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

Tomomi Kamba, Betty Y. Y. Tam, Hiroya Hashizume, Amy Haskell, Barbara Sennino, Michael R. Mancuso, Scott M. Norberg, Shaun M. O’Brien, Rachel B. Davis, Lori C. Gowen, Keith D. Anderson, Gavin Thurston, Shuji Joho, Matthew L. Springer, Calvin J. Kuo, and Donald M. McDonald. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am J Physiol Heart Circ Physiol 290: H560–H576, 2006. First published September 19, 2005; doi:10.1152/ajpheart.00133.2005.—Unlike during development, blood vessels in the adult are generally thought not to require VEGF for normal function. However, VEGF is a survival factor for many tumor vessels, and there are clues that some normal blood vessels may also depend on VEGF. In this study, we sought to identify which, if any, vascular beds in adult mice depend on VEGF for survival. Mice were treated with a small-molecule VEGF receptor (VEGFR) tyrosine kinase inhibitor or soluble VEGFRs for 1–3 wk. Blood vessels were assessed using immunohistochemistry or scanning or transmission electron microscopy. In a study of 17 normal organs after VEGF inhibition, we found significant capillary regression in pancreatic islets, thyroid, adrenal cortex, pituitary, choroid plexus, small-intestinal villi, and epididymal adipose tissue. The amount of regression was dose dependent and varied from organ to organ, with a maximum of 68% in thyroid, but was less in normal organs than in tumors in RIP-Tag2-transgenic mice or in Lewis lung carcinoma. VEGF-dependent capillaries were fenestrated, expressed VEGFR-1, and were sensitive to VEGF inhibition, accompanied some regimens of VEGF inhibition. Strikingly, most capillaries in the thyroid grew back within 2 wk after cessation of treatment for 1 wk. Our findings of VEGF dependency of normal fenestrated capillaries and rapid regrowth after regression demonstrate the plasticity of the adult microvasculature.

Address for reprint requests and other correspondence: D. M. McDonald, Dept. of Anatomy, S1363, Univ. of California, 513 Parnassus Ave., San Francisco, CA 94143-0452 (e-mail: dmc@itsa.ucsf.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ous effects of VEGF inhibition in certain vascular beds, we sought to compare the anti-VEGF sensitivity of normal capillaries in various organs with that of tumor vessels. Subsequently, we sought to identify some of the phenotypic features of blood vessels that are especially sensitive to VEGF inhibition, with a focus on pericyte coverage, the presence of endothelial fenestrations, and the level of VEGFR expression, as well as whether these features are changed after VEGF inhibition. We also investigated whether the loss of VEGF-dependent capillaries is accompanied by functional changes in affected organs. Finally, we sought to determine whether vascular changes induced by VEGF inhibition are reversible. Our experiments revealed that fenestrated capillaries in many organs regressed after inhibition of VEGF signaling. Most capillaries that regressed in the thyroid during 1 wk of treatment grew back within 2 wk after withdrawal of inhibition. Our findings regarding the VEGF dependency of normal fenestrated capillaries demonstrate the plasticity of the adult microvascularature.

**MATERIALS AND METHODS**

**Animals and tumor implantation.** Wild-type C57BL/6 mice, 9–13 wk old, were treated with AG-013736 or adenoviral vectors at the University of California, San Francisco (UCSF). Wild-type FVB/N or C57BL/6 mice, 8 wk old, were treated with VEGF Trap (also called VEGF TrapR1R2) at UCSF or Regeneron. Dose-response studies of AG-013736 were conducted with 10-wk-old wild-type C57BL/6 mice that had a 1-mm³ piece of Lewis lung carcinoma (LLC) implanted under the dorsal skin (30), when the tumors reached a diameter of 8–12 mm, and in RIP-Tag2-transgenic mice of C57BL/6 background (25) at 10–11 wk of age. All procedures were approved by the UCSF Institutional Animal Care and Use Committee.

**VEGF inhibition.** Four different approaches were used to inhibit VEGF-VEGFR signaling. 1) AG-013736, a small-molecule VEGFR tyrosine kinase inhibitor (IC₅₀ = 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) (27, 30). Mice were injected intraperitoneally (ip) with AG-013736 (25 mg/kg body wt) twice daily for 7, 14, or 21 days or with its vehicle for 21 days. For dose-response studies, mice were administered orally by gavage 1, 10, or 100 mg/kg body wt of AG-013736 twice daily for 7 days. For the regrowth study, mice were administered ip injections of AG-013736 (25 mg/kg body wt) twice daily for 7 days, followed by withdrawal of treatment for 14 days. 2) Ad-sVEGFR-1, and 3) Ad-sVEGFR-2, which act as soluble decoy receptors for VEGF, were the products of adenoviral vectors that express the extracellular domains of murine VEGFR-1 and VEGFR-2, respectively (38). The mice underwent injection of 1 × 10⁹ plaque-forming units of Ad-VEGFR-1, Ad-VEGFR-2, or Ad-Fc via the tail vein. Adenoviral transgene expression in vivo was confirmed using Western blot analysis with plasma samples obtained retro-orbitally while the mice were under anesthesia (38). 4) VEGF Trap, a soluble inhibitor of VEGF consisting of the second Ig domain of human VEGFR-1 and the third Ig domain of human VEGFR-2 fused to the Fc fragment of human IgG1, was supplied by Regeneron Pharmaceuticals (Tarrytown, NY) (26). Mice underwent ip injection with VEGF Trap (25 mg/kg body wt) or its vehicle every 2 or 3 days for 10 or 14 days.

The dose of each agent was determined on the basis of previous studies performed in mice to assess inhibition of VEGF-VEGFR signaling (26, 27, 30, 38). Doses were chosen to be high enough to push the system, not necessarily to conform to clinical levels. Indeed, some of the doses that we used may have been higher than necessary to achieve the maximal effect in preclinical tumor models. The 1- to 3-wk duration of treatment was chosen to favor detection of early cellular responses of the microvasculature to inhibition of VEGF signaling. This duration also corresponded to the time course examined in a previous study in which the regression of blood vessels in both tumors and normal thyroid of adult mice occurred during the first week or two of treatment (30).

