Angiopoietin 1 causes vessel enlargement, without angiogenic sprouting, during a critical developmental period

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Summary

Early in development, endothelial cells proliferate, coalesce, and sprout to form a primitive plexus of undifferentiated microvessels. Subsequently, this plexus remodels into a hierarchical network of different-sized vessels. Although the processes of proliferation and sprouting are well studied and are dependent on the angiogenic growth factor VEGF, the factors involved in subsequent vessel remodeling are poorly understood. Here, we show that angiopoietin 1 can induce circumferential vessel enlargement, specifically on the venous side of the circulation. This action is due to the ability of angiopoietin 1 to promote endothelial cell proliferation in the absence of angiogenic sprouting; vessel growth without sprouting has not been ascribed to other vascular growth factors, nor has specificity for a particular segment of the vasculature. Moreover, angiopoietin 1 potently mediates widespread vessel enlargement only during a brief postnatal period, in particular, prior to the fourth postnatal week, corresponding to stages in which VEGF inhibition causes widespread vessel regression. These findings show that angiopoietin 1 has a potentially unique role among the vascular growth factors by acting to enlarge blood vessels without inducing sprouting, and also define a critical window of vascular plasticity in neonatal development. Finding the key molecular factors that regulate this plasticity may prove crucial to the further development of pro- and anti-angiogenic therapies.

Key words: Angiogenesis, TIE2 receptor, Endothelial cells, Venules

Introduction

The blood vasculature is formed during early embryonic development when endothelial cells coalesce and sprout to form networks of small, undifferentiated vessels (Risau, 1997). Soon after their formation, these undifferentiated vessels undergo changes in their size and structure, resulting in a hierarchy of arteries, capillaries and veins. These changes are required to meet increased oxygen and metabolic requirements as the embryo grows.

Most studies of blood vessel development have focused on the sprouting phase of angiogenesis. The best-characterized angiogenic agent, vascular endothelial growth factor (VEGF), plays a key role in the formation of a primitive vascular plexus by promoting endothelial cell proliferation, sprouting and initial tube formation (Nehls et al., 1994; Wilting et al., 1993). Genetic deletion studies have confirmed that VEGF is required for these developmental processes in vivo (Carmeliet et al., 1996; Ferrara et al., 1996). Furthermore, even when VEGF is administered to adult animals, it retains its ability to induce sprouting and the formation of new vessels in normal tissues (Pettersson et al., 2000; Springer et al., 1998; Springer et al., 2000). Reciprocally, inhibition of VEGF signaling potently inhibits angiogenic sprouting in many situations of normal or pathological angiogenesis, such as that associated with tumors (Asano et al., 1995; Holash et al., 2002; Kim et al., 1993; Wood et al., 2000).

In contrast to abundant data indicating that VEGF is a crucial mediator of sprouting angiogenesis, much less is known about which factors may be involved in subsequently regulating the diameter and remodeling the structure of the primitive vessels, thereby allowing them to become specialized for their position in the vascular network. On the arterial side of the circulation the remodeling process is known as arterialization, and appears to involve the interactions of flow, pressure, and agents such as platelet derived growth factor B (PDGFB) that promote the interaction of endothelial tubes with smooth muscle cells (Hellstrom et al., 1999; Lindahl et al., 1997). An analogous process presumably occurs on the venous side, although even less is known about this process.

Gene deletion studies have shown that vascular-specific growth factors or receptors, such as angiopoietin 1 (Davis et al., 1996; Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996), angiopoietin 2 (Gale et al., 2002) and ephrin B2 (Shin et al., 2001; Wang et al., 1998), act later than VEGF during embryonic vascular remodeling and maturation. Genetic deletion of these growth factors or their receptors results in embryonic lethality and/or defects in vascular remodeling subsequent to the key initial vasculogenic and angiogenic steps that are dependent on VEGF. Although the precise roles of

these other factors in regulating the vasculature have yet to be clearly defined, gene deletion studies suggest that angiopoietin 1 (ANG1; ANGPT1 – Mouse Genome Informatics/Human Gene Nomenclature Database) and its receptor TIE2 (TEK – Mouse Genome Informatics/Human Gene Nomenclature Database) are involved in establishing a hierarchy of vessels, and are required for the normal interactions between perivascular cells and endothelial cells (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). While potent inhibitors are not yet available for the ANG1/TIE2 system, treatment of adult mice with soluble TIE2 receptor (a weak inhibitor) does not cause obvious vascular changes in normal organs (Lin et al., 1997) (G.T., D.J.-G. and Q.W., unpublished).

Studies using overexpression systems have shed some light on the functions of the ANG1/TIE2 signaling system in vivo, but have also raised key questions. Constitutive transgenic overexpression of angiopoietin 1 in the skin of mice (K14-ANG1 mice), starting in the early embryo, results in a dramatically reddened appearance due to increased numbers of enlarged dermal microvessels (Suri et al., 1998; Thurston et al., 1999). These vessels are also resistant to leakage induced by VEGF or inflammatory agents (Thurston et al., 1999). However, angiopoietin 1 treatment of adult mice does not change the morphology of the skin vessels nor make the mice red, although it does make the dermal blood vessels resistant to plasma leakage (Thurston et al., 2000b).

In contrast to the effects in adult mice, we now report that systemic angiopoietin 1 treatment of neonatal mice and rats results in conspicuously reddened pups containing enlarged blood vessels in the skin and in numerous other organs. The dramatic increases in vessel diameter are apparently caused by endothelial cell proliferation in the absence of vessel sprouting. Notably, the enlargement is largely restricted to the venous side of the microvasculature, and is not accompanied by changes in the number or pattern of vessels. By postnatal day (P) 30, vessels in most organs no longer enlarge in response to angiopoietin 1, indicating the passing of a critical period for vessel plasticity. VEGF-dependency of the vasculature in some organs of neonatal mice corresponds to a similar critical period of vessel plasticity. These findings show that angiopoietin 1 has a potentially unique role among the vascular growth factors, by acting to specifically increase blood vessel diameters without inducing sprouting, and also reveal a window of vascular plasticity in neonatal mice for multiple growth factors in multiple organs.

Materials and methods

Angiopoietin 1 reagents

For most treatments, a recombinant form of angiopoietin 1 called ANG1^{4FD}, which contains two human angiopoietin 1 fibrinogen-like receptor-binding domains coupled to a human Fc domain of IgG [also designated Ang-F1-Fc-F1 (Davis et al., 2003)], was produced in transfected Chinese hamster ovary (CHO) cells. Prolonged treatments of adult mice with angiopoietin 1 were generally performed using a single intravenous injection of adenovirus encoding a genetically engineered version of human angiopoietin 1, referred to as ANG1*, which contains the receptor-binding domain of ANG1 coupled to the coiled-coil oligomerization domain of ANG2 (Thurston et al., 2000b). ANG1* has similar receptor binding and activation properties as ANG1. Adenovirus expressing green fluorescent protein (Ad-GFP) was used as a control. ANG1^{2FD}, which contains only one angiopoietin 1 fibrinogen-like receptor-binding domain coupled to a human Fc domain of IgG, binds to the activating site of TIE2 but does not cluster the receptor, and therefore acts as a competitive inhibitor (Davis et al., 2003). ANG1^{2FD} was also produced in transfected CHO cells. VEGF-Trap was produced as described elsewhere (Holash et al., 2002).

