The Role of PDGF Receptor Inhibitors and PI3-Kinase Signaling in the Pathogenesis of Corneal Neovascularization

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**PURPOSE.** Corneal neovascularization remains an unsolved therapeutic problem. Platelet-derived growth factor (PDGF) is directly linked to vessel formation and stabilization. This study was undertaken to elucidate the mechanisms by which PDGF exerts its effects on corneal angiogenesis.

**METHODS.** Corneal neovascularization was induced in C57 mice by removal of the limbal epithelium. When mature vessels appeared after 7 days, mice were treated with the PDGF receptor-β inhibitor AG 1296 or the phosphatidylinositol 3-kinase (PI3-K)-inhibitors wortmannin and LY294002, respectively, using an intraperitoneally implanted miniosmotic pump. At day 14 after scraping, corneas of treated and untreated (control) mice were dissected and immunostained with FITC-CD31 antibody for endothelial cells and with Cy3-SMA (smooth muscle actin) for pericytes. VEGF (vascular endothelial growth factor), ang1/2 (angiopoietin 1 and 2), and PDGF mRNA levels of treated and untreated corneas were determined by real-time RT-PCR.

**RESULTS.** Mice treated with the PDGF inhibitor AG 1296 showed an inhibition of corneal neovascularization of 21.1% and a reduction of pericytes of 52% in the newly formed vessels compared with untreated animals. VEGF, ang1, ang2, and PDGF mRNA expression was reduced in the corneas of AG 1296-treated mice compared with the respective control. Treatment with the PI3-K inhibitors wortmannin and LY29002 had similar effects, inducing a decrease in corneal neovascularization and a reduction of VEGF, ang1, ang2, and PDGF mRNA levels.

**CONCLUSIONS.** Inhibition of the PDGF signal pathway results in loss of pericytes and a reduction in vessel density in the neovascularized cornea that correlates with reduced expression of PDGF, ang1/2, and VEGF mRNA. Furthermore, PI3-K was shown to be involved in the regulation of VEGF, ang1, and PDGF, as the PI3-K inhibitors wortmannin or LY29002 had similar effects. Because PDGF is a known stimulus for PI3-K activation, it can be postulated that the observed decrease in VEGF, ang1/2, and PDGF mRNA levels on administration of the PDGF inhibitor is caused by the decreased activation of the PI3-K signaling cascade. (Invest Ophthalmol Vis Sci. 2006;47:1928–1937) DOI:10.1167/iovs.05-1071

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death. Nevertheless, the direct interaction of PDGF with VEGF and its receptors in the regulation of corneal neovascularization is as yet unknown.

In recent years, several RTK inhibitors with in vivo activity have been developed. Most of these inhibitors have focused on targeting the signaling pathways of VEGF, but diverse chemical compounds were also developed as selective PDGFR kinase inhibitors. Many of these PDGFR kinase inhibitors, including several quinoxalines were found to be highly potent and selective toward the PDGFR and its family members, kit and Flt3. The quinoxaline AG 1296 is supposed to have an effect on downstream PDGFR receptor signaling, when PDGF stimulates binding of phosphatidylinositol 3-kinase (PI3-K) to the PDGFR receptor. Receptor-associated PI3-K activity is blocked by the quinoxaline to the same degree as the inhibition of PDGFR receptor autophosphorylation and the inhibition of phosphorylation of Tyr 751, which is involved in PI3-K binding to the receptor. The importance of the PI3-K in PDGFR signaling, as determined in mutant mice, was recently reviewed by Tallquist and Kazlauskas. In the present study, we examined whether PDGF-RTK inhibition with AG 1296 and PI3-K inhibitors block the catalytic activity of PI3-K without affecting the binding to the receptor.

## Material and Methods

### Animals

All protocols were approved by the Animal Care and Use Committee of the Regierungspräsidium (Cologne, Germany) and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57/Bl6 wild-type mice were purchased from Charles River Laboratories (Sulzfeld, Germany). For local anesthesia, we used oxybuprocaine (Novaris, Nürnberg, Germany). Corneal epithelial and limbal debridement was performed by application of 1.5% ophthalmic ointment consisting of neomycin sulfate 3.5 IE/mg, bacitracin 0.3 IE/mg, and polymyxin B sulfate 7.5 IE/mg (Polyspectran; Alcon Pharma GmbH, Freiburg, Germany) was applied to the cornea immediately after scraping. After epithelial debridement, the animals were randomized to the treatment groups.

