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Kinetics of Angiogenic Responses in Corneal Transplantation

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Abstract

Purpose—To delineate and compare the kinetics of corneal angiogenesis after high-risk (HR) versus low-risk (LR) corneal transplantation.

Methods—In mice intrastromal sutures were placed in the recipient graft bed two weeks before allogeneic transplantation to induce angiogenesis and amplify the risk for graft rejection. Control (LR) graft recipients did not undergo suture placement, and thus the host bed remained avascular at the time of transplantation. Graft hemangiogenesis and opacity scores were evaluated for 8 weeks by slit-lamp biomicroscopy. Immunohistochemistry was used to measure CD31^{hi} (blood vessels) and LYVE-1^{hi} (lymphatic vessels) cells.

Results—A biphasic kinetics was observed for hemangiogenesis in both HR and LR transplant recipients using clinical and immunohistochemical assessments. The biphasic kinetics was composed of a rise-fall (phase 1) followed by a second rise (phase 2) in the degree of vessels. Compared to LR recipients, HR recipients showed higher hemangiogenesis (whole cornea and graft) throughout 8 weeks. Analyzing grafts revealed sustained presence of lymphatic vessels in HR recipients; however, lymphatic neovessels regressed in LR recipients two weeks post-transplantation. In contrast to HR host beds, the LR host bed microenvironment cannot sustain the growth of lymphatic neovessels in allografts while it can sustain continued hemangiogenesis.

Conclusion—The sustained presence of lymphatic vessels in the HR host beds can facilitate host immunity against allografts and is likely associated with ongoing higher risk of rejection of these grafts in long-term, suggesting that therapeutic interventions targeting inflammation and lymphatic vessels need to be sustained long-term in the HR corneal transplant setting.

Keywords

cornea transplantation; angiogenesis; hemangiogenesis; lymphangiogenesis; allograft rejection

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INTRODUCTION

Neovessel formation is often a physiological process involved in tissue repair. Tissue damage is typically followed by an inflammatory response that protects the tissue from infection and promotes wound healing.¹ The innate arm of the immune system, in particular macrophages,²⁻⁴ facilitates wound healing by generating proangiogenic factors leading to angiogenesis. For example, we have previously shown that corneal injury leads to the recruitment of vascular endothelial growth factor (VEGF)-A-secreting macrophages and induction of hemangiogenesis.⁵ Blood vessels sprout at the edge of the injury, enabling the formation of granulation tissue, which consists of fibroblasts and collagen.⁶ Growth and maturation of blood vessels at the site of injury is usually a tightly controlled process and once the tissue is repaired the blood vessels regress.⁷ Numerous physiological antiangiogenic factors, such as angiostatin, endostatin, tumstatin, vasohibin, fragments of prolactin, and growth hormones, facilitate this regression.⁸ The regression of the vessels is in turn tightly associated with resolution of the inflammation.⁹

In the context of adaptive immune response, including in various immunopathologies seen in infection, allergy, autoimmunity, and graft rejection, inflammation and hemangiogenesis are related to the presence of antigens.¹⁰⁻¹² Syphilitic interstitial keratitis is an example during which blood vessels are formed upon exposure to antigen and regress once viral antigens are cleared.¹³ In atopic keratoconjunctivitis, chronic inflammation due to persistent exposure to allergen(s) promotes progressive corneal neovessel formation.¹⁴

Neovascularization (NV) is commonly seen after corneal transplantation, especially when grafts are placed in inflamed host beds; the NV caused by both mechanical injury and the inflammatory response to foreign (graft) antigens.¹⁵ Importantly, the survival of corneal transplants can be significantly affected by the degree of the angiogenic response.¹⁶⁻²² This is due to lymphatic and blood vessels acting as mediators of the afferent and efferent arms of the immune system, respectively, whereby lymphatics mediate the trafficking of alloantigen and immune cells from the graft to lymphoid tissues, and blood vessels facilitate the homing of immune cells to the graft.²³⁻²⁶ In the current study, we characterize the kinetics of vessel formation in the cornea in a mouse model of corneal transplantation in order to determine how the kinetics of neo-hemangiogenesis and neo-lymphangiogenesis differ in the two distinct settings: (i) low-risk recipients that have no pre-existing corneal blood and lymphatic vessels, and (ii) high-risk recipients whose corneas have ample blood and lymphatic vessels prior to transplantation.²⁷