**Fixation by vascular perfusion.** At the end of the treatment period, mice were anesthetized by ip injection with ketamine (100 mg/kg body wt) plus xylazine (10 mg/kg body wt). Mice underwent systemic perfusion via the aorta with 1% paraformaldehyde in PBS, for immunohistochemistry, with 3% glutaraldehyde in 75 mM cacodylate buffer, pH 7.1, 4% polyvinylpyrrolidone, 0.05% CaCl₂, and 1% sucrose for transmission electron microscopy (TEM) or with 2% glutaraldehyde in 100 mM phosphate buffer for scanning electron microscopy (SEM) as described previously (30). After perfusion, 17 organs and tissues, including brain, choroid plexus, retina, anterior pituitary, posterior pituitary, heart, lung, thyroid, pancreatic islets, pancreatic acini, adrenal cortex, adrenal medulla, kidney glomeruli, kidney peritubular region, small intestine, tongue, and epididymal adipose tissue, were collected for subsequent studies.

**Immunohistochemistry.** The organs and tumors used for immunohistochemistry were rinsed several times with PBS, infiltrated with 30% sucrose, and frozen in optimal cutting temperature compound (30). Cryostat sections were cut at 80-μm thickness for all studies except analysis of area density and fluorescence intensity, where section thickness was optimized in each organ (10 μm for kidney, adrenal, and pituitary, 80 μm for small intestine, epididymal adipose tissue, heart, and tumors and 20 μm for others). Sections were stained with the following primary antibodies: CD31 (clone MEC 13.3) from BD Pharmingen (San Diego, CA), CD31 (clone 2H8) and NG2 from Chemicon International (Temecula, CA), type IV collagen (Cosmo Bio, Tokyo, Japan), VEGF and VEGFR-3 from R&D Systems (Minneapolis, MN), insulin from Dako (Carpinteria, CA), VEGFR-2 from Rolf Breken and Philip Thorpe (University of Texas Southwestern Medical Center, Dallas, TX), and PDGF receptor-β (PDGFR-β) from Akiyoshi Uemura (Kyoto University, Kyoto, Japan), combined with fluorescent or biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA) (30). Most specimens were prepared for immunofluorescence imaging. Some VEGF Trap-treated samples were stained by the diaminobenzidine-peroxidase reaction for bright-field imaging. Specimens were examined using a Zeiss Axioshot microscope equipped with a high-sensitivity, three-chip, charge-coupled device camera (SciMeasure Analytical Systems, Atlanta, GA) for fluorescence or bright-field imaging or with a Zeiss LSM 510 confocal microscope (30).

**Electron microscopy.** After being fixated using vascular perfusion, tissues were processed and embedded in epoxy resin for TEM as described previously (30). Sections 60 to 80 nm in thickness were stained with lead citrate and examined with a Zeiss EM-10C electron microscope (30). After being fixated, tissues were cut with a microslicer (Dosaka EM, Kyoto, Japan) to make 100-μm thick sections for TEM. Some specimens were treated with 30% potassium hydroxide to remove extracellular matrix around blood vessels. The specimens were processed and critical point dried in liquid CO₂ (30). Dried specimens were coated with OsO₄ in an Osmium Plasma Coater (Vacuum Device, Ibaragi, Japan) and examined with a Hitachi S-4300N or S-5000 scanning electron microscope.

**Fluorescence intensity measurements.** The intensity of immunofluorescence was measured as an estimate of the expression of VEGFRs. The fluorescence intensity of blood vessels in cryostat sections was measured on digital images of specimens of pancreas (20 μm in thickness), thyroid (20 μm), or small intestine (80 μm) stained for VEGFR-2 or VEGFR-3 immunoactivity using ImageJ software (30). A fluorescence intensity of 15 (range, 0–255) was established as the threshold for distinguishing pixels of the vasculature from those of the background. The fluorescence intensity of the vasculature represented the average brightness of all vessel-related pixels. This value was calculated from all pixels with fluorescence intensities ≥15 as...
described previously (30). The mean value was calculated for four images of the regions of interest stained with each antibody in each mouse. The distribution of intensity values in the regions of interest was visualized using the Surface Plot function of ImageJ software (30).

**Vascular density measurement.** Our previous studies demonstrated that CD31 immunoreactivity is an effective marker for microvascular endothelial cells in mice (6, 30). An index of area density (proportion of sectional area) of 10- to 80-μm cryostat sections stained for CD31 immunoreactivity was measured in digital fluorescence or bright-field microscopic images to quantify vascular density (30). In brief, blood vessel immunoreactivity was analyzed in images captured from four or five regions of interest in each mouse. On the basis of pixel brightness values ranging from 0–255, blood vessels were distinguished from background using empirically determined threshold values that included only blood vessels in specimens. The area density of blood vessels stained with CD31 was calculated as the proportion of pixels having a brightness value equal to or greater than the corresponding threshold (30). The mean value was calculated for each mouse.

**Number of endothelial fenestrations and caveolae.** Endothelial fenestrations and caveolae in islet capillaries were counted on TEM images (30). For comparison of diaphragm fenestrations and caveolae, counts were made on TEM images of thin, anulcuar segments of endothelial profiles (8–14 vessels per mouse, 3–5 mice per group, total magnification ×56,000). Diaphragm fenestrations were identified as diaphragm-covered openings of ~70-nm diameter, and diaphragm caveolae were identified as flask-shaped plasma membrane invaginations of ~70-nm average outer diameter with stomatal diaphragms in endothelial cells (62). The mean numbers of diaphragm fenestrations, diaphragm caveolae, and total diaphragm organelles (fenestrations + caveolae) per 100 μm of vessel perimeter were calculated. In addition, fenestrations were counted on SEM images of the luminal surface of renal glomerular capillaries (7–11 vessel segments per mouse, 4 mice per group, magnification about ×15,000–30,000). Fenestrations were identified as simple round openings of ~70-nm diameter. The mean number of fenestrations per 1-μm² endothelial surface area was calculated.

**Echocardiography and measurement of systolic blood pressure.** Transthoracic echocardiography was performed on conscious mice using the Acuson Sequoia c256 ultrasound system (Siemens, Mountain View, CA) with a 15-MHz linear array transducer (32). The thickness of interventricular septum and LV posterior wall and LV internal dimension were measured at end diastole and at end systole from at least three beats. LV fractional shortening, LV ejection fraction, and cardiac output were calculated as described previously (32). The systolic blood pressure of mice was measured while mice were in the conscious state using a noninvasive, computerized tail-cuff system (NIHB-8; Columbus Instruments, Columbus, OH) (48). The reported values are the means of at least three recordings of 20 cardiac cycles per recording.

