Vascular Biology, Atherosclerosis and Endothelium Biology

Regulated Angiogenesis and Vascular Regression in Mice Overexpressing Vascular Endothelial Growth Factor in Airways

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Angiogenesis and vascular remodeling occurs in many inflammatory diseases, including asthma. In this study, we determined the time course and reversibility of the angiogenesis and vascular remodeling produced by vascular endothelial growth factor (VEGF) in a tet-on inducible transgenic system driven by the CC10 promoter in airway epithelium. One day after switching on VEGF expression, endothelial sprouts arose from venules, grew toward the epithelium, and were abundant by 3 to 5 days. Vessel density reached twice baseline by 7 days. Many new vessels were significantly larger than normal, were fenestrated, and penetrated the epithelium. Despite their mature appearance at 7 days suggested by their pericyte coat and basement membrane, the new vessels started to regress within 3 days when VEGF was switched off, showing stasis and luminal occlusion, influx of inflammatory cells, and retraction and apoptosis of endothelial cells and pericytes. Vessel density returned to normal within 28 days after VEGF withdrawal. Our study showed the dynamic nature of airway angiogenesis and regression. Blood vessels can respond to VEGF by sprouting angiogenesis within a few days, but regress more slowly after VEGF withdrawal, and leave a historical record of their previous extent in the form of empty basement membrane sleeves. (*Am J Pathol* 2004, 165:1071–1085)

Angiogenesis plays an important part in many physiological and pathological processes, including the female reproductive cycle, inflammation and wound healing, var-

ious eye and skin diseases, and in the growth of tumors.¹ Preventing or reversing angiogenesis is a promising avenue for the treatment of tumors² and promoting angiogenesis by therapeutic administration of exogenous growth factors is a potential therapy for the repair of ischemic or damaged tissues.^{3,4} In comparison with angiogenesis, however, the cellular events that accompany vessel regression have received much less attention. The factors that make some vessels mature and stable and allow others to die are gradually becoming understood.³ One notion that has arisen from studies of vessel regression in tumors and in diseases of the eye is that newly formed vessels are easily susceptible to regression on withdrawal of growth factors, whereas established vessels are more resistant.⁴ In this context, it has been proposed that pericytes and perhaps the basement membrane somehow stabilize newly formed vessels. However, it is not clear how widely this concept applies to different angiogenic situations.

Therefore, a clear need exists for simple and convenient experimental systems in which to examine these issues *in vivo*. The desirable features of such a model system should include: 1) the ability to overexpress and withdraw a single growth factor in an easily regulated manner; 2) the ability to express the growth factor in an orthotopic tissue-specific manner in adult animals, preferably in those with an intact immune system; and 3) the ability to examine the blood vessels and neighboring cells at multiple levels, ranging from the entire microvasculature to individual identified segments of the vasculature observed at the cellular and molecular level.

One new approach that satisfies these criteria is the expression of growth factors driven by tissue-specific promoters and regulated by tetracycline in so-called "tet-on" or "tet-off" systems. We took advantage of a recently

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developed tet-on transgenic mouse in which vascular endothelial growth factor (VEGF) is selectively overexpressed in the airways under the control of the Clara cell CC10 promoter and is regulated by doxycycline.⁵ In this study, we characterized the model to determine how quickly blood vessels grow in response to sustained VEGF overexpression in adult airways. Next, we examined how the morphology and phenotype of the newly formed vessels compare to that of normal vessels. In particular, we examined features that are regarded as evidence of maturity, such as the investment of endothelial cells by pericytes and basement membrane, and the expression of receptors for growth factors involved in vessel growth. Finally, we determined the time course of vascular regression after withdrawal of VEGF, whether pericyte and basement membrane investment protect newly formed vessels from regression, and whether these elements withdraw concurrently with regressing endothelial cells.

Materials and Methods

Production and Genotyping of Transgenic Mice

Mice overexpressing VEGF in the airways were generated at Yale University as described.⁵ Briefly, the Clara cell 10-kd (CC10) promoter and two transgenic constructs were used to target human VEGF165 to the murine lung in an externally regulatable manner.⁶ The gene for human VEGF165 was used because the ligand binds and activates the mouse receptors, and permits the analysis of potential autoregulatory pathways using techniques that differentiate the human transgene and the endogenous mouse gene. Construct 1, CC10-rtTA-hGH, contains the CC10 promoter, the reverse tetracycline transactivator (rtTA), and human growth hormone (hGH) intronic and polyadenylation sequences. Construct 2, tet-O-CMV-VEGF-hGH, contains a polymeric tetracycline operator (tet-O), minimal cytomegalovirus promoter, human VEGF165, cDNA, and hGH intronic and polyadenylation signals. Both constructs were purified and were simultaneously microinjected into pronuclei as described.⁷ Because Clara cells are the most abundant cells in the epithelium of the mouse trachea and bronchi,⁸ overexpression of VEGF occurred in the airway epithelium. Male transgenic mice were housed under barrier conditions at the University of California, San Francisco, and were bred with wild-type (WT) C57BL/6 female mice. Tail tips were genotyped by polymerase chain reaction to identify the VEGF165 transgenic (VEGF-Tg) mice.⁵ The University of California, San Francisco, Committee on Animal Research approved all animal procedures.