### Corneal Neovascularization Model

C57/Bl6-mice were anesthetized by 10 mg/kg xylazine 2% (Riemser Arzneimittel AG, Riems, Germany) and 50 mg/kg ketamine (Ketanest; Parke-Davis, Berlin, Germany). For local anesthesia, we used oxybuprocaine (Novaris, Nürnberg, Germany). Corneal epithelial and limbal debridement was performed by application of 1.5 μL of 0.15 mM NaOH to the central cornea. The corneal and limbal epithelia were removed by scraping with a Toeke corneal knife (Arista Surgical Supply, New York, NY). An anti-biotic ophthalmic ointment consisting of neomycin sulfate 3.5 IE/mg, bacitracin 0.3 IE/mg, and polymyxin B sulfate 7.5 IE/mg (Polyspectran; Alcon Pharma GmbH, Freiburg, Germany) was applied to the cornea immediately after scraping. After epithelial debridement, the animals were randomized to the treatment groups.

### Inhibitor Pump Implantation

The PDGF inhibitor AG 1296 (5 mg/100 μL in DMSO; Calbiochem, Schwalbach, Germany) was diluted in sterile PBS to a final concentration of 10 ng/mL. AG 1296 inhibits PDGF receptor kinase and blocks PDGF α and β receptors without affecting the binding to the receptor. The PI3-K inhibitors wortmannin (1 mg/100 μL in DMSO; Calbiochem) and LY294002 (1 mg/325 μL in DMSO; Calbiochem) were also diluted in sterile PBS to a final concentration of 500 nM. Wortmannin and LY294002 block the catalytic activity of PI3-K without affecting the upstream signaling events. Seven days after the corneas were scraped, the mice were anesthetized as described earlier. Mini osmotic pumps (Alzet model 2001; Alza Corp., Mountain View, CA) were charged with 200 μL of AG 1296, wortmannin, and LY294002. The pumping rate is 1.0 μL/hr, lasting for 7 days. These pumps were implanted in the intraperitoneal cavity of the mice. Control animals received miniosmotic pumps delivering equal amounts of sterile PBS. One week after pump implantation, the animals were killed and their corneas dissected.

### Labeling of Corneal Neovascularization

Mice were killed by cervical dislocation while under anesthesia and the eyes were enucleated to prepare corneal flatmounts for immunohistochemical staining. Corneas were dissected at the limbus and flattened by four radial cuts, rinsed in PBS for 30 minutes, and fixed in 100% aceton (Sigma-Aldrich, Deisenhofen, Germany) for 20 minutes. After they were washed in PBS, the corneas were immunostained with fluorescein isothiocyanate conjugated-coupled monoclonal anti-mouse CD31 antibody (BD-PharMingen, Heidelberg, Germany) at a concentration of 1:500 and double-labeled with Cy3 coupled anti-mouse smooth muscle actin (SMA) antibody (Sigma-Aldrich) in 0.1 M PBS, 2% albumin for 1 hour at 4°C overnight. The CD31 antibody was used for visualization of vascular endothelial cells and the SMA antibody to show vascular pericytes. After they were washed in PBS, the corneas were mounted on slides with an antifading agent (Fluorescent Mounting Medium; DakoCytomation, Hamburg, Germany), and examined by fluorescence microscopes (Axioplan2; Carl Zeiss Meditec, Inc., Oberkochen, Germany; and MZ FLII; Leica, Bensheim, Germany).

### Quantification of Corneal Neovascularization

Digital quantification of corneal neovascularization has been described. Images of the corneal vasculature were captured with a digital camera (ORCA ER; Hamamatsu, Hamamatsu City, Japan) attached to the fluorescent microscopes. The images were viewed on computer (model G4; Apple Computer, Cupertino, CA), resolved at 624 × 480 pixels, and converted to tagged information file format (.tif) files. NIH Image 1.62 (available by ftp at zippy.nimh.nih.gov) or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) was used for the image analysis. The neovascularization was quantified by setting a threshold level of fluorescence, above which only vessels were captured. The entire mounted cornea was analyzed to minimize the sampling bias. The total corneal area was outlined by using the innermost vessel of the limbal arcade as the border. The total area of neovascularization was then normalized to the total cornea area and the percentage of the cornea covered by vessels calculated. All quantitation was performed in a masked manner.

