Tie2 Receptor Ligands, Angiopoietin-1 and Angiopoietin-2, Modulate VEGF-Induced Postnatal Neovascularization

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Abstract—Angiopoietin-1 (Ang1) has been recently identified as the major physiological ligand for the tyrosine kinase receptor Tie2 and assigned responsibility for recruiting and sustaining periendothelial support cells. Angiopoietin-2 (Ang2) was found to disrupt blood vessel formation in the developing embryo by antagonizing the effects of Ang1 and Tie2 and was thus considered to represent a natural Ang1/Tie2 inhibitor. In vivo effects of either angiopoietin on postnatal neovascularization, however, have not been previously described. Accordingly, we used the cornea micropocket assay of neovascularization to investigate the impact of angiopoietins on neovascularization in vivo. Neither Ang1 nor Ang2 alone promoted neovascularization. Pellets containing vascular endothelial growth factor (VEGF) alone induced corneal neovasculature extending from the limbus across the cornea. Addition of Ang1 to VEGF (Ang1+VEGF) produced an increase in macroscopically evident perfusion of the corneal neovasculature without affecting macroscopic measurements of length (0.58±0.03 mm) or circumferential neovascularity (1.36±1.0°). In contrast, pellets containing Ang2+VEGF promoted significantly longer (0.67±0.05 mm) and more circumferential (160±15°) neovascularity than VEGF alone or Ang1+VEGF (P<0.05). Excess soluble Tie2 receptor (sTie2-Fc) precluded modulation of VEGF-induced neovascularization by both Ang2 and Ang1. Fluorescent microscopic findings demonstrated enhanced capillary density (fluorescence intensity, 2.55±0.23 e−9 versus 1.23±0.17 e−9, P<0.01) and increased luminal diameter of the basal limbus artery (39.0±2.8 versus 27.9±1.3 μm, P<0.01) for Ang1+VEGF compared with VEGF alone. In contrast to Ang1+VEGF, Ang2+VEGF produced longer vessels and, at the tip of the developing capillaries, frequent isolated sprouting cells. In the case of Ang2+VEGF, however, luminal diameter of the basal limbus artery was not increased (26.7±1.9 versus 27.9±1.3, P=NS). These findings constitute what is to our knowledge the first direct demonstration of postnatal bioactivity associated with either angiopoietin. In particular, these results indicate that angiopoietins may potentiate the effects of other angiogenic cytokines. Moreover, these findings provide in vivo evidence that Ang1 promotes vascular network maturation, whereas Ang2 works to initiate neovascularization. (Circ Res. 1998;83:233-240.)

Key Words: angiopoietin ■ Tie2 receptor ■ vascular endothelial growth factor ■ angiogenesis ■ endothelial cell
neovascularization has led to the dual inferences that Ang1 may induce maturation and stabilization of developing neo-
vasculature, whereas Ang2 may cause destabilization re-
quired for additional sprout formation. In vivo effects of
either angiopoietin on postnatal neovascularization, however, have not been previously described.

Accordingly, in the present series of experiments, we used the mouse corneal micropocket assay to determine the func-
tion of these novel endothelial cell–specific cytokines on postnatal neovascularization in vivo.

Materials and Methods

Reagents

Human Ang1, Ang2, and sTie2-Fc were kindly supplied by Regen-
eron Pharmaceuticals, Inc. The recombinant protein was formulated in buffer consisting of 0.05 mol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% Chaps. Ang1 is a genetically engi-
neered variant of naturally occurring Ang1 that retains similar properties in all assays. Soluble Tie2-Fc is a recombinant fusion protein consisting of the ectodomain of the Tie2 receptor fused to the Fc portion of human IgG1. Previous studies have shown that sTie2-Fc binds to Ang1, Ang2, and mouse angiopoietins. Recombin-

ant human VEGF165 was a generous gift of Dr Bruce Keyt (Genentech Inc, South San Francisco, Calif).

