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TRANSIENT POSTOPERATIVE VEGF-NEUTRALISATION IMPROVES GRAFT SURVIVAL IN CORNEAS WITH PARTLY REGRESSED INFLAMMATORY NEOVASCULARISATION

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Abstract

Background: High-risk keratoplasties normally are performed after an uninflamed and quiescent interval in corneas with partly regressed blood and lymphatic vessels. We analysed whether the inhibition of postkeratoplasty revascularisation in mice with partly regressed corneal vessels (“intermediate-risk”) improves graft survival.

Methods: Three interrupted stromal sutures (11-0) in corneas of Balb/c mice (6-8 weeks) were placed for 6 weeks. Six months after suture removal, penetrating keratoplasty was performed with C57BL/6 donors. The treatment group received a VEGF-A specific cytokine trap (VEGF Trap) intraperitoneally at days 0, 4, 7 and 14 after keratoplasty (25 mg/kg/mouse; controls received equal amounts Fc-protein). Pathological hem- and lymphangiogenesis prior to as well as three days or 8 weeks after keratoplasty and graft survival were analysed. **Results:** Three days after keratoplasty corneal revascularisation was sufficiently reduced by VEGF Trap (hem-vascularised areas 42.7% reduction; lymph-vascularised areas 54.7% reduction). Survival proportions 8 weeks after keratoplasty were 36 % in the treatment group compared to 9% in the control group (n=11; $p<0.05$). At that time no differences in hem- or lymphangiogenesis were observed between the two groups.

Conclusion: Early transient postoperative induction of hem- and lymphangiogenesis and reformation of regressed corneal blood and lymphatic vessels are important for transplant rejections after “intermediate-risk” corneal transplantation.

Introduction

Corneal neovascularisation (CNV) occurs in many pathologic conditions, including chemical burns, infections and many ocular surface disorders.[1 2] Although CNV is beneficial in some instances (eliminating inflammation, promoting wound healing and inhibiting corneal melting) on the other hand CNV has become one of the major reasons for corneal blindness.[3] Once stable CNV has established, often the only possible treatment option for achieving a clear cornea is perforating keratoplasty. The role of preexisting CNV has long been established as a strong risk factor for immune rejections following keratoplasty. However, the role of CNV occurring after keratoplasty and its strong influence on inducing transplant rejection has just recently been elucidated in a mouse model of low risk keratoplasty and more recently also in a high risk setup.[4 5]

The time course of CNV occurring after keratoplasty might be important for the development of strategies against corneal graft rejection. The early growth of vessels after keratoplasty might interfere with tolerogenesis and ultimately graft survival. We hypothesize, that an initial transient inhibition rather than an ongoing inhibition of hem- and lymphangiogenesis following high-risk keratoplasty is important for the prolongation of graft survival.

We could recently demonstrate that murine corneal neovascularisation after a transient inflammatory stimulus starts to regress after a few weeks and that after six months already there is no evidence of lymphatics and only some blood vessels are still remaining.[6] In human corneas with a history of CNV of less than 3 months lymphatic vessels are detectable whereas none are present in corneas with a history of CNV of more than 6 months.[7] Additionally, there is a significant positive correlation between the degree of human corneal hemangiogenesis and lymphangiogenesis.

Since in the clinical setting keratoplasties are most of the time not performed in freshly vascularised and inflamed eyes, but rather later after a quiescent and uninflamed interval with at least partly regressed CNV, we wanted to test the ability of VEGF-A-blockade to inhibit postoperative revascularisation and to improve graft survival after keratoplasty in corneas with partly regressed blood and lymphatic vessels. Therefore we developed a new mouse model of intermediate-risk keratoplasty which more closely resembles the human situation than the standard mouse model.

Here we show that inhibition of post-keratoplasty reconstitution of regressed corneal blood and lymphatic vessels by VEGF-A blockade can significantly prolong graft survival. This supports the concept of an important role for early postoperative induction of hem- and lymphangiogenesis for immune responses after corneal transplantation.