**Assessment of kidney and thyroid function and glucose metabolism.** Blood samples were obtained from a jugular vein while the mice were under anesthesia before vascular perfusion. Fresh urine samples were obtained by collection at urination or by performing needle aspiration of the bladder. In some experiments, mouse urine was collected for 24 h using the Nalgene Metabolic Cage system (Tecniplast). Kidney function was assessed by measuring serum creatinine and urine 24 h using the Nalgene Metabolic Cage system (Tecniplast). Kidney and thyroid function and glucose metabolism were performed on urine samples collected overnight before vascular perfusion. Fresh urine samples were analyzed by SYVA Dimension (Siemens, Mountain View, CA) or by Anilysys (Gaithersburg, MD). Pancreatic islet function was assessed by glucose and insulin tolerance tests. For glucose tolerance tests, mice were fasted overnight for at least 16 h and then challenged with intravenous (iv) or oral administration of 1 or 3 g of glucose/kg body wt, respectively (1, 47). For insulin tolerance tests, fed mice underwent ip injection with 0.75 U of regular human insulin/kg body wt (Novolin R; Novo Nordisk, Clayton, NC) (44). Pre- and postchallenge blood glucose levels were measured using Ascensia ELITE XL (Bayer, Mishawaka, IN) with blood obtained by performing tail-tip bleeding.

**Statistics.** Values are expressed as means ± SE. Experimental groups consisted of three to five mice per group unless otherwise indicated. The significance of differences between means was assessed using ANOVA followed by the Dunn-Bonferroni test, with P < 0.05 considered statistically significant.

**RESULTS**

**Effects of multiple methods of inhibiting VEGF signaling on microvasculature.** To help distinguish the biological effects of VEGF inhibition on the microvasculature from the pharmacological or pharmacokinetic properties of individual agents, we compared multiple approaches for blocking VEGF signaling. Phosphorylation of VEGFRs was blocked by the small-molecule receptor tyrosine kinase inhibitor AG-013736 (27, 30). Alternatively, VEGF ligand was absorbed by administration of decoy receptors, including soluble constructs of VEGFR-1 and VEGFR-2, delivered by adenovirus (Ad-sVEGFR-1 or Ad-sVEGFR-2) (38) or by a recombinant protein (VEGF Trap) (26). All of these approaches induced remarkably similar effects on the microvasculature of adult mice during the first few weeks.

The dense network of capillaries in pancreatic islets was conspicuously reduced in density and simplified in pattern after addition of all inhibitors of VEGF signaling (Fig. 1, A–E). Sleeves of vascular basement membrane, identified by type IV collagen immunoreactivity (30), which persisted after blood vessels had regressed, served as a historical record of the original blood vessel location (Fig. 1F). All agents produced approximately the same magnitude of regression (46–60% reduction) of islet capillaries, except for Ad-sVEGFR-2, which produced only a 21% loss (Table 1 and Fig. 1G).

**Identification of VEGF-dependent vascular beds in adult mice.** Having found that all agents used to inhibit VEGF signaling produced qualitatively similar changes in pancreatic islet capillaries, we addressed the question of how widely VEGF-dependent capillaries are distributed in the microvasculature of normal adult mice. The approach that we used was to examine the effects of VEGF inhibition on CD31-immunoreactive blood vessels in 17 different organs or tissues, including brain, choroid plexus, retina, anterior pituitary, posterior pituitary, heart, lung, thyroid, pancreatic islets, pancreatic acini, adrenal cortex, adrenal medulla, kidney glomeruli, kidney peritubular region, small intestine, tongue, and epididymal adipose tissue. The effects of two or more agents administered for 2 or 3 wk were assessed in most organs.

As with islet capillaries, significant capillary regression after Ad-sVEGFR-1 for 14 days was found in multiple organs, including thyroid, small-intestinal villi, and epithidymal adipose tissue (Fig. 2, A–C and E–G). Few changes were found in arterioles and venules. Pruning of capillaries simplified the normally dense capillary networks in these organs, but the extent of vascular regression varied from organ to organ.

After Ad-sVEGFR-1 for 14 days, overall vascular density decreased 59% in thyroid, 54% in islets, 36% in adrenal cortex, 34% in villi of small intestine, 29% in anterior pituitary, 29% in posterior pituitary, 25% in epithidymal adipose tissue, and 22% in chorid plexus (Fig. 2f and Table 1). Capillary loss was
uniformly distributed and resulted in a generalized thinning of the microvasculature in all affected organs with the exception of epididymal adipose tissue, in which capillary regression was patchy, ranging from 51% to none, with an average decrease of 25% (Fig. 2).

Approximately the same amount of capillary regression was also found in these organs after AG-013736 treatment for 3 wk. The vasculature of thyroid (68%) and islets (60%) had the greatest regression. Capillaries of several other organs also underwent significant regression (Table 1): adrenal cortex (46%), villi of small intestine (46%), choroid plexus (45%), posterior pituitary (41%), and anterior pituitary (24%). After VEGF Trap treatment for 2 wk, capillary regression was greatest in islets (46%) and thyroid (37%) and less in small intestine (20%), anterior pituitary (19%), and posterior pituitary (14%) (Table 1). Reductions in peritubular capillaries (30%) and glomerular capillaries (10%) were found in kidneys of mice treated with AG-013736 for 21 days but not after Ad-sVEGFR-1 or Ad-sVEGFR-2 treatment (Table 1). Despite the reduced number and simplified branching pattern of the
microvasculature, >30% of capillaries were still present in even the most severely affected organs, regardless of the agent used.

Despite the conspicuous reduction in capillaries in these organs, no significant change in vascular density was evident in many organs after treatment with any of the VEGF inhibitors used. For example, the vasculature of heart muscle showed no detectable change after Ad-sVEGFR-1 for 14 days (Table 1 and Fig. 2, D, H, and I). Similarly, the vascular density of brain, retina, lung, acinar pancreas, adrenal medulla, and skeletal muscle (tongue) showed no significant reduction after treatment with Ad-sVEGFR-1 or Ad-sVEGFR-2 for 14 days, AG-013736 for 21 days, or VEGF Trap for 14 days (Table 1).

Greater sensitivity of tumor vessels than normal vessels to VEGF inhibition. The relative sensitivities of normal pancreatic islet capillaries and tumor vessels to inhibition of VEGF signaling were compared in dose-response studies of AG-013736. Adult mice with implanted LLC or tumor-bearing RIP-Tag2-transgenic mice were administered one of three doses of AG-013736 (1, 10, or 100 mg/kg body wt, gavage administration) twice daily for 7 days. Vascular density measurements after staining for CD31 immunoreactivity revealed that in LLC, even the lowest dose of AG-013736 induced significant vascular regression (52%) and the highest dose caused even greater regression (67%) of the tumor vasculature (Fig. 2J). In RIP-Tag2-transgenic mice, significant reductions in tumor vasculature were found after both the intermediate (28% reduction) and largest doses (55% reduction) (Fig. 2J). By comparison, only the highest dose induced a significant decrease in vascular density of pancreatic islets, and under these circumstances, the maximal reduction was only 30% reduction (Fig. 2J). Similar dose-response patterns were observed in the thyroid and small-intestinal villi (data not shown).

