Photodynamic Therapy Leads to Time-Dependent Regression of Pathologic Corneal (Lymph) Angiogenesis and Promotes High-Risk Corneal Allograft Survival

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METHODS. BALB/c mice were used for suture-induced inflammatory corneal neovascularization to induce combined hem- and lymphangiogenesis. The treated group received PDT 3 minutes, 1 hour, and 24 hours after an i.v. verteporfin injection (control group: phosphate buffered saline). Corneal flatmounts were excised 3 days, 1 week, and 2 weeks after corneal PDT and stained with cluster of differentiation 31 (CD31) and lymphatic vessel endothelial hyaluronan receptor 1 antibodies (LYVE-1) to quantify hem- and lymphangiogenesis. Graft survival rates were compared between high-risk recipients with and without preoperative PDT.

RESULTS. Corneal blood vessels were significantly reduced when PDT was performed 3 minutes after i.v. verteporfin injection, whereas lymphatic vessels showed no significant difference. Both blood and lymphatic vessels were regressed when PDT was performed 1 hour or 24 hours after i.v. verteporfin application. Long-term allograft survival increased significantly in PDT-pretreated eyes when compared with controls.

CONCLUSIONS. PDT after i.v. verteporfin injection can selectively regress pre-existing corneal blood vessels or both blood and lymphatic vessels depending on the timing of PDT after verteporfin injection. The pretreatment of recipients with PDT and i.v. verteporfin might be a promising new method to improve graft survival in high-risk eyes.

Keywords: photodynamic therapy, angiogenesis, lymphangiogenesis, corneal transplantation

The healthy cornea is an immune privileged tissue that actively remains avascular and is devoid of blood vessels (BV) and lymphatic vessels (LV) as a result of the expression of antiangiogenic and antilymphangiogenic factors. Transparency and avascularity of the cornea is a prerequisite for good vision and a requirement for transplant tolerance. However, there are several corneal disorders, such as infections, chemical burns, prior trauma, and immunological diseases, which can destroy this avascular status and cause the ingrowth of pathological BV and LV into the central cornea.

Corneal neovascularization is a major cause of blindness worldwide and together with pathologic LV interferes with the “immune privileged” status. The ingrowth of LV reduces graft survival, especially after surgery. Therefore it is crucial to find efficient strategies to treat hemangiogenesis and lymphangiogenesis preoperatively in pathologically vascularized corneas.

Several approaches to stop progressive neovascularization have reached the clinic, and unfortunately limitations still exist regarding the regression of already established vessels. Corticosteroids have been shown to stop vascularization in the cornea. In contrast, they cannot regress preexisting BV and LV. New options to reduce progressive angiogenesis are, for example, antisense oligonucleotide eye drops, which have been tested in phase II and III clinical trials and have shown to effectively inhibit and (partially) also regress active corneal neovascularization. An anti-VEGF-B antibody fragment has been found to regress established corneal BV in rats. Recently, fine-needle diathermy united with anti-VEGFs seems to be helpful in treating corneal neovascularization, but complications still need to be considered, including intrastromal bleeding and crystalline deposits, which can occur and require repeated treatments. Hence, simple and effective therapies with less injuries to adjacent tissue and fewer side effects to regress pathological corneal neovascularization are still needed.

Photodynamic therapy (PDT) using the photosensitizer verteporfin has been used for several angiogenic diseases in the clinic, such as certain cancers, and in ophthalmology against subfoveal choroidal neovascularization. Moreover, PDT using verteporfin given intravenously (i.v.) produces a significant reduction of corneal neovascular vessels, but whether this method can regress corneal LV is unknown. Interestingly, PDT after corneal intrastromal injection of...
verteporfin can selectively reduce corneal LV without affecting preexisting BV. However, to date it is unknown whether PDT after systemic injection of verteporfin can regress both corneal mature BV and LV. Therefore, whether this PDT pretreatment improves graft survival after subsequent corneal transplantation still needs to be demonstrated. Here we analyzed whether PDT after verteporfin i.v. injection can regress corneal BV and LV, what is the influence of timing of PDT after verteporfin injection, and how this affects graft survival in subsequent experimental murine high-risk keratoplasty.