Mouse Cornea Neovascularization Assay

Age-matched (8 weeks) C57BL/6J male mice (Jackson Labs, Bar
Harbor, Me) were used for all experiments. Preoperative anesthesia was limited to intraperitoneal pentobarbital injection (160 mg/kg). Corneal pockets were created in the eyes of each mouse using a modified von Graefe cataract knife. A 0.34×0.34-mm sucrose aluminum sulfate (Bukh Meditec) pellet coated with hydron polymer type NCC (IFN Science) containing 1 of the agents indicated below was implanted into the corneal pocket. Pellets were positioned 1.0 mm from the corneal limbus, and erythromycin ophthalmic ointment (E. Foufara) was applied to each operated eye. The corneas of all mice were routinely examined by slit-lamp biomicroscopy on postoperative days 4 or 6 after pellet implantation. Vessel length and the arc of corneal circumference measured on the sixth postoperative day when all corneas were photographed. After these measurements were com-
pleted, mice received an intravenous injection of 500 µg of the endothelial cell–specific marker BS-1 lectin conjugated to FITC (Vector Laboratory). Thirty minutes later, the animals were killed. The eyes were enucleated and fixed in 100% methanol solution for immunohistochemical staining.

Study Design

Age-matched (8 weeks) C57BL/6J male mice (Jackson Labs, Bar Har-
bor, Me) were divided into 9 groups. Each mouse underwent surgical implan-
tation of pellets containing 1 of the following: control buffer (0.05 mol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Chaps) alone; Ang1 alone (300 ng/pellet); Ang2 alone (300 ng/pellet); control buffer with VEGF (control+VEGF, 300 ng/pellet); Ang1 with VEGF (Ang1+VEGF, 300 ng/pellet); Ang2 with VEGF (Ang2+VEGF, 300 ng/pellet); sTie2-Fc with control buffer and VEGF (sTie2-Fc+control+VEGF, 300 ng/pellet); sTie2-Fc with Ang1 and VEGF (sTie2-Fc+Ang1+VEGF, 300 ng/pellet); and sTie2-Fc with Ang2 and VEGF (sTie2-Fc+Ang2+VEGF, 300 ng/pellet).

Quantification of Corneal Neovascularization

Integrated optical density (IOD) from the cornea-derived fluorescent slides was measured using a Cooke SVGA cooled CCD camera attached to a Nikon Diaphot inverted microscope. The digital images were processed using Media Cybernetics Image Pro Plus 3.0 soft-
ware. Initially, all fluorescent images from different groups were acquired directly from the microscope to the computer screen using the same 2-second exposure time. The contour of the vascular area was traced, and the IOD was automatically calculated. The IOD equals the sum of all pixels, each of which has a value from 1 to 4096 gray levels, divided by the whole area. Results were expressed as mean±SEM. The differences between each individual group were evaluated by ANOVA. Differences were considered significant at P<0.05.

Immunohistochemistry

For immunohistochemical staining, the excised eyes were fixed immediately in 100% methanol overnight. After enucleation, the excised corneal hemispheres prepared under the dissecting micro-
scope were embedded in paraffin. Sections of 5 µm thickness, cut either in cross section or longitudinally, were used for immunohis-
tochemistry. Immunohistochemical staining for endothelial cells was car-
ried out using a rat monoclonal antibody against mouse CD-31 (Pharmingen). For detection of periendothelial cells, a mouse mono-
clonal antibody against SM α-actin conjugated with alkaline phos-
phatase (Sigma) was used. A polyclonal peroxidase–labeled anti-rat
immunoglobulin (Signet Laboratories) was used as secondary anti-
body for CD31. Vascular lumens bordered by CD31-positive cells were counted on cross sections of neovascularized cornea. To evaluate the frequency of periendothelial cell recruitment in corneal neovascularization, SM α-actin–positive cells were manually counted on tissue sections cut in cross section as well. The number of positive lumens in each case was expressed as mean±SEM. Group differences were analyzed by ANOVA. Differences were considered statistically significant at P<0.05.

