Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling

Fabienne Baffert,1 Tom Le,1 Barbara Sennino,1 Gavin Thurston,2 Calvin J. Kuo,3 Dana Hu-Lowe,4 and Donald M. McDonald1

1Cardiovascular Research Institute, Comprehensive Cancer Center, and Department of Anatomy, University of California, San Francisco; 2Regeneron Pharmaceuticals, Tarrytown, New York; 3Department of Medicine, Stanford University School of Medicine, Center for Clinical Sciences Research, Stanford; and 4Department of Research Pharmacology, Pfizer Global Research and Development, San Diego, California

Submitted 9 June 2005; accepted in final form 8 September 2005

Cellular changes in normal blood capillaries undergoing regression after inhibition of VEGF signaling. Am J Physiol Heart Circ Physiol 290: H547–H559, 2006. First published September 19, 2005; doi:10.1152/ajpheart.00616.2005.—The vasculature of the embryo requires vascular endothelial growth factor (VEGF) during development, but most adult blood vessels lose VEGF dependence. However, some capillaries in the respiratory tract and selected other organs of adult mice regress after VEGF inhibition. The present study sought to identify the sequence of events and the fate of endothelial cells, pericytes, and vascular basement membrane during capillary regression in mouse tracheas after VEGF signaling was blocked with a VEGF-receptor tyrosine kinase inhibitor AG-013736 or soluble receptor construct (VEGF Trap or soluble adenoviral VEGFR-1). Within 1 day, patency was lost and fibrin accumulated in some tracheal capillaries. Apoptotic endothelial cells marked by activated caspase-3 were present in capillaries without blood flow. VEGF inhibition was accompanied by a 19% decrease in tracheal capillaries over 7 days and 30% over 21 days. During this period, desmin/NG2-immunoreactive pericytes moved away from regressing capillaries onto surviving vessels. Empty sleeves of basement membrane, left behind by regressing endothelial cells, persisted for about 2 wk and served as a scaffold for vascular regrowth after treatment ended. The amount of regrowth was limited by the number of surviving basement membrane sleeves. These findings demonstrate that, after inhibition of VEGF signaling, some normal capillaries regress in a systematic sequence of events initiated by a cessation of blood flow and followed by apoptosis of endothelial cells, migration of pericytes away from regressing vessels, and formation of empty basement membrane sleeves that can facilitate capillary regrowth.

apoptosis; basement membrane; endothelial cells; platelet-derived growth factor; pericytes; trachea; vascular endothelial growth factor

VEGF and its receptors are expressed in many normal tissues (11, 25, 33, 37, 46, 60, 63, 73). Yet, the role of VEGF in the quiescent vasculature of the adult is only beginning to be understood. Unlike the VEGF-dependent vasculature of the embryo, most blood vessels in the adult are thought to be stable and do not require VEGF for survival. The infrequency of serious side effects in preclinical studies and in patients receiving VEGF inhibitors is consistent with this assumption (38, 41, 76). However, VEGF-dependent blood vessels are present in the ovary (30, 75) and in several other adult organs, including the trachea and thyroid (5, 42). VEGF may also be essential for the normal function of alveolar capillaries of the lung, glomerular capillaries of the kidney, and sinusoids of the liver (18, 23, 44).

The process of blood vessel regression has been examined in pre-and postnatal development (7, 35, 56), ovarian cycle (59), corneal pocket assay (4), ocular angiogenesis (16), models of conditional transgenic overexpression of VEGF (6), and inhibition of VEGF signaling in tumors and certain normal organs (5, 42). Steps in vascular regression induced by inhibition of VEGF signaling include cessation of blood flow followed by apoptosis and the detachment of endothelial cells from their basement membrane (5, 9, 42, 45, 50).

Pericytes are thought to stabilize blood vessels of the microcirculation and contribute factors that free endothelial cells from VEGF dependence (8, 36). However, the presence of pericytes does not protect blood vessels in tumors from regression after inhibition of VEGF signaling (42). Similarly, new blood vessels that grow in response to VEGF overexpression in the airways undergo regression after VEGF withdrawal despite the presence of pericytes (6). Pericytes are present on all tracheal capillaries in normal adult mice, but a subset of these vessels is VEGF dependent (5). Little is known of the fate of pericytes during vascular regression.

Empty sleeves of vascular basement membrane, sometimes described as acellular capillaries or basement membrane ghosts, form after endothelial cells regress and serve as a historical record of preexisting vessels. These sleeves have been described in regressing vessels of the tunica vasculosa lentis during postnatal eye development (22, 51), as part of the pathology of diabetes (70), and after inhibition of VEGF signaling in tumor models and in the trachea (5, 42).

There are still many unanswered questions about the cellular events associated with vascular regression and the response of

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is essential for blood vessel growth during embryonic development and in some forms of pathological angiogenesis in the adult (29). Studies that use selective inhibitors have shown that VEGF acts as a survival factor for newly formed blood vessels in tumors and in the neonatal retina (2, 9). Based on this property, promising strategies are now being used to inhibit VEGF signaling in the treatment of cancer and age-related macular degeneration (26, 41, 61, 68, 72, 76).

Address for reprint requests and other correspondence: D. M. McDonald, Dept. of Anatomy, Univ. of California, 513 Parnassus Ave., Rm. S-1363, San Francisco, CA 94143-0452 (e-mail: dmcd@itsa.ucsf.edu).

