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Vascular Biology, Atherosclerosis and Endothelium Biology

Vascular Endothelial Growth Factor Localization in the Adult

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Although vascular endothelial growth factor (VEGF) has been well studied in both developmental and pathological angiogenesis, its role in mature blood vessels is poorly understood. A growing body of observations, including the side effects of anti-VEGF therapies as well as the role of soluble VEGFR1 in preeclampsia, points to an important role for VEGF in maintenance of stable blood vessels. To better understand the potential function of VEGF in mature vessels, a survey of VEGF localization in adult mice was conducted. In adult *VEGF-lacZ* mice, VEGF was expressed in a cell-specific manner by cells overlying fenestrated and sinusoidal blood vessels, including podocytes, choroid plexus epithelium, and hepatocytes, as well as in tissues with high metabolic demands or with secretory functions, such as cardiac and skeletal myocytes, Leydig cells, prostatic epithelium, and salivary serous epithelium. VEGF was not detected in most endothelium but was specifically expressed by aortic endothelial cells where VEGFR2 was found to be phosphorylated, indicating an autocrine loop. Additionally, VEGFR2 was constitutively phosphorylated in the liver, lung, adipose, and kidney *in vivo*, providing evidence consistent with a role for VEGF in adult tissues. These observations support the concept that VEGF acts in the adult to stabilize mature vessels. ***(Am J Pathol 2006, 168:639–648; DOI: 10.2353/ajpath.2006.050834)***

Vascular endothelial growth factor (VEGF or VEGF-A) is proangiogenic both *in vitro* and *in vivo*. Its role in development is underscored by findings that mice heterozygous or homozygous for a mutation in the VEGF-A gene die during gestation.[1,2] VEGF is also important in the control of physiological angiogenesis, such as wound healing,[3] and in pathological angiogenesis, including tumor vascularization and neovascularization associated with a number of ocular diseases.[4,5] During development, VEGF is critical for vasculogenesis, the process by which endothelial cell precursors coalesce to form the initial capillary network, and angiogenesis, the formation of new blood vessels from pre-existing blood vessels. Angiogenesis is the main mechanism involved in physiological and pathological neovascularization in the adult. Immature blood vessels either regress or are stabilized by supporting mural cells such as pericytes or smooth muscle cells, which are recruited by paracrine growth factor signaling.[6–8] The integrity and function of mature blood vessels are dependent, at least in part, on the interactions between the endothelial cells and surrounding mural cells.

In addition to its role as an angiogenic factor, VEGF is a potent mediator of vascular permeability.[9] Application of exogenous VEGF to vasculature *in vivo* promotes edema and vessel leakiness.[10] Furthermore, VEGF has been shown to induce fenestrations in endothelial cells *in vitro.*[11–13] Fenestrated blood vessels are found in many secretory organs, including kidney, choroid plexus, and the choroid layer of the eye. Recent evidence has demonstrated a loss of tumor vessel fenestrations in pancreatic tumors after VEGF receptor blockade.[14] However, whether VEGF neutralization leads to loss of normal vascular fenestration *in vivo* remains unknown.

The dependence of tumors on dynamic blood vessel recruitment and growth has made the tumor vasculature an attractive target for anti-neoplastic therapies. Because of the limits of oxygen diffusion, cells, including tumor cells, must be within ~100 μm of a blood vessel. In response to growth factors and hypoxia, solid tumors express high levels of VEGF, which is important for their vascularization.[15] Many therapeutic strategies aimed at

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inhibiting VEGF signaling by either neutralization of VEGF or inhibition of its receptor are under development and in clinical trials.[16,17] The first anti-VEGF therapy, bevacizumab (Avastin; Genentech, San Francisco, CA), was approved by the Food and Drug Administration for the treatment of colorectal cancer in 2004.[18,19] Although reductions in tumor burden and disease severity have been observed, common side effects have been reported including hypertension and proteinuria.[19,20] These findings are consistent with our hypothesis that VEGF may play a role in the adult by acting as a maintenance factor for stable vasculature.

Further evidence that implicates a role for VEGF in the adult comes from observations that preeclampsia, in which endothelial dysfunction has been shown to be the result of VEGF neutralization,[21,22] is due at least in part to abnormally elevated levels of soluble Flt1 (sFlt1) (for a review of preeclampsia see Sibai and colleagues[23]). The increased sFlt1 levels correlate with a marked reduction of free circulating VEGF: the decrease is proportional with disease severity.[22] Widespread endothelial dysfunction in multiple vascular beds results in a range of clinical symptoms, including hypertension, headaches, loss of vision, edema, disseminated intravascular coagulation, and seizures, as well as pathological findings including proteinuria, glomerular damage, retinal detachment, hepatic damage, and pleural edema. The proteinuria and hypertension that characterize preeclampsia are reminiscent of the side effects of anti-VEGF therapies.[19,20] Experimental neutralization of free VEGF in rats by administration of sFlt1 results in endothelial dysfunction *in vivo.*[22] This experimental evidence, as well as the pathological findings in humans with preeclampsia, lends further support for a role of VEGF in maintaining normal blood vessels, and provides clues to the tissues where VEGF action is most important in the adult.

Although VEGF is well documented as an endothelial cell survival factor *in vitro*, its role *in vivo* is unclear. To understand its function in the adult, we performed a systematic survey of adult tissues to identify the tissues and cells that express VEGF. Previous observations of mRNA levels from adult tissues[24–26] demonstrated that VEGF is expressed in many adult tissues, but the cellular source was not identified. Using *VEGF-lacZ* adult mice, we have documented the localization of VEGF in a variety of adult tissues. Furthermore, we show that the VEGFR2 receptor in these tissues is constitutively phosphorylated, indicating that VEGF is actively signaling, an observation consistent with its role as a maintenance factor.