Induction of VEGF Overexpression and Its Quantitation

At 8 weeks of age, VEGF-Tg and WT littermate control mice of either sex were given normal drinking water or water containing 0.5 mg/ml of doxycycline hydrochloride (dox; Sigma Chemical Co., St. Louis, MO) and 40 mg/ml

of sucrose, as described previously.⁷ To switch off VEGF production, mice were again given normal water. To quantify the production of VEGF in the lungs and airways, mice were euthanized, the trachea was cannulated and aspirated with 2 vol of 1.0 ml of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. Each bronchoalveolar lavage fluid sample was centrifuged and the supernatants were stored at -70°C until used. The concentration of VEGF in bronchoalveolar lavage fluid was measured with a commercial enzyme-linked immunosorbent assay kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

Lectin Staining of Airway Microvasculature

Mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine i.p.) and the vasculature was labeled by perfusion with a biotinylated *Lycopersicon esculentum* lectin that binds uniformly to the luminal surface of endothelial cells and intravascular leukocytes, and was detected by avidin-biotin histochemistry.^{9,10} In experiments designed to detect vascular leakage, mice were injected intravenously with 200 μg of biotin-labeled *Ricinus communis* agglutinin 1 lectin (RCA1; Vector Laboratories, Burlingame, CA) 20 minutes before fixation.⁹ Biotinylated RCA1 lectin was detected by Cy3- or Cy5-conjugated streptavidin and processed as described below for immunohistochemistry.

Transmission Electron Microscopy

Tissues were fixed by vascular perfusion with buffered fixative contained 1% paraformaldehyde and 3% glutaraldehyde and processed for transmission electron microscopy as described previously.¹¹

Immunohistochemistry

Tracheal whole mounts were stained using immunohistochemical procedures described previously.^{12,13} Endothelial cells were identified with hamster or rat monoclonal antibodies to CD31 (Chemicon, Temecula, CA, or BD Pharmingen, San Diego, CA) or with a rabbit polyclonal antibody to VEGFR-2 (Dr. R. Brekken, University of Texas Southwestern, Dallas, TX). Pericytes were identified with rabbit polyclonal antibodies to desmin (DAKO, Carpinteria, CA), or NG2 chondroitin sulfate proteoglycan (Chemicon), or monoclonal antibodies to α -smooth muscle actin (Sigma) or anti-platelet-derived growth factor receptor- β (Dr. A. Uemura, Kyoto University, Kyoto, Japan). Basement membrane was identified with rabbit polyclonal antibodies to type IV collagen (Cosmo Bio Co. Ltd., Tokyo, Japan) or laminin (Chemicon), or a rat monoclonal antibody to entactin/nidogen (Chemicon). Apoptotic cells were identified with a rabbit polyclonal antibody to activated caspase-3 (R&D Systems). After several rinses with PBS, specimens were incubated for 6 hours at room temperature with fluorescent (fluorescein isothiocyanate, Cy3, or Cy5) secondary antibodies (goat or donkey anti-rat, anti-hamster, or anti-rabbit) (Jackson ImmunoRe-

search, West Grove, PA). They were then examined with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a low-light, three-chip CoolCam CCD camera (SciMeasure Analytical Systems, Atlanta, GA) or a Zeiss LSM 510 confocal microscope. In some cases artificial colors were assigned to the emission wavelengths to make the images easier to visualize, eg, Cy5 emission fluorescence was displayed in the red channel. The intensities of fluorescent images were analyzed with ImageJ software (<http://rsb.info.nih.gov/ij/>) as described.¹⁴ Measurements were made on immunohistochemically stained tracheal whole mounts from WT mice or VEGF-Tg mice treated with dox-water for 7 days. In brief, digital images were captured from WT and VEGF-Tg specimens using the same acquisition settings for threshold and gain. The information for platelet-derived growth factor (PDGF)-R β or VEGFR-2 pixels (red channel of the RGB images) was converted to 8-bit gray scale images (fluorescence intensity range, 0 to 255) and analyzed with the surface contour tool of ImageJ, using a lookup table that color coded greater pixel intensities as warmer (redder) colors. At least four tracheal whole mounts were stained for each antibody and treatment group.

Morphometric Measurements

We identified blood vessels, nerves, and lymphatics in tracheal whole mounts based on their position, staining properties, and cellular architecture. Blood vessels formed regular arcades over the tracheal cartilage rings.^{15,16} Arteries were relatively straight and unbranched vessels of uniform diameter with continuous smooth muscle coats, whereas capillaries traversed the cartilage rings and led to venules located between the cartilage rings. Lymphatics were large diameter vessels with thin walls located between the cartilage rings, as identified by toluidine blue staining, and by very weak immunohistochemical staining for CD31 and basement membrane markers and for specific lymphatic markers (data not shown). Nerves were identified by architecture and distribution, as described previously.¹⁷ The diameter of blood vessels and percentage area densities of the lectin-stained vasculature was assessed by morphometric methods in tracheal whole mounts observed with a Zeiss Axiophot microscope connected to display screen and a graphic tablet, as described previously.¹⁵ For vessel counting, 10 regions of 1.4 μm^2 each (total area of 14 μm^2) were measured in each trachea at a final magnification of 184, and the average percentage area of mucosa occupied by vessels was calculated. For measurements of vessel diameter, 100 vessels were measured in each group (25 vessels per mouse) at a final magnification of 735. The thickness of vascular basement membrane was measured using the graphics tablet on transmission electron micrographs of blood vessels at a final magnification of 56,000. To avoid errors resulting from tangential sections, we selected vessels that were sectioned approximately cross-sectioned with respect to the long axis of the vessel.

Statistics

Values are expressed as means $\pm$ SEM (SE). Group sizes consisted of four or more mice per group. The significance of differences between means was assessed by the *t*-test or by analysis of variance followed by the Bonferroni/Dunn test for multiple comparisons, with statistical significance judged as $P < 0.05$.

