Inflammatory Corneal (Lymph)angiogenesis Is Blocked by VEGFR-Tyrosine Kinase Inhibitor ZK 261991, Resulting in Improved Graft Survival after Corneal Transplantation

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PURPOSE. To analyze whether tyrosine kinase inhibitors blocking VEGF receptors (PTK787/ZK222584 [PTK/ZK] and ZK261991 [ZK991]) can inhibit not only hemangiogenesis but also lymphangiogenesis and whether treatment with tyrosine kinase inhibitors after corneal transplantation can improve graft survival.

METHODS. Inflammatory corneal neovascularization was induced by corneal suture placement. One treatment group received PTK/ZK, and the other treatment group received ZK991. Corneas were analyzed histomorphometrically for pathologic corneal hemangiogenesis and lymphangiogenesis. The inhibitory effect of tyrosine kinase inhibitors on lymphatic endothelial cells (LECs) in vitro was analyzed with a colorimetric (BrdU) proliferation ELISA. Low-risk allogeneic (C57Bl/6 to BALB/c) corneal transplantations were performed; the treatment group received ZK991, and grafts were graded for rejection (for 8 weeks).

RESULTS. Treatment with tyrosine kinase inhibitors resulted in a significant reduction of hemangiogenesis (PTK/ZK by 30%, P < 0.001; ZK991 by 53%, P < 0.001) and lymphangiogenesis (PTK/ZK by 70%, P < 0.001; ZK991 by 71%, P < 0.001) in vivo. Inhibition of proliferation of LECs in vitro was also significant and dose dependent (PTK/ZK, P < 0.001; ZK991, P < 0.001). Comparing the survival proportions after corneal transplantation, treatment with ZK991 significantly improved graft survival (68% vs. 53%; P < 0.02).

CONCLUSIONS. Tyrosine kinase inhibitors blocking VEGF receptors are potent inhibitors not only of inflammatory corneal hemangiogenesis but also lymphangiogenesis in vivo. Tyrosine kinase inhibitors seem to have the ability to restrain the formation of the afferent and efferent arm of the immune reflex arc and are therefore able to promote graft survival after corneal transplantation. (Invest Ophthalmol Vis Sci. 2008;49:1836–1842) DOI:10.1167/iovs.07-1314

The healthy cornea is devoid of blood and lymphatic vessels; however, inflammatory conditions such as herpes infection can lead to a breakdown of this angiogenic privilege. Consequently, blood and lymphatic vessels grow into the avascular cornea, reducing transparency and visual acuity. Furthermore, corneal neovascularization is the main risk factor for immune rejection after corneal transplantation. When corneal grafts are placed into an avascular recipient bed (low-risk keratoplasty), 2-year graft survival rates approach 90% under cover of topical steroids, even without HLA matching. In contrast, survival rates of corneal grafts placed into vascularized, not immune-privileged, recipient beds (high-risk keratoplasty) decrease significantly below 50%. In addition, even in the low-risk setting, mild corneal hemangiogenesis and lymphangiogenesis develop after keratoplasty and increase the risk for subsequent immune rejection. Corneal lymphatic vessels enable effective access of donor and host antigen-presenting cells and antigenic material to regional lymph nodes, where accelerated sensitization to graft antigens occurs (afferent arm of immune response). Neovascularization is not only an important factor in mediating immune reactions after (corneal) transplantation, it occurs in other ocular diseases, such as retinal neovascularization, and plays a crucial role in cancer development. Lymphangiogenesis and hemangiogenesis facilitate tumor metastasis and are necessary for the growth of tumors.

Thus, antiangiogenic therapy is a new and promising approach not only to reduce corneal complications secondary to inflammation and immune rejection after corneal transplantation but also to avoid visual loss in retinal neovascularization and to reduce tumor growth and metastasis.

Hemangiogenesis and lymphangiogenesis are driven by the production of angiogenic growth factors. The vascular endothelial growth factor (VEGF) family is central to the process of angiogenesis. The actions of these factors are mediated through receptors possessing intrinsic tyrosine kinase activity: VEGFR-1 (Flt-1), VEGFR-2 (Kdr/Fk-1), and VEGFR-3 (Flt-4). Various antiangiogenic therapy strategies have been used to interfere with the VEGF system in the different pathologic processes of neovascularization. Among others, these approaches comprise VEGF neutralizing antibodies, VEGF receptor antibodies, recombinant soluble VEGF receptor proteins, and receptor tyrosine kinase inhibitors.