Materials and Methods

Mouse Model

Mice expressing the β-galactosidase (*lacZ*) reporter gene cDNA with a nuclear localization signal and an internal ribosome entry site inserted into the 3′ untranslated region (3′UTR) of the *VEGF* gene[27] (generously provided by Dr. Andras Nagy, Samuel Lunenfield Institute, Toronto, Canada) were used in these studies. This gene yields a bicistronic mRNA that produces both functional VEGF and a reporter β-galactosidase (β-gal) protein. Previous experiments in our laboratory using *in situ* hybridization techniques to identify VEGF mRNA in the lung[25] and the retina[28] substantiate the *VEGF-lacZ* mouse as an accurate system in which to investigate the expression of VEGF. Adult heterozygous mice either from the inbred C57BL/6 background or outbred SW background were used for immunohistochemical analysis.

Tissue Preparation and Immunohistochemistry

Tissues were harvested from mice that were euthanized by CO_2 inhalation. Tissues were fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS) containing magnesium chloride and calcium chloride (Sigma Chemical Co., St. Louis, MO) overnight at 4°C, then washed in PBS, and placed into PBS containing 30% sucrose for 1 hour. Tissues were then embedded in OCT compound, frozen on dry ice, and serial 10-μm cryosections were cut.

For β-gal staining, tissue sections were air-dried, washed in PBS, and then incubated overnight at 37°C with x-gal substrate using an *in situ* β-galactosidase kit (Strategene, La Jolla, CA) according to the manufacturer's guidelines. Subsequently, tissues were blocked overnight at 4°C in blocking buffer (PBS, 0.1% Triton, 0.1% saponin, 0.02% azide, 2% goat serum, and 2% donkey serum) and incubated overnight at 4°C in an anti-PECAM monoclonal rat anti-mouse antibody (JD Biosciences Pharmingen) diluted 1:100 in blocking buffer, followed by washing with PBS. Sections were then incubated for 45 minutes at room temperature with a biotinylated secondary antibody (rabbit, anti-rat 1:300; Vector Laboratories, Burlingame, CA) diluted in blocking buffer, washed in PBS, then incubated in 0.3% H_2O_2/PBS, followed by horseradish peroxidase (Vectastain ABC kits, Vector Laboratories). Amino-ethylcarbazole (Sigma) was used to visualize peroxidase activity, and then the sections were washed in PBS and mounted. Processing without a primary antibody was used as a negative control. Age-matched wild-type mouse tissue was processed as above and subjected to β-gal staining as a negative control for *lacZ* expression (data not shown).

For adipose and muscle, tissue was stained for β-gal without sectioning. Pieces of tissue (1 cm^3) were permeabilized with 1% trypsin for 30 minutes and washed in PBS, and trypsin was inactivated by blocking overnight at 4°C in blocking buffer. These tissues were then treated with x-gal substrate overnight at 4°C, postfixed in 4% paraformaldehyde and mounted. Muscle tissue stained as above was also incubated with fluorescein-labeled *Griffornia simplicifolia* lectin 1 (1:50, Vector Laboratories) to localize the vasculature, followed by visualization by fluorescence microscopy.

For visualization of VEGFR2 in the aorta, an aorta with adjoining inferior vena cava (IVC) from a *VEGF-lacZ* mouse was sectioned and stained as above for β-gal expression. Immunohistochemical localization of VEGFR2 was conducted using a monoclonal rat anti-

mouse antibody (BD Pharmingen, San Diego, CA) diluted 1:100 in blocking buffer by the methods described above. Processing without primary antibody was used as a negative control. Images were captured with a Zeiss Axioskop 2 MOT plus microscope, and composites generated using Adobe Photoshop and Illustrator.

Analysis of VEGFR2 Activation Status in Vivo

Adult wild-type mice were anesthetized using a ketamine: xylazine mix (150 mg/kg, and 20 mg/kg, respectively) and then perfused via the left ventricle with cold PBS containing 1 mmol/L sodium orthovanadate. Tissues were dissected, excess fascia was removed, and cleaned tissue was flash-frozen in liquid nitrogen. Frozen tissues were then thawed on ice in the presence of a 1:2 ratio of tissue mass in lysis buffer, pH 7.2 [1% Triton 100, 10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L ethylenediaminetetraacetic acid, 50 mmol/L NaCl, 50 mmol/L NaF, 1:100 anti-proteinase (Sigma) and 1:100 anti-phosphatase (Sigma)]. After brief sonication, cell lysates were spun for 30 minutes at 4°C at 14,000 rpm, the supernatants were transferred to new tubes, and protein was quantified relative to bovine serum albumin using a protein quantification kit (Bio-Rad, Hercules, CA) according to the manufacturer's guidelines. For Western blot analysis, 200 μg of protein lysates from liver, lung, kidney, and adipose were separated on an 8% sodium dodecyl sulfate-polyacrylamide gel, transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA), blocked overnight at 4°C with 5% bovine serum albumin-Tris buffered saline containing Tween (TBST), and incubated with anti-phospho-VEGFR2 [pY^{951}] (Biosource, Camarillo, CA) diluted at 1:1000 in 3% bovine serum albumin-TBST overnight at 4°C. The membrane was then washed three times with TBST and incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit antibody (Amersham, Arlington Heights, IL) diluted at 1:5000 in 3% bovine serum albumin-TBST for 1 hour. After three additional washes with TBST, enhanced chemiluminescence (Amersham) was used to detect signal, and specific bands were visualized on Hyperfilm (Amersham). Membranes were then stripped and immunoblotted using 1:1000 anti-VEGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a secondary antibody and development as described above. The positive control consisted of lysates (30 μg) of porcine aortic endothelial cells overexpressing VEGFR2[29] (PAE-VEGFR2), a gift from Dr. Lena Claesson-Welsh (Ludwig Institute, Uppsala, Sweden), incubated in serum-free Dulbecco's modified Eagle's medium containing 2 mmol/L sodium orthovanadate and then stimulated for 5 minutes with 10 ng/ml of recombinant murine VEGF (R&D Systems, Minneapolis, MN).