### Visualization of Leakage of Corneal Vessels

PBS (250 μL) containing 50 mg/mL fluorescein isothiocyanate (FITC)-dextran (2000 kDa; Sigma-Aldrich, Deisenhofen, Germany) were injected into the tail veins of inhibitor-treated (AG 1296, wortmannin, and LY294002) and untreated C57/Bl6-mice with scraped corneas. After the dye had circulated for 10 minutes, the mice were killed by cervical dislocation. The eyes were enucleated, and their corneas were fixed for 20 minutes in 100% aceton, to prepare flatmounts as described earlier. Corneas were double-labeled with anti-CD31 antibody (BD-PharMingen, Heidelberg, Germany) and double-labeled with Cy3 coupled anti-mouse smooth muscle actin (SMA) antibody (Sigma-Aldrich) in 0.1 M PBS, 2% albumin for 1 hour at 4°C overnight. The CD31 antibody was used for visualization of vascular endothelial cells and the SMA antibody to show vascular pericytes. After they were washed in PBS, the corneas were mounted on slides with an antifading agent (Fluorescent Mounting Medium; DakoCytomation, Hamburg, Germany), and examined by fluorescence microscopes (Axioplan2; Carl Zeiss Meditec, Inc., Oberkochen, Germany; and MZ FLII; Leica, Bensheim, Germany).

### Quantitative Real-Time RT-PCR

Fresh corneas were dissected and put into a cell lysis reagent (150 μL in DMSO; Calbiochem, Schwabach, Germany) was diluted in sterile PBS to a final concentration of 10 ng/mL. AG 1296 inhibits PDGF receptor kinase and blocks PDGF α and β receptors without affecting the binding to the receptor. The PI3-K inhibitors wortmannin (1 mg/100 μL in DMSO; Calbiochem) and LY294002 (1 mg/325 μL in DMSO; Calbiochem) were also diluted in sterile PBS to a final concentration of 500 nM. Wortmannin and LY294002 block the catalytic activity of PI3-K without affecting the upstream signaling events. Seven days after the corneas were scraped, the mice were anesthetized as described earlier. Mini osmotic pumps (Alzet model 2001; Alza Corp., Mountain View, CA) were charged with 200 μL of AG 1296, wortmannin, and LY294002. The pumping rate is 1.0 μL/hr, lasting for 7 days. These pumps were implanted in the intraperitoneal cavity of the mice. Control animals received miniosmotic pumps delivering equal amounts of sterile PBS. One week after pump implantation, the animals were killed and their corneas dissected.
The mRNA levels for VEGF, ang1, ang2, PDGFb, and GAPDH in the mouse corneas after inhibitor treatment were compared to corneas of untreated mice (Real-time RT-PCR, SYBR Green I; Invitrogen, Eugene, OR) on an thermocycler (iCycler; Bio-Rad Laboratories, Hercules, CA). Using the primer analysis software (Oligo, ver. 4.1; National Biosciences, Plymouth, MN), we selected gene-specific primers suitable for real-time RT-PCR (VEGF: CAG CTA TTG TGG TGA GTT TTG AT; ang1: CTG ATG AGC TGG GAA GAG AAC C and CCG AGA CAT CAG C GTG TGA AG; ang2: GAA GGA CTG GGA AGA CAA CAA and CCA CCA GCC TCC TGA GAG CAT C; PDGFb: CAG GGA GCA GGC AGC CAA GA and CCG AGG AGG GCA ACA ACA TTA TCA; GAPDH: AAC TTT GTG AAG CTC ATT TCC TGG TAT and CCT TGC TGG GCT GGG TGG T). The melting temperature (Tm) of the primers was chosen between 55°C and 58°C, and the expected fragment length was between 125 and 236 bp.

With these primers the mRNA expression of VEGF, together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as calibrator, were analyzed simultaneously in triplicate reactions. The analysis was repeated at least two times. Aliquots of the diluted cDNA of inhibitor treated or nontreated mouse corneas, corresponding to 2 ng initially used total RNA, were mixed with 10× reaction buffer containing Tris-HCl and KCl, (NH4)2SO4, 3.5 mM MgCl2, (Qiagen). 0.2 mM of each dNTP, 0.2 µM of each specific primer, 0.1× nucleic acid stain (Sybr Green 1) and 1 U Taq DNA polymerase (HotStarTaq; Qiagen) in a volume of 50 µL. The following PCR cycle parameters were used: Taq polymerase activation for 15 minutes at 95°C and up to 50 cycles at 95°C for 20 seconds, 57°C for 20 seconds, and 72°C for 20 seconds. Detection of the fluorescent product was performed during the last 90% of the cycles. To confirm amplification specificity, we subjected the PCR products from each primer pair to a melting-curve analysis (data not shown). Genomic DNA contamination was excluded by choosing primers hybridizing to different exons. Moreover, control amplification reactions that were performed with nontranscribed RNA as templates gave only background fluorescence. The quantification data were analyzed with the thermocycler system software (iCycler iQ; Bio-Rad Laboratories), as described. After PCR baseline subtraction performed by the software, the log-linear portion of the fluorescence versus cycle plot was extended, to determine a fractional cycle number at which a threshold fluorescence was obtained (threshold cycle, Ct) for each analyzed gene and GAPDH as the reference. The comparative Ct method was used for quantification of the target genes relative to GAPDH.