The magnitude of regression of islet capillaries is thought to be less in these experiments in which the agent was administered by gavage than in studies using ip injection (Fig. 1G and Table 1) because of pharmacokinetic and pharmacodynamic differences associated with route of administration and depot effect of the agent after ip injection.

Distinctive phenotypic features of VEGF-dependent capillaries. To gain insight into why VEGF inhibition had conspicuous effects on certain microvascular beds and not others, we sought to identify features of VEGF-dependent blood vessels that distinguish them from other vessels. In particular, we examined the position of the affected vessels in the hierarchy of the microcirculatory bed, the amount of pericyte coverage, the presence of endothelial fenestrations, and the expression levels of VEGFRs. Immunohistochemical studies of islets, thyroid, pituitary, choroid plexus, adrenal, small intestine, and adipose tissue revealed that most of the affected blood vessels were capillaries. Few if any changes were found in arterioles, venules, or larger vessels.

Pericytes were present on the affected capillaries and thus did not protect the vessels from regression induced by VEGF inhibitors. Pericytes identified by NG2 or PDGFR-β immunoreactivity were consistently present along the length of capillaries in pancreatic islets (Fig. 3A), thyroid (Fig. 3C), villi of the small intestine (Fig. 3E), and epididymal adipose tissue (Fig. 3G) as in other organs. After inhibition of VEGF with Ad-sVEGFR-1 for 14 days, many capillaries in these organs regressed, although NG2- or PDGFR-β-positive pericytes remained at sites where blood vessels were previously located (Fig. 3, B, D, F, and H). Similar observations were made after treatment with other VEGF inhibitors (data not shown).

The presence of pericytes on thyroid capillaries that underwent regression after treatment with AG-013736 for 7 days was confirmed by SEM examination of the vessels (Fig. 4, A and B).

A consistent feature of many VEGF-dependent capillaries was the presence of diaphragm-covered fenestrations. SEM images of normal thyroid capillaries showed distinct clusters of endothelial fenestrations (Fig. 4C) and confirmed that many fenestrations disappeared within 7 days of treatment with AG-013736 (Fig. 4D). Similarly, examination of capillaries in normal pancreatic islets using TEM showed characteristically thin endothelial cells with abundant diaphragm fenestrations (Fig. 4, E and G). By comparison, after treatment with AG-013736 for 21 days (Fig. 4, F and H) or with Ad-sVEGFR-1 for 14 days (data not shown), endothelial cells of islet capillaries were much thicker and had few diaphragm-covered fenestrations. However, caveolae with diaphragms were much more abundant after treatment (Fig. 4, F and H). Quantitative TEM studies (10 capillaries per mouse; n = 3 mice/group) revealed that the number of endothelial fenestrations in islet capillaries decreased by ~90% after AG-013736 treatment for 21 days and 75% after Ad-sVEGFR-1 treatment for 14 days (Fig. 4D). Yet, during the same period, the number of diaphragm-covered caveolae increased approximately threefold and the total of diaphragm-covered fenestrations plus diaphragm-covered caveolae was unchanged (Fig. 4, F and J). After finding such a robust change in diaphragm-covered fenestrations in thyroid and islet capillaries after VEGF inhibi-

---

Table 1. Percentage reduction in vascular density after VEGF inhibition

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pancreas</th>
<th>Small Intestinal Villi</th>
<th>Adrenal</th>
<th>Pituitary</th>
<th>Heart</th>
<th>Brain</th>
<th>Choroid Plexus</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Islets</td>
<td>Acini</td>
<td>Cortex</td>
<td>Medulla</td>
<td>Anterior</td>
<td>Posterior</td>
<td>Lung</td>
<td>Cortex</td>
</tr>
<tr>
<td>AG-013736, 21d</td>
<td>60%*</td>
<td>13%</td>
<td>68%*</td>
<td>46%*</td>
<td>46%*</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-sVEGFR-1, 14d</td>
<td>54%*</td>
<td>59%*</td>
<td>34%*</td>
<td>36%*</td>
<td>10%</td>
<td></td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Ad-sVEGFR-2, 14d</td>
<td>21%</td>
<td>7%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>VEGF Trap, 14d</td>
<td>46%*</td>
<td>5%</td>
<td>37%*</td>
<td>20%*†</td>
<td>19%*</td>
<td>14%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means (n = 3–5 mice per group). *P < 0.05, significantly different from corresponding vehicle-treated mice. Vascularity of epididymal adipose tissue decreased 25%* after Ad-sVEGFR-1 treatment for 14 days (14d). Measurement of vascular density is based on CD31 immunoreactivity, comparing relative magnitude of reduction among organs after four different treatments. Differences among treatments resulted partly because doses and durations (14 or 21 days) were not matched. †Data for intestine after 10 days of treatment with VEGF Trap. The thickness of cryostat sections was optimized in each organ (10 μm for kidney, adrenal, and pituitary; 80 μm for small intestine, epididymal adipose tissue, and heart; and 20 μm for others).
bition, we sought to determine whether similar changes would occur in endothelial fenestrations without diaphragms in renal glomeruli, in which few capillaries regressed after VEGF inhibition. TEM images of normal renal glomerular capillaries demonstrated thin endothelial cells with abundant fenestrations without diaphragms (Fig. 5A). After Ad-sVEGFR-1 treatment for 14 days, the endothelial cells were much thicker and had few fenestrations, but some of the remaining fenestrations had