To determine the activation status of VEGFR2 in aortic endothelium *in vivo*, sections of aorta from *VEGF-lacZ* mice were prepared as described above. Immunohistochemistry was performed using a phospho-VEGFR2 (Tyr951) monoclonal antibody (Cell Signaling, Beverly, MA). Briefly, sections were washed in TBST, endogenous peroxidases were blocked by incubating in 3% H_2O_2/TBST for 10 minutes; sections were then washed in TBST and incubated overnight at 4°C in blocking buffer (TBST, 5% goat serum, 5% donkey serum). Sections were incubated overnight at 4°C with 1:100 phospho-VEGFR2 [pY^{951}] in blocking buffer. Incubation with rabbit IgG (1:1000) in blocking buffer was included as a negative control. After overnight incubation, sections were washed in TBST and incubated with a biotinylated secondary antibody (goat anti-rabbit, 1:200; Vector Laboratories) for 30 minutes and washed in TBST. Sections were incubated with horseradish peroxidase (ABC/TBST) for 30 minutes. Diaminobenzidine (Vector Laboratories) was used to visualize peroxidase activity, and slides were washed in dH_2O and mounted.

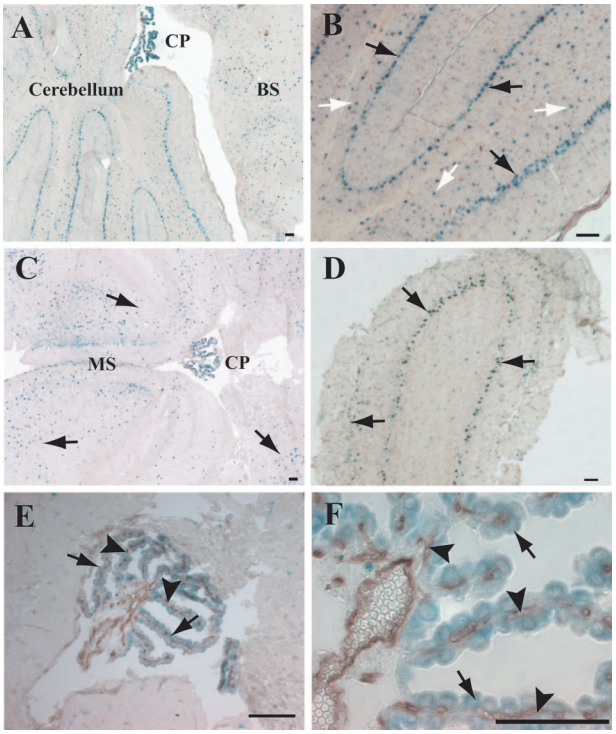
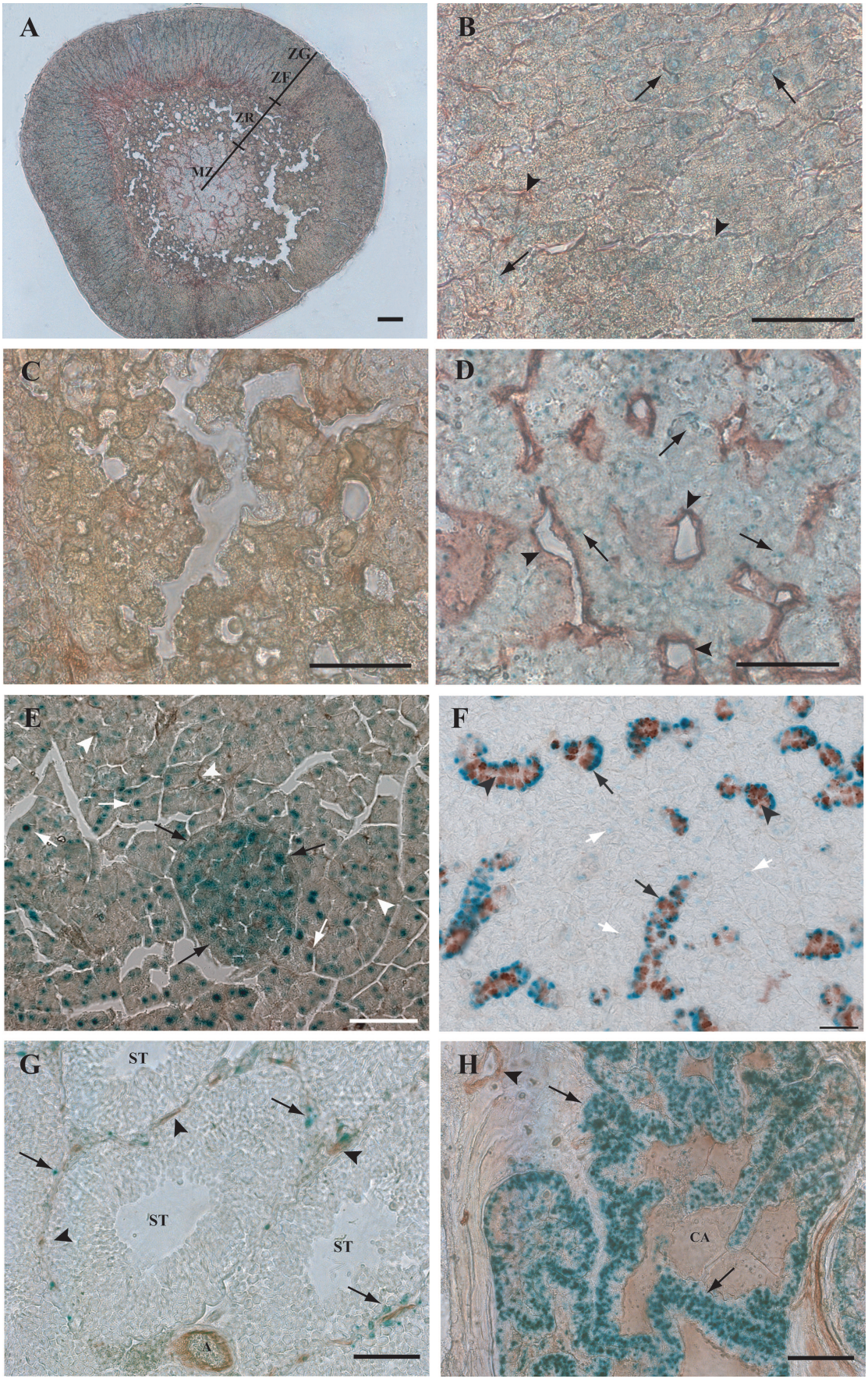