**Materials and Methods**

**Mice and Anesthesia**

Female BALB/c mice (6–8 weeks) were purchased from Charles River Laboratories (Sulzfeld, Germany) for the suture-induced inflammatory corneal neovascularization assay.21,22 A total of 5 BALB/c mice per group per timepoint were killed to quantify corneal BV and LV (40 mice in total), and 3 mice per group were killed for histology analysis of the cornea and retina (9 mice in total). A total of 12 C57BL/6 female mice aged 6 to 8 weeks (Charles River Laboratories) were used as corneal transplantation donors, and 23 prevascularized aged matched BALB/c mice were used as recipients. The number of total mice used in this study was 84. The mice were deeply anesthetized previous to surgery with an intraperitoneal injection of a mixture of 8 mg/kg ketanest (Godecke AG, Berlin, Germany) and 0.1 ml/kg rompun (Bayer, Leverkusen, Germany). All animal procedures in this study were approved by the local animal care committee and complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmologic and Vision Research.

**Murine Model of Suture-Induced Inflammatory Corneal Neovascularization**

The mouse model of suture-induced inflammatory corneal neovascularization was accomplished as previously described.21,22 Each mouse was deeply anesthetized before corneal suture placement. Three 11-0 nylon sutures (Serag Wiessner, Naila, Germany) extending more than 120° of corneal circumference each were then placed intrastromally with two stromal incursions (Fig. 1A). The outer suture point was chosen near the limbus and the inner point of suture placement was placed near the cornea center equidistant from the limbus to obtain standardized combined hem- and lymphangiogenic responses (Figs. 1B–C). The sutures were left in place for 14 days and were removed before additional procedures.

**Photodynamic Therapy After i.v. Injection of Verteporfin**

The mice were deeply anaeasthetized and all sutures were removed 14 days after suture placement. The treatment group received an i.v. injection via tail vein of 6 mg/m² verteporfin according to the manufacturer instructions (Novartis AG, Basel, Switzerland). The control mice received an i.v. injection of the same volume of phosphate buffered saline (PBS). Thereafter, the treatment group received laser treatment (t = 40 seconds, Power = 48 mW, Ø = 2 mm) of the corneas 3 minutes, 1 hour, or 24 hours after the i.v. injection of verteporfin with the Opal Photoactivator PDT laser (Lumenis GmbH, Dieburg, Germany), and the control mice received the same PDT laser treatment for 40 seconds each. The mice were killed, and the corneas were harvested 3 days, 1 week, and 2 weeks posttreatment to ascertain the timepoint of best effect of PDT treatment on the regression of hem- and lymphangiogenesis.

**Allogeneic Keratoplasty in Mice**

Corneal transplantation was applied 1 week after PDT, which was accomplished 24 hours after systemic administration of verteporfin (control group: PBS). Corneal allografting from 6- to 8-week-old female C57BL/6 mice to previous-treated BALB/c mice was performed as described previously (Fig. 1D).23,24 Donor corneas were marked in the center by using a 2.0-mm bore and then excised with curved Vannas scissors (Geuder AG, Heidelberg, Germany) from C57BL/6 mice. Corneal donor tissues were placed in phosphate-buffered saline (PBS) until grafting. BALB/c mice were anesthetized and used as recipients, and the graft bed was prepared by cutting out a 1.8-mm circle site in the central cornea on the right eye and then discarding the removed corneal tissue. Thereafter, the donor graft was immediately transplanted to the recipient and secured to the host bed with eight equidistant interrupted sutures (11-0 nylon; Serag Wiessner). Antibiotic ointment (FloxaL; Bausch & Lomb GmbH, Berlin, Germany) was applied on the recipient corneal surface and then the eyelids were sutured (tarsorrhaphy) with an 8/0 suture (Serag Wiessner) to prevent graft injury. Corneal sutures and tarsorrhaphy were removed after 1 week. At 2 weeks after transplantation, the grafts were examined once a week until 8 weeks after transplantation.