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the normal adult vasculature to VEGF inhibitors. Do different VEGF inhibitors have the same effect on normal capillaries? What is the mechanism of cessation of blood flow during regression? Is there a contribution of intraluminal fibrin accumulation? What is the fate of pericytes on regressing capillaries? Are pericytes responsible for the persistence of empty basement membrane sleeves? Do PDGF inhibitors cause regression of normal capillaries?

With these questions in mind, we sought to elucidate the sequence of cellular events during blood vessel regression in the adult by taking advantage of VEGF-dependent capillaries in the simple vascular network of the mouse trachea (5). This system made it possible to determine whether intravascular fibrin deposition contributes to the cessation of blood flow during regression and to identify the fates of endothelial cells, pericytes, and vascular basement membranes after inhibition of VEGF signaling. VEGF receptor (VEGFR) signaling was blocked by the receptor tyrosine kinase inhibitor AG-013736, a small molecule inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, and related tyrosine kinase receptors with potent antiangiogenic and antitumor effects (39, 42). Alternatively, VEGF ligand was sequestered by soluble VEGFR-1 delivered by adenosivirus (49) or by VEGF Trap (49), which is a recombinant soluble decoy receptor construct of VEGFR-1 and VEGFR-2 that has potent antiangiogenic activity in tumors (38). The specificity of the effect of these inhibitors was evaluated in parallel experiments wherein mice were treated with soluble PDGF receptor (PDGFR)-β, delivered by adenosivirus (49) or with a tyrosine kinase inhibitor (Gleevec) that blocks PDGFRs and certain other tyrosine kinases (14). Blood vessels in immunohistochemically stained tracheal whole mounts were examined by confocal microscopy.

MATERIALS AND METHODS

Animals and treatments. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California at San Francisco (UCSF). FVB/n mice 8 to 9 wk old (Charles River, Hollister, CA) or C57BL/6 mice 9 to 11 wk old (UCSF Animal Care Facility) were treated with one of five inhibitors or their respective control reagents. First, AG-013736, a small molecule inhibitor of VEGFR and related tyrosine kinases (IC_{50} = 1.2 nM for VEGFR-1, 0.16 nM for VEGFR-2, and 0.29 nM for VEGFR-3) was supplied by Pfizer Global Research and Development (San Diego, CA) (40, 42, 71). FVB/n mice were injected with AG-013736 (25 mg/kg) or its vehicle [5 μl/g; 3 parts of polyethylene glycol (PEG)-400 to 7 parts of acidified water, pH 2–3] twice daily intraperitoneally for 1, 2, 5, 14, or 21 days. Other groups of FVB/n mice received AG-013736 or vehicle twice daily intraperitoneally for 7 days followed by 7, 14, 28, or 40 days of withdrawal. Mice were perfused on the last day of treatment or withdrawal. Second, VEGF Trap, a recombinant decoy receptor for VEGF consisting of the second Ig domain of human VEGFR-1 and third Ig domain of human VEGFR-2 fused to Fc fragment of human IgG1, was supplied by Regeneron Pharmaceuticals (Tarrytown, NY) (38). FVB/n mice were injected with VEGF Trap (25 mg/kg ip) or its vehicle (Chinese hamster ovary cell-derived human Fc domain in 40 mM phosphate, 5 μl/g) on day 0, 3, and 6. Mice were perfused on day 7. Third, adenosivirus expressing soluble mouse VEGFR-1 (49) or, as a control, the constant Fc region of human IgG was injected intravenously into C57BL/6 mice in a dose of 1 × 10^6 plaque-forming units (pfu) (49). Mice were perfused on day 7. Adenoviral expression of VEGFR-1 (1–3) in mouse plasma was confirmed by Western blot analysis using rabbit anti-His (Santa Cruz Biotechnology) (49).

GAPDH was used as a reference. Blots developed with anti-rabbit-horseradish peroxidase conjugates were detected by chemiluminescence. Fourth, adenosivirus expressing soluble PDGFR-β or, as a control, the constant Fc region of human IgG was injected intravenously into C57BL/6 mice in a dose of 4 × 10^6 pfu. Mice were perfused on day 7. Expression of soluble PDGFR-β in mouse plasma was confirmed by Western blot analysis as for soluble VEGFR-1. Fifth, Gleevec (Novartis Pharma, Basel, Switzerland), an inhibitor of receptor tyrosine kinases, including PDGFR-α, PDGFR-β, v-Abl, and c-Kit (10, 14), was administered by gavage (50 mg/kg, twice daily to C57BL/6 mice for 7 or 21 days). Mice were perfused on the last day of treatment.

Blood vessel patency and fixation. At the end of the treatment or withdrawal period, mice were anesthetized with Nembutal (50 mg/kg ip), and the vasculature was perfused through the aorta via a cannula in the left ventricle with 1% paraformaldehyde (PFA) in PBS (pH 7.4) for 2 to 3 min at a pressure of 120 mmHg. Blood vessel patency was assessed in some mice by intravenous injection of FITC-labeled Lycopersicon esculentum lectin (100 μg in 100 μl 0.9% NaCl, Vector, Burlingame, CA) 2 min before the perfusion (42). After fixation was completed, tracheas were removed, immersed in fixative for 1 h, cut along the ventral longitudinal midline, pinned luminal surface up to Sylgard, and processed as whole mounts for immunohistochemistry.