Figure 1. VEGF expression in the brain. Adult *VEGF-LacZ* sections were stained for β-gal (blue) and PECAM (brown). In the cerebellum (**A**, **B**), VEGF is expressed in the Purkinje cell layer (**B**, **arrows**) and nonuniformly in other regions of the parenchyma (**B**, **white arrows**). VEGF is expressed in two discrete cell layers in the olfactory bulb (**D**, **arrows**). In the choroid plexus (**E**, **F**) VEGF is expressed in epithelial cells (**E**, **F**; **arrows**) overlying the fenestrated vessels (**E**, **F**; **arrowheads**). VEGF is also expressed nonuniformly in the cerebral hemispheres (**C**, **arrows**) and brainstem (**A**). BS, brainstem; CP, choroid plexus; MS, median sulcus. Scale bars, 50 μm. Original magnifications: ×62.5 (**A**, **C**); ×160 (**B**); ×125 (**D**); ×250 (**E**); ×800 (**F**).

Results

VEGF Expression in the Brain

To investigate VEGF localization in the brain, representative regions from *VEGF-lacZ* mouse brain, including the cerebellum, olfactory bulb, brainstem, choroid plexus, and cerebrum, were stained with x-gal. To understand the relationship between VEGF-expressing cells and vasculature, tissue sections were immunostained using an-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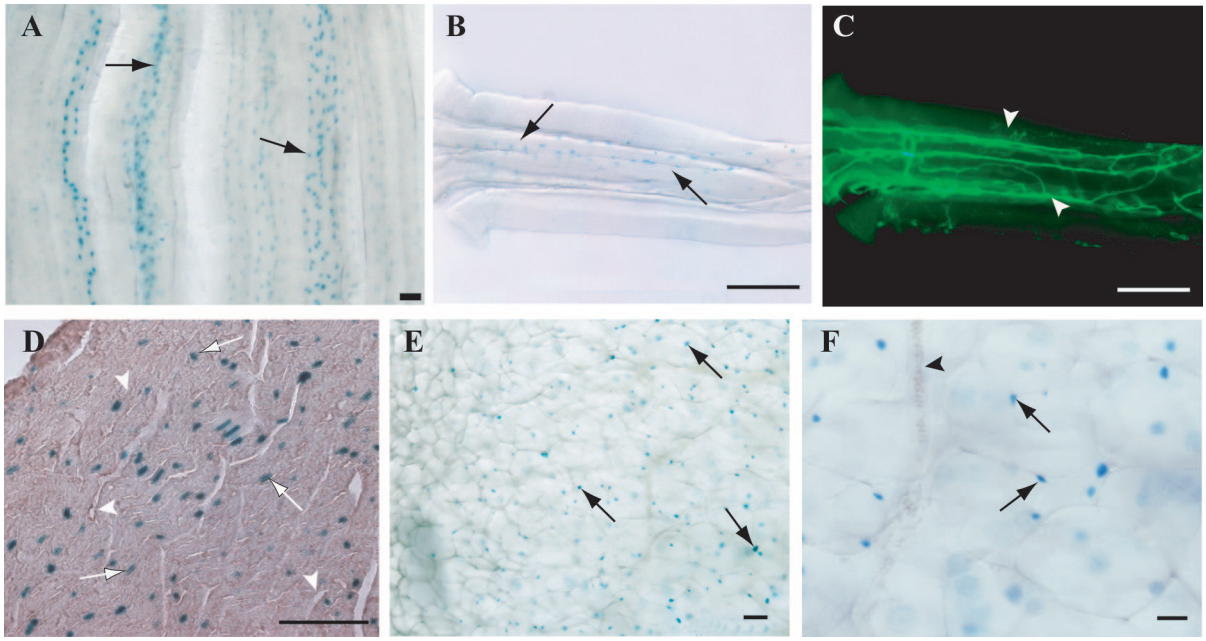

Figure 3. VEGF expression in muscle and adipose. Adult *VEGF-lacZ* mouse striated muscle, adipose, and heart were dissected, fixed in 4% PFA, and were either flat mounted (striated muscle and adipose) or sectioned into 10-μm-thick cryosections (cardiac muscle). Flat mounts of striated muscle were stained for β-gal (blue) and lectin-FITC (green), flat mounts for adipose were stained with β-gal only, and cardiac muscle sections were stained for β-gal and PECAM (brown). Uniform expression of VEGF is observed in skeletal (**A**, **B**; **arrows**) and cardiac (**D**, **arrows**) myocytes, as well as in adipocytes (**E**, **F**; **arrows**). Note the rich vascularity in proximity to VEGF-expressing myocytes in both striated muscles (**C**, **arrowheads**) and cardiac muscles (**D**, **arrowheads**), as well as the lack of VEGF-expressing nuclei in the large blood vessel in adipose tissue visible by phase contrast (**E**, **F**; **arrowheads**). Scale bars, 50 μm. Original magnifications: ×62.5 (**A**); ×400 (**B**, **C**); ×500 (**D**); ×125 (**E**); ×160 (**F**).