The dose dependency of AG 1296 was evaluated by quantitative real-time RT-PCR. To determine the mRNA expression of VEGF, ang1, ang2, and PDGF, miniosmotic pumps charged with the inhibitor or PBS as a control, were implanted 1 week after induction of neovascularization. The area covered by neovascularization was measured 1 week thereafter. The neovascularization decreased from 47.5% ± 2.5% of total area in control corneas to 26.4% ± 16.3% of total area in corneas after PDGF receptor inhibition (Fig. 1; n = 14; P < 0.01). Pericyte coverage was detected by a SMA staining. In control eyes, the ratio of pericytes to endothelial cells was significantly higher (68.9% ± 15%) than the ratio of pericytes to endothelial cells after treatment with AG 1296 (16.9% ± 12.4%; Fig. 2; n = 10; P < 0.0001). There was no difference between pericyte coverage near the limbus compared with the central third in eyes treated with AG 1296 or the control.

In nearly 20% of the treated animals, the lumina of the sprouting vessels were enlarged and formed pre-stage microaneurysms (Fig. 3B, white arrows) in comparison to corneal neovascularization of animals without application of the PDGF-inhibitor (Fig. 3A). In these cases, the mean vessel diameter increased significantly (P < 0.0001) from 14.53 (100%) ± 5.67 µm in control corneas up to 23.50 (161.7%) ± 11.86 µm after PDGF receptor inhibition (Fig. 3C). The newly formed vessels in the other treated corneas were of the same size as the control.

Leakage of Corneal Vessels. Corneal neovessels are leaky, and fluoresceinated dextrans with molecular mass of ≥2000 kDa can extravasate through them. Small-molecular-mass-fluoresceinated dextrans (molecular mass, ≥150 kDa) leaked rapidly from corneal vessels within the first minute, preventing a measurable difference in vascular leakage between control and treated mice (data not shown). Only 2000-kDa dextran showed a slower diffusion through the corneal vessels, which enabled us to differentiate the vessel leakage of control and AG 1296-treated mice (Fig. 4, white arrows). We observed an increase in leakage from 38.4% ± 7.5% in the control to 55.7% ± 7.0% in the AG 1296-treated corneas (n = 3; P > 0.05).

The overall results pooled from representative PCR reactions are shown in Figure 5. The target genes were quantified relative to the standard gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To compare relative expression between groups, all controls were set as 1 (arbitrary units, AU). The mRNA expression of ang1 was significantly reduced from 1.0 ± 0.28 to 0.21 ± 0.09 AU in the corneas of AG 1296-treated phospho-GSK-3β gives a hint of the presence of activated PKB/Akt-kinase. This kinase phosphorylates GSK-3β on serine 9. GSK-3β is unique in that it requires a substrate that has been phosphorylated by a distinct kinase (PKB/Akt-kinase) before it can phosphorylate a substrate. All measurements were performed in duplicate. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.
mice compared with the respective controls. Similarly, ang2 expression was measured at 1.0 ± 0.31 AU compared with 0.30 ± 0.15 AU in AG 1296-treated animals, and PDGF at 1.0 ± 0.27 AU compared with 0.4 ± 0.1 AU (Fig. 5A; \( P < 0.05 \) for all comparisons). There was a trend toward a decrease in VEGF mRNA expression from 1.0 ± 0.31 to 0.55 ± 0.12 AU (\( P < 0.05 \)).

As shown in Figure 6, quantitative real-time RT-PCR analysis was used to investigate the dose dependency of VEGF, ang1, ang2, and PDGF mRNA expression after PDGF inhibition with AG 1296. Concentrations of 0.1, 1, 10, 100, and 1000 ng/\( \mu \)L AG 1296 solved in PBS were tested and applied via miniosmotic pumps. To compare relative expression between groups, all controls were set as 1 (AU) as described earlier. For a better overview, control data are not shown in the graph.