Immunohistochemistry. Tracheas were incubated in 5% normal goat serum in PBS-0.3% Triton X-100 (PBS plus) for 1 h at room temperature and then in one or more primary antibodies overnight at 4°C. Endothelial cells were stained with hamster monoclonal anti-mouse CD31 (platelet endothelial cell adhesion molecule-1, clone 2H8, 1:1,000 in PBS plus, Chemicon, Temecula, CA). Vascular basement membrane was stained with rabbit polyclonal anti-mouse type IV collagen (1:10,000 in PBS plus, LSL/Cosmo Bio, Tokyo, Japan) or rat monoclonal anti-mouse nidogen (MAB9146, 1:1,000 in PBS plus, Chemicon). Pericytes were stained with a mixture of rabbit polyclonal anti-chicken desmin (A0611, 1:2,000 in PBS plus, Dako, Carpinteria, CA) and rabbit polyclonal anti-mouse NG2 proteoglycan (AB5320, 1:2,000 in PBS plus, Chemicon) in combination with anti-CD31 and anti-nidogen. Intravascular fibrin was stained with rabbit polyclonal anti-human fibrinogen/fibrin (A0080, 1:2,000 in PBS plus, Dako) in combination with anti-CD31. Apoptotic cells were stained with rabbit monoclonal anti-human/mouse-activated caspase-3 (AF835, 1:1,000 in PBS plus, R & D Systems, Minneapolis, MN) in combination with anti-CD31 and anti-nidogen.

After being washed in PBS plus, tracheas were incubated for 4 h at room temperature in fluorophore-labeled, species-specific secondary antibodies, including FITC- or Cy3-conjugated goat anti-hamster IgG (1:200 in PBS plus, Jackson ImmunoResearch, West Grove, PA), Cy3-conjugated goat anti-rabbit IgG (1:400 in PBS plus, Jackson ImmunoResearch), Cy5-conjugated goat anti-rat IgG (1:200 in PBS plus, Jackson ImmunoResearch), or Alexa Fluor 350 goat anti-rat IgG (1:200 in PBS plus, Molecular Probes, Eugene, OR). Tracheas were rinsed in PBS plus, fixed in 4% PFA for 10 min at room temperature, then mounted in Vectashield (Vector), and examined with a Zeiss Axiohot fluorescence microscope with single, dual, and triple fluorescence filters or a Zeiss LSM-510 confocal microscope with argon, helium-neon, and UV lasers.

Assessment of capillary regression. Measurements were made on digital images of mucosal capillaries overlaying the 10 rostral-most cartilaginous rings of tracheas after the vasculature was labeled by fluorescent lectin and stained for CD31 and type IV collagen or nidogen. Images (×10 objective) were captured with a low-light, externally cooled, three-chip charged-coupled device camera (480 × 640 pixel, RGB-color digital images, CoolCam, SciMeasure Analytical Systems, Atlanta, GA) on the fluorescence microscope. Measurements of real-time or captured images were made with a digitizing tablet (Digi-Pad, GTCC CalComp, Scottsdale, AZ) coupled to the video camera.
Changes in luminal diameter of regressing capillaries were measured on printed images of tracheal capillaries prepared after 1 day of treatment with AG-013736 (n = 4 mice). Sites of vascular regression were identified as focal regions of CD31-positive capillaries where lectin staining was faint or absent. The luminal diameter of tracheal capillaries stained with lectin under baseline conditions (1 day of vehicle treatment, n = 8 mice) was measured at three points where capillaries crossed the lateral borders and in the midpoint of the cartilaginous rings.

The patency of tracheal capillaries was assessed from the fluorescence intensity of lectin labeling. Selected regions, measuring 89 × 85 pixels, of fluorescent digital images showing lectin labeling or CD31 immunoreactivity were converted to an eight-bit gray scale, custom look-up tables were applied, and surface plots were made by using the surface plot tool of ImageJ software (http://rsb.info.nih.gov/ij/). Fluorescence intensity was represented in the z-axis.

Vessel length density (mm of capillary length/mm² of mucosal surface) was measured on prints of digital images of lectin, CD31, or type IV collagen staining with the use of image analysis software developed for this purpose in McDonald’s laboratory (55).

Measurement of pericyte coverage. Pericyte coverage of tracheal capillaries was analyzed in tracheas of mice treated with vehicle or with AG-013736 for 1, 7, 14, or 21 days. Tracheas triple-stained for CD31, desmin/NG2, and nidogen were viewed with the video camera on the fluorescence microscope (×20 objective), and measurements on real-time images were made with the attached digitizing tablet. Pericyte measurements were made on capillaries overlying five cartilage rings at the rostral end of each trachea. Pericyte cell bodies were identified as bulbous regions of desmin/NG2-positive cells closely associated with capillaries. Pericyte number was expressed as cell bodies per millimeter length of nidogen-positive capillary. Values were obtained separately for surviving capillaries (CD31-positive/nidogen-positive) and regressing capillaries (CD31-negative/nidogen-positive). Pericyte coverage was expressed as the percentage of capillary length accompanied by desmin/NG2-positive pericytes. Length per pericyte was estimated as the total nidogen-positive vessel length covered by desmin/NG2-positive pericytes divided by the number of pericyte cell bodies.

Data analysis and statistics. Values are means ± SE (n = 3–8 mice/group). The significance of differences among means was evaluated by using Student’s t-test or ANOVA (P < 0.05), followed by the Bonferroni-Dunn or Fisher test for multiple comparisons. P < 0.05 was considered significant, except when lower values were indicated in multiple comparison tests.