tisera against the pan-endothelial marker PECAM. VEGF was localized in specific patterns in the choroid plexus (Figure 1, A, C, E, and F), olfactory bulb (Figure 1D), and the cerebellum (Figure 1, A and B), whereas it was non-uniformly expressed in the cerebral parenchyma (Figure 1C) and brainstem (Figure 1A). In the cerebellum, which contains more than half of the brain's neurons, VEGF was expressed in the Purkinje cell layer (Figure 1, A and B; black arrows) and sporadically in other parenchymal cells (Figure 1B, white arrows). In the olfactory bulb (Figure 1D), VEGF was expressed in two layers of cell bodies (Figure 1D, arrows). Robust VEGF expression was seen in the choroid plexus (Figure 1, A, C, E, and F) in epithelial cells (Figure 1, E and F; arrows), which directly overlay fenestrated vessels (Figure 1, E and F; arrowheads).

VEGF Expression in the Secretory Organs

Of the secretory organs analyzed for VEGF expression, the adrenal gland, salivary gland, pancreas, testis, and prostate all displayed cell type-specific expression. In the adrenal gland, VEGF expression was localized to the zona glomerulosa and zona fasciculata (Figure 2, A and B; arrows), as well as in secretory cells of the medullary zone (Figure 2, A and D; arrows). VEGF expression was not observed in the zona reticularis (Figure 2, A and C).

In the pancreas, VEGF was expressed in islet cells (Figure 2E, black arrows) and in exocrine pancreatic epithelium (Figure 2E, white arrows). In the mixed salivary gland, serous epithelial cells (Figure 2F, black arrows), which are highly vascularized as evidenced by PECAM staining (Figure 2F, arrowheads), strongly expressed VEGF whereas the less vascularized mucous acinar cells revealed much weaker VEGF expression (Figure 2F, white arrows). In the testis there was cell-specific expression in the Leydig cells (Figure 2G, arrows), which lie either in clusters or as single cells in the interstitial space around the seminiferous tubules, the site of the blood-testis barrier (Figure 2G, arrowheads). Prostate epithelium uniformly expressed VEGF (Figure 2H, arrows).

VEGF Expression in Muscle and Adipose

VEGF was expressed uniformly in both cardiac and skeletal myocytes (Figure 3, A, B, and D), with both tissues richly vascularized (Figure 3, C and D; arrowheads). PECAM (cardiac muscle, Figure 3D) and lectin staining

Figure 2. VEGF expression in the secretory organs. Adult *VEGF-LacZ* sections were stained for β-gal (blue) and PECAM (brown). In the adrenal gland, VEGF is expressed in the zona glomerulosa and zona fasciculata (**A**, **B**) and in the medullary zone (**A**, **D**), which is vascularized with large collecting venules (**arrowheads**). The zona reticularis (**A**, **C**) does not express VEGF. In the pancreas, VEGF expression is detected in pancreatic epithelial cells (**E**, **white arrows**), as well as in islet cells (**E**, **black arrows**). In the salivary glands (**F**) VEGF is strongly expressed in serous epithelial cells (**black arrows**), which are highly vascularized (**black arrowheads**), and weakly in mucous epithelial cells (**white arrows**). In the testis (**G**), VEGF expression is localized to Leydig cells (**arrows**) that lie in the interstitial space around the seminiferous tubules. Prostatic epithelium (**H**, **arrows**) uniformly expresses VEGF. ZG, zona glomerulosa; ZF, zona fasciculata; MZ, medullary zone; ZR, zona reticularis; ST, seminiferous tubules; A, artery; CA, corpora amylacea. Scale bars: 100 μm (**A**), 50 μm (**B–H**). Original magnifications: ×62.5 (**A**); ×500 (**B–D**); ×250 (**E–H**).