Fig. 2. Differences in abundance of VEGF-dependent capillaries among organs of adult mice and comparison of sensitivity of normal capillaries and tumor vessels to VEGF inhibition. A–H: confocal microscopic images of CD31-immunoreactive capillaries in thyroid, small intestine, epididymal adipose tissue, and heart under baseline conditions (A–D) and 14 days after Ad-sVEGFR-1 treatment (E–H). VEGF inhibition resulted in pruning of the capillary network in thyroid, small intestine, and adipose tissue but not in heart. I: relative magnitude of reduction in vascular density in nine organs after Ad-sVEGFR-1 for 14 days. Control values are from mice 14 days after Ad-Fc. J: dose-response comparison of sensitivity of normal islet capillaries and tumor vessels in RIP-Tag2-transgenic mice and LLC to AG-013736 for 7 days. *P < 0.05, significantly different from corresponding control group (Ad-Fc or vehicle). †P < 0.05, significantly different from corresponding group of normal islets. Scale bars, 50 μm in A, B, E, and F and 100 μm in C, D, G, and H.
diaphragms (Fig. 5B, arrow). We made similar observations after AG-013736 treatment for 14 days (data not shown). SEM examination of the luminal surface of normal renal glomerular capillaries revealed clusters of endothelial fenestrations surrounded by thicker, nonfenestrated regions (Fig. 5, C and D). villi of small intestine (E and F), and epididymal adipose tissue (G and H). Capillaries in normal tissues had uniform pericyte coverage (A, C, E, and G), but after Ad-sVEGFR-1 treatment for 14 days, some endothelial cells regressed, leaving pericytes (arrowheads) at sites of regression (B, D, F, and H). Scale bars, 25 μm in A–F and 50 μm in G–H.

Endothelial cells of VEGF-dependent capillaries demonstrated unusually high expression of VEGFR-2 and VEGFR-3. In the normal pancreas, for example, intense VEGFR-2 immunoreactivity of endothelial cells of islet capillaries made them readily distinguishable from capillaries of the surrounding acinar pancreas (Fig. 6A). Inhibition of VEGF signaling by Ad-sVEGFR-1 treatment for 14 days reduced the expression of VEGFR-2 in islet capillaries to the level of acinar capillaries, thereby eliminating this distinguishing feature of the two types of capillaries (Fig. 6, B and C). Endothelial cells of islet capillaries also had VEGFR-3 immunoreactivity, but those of acinar capillaries did not (Fig. 6D). After Ad-sVEGFR-1 treatment for 14 days, the expression of VEGFR-3 was markedly reduced in islet capillaries but not in surrounding lymphatic vessels (Fig. 6, E and F, arrows).

Fluorescence measurements showed that under baseline conditions, VEGFR-2 immunoreactivity in islet capillaries was ~30% greater than in acinar capillaries (Fig. 6G). After Ad-
sVEGFR-1, VEGFR-2 immunoreactivity of surviving islet capillaries was reduced to the level found in capillaries of acinar pancreas, but VEGFR-2 immunoreactivity of acinar capillaries did not change (Fig. 6G). Ad-sVEGFR-1 reduced VEGFR-3 immunoreactivity of islet capillaries by >80% (Fig. 6H).

Similarly, VEGFR-2 immunofluorescence of thyroid and small-intestinal villi was strong under baseline conditions but was significantly lower 14 days after Ad-sVEGFR-1 (Fig. 6G). VEGFR-3 immunoreactivity was present in both of these vascular beds under baseline conditions but was essentially absent after Ad-sVEGFR-1 (Fig. 6H). Similar reductions in expression of VEGFR-2 and VEGFR-3 were found in the vasculature of these organs after AG-013736 for 21 days (data not shown).

Functional changes accompanying loss of VEGF-dependent capillaries. Overall, mice treated with inhibitors of VEGF signaling were healthy, and none died during any of the treatments. However, because of the regression of a significant
proportion of the capillaries in certain organs, multiple physiological parameters were examined to determine whether loss of VEGF-dependent capillaries was accompanied by functional changes.

Blood pressure and cardiac function. Cardiovascular function was evaluated by measurements of blood pressure and by echocardiography. Systolic blood pressure measured by tail cuff at 14 days after Ad-sVEGFR-1 was similar to control mice treated with Ad-Fc (116 ± 3 vs. 110 ± 1 mmHg) (Table 2). Echocardiographic evaluation of similar groups of mice revealed no abnormalities in most measures of myocardial function after Ad-sVEGFR-1. These included heart rate, LV fractional shortening, and ejection fraction (Table 2). Similarly, there was no difference in mean vascularity (Table 1) or wet weight of the heart (Table 2). However, cardiac output was reduced by 32% (Table 2). The mechanism of this decrease is unclear but seems not to have been associated with any obvious impairment of heart function.

Glucose tolerance. The functional impact of the reduced vascularity of pancreatic islets after VEGF inhibition was evaluated by performing oral glucose tolerance tests. We reasoned that if the loss of islet capillaries resulted in a significant impairment of glucose sensing or insulin release, the change should be reflected in the glycemic response to glucose challenge. Surprisingly, treatment of mice with Ad-sVEGFR-1 for 14 days resulted in flattening of the glucose
tolerance curve after oral challenge. In the Ad-Fc treated control group, blood glucose was sharply higher at 15 min and then decreased during the course of 60 min (Fig. 7A). By contrast, in the Ad-sVEGFR-1-treated group, blood glucose remained remarkably constant near baseline from 15 to 120 min after glucose challenge (Fig. 7A). In addition, blood glucose was significantly lower in these mice than it was in the Ad-Fc control group throughout the 120-min period of the test (Fig. 7A). Baseline blood glucose and the shape of the glucose tolerance curve after oral challenge were unchanged in mice treated with VEGF Trap for 14 days (data not shown). To examine the possibility that the flat response to oral glucose challenge after Ad-sVEGFR-1 resulted from delayed glucose absorption from the gastrointestinal tract, the studies were...
repeated with iv glucose challenge. After Ad-sVEGFR-1, mice had a similarly flattened response to iv glucose challenge (Fig. 7F). Mice treated with AG-013736 for 21 days also had a flattened response to iv glucose challenge (Fig. 7C). Next, we examined whether the response to insulin was impaired after VEGF inhibition by performing an insulin tolerance test in mice treated with Ad-sVEGFR-1 or Ad-Fc for 14 days. These experiments revealed no significant difference between the two groups in glucose clearance from blood in response to insulin challenge (Fig. 7D). However, the baseline blood glucose concentration before insulin challenge was significantly lower in the Ad-sVEGFR-1 group (108 ± 0.03 vs. 0.16 ± 0.04 mg/dl; *P < 0.05). This difference was not evident when values were plotted as percentage changes from baseline (Fig. 7A).

**Thyroid function.** As an index of thyroid function, the concentration of free thyroxin (T4) in serum did not change with AG-013736 (7D). Serum TSH concentration in mice treated with AG-013736 was 19 times the value for the corresponding vehicle group (2.12 ± 0.01 vs. 0.16 ± 0.04 µg/ml; *P < 0.05). This difference was not evident when values were plotted as percentage changes from baseline (Fig. 7D).