Results

Onset of Angiogenesis after Overexpression of VEGF

The vasculature could be observed in its entirety in lectin-stained tracheal whole mounts. In the tracheas of WT mice drinking normal or dox-water, a two-dimensional network of repeating arcades of blood vessels was present in the mucosa beneath the epithelium. Arterioles and venules were located in the connective tissue between the cartilages and were interconnected by capillaries crossing the cartilage rings (Figure 1A). Tracheas from VEGF-Tg mice given normal water were indistinguishable from tracheas from WT mice given dox-water (data not shown), and there were no significant differences between the percentage vascular area densities in tracheal whole mounts in mice from these experimental groups ($26.3 \pm 2.3\%$ versus $27.3 \pm 1.0\%$, respectively (mean $\pm$ SEM, $P > 0.05$), or in the diameter of the capillaries overlying the cartilages ($8.6 \pm 0.1 \mu\text{m}$ versus $9.1 \pm 0.3 \mu\text{m}$, $P > 0.05$).

Within 1 day of drinking dox-water, tracheal blood vessels in VEGF-Tg mice started to sprout, and multiple endothelial sprouts were conspicuous from 3 to 5 days (Figure 1B). The sprouts arose mostly from venules located in the spaces between the cartilages (Figure 1, B and C). At 7 days, blind ending sprouts, vascular loops, and bulbous expansions of vessels directed toward the epithelium were abundant (Figure 1D). Sprouts ranged in shape from blunt to sharp-pointed, were frequently only weakly stained by the lectin, and often had erythrocytes stuck at their blind endings (Figure 1, E and F). However, these new vessels were accessible to the circulation because their lumens were at least partially stained by intravenously injected lectin.

On average, the newly formed vessels were closer to the apical surface of the airway epithelium than capillaries in WT mice (Figure 1, G and H) and the newly formed bulbous expansions ($15.9 \pm 0.6 \mu\text{m}$ in diameter; range, 9 to 23 μm) were significantly larger than normal capillaries. Many vessels penetrated the elastic lamina at the base of the epithelium and appeared to press against the epithelium, displacing it into an abnormally thin sheet. Vacuoles were found in and between the epithelial cells. Some newly formed blood vessels appeared to be located entirely within the epithelium (Figure 1H).

The area density occupied by lectin-stained blood vessels in VEGF-Tg mice drinking dox-water increased rapidly in the first week, peaked at 14 days, reaching twice the area density of WT mice, then declined only slightly

Figure 2. Time course of onset of angiogenesis and VEGF expression after doxycycline. **A:** Percentage area density occupied by blood vessels perfused and stained with *L. esculentum* lectin in whole mount preparations of the mouse trachea (group size at least four mice per point). **B:** Concentration of VEGF in bronchoalveolar lavage fluid as measured by enzyme-linked immunosorbent assay. *, Significant difference from baseline ($P < 0.05$).

by 28 days (Figure 2A). It remained elevated for at least 56 days (vessel area density, $53.2 \pm 1.0\%$). The changes in the vessels followed the expression of VEGF. Baseline levels of VEGF protein levels in bronchoalveolar lavage fluid were at or below the level of detection of the assay (18 pg/ml). After dox-treatment VEGF levels were increased ~1000-fold, peaking at 7 days at 15 ± 2.5 ng/ml, and then stayed at a plateau throughout time (Figure 2B).

No mortality was observed in VEGF-Tg mice drinking dox-water for 56 days, the longest time point studied.

Do Newly Formed Blood Vessels Have a Normal Morphology?

Transmission electron microscopy confirmed that the normal pre-existing vessels of WT mice were located in the mucosa at some distance from the epithelium (Figure 3A), whereas, at 7 days of dox-treatment, the blood vessels of VEGF-Tg mice were much closer to the epithelium, and sometimes penetrated inside it (Figure 3B). The intraepithelial vessels were surrounded by empty-looking spaces separating them from the adjacent epithelial cells. Epithelial cells had vacuoles, and were often stretched and distorted, sometimes with only a thin rim of cytoplasm separating the intraepithelial blood vessels from the airway lumen (Figure 3B). Vessels of WT mice had endothelial walls of relatively uniform diameter, but with few fenestrations (Figure 3C). The newly formed endothelial cells of VEGF-Tg mice on dox-water varied in their wall thickness (Figure 3; D to H) and many had regions with numerous fenestrations (Figure 3, D and F). Other growing vessels had plump cytoplasm with abundant ribosomes and prominent rough endoplasmic reticulum, and slit-like lumens (Figure 3G), features characteristic of metabolically active endothelial cells. Coagulated fibrin-like material, stagnant erythrocytes, and platelets were common in the new vascular segments, and occasionally erythrocytes were found trapped between endothelial cell borders (Figure 3; B, D, F, H).

Electron microscopy showed that at 7 days, endothelial cells of newly formed vessels were already entirely ensheathed by a basement membrane (Figure 3D) that was somewhat thinner and more irregular than in normal vessels of the WT mice (85 ± 5 nm versus 135 ± 11 nm, $P < 0.05$) (Figure 3, E and F). The endothelial cells of normal vessels in WT mice and newly formed vessels in VEGF-Tg mice were usually closely associated with processes of pericytes that shared the endothelial basement membrane, and with more distant processes of fibroblast-like cells that did not have a basement membrane (Figure 3; A to H). At 7 days, the perimeter of newly formed vessels frequently consisted of two or more endothelial cells (Figure 3; B, D to H).