RESULTS

Regression of tracheal capillaries after inhibition of VEGF signaling. Under baseline conditions, lectin staining of the vascular lumen colocalized with CD31 immunoreactivity in all tracheal blood vessels (Fig. 1, A and B). Complete labeling by circulating lectin reflected the continuous patency and functionality of the normal microvasculature. Surface plots of the intensity of lectin fluorescence confirmed the uniformity of lectin labeling (Fig. 1C). By comparison, after 1 day of treatment with AG-013736, segments of some tracheal capillaries had little to no lectin staining (Fig. 1, D and E). Surface plots of fluorescence intensity illustrated the discrepancies between lectin staining and CD31 immunoreactivity indicative of narrowed or unperfused segments (Fig. 1F). Arterioles and venules of the tracheal mucosa appeared unaffected. Measurements showed that impaired lectin staining of capillary segments reflected on average a 56% reduction in luminal (lectin stained) diameter (8.6 ± 0.1 μm after vehicle, n = 8 mice, compared with 3.8 ± 0.3 μm after 1 day of AG-013736, n = 4 mice).

Measurements of capillary length densities showed a significant reduction in lectin binding and CD31 immunoreactivity after 2, 5, or 7 days of treatment with AG-013736 (Fig. 1G). At 1 and 2 days, the impact on lectin staining was greater than the loss of CD31-positive capillaries, consistent with vessel patency being lost before endothelial cells regress (Fig. 1G). At these times, apoptotic endothelial cells, identified by activated caspase-3 labeling, were present at sites of capillary regression (Fig. 1, H and I). By 7 days, length densities for lectin and CD31 staining were again similar, indicating that nonperfused capillaries (lectin negative/CD31-positive) had regressed, and surviving capillaries were patent and perfused (Fig. 1G).

A possible mechanism of luminal narrowing or obstruction was explored by determining whether fibrin accumulated within the lumen of regressing capillaries. Immunohistochemical studies revealed the presence of deposits of intravascular fibrin in narrowed regions of some capillaries lacking lectin staining after 1 day of treatment with AG-013736 (Fig. 2). Studies of the time course of loss of airway capillaries after inhibition of VEGF signaling by treatment with AG-013736 showed that the length density of CD31-positive capillaries decreased 4% at 1 day, 19% at 7 days, 25% at 14 days, and 30% at 21 days (Fig. 3A).

The specificity of the changes for inhibition of VEGF signaling by AG-013736, which also inhibits PDGFR and related receptors, was assessed by comparing two more selective inhibitors, VEGF Trap and adenovirally delivered soluble VEGFR-1, which both act as decoy receptors that bind VEGF. Both of these inhibitors resulted in a similar magnitude of reduction (~20%) in length density of tracheal capillaries over 7 days (Fig. 3, B and C). By comparison, inhibition of PDGFR by soluble PDGFR-β at 7 days after injection of adenovirus (Fig. 4B) or the receptor tyrosine kinase inhibitor Gleevec for 7 or 21 days did not have a measurable effect on the length density of tracheal capillaries (Fig. 3, D and E). The presence of adenoviral soluble VEGFR-1 and soluble PDGFR-β in plasma of transduced mice was confirmed by Western blot analysis (Fig. 4, A and B).

Fate of empty basement membrane sleeves. Under baseline conditions, tracheal capillaries had a continuous basement membrane as reflected by uniform colocalization of type IV collagen and CD31 immunoreactivities (Fig. 5A). In contrast, after treatment with AG-013736 for 7 days, some segments of type IV collagen lacked CD31 immunoreactivity. These segments were empty sleeves of basement membrane at locations previously occupied by tracheal capillaries (Fig. 5B, arrows), as reported previously (5, 42).

Vascular regrowth after cessation of VEGF inhibitor. Empty basement membrane sleeves may provide a scaffold for vascular regrowth in tumors and certain normal organs (5a). We, therefore, asked whether basement membrane sleeves that form after regression of tracheal capillaries persist long enough to serve as a scaffold for vascular regrowth in the airway mucosa. Mice were treated with AG-013736 for 7 days, treatment was stopped, and the fate of the tracheal vasculature and empty basement membrane sleeves was analyzed at 7, 14, 28, or 40 days thereafter. Vehicle-treated control mice were studied at the same time points. Empty sleeves of vascular base-
Fig. 1. Early loss of capillary patency after inhibition of vascular endothelial growth factor (VEGF) signaling. A–F: tracheal vasculature stained for CD31 immunoreactivity (red, A and D) and Lycopersicon esculentum lectin binding (green, B and E). In vehicle-treated mouse (A and B), 2 markers colocalize uniformly (B, inset, merged image), reflecting patency and perfusion of all vessels. Surface plots of CD31 and lectin fluorescence confirm uniform staining (C). In contrast, after 1 day of AG-013736, some capillaries have narrowed or have focal regions lacking CD31 staining (D, arrowheads). Same regions have little (E, arrow) or no lectin staining (E, arrowheads), indicative of loss of blood flow. Regions with impaired blood flow appear red-orange in merged image (E, inset). Surface plots show mismatch between CD31 fluorescence and lectin fluorescence (F). One region of D and E, also marked in E, has CD31 immunoreactivity but lacks lectin binding (arrow); 2 others lack both markers (arrowheads). G: comparison of vessel length densities for CD31 immunoreactivity and lectin binding under baseline conditions and after treatment with AG-013736 for 1 to 7 days. *P < 0.05, lectin binding is significantly different from corresponding values for CD31 immunoreactivity; †P < 0.05 for CD31 or #P < 0.05 for lectin after treatment is significantly different from corresponding value for vehicle. H and I: confocal microscopic images of tracheal capillaries after AG-013736 for 2 days showing uniform nidogen immunoreactivity of the vascular basement membrane. Focal region of activated caspase-3 immunoreactivity (I, red), indicative of endothelial cell apoptosis, is located in segment with little CD31 immunoreactivity. Scale bar in I applies to all panels; bar, 70 μm in A and B as well as D and E, 50 μm in B and E insets, and 25 μm in H and I.
Fig. 2. Fibrin deposit in nonpatent region of tracheal capillary after inhibition of VEGF signaling by AG-013736 for 1 day. A–C: confocal microscopic images of tracheal capillaries show fibrin immunoreactivity (A, arrow) in capillary segment that lacks lectin binding. Same region has nonuniform CD31 immunoreactivity (B, arrowheads), suggestive of early endothelial cell regression. Fibrin deposit coincides with region of interrupted CD31 immunoreactivity (C, arrow). Scale bar in C applies to all panels; bar, 20 μm.

