Age-Related Changes in Vascular Endothelial Growth Factor Dependency and Angiopoietin-1–Induced Plasticity of Adult Blood Vessels

Fabienne Baffert, Gavin Thurston, Michael Rochon-Duck, Tom Le, Rolf Brekken, Donald M. McDonald

Abstract—Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) are essential for vascular development, but this dependency has been assumed not to persist into adult life. In this study, we report that after 10 days of systemic treatment of 4-, 8-, and 16-week-old mice with VEGF-Trap, an inhibitor of VEGF, the number of capillaries in the tracheal mucosa was reduced by 39%, 28%, and 14%, respectively. The magnitude of the reduction decreased with age ($r^2=0.6$, $P<0.001$), but was still significant at 16 weeks. A corresponding age-related decrease in vascular endothelial growth factor receptor-2 (VEGFR-2) immunoreactivity suggests that diminished VEGFR-2 expression may contribute to resistance to VEGF signaling inhibition. VEGF-Trap further reduced VEGFR-2 expression in tracheal capillaries. By comparison, systemic treatment with adenovirus encoding Ang1 led to a significant enlargement of tracheal venules with little age effect (64%, 56%, and 49% increase in diameter at 10 days). When Ang1 was given in combination with VEGF-Trap, tracheal vessels presented the typical response to each factor, showing that the Ang1 effect was not VEGF-mediated, yet Ang1 seems to have a protective effect, as judged by prevention of VEGF-Trap–induced reduction in tracheal capillaries in the oldest group. Together, these findings indicate that VEGF and Ang1 participate in blood vessel survival and plasticity in adult life. (Circ Res. 2004;94:984–992.)

Key Words: angiogenesis • angiopoietin-1 • microvasculature • vascular endothelial growth factor • vascular endothelial growth factor receptor-2

The growth of new blood vessels requires the activation of specific signal transduction pathways mediated in endothelial cells by the vascular endothelial growth factor (VEGF) and angiopoietin families of growth factors.1,2 These growth factors are essential for blood vessel formation and maturation during embryonic development.3–8 VEGF is a mitogen and proangiogenic factor for endothelial cells both in vitro and in vivo.9–12 VEGF is also implicated in the survival of newly formed blood vessels and blood vessels in tumors.13–16 It has been assumed that the VEGF dependency of normal vessels does not persist into adult life. Published data show that treatment with VEGF inhibitors does not cause major changes in the normal vasculature after the fourth postnatal week.16 However, VEGF inhibitors may affect processes that require the formation of new blood vessels in the female reproductive system and wound healing.17,18 The loss of VEGF dependency is not completely understood, but newly formed blood vessels reportedly become independent of VEGF when covered by pericytes.13,19

Inhibition of VEGF signaling by VEGF-TrapR1/R2 (here referred to as VEGF-Trap), a soluble molecule consisting of the extracellular domains of VEGF receptors 1 and 2 coupled to the Fc portion of human IgG,20 leads to a reduction in the number of mouse tracheal capillaries during postnatal development through day 30 (G. Thurston, unpublished data, 2004). Moreover, dermal blood vessels are structurally abnormal and leaky in 8-week-old mice that transgenically overexpress VEGF in the skin.21

A role in blood vessel maturation for angiopoietin-1 (Ang1) and its receptor Tie-2 has been demonstrated in embryonic development.7,8 The vasculature continues to be sensitive to Ang1 in postnatal life. Treatment of mice from postnatal day 7 to 30 with Ang1F4D, the soluble fibrinogen-like receptor-binding domain of Ang1, or recombinant adenovirus encoding Ang1* (here referred to as Ad-Ang1), a genetically engineered version of human Ang1 that contains the receptor binding domain of Ang1 coupled to the oligomerization domain of Ang2,22 causes conspicuous enlargement of venules in the tongue, diaphragm, eye, and trachea of...
neonatal mice as a result of endothelial cell proliferation (G. Thurston, unpublished data, 2004).

In the present study, we investigated VEGF dependency of blood vessels in adult animals and a possible mechanism of change in VEGF sensitivity. We also determined whether Ang1 causes enlargement of venules in the adult and whether this effect is mediated by VEGF.