Angiopoietin 1 and VEGF-Trap treatments

For treatment of mice aged P7 to P49, pups from litters of C57BL/6 mice (Taconic Laboratories, NY) were randomized and injected intraperitoneally with ANG1^{4FD} (20, 50 or 200 μg) daily, or with PBS for controls. Rats (Sprague-Dawley, Taconic) aged P7 were injected either daily or every other day with ANG1^{4FD} [200 or 500 μg intraperitoneally (ip)]. Mice treated with VEGF-Trap were given ip injections of 25 mg/kg every second day. For experiments with adult mice, male pathogen-free FVB/N, C57BL/6 or CD-1 nude mice (Taconic Laboratories, NY, or Charles River, Hollister, CA) were used at age 7-12 weeks. For adenovirus treatments, mice were anesthetized with ketamine/xylazine, then injected into the jugular vein with 10^9 plaque forming units (pfu) of adenovirus encoding ANG1* diluted to 150 μl in sterile saline (Thurston et al., 2000b). Injection of Ad-ANG1* via the tail vein of mice had very similar effects on blood vessel morphology to jugular vein injection. In some mice, adenovirus was given locally, either via injections into the ear skin (2×10^8 pfu in 5-8 μl) or intranasally (1×10^9 pfu in 50 μl). Systemic VEGF was not used because of toxicity (Thurston et al., 2000b).

ELISA for ANG1^{4FD} and ANG1* in serum

To confirm delivery of angiopoietin reagents and measure circulating levels, blood (0.2 ml) was withdrawn from the right ventricle of anesthetized mice and rats immediately prior to perfusion, centrifuged to obtain plasma, and frozen until analysis. ANG1* and ANG1^{4FD} were measured by ELISA, using recombinant TIE2 for capture, and an antibody against the N terminus of ANG2 (which is present in ANG1*) or the human Fc domain of IgG (in ANG1^{4FD}) to report.

Staining and measurement of neonatal vessels

Tissues from mouse and rat pups were harvested and immersion-fixed in paraformaldehyde (1% in PBS, pH 7.4) for 1 hour to overnight. Tissues were permeabilized with 0.3% Triton-X100, stained as wholemounts with hamster anti-mouse PECAM antibody (Serotec, used at 1:500) or mouse RECA antibody (Serotec), and, for mice, Cy3-labeled mouse anti-α smooth muscle cell actin antibody (Sigma, used at 1:500), followed by FITC-labeled goat anti-hamster or goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA; used at 1:500), and mounted in Vectashield. Staining blood vessels with this method clearly labels the endothelial cells of the vessels, as well as any endothelial sprouts that emanate from the vessels into the interstitium. Confocal fluorescence images were collected using an inverted confocal microscope (Leica) and a 40× oil immersion lens. Measurements of vessel diameter were performed on the tracheal vasculature (McDonald, 1994; Thurston et al., 1998). Measurements were made on three tracheas per group on four representative regions across the cartilaginous rings. Average vessel diameter was expressed as mean±s.e.m.

BrdU labeling

A thymidine analog, 5-bromo-2′-deoxyuridine (BrdU; Sigma) was injected intravenously (1 mg in 100 μl PBS) into mice. Three hours later, mice were fixed by vascular perfusion of 1% paraformaldehyde in PBS. Tongues were removed, washed in PBS, embedded in paraffin wax, cut into 8-μm-thick sections and stained for BrdU (Staining Bulk Kit; Zymed Laboratories, San Francisco, CA). Retinas were removed and stained whole, using Cy3-labeled secondary antibodies and no counterstaining, or were counterstained with FITC-labeled lectin (GSL I – isolectin B4, Vector Laboratories). To quantify the number of nuclei labeled by BrdU, low magnification images of whole retinas

were recorded digitally, and software was used to demarcate the inner half of the retina. Labeled nuclei were counted in the inner half of the retinas for three mice per group, and the average number of labeled nuclei was expressed as mean±s.e.m.

Western blots for phosphotyrosine

One day after the final ip injection of ANG1^{4FD} or ANG1^{2FD}, mice were sacrificed, and the trachea and lungs were removed and rapidly frozen. Tissue was homogenized in buffer containing protease and phosphatase inhibitors plus 0.1% NP40, and protein levels in each homogenate were assessed using a micro-BCA assay (Pierce, Rockford, IL). TIE2 was immunoprecipitated overnight from 1 mg of lung lysate with 2 μg of the anti-TIE2 antibody mab33 (K. Peters, Proctor and Gamble) and protein G beads (Pharmacia), electrophoresed under reducing conditions, and transferred to Immobilon-P membranes (PVDF; Owl Separation Systems, Portsmouth, NH). Phosphotyrosine immunoreactivity was detected using an anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) and an HRP-conjugated goat anti-mouse secondary antibody (Promega, Madison, WI), followed by chemiluminescent detection (Amersham, Arlington Heights, IL). Total TIE2 protein was detected by stripping the blots and re-probing with the mab33 anti-TIE2 antibody.

Results

Angiopoietin 1 induces widespread vessel enlargement in pups, but not adult mice, in a time- and dose-dependent manner

Previously, we reported that K14-ANG1 mice had a reddened appearance (Fig. 1A,B) associated with increased numbers of enlarged dermal microvessels, and that these vessels were resistant to vascular leak (Thurston et al., 1999). The vasculature of internal organs was not affected in these mice, because of the restricted diffusion of ANG1 in the skin. By contrast, although acute or chronic treatment of adult mice with recombinant angiopoietin 1 proteins [ANG1* and ANG1^{4FD} (Davis et al., 2003; Thurston et al., 2000b)] replicated the anti-leak effects, these treatments did not induce an obvious reddened appearance of the mice (Fig. 1C), or obvious vascular morphologic changes (Davis et al., 2003; Thurston et al., 2000b). To address this apparent paradox, in which angiopoietin 1 induces hypervascularity when delivered transgenically but not when delivered to adults, we treated newborn mouse and rat pups with recombinant angiopoietin 1 (ANG1^{4FD}). In mouse pups treated daily beginning from P7, the snout, tongue, paws and ear skin began to obviously redden by 2 days after injection (P9), and were dramatically reddened after 7 days (P14, Fig. 1D). Similarly, rat pups treated from P7 were also dramatically reddened at P14 on the snout, tongue, paws and ears (Fig. 1E,F).

Associated with the angiopoietin 1-induced reddening, we observed enlargement of the blood vessels of numerous organs, such as the trachea (Fig. 2A,B), tongue (Fig. 2C,D), diaphragm (Fig. 2E,F), retina (Fig. 2G,H), skin of the snout and mucosa of the bladder (data not shown). Of the tissues examined [ear skin, snout skin, tongue (mucosal, muscle), eye (cornea, retina), pancreas (islets, acinar), small intestine (mucosal, submucosal, muscular), bladder (mucosal), trachea (mucosal), diaphragm (central tendon, muscular), esophagus (muscular), kidney (glomerulus, medulla)], the vasculature of the brain and intestine (data not shown) was not notably enlarged. In the affected organs, the enlarged vessels were obvious in whole-mount views, as well as in thick and thin sections (Fig. 2, and results not shown). Strikingly, the vessel enlargement was largely restricted to the venous side of the circulation, including venular capillaries, postcapillary venules and collecting venules (arrows in Fig. 2), whereas arterioles (arrowheads in Fig. 2) were not enlarged by angiopoietin 1 treatment.