The lowest dose of AG 1296 (0.1 ng/\( \mu \)L) had no effect on mRNA expression of VEGF (1.24 ± 0.41 AU) and ang1 (0.56 ± 0.50 AU), but stimulated the mRNA expression of ang2 (3.94 ± 0.99 AU) significantly (\( P < 0.05 \)) and the mRNA expression of PDGF (3.35 ± 2.89 AU) nonsignificantly (\( P > 0.05 \)), compared with the control (\( n = 8 \)). The dose of 1 ng/\( \mu \)L stimulated the mRNA expression of VEGF (1.56 ± 0.16 AU), ang1 (2.56 ± 0.11 AU), and ang2 (3.34 ± 0.57 AU) significantly (\( P < 0.05 \)). The slight increase of PDGF mRNA expression was not significant (1.32 ± 0.35 AU; \( P > 0.05 \); \( n = 8 \)). The dose of 10 ng/\( \mu \)L AG 1296 inhibits the mRNA expression of VEGF, ang1, ang2, and PDGF, as described earlier and shown in Figure 5A).

Higher doses of AG 1296 (100 and 1000 ng/\( \mu \)L, \( n = 8 \) for all comparisons) also inhibited the mRNA expression of VEGF (0.78 ± 0.16 AU; 0.67 ± 0.14 AU, respectively; \( P > 0.05 \)), ang1 (0.66 ± 0.14 AU; 0.57 ± 0.20 AU; \( P < 0.05 \)), and PDGF (0.32 ± 0.12 AU; 0.24 ± 0.09 AU; \( P < 0.05 \)). At 100 ng/\( \mu \)L AG 1296, we observed no effect on ang2 mRNA expression (1.14 ± 0.53 AU; \( P > 0.05 \)), but 1000 ng/\( \mu \)L inhibited ang2 mRNA expression (0.64 ± 0.19 AU; \( P < 0.05 \)).

**Measurement of Akt-Kinase Activity by Phospho-GSK-3\( \beta \) ELISA.** Phospho-GSK-3\( \beta \) was quantified from protein extracts of cornes by using a sensitive ELISA (Fig. 7). To compare the concentration of phospho-GSK-3\( \beta \) between groups accurately, all samples were corrected for protein concentration (picograms pGSK-3\( \beta \)/mg total protein). There was no significant difference in phospho-GSK-3\( \beta \) between the AG 1296-treated mice 19.5 ± 3.7 pg and their respective controls 16.9 ± 5.3 pg (\( P > 0.05 \)). As activated PKB/Akt phosphorylates GSK-3\( \beta \) to phospho-GSK-3\( \beta \), we can conclude from these results that there was no difference in the activity of Akt kinase between the animal groups.

**Effect of Inhibition of the PI3-Kinase with Wortmannin**

To investigate the effect of the PI3-K inhibitor wortmannin, we treated the mice systemically with the inhibitor as described in the Methods section.
Immunostaining of Corneal Flatmounts. On day 7 after treatment with wortmannin (day 14 after induction of neovascularization), a significant decrease of the neovascularized area was observed (Fig. 1A). The vascularized area in treated mice was 20.2% ± 11.7% compared with 33.2% ± 21.2% for the control ($P < 0.05, n = 23$ Fig. 1B). We did not observe...
enlargement and dilation of the newly formed vessels similar to the effects described for AG 1296. Nevertheless, the pericyte coverage was reduced from 68.9% to 27.7% (P < 0.05; n = 3).

mRNA Expression of VEGF, Ang1, Ang2, and PDGF.
Similar to the RTK inhibition by AG 1296, the inhibition of the PI3-K with wortmannin led to a reduction of VEGF, ang1, ang2, and PDGF mRNA expression (Fig. 5B). VEGF mRNA expression decreased from 1.0 ± 0.15 to 0.56 ± 0.17 AU after treatment with wortmannin. Similarly, ang1 mRNA expression decreased from 1.0 ± 0.33 to 0.54 ± 0.23 AU, Ang2 from 1.0 ± 0.36 to 0.51 ± 0.14 AU (P < 0.05 for all measurements). The reduction of PDGF mRNA expression from 1.0 ± 0.26 to 0.64 ± 0.47 by wortmannin was not significant (P > 0.05).

Measurement of Akt-Kinase Activity by Phospho-GSK-3β ELISA. As shown in Figure 7, the difference between the wortmannin treatment group (15.6 ± 2.8) and the control group (19.6 ± 3.5) was not significant (P > 0.05).

Effect of Inhibition of PI3-Kinase with LY294002
To confirm and further investigate the mechanism of PI3-K inhibition, we tested a second PI3-K inhibitor, LY294002.