To detect relatively subtle effects of VEGF inhibition and Ang1 on blood vessels of adult mice, we focused on the microvasculature of the trachea, which is distinguished by its simplicity and monolayer nature. VEGF dependency was studied by using VEGF-Trap to block VEGF signaling, whereas Ang1-induced venular plasticity was examined by systemic administration of Ad-Ang1. Mice from 4 to 16 weeks in age were treated with VEGF-Trap, Ad-Ang1, or a combination of the two. Changes in vascular endothelial growth factor receptor-2 (VEGFR-2) expression were assessed by quantifying immunofluorescence. The effects of the treatment were quantified in tracheal whole mounts after the vasculature was stained in situ with *Lycopersicon esculentum* lectin. Our experiments showed that VEGF dependency of blood vessels in adult mice was focused on the microvasculature of the trachea, which is distinguished by its simplicity and monolayer nature. VEGF dependency was studied by using VEGF-Trap to block VEGF signaling, whereas Ang1-induced venular plasticity was examined by systemic administration of Ad-Ang1. Mice from 4 to 16 weeks in age were treated with VEGF-Trap, Ad-Ang1, or a combination of the two. Changes in vascular endothelial growth factor receptor-2 (VEGFR-2) expression were assessed by quantifying immunofluorescence. The effects of the treatment were quantified in tracheal whole mounts after the vasculature was stained in situ with *Lycopersicon esculentum* lectin.

### Materials and Methods

#### Animals and Treatment

Male pathogen-free FVB/n mice, 4 to 16 weeks old, were purchased from Charles River, Hollister, Calif. Mice were treated with VEGF-Trap, its vehicle, Ad-Ang1, VEGF-Trap and Ad-Ang1, or nothing. Mice were treated on day 0, 3, 6, and 9 with VEGF-Trap (25 mg/kg, IP) or its vehicle (5 mmol/L phosphate, 5 mmol/L citrate, pH 6.0, 100 mmol/L NaCl, 20% glycerol, and 0.1% Tween-20) and studied on day 10. For injection with Ad-Ang1, mice were anesthetized by intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg), and 10^7 pfu of adenovirus diluted in 150 mmol/L NaCl was injected intravenously. Other groups received both VEGF-Trap and Ad-Ang1. No significant loss of body weight or change in health was observed among various age-matched groups. All experiments were performed in accordance with the guidelines of the Committee for Animal Research of the University of California, San Francisco (UCSF).

#### Lectin Injection and Perfusion Fixation of Vasculature

At 10 days after treatment, mice used for blood vessel counts and measurements were anesthetized with Nembutal (50 mg/kg, IP) and injected intravenously with biotinylated *L. esculentum* lectin (100 μg lectin in 100 μL saline, Vector Laboratories Inc) followed 2 minutes later by vascular perfusion of 1% paraformaldehyde (PFA) and 0.5% glutaraldehyde in phosphate buffer saline (PBS). Untreated mice were handled similarly. Tracheas were stained with avidin-biotin complex/diaminobenzidine (Vector), dehydrated, mounted, and examined by light microscopy.

#### Measurement of VEGF-Trap and Ang1 Effects in the Trachea

Changes in number and size of blood vessels were quantified in lectin-stained tracheal whole mounts. Vessels were analyzed on real-time brightfield color video images using a Zeiss Axioshot microscope equipped with a low-light, 3-chip CCD camera (Coolcam, Science Measure Analytical Systems) coupled to a digitizing tablet (Digi-Pad, GTCO CalComp) with custom software written in the laboratory. The diameter of blood vessels overlying the five rostral-most cartilage rings was measured. Using an overlay grid with 15-mm spacing, the diameters of arterioles, capillaries, and postcapillary venules that crossed vertical lines on each side of the trachea and down the middle of the cartilage rings were measured. Vessel number (number of capillaries per unit length) was estimated by counting the vessels intersecting a line drawn along the midline of each cartilage ring (Figure 1, dashed line). Capillary branch points (branch points per unit area) overlying the cartilage rings were also counted (Figure 1, asterisk).

