Vascular Endothelial Growth Factor-A Specifies Formation of Native Collaterals and Regulates Collateral Growth in Ischemia

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Abstract—The density of native (preexisting) collaterals and their capacity to enlarge into large conduit arteries in ischemia (arteriogenesis) are major determinants of the severity of tissue injury in occlusive disease. Mechanisms directing arteriogenesis remain unclear. Moreover, nothing is known about how native collaterals form in healthy tissue. Evidence suggests vascular endothelial growth factor (VEGF), which is important in embryonic vascular patterning and ischemic angiogenesis, may contribute to native collateral formation and arteriogenesis. Therefore, we examined mice heterozygous for VEGF receptor-1 (VEGFR-1<sup>−/−</sup>), VEGF receptor-2 (VEGFR-2<sup>−/−</sup>), and overexpressing (VEGF<sup>hi/+</sup>) and underexpressing VEGF-A (VEGF<sup>lo/+</sup>). Recovery from hindlimb ischemia was followed for 21 days after femoral artery ligation. All statements below are P<0.05. Compared to wild-type mice, VEGFR-2<sup>−/−</sup> showed similar: ischemic scores, recovery of hindlimb perfusion, pericollateral leukocytes, collateral enlargement, and angiogenesis. In contrast, VEGFR-1<sup>−/−</sup> showed impaired: perfusion recovery, pericollateral leukocytes, collateral enlargement, worse ischemic scores, and comparable angiogenesis. Compared to wild-type mice, VEGF<sup>hi/+</sup> had 2-fold lower perfusion immediately after ligation (suggesting fewer native collaterals which was confirmed by angiography) and blunted recovery of perfusion. VEGF<sup>lo/+</sup> mice had 3-fold greater perfusion immediately after ligation, more native collaterals, and improved recovery of perfusion. These differences were confirmed in the cerebral pial cortical circulation where, compared to VEGF<sup>hi/+</sup> mice, VEGF<sup>lo/+</sup> formed fewer collaterals during the perinatal period when adult density was established, and had 2-fold larger infarctions after middle cerebral artery ligation. Our findings indicate VEGF and VEGFR-1 are determinants of arteriogenesis. Moreover, we describe the first signaling molecule, VEGF-A, that specifies formation of native collaterals in healthy tissues. (Circ Res. 2008;103:1027-1036.)

Key Words: arteriogenesis ■ angiogenesis ■ cerebral circulation ■ cerebral infarction ■ vascular development

Ischemic vascular disease is the leading cause of morbidity and mortality in the developed world. The number and diameter of native (preexisting) collaterals in healthy tissue and their capacity to enlarge (remodel) are critical determinants of the severity of ischemic injury following arterial obstruction. Whereas molecules regulating collateral remodeling in ischemia are beginning to be understood, nothing is known regarding when native collaterals form and the responsible signaling mechanisms.

Collateral enlargement in ischemia requires proliferation of endothelial and mural cells, leukocyte recruitment, and remodeling of extracellular matrix. Evidence suggests vascular endothelial growth factor (VEGF) participates in these processes. However, trials testing whether exogenous VEGF can augment collateral enlargement have met with mixed results. The multiple VEGF isoforms, receptors, and intracellular pathways, plus the inaccessibility of the collateral circulation, make it difficult to determine a direct effect on collateral remodeling. Most studies have administered a single VEGF isoform with limited control of concentration, leading to defective neovascularization, tissue edema and impaired vessel growth. Recent studies have shown that multiple gradient-forming isoforms are expressed within narrow concentration windows that, if exceeded too greatly in either direction, lead to aberrant vessel formation, embryonic lethality or disturbed vessel maintenance and growth in the adult. Interestingly, Yu et al and Xie et al have shown that administration of an engineered zinc finger transcription factor that drives expression of multiple VEGF isoforms improved recovery of limb perfusion following femoral artery ligation. Studies by Jacobi et al, Lloyd et al, and Toyota et al using VEGF antagonists given systemically suggest that endogenous VEGF may contribute to ischemic collateral remodeling. However, no study has examined this question with a genetic approach or identified the responsible VEGF receptor type. VEGF acts primarily through VEGF receptor (VEGFR)-1 (flt-1) and VEGFR-2 (flk-1/KDR). VEGF-
induced angiogenesis is mediated predominantly by VEGFR-2. Experiments in adult mice using VEGFR-1 specific agonists, placental growth factor and VEGF-B, and targeted mice lacking the kinase domain of VEGFR-1 demonstrated a positive role for VEGFR-1. However, during embryonic development, alternately transcribed soluble VEGFR-1 (sFlt-1) participates as a negative regulator of VEGF signaling. In this study, we examined the effect of targeted over- and underexpression of VEGF, VEGFR-1, and VEGFR-2 on collateral remodeling in ischemia.