Immunohistochemical Studies of Normal and Growing Vessels

Immunohistochemical staining for CD31 and for desmin confirmed that periendothelial cells were present on all

Figure 1. VEGF overexpression induces angiogenesis of airway blood vessels. **A–F:** Blood vessels stained by perfusion of biotinylated *L. esculentum* lectin in flattened whole mounts of mouse tracheas. **A:** WT control mouse drinking normal water. Blood vessels form regular arcades of arterioles (A) and venules (V) in regions between the cartilages and capillaries (arrows) overlying the cartilages. **B:** VEGF-Tg mouse, 5 days of drinking doxycycline (dox)-water. Endothelial sprouts arise from postcapillary venules (arrows). **C:** Enlargement of part of B showing endothelial sprouts (arrows). **D:** VEGF-Tg mouse, 7 days dox-water. Numerous endothelial loops and sprouts increase the vessel area density. **E and F:** Nomarski images of enlarged regions of D. **E:** Endothelial sprouts have various shapes, including pointed (arrow) and blunt (arrowheads). Some are field with stagnant erythrocytes. **F:** Numerous endothelial loops form bulbous protrusions (arrows) toward the epithelium. **G and H:** Toluidine blue-stained sections of tracheal mucosa. **G:** WT control, normal water. Blood vessels (arrows) are located far from the epithelium in the connective tissue overlying the cartilage (C). **H:** VEGF-Tg mouse, 7 days dox-water. Blood vessels (arrows) are more abundant and are located much closer to, or even within, the epithelium. Scale bar: 200 μ m (A, B, D); 50 μ m (C, E, F); 25 μ m (G, H).

segments of the tracheal microvasculature of WT mice (Figure 4A). In general, the endothelial tubes in WT mice had relatively smooth cylindrical or tapering outlines. In contrast, the shape of the periendothelial cells changed abruptly from the short circumferentially oriented smooth muscle cells on arterioles (Figure 4B), to spindle- or spider-shaped pericytes longitudinally oriented on capillaries, and then more gradually to periendothelial cells of intermediate shape on venules. Indeed, each type of vessel could be recognized from the shape and orientation of periendothelial cells alone (Figure 4B). Staining for α -smooth muscle actin was not an effective way to detect all pericytes; only smooth muscle cells on arterioles and pericytes on larger venules had α -smooth muscle actin immunoreactivity (Figure 4, C and D), but the pericytes on capillaries did not. NG2, another pericyte marker, had a reciprocal distribution, and was strongly expressed on pericytes on capillaries, but was only weakly expressed on larger vessels (Figure 4, C and D). VEGFR-2 was expressed more strongly on capillaries and small post-capillary venules than on arterioles and larger venules (Figure 4E).

Normal tracheal blood vessels of WT mice were entirely covered by a relatively smooth basement membrane, as shown by collagen type IV immunoreactivity (Figure 4F). The nuclei of pericytes were also outlined by collagen type IV immunoreactivity as smooth bubble-shaped domes. However, type IV collagen immunoreactivity was not distributed uniformly over the entire vessel surface, but showed denser and weaker patches (Figure 4F). Weak patches of type IV collagen immunoreactivity were even found on some lymphatic vessels and autonomic nerve bundles. Focal spikes of collagen type IV immunoreactivity occasionally projected away from blood vessels (Figure 4F). Of the markers used to localize basement membrane, type IV collagen proved the most effective. Laminin and nidogen/entactin were also found around blood vessels, but were more strongly expressed around other cells, such as nerve fibers, and thus were less useful.

At the early stages of angiogenesis, eg, at 3 days, blood vessels and endothelial cells in VEGF-Tg mice had irregular jagged contours (Figure 5; A to C). In contrast to WT mice, after overexpression of VEGF, the basement membrane sleeves were ruffled and much more irregular (Figure 5; A to C) and projected away from endothelial surface to a much greater extent (Figure 5, B and C). However, almost all endothelial spouts appeared to be entirely coated by collagen type IV immunoreactivity (Figure 5C). By 7 days, newly formed blood vessels, including those present within the epithelium, were entirely coated by collagen type IV immunoreactivity (Figure 5D).

Similar results were found for laminin and nidogen/entactin staining (data not shown).

A few tips of endothelial sprouts were devoid of pericytes stained for desmin or NG2 immunoreactivity (Figure 5, E and F), but most vessels appeared to be covered. By 7 days, most newly formed vessels, including the loops and bulbous expansions found within the epithelium were invested with slender spindle-shaped pericytes staining for desmin immunoreactivity (Figure 5G), but not for α -smooth muscle actin (not shown). Most of the pericytes were closely associated with individual endothelial tubes, but a few pericytes bridged different endothelial loops via long cellular processes. PDGFR- β and VEGFR-2 are tyrosine kinase receptors associated with pericytes and endothelial cells, respectively. We found that there was a marked increase in the staining intensity of both receptors in newly formed vessels (Figure 5; H to M). Staining for RCA1 lectin that had been injected intravenously before fixation revealed the pattern of endothelial cell borders and some sites of leakage of lectin into the connective tissue surrounding newly formed vessels in dox-treated VEGF-Tg mice (Figure 5N).

Time Course and Mechanism of Regression of Newly Formed Vessels

Because newly formed vessels were already well developed by 7 days of dox-treatment and had almost reached their peak density, we chose this time point as the starting point for studying the effects of withdrawal of doxycycline. Newly formed vessels, as assessed by lectin staining, started to regress within a few days of switching off VEGF production (Figure 6A). The initial regression of vessels was rapid, with approximately half of newly formed vessels disappearing within 3 days, including almost all of the abnormal bulbous sprouts and loops in or near the epithelium. In comparison, production of VEGF in VEGF-Tg mice fell rapidly within 1 day and reached baseline levels within 3 days of withdrawal of doxycycline (Figure 6B). A conspicuous early feature of apparently regressing vessels was stasis of erythrocytes and weak or absent lectin staining of the lumen beyond the point of blockage (Figure 7; A to D). The vascular density continued to decline gradually, so that from 3 days of withdrawal of doxycycline onwards the number of perfused vessels was not significantly greater than in control tracheas (Figure 6A). The area density of perfused vessels reached baseline values within 28 days (Figure 6A and Figure 7, C and D).