#### Immunohistochemistry of Tracheal Blood Vessels

After fixation by vascular perfusion with 1% PFA in PBS, tracheas were incised along the ventral midline and processed as whole mounts. After three washes in PBS, specimens were incubated for 1 hour in a blocking solution containing 5% normal goat serum (Jackson ImmunoResearch) in PBS-0.3% Triton X-100 (PBS-plus) and then, incubated overnight at 4°C in primary antibody solution. Primary antibodies were used as a combination of hamster monoclonal anti-mouse PECAM-1 (CD31) antibody (clone 2H8, 1:1000 in PBS-plus, Chemicon) plus another antibody. VEGFR-2 immunofluorescence of tracheal blood vessels was evaluated by immunohistochemistry using a rabbit polyclonal antibody (TO14, 1:2000, Rolf Brekken and Philip Thorpe, University of Texas Southwestern Medical Center). Tie-2 receptors were immunostained using a rat antibody (1:400, mTEK, generous gift from T. Suda, Keio University, School of Medicine, Tokyo, Japan). Pericytes were stained using rabbit polyclonal anti-desmin (A0611, 1:2000 in PBS-plus, DAKO) plus rabbit polyclonal anti-Ng2 proteoglycan antibodies (AB5320, 1:2000 in PBS-plus, Jackson) or a Cy3-conjugated rat polyclonal anti-α-smooth muscle actin antibody (α-SMA, clone IA4, 1:1000 in PBS-plus, Sigma-Aldrich). Apoptotic cells were visualized by a rabbit monoclonal antibody raised against activated caspase-3 (AF835, 1:1000 in PBS-plus, R&D Systems) in combination with a rat polyclonal anti-mouse nidogen antibody as a marker of basement membrane (MAB1946, 1:1000 in PBS-plus, Jackson). After several washes in PBS-plus, specimens were incubated for 4 hours at room temperature with secondary antibodies, including FITC-conjugated goat anti-hamster (1:200 in PBS-plus, for CD31, Jackson), Cy3-conjugated goat anti-rabbit (1:200 in PBS-plus for VEGFR-2, 1:400 for desmin/Ng2 or caspase-3, Jackson), or Cy5-conjugated goat anti-rat (1:200 in PBS-plus for nidogen, Jackson). Specimens were rinsed several times in PBS-plus, flattened, and mounted in Vectashield (Vector). Specimens were analyzed and digital images obtained with a Zeiss Axioshot fluorescence microscope with a low-light CCD camera (Coolcam) and with a Zeiss LSM 510 confocal microscope with Arcon, Argon-Neon, and UV lasers.

#### BrdU Labeling

5-bromo-2′-deoxyuridine (BrdU, Sigma) was injected intravenously (1 mg in 100 μL PBS), and 3 hours later, mice were perfused with 1% PFA in PBS. Tracheas were removed, frozen in liquid nitrogen, and cut into 14-μm-thick cryosections. Sections were digested in 0.005% pepsin (Sigma) in 0.01 N HCl at 37°C for 10 minutes and
then immersed in 4 N HCl for 30 minutes at room temperature. Sections were incubated with a mouse monoclonal anti-BrdU antibody (1:200, DAKO) for 2 hours, and then with alkaline phosphatase–conjugated goat anti-mouse antibody (1:200, Jackson) for 30 minutes. Alkaline phosphatase was visualized with the Vector Red substrate kit (Vector). The sections were lightly counterstained with hematoxylin and then mounted in GelMount (Biomeda Corp).

**Measurement of VEGFR-2 Immunofluorescence**

Measurements of fluorescence intensity were made on fluorescence microscopic digital images measuring 960×1280 μm (10× objective, 1× Optovar) obtained from tracheal whole mounts stained with VEGFR-2 from 4- to 16-week-old mice after 10 days of treatment with VEGF-Trap or its vehicle using ImageJ software (http://rsb.info.nih.gov/ij). VEGFR-2 fluorescence intensity was analyzed in regions over cartilage rings where individual capillaries are readily distinguishable (n=4 mice/group, five regions/trachea). The measurement involved three steps: (1) 480×640 pixel RGB color images of capillaries were acquired with a background fluorescence (non-specific fluorescence) set at a constant level by adjusting the gain of the CCD camera; (2) color images were converted to 8-bit gray scale (fluorescence intensity from 0 to 255) using ImageJ; (3) mean intensity in the region of tracheal capillaries was calculated from pixels with intensity values >50, a value selected to exclude all background fluorescence. Fluorescence intensity values for all groups were normalized to the mean intensity of VEGFR-2 immunofluorescence in tracheas from untreated 4-week-old mice.