Fig. 3. Magnitude and time course of tracheal capillary regression after inhibition of VEGF or platelet-derived growth factor (PDGF) signaling. A: comparison between the length density of CD31 and type IV collagen immunoreactivities under baseline conditions and after inhibition of VEGF signaling by AG-013736 for 1 to 21 days (d). After AG-013736 treatment, CD31 values are consistently lower than those for type IV collagen, reflecting more rapid regression of capillary endothelial cells than basement membrane. B and C: significant reduction in tracheal capillary CD31 immunoreactivity after VEGF Trap (B) or adenoviral soluble (s)VEGF receptor (R)-1 (C) for 7 days. D and E: in contrast to inhibition of VEGF signaling, inhibition of PDGF signaling by adenoviral sPDGFR-β for 7 days (D) or Gleevec for 7 or 21 days (E) did not decrease CD31 immunoreactivity of tracheal vasculature. *P < 0.005, significantly different from corresponding CD31 value for vehicle; †P < 0.005, significantly different from CD31 value after AG-013736 for 1 day; #P < 0.005, type IV collagen value significantly different from CD31 value at same time point; $P < 0.001, type IV collagen value at 21 days significantly different from corresponding values for vehicle, AG-013736 for 1 day, and AG-013736 for 7 days.
ment membrane were visible after 7 days of AG-013736 (Fig. 5B, arrows), but at 14 days after treatment ended, the sleeves were thinner or discontinuous in some places (Fig. 5C, arrows). At 28 days, no empty basement membrane sleeves were visible (Fig. 5D).

In these experiments, the length density of CD31-positive tracheal capillaries decreased 29% after AG-013736 for 7 days (Fig. 5E). This value was in the same range as reductions after inhibition of VEGF signaling by adenoviral soluble VEGFR-1 or VEGF Trap for 7 days (Fig. 5F). Interestingly, capillary length density continued to decrease over the first week after cessation of AG-013736, reaching a nadir of 46% reduction (Fig. 5E). The continued decrease suggested ongoing activity of the inhibitor for several days due to the sustained depot effect of the vehicle (PEG-400 in acidified water) after repeated intraperitoneal injection. Thereafter, regrowth of tracheal capillaries was evident, with capillary length density increasing to 22% below baseline at 4 wk (Fig. 5E). At that point, the amount of regrowth plateaued; the capillary length density at 40 days was still 19% less than the corresponding vehicle control value.

Measurement of the length density of type IV collagen immunoreactivity showed a reduction of only 6% after the 7-day treatment with AG-013736 and a maximal reduction in the range of 20% to 25% during the 40-day period after the treatment ended. The maximal discrepancy between the values for CD31 and type IV collagen, which reflected the presence of empty basement membrane sleeves, was 22% at the end of the 7-day treatment. The discrepancy was a bit less (16%) at 14 days after the treatment ended, but at 28 and 40 days, the length densities for CD31 and type IV collagen were again equal, consistent with the disappearance of empty basement membrane sleeves.

Tracheal capillaries did not completely grow back after cessation of treatment with AG-013736. The capillary length density at 40 days was ~20% less than the corresponding control value (Fig. 5E). Matching of CD31 and type IV collagen length densities at values 20% less than normal is consistent with partial regrowth of tracheal capillaries to a point limited by the amount of remaining basement membrane sleeves. Disappearance of empty basement membrane sleeves may, therefore, result from two opposing processes: regrowth of endothelial cells into some sleeves left at sites of endothelial cell regression (<27% regrowth) accompanied by dissolution of other sleeves (~20% loss). These changes resulted in a ~20% net loss of capillaries.

Fate of pericytes after regression of capillary endothelial cells. Desmin/NG2-positive pericytes formed a continuous chain on all intact tracheal capillaries, both under baseline conditions (Fig. 6, A and B) and after treatment with AG-013736 for 1 to 21 days (Table 1). However, pericytes were less numerous in CD31-negative sleeves of basement membrane left behind where capillaries regressed (Fig. 6, C and D). After 2 days of treatment, some regions of empty basement membrane sleeves contained pericyte processes; others did not (Fig. 6, C and D). At 7 days, the sleeves contained even fewer pericyte processes (Fig. 6, E and F). Measurements showed that pericytes accompanied 92% of the length of capillaries under baseline conditions but occupied considerably less of empty basement membrane sleeves. Expressed as a proportion of sleeve length, pericyte occupancy was only 57% after 1 day of treatment with AG-013736, 32% after 7 days, and 22% after 21 days (Table 1). This sparseness of pericytes indicates that persistence of empty basement membrane sleeves was not explained solely by the presence of pericytes left behind by regressing endothelial cells.