The network of blood vessels, as revealed by immunohistochemical staining for CD31, appeared similar to

Figure 3. Ultrastructural features of newly formed airway blood vessels. **A:** WT control, normal water. Capillary (*) distant from epithelium. **B:** VEGF-Tg mouse, 7 days dox-water. Newly formed blood vessels (*) are close to and within the distorted epithelium containing vacuoles (arrowhead). Endothelial cells are accompanied by pericytes and periendothelial cells (arrow) and are separated by empty-looking space from epithelium. **C:** WT control, normal water. Endothelial cells of normal capillary are accompanied by basement membrane (arrowheads) and processes of a pericyte (arrow) and periendothelial cell beyond (double arrow). **D:** VEGF-Tg mouse, 7 days dox-water. **D:** Capillary has many fenestrations and delicate basement membrane (arrowheads). Thin processes of a pericyte (arrow) and periendothelial cells (double arrow) accompany the new blood vessel. A platelet (*), erythrocyte, and coagulated material (**) appear trapped inside lumen. **E** and **F:** Enlarged regions of **C** and **D**. **G:** Growing blood vessel has a narrow slit-like lumen (arrowheads). **H:** Another growing blood vessel has plump endothelial cells with many ribosomes. An erythrocyte is trapped between the endothelial cell junctions (*). Pericyte processes (arrows) accompany blood vessel. Scale bar: 5 μ m (**A**, **B**); 1 μ m (**C**, **D**); 0.5 μ m (**E**, **F**); 2 μ m (**G**, **H**).

Figure 4. Immunohistochemical features of normal endothelial cells and pericytes. **A:** Overview of a whole mount preparation of tracheal microvascular arcades stained for endothelial cells (hamster anti-mouse CD31, green) and pericytes (desmin, red). **B:** Higher magnification view of desmin-positive smooth muscle cells (red) on arteriole (A) and pericytes on capillaries (C) and venules (V) stained with rat anti-mouse CD31 antibody (green). **C and D:** Periendoneurial cells of tracheal microvascular arcades stained for α -smooth muscle actin (red) and NG2 (green). Arterioles (arrow) and venules are stained for α -smooth muscle actin, but NG2 immunoreactivity is weak. Pericytes on normal capillaries (arrowheads) are strongly positive for NG2, but negative for α -smooth muscle actin immunoreactivity. **E:** Staining for endothelial cells (hamster anti-mouse CD31, green) and VEGFR-2 (red). VEGFR-2 is more strongly expressed on capillaries (arrowheads) than on arterioles (A) or venules (V). VEGFR-2 is also expressed on lymphatic vessels (L). **F:** Type IV collagen immunoreactivity (red) on normal blood vessels that have relatively smooth outlines. Bubble-like bulges (arrowheads) indicate location of pericyte nuclei. Weak staining for type IV collagen is also present on nerve bundles (N) and in patches on lymphatic vessels (L). Scale bar: 65 μ m (A); 50 μ m (B, D); 200 μ m (C); 100 μ m (E); 36 μ m (F).

that seen by lectin staining. By 7 days after withdrawal of doxycycline the bulbous vascular sprouts and loops had entirely disappeared, but the overall vascular density still appeared greater than in control tracheas, especially over the cartilaginous portions (Figure 7, E and F). Both of the anti-CD31 antibodies we used also stained leukocytes (mainly granulocytes and monocytes) in addition to the endothelium. This fortuitous property showed that many inflammatory cells were present in the connective tissue spaces around the blood vessels (Figure 7; E to I). In our model, extravascular leukocytes were consistently more abundant during vessel regression than during vessel growth.

Double staining for type IV collagen and CD31-immunoreactivity of the regressing vasculature showed that type IV collagen immunoreactivity was co-localized over the entire vascular network. Many vascular segments had endothelial cells that were thinned, shriveled, or partially retracted. In addition, staining for type IV collagen labeled tubular segments and loops of the microvascular network that were totally lacking CD31 immunoreactivity, ie, the segments consisted of empty sleeves of basement membrane (Figure 7; E to G). In an attempt to give the newly formed vessels more time to become mature and stabilized, we treated some VEGF-Tg mice with doxycycline for 28 days rather than 7 days. Even so, the newly formed vessels still regressed on withdrawal of doxycycline (Figure 7G).

Double staining for desmin and CD31-immunoreactivity of the regressing vasculature showed that all desmin immunoreactivity was confined to pericytes associated with the surviving vasculature, ie, desmin-immunoreactive pericytes were not found lingering at sites of former blood vessels that had regressed (Figure 7, H and I). The pericytes on surviving vessels were normal in their appearance and in packing density, and did not appear to be bunched up along the vessels.

Triple-staining for CD31 as a marker of endothelial cells, nidogen/entactin as a marker of basement membrane, and activated caspase-3 as a marker of apoptosis, revealed that both endothelial cells (Figure 7, J and K) and pericytes (Figure 7, L and M) underwent apoptosis. Sometimes the dying cell was located in or near a vascular segment that was shriveled or had endothelial cells lacking entirely, but still had an empty basement membrane sleeve (Figure 7, K and M). The antibody to nidogen/entactin also stained ring-like structures at the base of the epithelium (Figure 7K), and nerve bundles (Figure 7M).