Methods

Mice and anesthesia

Six- to eight-weeks-old female BALB/c mice (Charles River Germany, Sulzfeld, Germany) were used for vessel inducing suturing of the corneal stroma and further on served as recipients for corneal transplantations. Donor buttons were generated by trephination of corneas from six- to eight-weeks-old female C57Bl/6. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. No animal showed any symptoms suggesting of systemic disease due to the experiment. For surgical procedures, mice were anaesthetized using a mixture of ketamine and xylazine (120 mg/kg and 20 mg/kg body weight, respectively).

Vascular regression in high-risk corneas

In a prior study we demonstrated a positive effect of VEGF blocking on transplant survival in high-risk corneas with only minimally regressed vascularisation when keratoplasty was performed three weeks after removal of neovascularisation inducing sutures.[4] To obtain donor mice with a more pronounced regression of corneal blood and lymphatic vessels more closely mimicking the human setting corneal stromas of 6-8 weeks old BALB/c mice were sutured with three interrupted 11-0 sutures (nylon; Serag Wiessner, Naila, Germany) and left in place for six weeks. Penetrating corneal transplantation was performed six months after suture removal. According to previous studies from our group almost no lymphatic vessels and only few blood vessels are detectable at this time point (corneal sutures in that study remained 2 weeks only).[6] Keratoplasty was performed as described previously.[8 9] Briefly, donor corneas were excised by trephination using a 2.0 mm bore and cut with curved Vannas scissors. Until grafting, corneal tissue was placed in chilled phosphate-

buffered saline (PBS). Recipients were anaesthetized, and the graft bed was prepared by trephining a 1.5-mm site in the central cornea of the right eye and discarding the excised cornea. The donor cornea was immediately applied to the bed and secured in place with eight interrupted 11-0 sutures. Antibiotic ointment (Refobacin, Merck KGaA, Darmstadt, Germany) was placed on the corneal surface and the eyelids were closed with 8-0 suture (Serag Wiessner, Naila, Germany).

To study graft survival proportions tarsorrhaphy and corneal sutures were removed after 7 days and grafts with technical difficulties (hyphema, cataract, infection, loss of anterior chamber) were excluded from further consideration. Grafts were then examined at least twice a week until week 8 after transplantation by slit lamp microscopy and scored for opacity (Score 0 = clear cornea, 1 = minimal superficial opacity, 2 = minimal, deep (stromal) opacity (pupil margin and iris vessels visible, 3 = moderate stromal opacity (only pupil margin visible), 4 = intense stromal opacity (only a portion of pupil margin visible), 5 = maximum stromal opacity, anterior chamber not visible) as described previously.[8] Grafts with opacity scores 2 or greater after 2 weeks were considered to have been rejected.

Corneal flatmounts and morphological determination of hemangiogenesis and lymphangiogenesis

To measure the influence of VEGF deprivation on the amount of CNV corneas were excised either prior to or three days after keratoplasty for investigation of the immediate influence of VEGF Trap or 8 weeks after keratoplasty for the long term effect. The corneas were processed for morphometric analysis of both hem- and lymphangiogenesis in corneal flatmounts as described previously.[5 10] The corneas were rinsed in PBS and fixed in acetone for 30 minutes. After three additional washing steps in PBS and blocking with 2 % BSA in PBS for 2 hours the corneas

were stained overnight at 4°C with rabbit anti-mouse LYVE-1 (1:500; Angiobio, Del Mar, California, USA). On day two the tissue was washed, blocked and stained with FITC-conjugated rat anti-CD31 (Acris Antibodies GmbH, Hiddenhausen, Germany) antibody overnight at 4°C. After a last washing and blocking step on day three, LYVE-1 was detected with a Cy3-conjugated secondary antibody (goat anti rabbit, 1:100; Dianova, Hamburg, Germany).