PTK787/ZK222584 (PTK/ZK), a small molecule tyrosine kinase inhibitor codeveloped by Novartis (Basel, Switzerland) and Bayer-Schering-Pharma AG (Berlin, Germany), is a potent oral angiogenesis inhibitor that specifically targets all vascular endothelial growth factor receptor tyrosine kinases, including the lymphangiogenic receptor VEGFR-3. Its properties have been described in detail elsewhere. ZK 261991 (ZK911) is a novel oral small molecule VEGF receptor tyrosine kinase inhibitor developed by Bayer-Schering-Pharma AG. It has already been shown that this substance is an inhibitor of
VEGFR-2 (in vitro VEGFR-2 kinase inhibition with an IC_{50} of 5 nM and blockade of cellular receptor autophosphorylation in KDR-PAECs with an IC_{50} of 2 nM; Thierauch K-H, et al., manuscript in preparation).

It has thus far not been analyzed whether small molecule VEGF receptor tyrosine kinase inhibitors can inhibit lymphangiogenesis in vivo, nor is it known whether PTK/ZK and ZK991 are able to do this.

Hence we analyzed the hypothesis that tyrosine kinase inhibitors can reduce the outgrowth of blood and lymphatic vessels in an inflammatory corneal neovascularization assay. Furthermore, we used a lymphatic endothelial cell (LEC) proliferation assay as an in vitro model to analyze a direct inhibiting effect of tyrosine kinase inhibitors on lymphatic endothelial cells. Finally, we investigated whether inhibition of hemangiogenesis and lymphangiogenesis by ZK991 promotes long-term allograft survival in the murine model of corneal transplantation.

METHODS

Animals and Anesthesia

All animal protocols were approved by the local animal care committee and were in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with an intraperitoneal injection of a combination of 8 mg/kg ketamine (Ketanest S; Gödecke AG, Berlin, Germany) and 0.1 mL/kg xylazine (Rompun; Bayer, Leverkusen, Germany). For the suture-induced, inflammatory corneal neovascularization assay, female BALB/c mice were used. For corneal transplantation, donor corneas were excised from female C57Bl/6 mice, and recipients were female BALB/c mice (all mice aged 6 – 8 weeks; Charles River, Sulzfeld, Germany).

Suture-Induced Inflammatory Corneal Neovascularization Assay

The mouse model of suture-induced inflammatory corneal neovascularization was used as previously described.37 Before corneal neovascularization, each animal was deeply anesthetized. Three 11–0 nylon sutures (Serag Wiessner, Naila, Germany) were placed intrastromally with two stromal incursions extending over 120° of corneal circumference each. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen near the corneal center equidistant from the limbus to obtain standardized angiogenic responses. Sutures were left in place for 14 days. The first treatment group received the tyrosine kinase inhibitor ZK991 (50 mg/kg, orally, twice daily), and control mice received equal amounts of the substance vehicle. Corneal sutures were removed 7 days after transplantation, and grafts were then examined microscopically every week until week 8 after transplantation and were scored for opacity as described previously.38 Clinical opacity scores of corneal grafts 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 of +2 or greater after 8 weeks were considered to have been rejected.38 Rejection time was then considered the first time with a score of +2 or greater.

The survival experiment was performed twice and included 13 (control group) and 14 (treatment group) mice in the first experiment and 10 (control group) and 9 (treatment group) mice in the second experiment.

Corneal Wholemounts and Morphologic Determination of Hemangiogenesis and Lymphangiogenesis, Morphologic Determination of Macrophages

Excised corneas from the corneal neovascularization assay were rinsed in PBS and fixed in acetone for 30 minutes. After three additional washing steps in PBS and blocking with 2% bovine serum albumin (BSA) in PBS for 2 hours, the corneas were stained overnight at 4°C with rabbit anti-mouse LYVE-1 antibody (1:50; AngioBio, Del Mar, CA). On day 2, the tissue was washed, blocked, and stained with FITC-conjugated rat anti-mouse CD31 antibody (1:50; Acris Antibodies GmbH, Hiddenhausen, Germany) overnight at 4°C. After a last washing and blocking step on day 3, LYVE-1 was detected with a Cy3-conjugated secondary goat anti-rabbit antibody (1:100; Dianova, Hamburg, Germany). Corneas from the corneal cell recruitment assay were excised, rinsed in PBS, and fixed in acetone for 30 minutes. For 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 FITC-conjugated rat anti-mouse CD11b antibody (1:100; Serotec, Oxford, UK). All corneas were moved to slides (SuperFrost; Menzel-Glaser, Braunschweig, Germany) and covered with Dako (Glostrup, Denmark) fluorescence mounting medium and stored at 4°C in the dark.