MATERIAL AND METHODS

Animals

Six-week-old BALB/c (H-2d) and C57BL/6 (H-2b) male mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in the Schepens Eye Research Institute animal vivarium and treated according to the guidelines set forth by the Association for Research in Vision and Ophthalmology (ARVO). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee.

Suture-induced Inflamed Graft Bed Preparation

Inflamed and neovascularized (high-risk, HR) host beds were created as detailed previously.²⁷ First, a 1.5 mm trephine was used to mark the central corneas of BALB/c host mice. Three intrastromal figure-of-eight suture knots were placed into the central cornea using 11-0 nylon sutures (AB-0550S, MANI, Tochigi, Japan) 14 days before corneal transplantation to induce extensive neovascularization in the host beds. Mice with unmanipulated (low-risk, LR) host beds served as controls.

Allogeneic Corneal Transplantation

Allogeneic orthotopic corneal transplantation was performed as described previously.^{27, 28} Briefly, the central cornea (2-mm diameter) was excised from a fully disparate donor C57BL/6 mouse using scissors (Vannas; Storz Instruments, San Dimas, CA). The graft bed was prepared by excising a 1.5-mm site of the central cornea of a BALB/c host mouse. The donor button was then placed onto the recipient bed and secured with eight interrupted 11-0 nylon sutures. After surgery host eyelids were closed for 3 days and interrupted corneal sutures were removed 7 days after surgery. Grafts' hemangiogenesis, opacity score, and survival rate were evaluated once a week for 8 weeks by slit-lamp biomicroscopy (magnification, $\times 25$). We used a standardized scoring system to assess opacity (range, 0–5+) and hemangiogenesis (range, 0–8+).²⁷ Corneas with an opacity score of 2+ for two consecutive examinations were considered rejected.²⁷ To exclude grafts undergoing primary failure, only grafts with scores of <2 at day 14 after transplantation were used for further analysis. Immune response-mediated rejection occurs after 14 days post-transplantation.

Corneal Whole Mount and Immunofluorescent Staining

Freshly excised corneas were washed in phosphate-buffered saline. The corneal epithelium was removed after incubation with 20mM EDTA for 60 minutes in 37°C; fixed in acetone for 15 minutes at room temperature, blocked in 2% bovine serum albumin for 60 minutes. Then corneas were double stained with CD31 (Santa Cruz Biotechnology, Dallas, TX, USA) and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (R&D AF2125) as described previously using goat anti-mouse CD31 FITC (1:100), LYVE-1 purified goat-anti mouse (1:400) overnight. Cy3 conjugated donkey anti-goat (1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was then added as secondary antibody and incubated for 2 hours. Stained wholemount corneas were mounted in Vectashield with DAPI. The area covered by blood or lymphatic vessels of the total cornea or graft was then analyzed using ImageJ (See Supplementary Fig. 1 for representative image of CD31/LYVE-1 staining and calculated vessel area [%]).

Statistical analyses

Two-way ANOVA test with Bonferroni's multiple comparison test was used to compare between two groups. The correlation of neovascularization and opacity score was analyzed using the Spearman's rank correlation at 14 days post transplantation. Kaplan-Meier analysis was used to construct survival curves, and the log-rank test was used to compare corneal graft survival. Data are presented as mean $\pm$ standard error of mean (SEM) and considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS