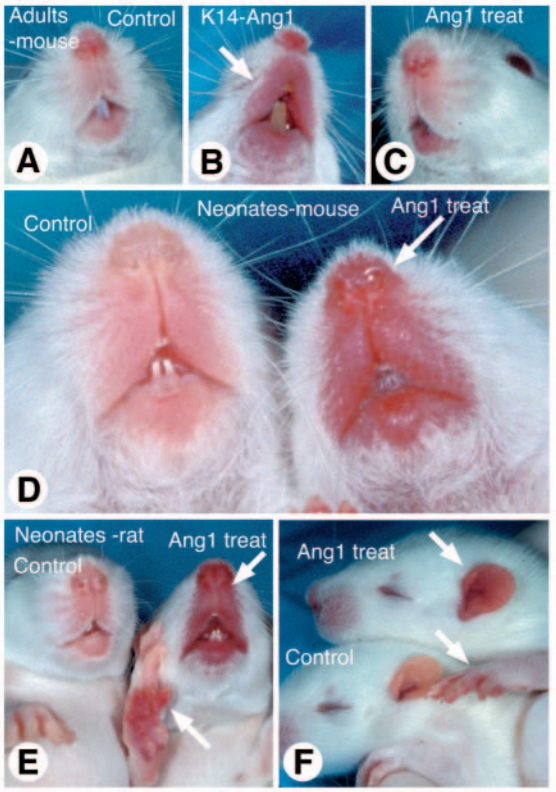

Fig. 1. Reddened skin in mouse and rat pups treated with angiopoietin 1. (A-C) Snouts of wild-type adult mice (A) are pale compared with the reddened snout of the transgenic K14-ANG1 mouse (B), which overexpresses angiopoietin 1 in the skin. Prolonged treatment (10 days) of normal adult mice with ANG1^{4FD} protein (C) or Adeno-ANG1* adenovirus (not shown) does not cause reddening. (D) Snouts of mouse pups treated for 7 days (beginning at P7) with PBS or ANG1^{4FD} (200 μg ip). The snout (arrow), gums and tongue of the pups treated with ANG1^{4FD} are markedly reddened compared with the PBS-treated pup. (E,F) Snouts and ears of rat pups treated for 7 days (beginning at P7) with PBS or ANG1^{4FD}. The snouts (arrow), gums, tongues, paws (arrow) and ears (arrow) of rat pups treated with ANG1^{4FD} (200 μg ip) are markedly reddened compared with PBS-treated pups.

The enlargement of the vessels following treatment of neonatal mice with recombinant angiopoietin 1 occurred in a time-dependent (Fig. 3A) and dose-dependent (Fig. 3B) fashion. The maximum dose of angiopoietin 1 (200 micrograms per day) that was used in neonatal mice and the longest treatment duration (7 days) resulted in a 7-fold increase in average vessel diameter (Fig. 3A). In contrast to the dramatic increases in venule size, arterioles did not increase in size (not shown), nor did the pattern or the number of vessels change. Thus, angiopoietin 1 increased vessel diameter in the absence of angiogenic sprouting.

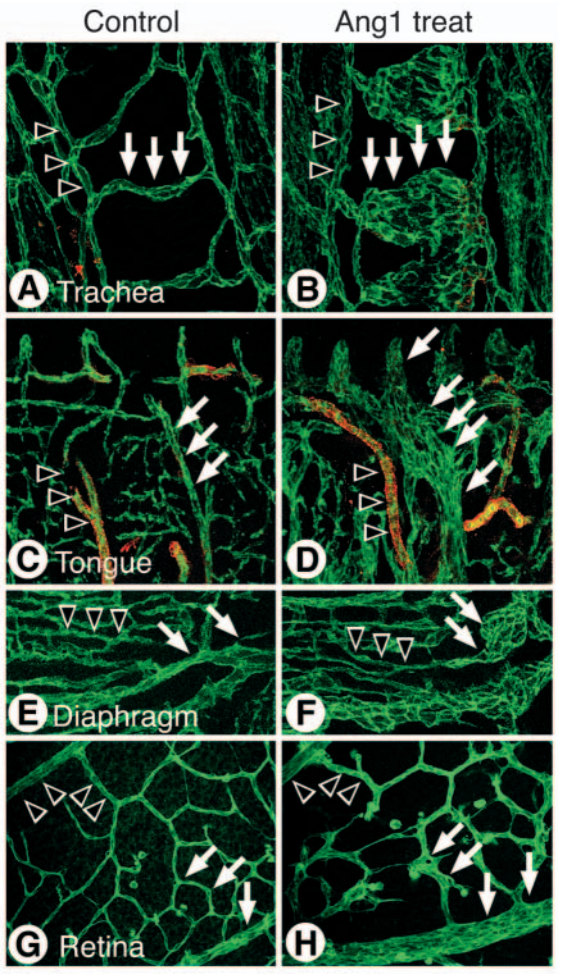

Fig. 2. Vessel morphology in mouse tissues after treatment with angiopoietin 1. Mouse pups were treated daily for 7 days with 200 μg of ANG1^{4FD} protein. Arteries and veins were readily identified in wholemounts by virtue of the differential coating of the smooth muscle cell layer (arterioles had a regular layer of elongated smooth muscle cells wrapped circumferentially around the vessels, whereas venules had an irregular layer of spread, branched smooth muscle cells associated with the vessels) and by vessel morphology (arteries were straighter and smaller in diameter than the corresponding veins). (A,B) Whole-mount views of tracheas from P14 mice, with blood vessels immunostained for PECAM (green) and α-smooth muscle cell actin (red/orange). The straight capillaries traverse the cartilaginous rings. The venular ends of the capillaries are enlarged in the tracheas of ANG1^{4FD}-treated mice (arrows, B) compared with PBS controls (A), and the draining venules are also enlarged, whereas the arterioles (arrowheads) are not enlarged. (C,D) Cross-sections of tongue from P14 mice, with blood vessels immunostained for PECAM (green) and α-smooth muscle cell actin (red/orange). The upper epidermal surface of the tongue is at the upper part of the image. The draining venules and the vessel loops in the dermal papillae (arrows) in the muscosa and muscle layers are enlarged in the tongues of ANG1^{4FD}-treated mice (D) compared with PBS controls (C). By contrast, the feeding arterioles (arrowheads) are similar in size in both ANG1^{4FD}-treated and control mice. (E,F) Whole-mount views of diaphragm from P14 mice, with blood vessels immunostained for PECAM (green) and α-smooth muscle cell actin (red/orange). The straight capillaries are in the skeletal muscle and the draining venules (arrows) are at the boundary with the central tendon. The venules (arrows) in the central tendon are enlarged in the diaphragms of ANG1^{4FD}-treated mice (F) compared with PBS controls (E). By contrast, the muscle capillaries in ANG1^{4FD}-treated mice and control mice are similar in size. (G,H) Whole-mount views of retinas from P14 mice, with blood vessels stained with *Griffonia simplicifolia* isolectin B4 (green). The capillaries traverse between arterioles (arrowheads) and venules (arrows). The venular networks (arrows) are enlarged in retinas of ANG1^{4FD}-treated mice (H) compared with PBS controls (G).

Angiopoietin 1-induced vessel enlargement is blocked by specific antagonists

The ability of recombinant angiopoietin 1 to induce vessel enlargement correlated with its ability to promote phosphorylation of the TIE2 receptor in vivo (Fig. 3C). To verify the specificity of the vessel response to treatment with recombinant angiopoietin 1, and to determine whether potential inhibitors of angiopoietin 1 would cause vessel shrinkage, we examined the effects on neonatal vessels of active and inactive versions of angiopoietin 1. Daily treatment of P7 pups with ANG1^{2FD}, an inactive dimeric form of angiopoietin 1 that does not induce TIE2 phosphorylation in vitro (Davis et al., 2003) or in vivo (Fig. 3C), did not cause obvious changes in vessel morphology in the tongue or the trachea (Fig. 3D,E,H). Moreover, when co-administered with ANG1^{4FD}, ANG1^{2FD} inhibited ANG1^{4FD}-induced TIE2 phosphorylation in vivo (Fig. 3C), as it does in vitro (Davis et al., 2003), and correspondingly reduced the redness of the snout caused by ANG1^{4FD} (not shown), and reduced the amount of vessel enlargement in the trachea and tongue (Fig. 3D-J).