**Renal function.** Mice treated with AG-013736 (25 mg/kg body wt ip twice daily) for as long as 21 days had normal levels of serum creatinine (0.38 ± 0.02 mg/dl), as did mice treated with the vehicle (0.44 ± 0.02 mg/dl). However, some mice treated with AG-013736 or Ad-sVEGFR-1 had moderately elevated concentrations of albumin in urine assessed by Albusix readings (Fig. 7F). The proportion of mice with mild albuminuria after AG-013736 treatment for 7 days was dose related (Fig. 7F). The proportion of mice with elevated urine albumin (Albusix reading of ++ or greater, representing ≥100 mg/dl albumin in urine) was 20% at 1 mg/kg body wt, 43% at 10 mg/kg body wt, and 63% at 100 mg/kg body wt (administered by gavage twice daily). Albuminuria was not severe at any dose, because only one mouse had a value of ++++ (≥300 mg/dl) and none had a reading of +++++ (≥2,000 mg/dl). Only 1 of 33 (3%) of mice treated with vehicle for 7 days had + albuminuria (Fig. 7F). At 14 days after Ad-sVEGFR-1 treatment, 25% of mice had urine with an Albusix reading of ++. The amount of protein in urine of mice treated with VEGF Trap for 14 days was similar to that in mice treated with vehicle (Fig. 7F).

**DISCUSSION**

The goal of this study was to determine which vascular beds, if any, in normal adult mice depend on VEGF for survival. Multiple approaches were used to inhibit VEGF signaling to exclude possible nonselective effects of individual agents. Although blood vessels in most organs did not regress, significant capillary regression was observed in pancreatic islets, thyroid, adrenal cortex, pituitary, choroid plexus, villi of small intestine, and epididymal adipose tissue after VEGF inhibition for 1–3 wk. Vascular densities decreased by as much as 68%. The magnitude of the change was dose dependent, and vessels in normal organs were less sensitive to VEGF inhibition than were vessels in two tumor models: implanted Lewis lung carcinomas and spontaneous tumors in RIP-Tag2 mice. Most VEGF-dependent capillaries were fenestrated and had relatively high expression of VEGFR-2 and VEGFR-3. Capillaries surviving VEGF inhibition in affected organs did not have these features. Importantly, capillaries in the thyroid grew back within 2 wk after cessation of treatment for 1 wk, demonstrating the VEGF-dependent plasticity of certain vascular beds in the adult. Mice treated with VEGF inhibitors showed no obvious health impairment, but certain physiological changes accompanied the loss of VEGF-dependent capillaries.

**Agent-independent actions of inhibitors of VEGF signaling.** All the agents used in the present studies are strong inhibitors of VEGF signaling but have differences in chemical structure, size, targets, and route of administration (26, 27, 30, 38), and none inhibits the action of VEGF alone. AG-013736 inhibits multiple tyrosine kinases, including VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, and c-kit (27, 30). Soluble VEGFR-1 and VEGF Trap can bind placental growth factor and VEGF-B, and soluble VEGFR-2 can potentially bind VEGF-C and VEGF-D as well as VEGF (12). However, all of these agents caused capillary regression in organs such as thyroid and pancreatic islets without discernible changes in the vasculature of brain, heart, and skeletal muscle. These agent-independent effects are interpreted as biological consequences of inhibiting VEGF signaling. Because time-course and dose-response studies were not performed in most cases, differences among the agents in the magnitude of vascular regression produced did not necessarily reflect the strength of VEGF inhibitory effect. Also, differences in pharmacokinetics and pharmacodynamics were not taken into account.

The question of whether certain normal capillaries are as sensitive as tumor vessels to inhibition of VEGF signaling was
examined in dose-response studies with AG-013736. These experiments revealed that blood vessels in two tumor models were at least 10-fold more sensitive than normal capillaries to VEGF inhibition. Lower doses of AG-013736 (1 or 10 mg/kg body wt by mouth twice daily) produced significant regression of blood vessels in Lewis lung carcinoma and RIP-Tag2 tumors but had little or no effect on normal capillaries. Significant vascular regression in some normal organs occurred only with the highest dose tested (100 mg/kg body wt by mouth twice daily), but even then the amount of capillary dropout was less than that in the tumors. Thus, like tumor vessels, some normal capillaries depend on VEGF for survival, but in the models examined, tumor vessels were more sensitive to reduction in VEGF signaling.

Fig. 7. Physiological changes accompanying treatment with VEGF inhibitors. A–D: glucose tolerance tests (A–C) and insulin tolerance test (D) showing improved glucose handling in mice after Ad-sVEGFR-1 treatment for 14 days or AG-013736 treatment for 21 days (*n = 3–5/group). Baseline blood glucose after Ad-sVEGFR-1 but not after AG-013736 treatment was significantly lower than that in the corresponding vehicle group. E: serum TSH concentration as a measure of thyroid function after treatment with AG-013736 for 21 days, Ad-sVEGFR-1 for 14 days, or VEGF Trap for 14 days (*n = 4–5/group). TSH values after AG-013736 treatment were significantly increased, but those for the other agents were not. F: bar graphs showing amount of proteinuria after treatment with AG-013736 for 7 days, with Ad-sVEGFR-1 for 14 days, with VEGF Trap for 14 days, or with corresponding vehicles. Values for 3 doses of AG-013736 and for Ad-sVEGFR-1 show proportion of mice with Albustix values of + + or greater (≥100 mg albumin/dl of urine). Values for VEGF Trap are urinary protein concentrations. Numbers above bars represent the number of mice per group. *P < 0.05, significantly different from corresponding control.
Fenestrations and VEGF receptor expression in endothelial cells. The present study revealed that most normal capillaries that regressed after VEGF inhibition had endothelial fenestrations. Fenestrated capillaries are abundant in endocrine glands, choroid plexus, kidney, and small intestine, where extensive water and solute exchange takes place (49, 54, 59).