The graft opacity was scored as previously described.24 Clinical scores of corneal grafts for opacity were as follows: 0, clear; +1, minimal, superficial (nonstromal) opacity; pupil margin and iris vessels readily visible through the cornea; +2,
minimal, deep (stromal) opacity; pupil margins and iris vessels visible; +3, moderate stromal opacity; only pupil margin visible; +4, intense stromal opacity; only a portion of pupil margin visible; and +5, maximum stromal opacity; anterior chamber not visible. Grafts with opacity scores greater than +2 were considered to be rejected.

The treated group and control group were blinded, and the graft opacity scores were graded by two independent investigators who were unaware of grouping.

Immunohistochemistry and Morphological Analysis of Hemangiogenesis and Lymphangiogenesis

The corneas were excised and corneal flatmounts were double stained to quantify hemangiogenesis and lymphangiogenesis as previously described.21-27 Excised corneal flatmounts were rinsed twice in PBS, fixed in acetone, rinsed three times in PBS, blocked in 2% bovine serum albumin (BSA), stained with rat anti-mouse FITC-conjugated CD31 antibody (1:100, BD Pharmingen; BD Biosciences, San Diego, CA, USA) and rabbit anti-mouse lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) antibody (1:200; AngioBio, Del Mar, CA, USA) overnight (4°C, in dark), washed with PBS, stained with cyanine 3 (Cy3)-conjugated goat anti-rabbit secondary antibody (1:100, dianova; Jackson ImmunoResearch, West Grove, PA, USA), and analyzed with a fluorescence microscope (BX53; Olympus Optical Co., Hamburg, Germany). A total of 9 to 12 digital pictures of double-stained flatmounts were taken automatically at 100× magnification with a fluorescence microscope (BX53; Olympus) and assembled to one whole image (multi-image alignment; Fig. 1C). The areas covered by BV or LV were then detected with an algorithm established in the image analyzing program cell^A^F (Olympus Soft Imaging Solutions GmbH, Münster, Germany) as described previously.21-27 Briefly, individual filters were used to modify grayscale images of wholemount staining images before analysis to contrast the vessels and make them more detectable. The area covered by vessels was determined and calculated by setting a threshold including bright vessels and excluding a dark background, and the total corneal area was defined with the innermost vessel of the limbal arcade serving as the border. Then the percentage of the wholemounts covered by BV or LV was calculated. Afterward, the mean percentage of hemangiogenesis and lymphangiogenesis was normalized, as the original mean percentage in the control group was defined as 100% and then the percentage in other groups were correlated with the control group (vessel ratio).

Histology Analysis of the Cornea and Retina

Three eyeballs from each group, the PBS control, and the PDT 24-hour group (laser treatment performed 24 hours after verteporfin i.v. injection) were excised 1 week after corneal PDT. In addition, three naïve eyes were harvested as normal morphological controls. The excised eyeball was fixed, dehydrated, and paraffin embedded. Afterward, sections were cut at 4 μm and collected on slides. The sections were deparaffinized in xylene, rehydrated in a descending alcohol series to water, and then stained with hematoxylin and eosin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), rinsed in water; dehydrated through an ascending series of alcohols and xylenes, and mounted in Neo-Mount (Merck KGaA, Darmstadt, Germany) and coverslipped. The stained sections were examined with an Olympus light microscope (BX53; Olympus). The region of the central cornea and retina were identified in sections, and five sections were imaged in the central cornea and retina region from a section series at 400× magnification with a digital camera (XM10; Olympus) in each eye. These central cornea images were used to count the keratocytes and corneal endothelial cells manually. The thickness of the retina was measured in the retina region with ImageJ 1.50i (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data are shown as mean ± SEM and analyzed using one-way ANOVA test and Student’s t-test. *P < 0.05 was considered a statistically significant difference. Postoperative corneal allo-grafts survival was analyzed with Kaplan–Meier survival curves and the Logrank test. All statistical analyses and graphs were performed using Prism 6 version 6.07 (GraphPad Software, San Diego, CA, USA).