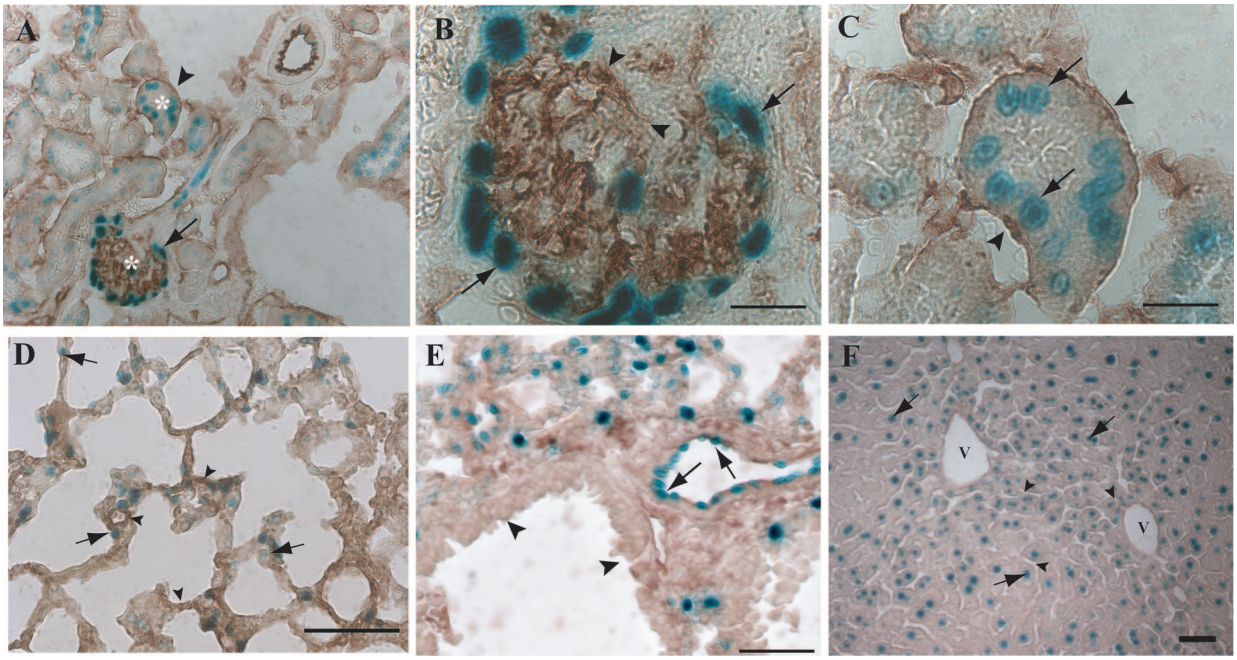

Figure 4. VEGF expression in the kidney, lung, and liver. Adult *VEGF-lacZ* mouse tissues were sectioned and stained as in Figure 2. In the kidney, VEGF is strongly localized to podocytes (**A**, **B**; **arrows**), which overlay the fenestrated glomerular blood vessels (**B**, **arrowheads**); weaker expression is visible in the proximal convoluted tubule epithelium (**A**, **arrowhead**; **C**, **arrows**), which is also richly vascularized (**C**, **arrowhead**). **Asterisks** in **A** indicate regions shown in higher magnification in **B** and **C**. In the lung, VEGF localizes in the alveoli (**D**) to type II pneumocytes (**arrows**), which directly abut the gas-exchange blood vessels (**arrowhead**). VEGF is also localized in epithelium of smaller bronchioles (**E**, **arrow**) but not in the epithelium of larger bronchioles (**E**, **arrowheads**). In the liver (**F**) VEGF is expressed in hepatocytes (**arrows**), which lie adjacent to the sinusoidal blood vessels (**arrowheads**). Scale bars: 20 μm (**B**, **C**); 50 μm (**D–F**). Original magnifications: ×160 (**A**); ×800 (**B**, **C**); ×500 (**D**, **E**); ×125 (**F**).

(skeletal muscle, Figure 3C) highlight the rich vascular networks of these tissues and indicate the proximity of the source of VEGF to the vessels. In fat, VEGF was expressed uniformly by adipocytes (Figure 3, E and F; arrows).

VEGF Expression in the Lung, Liver, and Kidney

VEGF was robustly expressed in the kidney by podocytes (Figure 4, A and B; arrows) and to a lesser extent by the proximal convoluting tubule (PCT) epithelium [Figure 4, A (arrowhead) and C (arrows)]. The podocytes extend foot processes that contact the basement membrane of the fenestrated glomerular endothelium (Figure 4B, arrowheads). The PCT is also lined by a rich vascular network arising from the efferent arteriole of the glomerulus (Figure 4C, arrowheads).

VEGF expression in the lung was detected in the terminal gas exchange alveoli (Figure 4D) and was also observed in the epithelium of small bronchioles (Figure 4E, arrows) but not in large bronchioles (Figure 4E, arrowheads). Previous work in our laboratory has identified type II pneumocytes as the site of VEGF expression in the alveolus[25] (Figure 4D, arrow).

The liver, which is vascularized by a sinusoidal vascular bed[13,30] (Figure 4F, arrowheads), uniformly expressed VEGF in hepatocytes (Figure 4F, arrows). The hepatocytes abut the sinusoidal vasculature and are responsible for extracting nutrients and toxins from the blood as it makes its way from peripheral arteries to the central collecting vein (Figure 4F). Although a relative zone of hypoxia is created between the peripheral arteries and the central vein, with the central vein region being the most hypoxic, no qualitative difference in VEGF expression was seen.

VEGF Expression in the Large Blood Vessels

All blood vessels from the aorta to the smallest capillaries are lined with endothelium. Given the thickness of large blood vessels, we hypothesized that if VEGF is required in the endothelium of these vessels too, then mural cells of the blood vessels might also express VEGF. To investigate VEGF expression in the large vessels, the thoracic aorta, along with the adjoining IVC, was stained for β-gal expression (Figure 5, A–E) or was doubled stained for β-gal expression and PECAM (Figure 5, B and E). In the IVC, VEGF was expressed in the medial layer (Figure 5, A and C; arrows). Surprisingly, VEGF was expressed in the intimal layer of the aorta (Figure 5, A, D, and E; arrows) and was co-localized with the endothelium, as evidenced by PECAM immunostaining (Figure 5, B and E; arrowheads). However, VEGF was not expressed in endothelium in the IVC (Figure 5, A and C). Staining the aorta with an anti-VEGFR2 (Figure 5F) antibody revealed that both VEGF and VEGFR2 (Figure 5F, arrowheads) are expressed in the intimal layer of the aorta, suggesting an autocrine function in these endothelial cells.