Functional and Statistical Analyses

Stained wholemounts were analyzed 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). Each wholemount picture was assembled out of 9 or 12 pictures (depending on the size of the cornea) taken at 100× magnification. The area covered with blood or lymphatic vessels was detected with an algorithm established in the image analyzing program (cellF; Soft Imaging System). Before analysis, gray 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. The mean vascularized area of the control wholemounts (both PTK/ZK and ZK991) from C57Bl/6 mice were excited using a 2.0-mm diameter trephine and cut out with scissors. Until grafting, corneal tissue was placed in saline solution. BALB/c recipients were anesthetized, and the graft bed was also prepared by trephining the right eye and discarding the excised cornea. The donor cornea was immediately applied to the bed and was secured in place with eight interrupted sutures (11–0 nylon; Serag Wiessner). Antibiotic ointment (Gentamycin; Merck Pharma GmbH, Darmstadt, Germany) was placed on the corneal surface, and the eyelids were closed with 7–0 sutures. For the first 2 weeks after corneal transplantation, the treatment group received the tyrosine kinase inhibitor ZK991 (50 mg/kg, orally, twice daily), and control mice received equal amounts of the substance vehicle. Corneal sutures were removed 7 days after transplantation, and grafts were then examined microscopically every week until week 8 after transplantation and were scored for opacity as described previously.39
LYVE-1 (103 cells/well). After 6 hours, medium was replaced with serum-free endothelial cell growth factors such as VEGF-A and bFGF. For ELISA, cells were seeded in 96-well plates in EGM-2 MV medium at a density of 2 x 105 cells/well. After 6 hours, medium was replaced with serum-free medium and was left overnight to attach. On day 2, BrdU (10 μL/mL; Cell Proliferation ELISA, BrdU; Roche, Penzberg, Germany) was added and incubated for 6 hours at room temperature. After six washing steps, BM chemiluminescence was measured with an ELISA reader (Spectra; SLT Labinstruments Deutschland, Gurt, Germany) were coated overnight at 4°C with rabbit anti-human Flt-4 antibody (1:300 in coating buffer; Santa Cruz Biotechnology, Santa Cruz, CA), and graphs were drawn (Prism4, Version 4.03; GraphPad Software).

LEC Proliferation ELISA. Human lymphatic microvascular endothelial cells (HLMVECs; Cambrex Bio Science, Walkersville, MD) were cultured in EGM-2 MV medium (Cambrex Bio Science) according to the manufacturer's instructions. EGM-2 MV medium contains endothelial cell growth factors such as VEGF-A and bFGF. For ELISA, cells were seeded in 96-well plates in EGM-2 MV medium at a density of 2 x 105 cells/well. After 6 hours, medium was replaced with serum-free medium and was left overnight to attach. On day 2, BrdU (10 μL/mL; Cell Proliferation ELISA, BrdU; Roche, Penzberg, Germany) and a tyrosine kinase inhibitor (10 nM and 20 nM) were used. Control cells received the substance vehicle. Cells were fixed and stained after 3 days according to manufacturer's instructions (Cell Proliferation ELISA, BrdU; Roche). Colorimetric analysis was performed with an ELISA reader (Spectra; SLT Labinstruments Deutschland GmbH, Crailsheim, Germany). The mean extinction of the wells per group was as follows: PTK/ZK control group, n = 18; PTK/ZK 10 nM, n = 18; PTK/ZK 20 nM, n = 24; ZK991 control group, n = 18; ZK991 10 nM, n = 18; ZK991 20 nM, n = 24.

