time PCR analysis showed increased CD4 and CD8 expression in the PBS-treated group compared with the endostatin-treated group. (C and D) Immunostaining of T cells showed that there were more CD4+ and CD8+ T cells infiltrated into the corneal stroma of the PBS-treated group compared with the endostatin-treated group \( (n = 3 \text{ mice/group}) \). (E and F) DAPI nuclear staining of corneal grafts. The stroma of endostatin-treated allografts maintained its structural integrity, whereas PBS-treated allografts had thicker and structurally disorganized stroma. Original magnification ×20.
To provide direct evidence that endogenous endostatin upregulation is part of the mechanism that prevents graft failure, we were interested in assessing endostatin levels in a setting in which all grafts are not rejected. To do this, we performed corneal transplantation with Rag2−/− mice, which are T cell and B cell deficient. As hypothesized, all of the allogenic and syngeneic transplants survived, and endostatin production remained high in both groups (p < 0.01 versus WT allografts; Fig. 3A, 3B). Although we cannot explain why the endostatin levels in Rag2−/− mice are higher than in WT mice, regardless of donor tissue, one simple explanation is a strain-specific difference, even though the Rag2−/− mice were on the same BALB/c background. The Rag2−/− mice may have a slightly different wound-healing response that is reflected in greater endostatin production. In addition, we cannot rule out a possible role of innate immune cells that may be recruited to the grafts.

The Rag2−/− system also allowed us to directly test the hypothesis that allospecific T cells are indeed responsible for killing endostatin-producing cells by selective adoptive transfer of these cells after transplantation. Forty days after T cell transfer, the allografts started rejecting, and 75% were rejected by POD50 (60 d after T cell transfer), whereas all of the syngeneic grafts survived, even after transfer of T cells (Fig. 3C). Interestingly, ELISA data showed that endostatin production decreased dramatically in rejected allografts, whereas it remained high in syngeneic grafts (p < 0.01; Fig. 3D). To our knowledge, this is the first demonstration that allospecific T cells destroy endostatin-producing cells, which represents a previously unknown aspect of allograft rejection. In the syngeneic grafts, there is no allospecific T cell recruitment; the functional endostatin-producing cells maintain a high concentration of endostatin, which keeps the syngeneic grafts avascular, and no massive infiltration of effector T cells occurs. It will be interesting to explore whether and how the paradigm of T cell-mediated killing of endostatin-producing cells applies to other types of solid organ transplantation.

Endostatin is one of the broad-spectrum angiostatic factors that suppresses angiogenesis by blocking general mechanisms that govern endothelial cell growth. In studies from different investigators, many animal and human tumors in mice have been inhibited by administration of endostatin protein. Tumor responses ranged from 47 to 91% inhibition by endostatin doses of 10–100 mg/kg/d (36). Because corneal angiogenesis represents a major reason for corneal graft rejection (6, 21), we hypothesize that suppressing neovascularization favors corneal graft survival. Data from our laboratory and that of other investigators showed that administration of exogenous endostatin inhibited corneal neovascularization (37, 38). Our group noted prolonged corneal allograft survival when mice were treated with exogenous endostatin (Fig. 4A), which correlated with fewer blood vessels, and lower CD4+ and CD8+ T cell infiltration in endostatin-treated allografts (Figs. 5, 6). We hypothesize that minimal or no T cell infiltration is the reason for prolonged graft survival. To address whether the phenomenon of lower T cell infiltration into the corneal grafts after treatment with endostatin is secondary to the inhibition of neovascularization or a direct effect of endostatin on T cells, we performed in vivo and in vitro experiments to exclude the direct effect on T cells. The in vivo experiment involved endostatin treatment after vascularization was established. Because vascularization of the allograft has already occurred, the sole effect of endostatin on T cells could be elucidated. Not surprisingly, all of the grafts started rejecting around POD30; no difference existed between the endostatin-treated group and the PBS-treated group (p > 0.05, Fig. 4C). Our in vitro stimulation experiment used CD4+ T cells harvested from OT-II mice. These cells were activated using OVA peptide in the presence or absence of endostatin. After 4 d of culture, supernatants were collected, and IL-2 production was measured by ELISA; T cell activation was also determined by staining for the activation marker CD69 and flow cytometry. The results showed that IL-2 production and the percentage of CD69+/CD4+ cells increased after stimulation with OVA peptide as expected, but endostatin had no effect on T cell activation in vitro (data not shown). We conclude that the protective effect of endostatin on allografts appeared to be secondary to inhibition of corneal graft neovascularization and not a direct effect on T cell activation, resulting in significantly decreased recruitment of effector T cells into the site of transplantation. Although antiangiogenic therapy appears to help in corneal allograft survival, the need to control immunological responses is still crucial in the protection of corneal grafts. This has major implications in the development of novel therapies to prevent graft rejection, where agents that block both angiogenic and immunological signals will be more effective in maintaining corneal allograft survival.

Endostatin is a promising strategy to prevent graft rejection. Likewise, Perkins et al. (39) suggested that endostatin levels might be used as a measure of alveolar capillary damage and support a potential pathophysiological role in acute lung injury. We also suggest that impending failure of corneal grafts might be fore-shadowed by monitoring endostatin levels. Questions still exist that need to be addressed. Particularly, which cell type(s) produce endostatin in the corneal grafts? How do infiltrated allospecific T cells kill the allografts? Do the early infiltrated allospecific T cells produce VEGF? We are currently dedicating efforts to understand these mechanisms. However, the data reported in this article point to a new two-step mechanism of allograft rejection: a first wave of attack that eliminates inhibitors of angiogenesis and migration, followed by a second wave that effectively kills the graft. This information can lead to new approaches in the treatment and prevention of immunological failure of transplantation.

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References