**Measurement of Pericycle Coverage**

Pericycle coverage of capillaries in tracheal whole mounts stained for CD31 and desmin/NG2 immunoreactivities was analyzed on real-time fluorescent microscopic images (20× objective) by using the Coolcam CCD camera and digitizing tablet. Tracheas stained for CD31 and α-SMA immunoreactivities were examined for comparison. Measurements were made on capillaries overlying five cartilage rings at the rostral end of tracheas of four control 8-week-old mice. The total length of CD31-immunoreactive capillaries and length of pericycle coverage of the same capillaries were measured. Values were expressed as the percentage of total vessel length covered by pericycle cell bodies and/or processes.

**Statistical Analysis**

Data are expressed as mean±SE (n=4 to 7 mice per group). The significance of differences among means was evaluated using analysis of variance (P<0.05), followed by Bonferroni-Dunn’s or Fisher’s PLSD test for multiple comparisons.

**Results**

**Age-Related VEGF Dependency of Tracheal Capillaries**

VEGF dependency of blood vessels was investigated by examining the microvasculature of the tracheal mucosa in mice at 4, 8, and 16 weeks of age. The architecture of the tracheal microvasculature consists of arterioles located between cartilage rings, capillaries across cartilage rings, and postcapillary venules at the edge of the rings (Figure 1). After treatment with VEGF-Trap for 10 days, the number of tracheal capillaries was conspicuously reduced (Figures 2A and 2B). The magnitude of the reduction was age-dependent: 39%, 28%, and 14%, respectively, at ages 4, 8, and 16 weeks (Figure 2C). The inverse relationship between age and effect of VEGF-Trap on capillary number was significant (r²=0.6, P<0.001). However, the reduction at 16 weeks was still significant. Additionally, VEGF-Trap treatment significantly reduced the number of capillary branch points at all ages, with the largest change at 4 weeks (Figure 2D).
VEGF Inhibition-Induced Apoptosis in Endothelial Cells

Assuming that VEGF-mediated signaling is essential for endothelial cell survival in some tracheal capillaries, we reasoned that the cells should begin to undergo apoptosis soon after the onset of VEGF-Trap treatment. Indeed, activated caspase-3 immunoreactivity was present in some capillary endothelial cells undergoing regression, after 2 days of treatment (Figure 3A, arrow). These sites were identified by strands of basement membrane lacking CD31 staining (Figure 3B, arrow).

VEGF Dependency of Vessels Despite Presence of Pericytes

To determine whether VEGF dependency results from the absence of mural cells, we systematically assessed the coverage of tracheal blood vessels by pericytes or smooth muscle cells using α-SMA, desmin, and NG2 proteoglycan as markers. In the trachea of 8-week-old mice, mural cells with α-SMA immunoreactivity were abundant on arterioles and venules but absent on capillaries (Figure 4A). However, pericytes with desmin/NG2 immunoreactivity covered almost the entire length of the capillaries as well as other vessels (Figures 4B and 4C). Measurements showed that 92.7 ± 1.0% (n = 4) of the length of tracheal capillaries was covered by pericytes. The extent of the coverage indicates that most capillaries that regress in response to VEGF-Trap (28% in this age group) are likely to have pericytes.

Age-Related Decrease in VEGFR-2 Expression

To investigate the mechanism of the effect of age on VEGF dependency, VEGFR-2 immunoreactivity of tracheal blood vessels was evaluated in either control mice or mice treated with VEGF-Trap in each of the three age groups. Under
baseline conditions, VEGFR-2 expression was highest in capillaries. Plot profiles of VEGFR-2 immunofluorescence of individual capillaries, as a measure of receptor expression, illustrated obvious differences among mice of different age (Figures 5A through 5D). With values in 4-week-old vehicle-treated mice as a reference, VEGFR-2 immunofluorescence of capillaries was 40% less at 8 weeks and 60% less at 16 weeks (Figure 6). The sensitivity of VEGFR-2 expression to VEGF-Trap was shown in further experiments where treatment for 10 days caused a large reduction in VEGFR-2 immunoreactivity (Figures 5B through 5D). VEGF-Trap reduced VEGFR-2 immunoreactivity in all age groups, with the largest effect in the 4-week group. This effect was not age-dependent (Figure 6).