LEC Cytotoxicity Assay. HLMVECs (Cambrex Bio Science) were cultured in EGM-2 MV medium (Cambrex Bio Science) according to the manufacturer's instructions. For the cytotoxicity assay, cells were seeded in 96-well plates in EGM-2 MV medium at a density of 5 x 105 cells/well. Medium was changed every second day, and cells were cultured until reaching confluence. Then the medium was replaced with serum-free medium containing a tyrosine kinase inhibitor (PTK/ZK or ZK991; concentration 20 nM) and left overnight. Control cells received the substance vehicle. The next day, cytotoxicity assay (WST-1 based) was performed according to the manufacturer's instructions (Cell Viability and Cytotoxicity Assay, WST-1; Roche). Colorimetric analysis was performed with an ELISA reader (Spectra; SLT Labinstruments Deutschland GmbH). Subsequent statistical analysis was performed (Excel 2000 [Microsoft]; InStat 3 Version 3.06 [GraphPad Software]), and graphs were drawn (Prism4, Version 4.03 [GraphPad Software]). The number of wells per group was as follows: PTK/ZK control group, n = 30; PTK/ZK 20 nM, n = 30; ZK991 control group, n = 30; ZK991 20 nM, n = 30.

VEGFR-3 (Flt-4) Autophosphorylation ELISA. MTP plates (Fluoronic MaxiSorp; 3204006; Zinsser Analytic GmbH, Frankfurt, Germany) were coated overnight at 4°C with rabbit anti-human Flt-4 antibody (1:300 in coating buffer; Santa Cruz Biotechnology, Heidelberg, Germany). On day 2, the plates were washed three times with washing buffer (PBS, 0.1% Tween 20) and blocked with 250 μL blocking buffer (1:10; Roti Block; Carl Roth GmbH, Karlsruhe, Germany) for 1 hour at room temperature. After three washing steps, cell lysates of microvascular endothelial cells (MVECs) were added and incubated overnight at 4°C (before cell lysis, MVECs had been stimulated with 5 nM VEGF-C, and different concentrations of ZK991 had been added). On day 3, the plates were washed three times, and HRP-conjugated antiphosphotyrosine antibody (1:20,000 in TBST+3%; 16–105; Upstate, Charlotteville, VA) was added and incubated for 6 hours at room temperature. After six washing steps, BM chemiluminescence ELISA reagent (1582950; Roche) was added, and luminescence intensity was measured according to the manufacturer's instructions.

RESULTS

Systemic Treatment with Tyrosine Kinase Inhibitors Blocks Corneal Hemangiogenesis and Lymphangiogenesis In Vivo

Treatment with VEGF receptor tyrosine kinase inhibitors resulted in a significant reduction of hemangiogenesis and lymphangiogenesis in vivo. The mean vascularized area of the control wholemounts was set as 100%; vascularized areas were then related to this value (vessel ratio). In comparison with controls, hemangiogenesis was inhibited by 30% (PTK/ZK; P < 0.001) and 53% (ZK991; P < 0.001), and lymphangiogenesis was inhibited by 70% (PTK/ZK; P < 0.001) and 71% (ZK991; P < 0.001) (Fig. 1).

Because of the stronger in vivo effect on angiogenesis despite lower used doses of ZK991 (75 mg/kg, PTK/ZK vs. 50 mg/kg, ZK991) in the corneal neovascularization assay, we decided to perform the inflammatory cell recruitment assay, the VEGFR-3 autophosphorylation assay, and corneal transplantsations only with this substance (although PTZ/ZK showed slightly less LEC proliferation than ZK991 in vitro; Fig. 2).

Tyrosine Kinase Inhibitors Suppress Lymphatic Endothelial Cell Proliferation In Vitro

To analyze whether tyrosine kinase inhibitors have a direct effect on LECs, we studied their effect on LEC proliferation in vitro. Statistical analysis showed that the proliferation of the LECs was significantly suppressed because of treatment with PTK/ZK or ZK991. The inhibitory effects of both tyrosine kinase inhibitors were dose dependent (PTK/ZK 10 nM, inhibition by 46%, P < 0.001; PTK/ZK 20 nM, inhibition by 58%, P < 0.001; ZK991 10 nM, inhibition by 54%, P < 0.001; ZK991 20 nM, inhibition by 59%, P < 0.001; PTK/ZK 10 nM vs. PTK/ZK 20 nM, not significant; ZK991 10 nM vs. ZK991 20 nM, P < 0.001). The cytotoxicity assay showed no toxic effect (PTK/ZK 20 nM vs. control: no significant difference in toxicity, P > 0.8; ZK991 20 nM vs. control: no significant difference in toxicity, P > 0.5).