Discussion

In this study, we examined the growth, maturity, and death of vessels in the mouse trachea in a system in which the overexpression of VEGF could be controlled in a simple, reliable, and reversible manner. We found that endothelial sprouts grew within a few days of VEGF overexpression and that newly formed vessels persisted as long as the stimulus was present. Newly formed vessels were larger than normal capillaries, were frequently fe-

nestrated, leaky, and abnormally close to the epithelium—the source of VEGF. The newly formed vessels were covered by a coat of basement membrane and pericytes within 7 days of VEGF exposure. These features, which are sometimes interpreted as signs of vessel maturity, failed to protect the newly formed vessels from total regression on withdrawal of VEGF. Regressing vessels showed a characteristic sequence of events. Blood flow stopped first, followed by retraction, death, and fragmentation of endothelial cells. Pericytes also died by apoptosis, or perhaps dedifferentiated and became no longer detectable by immunohistochemical staining for their former marker molecules. Empty basement membrane sleeves persisted around former vessels for some time after endothelial cells had disappeared. Eventually, phagocytes cleared the cell debris, and the tracheal microvasculature returned to its baseline condition.

Comparison of Present Model with Other Models of Angiogenesis

In our model, overexpression of VEGF caused rapid vessel growth, with a time course similar to that observed in genetic overexpression of the retina.¹⁸ In contrast, overexpression of VEGF in the liver and in the heart led to a slower growth of sac-like vessels with large lumens and the eventual formation of hemangioma-like vascular sacs.¹⁹ Growth of new tracheal vessels was completely reversible on removal of VEGF. We found no evidence for leakage of the VEGF-induced phenotype in VEGF-Tg mice in the absence of doxycycline, suggesting that the angiogenic switch could be efficiently turned on and off. Compared to the large abnormal vessels described as “hemangiomas” in heart and liver¹⁹ or “glomeruloid bodies” and “mother vessels” formed in the skin in response to adenovirus-mediated VEGF,²⁰ the increase in size of the newly formed tracheal vessels was more modest, with only a twofold increase in diameter. The reasons for this difference are not clear, but may involve local concentrations of VEGF attained in the different organs or differential tissue responsiveness.²¹ An unusual feature of angiogenic vessels in the trachea was the penetration into the epithelium, a tissue that normally excludes blood vessels in all but special circumstances, such as cancer. This may be explained by the directional growth of the blood vessels toward the source of the VEGF from Clara cells, which are abundant in tracheal epithelium.⁸

Ultrastructural Features of Growing Endothelial Sprouts

In the present study, the vessels growing in response to overexpression of VEGF had many of the ultrastructural features described for angiogenic vessels in other systems. Growing vessels had an abundance of cytoplasmic apparatus for protein synthesis and secretion, ie, rough endoplasmic reticulum, ribosomes, and membrane-bound vesicles in plump embryonic-looking endothelial cells and narrow slit-like lumens.^{22–24} In the same

Figure 6. Time course of regression of blood vessels and VEGF after withdrawal of doxycycline. VEGF-Tg mice were given dox-water for 7 days, and then normal water. **A:** Percentage area density occupied by blood vessels stained by biotinylated *L. esculentum* lectin in whole mount preparations of the trachea. **B:** Concentration of VEGF in bronchoalveolar lavage fluid as measured by enzyme-linked immunosorbent assay. *, Significant difference from baseline; †, significant difference from initial peak value at 7 days of doxycycline treatment ($P < 0.05$).

Figure 5. Immunohistochemical features of growing endothelial cells and pericytes. **A:** Low magnification of blood vessels from VEGF-Tg mouse treated with dox-water for 3 days and stained for endothelial cells (CD31, green) and type IV collagen immunoreactivity (red). Profiles of blood vessels are rough and jagged. Regions indicated by **arrows** and **arrowheads** are enlarged in **B** and **C**, respectively. Endothelial sprouts (green, **arrowheads**) are covered by a delicate irregular coat of type IV collagen immunoreactivity (red). **D:** Section of tracheal wall from VEGF-Tg mouse treated for 7 days with dox-water stained for endothelial cells (green) and type IV collagen immunoreactivity (red). Newly formed blood vessels are located close to the epithelial basement membrane (**red line**, **arrowheads**) or penetrate (**arrows**) into the epithelium (E). **E–K:** Pericytes (red) on growing blood vessels stained for CD31-immunoreactivity (green). **E** and **F:** VEGF-Tg mouse, 3 days dox-water. Pericytes (red) stained for desmin in **E**, and NG2 in **F**, are absent from the extreme tips of growing endothelial sprouts (**arrows**). **G:** VEGF-Tg mouse, 7 days dox-water. Pericytes stained for desmin are present on most endothelial loops and sprouts (**arrows**). Some pericytes cross between adjacent endothelial loops. **H–M:** Up-regulation of PDGFR- β and VEGFR-2 receptor fluorescence intensity (red) in newly formed blood vessels. Endothelial cells labeled with CD31 (green). Pairs of photos are printed with the same contrast and intensity settings, so intensity is a measure of receptor expression. **H** and **I:** Expression of PDGFR- β immunoreactivity (red) in WT mice (**H**) and VEGF-Tg mice treated with dox-water for 7 days (**I**). PDGFR- β immunoreactivity is weak in blood vessels of WT mice, but is stronger in newly formed blood vessels. **J** and **K:** VEGFR-2 expression (red) is present on endothelial cells of WT mouse (**J**) and is greatly increased in VEGF-Tg mouse (**K**) treated with dox-water for 7 days. **L** and **M:** Surface plots of fluorescence intensities of PDGFR- β and VEGFR-2 immunoreactivity shown in **H** to **K**. Warmer, redder colors indicate greater pixel intensities. **N:** Leakage of RCA1 lectin (red, **arrows**) from blood vessels in VEGF-Tg mouse treated with dox-water for 7 days. Scale bar: 50 μ m (**A**, **E**, **F**, **H–K**); 20 μ m (**B**, **C**); 40 μ m (**D**); 25 μ m (**G**, **N**).