The number of pericyte cell bodies in basement membrane sleeves decreased much more than was reflected by the value for pericyte occupancy, which represented mainly pericyte processes (Table 1). An average of 10.7 cell bodies were present per millimeter of normal capillary, but only 0.7 to 1.2 cell bodies per millimeter were found in empty basement membrane sleeves after treatment (Table 1). Pericyte cell
bodies, though rarely located in empty basement membrane sleeves, were often positioned where the sleeves joined surviving (CD31-positive/nidogen-positive) capillaries, suggesting that the cell bodies migrated away from regressing onto surviving capillaries.

The number of pericyte cell bodies expressed per millimeter length of surviving capillaries gradually decreased during treatment with AG-013736 despite the amount of pericyte coverage remaining relatively constant. At 7 days, the pericyte number was 19% less than the baseline value, and at 21 days, it decreased by 42% (Table 1). The pericyte coverage on surviving vessels was maintained by an increase in the average length of pericytes on surviving capillaries, which offset the reduction in cell number. After 21 days of treatment, mean pericyte...
length increased to 157 μm, compared with 93 μm in the vehicle group (Table 1).

Treatment with VEGF Trap for 7 days caused a reduction in pericyte coverage of tracheal capillaries generally similar to that produced by AG-013736 for 7 days, with only 28% of the length of empty basement membrane sleeves accompanied by pericyte processes. VEGF Trap for 7 days had little affect on the overall number of pericyte cell bodies on tracheal capillaries (11.7 ± 0.3 cell bodies/mm in vehicle group vs. 11.5 ± 0.4 cell bodies/mm after VEGF Trap).

**DISCUSSION**

The goal of this study was to identify the sequence of cellular changes occurring in normal capillaries that regress after inhibition of VEGF signaling with a particular focus on the fate of endothelial cells, pericytes, and their basement membrane. The study built on observations reported previously on blood vessels in tumors and sites of VEGF-induced angiogenesis after VEGF withdrawal (6, 42). We used as a model system the regression of VEGF-dependent capillaries in the...
simple vascular network of the mouse trachea (5, 69). In this vascular bed, some normal capillaries regress after VEGF inhibition, and the proportion affected is age dependent, ranging from 100% at 2 wk of age, 39% at 4 wk, 28% at 8 wk, to 14% at 16 wk (5, 69).

In the present study, three different VEGF inhibitors, two binding VEGF ligand and the other inactivating VEGFRs, were used to distinguish vascular regression attributed to VEGF dependence from possible non-VEGF effects. The three inhibitors were used in doses previously shown to be efficacious at blocking VEGF in tumors or other models in mice (5, 38, 40, 42, 49, 69, 71). All three inhibitors caused regression of ~20% of capillaries in the tracheas of normal mice 8–10 wk of age treated for 1 wk, a finding more consistent with VEGF dependence than a non-VEGF-related side effect. Inhibition of PDGF signaling did not trigger regression of tracheal capillaries. None of the mice had weight loss or an obvious health impairment.

Vessel narrowing and cessation of blood flow, detected by lectin staining, were among the earliest changes in tracheal capillaries after inhibition of VEGF signaling. Accumulation of intravascular fibrin accompanied the interruption of blood flow during the first day or two suggested a contribution of fibrin to the loss of capillary patency.

Pericytes were present on all tracheal capillaries under baseline conditions, indicating that their presence was not sufficient to prevent vascular regression. During regression after VEGF inhibition, desmin/Ng2-immunoreactive pericytes retracted from regressing capillaries onto surviving capillaries, and some eventually degenerated. Empty sleeves of basement membrane, left behind by regressing endothelial cells, initially contained pericyte processes but rarely pericyte cell bodies. Some basement membrane sleeves regressed over a period of weeks; others served as a scaffold for partial regrowth of tracheal capillaries after cessation of treatment. The extent of regrowth appeared to be limited by the number of surviving basement membrane sleeves.

Loss of capillary patency and endothelial cell apoptosis. Cessation of blood flow was an early sign of regression of tracheal capillaries after VEGF inhibition, as has been reported previously (42). Focal regions of some tracheal capillaries marked by CD31 immunoreactivity were not stained by intravascular lectin during the first 2 days of treatment. These regions were sites of endothelial cell apoptosis indicated by expression of activated caspase-3. By 5 days, lectin staining and CD31 immunoreactivity were equally reduced, consistent with the disappearance of VEGF-dependent capillaries and normal functioning of the surviving ones. Regression of capillary segments left blind-ended projections at connection points with other vessels, as described in other conditions of vascular regression (77). Similar features have been observed in the eye’s pupillary membrane where cessation of blood flow precedes of endothelial cell apoptosis (56). Inhibition of VEGF signaling also causes endothelial cell apoptosis in hyaloid vessels of the eye (57, 58).

Reduced lectin staining in tracheal capillaries resulted from luminal narrowing or obstruction. Intraluminal fibrin deposition and apoptotic endothelial cells, as demonstrated by activated caspase-3 staining, were present in these narrowed vessels. All of these changes were present within the first 2 days of treatment by the VEGF inhibitor. Cause versus effect and the chronology of events are difficult to determine by the approach we used, because intravascular fibrin deposition, loss of vessel patency, and endothelial cell apoptosis occurred about the same time. Published reports provide evidence that regions of low shear stress promote endothelial cell apoptosis (20), and apoptotic endothelial cells become procoagulant (4, 13, 21). One scenario, thought to occur in capillary regression during development, begins with endothelial cell apoptosis that narrows the vessel lumen, thereby reducing blood flow (56). Reduced blood flow promotes apoptosis of other endothelial cells, creating a procoagulant surface that initiates thrombus formation.