Results

On the sixth postoperative day after pellet implantation, corneal neovascularization was evaluated qualitatively (Figure 1) and quantitatively using slit-lamp biomicroscopy to measure vessel length and circumferential neovascularity (Figure 2). Neither the control buffer pellet nor pellets containing Ang1 or Ang2 alone induced neovascularization. Pellets containing VEGF alone induced corneal neovascularity extending from the limbus across the cornea (Figure 1); these vessels constituted 120±9° of the corneal circumference and measured 0.52±0.03 mm in length. The addition of Ang1 to VEGF (Ang1+VEGF) produced an increase in macroscopically evident perfusion of the corneal neovascular core (Figure 3) without affecting macroscopic mea-
surements of length (0.58±0.03 mm) or circumferential neovas-
cularity (136±15°). In contrast, pellets containing Ang2+VEGF promoted significantly longer (0.67±0.05 mm) and more cir-

cumferential (160±15°) neovascularity than VEGF alone or Ang1+VEGF (P<0.05). An excess of sTie2-Fc precluded modulation of VEGF-induced neovascularization by either angiopoietin (Ang2 as well as Ang1). Neovascularization induced by control buffer+VEGF was not attenuated by sTie2-Fc,
excluding a potential contribution from endogenous angiopoietins.

Fluorescence microscopic findings resulting from pretreatment immunostaining with BS-1 lectin permitted more detailed histopathologic analysis. Compared with control + VEGF, Ang1 + VEGF enhanced capillary density and luminal diameter of the basal limbus artery (Figure 3); this corresponded to a significant increase in fluorescence intensity ($2.55 \pm 0.23$ e$^{-1}$ versus $1.23 \pm 0.17$ e$^{-1}$, $P < 0.01$; Figure 4) and luminal diameter of the basal limbus artery ($39.0 \pm 2.8$ versus $27.9 \pm 1.3$ μm, $P < 0.01$; Figure 5). In contrast to Ang1 + VEGF, Ang2 + VEGF produced longer vessels and, at the tip of the developing capillaries, frequent isolated sprouting cells (Figure 3). These findings were not observed with Ang1 + VEGF. In the case of Ang2 + VEGF, however, luminal diameter of the basal limbus artery was not increased ($26.7 \pm 1.9$ versus $27.9 \pm 1.3$, $P = \text{NS}$; Figure 5). The differential effects observed for Ang1 versus Ang2 in combination with VEGF were apparent at day 4 (Figure 6).

When sTie2-Fc was included with VEGF and control buffer in the implanted pellets, histopathologic vascular morphometry was unchanged. In contrast, sTie2-Fc obviated modulation of corneal neovascularization resulting from addition of Ang1 and Ang2 to the implanted pellets. These findings suggest an agonist function for both Ang1 and Ang2 in modulating VEGF-induced corneal neovascularization.

Light microscopic sections stained with hematoxylin and eosin disclosed only rare inflammatory cells associated with corneal neovascularization. A similar extent of low-level inflammation was observed after implantation of the control buffer pellet or with pellets containing Ang1 or Ang2 alone. Pellets containing VEGF alone induced corneal neovascularization extending from the limbus across the cornea. The combination of Ang1 and VEGF showed enriched vascularity, although length (distance from limbus toward pellet) of the new vessels was similar to that seen with VEGF alone. Ang2 + VEGF resulted in vessels with increased length (as well as circumferential extent of neovascularity; see Figure 2) compared with VEGF alone or Ang1 + VEGF. Addition of sTie2-Fc did not change vascularity induced by VEGF but reduced both vascularity seen with Ang1 + VEGF and increased vessel length seen with Ang2 + VEGF.