VEGFR2 Is Expressed and Activated in Tissues Expressing VEGF

To further investigate the hypothesis that VEGF expression in the adult serves as a maintenance factor, we

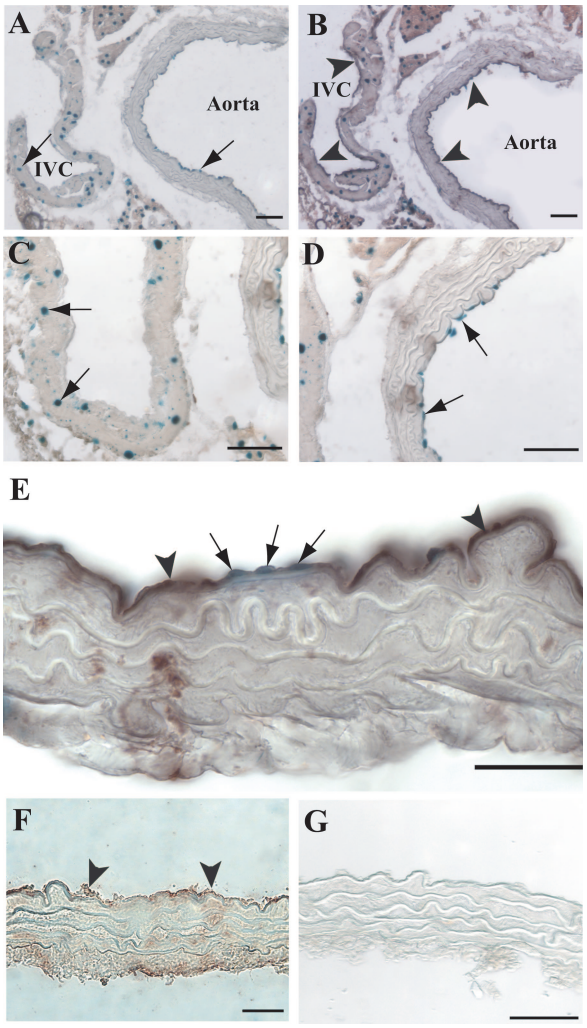

Figure 5. VEGF expression in the large blood vessels. Adult *VEGF-lacZ* mouse aorta and IVC were dissected, sectioned, and stained as in Figure 2. VEGF is localized to the medial layer of the IVC (**A**, **C**; **arrows**), while it is expressed in the intimal layer of the aorta (**A**, **D**, **E**; **arrows**). Co-staining against PECAM (**B**, **E**; **arrowheads**) reveals that a subset of endothelial cells themselves express VEGF in the aorta as evidenced by co-localization with β-gal expression (**A**, **D**, **E**; **arrows**) in that layer. VEGFR2 is also expressed in the intima (**F**, **arrowheads**) of the aorta as evidenced by immunohistochemistry toward VEGFR2. **G** is a negative control for VEGFR2 immunohistochemistry. Scale bars, 50 μm. Original magnifications: $\times$200 (**A**, **B**); $\times$400 (**C**, **D**, **F**); $\times$500 (**E**, **G**).

examined the expression and activation status of VEGFR2 in a subset of adult tissues investigated above. VEGFR2 phosphorylation was examined in the liver, kidney, lung, and adipose. Western blotting using a phosphorylation-specific anti-VEGFR2 antibody, followed by a Western blot using an anti-VEGFR2 antibody to detect total VEGFR2, revealed that VEGFR2 in the lung, liver, kidney, and adipose is constitutively activated (Figure 6A).

Motivated by the observation of both VEGF and VEGFR2 expression by aortic endothelium, we investigated the activation status of VEGFR2 in aortic endothelium. Because the limited amount of tissue makes immunoprecipitation impossible, immunohistochemistry using a phoshpo-specific VEGFR2 antibody was used. Staining revealed that VEGFR2 was indeed activated (Figure 6B),

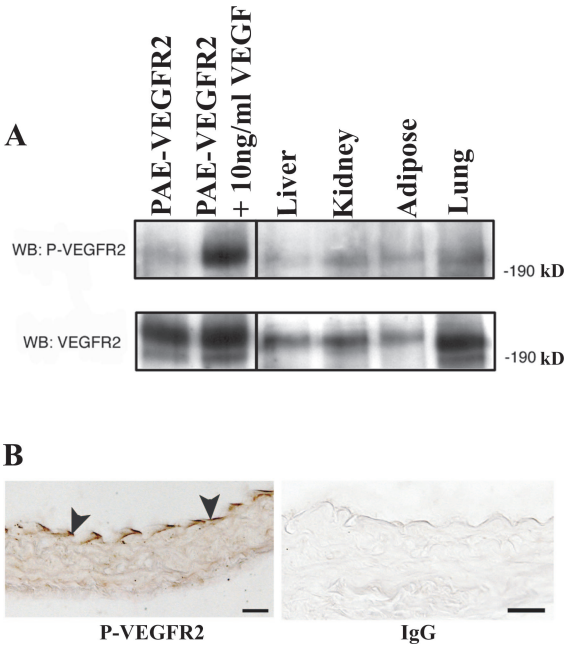

Figure 6. VEGFR2 expression and activation in adult mouse tissue. **A:** Western blots of adult mouse tissue lysates were immunoblotted with a phospho-specific anti-VEGFR2 antibody. PAE-VEGFR2 cells were used as a control for VEGFR2 expression, and PAE-VEGFR2 + 10 ng/ml VEGF cell lysates were used as a control for VEGFR2 activation. Membranes were then stripped and blotted for anti-VEGFR2. As revealed by Western blotting, VEGFR2 is not only expressed in liver, kidney, adipose, and lung, but the receptor is also activated in these adult tissues. **B:** Sections of adult *VEGF-lacZ* aorta were stained using a phospho-VEGFR2 (Tyr951) antibody and visualized with diaminobenzidine (brown). As a negative control, sections were incubated with rabbit IgG (**B**, **right**). As revealed by immunohistochemistry, VEGFR2 is activated in the aortic endothelium (**B**, **left**; **arrowheads**). Scale bars, 20 μm. Original magnifications, $\times$400.

supporting an autocrine signaling pathway of VEGF in aortic endothelium.