Thrombotic events have also been found with other antiangiogenic treatments for cancer (15). Furthermore, rapamycin, which augments VEGF-induced tissue factor expression in endothelial cells, causes intravascular coagulation in tumors (34). Thrombotic events have been observed in patients receiving chemotherapy in association with SU-5416, a tyrosine kinase inhibitor selective for VEGFR-1 and VEGFR-2 (47, 48). SU-5416 may activate endothelial cells and increase the potential for thrombus formation.

Empty basement membrane sleeves and capillary regrowth. Endothelial cell regression after treatment with AG-013736, VEGF Trap, or adenoviral soluble VEGFR-1 left empty sleeves of vascular basement membrane in the tracheal mucosa. The sleeves, which served as a historical record of sites where capillaries regressed, were most abundant after 7 days of treatment when the difference in regression of endothelial cells and basement membrane was greatest. At that point, the

Table 1. Effect of inhibition of VEGF signaling on pericytes of tracheal capillaries

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<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surviving capillaries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pericyte coverage, %</td>
<td>92 ± 1.1</td>
<td>92 ± 0.4</td>
<td>91 ± 0.9</td>
<td>91 ± 1.0</td>
<td>90 ± 0.8</td>
</tr>
<tr>
<td>Pericyte cell bodies/mm</td>
<td>10.7 ± 0.7</td>
<td>10.2 ± 10</td>
<td>8.7 ± 1.1</td>
<td>7.8 ± 0.8*</td>
<td>6.3 ± 0.6*</td>
</tr>
<tr>
<td>Pericyte length, μm</td>
<td>95 ± 6</td>
<td>101 ± 10</td>
<td>129 ± 17*</td>
<td>129 ± 11*</td>
<td>157 ± 11*</td>
</tr>
<tr>
<td><strong>Regressing capillaries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pericyte coverage, %</td>
<td>57 ± 7</td>
<td>52 ± 3†</td>
<td>32 ± 3†</td>
<td>33 ± 4†</td>
<td>22 ± 4†</td>
</tr>
<tr>
<td>Pericyte cell bodies/mm</td>
<td>0.7 ± 0.5</td>
<td>1.2 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Pericytes on surviving capillaries (CD31-positive, nidenogen-positive) or regressing capillaries (CD31-negative, nidenogen-positive) with AG-013736 treatment were analyzed in real-time fluorescence microscopic images (×20 objective) of tracheal mucosal overlying 5 cartilaginous rings in 3–6 mice/group. Pericyte coverage is expressed as proportion of capillary length, determined by presence of CD31 and/or nidenogen immunoreactivities, covered by desmin/Ng2-positive pericyte cell bodies and processes. Number of pericyte cell bodies is expressed per millimeter of capillary length. Pericyte length is estimated from total length of pericyte-covered capillaries divided by number of pericytes. *P < 0.05, significantly different from corresponding value for vehicle; †P < 0.05, significantly different from value for treatment with AG-013736 for 1 day.
sleeves accounted for 22% of the original tracheal capillaries. Similar sleeves of basement membrane have been described in the tunica vasculosa lentis (22) and in tumors treated with AG-013736 or VEGF Trap (42).

Empty sleeves of vascular basement membrane may serve as a scaffold for capillary regrowth (70). We examined this issue by inhibiting VEGF signaling for 7 days and then by examining the tracheal vasculature at multiple intervals over the next 40 days without treatment. These experiments revealed that the sleeves were present for about 2 wk and then gradually disappeared. Loss of the sleeves appeared to involve regression of some sleeves and regrowth of tracheal capillaries into others. Measurements of length densities of CD31 and type IV collagen immunoreactivities suggested that capillaries regrew from a 46% deficit at 7 days to a 22% deficit at 28 days, but then regrowth was limited by the number of remaining basement membrane sleeves. At 40 days, the values for CD31 and type IV collagen matched at ~20% below normal. The gradual regression of some basement membrane sleeves in the trachea over 2 wk differed from observations in tumors where they persisted during 3 wk of treatment with an inhibitor of VEGF signaling (42).

Change in pericyte coverage after VEGF inhibition. Pericytes are mural cells that share a basement membrane with endothelial cells and are thought to provide structural and growth factor support for nascent and established capillaries and venules (19, 62). Pericytes are thought to stabilize blood vessels by inhibiting endothelial cell proliferation, contributing to the extracellular matrix, and favoring vessel maturation (3, 17, 32, 53).

Despite well-documented evidence of the importance of pericytes in vascular development and function (1, 12, 32, 64), little is known about the fate of these cells during vascular regression. We reported previously that the presence of pericytes is not sufficient to prevent regression of VEGF-dependent tracheal capillaries (5). Most pericytes in tumors treated with VEGF inhibitors survive even when as many as 80% of tumor vessels undergo regression (42). Pericytes can persist at least temporarily in empty basement membrane sleeves (6). However, pericytes on some regressing blood vessels undergo apoptosis (22, 67).

The present study addressed the question of whether pericytes help in the maintenance of the basement membrane sleeves left at sites of endothelial cell regression. The vasculature of the mouse trachea provided a favorable model to examine this issue, because >90% of the capillary length is normally covered by pericytes (5). Although pericytes on these vessels do not express α-smooth muscle actin, they do express desmin and NG2 and thus are readily identified by immunohistochemistry (5). After the inhibition of VEGF signaling, the proportion of basement membrane sleeves occupied by pericytes was only 57% at 1 day and decreased to 22% at 21 days. Pericyte cell bodies were rarely present in the sleeves at any time point. Therefore, the persistence of the sleeves could not be explained solely by the presence of pericytes. The observation that the sleeves eventually disappeared raised the possibility that the absence of pericytes did influence the ultimate fate. Pericyte retraction was likely to be an indirect effect of inhibiting VEGF signaling because the change was found with VEGF Trap as well as with AG-013736.