Treatment with ZK991 Blocks the Recruitment of Inflammatory Macrophages into the Cornea In Vivo

To test whether the anti(lymph)angiogenic effect of these tyrosine kinase inhibitors was, at least partially, also caused by an indirect effect on macrophages (which have been shown to be essential mediators of inflammatory corneal hemangiogenesis and lymphangiogenesis23,25), we analyzed the effect of ZK991 on macrophage recruitment to the cornea. Treatment with ZK991 resulted in a significantly reduced recruitment of CD11b+ and LYVE-1+ cells into the murine cornea. The mean number of macrophages of the control wholemounts was set as 100%, and the number of macrophages per wholemount was then related to this value (cell ratio). In comparison with controls, recruitment of CD11b+ cells was inhibited by 25% (P < 0.001), and recruitment of LYVE-1+ cells was inhibited by 52% (P < 0.001) (Fig. 3).

ZK991 Inhibits VEGFR-3 Autophosphorylation in MVECs after Stimulation with VEGF-C

VEGFR-3 is considered to be a major signal pathway that results in processes leading to lymphangiogenesis. For PTK/ZK, the inhibitory action on VEGFR-3 signaling was published previ-
To demonstrate the inhibitory action of ZK991 on the VEGF receptor 3, we analyzed the inhibition of VEGFR-3 autophosphorylation after stimulation with VEGF-C. It is shown in Figure 4 that ZK991 inhibits VEGFR-3 autophosphorylation concentration dependently with an IC50 of 20 nM. The inhibitory effect of ZK991 on VEGFR-2 has previously been shown (Thierauch K-H, et al., manuscript in preparation).

Treatment with VEGF Receptor Tyrosine Kinase Inhibitor ZK991 Improves Graft Survival after Low-Risk Corneal Transplantation

Comparing the survival proportions after corneal transplantation, treatment with ZK991 significantly improved the graft survival.
survival rates: ZK991, 68%; control, 33%; \( P < 0.02 \) (Fig. 5).

**DISCUSSION**

The experiments performed in this article allow the following conclusions to be drawn. First, VEGF receptor tyrosine kinase inhibitors (PTK/ZK and ZK 991) potently inhibit not only hemangiogenesis but (and this has not been shown before) also lymphangiogenesis in vivo. Second, because VEGF receptor tyrosine kinase inhibitors both inhibit the recruitment of macrophages into the cornea in vivo and directly impair LEC proliferation in vitro, their potent antiangiogenic and antilymphangiogenic effect in vivo seems to be attributed to multiple mechanisms. Given that the cytotoxicity assay ruled out a toxic effect of both substances on LECs, the effect in the performed assay was in fact caused by the inhibition of LEC proliferation. Third, VEGF receptor tyrosine kinase inhibitors are potent pharmacologic agents that inhibit corneal inflammation- and transplantation-associated neovascularization and that promote corneal allograft survival.

Many clinical indications exist for antiangiogenic treatment in ocular diseases, especially of the cornea, among them stopping vision-threatening corneal neovascularization and to improving graft survival after corneal transplantation. In

**FIGURE 3.** Treatment with the VEGF receptor tyrosine kinase inhibitor ZK991 significantly diminishes the recruitment of macrophages to the cornea. In response to an inflammatory stimulus, treated mice show significantly decreased CD11b+ and LYVE-1+ cells in the cornea compared with control. (a–d) Representative sections from corneal wholemounts. Original magnification, 200×. (a, c) CD11b/FITC+ cells. (b, d) LYVE-1/Cy3+ cells. (e) Comparison of CD11b+ cells; recruitment of CD11b+ cells into the cornea was inhibited by 25% (\( P < 0.001 \)). (f) Comparison of LYVE-1+ cells; recruitment of LYVE-1+ cells into the cornea was inhibited by 52% (\( P < 0.001 \)).

**FIGURE 4.** VEGF receptor tyrosine kinase inhibitor treatment (ZK991) suppresses VEGFR-3 autophosphorylation after stimulation with VEGF-C. The inhibition of VEGF receptor autophosphorylation is dose dependent and has an IC_{50} of 20 nM. Autophosphorylation was measured with a chemiluminescence ELISA with HRP.
hemangiogenesis but also lymphangiogenesis in the cornea and are able to promote graft survival after corneal transplantation.

References