Slitlamp examination revealed biphasic kinetics for hemangiogenesis (Fig. 1A, B). As expected, there was a higher degree of hemangiogenesis in the corneas of HR versus LR recipients at baseline on the day of transplantation. One week post-transplantation, hemangiogenesis increased in both groups with the magnitude of the difference between the two groups being reduced. Interestingly, we observed a reduced hemangiogenic response 2 weeks post-transplantation (one week after the suture removal) in both groups. A second rise in hemangiogenesis was noted in both groups 3 weeks post-transplantation, which was sustained for 8 weeks. This rise was more prominent in the HR as compared to the LR recipient group (Fig. 1B, $n=10$, *** $p<0.0001$). The second increase of hemangiogenesis directly correlated with increased opacity scores (Fig. 1C, $n=10$. *** $p<0.0001$, Fig. 1D, $n=140$, $\gamma=0.84$, $p<0.0001$). Graft survival was significantly decreased in the HR compared to the LR transplants (Fig. 1E, $n=10$. *** $p=0.0002$).

Immunohistochemical analyses confirmed a biphasic kinetic response in blood vessel formation (Fig. 2A and 2B). While in the naïve cornea we detected only a small limbal area covered with vessels (See Supplementary Fig. 2A and 2B for the area of blood vessels in naïve cornea), in grafted corneas we observed increased angiogenesis using fluorescence microscopy (Fig. 2A and 2B. $n=10$, ** $p<0.01$, *** $p<0.001$). We noted increased vessel formation in the graft, which was significantly more pronounced in HR vs. LR recipients (Fig. 2C and 2D, $n=10$, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$). In LR recipients, the fraction of the graft area that was covered by blood vessels was only slightly increased shortly after transplantation, and remained constant for nearly two weeks. A fast and significant angiogenic response was seen in HR recipients that saturated within a week after transplantation.

In contrast to blood vessels, the lymphangiogenic response was similar in HR and LR recipients when the entire corneal surface was analyzed (Fig. 3A and 3B, $n=10$). However, when only the graft itself was analyzed, distinct dynamics for lymphatic ingrowth was observed (Fig. 3C and 3D, $n=10$, *, $p<0.05$, ***, $p<0.001$). Compared to LR recipients, a significantly higher amount of lymphatic vessels was observed in grafted corneas in HR recipients one week after transplantation, which stayed higher throughout the follow-up (Fig. 3C and 3D). HR recipients showed a monotonically increasing trend with three distinct phases, a fast rise during the first week, a shallow rise during 2nd and 3rd weeks, and finally a delayed rise during the 4th week. The delayed rise started after 3 weeks in HR recipients, while in LR recipients the lymphatic vessels regressed in both the graft and the host bed over 3 to 4 weeks post-transplantation (Fig. 3C and 3D).

DISCUSSION

Trafficking of immune cells to and from the graft site is essential for the adaptive immune response to allografts, and is established by blood and lymphatic vessels, which mediate the efferent and afferent arms of the host alloimmune response, respectively.²⁹ The afferent arm refers to trafficking of antigen and antigen-laden immune cells that lead to host allosensitization, and the efferent arm to the targeting of the graft principally by alloreactive

T cells. Suppressing the amount of vessels prior to,²² or at the time of transplantation,²⁰ can lead to significantly increased graft survival. Blood and lymphatic vessels also contribute to wound healing and resolution of edema, respectively. As such, understanding the dynamics of vessel formation and maintenance of neovessels is essential to understanding the timecourse of postsurgical recovery, as well as short and long term immune response to the grafted corneas.

High-risk recipients of corneal grafts are at enhanced risk for graft rejection resolution of surgery-induced tissue edema (Fig. 1B and 1E). At the very early stage post-grafting, the difference between the survival rates of HR and LR grafts could potentially be attributed in part to the significantly different amounts of blood vessels already present in the host beds. However, previous studies using integrin blockade to fully suppress lymphatic vessels prior to transplantation resulted in reduced rejection in the high-risk setting, suggesting that blood vessels are not the exclusive mediators of immune rejection.²² In contrast, our study focuses on post-transplantation angiogenic responses, and reveals that low-risk and high-risk hosts have comparable lymphatic ingrowth early on, while they differ significantly in the amount of blood vessel ingrowth. In a recent publication we have reported that inhibition of vessel formation reduces the alloimmune response and promotes graft survival.²⁰