Angiopoietin 1-induced vessel enlargement is associated with endothelial cell proliferation

To determine whether the vessel enlargement induced by angiopoietin 1 was a result of vasodilation or remodeling (i.e. increased vessel size without more endothelial cells or increased the number of endothelial cells), we determined whether the vessels contained more endothelial cells and whether the enlargement was accompanied by endothelial cell proliferation. The number of endothelial cells was estimated in tracheal wholemounts stained for PECAM immunoreactivity, which outlines the endothelial cell junctions (see Fig. S1 in the supplementary material). Normal postcapillary venules in the airways were lined circumferentially by one or two endothelial cells. By comparison, after angiopoietin 1 treatment, the enlarged vessels had three or more endothelial cells lining their circumference (see Fig. S1 in the supplementary material). In addition, BrdU-labeled endothelial cells were more abundant in enlarged vessels of the retina (Fig. 4A,B) and tongue (see Fig. S1) in mice treated with angiopoietin 1 than in the corresponding vessels from control pups. The increased numbers of BrdU-labeled endothelial cells were clearly preferentially distributed on the venous side of the circulation (Fig. 4C,D, arrows). Because the retina was amenable to whole-mount BrdU staining, we quantified BrdU labeling in retinas from mouse pups, and found that angiopoietin 1 treatment resulted in 4-fold more BrdU-labeled endothelial cells in the retina, when compared with controls (Fig. 4E). Thus, vessel enlargement induced by ANG1 results from

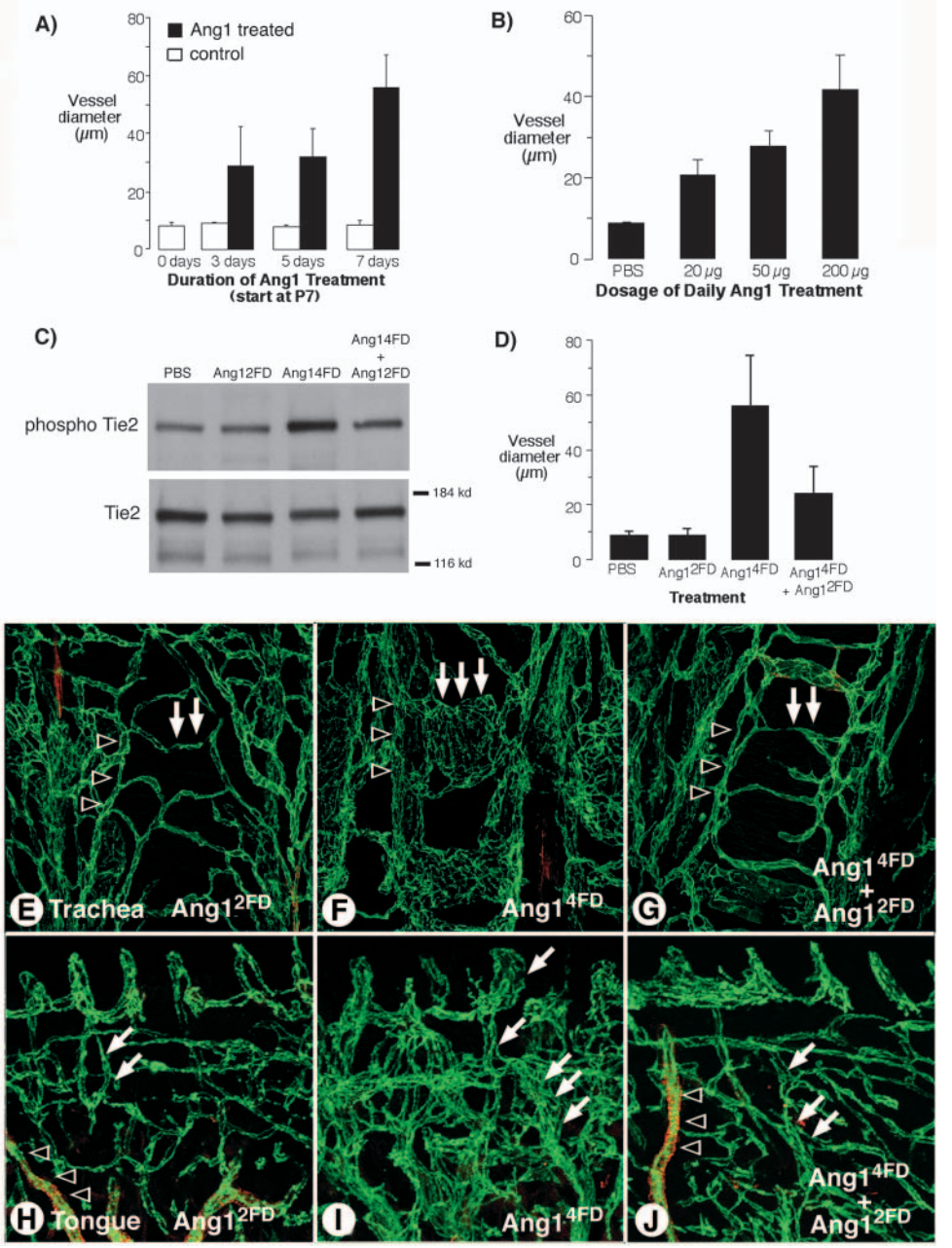

Fig. 3. (A,B) Time course and dose-response analysis of vessel enlargement after treatment with ANG1^{4FD}. The diameter of tracheal vessels on the edge of cartilaginous rings was measured in confocal micrographs of PECAM-stained tracheal wholemounts. (A) Time course of venule enlargement in mice treated daily with 200 μg/day of ANG1^{4FD}, starting at P7. Venules increased in diameter with treatment (black bars), whereas untreated venules (white bars) did not change in diameter. Arterioles and venules were identified as described for Fig. 2. (B) Dose-response analysis of vessel enlargement. Mice treated daily for 7 days (starting at P7) with 20, 50 or 200 μg/day of ANG1^{4FD}. Results are mean±s.e.m.; three mice per group. (C) TIE2 phosphorylation after treatment with ANG1^{4FD}, and inhibition by ANG1^{2FD}. Lungs of mice treated daily for 7 days starting at P7 were excised, extracted, immunoprecipitated for TIE2, and immunoblotted for phosphotyrosine (upper panel) or TIE2 (lower panel). Mice were treated with PBS, ANG1^{2FD}, ANG1^{4FD}, or ANG1^{2FD} plus ANG1^{4FD}. TIE2 from lung shows a basal level of tyrosine phosphorylation in PBS-treated mice, which was not significantly altered by treatment with ANG1^{2FD}. ANG1^{4FD} induced increased TIE2 phosphorylation, which was largely inhibited by ANG1^{2FD}. Results shown are from one mouse per group, but are representative of a total of six mice per group analyzed for TIE2 phosphorylation. (D) Reduced vessel diameter with inhibitor of angiopoietin 1. Diameter of tracheal vessels on the edge of cartilaginous rings was measured in confocal micrographs of PECAM-stained tracheal wholemounts. Mice treated daily for 7 days with 200 μg/day of ANG1^{2FD}, ANG1^{4FD}, or both, starting at P7. Results are mean±s.e.m.; three mice per group. (E-J) Reduced vessel enlargement with an inhibitor of angiopoietin 1. Blood vessels were immunostained for PECAM (green) and α-smooth muscle cell actin (red/orange). (E-G) Whole-mount views of tracheas from P14 mice. Daily treatment of mice with ANG1^{2FD} (200 μg/day) for 7 days had no obvious effect on vessel morphology (E). The enlargement of the venules near the cartilaginous rings (arrows) induced by ANG1^{4FD} treatment (F) was largely inhibited by co-treatment with ANG1^{2FD} (G), whereas arterioles (arrows) were not affected by ANG1^{4FD}. (H-J) Cross-sections of tongue from P14 mice. The epithelial surface of the tongue is at the upper part of the image. Daily treatment of mice with ANG1^{2FD} (200 μg/day) for 7 days (H) had no obvious effect on vessel morphology. The enlargement of the vessels in the dermal papillae (arrowheads) and draining venules (arrows) induced by ANG1^{4FD} treatment (I) was largely inhibited by co-treatment with ANG1^{2FD} (J), whereas arterioles (arrows) were not affected by ANG1^{4FD}.

increased numbers of endothelial cells and endothelial cell proliferation, and is not due to vasodilation alone.