It is not known when the native collateral circulation develops or what pathways specify formation of these rare arteriole-to-arteriole anastomoses that interconnect adjacent arterial trees. We previously found that compared to C57BL/6 mice, the BALB/c strain is markedly deficient in collateral density, and identified an expression quantitative trait locus at or near Vegfa that positively correlated with low VEGF expression at the BALB/c allele. Thus, in the present study, we tested the hypothesis that VEGF expression is a determinant of collateral formation.

Materials and Methods
Twelve- to 16-week-old mice were randomized, and procedures were conducted blindly. Femoral artery ligation was distal to the lateral caudal femoral and superficial epigastric arteries. Laser Doppler perfusion imaging and arterial pressure (cannulated femoral artery) determinations were obtained under light isoflurane (1.125%) and oxygen anesthesia. Histology, x-ray angiography, and cerebral arteriography were done after pressure-perfused maximal dilation and fixation. RNA was extracted after perfusion with RNA-later. Data are means±SEM and were subjected to ANOVA, followed by Dunn–Bonferroni corrected t tests or Student’s t tests where appropriate or by Mann–Whitney U tests for capillary number-to-muscle fiber ratios and appearance scores. For additional details, see the online data supplement at http://circres.ahajournals.org.

Results
Reduced Recovery After Femoral Ligation in VEGFR-1 Heterozygous Mice
Although VEGFR-1−/− or VEGFR-2−/− mice are embryonic lethal, heterozygous mice appear normal but have defects in cardiac preconditioning. Compared to wild type, VEGFR-1−/− had larger paw appearance scores, indicating greater ischemia (Figure 1a). In contrast, VEGFR-2−/− showed no differences (Figure 1b). In agreement, recovery of plantar perfusion (Figure 1c), which correlates with overall hindlimb blood flow, was attenuated in VEGFR-1−/− but not in VEGFR-2−/− mice (Figure 1d and 1e).
To investigate mechanisms underlying the perfusion differences, we measured capillary density in gastrocnemius. Whereas capillary-to-muscle fiber number ratios at baseline were comparable among groups, ratios significantly increased at day 21 in VEGFR-2/+/ mice and trended similarly in VEGFR-1/+/ mice. (Figure 2a, 2d, and 2g). The increase in VEGFR-2/+ was associated with slightly greater capillary density and muscle atrophy (supplemental Figure Ib and If). Capillary density and muscle atrophy were not different in VEGFR-1/+/ mice (Figure Ia and Ie in the online data supplement). We next examined collateral diameters in the anterior and posterior gracilis muscles and perfusion in the superficial adductor region containing these collaterals. Lumen diameter was not different at baseline. However, outward remodeling at day 21 was less in VEGFR-1/+ (P=0.07, 2-tailed) (Figure 2b, 2e, and 2h). In addition, VEGFR-1/+ had reduced adductor perfusion (supplemental Figure IId). Collateral remodeling and perfusion were not different in VEGFR-2/+ mice (Figure 2h and supplemental Figure I I e). Leukocytes contribute importantly to collateral artery growth.\(^{1–3}\) Pericollateral CD45\(^+\) leukocytes were not different in VEGFR-2/+ but were reduced in VEGFR-1/+ mice (Figure 2c, 2f, and 2i), consistent with their attenuated collateral remodeling. In addition, circulating leukocytes, lymphocytes, and monocytes were lower 3 days after ligation in VEGFR-1/+ (supplemental Table I). These findings indicate that reduced expression of VEGFR-1 results, after femoral artery occlusion, in fewer circulating and pericollateral leukocytes, reduced collateral enlargement and flow, reduced recovery of hindlimb flow, and greater tissue ischemic injury suggesting VEGFR-1 is important in collateral remodeling.

The VEGF Hypermorphic Allele Augments, and Hypomorphic Allele Attenuates, Recovery After Femoral Ligation

Nagy and colleagues have constructed unique VEGF-A lacZ reporter mice with hypermorphic or hypomorphic VEGF-A alleles.\(^{8,9,22}\) Homozygosity of either allele results in embry-
onic lethality. However, VEGF$^{hi+}$ and VEGF$^{lo+}$ are viable and appear normal despite expressing VEGF at intermediate levels to homozygous and wild-type counterparts. Because data are lacking, we assessed expression of the VEGF isoforms, VEGF120, VEGF164, and VEGF188 by quantitative RT-PCR in calf and collateral zone adductor muscles.

Baseline VEGF expression in calf from the nonligated leg was 2-fold higher in VEGF$^{hi+}$ and 25% to 75% lower in VEGF$^{lo+}$ compared to wild type (supplemental Figure IIIa). Thirty-six hours after femoral ligation, expression in all 3 strains for VEGF120 (Figure 3a through 3c). In contrast, expression in the adductor of the ligated leg, which does not experience detectable ischemia in this model,23 showed upregulation of the high-molecular-weight isoforms, VEGF-164 and -188, in wild-type and VEGF$^{hi+}$ mice (Figure 3d and 3e). Interestingly, all VEGF isoforms decreased in the VEGF$^{lo+}$ mice (Figure 3f).