![Figure 1](image1.png)  
**Figure 1.** Macroscopic photographs of mouse cornea obtained by slit-lamp biomicroscopy 6 days after pellet implantation. Macroscopic evidence of corneal neovascularization was not observed with control buffer pellet or with pellets containing Ang1 or Ang2 alone. Pellets containing VEGF alone induced corneal neovascularization extending from the limbus across the cornea. The combination of Ang1 and VEGF showed enriched vascularity, although length (distance from limbus toward pellet) of the new vessels was similar to that seen with VEGF alone. Ang2 + VEGF resulted in vessels with increased length (as well as circumferential extent of neovascularity; see Figure 2) compared with VEGF alone or Ang1 + VEGF. Addition of sTie2-Fc did not change vascularity induced by VEGF but reduced both vascularity seen with Ang1 + VEGF and increased vessel length seen with Ang2 + VEGF.

![Figure 2](image2.png)  
**Figure 2.** Vessel length (millimeters) and circumferential extent (degrees) of neovascularization measured macroscopically by slit-lamp biomicroscopy 6 days after surgery. Neovascularization induced by Ang2 + VEGF is characterized by a statistically significant increase in both vessel length and circumferential extent of neovascularity compared with control (Cont) pellet or Ang1 + VEGF. Introduction of sTie2-Fc obviates both findings ($P < 0.05$).
buffer pellet as well as pellets containing Ang1 or Ang2 alone, none of which induced neovascularization. This suggests that an inflammatory response is not a prerequisite for neovascularization in this cornea model, as suggested by others.16

To investigate specific histoarchitectural differences in neovascularization resulting from Ang1 versus Ang2 in combination with VEGF, we analyzed light microscopic sections prepared for immunohistochemical staining with antibodies to CD31 and SM α-actin. Immunostaining for CD31 disclosed an increased number of vascular lumens per cross section in tissue sections retrieved from animals receiving Ang1+VEGF versus control buffer+VEGF (155.8±32.1 versus 33.0±3.9 per cross section, P<0.05). Sections retrieved from the Ang2+VEGF group also disclosed an increased number of vascular lumens compared with the control+VEGF group (80.6±16.2 versus 33.0±3.9 per cross

![Figure 3](image)

**Figure 3.** In situ BS-1 lectin fluorescent staining of corneal limbus vessels 6 days after pellet insertion. Limbus vessels were unchanged in response to pellets containing control buffer, Ang1 alone, and Ang2 alone. Pellet containing VEGF alone resulted in corneal neovascularization. Further enhancement of vascularity and limbus arterial diameter (arrowhead) was observed with the combination of Ang1+VEGF; note that the length of the new vascular sprouts, however, is similar to those seen with VEGF alone. In contrast, the length of the vascular sprouts was observed to be increased in response to Ang2+VEGF; moreover, frequent isolated endothelial cells (arrows) were observed at the growing edge of the capillary tips. The fluorescent photomicrographs confirm macroscopic observations regarding addition of sTie2-Fc: no change in VEGF alone but substantial attenuation of vascularity in the case of Ang1+VEGF and vessel length as well as vascularity in the case of Ang2+VEGF.

![Figure 4](image)

**Figure 4.** Quantitative analysis of neovascularature induced by control buffer (Cont)+VEGF, Ang1+VEGF, or Ang2+VEGF. Measurement of integrated optical density from fluorescent photomicrograph of cornea with digital image analysis was used to determine fluorescence density. Both Ang1+VEGF and Ang2+VEGF revealed significant increase in fluorescence density in comparison to VEGF alone (*P<0.01).
section), but this failed to achieve statistical significance ($P=\text{NS}$).