**Ang1-Induced Enlargement of Venules**

The question of whether Ang1 causes an enlargement of venules in the adult mouse was investigated by examining the effects of Ad-Ang1 on the tracheal microvasculature of mice at 4, 8, and 16 weeks of age. Treatment with Ad-Ang1 resulted in enlargement of tracheal venules and the venular end of capillaries at all ages (Figures 7A and 7B, arrows). After treatment with Ad-Ang1, no change in the size of arterioles was detected (Figure 7C). However, capillaries were significantly enlarged at all ages (Figure 7C). The largest effect of Ad-Ang1 was in postcapillary venules, which enlarged by 64%, 56%, and 49% in the three age groups (Figure 7C). Age did not have a significant effect on the magnitude of enlargement ($r^2=0.07, P=0.3$).
BrdU was incorporated into endothelial cells of tracheal blood vessels after Ad-Ang1 treatment, consistent with the enlargement resulting from endothelial cell proliferation (Figure 7D). No BrdU labeling was found in tracheal vessels under baseline conditions (data not shown).

The basis of the effects of Ang1 on venules was investigated by determining the pattern of Tie-2 immunoreactivity in the tracheal vasculature. Tie-2 immunoreactivity was strong in enlarged venules but weak or undetectable in other tracheal blood vessels (Figures 7E and 7F).

**Is Ang1-Induced Venule Enlargement Mediated by VEGF?**

Ang1 is not known as an endothelial cell mitogen, but VEGF clearly has this action. Therefore, we determined whether the endothelial cell proliferation leading to Ang1-induced venular enlargement was mediated by VEGF by treating mice at 4, 8, and 16 weeks of age for 10 days with Ad-Ang1 (on day 0) and with VEGF-Trap (on day 0, 3, 6, and 9). Tracheal vessels showed the typical response to each factor, as evidenced by reduction in number of capillaries and enlargement of venules (Figures 8A through 8C). In 4- and 8-week-old mice that received both agents, the number of capillaries was reduced by 26% and 21%, respectively (Figure 8D). However, at 16 weeks, there was no reduction in capillaries, unlike the situation found after VEGF-Trap alone, suggesting a protective effect of Ang1. Treatment with Ad-Ang1 in combination with VEGF-Trap also led to enlargement of venules, by 62%, 65%, and 46% at the three ages (Figure 8E). The combination produced no significant change in the magnitude of the Ang1-induced enlargement.
Discussion

This study demonstrates that some blood vessels—other than those in the reproductive tract—are dependent on VEGF and responsive to Ang1 in adult mice. VEGF dependency of tracheal capillaries was age-dependent, with a 39% reduction at 4 weeks diminishing to 14% at 16 weeks, but was still significant in the oldest group. The reduced effect of VEGF-Trap coincided with an age-related decrease in VEGFR-2 expression, as measured by immunofluorescence histochemistry. Reduction in VEGFR-2 expression by VEGF-Trap in tracheal capillaries may reflect decreased VEGFR-2 signaling in these vessels. Furthermore, systemic treatment with Ad-Ang1 led to an enlargement of postcapillary venules in the trachea of mice through at least 16 weeks of age. This Ang1 effect was specific to venules that had strong Tie-2 immunoreactivity and resulted from endothelial cell proliferation that was not mediated by VEGF. Activated caspase-3 immunoreactivity was present in some capillaries undergoing regression, suggesting that VEGF-mediated signaling is essential for endothelial cell survival in those vessels.