Functional and statistical analysis

Double stained wholemounts were analysed with a fluorescence microscope (BX51, Olympus Optical Co., Hamburg, Germany) and digital pictures were taken with a 12-bit monochrome CCD camera (F-View II, Soft Imaging System, Münster, Germany). The areas covered with blood or lymphatic vessels were detected with an algorithm established in the image analyzing program analySIS^B (Soft Imaging System, Münster, Germany) as described previously [11]: prior to analysis grey value images of the wholemount pictures were modified by several filters. Blood and lymphatic vessels were detected by threshold setting including the bright vessels and excluding the dark background. Quantitative analysis was performed using rectangles of a standardized size (1.11mm^2) aligned along the limbus. Blood or lymphatic vessels in each rectangle were measured and correlated with the rectangle area (vessel ratio). Statistical differences were analysed using the Mann-Whitney U test and Kaplan-Meier survival curves. Graphs were drawn using Excel, Microsoft Corporation.

Results

Regressed corneal blood and lymphatic vessels in older mice have a capability for vascular reformation after keratoplasty

To evaluate whether a keratoplasty causes an additional ingrowth of blood and lymphatic vessels in a cornea with almost completely regressed prevascularisation (6 months after suture removal after 6 weeks of stromal sutures in place), we compared the density of vascularisation immediately prior to transplantation with that observed three days after allografting (n=4). Pre-operatively vital microscopy revealed a calm cornea with little vascularised area and without any sign for an ongoing inflammation. Immunohistochemical staining using LYVE-1 as a lymphatic vessel endothelial specific marker confirmed that blood vessels (CD31⁺⁺⁺ /LYVE-1⁻) were present in small numbers and only very few lymphatic vessels (CD31⁺ /LYVE-1⁺⁺⁺) appeared adjacent to physiologically vascularised limbal arcade. Three days after corneal transplantation a considerable increase in blood vessel density and a small additional ingrowth of lymphatic vessels was observed. Quantitative analyses revealed that immediately pre-operatively $6.95 \pm 5.44\%$ of the corneal surface was covered by pathological blood vessels and only $0.43 \pm 0.76\%$ of the cornea was covered by lymphatic vessels. Three days after corneal transplantation, there was a significant increase in both blood vessel density, covering $14.10 \pm 1.37\%$ ($p < 0.001$) and lymphatic vessel density, covering $1.72 \pm 1.07\%$ ($p < 0.05$) of the cornea (**Figures 1a, b and Figures 3a, b, d, e**).

Additional vascularisation can be blocked by initial VEGF deprivation after keratoplasty in corneas with regressed blood and lymphatic vessels

Blocking of VEGF-A using VEGF Trap has been proven to inhibit corneal hem- and lymphangiogenesis following a corneal inflammatory stimulus as well as after low-risk and “classical” high-keratoplasty. VEGF Trap binds and neutralizes all isoforms of VEGF-A, as well as placental growth factor (PIGF), but does not directly block VEGF-C or -D.[12] In the context of pathological angiogenesis PIGF mainly acts as enhancer of the stimulation of VEGFR-2 by VEGF-A. Therefore, we utilized VEGF Trap to evaluate the extent to which combined hem- and lymphangiogenesis occurring after intermediate-risk corneal transplantation also depend on VEGF-A. Three days after corneal transplantation, vital microscopy revealed that a single injection of VEGF Trap on day of surgery effectively suppressed additional corneal blood vessel ingrowth, compared to control mice treated with hFc. Quantitative, immunohistochemical and morphometric analyses showed that 3 days following high-risk corneal transplantation, the density of blood vessels was statistically reduced. Although even the additional ingrowth of lymphatic vessels in corneas of VEGF Trap treated mice was markedly reduced it did not reach the level of significance (hem-vascularised areas 6.02 ± 2.46 % versus 14.10 ± 1.37 % in Fc controls, $p < 0.0001$ and lymph-vascularised area in the treatment group: 0.94 ± 0.91 % versus 1.72 ± 1.07 % Fc treated, $p = 0.15$) (**Figures 2a, b and Figures 3b, c, e, f**).