Discussion

In the present study, we have begun to investigate the hypothesis that VEGF is required for maintenance of the vasculature in adult tissues by performing a comprehensive survey of VEGF expression in the adult mouse. In addition to demonstrating tissue- and cell-specific expression patterns for VEGF synthesis, we have shown that VEGFR2, the predominant signaling receptor for VEGF, is expressed and constitutively activated in these VEGF-expressing tissues. Although previous studies have demonstrated that VEGF is expressed in adult tissues[31–35] and that both total amounts and isoform levels vary, these observations have been primarily confined to single organs and have been limited by the lack of sensitivity and specificity of the reagents and immunohistochemical techniques available. Furthermore, an understanding of the spatial relationship between VEGF-producing cells and the tissue's vasculature would be useful to determine the role of VEGF in these tissues. β-gal histochemistry in adult *VEGF-LacZ* mice allowed us to accurately identify cells expressing VEGF, and co-labeling for PECAM provided information on their relation to the vascular endothelium.

Observations from this survey demonstrate that VEGF is expressed in a cell-specific manner in virtually all vascularized adult tissues. In general, VEGF was expressed in epithelium within close proximity to fenestrated blood vessels, such as in the choroid plexus, glomerulus of the kidney, and endocrine tissues. VEGF was also expressed in cells that abut sinusoidal vessels, such as in the liver and spleen (data not shown). In addition to its expression in tissues with permeable blood vessels, VEGF expression was also detected in skeletal and cardiac myocytes, with both tissues being richly vascularized and having high metabolic demands. The expression of VEGF in endocrine cells such as in the salivary gland, as well as in the relatively metabolically low demanding adipose, suggests that VEGF also acts in a juxtacrine manner at sites distant from its expression. Indeed, both normal serum[36] and saliva[37,38] contain free VEGF, an observation consistent with a potential juxtacrine role for VEGF. VEGF was not expressed in the cornea (data not shown), a tissue that is avascular in its normal state, and was sparsely expressed in the zona reticulosa, the retina (data not shown),[28] cerebral parenchyma, and testis. Interestingly, the cerebral and retinal vasculatures are sites of the blood brain barrier and as such are relatively impermeable.[39] Additionally, the testis contains a blood-testis barrier,[40] which is also relatively impermeable compared to other capillaries. This correlation between low VEGF levels and impermeable vasculatures raises the possibility that low levels of VEGF are required for endothelial cell survival, while higher levels are important for increased permeability and fenestration in addition to endothelial cell survival.

VEGF is most commonly expressed by mesenchymal, epithelial, or tumor cells and is thought to act on nearby endothelium.[41,42] Under pathological situations, such as extreme hypoxia, endothelial cells *in vivo* have been reported to express VEGF.[43] In general, however, endothelial cells were believed not to make VEGF[41,42] *in vivo* under normal conditions because production of VEGF by these cells might be expected to lead to a dangerous autocrine loop, such as reported in hemangioma endothelium[44] and, more recently, in tumor microvessel endothelial cells.[43,45] In the current study, VEGF was found to be expressed by the endothelium in the intimal layer of the aorta, but not by the endothelium of the IVC. The co-expression of VEGF along with activated VEGFR2 in aortic endothelium suggests the possibility of an autocrine signaling pattern. One obvious distinction between arterial and venous endothelial cells is the difference in shear stress and/or transmural stretch experienced by the two *in vivo*. Previous evidence has shown that exposure of endothelial cells to either arterial or venous forces results in differential gene expression patterns and phenotype.[46] These forces have both been reported to upregulate HIF-1α,[47] a common mediator of VEGF expression. It is therefore possible that VEGF expression in the aortic endothelium is the result of these specific forces.

VEGF has been postulated to play an important role in the maintenance and development of fenestrations. *In vitro*, VEGF has been shown to mediate the formation of fenestrations by choroid plexus[11] and liver endothelial cells.[13] A recent report[48] describes the loss of fenestrations in pancreatic islet endothelial cells after Cre-mediated deletion of VEGF-A, but in this experiment VEGF-A was deleted during development and its role in the adult was not addressed. Fenestrations have also been shown to be diminished in pancreatic tumors after VEGF neutralization.[14] *In vivo*, neutralization of VEGF in the kidney leads to proteinuria and glomerulosis, suggesting breakdown in the fenestrations of glomerular capillaries.[22,49] However, whether neutralization of VEGF *in vivo* leads to a loss of fenestration in normal, mature vessels is unclear. Observations from our study are consistent with the concept that VEGF expressed in the choroid plexus epithelial cells, hepatocytes, and podocytes acts locally to maintain the underlying fenestrated vessels.

VEGF has been shown to play a survival role *in vitro*.[28] In this study, we have demonstrated that VEGF is expressed in the adult, and that its receptor, VEGFR2, is constitutively activated in adult organs. Our data are consistent with several studies that suggest a role for VEGF as a survival factor *in vivo*. VEGF neutralization in the lung has been reported to result in alveolar endothelial cell apoptosis, eventually leading to alveolar septal destruction.[50] In addition, systemic VEGF neutralization leads to loss of tracheal vessels and endothelial cell death, leaving behind the underlying basement membrane.[14] Finally, loss of peritubular capillary endothelium has been observed secondary to VEGF neutralization.[51] In addition, the increased sFlt1 levels and decreased circulating VEGF levels in the disease preeclampsia provide additional evidence that VEGF neutralization can lead to major systemic endothelial dysfunction.

In addition to its potential role in vascular maintenance, VEGF in the adult may act on other cell types because VEGF receptors are expressed by many nonendothelial cells.[52,53] A thorough understanding of VEGF's role in the adult will shed important insight into pathogenesis such as preeclampsia, and will allow modifications of therapies aimed at either neutralization or stimulation of VEGF.

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