RESULTS

Time-Dependent Differential Regression of Hemangiogenesis and Lymphangiogenesis by PDT

To investigate the effect of PDT on corneal hemangiogenesis and lymphangiogenesis and to identify the best timepoint to perform PDT laser treatment, suture-induced neovascularized corneas were treated with PDT laser 3 minutes, 1 hour, or 24 hours after i.v. injection of the photosensitizer verteporfin and then harvested 3 days posttreatment. As shown in Figure 2, suture-induced corneal angiogenesis was reduced by 24% in mice that received PDT treatment 3 minutes after verteporfin injection intravenously compared to the control mice (PBS injected), whereas the LV in the PDT-treated group had no significant difference when compared with the control group 3 days after treatment. Interestingly, when PDT laser treatment was delivered 1 hour or 24 hours after the i.v. verteporfin injection, both corneal BV and LV were significantly regressed 3 days after PDT: 26.9% mature corneal BV and 43.1% LV in the PDT 1-hour group were regressed when compared with the control (n = 5; *P < 0.05). In addition, a 41.8% reduction of corneal BV and 62.7% of LV were observed in the PDT 24-hour group when compared with the control (BV: n = 5, *P < 0.001; LV: n = 5, *P < 0.01). This shows that the time interval between injection of the photosensitizer and PDT determines whether corneal PDT induces a selective regression of only BV or a combined regression of both BV and LV.

Furthermore, to assess the long-time outcomes of PDT treatment, high-risk corneas that received PDT 24 hours after a verteporfin i.v. injection were killed 1 week and 2 weeks later. As shown in Figure 3, the BV were regressed by 23.1% (Fig. 3A) and the LV were regressed by 56.9% (Fig. 3C) in the treated group 1 week post-PDT treatment. The differences of both vessel types were significant when compared with the control group (BV: n = 5; *P < 0.001; LV: n = 5, *P < 0.05). After 2 weeks, there was a reduction of BV by 19.5% (Fig. 3B) and a reduction of LV by 14.2% (Fig. 3D) in the treated group when compared with the control group (n = 5; *P > 0.05, not significant). This shows that PDT only causes a temporary regression of both vessel types (at least in an inflamed environment).

No Visible Change of Corneal and Retinal Tissue After PDT With Verteporfin i.v. Injection

At 1 week after PDT treatment, no visible morphologic difference of the cornea and retina were found in both the PBS-treated control group and the verteporfin-treated PDT, 24-hour group when compared with naïve eyes on the light microscopic level (Fig. 4). Cell counts measured in the control and PDT 24-hour groups including keratocytes and corneal endothelial cells showed no significant difference (keratocytes: control [PBS], 29.83 ± 4.19 cells/visual field; PDT 24 hours,
30.06 ± 1.9 cells/visual field; naïve, 30.94 ± 1.87 cells/visual field [Fig. 4H]; endothelial cells: control [PBS], 18.22 ± 1.27 cells/visual field; PDT 24 hours, 19.39 ± 0.81 cells/visual field; naïve, 20.06 ± 0.84 cells/visual field [Fig. 4I]). The thickness of the retina was 1160.83 ± 155.36 pixels/visual field in control group, 1326.27 ± 19.91 pixels/visual field in the PDT 24-hour group, and 1359.65 ± 27.54 pixels/visual field in the naïve group with no statistical difference (n = 3; P > 0.05; Fig. 4J).