Most vascular beds lose responsiveness to angiopoietin 1 by postnatal day 30

The above data indicate that although blood vessels in many organs of mouse and rat pups can dramatically enlarge in response to angiopoietin 1, the blood vessels of adults generally do not. To determine the age when most vessel beds lose their responsiveness to angiopoietin 1, mice were treated for 7 days starting at ages P14, P21, P30 and P49. The mice in the P14 group (Fig. 5A) and the P21 group (not shown) became notably reddened, although not as conspicuously as when treatment was initiated earlier. However mice in the P30 (Fig.

5B) and P49 groups (not shown) remained relatively pale, similar to control mice. Whole-mount views of the blood vessels confirmed that most vascular beds, including the tongue, exhibited notable enlargement responses in P14 mice but not in P30 mice (Fig. 5F-H). These findings show that during the third to fourth weeks of postnatal development, the vessels of most organs lose their responsiveness to angiopoietin 1. However, one notable exception was the vasculature of the airways: whole-mount views of the trachea revealed that these vessels enlarged in response to angiopoietin 1 in P30 mice (Fig. 5C-E) and, in fact, in adult mice (Baffert et al., 2004), although not quite as dramatically as in younger pups.

The tracheal vessels in adult mice were also responsive to local delivery of angiopoietin 1. When delivered intranasally into adult mice, adenovirus encoding ANG1* resulted in infection of the airway epithelium and caused enlargement of the tracheal venules without sprouting. Intranasal delivery of control adenoviruses encoding green fluorescent protein did not cause changes in vessel morphology (Fig. 5I,J). By contrast, the same angiopoietin-encoding adenovirus injected into the ear skin did not induce enlargement of the skin microvessels (data not shown).

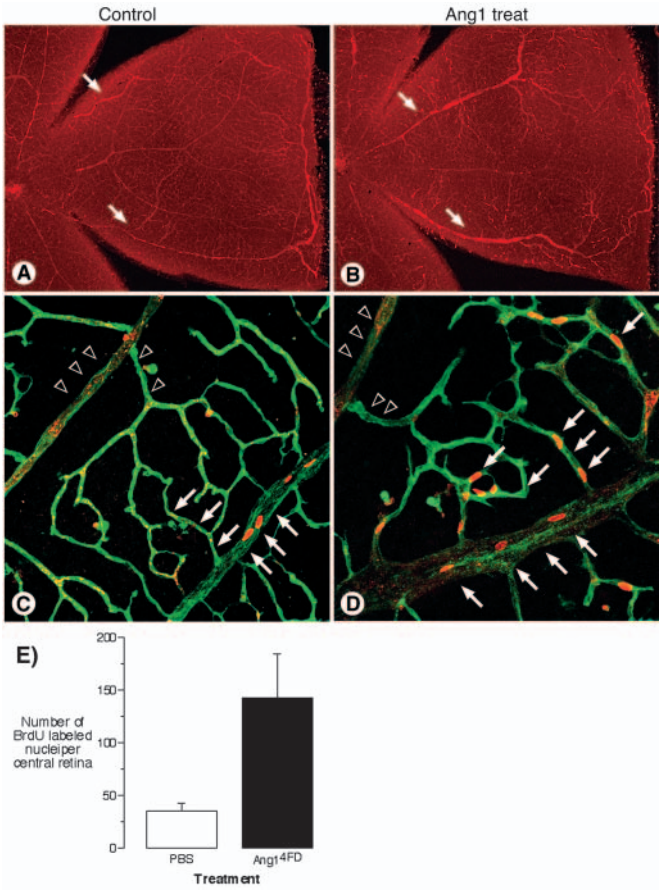

Fig. 4. Vessel enlargement is associated with increased numbers of endothelial cells and endothelial cell proliferation. Retinal vessels in wholemounts of PBS- or ANG1^{4FD}-treated mice, stained with BrdU and Cy3-labeled secondary antibody. (A) In control mice, BrdU label (red) is apparent near the retinal periphery where active angiogenesis occurs in P14 mice, as well as in veins (arrows). (B) In ANG1^{4FD}-treated mice, intense BrdU labeling is apparent in veins (arrows) and in microvessels near veins. (C) Higher magnification of retinal vessels (labeled with isolectin B4, green) shows occasional BrdU-labeled (red) endothelial cells in veins (arrow), and few BrdU cells in arterioles (arrowhead) in control mice. (D) By contrast, in retinal vessels of mice treated with ANG1^{4FD}, BrdU-labeled endothelial cells are abundant in venous capillaries and veins (arrows). (E) Quantification of BrdU labeling shows approximately four times as many BrdU-labeled endothelial cells in retinal vessels from mice treated with ANG1^{4FD} as in PBS-treated mice.

Reduced number of vessels in mouse pups treated with VEGF-Trap

The above findings, namely that most vessels can enlarge in response to angiopoietin 1 during the first few postnatal weeks but not thereafter, are analogous to previous findings that inhibition of VEGF or conditional VEGF-gene deletion has much more profound effects on body growth and tissue function in young mice than in adult mice (Gerber et al., 1999). In other settings of angiogenesis, such as in the retina of neonatal mice, inhibition of VEGF results in the regression of newly formed vessels (Benjamin et al., 1999; Benjamin et al., 1998; Benjamin and Keshet, 1997; Kim et al., 2002). Collectively, these findings raise the possibility of a 'plasticity window' in early postnatal development, during which blood vessels remain relatively unstable, as marked by their continued dependence on VEGF for maintenance and their continued responsiveness to the vessel-enlarging actions of angiopoietin 1. To further explore this possibility, we characterized the actions of a potent VEGF inhibitor, termed VEGF-Trap (Holash et al., 2002), on vascular beds during the time they retained responsiveness to the vascular-enlarging actions of angiopoietin 1. Whole-mount views revealed that angiopoietin-responsive vascular beds in neonatal mice displayed profound vascular regression in response to VEGF-Trap treatment (Fig. 6). In the trachea of pups treated for 7 days with VEGF-Trap, the capillaries across the cartilaginous rings were completely absent (Fig. 6B, asterisks). In the tongue, the capillary loops into the dermal papillae were virtually absent, and the submucosal plexus was diminished (Fig. 6D, asterisks). Interestingly, and once again consistent with the preferential effects of angiopoietin 1 on the venous side of the circulation, regression induced by the VEGF-Trap may be associated with the relative sparing of arterial vessels (Fig. 6D). Adult vascular beds, such as heart, brain, kidney and lung, that were not responsive to angiopoietin 1 did not undergo obvious regression with VEGF-Trap treatment (data not shown) (see also Baffert et al., 2004). Combination treatment of mouse pups with angiopoietin 1 and VEGF-Trap resulted in independent effects of both reagents: many vessels regressed but the remaining vessels were enlarged (data not shown). The combination treatment suggests that the increase in endothelial cell number induced by angiopoietin 1 is independent of VEGF. Thus, during the period of postnatal development when blood vessels are responsive to the vessel-enlarging actions of angiopoietin 1, they are also dependent on VEGF for their maintenance, defining a critical window of vascular plasticity in neonatal mice for multiple growth factors in multiple organs.