**Effect of PDGF on Corneal Angiogenesis**

**Figure 3.** Increased vessel diameter after PDGF inhibition. (A) Corneal neovascularization 14 days after corneal scraping, without treatment. (B) Corneal neovascularization (arrows) 14 days after corneal scraping with 7 days of systemic treatment with the PDGF receptor inhibitor AG 1296. (C) The mean vessel diameter increased significantly (P < 0.001) from 14.53 ± 5.67 μm (100%) in control corneas to 23.50 ± 11.86 μm (161.74%) after PDGF receptor inhibition (n = 130 reading points per image).

**Figure 4.** Visualization of corneal vessel leakage. (A) Increased leakage (white arrows) of FITC-dextran (2000 kDa) through corneal neovessels after systemic treatment with AG 1296, wortmannin, and LY294002. (B) Threshold analysis was performed by NIH Image. Fluorescent area = leakage area + vascularized area (n = 3; P > 0.05).
Immunostaining of Corneal Flatmounts. Systemic treatment with LY294002 was performed 7 days after induction of neovascularization, as described for AG 1296. The neovascularized area decreased from 45.1% ± 19.8% of total area in control corneas to 20.7% ± 8.1% of total area in corneas after treatment with LY294002 (Fig. 1, n = 11; P < 0.01). An enlargement of the newly formed vessels was not observed. As demonstrated in Figure 2, pericyte coverage was reduced from 68.9% ± 15% to 44.5% ± 17% (P < 0.05, n = 10).

Leakage of Corneal Vessels. The leakage within the neovascularized area in LY294002-treated corneas increased compared with the control from 38.4% ± 7.5% to 52.1% ± 8.2% (Fig. 4; P < 0.05, n = 3).

mRNA Expression of VEGF, Ang1, Ang2, and PDGF. Inhibition of the PI3-K with LY294002 reduced, similar to the treatment with wortmannin, the mRNA expression of VEGF, ang1, ang2, and PDGF (Fig. 5C). VEGF mRNA expression decreased from 1.0 ± 0.33 to 0.62 ± 0.10 AU after treatment with LY294002 (P < 0.05). There was also a reduction of ang1 mRNA expression from 1.0 ± 0.32 to 0.12 ± 0.07 AU, and of ang2 from 1.0 ± 0.18 to 0.47 ± 0.22 AU (P < 0.05). There was no significant inhibition of PDGF mRNA expression by LY294002 (1.0 ± 0.35 vs. 0.48 ± 0.17 AU; P > 0.05).

Measurement of Akt-Kinase Activity by Phospho-GSK-3β ELISA. The activity of Akt (concentration of phospho-GSK3β) did not differ significantly between the LY294002 treated animals and control animals (18.1 ± 1.3 vs. 14.1 ± 5.6 pg; P > 0.05, Fig. 7).

Effect of a Combination of AG 1296 and Wortmannin

To investigate the synergistic effects and to further delineate the mechanism of RTK inhibition, we tested the PDGF recep-

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932937/)
tor inhibitor AG 1296 in combination with the PI3-K inhibitor wortmannin.

**Immunostaining of Corneal Flatmounts.** A combined systemic treatment with both AG 1296 and wortmannin was performed on scraped corneas as described for AG 1296 alone. The neovascularized area decreased from 45.4% ± 8.4% of total area in control corneas to 20.5% ± 12.4% of total area in corneas after PDGF receptor inhibition (Fig. 1, n = 8; P < 0.01). As shown in Figure 2, the pericyte coverage was significantly reduced from 68.9% ± 15% to 21.2% ± 5% (P < 0.05, n = 10). An enlargement of the newly formed vessels was not observed.

**mRNA Expression of VEGF, Ang1, Ang2, and PDGF.** Inhibition of both the RTK with AG 1296 and the PI3-K with wortmannin resulted in expression levels similar to those described earlier. The combined treatment reduced the mRNA expression of VEGF, ang1, ang2, and PDGF (Fig. 5D). In detail, VEGF mRNA expression decreased from 1.0 ± 0.33 to 0.62 ± 0.10 AU (P > 0.05). There was a significant reduction of ang1 mRNA from 1.0 ± 0.52 to 0.12 ± 0.07 AU and of ang2 from 1.0 ± 0.18 AU to 0.47 ± 0.22 AU (P < 0.05). The inhibition of PDGF mRNA expression from 1.0 ± 0.35 to 0.48 ± 0.17 AU by AG 1296 and wortmannin was not significant (P > 0.05).

**Measurement of Akt-Kinase Activity by Phospho-GSK-3β ELISA.** Both, the AG 1296/wortmannin-treated mice and their respective controls demonstrated a similar amount of phospho-GSK-3β (17.2 ± 0.4 pg vs. 18.1 ± 4.0 pg; P > 0.05). We conclude that there is no difference in the activity of Akt between the animal groups.