**FIGURE 2.** Time-dependent differential regression of blood and lymphatic vessels by PDT. Corneal wholemounts stained with FITC-conjugated CD31 and LYVE-1 followed by Cy3-conjugated second antibody 3 days after PDT with i.v. verteporfin or PBS (control) injection. By performing PDT 3 minutes after i.v. injection of verteporfin blood vessels (F, M) were regressed by 24.0% while lymphatic vessels (J, N) showed no significant difference when compared with the control group (J, N) 3 days after PDT. Both corneal blood and lymphatic vessels showed significant reduction when PDT was applied 1 hour (BV: 26.9% [G, M]; LV: 43.1% [K, N]) or 24 hours (BV: 41.8% [H, M]; LV: 62.7% [L, N]) after i.v. injection of verteporfin 3 days after PDT. A–L magnification: ×100; scale bar: 500 µm. Green, blood vessels; red, lymphatic vessels. M–N: n = 5. *P < 0.05; **P < 0.01; ***P < 0.001.

**PDT Pretreatment-Induced Temporary Regression of Both Vessel Types Improves Allograft Survival in Subsequent Murine High-Risk Corneal Transplantation**

As hemangiogenesis and lymphangiogenesis showed the highest reduction rate in mice that received PDT treatment 24 hours after i.v. injection of verteporfin among the three
groups in contrast to the control group (Fig. 2), and only a mild recurrence of both vessel types 2 weeks after treatment in the PDT 24-hour group (Fig. 3), we determined whether the preoperative regression of hemangiogenesis and lymphangiogenesis in high-risk recipients by PDT improves allograft survival after subsequent corneal transplantation. The long-term survival of C57BL/6 donor corneas transplanted into prevascularized BALB/c recipient beds was compared between mice receiving photodynamic therapy 24 hours after i.v. injection of verteporfin, and those receiving PBS injection. As shown in Figure 5, preoperatively PDT-treated, vascularized, high-risk mice achieved a significantly enhanced long-term allograft survival proportion at 8 weeks after operation (75%; n = 12) when compared with the allografts in the eyes of PBS-injected control mice (27%; n = 11; P < 0.05).

DISCUSSION

Several conclusions can be drawn from this study. First, corneal PDT can time-dependently regress selectively corneal hemangiogenesis or both corneal hem- and lymphangiogenesis. Mature corneal BV could be selectively regressed via PDT at an early stage after i.v. injection of verteporfin, and later both pathologic BV and LV could be regressed. Second, PDT used in the herein applied conditions did not cause obvious structural damage to the cornea and retina, although the photosensitizer verteporfin was applied systemically. This method therefore may be an option to be transferred to clinical application. Third, preoperative hemangio- and lymphangio-regression via PDT with i.v. verteporfin injection significantly promotes allograft survival in high-risk, prevascularized eyes after subsequent transplantation. Only temporary regression of both vessel types preoperatively is sufficient to achieve improved graft survival.

Because both preexisting corneal BV and LV play crucial roles in immune-mediated graft rejection after corneal transplantation,3,23,28 effective and safe corneal angio- and lymphangio-regressive therapeutic strategies to promote corneal graft survival especially in prevascularized high-risk recipients that normally have a very poor graft survival is needed. This
study establishes a novel PDT strategy that can regress not only preexisting corneal BV but also LV in vivo. Interestingly, performing PDT 3 minutes after i.v. injection of verteporfin selectively regressed corneal pathologic BV, whereas performing PDT 1 hour or 24 hours after the injection can reduce both BV and LV on day 3 posttreatment (Fig. 2). This finding is partly consistent with previous studies in which corneal BV could be regressed via PDT with i.v. verteporfin injection; LV were not examined in rabbits. Comparing with the results of PDT after intrastromal verteporfin injection study, here we regressed both corneal blood and lymphatic vessels via PDT with i.v. verteporfin injection instead of only LV, which may be because of the different applications of verteporfin.