Tracheal capillaries had strong VEGFR-2 immunoreactivity at 4 weeks of age. We reasoned, therefore, that a decrease in receptor expression could contribute to the relatively high VEGF dependency at this age. Measurements in the three age groups showed a progressive reduction in VEGFR-2 immunoreactivity of tracheal capillaries from 4 to 16 weeks of age. Thus, tracheal capillaries become more resistant to inhibition of VEGF signaling as VEGFR-2 expression in endothelial cells decreases with age. Our finding that VEGF-Trap treatment led to a reduction in VEGFR-2 immunoreactivity is consistent with downregulation of receptor expression in capillaries that survive the treatment, as reported in other models.24,25

Continued expression of VEGF in adult tissues suggests that the growth factor may play a role in the maintenance of normal vessel physiology and serve additional functions yet to be defined.26 Our results indicate that VEGF is a survival factor for some capillaries in the trachea. Factors that determine which vessels exhibit VEGF dependency are still unclear. One possibility is that capillaries with the highest VEGFR-2 expression are most sensitive to inhibition of VEGF signaling and thus are most vulnerable to inhibition of VEGF inhibitors. However, only ~20% of the tracheal capillaries...
exhibited this vulnerability in adult mice. Why? The limiting factor may be that the capillary network is pruned only to the minimum number of vessels necessary to maintain tissue oxygenation and nutrition. In the trachea, this may be about 80% of normal. The existence of such a limit is consistent with our finding of no obvious functional impairment. Indeed, the treated mice had no weight loss or evidence of impaired health. This absence of serious side effects is in accordance with the results of clinical trials in which anti-VEGF therapies appear to be well tolerated. Further experiments are needed to examine the VEGF dependency of capillaries in other organs of adult mice and to compare the sensitivity to VEGF signaling inhibition in normal organs to that in tumors.

Studies of the reproductive system and retina suggest that recruitment of mural cells is a critical step in vessel maturation. However, the presence of pericytes may not be a valid indication of the state of blood vessel maturity, as judged by VEGF dependency. Pericytes with desmin/NG2 immunoreactivity covered more than 90% length of tracheal capillaries. Pericyte-free segments of tracheal capillaries were too sparse to explain the 20% reduction in capillaries we found. A report that VEGF-Trap eliminated pericyte-coated blood vessels in a tumor model adds to evidence that presence of pericytes does not guarantee protection against VEGF inhibition.25

Systemic treatment of adult mice with Ang1 led to an enlargement of tracheal venules by an average of 60%. This change was not age-dependent. Tie-2 immunoreactivity was strong in regions of venular enlargement. This enlargement appears to result from endothelial cell proliferation, not vasodilatation, as shown by the presence of BrdU-positive cells after Ad-Ang1 treatment.

Unlike VEGF, Ang1 seems not to be an endothelial cell mitogen but is able to induce sprouting in vitro and control vascular development in vivo.1,2 Could the venular enlargement produced by Ang1 treatment be a consequence of VEGF-mediated endothelial cell proliferation? To address this question, we determined whether the Ang1 effect could be blocked with VEGF-Trap. Because VEGF-Trap did not alter the Ang1-induced venular enlargement, it appears that VEGF does not mediate this effect. The dose of VEGF-Trap used was previously demonstrated to inhibit VEGF signaling in a variety of subcutaneous tumor models. Moreover, this dose was sufficient to reduce the number of tracheal capillaries.

Previous studies indicate that Ang1 can protect the vasculature from VEGF-induced leakage in the adult mouse despite an abnormal phenotype.22 In this study, we show that Ang1 could be implicated in the control of vessel diameter by a mechanism involving endothelial cell proliferation but is otherwise uncharacterized. Ang1 also seems to have a protective effect, as judged by the prevention of VEGF-Trap–induced reduction in tracheal capillaries. A more definitive answer regarding the role of Ang1 in vessel maintenance requires further investigation.
In conclusion, we showed that the vasculature of the airways exhibits both VEGF dependency and plasticity to Ang1 in the adult. Decreased VEGFR-2 expression in tracheal capillaries may contribute to the age-related reduction in VEGF dependency. Furthermore, venules in the trachea enlarged after Ad-Ang1 treatment, an effect that is neither age-dependent nor mediated by VEGF. These findings could lead to new perspectives regarding the role of VEGF and Ang1 in blood vessel survival and maintenance of diameter in adult life.

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