Discussion

Compared with the abundant information on angiogenic sprouting, the processes and growth factors involved in

adjusting the size of developing vessels remain relatively obscure. Here, we show that angiopoietin 1 can rapidly induce circumferential vessel enlargement and that the enlargement is relatively specific to the venous side of the circulation. This action is apparently caused by the ability of angiopoietin 1 to promote endothelial cell proliferation in the absence of angiogenic sprouting. To our knowledge, vessel growth without sprouting has not been ascribed to other vascular growth factors, nor has specificity for a particular segment of the vasculature. Angiopoietin 1 can mediate widespread vessel enlargement only during a brief postnatal period, before approximately 4 weeks of age in mice, corresponding to developmental stages in which we find that treatment with a VEGF inhibitor causes dramatic vessel regression, similar to that described in previous studies (Gerber et al., 1999). These findings show that angiopoietin 1 has a potentially unique role among the vascular growth factors by acting to enlarge vessel diameters without inducing any associated angiogenic sprouting, and also define a critical window of vascular plasticity in neonatal mice for multiple growth factors in multiple organs.

Does angiopoietin 1 play a role in the normal regulation of vessel size? Genetic deletion of either angiopoietin 1 or its receptor TIE2 seems to support this possibility, as the vasculature of the resulting embryos fails to properly remodel from the initial rather primitive and homogenous plexus. However, these embryos suffer from early lethality, making it difficult to define more precisely the roles of angiopoietin 1 during later remodeling. Further support comes from the report that venous malformations, an abnormal vascular enlargement in segments of the venules and small veins, can arise in cases of constitutive activation of TIE2 due to a point mutation in the kinase domain (Vikkula et al., 1996). Thus, angiopoietin 1 may play a particular role in remodeling the venous side of the circulation, where the forces of blood flow and pressure are not as significant, and where we see vessel enlargement in response to exogenous angiopoietin 1. Unfortunately, although current inhibitors of angiopoietin 1 (such as TIE2-Fc and ANG1^{2FD}) can block exogenously added angiopoietin 1 from activating endogenous TIE2 receptors, they do not seem to be capable of competing with endogenous angiopoietin 1 to block the ongoing constitutive phosphorylation of the TIE2 receptors. Further exploration of the role of angiopoietin 1 during remodeling awaits the development of better inhibitors, or the conditional deletion of the angiopoietin 1 or TIE2 genes.

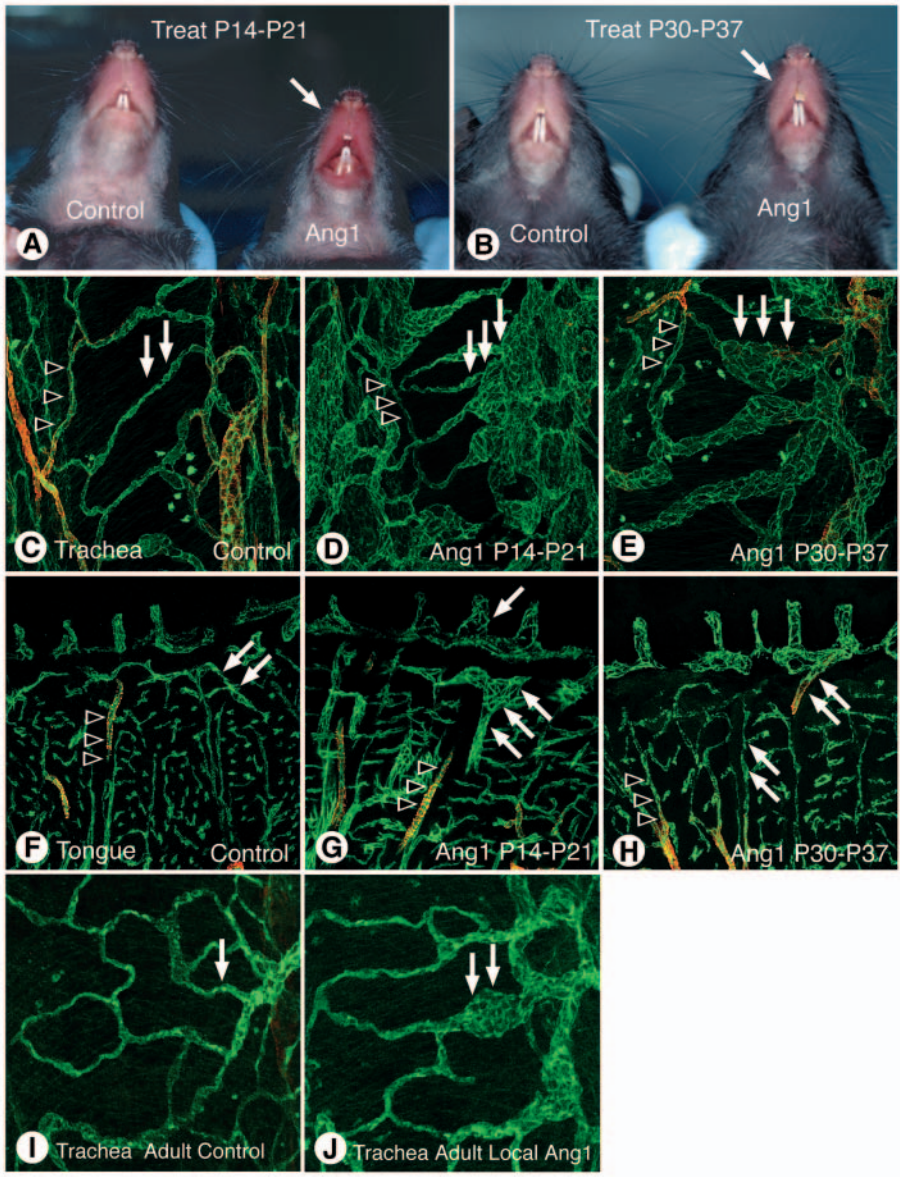

Fig. 5. Most vessels lose responsiveness to ANG1^{4FD} between P14 and P30. Mice were injected daily for 7 days starting at P7, P14, P30 and P49 with ANG1^{4FD}. Vessels were immunostained for PECAM (green) and α-smooth muscle cell actin (red). (A,B) Snouts of mice treated with ANG1^{4FD} (arrows) were dramatically reddened in mice treated at P14 (A), but not at P30 (B). (C-E) Enlarged tracheal vessels in ANG1^{4FD}-treated P14 (D) and P30 (E) mice compared with control mice (C). Enlargement occurred in venular capillaries, in postcapillaries (arrows) and in collecting venules, whereas arterioles (arrowheads) appeared to be unchanged. (F-H) Vessels in the tongue of P14 ANG1^{4FD}-treated mice (G) are enlarged compared with controls (F), but those from P30 mice (H) are only slightly enlarged. Enlargement was apparent in vessels of dermal papillae and in draining venules (arrows), whereas arterioles (arrowheads) appear to be unchanged. (I,J) Tracheal vessels of adult mice (12 wk) at 14 days after intranasal administration of adenovirus encoding green fluorescent protein (I) or angiopoietin 1* (J). Angiopoietin 1* caused enlargement of airway venules (arrows).