VEGF-Tg specimens, other more differentiated vessels had abundant endothelial fenestrations that were uncommon in WT controls. The induction of fenestrations is a well-known effect of VEGF on blood vessels.^{25,26} Electron microscopy showed that newly formed vessels were associated with pericyte processes surrounded by basement membrane and fibroblast-like processes. The fibroblast-like cells may represent mesenchymal cells being recruited toward newly formed blood vessels to become pericytes. Growing blood vessels are thought to recruit such cells by secretion of PDGF-B that acts on PDGFR- β receptors on the migrating cells.^{27–29}

Increased Leakiness of Newly Formed Vessels

Our studies with intravenously injected RCA1 lectin suggested that the newly formed tracheal vessels were leaky, as has been described in inflammatory conditions.⁹ In related studies of the lung, extravasation of Evans blue and lung weight wet-to-dry ratio increased in VEGF-Tg mice compared to WT controls.⁵ Electron microscopy showed edematous spaces around some of the vessels, especially those in the epithelium, and imperfectly sealed endothelial gaps. The deposits of a felt-like material observed are similar to fibrin or fibrinoid described in other situations, thought to establish a provisional matrix in which angiogenic vessels can thrive.^{24,30} Our conclusion that the newly formed vessels are leaky is hardly surprising because VEGF was originally described as vascular permeability factor.³¹ The ability of VEGF to up-regulate the expression of VEGFR-2 receptors may also contribute to the increased leakiness of the newly formed vessels.³²

Basement Membrane and Pericytes Are Not Sufficient to Make Vessels Mature and Stable

It is often assumed that growing blood vessels are deficient in basement membrane.³³ We used electron microscopy to show that basement membrane was indeed present on vessels by 7 days of VEGF overexpression, although it was somewhat thinner on new vessels than on established ones. Our finding is in keeping with a detailed electron microscopic study of angiogenesis in the growing rat mesentery, where basement membrane was present on all vessels except at the extreme tips of endothelial sprouts.²⁴ Our immunohistochemical studies with

multiple markers confirmed that basement membrane covered essentially all vessels, but that the coat was rougher and more ragged in growing vessels. These findings suggest that rather than being totally absent, the basement membrane is still in the process of being generated, and is in a state of flux on actively growing vessels.

Similar considerations apply to the identification of pericytes. Although the precise definition of what exactly a pericyte is and what is a good marker for it are not entirely resolved, their importance in the microvasculature is now recognized.^{27,29,34} Pericytes share the basement membrane of a capillary or postcapillary venule, but the exact distinction between them and true smooth muscle cells is not clear. Likewise, the delicate fibroblast-like cells we observed may represent mesenchymal cells being recruited toward the newly formed blood vessels, perhaps via secretion of PDGF acting on PDGFR- β receptors on the migrating cells.^{27–29} It appears that different angiogenic stimuli result in differing degrees of pericyte investment of capillary sprouts in the rat mesentery.³⁵

Currently, there is no immunohistochemical marker that exclusively labels all pericytes. Smooth muscle actin is not an adequate marker of pericytes in the tracheal microvasculature because it fails to identify pericytes on capillaries in normal tissues, but this marker can be up-regulated in pericytes in tumors.¹² Desmin appears to be a more consistent marker, but is also strongly expressed by smooth muscle cells,^{12,35} whereas NG2^{20,36} is only expressed by pericytes on capillaries. Regardless of these issues, we showed conclusively that pericytes invest newly formed vessels in mice overexpressing VEGF. Moreover, we found that such vessels with indisputable pericytes were still subject to regression on withdrawal of VEGF. We conclude that although pericytes may contribute to vessel maturity, their mere physical presence is not sufficient to protect such vessels from regression and other factors involving their relationship to endothelial cells may be important for stabilizing newly formed vessels. Indeed, it is possible that newly formed vessels are susceptible to rapid degeneration on withdrawal of growth factor because their interactions with pericytes and/or basement membrane are abnormal. Further studies are needed to determine whether only newly formed vessels regress on VEGF withdrawal, and whether any of the newly formed vessels eventually become VEGF-independent.

Mechanism of Blood Vessel Regression

Although many studies have investigated blood vessel growth, relatively few studies have addressed the mechanisms of vascular regression. Such studies are needed for two applications: eliminating undesirable new blood vessels in growing tumors and sustaining desirable new blood vessels induced by growth factor therapy in ischemic tissues.^{2–4} In the present model, the time course of vascular regression was somewhat slower than for vessel growth. The regression appears to have two phases, with a rapid cessation of blood flow to newly formed vessels, then a more gradual removal of the various components of the vessel wall. Cessation of blood flow may be an early predictor of which individual blood vessels are destined to regress. In our study, staining for CD31 consistently seemed to label more vessels than perfusion of *L. esculentum* lectin, but many of the CD31-labeled endothelial cells were in various stages of shrinkage or retraction. Destruction of the vessel wall proceeded in two steps, with a time lag. Endothelial cells quickly shriveled, retracted, fragmented, and died by apoptosis, but the basement membrane sleeves that originally surrounded them persisted for longer, leading to the presence of numerous distinct empty basement membrane sleeves. Such empty basement membrane sleeves have been observed previously by electron microscopy in regressing capillaries in injured tissues and in tunica vasculosa lentis of the eye.^{37,38} Recently, such empty sleeves, termed basement membrane ghosts have been observed in tumors treated with anti-angiogenic drugs.³⁹ The empty basement membrane sleeves testify as a historical record indicating where degenerating vessels once existed—a form of forensic histopathology. Such empty sleeves could provide fast-track pathways for the regrowth of vessels should the balance of pro- and anti-angiogenic factors tilt in favor of growth. The empty sleeves could also facilitate chronic reoccurrence of episodes of inflammation and the persistence of remodeled abnormal vessels in asthma.