The reason for time-dependent differential effects of PDT could be that after the i.v. application, verteporfin slowly accumulates in the corneal stroma as a result of leakage from immature vessels. As the size of verteporfin is ideal to be taken up by lymphatics, the linked verteporfin might be taken up by blind-ending LV from the corneal stroma toward the limbal arcades. The porphyrin-derivate verteporfin is then taken up by low-density lipoprotein receptors, activated by laser energy that leads to liberated highly reactive cytotoxic oxygen radicals. Therefore, the liberation of highly reactive cytotoxic oxygen radicals cause damage of endothelial cells and results in coagulation and thrombocyte aggregation and then leads to occlusion of the vessels. With regard to the nonactivated form of verteporfin, the elimination of vertepor-

Figure 4. No significant histologic effect of PDT on corneal and retinal tissues. (A–G) Light microscopy of a representative excised eyes 1 week after PDT, which was performed 24 hours after verteporfin i.v. administration. The central corneal region is marked as box with black arrow and central retina as box with red arrow in A. The amount of keratocytes and corneal endothelial cells in the PDT-treated group (C) and the control group (B) was similar to that observed in naive eyes (D). No significant difference regarding retinal thickness or light microscopic changes was observed when comparing the control group (E), PDT group (F), and naive eyes (G; magnification: 400×; scale bar: 50 µm). (H–J) Quantitative analysis of keratocyte density, endothelial cell count, and retinal thickness 1 week after PDT treatment, which was administered 24 hours post-verteporfin i.v. injection. There was no statistically significant difference of keratocyte, endothelial cell number, and retinal thickness between the control group (PBS injected), PDT group (verteporfin injected), and naive group (n=5; P>0.05).

Figure 5. PDT pretreatment improves subsequent graft survival in high-risk eyes. PDT prior to corneal transplantation significantly improved corneal allograft survival in pretreated high-risk eyes. At 2 weeks after corneal suture placement, PDT was performed 24 hours after verteporfin i.v. injection in the treated group or i.v. PBS injection in the control group. All pretreated mice received orthotopic transplants from C57BL/6 donors 1 week after the treatment and were subsequently observed and scored for allograft opacity once a week. Survival proportions in the PDT-treated mice were 75%, which was significantly higher than in the control mice (25%) 8 weeks after corneal transplantation (control, n=11; PDT, n=12; *P<0.05).
PDT and the Effect on Graft Survival in High-Risk Keratoplasty

fin is primarily through the liver (99.5% of an administered dose), with less than 0.01% of the dose excreted in urine.\textsuperscript{31-36} Most of a verteporfin dose appears to be excreted as unchanged drug in bile.\textsuperscript{31-36}

In our study, PDT administrated 24 hours post-verteporfin injection reduced 41.8% of the BV and 62.7% of the LV (Fig. 2), therefore being the most effective strategy to regress corneal mature BV and LV. Furthermore, even the most effective PDT treatment has no side effect on other corneal and retinal structures, although the photosensitizer verteporfin was applied systemically (Fig. 4), which suggests that this new PDT strategy should be a safe method for use. In addition, as it was previously shown that LV are key for the induction of immune responses after transplantation,\textsuperscript{5} the reduction of LV by PDT significantly promotes graft survival (Fig. 5). Only the temporary preoperative regression of both vessel types is sufficient to achieve improved graft survival. Therefore, this PDT treatment can be an effective and safe corneal angi- and lymphango-regressive therapeutic strategy to promote corneal graft survival in prevascularized high-risk recipients and to improve the outcome of so-called high-risk keratoplasties.

In conclusion, our findings showed that corneal PDT after i.v. verteporfin injection time-dependently regresses mature corneal BV and LV and promotes allograft survival after subsequent high-risk corneal transplantation. This approach can be a promising option in the clinic to treat inflammatory ocular disease and improve the outcomes of high-risk corneal transplantation.

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