Early blocking of VEGF leads to prolonged graft survival after keratoplasty in corneas with regressed blood- and lymphatic vessels

To test whether a decrease of additional ingrowth of blood and lymphatic vessels after intermediate-risk corneal transplantation by VEGF-A blocking leads to a reduced transplant rejection rate, VEGF Trap was used immediately for two weeks

after keratoplasty. Six months after temporary suture induced corneal inflammation keratoplasty was performed and mice were treated with VEGF Trap (n=11) on the day of surgery, and 3, 7 and 14 days thereafter. The animals showed significantly prolonged long term graft survival ($p<0.05$) relative to control mice injected with hFc. Notably, in the VEGF Trap treated group, 36 % of the grafts survived through the end of the 8 week observation period. In marked contrast, 91% of the grafts were rejected within 2 weeks of transplantation in the Fc control group (**Figure 4**).

Initial transient blocking of VEGF after keratoplasty has no effect on final corneal revascularisation

To investigate, whether temporary inhibition of VEGF-A influences the long term degree of corneal revascularisation, we quantified the amount of revascularisation 8 weeks after keratoplasty in corneas with regressed blood and lymphatic vessels (**Figures 5a, b**). Mice who received an initial treatment with VEGF-Trap or Fc-protein the first two weeks after keratoplasty had the same amount of corneal blood- (36.0 ± 5.8 % VEGF-Trap treated vs. 37.0 ± 8.9 % Fc-protein treated, $p=0.76$) and lymphatic vessels (15.1 ± 4.3 % VEGF-Trap treated vs. 13.9 ± 2.3 % Fc-protein treated, $p=0.42$).

Discussion

Allogenic graft rejection strongly depends on the initiation of an immune response by contact of antigen presenting cells with T-Lymphocytes in the corresponding lymph nodes.[13-17] In rodent low-risk keratoplasties, allosensitisation occurs 2-4 weeks after corneal transplantation, presumably through lymphatic drainage pathways.[5 10] 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 sensitisation and a 100% rejection rate in as little as one week.[8 18 19] The physiological blood and lymphatic vessel free cornea does not offer an immediate pathway for allogenic material to the draining lymph nodes after keratoplasty leading to a very low transplant rejection rate of less than 10% in humans.[20-22] Draining cervical lymph nodes play a critical role in allosensitisation and subsequent rejection but seem not responsible for tolerance induction after keratoplasty. This suggests that the initiation of tolerogenic mechanisms leading to long term acceptance of the graft may be achieved when the graft remains concealed for the local lymph nodes for a certain time.[23] Additionally, although many other factors besides avascularity are important for the corneal "immune privilege" the graft rejection rate increases dramatically when the transplantation is performed in a freshly prevascularised cornea. The grafted material gets instantaneous connection to the draining lymphatic system leading to a transplant rejection rate of more than 50%.[24]

The beneficial effect of an early transient antiangiogenic treatment after keratoplasty on long term graft survival has been proven in two publications only so far.[4 5] In a low-risk as well as in a classical high-risk setup of murine corneal transplantation a beneficial effect for the reduction of postkeratoplasty ingrowth of blood and lymphatic vessels by postoperative blocking of VEGF was observed. Additionally, in both models VEGF deprivation led to an improved graft survival in comparison with their

respective control group. The model of the current work utilises hosts where the corneal prevascularisation ranks between the two extremities of the traditional murine model of low-risk and high-risk keratoplasty. We were able to prove, that even in this setup, which more closely mimics the human high-risk situation, decreased influence of VEGF reduces postoperative neovascularisation and promotes graft survival. Additionally, whether transient early antiangiogenic treatment leads to a long standing diminished corneal vascularisation has been unclear to date. We showed for the first time that transient early deprivation of VEGF following keratoplasty leads to impaired early vascularisation whereas long term vascularisation is not altered. Moreover, this reduced early vascularisation is coincidental with an improved long standing graft survival. Our findings underscore that early transient and not necessarily the long term avoidance of contact of antigen presenting cells to the draining lymphatic vessel system leads to long-standing graft tolerance.