Despite previous reports that angiopoietin 1 might be able to induce vessel sprouting and migration in vitro (Koblizek et al., 1998), the data herein indicate that any such effects would

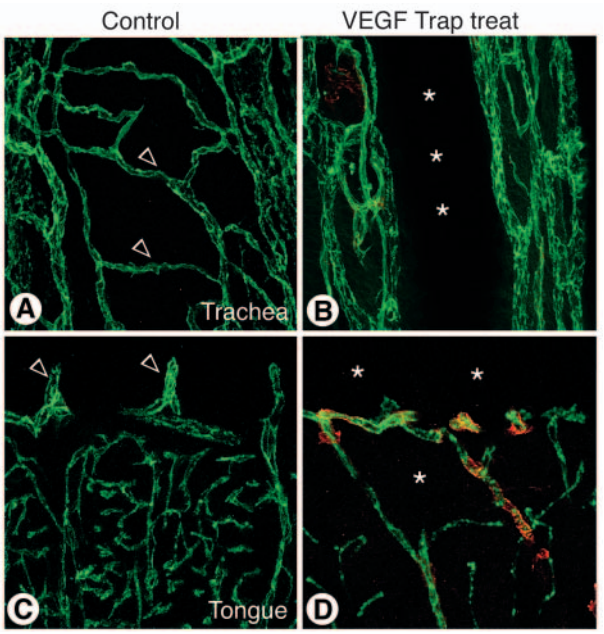

Fig. 6. Vessel morphology in mouse tissues treated with VEGF-Trap. Mouse pups were treated every 2 days for 7 days with 25 mg/kg ip of VEGF-Trap protein. Tissue is from P14 mice, with blood vessels immunostained for PECAM (green) and α smooth muscle cell actin (red/orange). (A,B) Whole-mount views of tracheas, showing straight vessels (arrowheads) across the cartilaginous rings in control mice (A). The vessels across the cartilaginous region (asterisks) in tracheas of VEGF-Trap-treated mice (B) are completely absent. (C,D) Cross-sections of tongue, with the upper epidermal surface of the tongue at the upper part of the image. The vessel loops in the dermal papillae (C, arrowheads) in tongues of VEGF-Trap-treated mice are almost abolished (D, asterisks), and the vascularity of the dermis and muscle layers is also reduced.

be minor when compared with the ability of angiopoietin 1 to regulate vessel diameter. Thus, as previously suggested, VEGF and angiopoietin 1 are complementary in their actions – that is, while VEGF seems to promote early phases of vasculogenesis and angiogenic sprouting, angiopoietin 1 seems to be more relevant to the subsequent processes that regulate vessel size and maturation. In addition, angiopoietin 1 is able to mediate its actions independently of VEGF. Interestingly, although both factors have relatively minor proliferative actions on cultured endothelial cells when compared with other growth factors such as FGF, they both seem to be capable of promoting endothelial proliferation in vivo, whereas the in vivo proliferative actions of FGF remain to be validated.

The limitations of current in vitro systems to accurately reflect actions of growth factors in vivo makes it more difficult to define the mechanistic differences underlying the differential growth effects of VEGF versus angiopoietin 1. One clue may come from the finding that angiopoietin 1, unlike VEGF, may lack the ability to induce protease activation in endothelial cells (Mandriota et al., 1995; Zucker et al., 1998). Because sprouting through the vascular basement membrane is likely to require the induction of specific proteases that can degrade extracellular matrix, the inability of angiopoietin 1 to induce protease activation may force dividing endothelial cells to remain within their original vessel, thus allowing angiopoietin 1 to regulate vessel size without causing angiogenic sprouting. By contrast, VEGF may empower proliferating endothelial cells to break through the vessel wall by inducing necessary proteases, thereby allowing for sprouting angiogenesis.

The vessel enlargement induced by angiopoietin 1 appears to be rather specific to the venous side of the microcirculation. Venules are specialized functionally, morphologically and molecularly (Thurston et al., 2000a). Functionally, venules are most leaky to plasma proteins under baseline conditions and are the site of inflammation-induced plasma leak (Majno et al., 1969). Venular endothelial cells have a distinct molecular profile, including increased expression of P-selectin, von Willebrand factor (Thurston et al., 2000a), and receptors for inflammatory mediators (Bowden et al., 1994; Heltianu et al., 1982). In addition, venules are the segment most likely to sprout during angiogenesis (Folkman, 1982; Phillips et al., 1991). Previous studies have suggested that the venous side of the circulation may be most responsive to, or dependent upon, angiopoietin 1 during development (Loughna and Sato, 2001; Moyon et al., 2001; Thurston et al., 1999). The enlargement of the venules could be due to an abundance of TIE2 receptors on the endothelial cells of venules, to increased accessibility to the abluminal surface, to localized expression of angiopoietin 1, or to general plasticity due to specialized pericytes and the basement membrane. Alternatively, previous studies have noted that angiopoietin 2, which can act as an antagonist of angiopoietin 1, is expressed in arterial smooth muscle cells, with much weaker expression on the venous side (Gale et al., 2002; Moyon et al., 2001). Thus, the arterial expression of angiopoietin 2 may act to inhibit angiopoietin 1 in these vessels, and thus may explain why the venous side of the circulation is more responsive to angiopoietin 1 stimulation.

Angiopoietin 1 appears to be able to regulate vessel size in most organs only during a critical developmental window. This window coincides with a period during which vessels in many organs are dependent on VEGF for survival. Subsequent maturation of the vessels in many organs makes them less responsive to vessel enlargement after angiopoietin 1 treatment and less dependent on VEGF for survival. The evidence strongly suggests that these mature vessels do not lose all responsiveness to angiopoietin 1, because angiopoietin 1 treatment of mature vessels results in a reduced plasma-leakage response (Thurston et al., 2000b). Thus, the developmental window appears to be a period when vessels maintain plasticity to remodel morphologically in response to angiopoietin 1. By comparison, even mature vessels appear to maintain plasticity to remodel in response to VEGF, because robust angiogenesis occurs when exogenous VEGF is applied to adult tissues that were not responsive to angiopoietin 1, for example, the skin and the heart (Pettersson et al., 2000) (data not shown).

So what reduces plasticity as vessels mature? Based on previous studies (Benjamin et al., 1998; Hirschi and D'Amore, 1997), it is possible that vessel maturation involves changes in the association of endothelial cells with the surrounding perivascular support cells. Although our preliminary data indicate that the neonatal vessels responsive to angiopoietin 1 are already associated with perivascular cells, it is likely that the interactions between endothelial cells and perivascular cells continue to mature after initial investment. Indeed, even in adulthood, the blood vessels of the airways continue to respond to angiopoietin 1 by enlarging, although these vessels are

covered by seemingly mature pericytes. The response of the vasculature does not seem to depend on the route of delivery, because the ear skin vessels are unresponsive to both local and systemic delivery of ANG1, whereas the tracheal vessels are responsive to both local and systemic delivery. Thus, the responsiveness of blood vessels to angiopoietin 1 may be regulated by complex and poorly understood interactions between endothelial and support cells, and/or basement membrane.

The ability to further characterize the maturity of blood vessels, the nature of the interactions between endothelial cells and pericytes and basement membrane, and the signals that underlie responsiveness to VEGF and angiopoietin 1, seems likely to have important therapeutic implications. The success of pro- and anti-angiogenic approaches in the clinic may well depend on the ability to manipulate the state of vessel maturation, i.e. to revert mature vessels to a more plastic state, or to induce plastic vessels to mature. For example, in ischemic settings in which it is desirable to promote vessel sprouting as well as increases in vessel size [such as of collaterals feeding the ischemic tissue (Carmeliet, 2000)], one would want to induce a greater state of vessel plasticity. Similarly, in tumors and other settings in which it may be desirable to regress an existing vasculature, it may be useful to once again induce vessel plasticity that would be associated with vascular instability and increased responsiveness to VEGF blockade. Finding the key molecular and cellular factors that regulate this plasticity switch may prove crucial to the further development of pro- and anti-angiogenic therapies.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/14/3317/DC1

References

Asano, M., Yukita, A., Matsumoto, T., Kondo, S. and Suzuki, H. (1995). Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor121. *Cancer Res.* **55**, 5296-5301.