While both Ang1 and Ang2 resulted in an increased number of vascular lumens, the neovasculature developing in response to each of these 2 cytokines differed in 2 respects. First, the lumens of vessels constituting the neovasculature of Ang1+VEGF group were typically larger than those of the control+VEGF group; those vessels that developed in response to Ang2+VEGF, however, were of lesser luminal diameter than those in the Ang1+VEGF group and in this respect similar to those of the control+VEGF group (Figure 7). While this feature was difficult to quantify because of the diminutive size of the lumens in general, it is illustrated qualitatively in the representative histological sections shown in Figure 7. Second, the neovasculature of these 2 groups was characterized by a marked difference in periendothelial cell frequency (Figure 8). Recruitment of periendothelial cells was more frequent in Ang1+VEGF (50.0±14.2 per cross section) than in Ang2+VEGF (11.6±1.4) or control+VEGF (5.4±1.4) groups.

**Discussion**

We used the cornea micropocket assay of neovascularization to investigate the impact of angiopoietins on neovascularization in vivo. This cytokine-containing sucralfate assay is unique by virtue of the fact that it yields a predictable, persistent, and aggressive neovascular response that is dependent on direct stimulation of blood vessel development rather than indirect neovascularization induced by inflammation.16 We observed only very few inflammatory cells in association

![Figure 5. Luminal diameter of cornea limbus arteries treated with control buffer (Cont) +VEGF, Ang1+VEGF, or Ang2+VEGF. The luminal diameter of basal limbus artery in response to Ang1+VEGF was significantly increased in comparison with VEGF alone or Ang2+VEGF (P<0.05).](image)

![Figure 6. Corneal neovascularization induced by VEGF with control buffer, Ang1, or Ang2 and evaluated by in situ BS-1 lectin fluorescence staining 4 days after pellet insertion. A, Appearance of nascent vascular sprouts induced by VEGF. B, More complex vascularity induced by combination of Ang1+VEGF. C, Ang2+VEGF also results in more robust vasculature, including increased length of sprouts and plethora of isolated endothelial cells at the growing tips of the sprouts. D, Higher-power photomicrograph of Ang1+VEGF showing lumen development at 4 days. E, Higher power view of Ang2+VEGF showing isolated endothelial cells (arrows) at sprout tips.](image)
with corneal neovascularization. The fact that a similarly limited number of inflammatory cells was observed after implantation of the control buffer pellet as well as pellets containing Ang1 or Ang2 alone—none of which induced neovascularization but failed to achieve statistical significance ($P > 0.05$) suggests that an active inflammatory response is not a prerequisite for neovascularization in this cornea model, as suggested by others. Neovascularity related to pellet implantation was thus exclusively cytokine dependent.

Previous reports have suggested that angiopoietins alone were not mitogenic for endothelial cells in culture nor did they induce tube formation within a collagen substrate. In the present experiments, neither Ang1 nor Ang2 alone promoted neovascularization. In contrast, however, when coadministered with VEGF, Ang1 had an important modulatory effect on corneal neovascularization. The increase in vascular density seen with Ang1+VEGF is consistent with previous studies showing that combined administration of endothelial mitogens may have a synergistic effect on neovascularization. The increase in luminal diameter of vessels constituting the neovasculature as well as the limbus artery may represent an additional modulatory effect, although the possibility that this was due to vasodilation resulting from augmented flow into the enriched plexus of corneal vessels.

![Figure 7](image)

**Figure 7.** Immunohistochemical staining of neovasculature induced by VEGF, Ang1+VEGF, and Ang+VEGF. A through C, Representative photomicrographs of tissue sections stained with antibody to CD31. The number of vascular lumens in Ang1+VEGF was significantly ($P < 0.05$) higher than that observed with control buffer+VEGF. Ang2 increased the number of vascular lumens compared with control buffer+VEGF but failed to achieve statistical significance ($P > 0.05$). Note that Ang1, but not Ang2, also resulted in increased size of vascular lumens. D through F, Sections stained with antibody to SM α-actin show that Ang1, but not Ang2, also increased recruitment of periendothelial cells.