Earlier work demonstrated that temporary placement of sutures in the corneal stroma induced an inflammatory response with pathological corneal hem- and lymphangiogenesis, but that the vessels regressed over time.[6] In that study, the sutures were left for two weeks only before being removed. The results showed that lymphatic vessels regressed earlier and to a greater extent compared with blood vessels. By six months after suture removal, no lymphatic vessels were observed while there were only some partially perfused and nonperfused ("ghost") blood vessels. Similar results indicate that the regression of blood and lymphatic vessels after a CNV inducing stimulus also appears in human corneas.[7] This bears analogy with our modified intermediate-risk model. When corneal stromal sutures were left for 6 weeks only few blood and almost no lymphatic vessels were visible after 6 months. This model with regressed blood and lymphatic vessels is closer to the clinical situation where - whenever possible - one would wait a certain time after an

angiogenic stimulus before performing the corneal transplantation. Graft survival in this model of intermediate-risk keratoplasty was slightly better in both the treatment and the control group compared to our recent experiment, where the keratoplasty was performed three weeks after suture removal.[4] This better graft survival indicates that, if possible, one should always wait a certain inflammatory free interval before performing a keratoplasty.

One would still expect a higher capacity for the reformation of regressed blood or lymphatic vessels than seen in our model. In addition to the assimilation of corneas with regressed vascularisation to corneas without pathological vascularisation there might be a further factor contributing to the very low observed additional ingrowth of blood and lymphatic vessels after keratoplasty: Recent findings suggest that corneas of young mice have an increased capacity of neovascularisation after stromal suturing compared with older mice.[25] Older mice thereby have a reduced angiogenic response to an inflammatory corneal stimulus.

In conclusion our findings indicate that early transient anti-VEGF treatment after keratoplasty has a benefit for limiting the early and not the long term ingrowth of vessels. Early VEGF deprivation improves long term graft survival in the “intermediate-risk” murine model of corneal transplantation which more closely mimics the human scenario.

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Figure legends

Figure 1a+b)

Blood (a) and lymphatic (b) vessels prior to and three days after keratoplasty in corneas 6 months after a transient inflammatory stimulus of six weeks duration.

Figure 2a+b)

Blood and lymphatic vessels three days after keratoplasty in corneas 6 months after a six week inflammatory stimulus ("sutures") treated either with VEGF Trap or with hFc-control protein.

Figure 3a-f)

Immunohistochemical preparations of corneal flatmounts stained with antibodies against CD31 as a hemangiogenic marker (a-c) and LYVE-1 as a lymphangiogenic marker (d-f). Corneas were excised either prior (a+d) to or three days after keratoplasty (b, c, e, f). Immediately pre-operatively few of the corneal surface was covered by pathological blood vessels and only very little of the corneal surface adjacent to the limbal area was covered by lymphatic vessels. The additional postoperative ingrowth of blood (b+c) and lymphatic (e+f) vessels was reduced by VEGF Trap treatment.

Figure 4:

Temporary, early postoperative neutralisation of VEGF-A improves long-term graft survival when performed in corneas with regressed blood- and lymphatic vessels 6 months after temporary suture-induced inflammation.

Figure 5a+b):

Despite early transient blockade of VEGF leads to reduced initial corneal neovascularisation, no effect on long term ingrowth of both blood (a) and lymphatic (b) vessels was detectable 8 weeks after keratoplasty.

Fig. 1a

Fig. 1b

Fig. 2a

Fig. 2b

Fig. 3

**Immediately prior to
keratoplasty**

**Control group three days
after keratoplasty**

**VEGF Trap treated group
day 3 after keratoplasty**

CD31

LYVE-1

Fig. 4

Fig. 5a

Fig. 5b