Other features associated with the vessel regression observed in the present study resembled those reported in similar cases of regression—blood stasis, influx of inflammatory cells, and eventual clearing of the debris by phagocytes.⁴⁰ Apoptosis is a likely fate for both endothelial cells and pericytes, as reported also for degenerating vessels in the pupillary membrane^{41,42} and in VEGF-dependent tumors on withdrawal of VEGF.⁴³ The staining

Figure 7. Features of regressing endothelial cells and pericytes. **A–D:** Low (**A, C**)- and high (**B, D**)-magnification views and of blood vessels in tracheal whole mount preparations stained by perfusion of *L. esculentum* lectin in VEGF-Tg mouse treated with dox-water for 7 days, and then with normal water for 3 days (**A, B**) or 28 days (**C, D**). Three days after withdrawal of doxycycline (**A, B**), fewer endothelial loops and many blood vessels are blocked with erythrocytes (**arrows**) and are weakly stained by lectin. At 28 days after doxycycline withdrawal (**C, D**), the vasculature has almost returned to the baseline appearance of Figure 2A. A few vessels are blocked by erythrocytes and are weakly stained by lectin (**arrows**). **E–I:** VEGF-Tg mouse treated with dox-water for 7 days (**E, F, H, I**) or 28 days (**G**) and then normal water for 7 days. Endothelial cells are stained for CD31 (green) and (**E–G**) basement membrane for type IV collagen (red), and (**H, I**) pericytes for desmin (red). Many type IV collagen-immunoreactive sleeves devoid of CD31 staining (**arrows** in **E–G**) and CD31-positive cells are present (**arrowheads**). Desmin-positive pericytes (**arrows** in **H** and **I**) are confined to blood vessels. **J–M:** Two pairs of images from trachea of VEGF-Tg mouse on dox-water for 7 days, then normal water for 7 days. Triple staining for CD31 (green in all panels) activated caspase-3, an apoptosis marker (red in **J** and **L**), and nidogen/entactin immunoreactivity, a marker for basement membrane (red in **K** and **M**). Endothelial cell (**arrow** in **J**) and pericyte (**arrow** in **L**) are labeled for activated caspase-3. **Double arrows** indicate segments of empty basement membrane sleeves. Nerve bundle (N) in **M** also has basement membrane. Scale bar: 200 μ m (**A, C**); 50 μ m (**B, D**); 160 μ m (**E, H**); 40 μ m (**F, G, I, J–M**).

for activated caspase-3 is consistent with this mechanism, because VEGF can promote survival and protect cells from entering the apoptotic cascade by inhibiting the activation of the key enzyme caspase-3.⁴⁴

Our finding that the presence of pericytes and basement membrane were not sufficient to protect the newly formed vessels from regression on withdrawal of VEGF differs from the postnatal remodeling of the retina and in VEGF-dependent tumors.^{43,45} Furthermore, in the conditional VEGF-overexpressing system in the heart and liver, short-term exposure to VEGF resulted in vessel growth followed by almost complete regression, but long-term VEGF exposure (4 weeks) resulted in vessel persistence after VEGF withdrawal.¹⁹ In our model, we consistently observed vessel regression regardless of whether the VEGF exposure was long or short term—there was no rescue. The reasons for this difference are unclear, but may include tissue- and strain-specific features such as the cellular sources and balance of growth factors and inhibitors, and the degree of pericyte coverage. Two examples of such differences suffice. The same stimulus can lead to different amounts of angiogenesis. Ischemia induces significant retinal neovascularization in Brown Norway rats, but not in Sprague-Dawley rats,⁴⁶ whereas VEGF164-mediated inflammation is required for pathological, but not for physiological, ischemia-induced angiogenesis in mouse retinas, which appears to be driven by the VEGF120 and/or VEGF188 isoforms.⁴⁷ And the precise trigger for vascular regression is not well understood even in tissues as much studied as the tunica vasculosa lentis, where the fetal vasculature regresses despite the apparent continued expression of VEGF at high levels by the lens.⁴⁸

A further mystery is the fate of pericytes of newly formed vessels when those vessels die. Some pericytes may die by apoptosis and be cleared by phagocytes. Another possibility is that they dedifferentiate into fibroblasts and return to the connective tissue that they originated from. The malleability of the pericyte phenotype is confirmed in the present study in the up-regulation of PDGFR- β receptors. Other studies suggest that during wound healing pericytes can detach from blood vessel walls and become fibroblast-like cells secreting type I collagen.⁴⁹

Conclusion

In summary, transgenic mice overexpressing VEGF in the airways under the control of the Clara cell CC10 promoter in a doxycycline-regulated manner are a novel and powerful model for investigating angiogenesis and vascular regression. Blood vessels grow in response to VEGF by sprouting angiogenesis within a few days, but regress more slowly after VEGF withdrawal, and dying vessels leave a historical record of their previous existence in the form of empty basement membrane sleeves. We conclude that the presence of basement membrane and a pericyte coat does not stabilize the vessels sufficiently to protect them from regression when VEGF is withdrawn.

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