Baffert, F., Thurston, G., Rochon-Duck, M., Le, T., Brekken, R. and McDonald, D. M. (2004). Age-related changes in vascular endothelial growth factor dependency and angiopoietin-1-induced plasticity of adult blood vessels. *Circ. Res.* **94**, 984-992.

Benjamin, L. E. and Keshet, E. (1997). Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc. Natl. Acad. Sci. USA* **94**, 8761-8766.

Benjamin, L. E., Hemo, I. and Keshet, E. (1998). A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* **125**, 1591-1598.

Benjamin, L. E., Golijanin, D., Itin, A., Pode, D. and Keshet, E. (1999). Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J. Clin. Invest.* **103**, 159-165.

Bowden, J. J., Garland, A. M., Baluk, P., Lefevre, P., Grady, E. F., Vigna, S. R., Bunnett, N. W. and McDonald, D. M. (1994). Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. *Proc. Natl. Acad. Sci. USA* **91**, 8964-8968.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* **6**, 389-395.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C. et al. (1996). Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87**, 1161-1169.

Davis, S., Papadopoulos, N., Aldrich, T. H., Maisonpierre, P. C., Huang, T., Kovac, L., Xu, A., Leidich, R., Radziejewska, E., Rafique, A. et al. (2003). Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. *Nat. Struct. Biol.* **10**, 38-44.

Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A. and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897-1909.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.

Folkman, J. (1982). Angiogenesis: initiation and control. *Ann. New York Acad. Sci.* **401**, 212-227.

Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D. et al. (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Dev. Cell* **3**, 411-423.

Gerber, H. P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G. A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M. and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. *Development* **126**, 1149-1159.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A. and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126**, 3047-3055.

Heltianu, C., Simionescu, M. and Simionescu, N. (1982). Histamine receptors of the microvascular endothelium revealed in situ with a histamine-ferritin conjugate: characteristic high-affinity binding sites in venules. *J. Cell Biol.* **93**, 357-364.

Hirschi, K. K. and D'Amore, P. A. (1997). Control of angiogenesis by the pericyte: molecular mechanisms and significance. *EXS* **79**, 419-428.

Holash, J., Davis, S., Papadopoulos, N., Croll, S. D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E. et al. (2002). VEGF-Trap: A VEGF blocker with potent antitumor effects. *Proc. Natl. Acad. Sci. USA* **99**, 11393-11398.

Kim, E. S., Serur, A., Huang, J., Manley, C. A., McCrudden, K. W., Frischer, J. S., Soffer, S. Z., Ring, L., New, T., Zabski, S. et al. (2002). Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. *Proc. Natl. Acad. Sci. USA* **99**, 11399-11404.

Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **362**, 841-844.

Koblizek, T. I., Weiss, C., Yancopoulos, G. D., Deutsch, U. and Risau, W. (1998). Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr. Biol.* **8**, 529-532.

Lin, P., Polverini, P., Dewhirst, M., Shan, S., Rao, P. S. and Peters, K. (1997). Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. *J. Clin. Invest.* **100**, 2072-2078.

Lindahl, P., Johansson, B. R., Leveen, P. and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277**, 242-245.

Loughna, S. and Sato, T. N. (2001). A combinatorial role of angiopoietin-1 and orphan receptor TIE1 pathways in establishing vascular polarity during angiogenesis. *Mol. Cell* **7**, 233-239.

Majno, G., Shea, S. M. and Leventhal, M. (1969). Endothelial contraction induced by histamine-type mediators: an electron microscopic study. *J. Cell Biol.* **42**, 647-672.

Mandriota, S. J., Seghezzi, G., Vassalli, J. D., Ferrara, N., Wasi, S., Mazzieri, R., Mignatti, P. and Pepper, M. S. (1995). Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. *J Biol. Chem.* **270**, 9709-9716.

McDonald, D. M. (1994). Endothelial gaps and permeability of venules in rat tracheas exposed to inflammatory stimuli. *Am. J. Physiol.* **266**, L61-L83.

Moyon, D., Pardanaud, L., Yuan, L., Breant, C. and Eichmann, A. (2001). Selective expression of angiopoietin 1 and 2 in mesenchymal cells surrounding veins and arteries of the avian embryo. *Mech. Dev.* **106**, 133-136.

Nehls, V., Schuchardt, E. and Drenckhahn, D. (1994). The effect of fibroblasts, vascular smooth muscle cells, and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system. *Microvasc. Res.* **48**, 349-363.

Pettersson, A., Nagy, J. A., Brown, L. F., Sundberg, C., Morgan, E., Jungles, S., Carter, R., Krieger, J. E., Manseau, E. J., Harvey, V. S. et al. (2000). Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. *Lab. Invest.* **80**, 99-115.

Phillips, G. D., Whitehead, R. A. and Knighton, D. R. (1991). Initiation and pattern of angiogenesis in wound healing in the rat. *Am J. Anat.* **192**, 257-262.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70-74.

Shin, D., Garcia-Cardena, G., Hayashi, S., Gerety, S., Asahara, T., Stavrakis, G., Isner, J., Folkman, J., Gimbrone, M. A., Jr and Anderson, D. J. (2001). Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev. Biol.* **230**, 139-150.

Springer, M. L., Chen, A. S., Kraft, P. E., Bednarski, M. and Blau, H. M. (1998). VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol. Cell* **2**, 549-558.

Springer, M. L., Hortelano, G., Bouley, D. M., Wong, J., Kraft, P. E. and Blau, H. M. (2000). Induction of angiogenesis by implantation of encapsulated primary myoblasts expressing vascular endothelial growth factor. *J. Gene. Med.* **2**, 279-288.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N. and Yancopoulos, G. D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171-1180.

Suri, C., McClain, J., Thurston, G., McDonald, D. M., Zhou, H., Oldmixon, E. H., Sato, T. N. and Yancopoulos, G. D. (1998). Increased vascularization in mice overexpressing angiopoietin-1. *Science* **282**, 468-471.

Thurston, G., Murphy, T. J., Baluk, P., Lindsey, J. R. and McDonald, D. M. (1998). Angiogenesis in mice with chronic airway inflammation: strain-dependent differences. *Am. J. Pathol.* **153**, 1099-1112.

Thurston, G., Suri, C., Smith, K., McClain, J., Sato, T. N., Yancopoulos, G. D. and McDonald, D. M. (1999). Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286**, 2511-2514.

Thurston, G., Baluk, P. and McDonald, D. M. (2000a). Determinants of endothelial cell phenotype in venules. *Microcirculation* **7**, 67-80.

Thurston, G., Rudge, J. S., Ioffe, E., Zhou, H., Ross, L., Croll, S. D., Glazer, N., Holash, J., McDonald, D. M. and Yancopoulos, G. D. (2000b). Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat. Med.* **6**, 460-463.

Vikkula, M., Boon, L. M., Carraway, K. L., 3rd, Calvert, J. T., Diamonti, A. J., Goumnerov, B., Pasyk, K. A., Marchuk, D. A., Warman, M. L., Cantley, L. C. et al. (1996). Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* **87**, 1181-1190.

Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**, 741-753.

Wilting, J., Christ, B., Bokeloh, M. and Weich, H. A. (1993). In vivo effects of vascular endothelial growth factor on the chicken chorioallantoic membrane. *Cell Tissue Res.* **274**, 163-172.

Wood, J. M., Bold, G., Buchdunger, E., Cozens, R., Ferrari, S., Frei, J., Hofmann, F., Mestan, J., Mett, H., O'Reilly, T. et al. (2000). PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res.* **60**, 2178-2189.

Zucker, S., Mirza, H., Conner, C. E., Lorenz, A. F., Drews, M. H., Bahou, W. F. and Jesty, J. (1998). Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. *Int. J. Cancer* **75**, 780-786.