Endothelial fenestrations are induced by VEGF in vitro and in vivo (16, 55), and VEGF inhibition reduces the number of fenestrations in blood vessels of the normal thyroid and in islet cell tumors (30). Endothelial cells of surviving capillaries in most organs affected by VEGF inhibitors were unusually thick, had greatly reduced numbers of fenestrations, and contained abundant caveolae with diaphragms. Similar changes were observed in islet blood vessels of mice that had targeted deletion of VEGF in the pancreas (40). Interestingly, the combined total of diaphragm fenestrations plus the number of diaphragm caveolae in islet capillaries was unchanged by VEGF inhibition. Through the presence of the distinctive protein PV-1 [plasmalemma vesicle-associated protein (PLVAP) and MECA-32 antigen], fenestral diaphragms are biochemically related to stomatal diaphragms of caveolae, transendothelial channels, and vesiculovacuolar organelles (63). Thus diaphragm fenestrations and caveolae may be interchangeable (Fig. 4f), although this concept is controversial (61). Another link of VEGF to fenestrated capillaries is evidence that activation of VEGFR-2 signaling by VEGF-165 upregulates PLVAP in endothelial cells in culture (64).

Endothelial cells of fenestrated capillaries have high expression of both VEGFR-2 and VEGFR-3 as observed in this study and in studies conducted by other investigators (10, 16, 51, 55, 68). Upregulation of VEGFR-2 and VEGFR-3 has been found in tumor vasculature (50, 52, 66) and is considered a feature of the angiogenic phenotype (9, 69). Indeed, VEGF may increase expression of VEGFR-2 (58).

The present observations are consistent with earlier reports that expression of VEGFR-2 and VEGFR-3 is downregulated by inhibition of VEGF signaling (28, 30, 75). The mechanism of the downregulation is unclear. A recent report demonstrated that VEGF can trigger heterodimerization of VEGFR-3 with VEGFR-2 and phosphorylation of VEGFR-3 (2). Thus there might be cross talk between VEGFR-2 and VEGFR-3 in VEGF-dependent capillaries. Most normal continuous capillaries, which lack endothelial fenestrations and are largely insensitive to VEGF inhibition, have low VEGFR-2 immunoreactivity and no VEGFR-3 immunoreactivity. Perhaps blood vessels that strongly coexpress VEGFR-2 and VEGFR-3 have more angiogenic capacity and are more sensitive to VEGF inhibition.

The kidney seems to be an exception in our study because glomerular and peritubular capillaries have abundant endothelial fenestrations and strongly express VEGFR-2 and VEGF-3 but did not show significant regression after inhibition of VEGF signaling. However, VEGF inhibition by AG-013736 and Ad-sVEGFR-1 did cause a significant reduction in the

Fig. 8. Regression of thyroid capillaries during VEGF inhibition followed by regrowth after withdrawal of therapy. A–C: fluorescence micrographs of thyroid capillaries stained for CD31 immunoreactivity showing dense vascularity under baseline conditions (A), loss of one-half of the capillaries during AG-013736 treatment for 7 days (B), and complete recovery of dense vascularity during 14 days after cessation of treatment (C). D: measurements of area density of CD31-positive vasculature of thyroid under the same conditions shown by fluorescence photomicrographs. *P < 0.05, significantly different from vehicle. Scale bar, 160 μm (A–C).
number of fenestrations in renal glomerular capillaries and in some cases led to proteinuria. Thus our data are consistent with other evidence of the importance of VEGF in the maintenance of glomerular fenestrations (37, 65), but factors other than VEGF may participate in the survival of capillaries in the kidney (41).

VEGF inhibition causes regression of some capillaries in the trachea (5), but it is unclear whether these VEGF-dependent capillaries have endothelial fenestrations. A small and regionally variable dropout of capillaries was found in adipose tissue. Although some capillaries in fat are fenestrated, they are not the dominant phenotype, and the number of fenestrations appears to be relatively small (10). Therefore, we cannot exclude that some VEGF-dependent normal capillaries lack endothelial fenestrations.

Not every vascular bed potentially affected by inhibition of VEGF signaling was examined in the present study. Although we studied the choroid plexus, which is a major source of cerebrospinal fluid (57), other regions of the brain with fenestrated capillaries should be examined. It is also important to determine whether sinusoids of liver and bone marrow are affected. The microvasculature of bone marrow is less well understood than that of many other organs (60), and small changes may be challenging to detect; yet, future experiments should determine whether inhibition of VEGF signaling alters these important blood vessels.

Capillary regrowth after regression. Vascular regression in the thyroid after treatment with AG-013736 was completely reversed within 2 wk by the regrowth of capillaries. Rapid capillary regrowth after withdrawal of VEGF inhibition may have been facilitated by the empty sleeves of basement membrane (30). The empty sleeves remain during several weeks of treatment (30) but rapidly disappear as new capillaries grow after cessation of VEGF inhibition, consistent with the putative role of basement membrane as a scaffold for new capillary growth.

In the present experiments, the feasibility of capillary regrowth was tested after only 1 wk of treatment because of the known rapidity of vascular regression in the thyroid (30). This brief treatment clearly does not match the current prolonged use of VEGF inhibitors in the treatment of cancer or angiogenic eye diseases, in which treatment could last for as long as the disease responds or remains stable. Indeed, there could be a critical duration of treatment after which empty basement membrane sleeves would no longer be present or other changes could occur that might prevent vascular regrowth. In light of evidence that prolonged VEGF overexpression leads to more stable blood vessels than does brief exposure (13), the reversibility of vascular regression should be examined after longer periods of treatment. The properties of capillary regrowth in organs other than thyroid also need to be characterized.

Functional consequences of inhibition of VEGF signaling. Our experiments, using multiple different VEGF inhibitors, identified several physiological changes that accompanied the loss of VEGF-dependent capillaries after particular treatment regimens. Some investigators who used a single agent to inhibit VEGF reported proteinuria or conditions resembling emphysema or preeclampsia after VEGF inhibition in adult rodents (34, 45, 65), but others reported little or no loss of body weight, changes in blood biochemical parameters, or histopathological abnormalities in major organs (22, 23).

Glucose tolerance tests were performed in our studies to determine whether glucose handling was impaired by the reduction in endothelial fenestrations or in the vascularity of pancreatic islets after inhibition of VEGF signaling. This possibility seemed reasonable on the basis of the finding of mild defects in glucose sensing manifested by delayed glucose clearance after glucose challenge in mice genetically lacking VEGF in the pancreas (31, 40). The latter defect was attributed to vascular changes in pancreatic islets resembling those found in the present studies. However, we surprisingly found the opposite after treatment with AG-013736 and Ad-sVEGFR-1, which tended to improve glucose handling. Targeted deletion of VEGF differs from the present approach, because VEGF was absent during development and the deletion was restricted to the pancreas, whereas we inhibited VEGF signaling systematically in adult mice. Thus the changes that we observed in glucose handling appear to involve target organs other than or in addition to pancreatic islets, such as small intestine, adipose tissue, or liver (35, 73). Comprehensive studies of the effect of VEGF inhibition on liver function and insulin, glucagon, leptin, and gastric inhibitory polypeptide activity may help to increase the understanding of the mechanisms underlying the observed change in glucose handling.