A previous study using an alkali burn model has shown that corneal lymphangiogenesis develops 3 days post-injury, peaks at 2 weeks, and then decreases gradually.³⁰ Similarly to the alkali burn model LR graft recipients show regression of lymphatic neovessels soon after grafting (within a month). In contrast, HR recipients maintain lymphatic vessels for a long period. Our data show that LR host beds are able to maintain blood vessels but not lymphatic vessels, in contrast to HR recipients in which significant levels of both blood and lymphatic vessels are maintained. There are two major implications of these findings. First, our data clearly suggest that angiogenic homeostatic mechanisms are restored much earlier in LR, as opposed to HR graft recipients. Specifically, lymphatic responses regress early in non-inflamed LR hosts, as described similarly using an alkali burn model³⁰. Related to this, is the corollary that interventions targeting inflammation and lymphangiogenesis need to be maintained long-term in high-risk transplantation given that hosts with long-term unrestrained lymphatics will remain at risk for ongoing allo-sensitization and rejection for prolonged periods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Neovascularization, opacity, and graft survival in low-risk and high-risk transplant recipients

High-risk (HR) recipients underwent three intrastromal suture placements 14 days prior to corneal transplantation. Low-risk (LR) recipients received no sutures prior to transplantation and the corneas were left avascular and alymphatic. Corneal grafts were harvested from C57BL/6 donors and transplanted to BALB/c recipients, and the outcomes were assessed weekly for 8 weeks. (A) Representative slit-lamp images showing corneas post-transplantation *in vivo*. (Magnification $\times 25$) (B) Neovascularization scores were markedly higher in HR grafts compared to LR (Two-way ANOVA, $n=10/\text{group}$, $***p<0.0001$). We included all grafts in this statistics, irrespective of their survival scores. (C) Graft opacity scores were markedly higher in the HR grafts compared to LR (Two-way ANOVA, $n=10/\text{group}$, $***p<0.0001$). (D) The correlation between neovascularization and graft opacity

scores were examined by Spearman's correlation test after 14 day corneal transplantation (n=140, $\gamma = 0.84$, $p < 0.0001$). (E) Kaplan-Meier survival curves show significantly decreased of survival of HR grafts compared with LR recipients (Log-rank Test, n=10/group, *** $p < 0.0002$). (F)

Figure 2. Immunohistochemical assessment of hemangiogenesis

(A) Representative images showing CD31 staining of the whole cornea. (B) The percentage of the corneal area covered with blood vessels (CD31^{high}LYVE-1^{low}) in LR and HR recipients is shown. (C) Representative images showing CD31 staining in the grafted cornea. (D) The percentage of the graft area covered with blood vessels (CD31^{high}LYVE-1^{low}) in LR and HR recipients is shown. Percentage of blood vessels was calculated as follows: area covered by vessels divided by total area of cornea or graft. All data were obtained from n=10 mice/group and representative data from three independent experiments are shown. *p* values were calculated using two-way ANOVA test. Scale bar, 500μm.

Figure 3. Immunohistochemical assessment of lymphangiogenesis

(A) Representative images showing LYVE-1 staining of the whole cornea. (B) The percentage of the corneal area covered with lymphatic vessels ($CD31^{low}LYVE-1^{high}$) in LR and HR recipients is shown. (C) Representative images showing LYVE-1 staining in the grafted cornea. (D) The percentage of the graft area covered with lymphatic vessels ($CD31^{low}LYVE-1^{high}$) in LR and HR recipients is shown. Percentage of lymphatic vessels was calculated as follows: area covered by vessels divided by total area of cornea or graft. All data were obtained from $n=10$ mice/group and representative data from three independent experiments are shown. p values were calculated using two-way ANOVA test. Scale bar, $500\mu m$.