**DISCUSSION**

In the present study we investigated the effect of a small inhibitor of PDGF on corneal neovascularization. The PDGF inhibitor AG 1296 inhibited the vascularized area by 21.2%. Inhibition of the PDGF signaling pathway further resulted in loss of pericytes and a decrease of vessels in the neovascularized cornea correlating with reduced expression of PDGF, ang1, ang2, and VEGF. Administration of wortmannin or LY294002 had similar results, proving that PI3-K is involved in the regulation of VEGF, ang1, ang2 and PDGF. Our findings are consistent with the view that PDGF-BB plays a critical role in maintaining the integrity of blood vessels, and highlights the importance of PDGF-BB and pericytes in the angiogenic process of corneal neovascularization.

PDGF-BB has an angiogenic effect on sprouting endothelial cells and is necessary for pericyte viability. In mice without PDGF-BB or its receptor, capillaries in the brain have few pericytes and develop microaneurysms. A similar situation seems to occur in the neovascularized cornea. We observed a lack of pericytes of 52% and pre-stages of microaneurysms as well as an increase of vessel leakage of 17% in the AG 1296-treated corneas compared with the control.

Of note, the PDGF inhibitor seems to be effective, not only in growing vessels, but also in existing vessels. In our model, treatment with the AG 1296 started on day 7 when corneal neovessels had already started growing from the limbal area. If the PDGF inhibitor had only inhibited the assembly between pericytes and endothelial cells, then pericyte coverage after treatment would have been unchanged in the areas closed to the limbus and would have diminished at the tips of the neovascularization. Our observation in contrast demonstrated a reduction of pericyte coverage independent throughout the neovascularization without a difference between the limbal region and the center of the cornea.

It is likely, that PDGF inhibitors act within a window of plasticity in which the final stability of a vessel is not yet achieved. This is in accordance with reports of the lack of pericyte recruitment in the development of PDGF-B and PDGF-β-deficient mice. Little is known about the plasticity of corneal neovascularization. In human specimens more than 80% of new corneal vessels were covered with pericytes as early as 2 weeks after the onset of neovascularization, and, after only 3 weeks, smooth muscles were observed in animal models, assuming morphologic features of arterioles. This result is in contrast to the clinical observation that even longstanding corneal neovascularization demonstrates fluorescein leakage during angiography, which is supported by our current data indicating that in mice fluoresceinated dextran up to 150 kDa leaked rapidly from corneal vessels within the first minutes. Only 2000-kDa dextran showed a slower diffusion through the corneal vessels and allowed for differentiation of the vessel leakage in control and treated mice.

In the current experiments we created neovascularization by limbal debridement after application of NaOH to the cornea. This leads to the damage of limbal stem cells and associated alterations of the corneal surface. We had previously demonstrated that VEGF expression and neovascular growth is closely related to corneal surface alterations in this model. The detailed effects of PDGF inhibition on limbal stem cell function remain to be elucidated; however, our current data suggest that PDGF rather directly affects vessel integrity and growth and may only indirectly affect limbal stem cell function.

It has been demonstrated, that VEGF is necessary for neangiogenesis in the mouse model of corneal neovascularization that we used for our experiments. Corneal leukocytes, via their own VEGF have been shown to constitute most of the VEGF gene expression in the injured cornea. The prevention of CD18- and ICAM-1-dependent leukocyte emigration leads to a reduction of leukocyte VEGF and thereby to a suppression of corneal neovascularization. The finding that polymorphonuclear leukocytes induce a PDGF release from IL-1β-treated endothelial cells and that PDGF increases mitogenic activity of endothelial cells is consistent with our data that inhibition of PDGF RTK leads to a reduced mRNA expression and vessel growth.

The role of PDGF in the regulation of corneal neovascularization is only partly understood. Homodimers of PDGF-BB, and PDGF-CC, and the heterodimeric PDGF-AB induce a similar angiogenic pattern in the mouse cornea if used in the corneal pocket assay. Blood vessels stimulated by all four isofoms of the PDGFs were dilated in addition to PDGF-AA, exhibiting a reduced density of vessels. We observed in approximately
20% of the treated animals enlarged lumina in the sprouting vessels. This finding was not associated with altered SMA staining in the respective corneas or with an altered expression of VEGF or angiopoietins. Potential explanations of this rather contradictory finding include a reduction of the pericyte coverage resulting in vessel instability and thus enlargement. Further, a solitary inhibition of certain PDGF isoforms may play a role, leaving other isoforms unaffected or even, as a counter-regulation, stimulating their expression. Still these two possibilities do not uncover why only 20% of the mice demonstrated these distinctive features. It seems unlikely that these enlarged vessels, which we found only in the treatment group, are a result of scraping, as similar alterations have not been described using this model before.\(^\text{3,35-39,40}\) We suspect that interindividual differences in the response to the agent or in the absorption after pump implantation may play a role. In this direction, heterogeneity of angiogenesis and blood vessel maturation has been reported in various tissues.\(^\text{31,32}\) Thus, the PDGF receptor inhibitor AG 1296 may exert a differential effect on corneal vessels due to a variable sensitivity to PDGF.