Pericyte coverage of surviving vessels was unchanged by the inhibition of VEGF signaling. Normal coverage was maintained by an increase in cell length despite an overall decrease in the number of pericyte cell bodies after AG-013736. The reduction in pericyte cell bodies after AG-013736, which inhibits both VEGFR and PDGFR, was probably an indirect effect of inhibiting VEGF signaling in the endothelial cells combined with a direct effect of blocking PDGFR activity in pericytes, because VEGF Trap lacked this effect. Inhibition of VEGF action on endothelial cells plus PDGF action on pericytes would be expected to have greater effects on blood vessels (10). This finding is also consistent with the interdependency and close interaction between the endothelial cells and pericytes during vascular remodeling in normal organs of adult animals (12, 32).

Effects of inhibiting VEGF signaling on normal capillaries. Findings of capillary regression in normal tracheas raise the question of whether other organs are similarly affected after VEGF inhibition. Such changes are relevant to possible side effects of VEGF inhibitors used in the treatment of cancer and age-related macular degeneration. Most blood vessels in the adult are assumed not to depend on VEGF as a survival factor. The relative freedom of side effects, apart from hypertension or proteinuria, in patients receiving VEGF inhibitors in the treatment of cancer is consistent with this assumption (31, 38, 41, 76). However, VEGF and VEGFRs are expressed in multiple adult organs, including the lung, kidney, endocrine glands, brain, and intestine (27, 28, 65, 74), and there is increasing evidence that VEGF-dependent components of the microvasculature are more widely distributed than previously appreciated. An emphysema-like condition with vascular thinning develops in rats after treatment with the VEGFR tyrosine kinase inhibitor SU-5416 (44). VEGF dependence of renal capillaries is suggested by the putative role of VEGF in the maintenance of the glomerular filtration barrier and by the role of soluble VEGFR-1 in renal disease associated with pre-eclampsia (24, 54, 66).

The distribution of VEGF-dependent capillaries has been examined in a study of 17 organs in normal adult mice (43). Significant reductions in vascular density were found in pancreatic islets, thyroid, adrenal cortex, pituitary, small intestine, choroid plexus, adipose tissue, and kidneys after treatment with each of several different VEGF inhibitors. As many as 68% of capillaries regressed over 3 wk of treatment. Common features of most VEGF-dependent capillaries are the presence of endothelial fenestrations and the expression of high levels of VEGFR-2 and VEGFR-3 (43). Similar findings have been made in blood vessels of spontaneous rat insulin promoter-T antigen 2 (RIP-Tag2) tumors and implanted Lewis lung carcinomas (42), but vessels in these tumors are about 10-fold more sensitive to VEGF inhibitors than normal capillaries (43). Similarly, doses of AG-013736 or VEGF Trap that produced a 20% reduction in tracheal capillaries over 7 days induced a 50–80% reduction in vascularity of RIP-Tag2 tumors and Lewis lung carcinomas (42). Importantly, capillaries in the thyroid grew back within 2 wk, after treatment was stopped for 1 wk (43).

The overall healthy condition of the mice treated with a VEGF inhibitor indicates that vascular changes had subtle effects on organ function (43). Cardiac function was largely unchanged, and renal function reflected by serum creatinine
was normal, but some mice had proteinuria (43). Serum thyr
roxin was normal, but thyroid-stimulating hormone was in-
creased. Glucose tolerance tests showed a paradoxical im-
provement of glucose handling with some VEGF inhibitors,
despite the significant reduction in vascularity of pancreatic
islets (43). It is unclear whether these findings also apply to
humans treated with inhibitors of VEGF signaling.

In conclusion, inhibition of VEGF signaling causes regres-
sion of a population of VEGF-dependent normal capillaries in
the airways of adult mice. The regression follows a systematic
series of steps beginning with the loss of vessel patency,
intraluminal fibrin deposition, and cessation of blood flow.
Apoptosis and loss of endothelial cells follow. Changes are
evident within a day and progress for about 2 wk. Pericytes
either retract from regressing endothelial cells onto adjacent
normal vessels, or they themselves undergo regression. Empty
sleeves of basement membrane remain temporarily at sites of
endothelial cell regression as a historical record of the number
and location of VEGF-dependent capillaries. About half of the
basement membrane sleeves serve a scaffold for capillary regrowth, and the others disappear. The amount of capillary
regrowth appears to be limited by the number of basement
membrane sleeves that persist.

ACKNOWLEDGMENTS

The authors thank collaborators at Pfizer Global Research and Develop-
ment, San Diego, for supplying AG-013736; collaborators at Regeneron
Pharmaceuticals for supplying VEGF Trap; Betty Tam and Frank Kuhnert
(Stanford University) for help with preparation of adenoviral vectors; and
Pharmaceuticals for supplying VEGF Trap; Betty Tam and Frank Kuhnert
(University of California, San Francisco) and David Shalinsky (Pfizer)
for many stimulating and helpful discussions.

GRANTS

This work was supported in part by National Heart, Lung, and Blood
Institute Grants HL-24136 and HL-59157 and a grant from Pfizer Global
Research and Development, San Diego.

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VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature.

Regression of blood vessels precedes cartilage differentiation during chick limb development.


Potent VEGF blocking effects of SU5416 in retinal neovascularization in diabetic rats and proteinuria in preeclampsia.