![Figure 8](image)

**Figure 8.** A, Quantification of vascular lumens per cross section. B, Quantification of SM α-actin-positive cells per cross section ($P < 0.05$ versus control+VEGF by ANOVA).
cannot be excluded. The development of a more complex vascular network characterized by vessels of increased luminal size and more frequent recruitment of periendothelial cells may be considered evidence for a maturation effect of Ang1 on VEGF-induced neovascularization. This idea was suggested by Suri et al., who characterized the vascular network of Ang1−/− mice as less complex and comprising fewer large arteries, most of which were of lesser caliber and more poorly invested by periendothelial cells than the vascularity of wild-type mice.

The modulatory impact of Ang2 on VEGF-induced neovascularization was morphologically distinct and thereby quite different from simple enhancement of vascular density induced by VEGF. The circumferential extent and the length of vessels cooperatively induced by Ang2+VEGF were significantly increased compared with control+VEGF or Ang1+VEGF. Moreover, histological examination disclosed isolated migrating endothelial cells at the leading tip of capillaries coursing toward the pellet. Neither the size of the limbus artery nor that of vessels constituting the neovascularature, however, were enhanced in the case of Ang2+VEGF. We interpret these findings to indicate that the effect of Ang2 on VEGF-induced neovascularization is to promote vascular destabilization and sprouting required to initiate neovascularization. Consistent with this interpretation, Maisonpierre et al. previously suggested that Ang2 may collaborate with VEGF at the leading edge of invading vascular sprouts by blocking constitutive stabilization or the maturation function of Ang1, thus allowing vessels to revert to and remain in a more plastic state in which they may be more responsive to sprouting signals provided by VEGF.

The effect of Ang1 or Ang2 on VEGF-induced neovascularization constitutes inferential evidence for Tie2 expression in the cornea micropocket assay. Coadministration of sTie2-Fc had no inhibitory effect on angiogenesis induced by VEGF alone, indicating that endogenous angiopoietins do not contribute to corneal neovascularization. Moreover, we found very few α-SM actin–positive periendothelial cells in VEGF-induced neovascularature, whereas abundant periendothelial cells were seen in the cornea of Ang1+VEGF group. This suggests that periendothelial cell recruitment in neovascular development is dependent on Ang1 and that VEGF-induced corneal neovascularization, in the absence of endogenous angiopoietin, does not include Ang1-dependent periendothelial cell recruitment for vascular maturation, at least up to day 6.

Second, coadministration of sTie2-Fc abolished the modulating effects of Ang1 and Ang2 on VEGF-induced neovascularization. This finding is consistent with the recent demonstration by Lin et al. that a soluble form of Tie2-Fc prevents the angiogenic activity of VEGF in vivo.

findings thus constitute the initial demonstration that Ang2 may activate the Tie2 receptor in vascular cells and support the notion that Ang2 may facilitate neovascularization.

Because Tie2 expression is restricted to endothelial lineage and certain phenotypes of immature hematopoietic cells, it is possible that the cells responding to Ang2 in the present experiment could be either activated endothelial cells or endothelial progenitor cells derived from circulating blood that have homed to the site of corneal neovascularization. We have found previously that endothelial progenitor cells express the Tie2 receptor and, more recently, shown that endothelial progenitor cells make a substantive contribution to VEGF-induced corneal neovascularization. Therefore, we must consider the possibility that Tie2-expressing cells play a role in the neovascularization seen in this model.

In summary, these findings constitute what is to our knowledge the first direct demonstration of angiopoietin bioactivity in postnatal animals. In particular, these results indicate that angiopoietins, as suggested by Wong et al., may potentiate the effects of other angiogenic cytokines. Moreover, these findings provide in vivo evidence that Ang1 promotes vascular network maturation, whereas Ang2 works to initiate neovascularization, concepts that were inferred from targeted genetic experiments or detection of gene expression in postnatal organs. Further studies will be required to elucidate the regulatory mechanisms by which angiopoietins modulate cells of endothelial lineage through the Tie2 receptor.

References


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