This effect was described for a distinctive kinase inhibitor targeting PDGF activity (imatinib mesylate) that specifically acts in tumor vessels, but does not affect healthy vessels of the organism.\(^\text{42}\) Imatinib mesylate (Gleevec; Novartis, East Hanover, NJ) also inhibits the RTKs for platelet-derived growth factor. In our experiments, we did not see any effect of AG 1296 on vessels, except in the growing corneal neovascularization.

It is highly plausible that the establishment of functional and stable vessels requires a combination of several growth factors.\(^\text{5,45-47}\) Besides the PDGF family, angiopoietins may play critical roles in recruiting mural cells (smooth muscle cells and pericytes) to endothelial cells.\(^\text{12,48}\) Angiopoietins 1 and 2 are growth factors that act specifically on endothelial cells. They are secreted by periendothelial cells such as vascular smooth muscle cells or pericytes.\(^\text{49,50}\) Angiopoietins are known to play a role in corneal neovascularization.\(^\text{51}\) Nevertheless, the interaction between the angiopoietin and PDGF pathways remains to be elucidated. We were able to demonstrate in the present study that angiopoietin expression in the mouse cornea can be dose-dependently modulated by the PDGF inhibitor AG 1296.

VEGF is the most widespread mediator of ocular angiogenesis. PDGF-BB induces VEGF expression in a time- and dose-dependent manner in perivascular cells by increased transcription of VEGF mRNA.\(^\text{52,55}\) In our murine scrape model of inflammatory corneal angiogenesis, inhibition of PDGF-RTK decreased VEGF mRNA expression and neovascularization. To maintain vascular homeostasis, the complex gene regulations include both positive and negative stimuli between endothelial cells and smooth muscle cells. The importance of the PI3-K in PDGF signaling, as determined in mutant mice, was recently reviewed by Tallquist and Kazlauskas.\(^\text{10}\) PDGF-BB stimulation upregulates angiopoietin-1 expression in smooth muscle cells through the PI3-K and PKC pathways.\(^\text{54}\) This finding is in agreement with our data demonstrating that inhibition of PDGF-RTK in vivo results in downregulation of angiopoietin-1 mRNA expression. Inhibition of PI3-K with wortmannin and LY294002 equally reduced the expression of angiopoietin-1, suggesting that PI3-K is indeed involved in our model. Similarly, we hypothesized that the observed decrease in VEGF expression on administration of the PDGF-RTK inhibitor is similarly caused by the decreased activation of the PI3 kinase signaling cascade. Both wortmannin and LY294002 did not reduce PDGF expression. Furthermore, they had no additive effect with AG 1296 in reducing corneal neovascularization and VEGF and angiopoietin expression. This confirms that PI3-K is a key mediator in the downstream signaling of PDGF. A downstream target of the PI3-K is protein kinase B (PKB/Akt). Akt kinases are known to be regulators of cell signaling and have been shown to be stimulated by PDGF.\(^\text{55-57}\) Chadhary et al.\(^\text{58}\) demonstrated that PDGF-BB increases the phosphorylation of Erk1/2 and Akt and that Akt is activated by PDGF-BB. Inhibition of the PDGF signaling pathway by both RTK and PI3-K inhibition did not result in a significant alteration of Akt–kinase activity in our model, indicating that alternative pathways are involved or PDGF via PI3-K acts directly on VEGF and angiopoietin expression.

Among the several PDGFK kinase inhibitors available, the quinoxaline AG 1296, which was used in our experiments, was found to be highly potent and selective toward the PDGFK.\(^\text{22}\) Because of its small molecular structure, it may have advantages over other PDGF kinase inhibitors in tissue penetration, which could be advantageous (e.g., for intraocular topical applications in neovangiogenic retinal or choroidal disease). Taken together, our data demonstrate that an RTK inhibitor is able to interact specifically with inflammatory corneal neovascularization. Further investigations are needed to elucidate the effectiveness on specific diseases.

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**References**


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