Some mice treated with AG-013736 had moderate albuminuria with no change in serum creatinine. The amount of albuminuria increased with dose, with ++ or greater Albustix readings, increasing from 20% of mice that were administered the lowest dose to 63% of mice that were administered the highest dose for 7 days. Proteinuria is frequently observed in mice after soluble VEGFR-1 and in humans treated with anti-VEGF antibody (65, 72). Such findings implicate VEGF in the maintenance of the glomerular filtration barrier that prevents leakage of plasma proteins into urine (15, 65).

Loss of endothelial fenestrations in glomerular capillaries seems to cause proteinuria indirectly. Podocytes are now considered the most important component of renal glomerular filtration barrier against protein leakage into urine, because fenestrated endothelial cells do not prevent albumin penetration and even large proteins can traverse the fenestrations (65). Maintenance of barrier function may involve reciprocal interactions between endothelial cells and podocytes via VEGF signaling (15).

Only 25% of the mice that received Ad-sVEGFR-1 had ++ or greater Albustix readings, and none of the mice treated with VEGF Trap had values outside the normal range, raising the question of whether non-VEGF actions have a role in the cause of proteinuria. However, the extent of inhibition of VEGF signaling at the level of glomerular endothelial cells is likely to contribute to the incidence of proteinuria. The presence of ++ or greater proteinuria in 80% of adult transgenic mice in which VEGF gene expression was switched off conditionally by the removal of doxycycline but in none of the controls (Kamba T, Zheng YW, Coughlin SR, and McDonald DM, unpublished data) and in some patients treated with anti-VEGF antibody (72) indicates the involvement of a VEGF-related mechanism. Dose-response effects of AG-013736 observed in the present study are also consistent with this possibility.

Implications of VEGF-dependent normal capillaries. Taken together, our findings indicate that some organs in adult mice have a population of VEGF-dependent capillaries and that endothelial fenestrations and high expression of VEGF-2 and...
VEGFR-3 are markers of this feature. The findings also show that VEGF-dependent capillaries have phenotypic plasticity whereby some may undergo regression and others lose fenestrations, downregulate VEGFR-2 and VEGFR-3, and survive by becoming insensitive to VEGF inhibition. Moreover, organ-specific differences in the sensitivity of fenestrated capillaries to VEGF inhibition and rapid regrowth of capillaries after cessation of inhibition suggest multiple levels of vascular plasticity in response to changes in local concentrations of VEGF.

The overall healthy condition of mice after VEGF inhibition indicates that any vascular changes have relatively subtle physiological consequences. Even after VEGF-dependent normal capillaries regressed, organs appeared to have sufficient vascular reserves to maintain normal health. However, the question of whether mice remain completely healthy during much longer-term treatment requires careful consideration, including assessment of pituitary, thyroid, pancreatic islet, and adrenal function. Because diverse systemically administered VEGF inhibitors are in various stages of preclinical and clinical development or have already been approved for use in the treatment of cancer (21, 53), patients have been treated continuously with these agents for months or even years. However, further work seems to be justified to learn more about the apparently greater sensitivity of tumor blood vessels to VEGF inhibition and to determine an optimal dose for targeting tumor vessels while minimizing changes in the normal vasculature and/or physiology.

In our study, we found that the reduction in tissue capillaries did not necessarily translate into a predicted physiological consequence. In fact, some changes in physiology may be contrary to what would be predicted from the vascular changes. Development of a better understanding of the biology underlying the structural and functional changes produced by VEGF inhibitors should also address possible non-VEGF actions and species-specific differences. Because we did not detect increased blood pressure in tail-cuffed mice or evidence of thrombosis that occurs in some patients undergoing anti-VEGF therapy (53), the extent of applicability to humans of the rapid onset and large magnitude of capillary regression in mice needs to be determined in future studies.

In conclusion, we have demonstrated that some capillaries of pancreatic islets, thyroid, adrenal cortex, pituitary, villi of small intestine, and epididymal adipose tissue are dependent on VEGF signaling for survival. The proportion of VEGF-dependent capillaries varies from organ to organ, with few being present in brain, retina, and heart; however, even the most sensitive normal capillaries are less sensitive to VEGF inhibitors than blood vessels in Lewis lung carcinoma or tumors in RIP-Tag-2 transgenic mice. VEGF-dependent capillaries are characterized by endothelial fenestrations and a relatively high level of expression of VEGFR-2 and VEGFR-3. Most surviving capillaries do not have these features. Capillaries that regress in the thyroid grow back within 2 wk after the end of 1 wk of treatment. These findings indicate that capillaries in certain microvascular beds in the adult exhibit VEGF-dependent plasticity.

ACKNOWLEDGMENTS

We thank Dana Hu-Lowe and David Shalinsky (Pfizer Global Research and Development) for kindly providing AG-013736, Douglas Hanahan (UCSF) for supplying breeding pairs for the colony of RIP-Tag2 mice, Jie Wei (UCSF) for genotyping the mice, Hilary Cox and Karen Thabet (Regeneron) for technical assistance, William Grossman (UCSF) for providing the equipment needed for assessing cardiovascular function in mice, Fabienne Baffert and Tom Le (UCSF) for providing tissue samples, Shaun Coughlin and Yao Wu Zheng (UCSF) for supplying the conditional VEGF-null mice, Rolf Breken and Philip Thorpe (University of Texas Southwestern Medical Center) for the anti-VEGFR-2 antibody, and Akiyoshi Uemura (Kyoto University, Japan) for the PDGFR-β antibody. We also thank Michael German, Yoshinori Shimajiri, Peter Baluk, Tsutomu Nakahara (UCSF), Stanley Wiegand (Regeneron), Tetsuichiro Inai (Kyushu University, Japan), and Frank Kuhntert (Stanford University) for many valuable discussions.

REFERENCES


GRANTS

This study was supported in part by National Heart, Lung, and Blood Institute Grants HL-24136 and HL-59157 and National Cancer Institute Grant P50-CA-90270 and by funding from AngelWorks Foundation and the Vascular Mapping Project (to D. M. McDonald).

DISCLOSURES

G. Thurston, K. Anderson, and L. Gowen are employed by Regeneron Pharmaceuticals and are holders of Regeneron stock and stock options. The research described in this article was supported in part by a grant from Pfizer Global Research & Development San Diego.