To determine involvement of hypoxia-inducible factor (HIF) signaling in this unique pattern of upregulation in the adductor collateral zone, we performed quantitative RT-PCR for HIF-1α, HIF-1β, and HIF-2α. Whereas expression was similar in all strains in the adductor of the nonligated leg (supplemental Figure IIIb), HIF-1α increased 2-fold, whereas HIF-1β was unaffected in the ligated leg (Figure 3g through 3i). Interestingly, HIF-2α decreased, suggesting an alternative role for this subunit. These data suggest that ischemia in calf promotes alternative splicing of VEGF favoring the soluble isoforms, whereas shear-induced collateral remodeling in adductor is associated with expression of matrix-bound isoforms.

Compared to wild-type mice, VEGF$^{hi+}$ exhibited less (Figure 4a), whereas VEGF$^{lo+}$ experienced greater (Figure 4b), ischemic appearance scores after femoral ligation. Likewise, VEGF$^{hi+}$ mice exhibited less reduction in perfusion immediately after ligation and better recovery thereafter (Figure 4c, 4e, and 4f), whereas VEGF$^{lo+}$ experienced greater reduction and worse recovery (Figure 4d through 4f); however, only the indicated time points differed significantly by Bonferroni tests. The milder ischemia in VEGF$^{hi+}$ is consistent with their trend toward a smaller increase in VEGF120 in
the calf, compared to wild type (Figure 3b). The slight
reduction of VEGF120 in VEGFlo/H11001, compared to wild type,
and the decrease of VEGF164 (Figure 3c) may reflect the
reduced stability of VEGF transcripts in the VEGFlo/H11001
strain.9 Because differences in arterial pressure could cause differences in Doppler perfusion measurements, we measured
arterial pressure under the same conditions of anesthesia used
for Doppler imaging. Mean arterial pressure did not differ in
VEGFhi/H11001 versus VEGFlo/H11001 (Figure 4g). Moreover, because
VEGF is a vasodilator, increased VEGF in VEGFhi/H11001 (versus
decreased VEGF in VEGFlo/H11001) favors lower (versus higher)
arterial pressure and less (versus more) perfusion immediately
after ligation (and thereafter), which is the opposite of
what we observed (Figure 4c and 4d).

Measurement of plantar perfusion immediately after ligation largely reflects anatomic differences in preexisting collateral
density and/or diameter and, to a lesser extent, capillary
density, because vascular tone is strongly inhibited in the ischemic limb.25 Thereafter, recovery of perfusion is primarily
determined by collateral remodeling, with a smaller contribution
of angiogenesis. To distinguish the potential effect of VEGF on these determinants, we first examined capillary-to-muscle fiber ratio in gastrocnemius at baseline
(Figure 5a and 5b). There was no difference between wild-
type and VEGFho/H11001 or VEGFhi/H11001 mice, suggesting that differences in collateral number and/or diameter underlie the
differences in perfusion immediately after ligation. Capillary-
to-fiber ratio increased at day 21 in VEGFho/H11001. Interestingly,
baseline fiber size was greater in VEGFho/H11001, consistent with
the role of VEGF in skeletal muscle development (supplemental Figure 1c and Ig).26 Capillary-to-fiber ratio decreased at day 21 in VEGFho/H11001 mice. Although capillary number-per-tissue-area of VEGFhi/H11001 did not differ from wild type, greater atrophy occurred (supplemental Figure 1d and 1h), resulting in reduced capillary-to-muscle fiber ratio.

Given the importance of collateral conductance following femoral ligation, we determined collateral diameter at baseline
and at 21 days postligation. Lumen diameters in VEGFho/H11001 mice were significantly smaller than wild type at both time points; however, percentage remodeling and adductor perfusion in VEGFhi/H11001 were not different (Figure 5c and supplemental Figure III). In contrast, baseline diameters in VEGFhi/H11001 mice did not differ from wild type; however, remodeling and adductor perfusion were reduced (Figure 5d and supplemental Figure II g). The baseline diameter data suggest (because baseline capillarity do not differ) that differences in collateral density may underlie the differences in perfusion immediately after ligation, ie, greater number of smaller diameter collaterals in VEGFho/H11001, versus fewer in VEGFhi/H11001. In addition, less remodeling in VEGFho/H11001 mice suggests VEGF contributes to collateral remodeling.

Immunofluorescence was performed against β-galactosidase
in adductor sections of VEGFhi/H11001 mice to localize VEGF
expression (Figure 5e). Low-level basal expression of VEGF
was evident in skeletal muscle surrounding noncollateral
arterioles and unremodeled collaterals (Figure 5e, left and
middle images), consistent with data from Maharaj et al describing VEGF expression pattern in these mice.24 How-

Figure 4. VEGFhi/H11001 mice have better and VEGFho/H11001 worse recovery after femoral ligation. a and b, Lower ischemic appearance score in VEGFhi/H11001 and more ischemia in VEGFho/H11001 (see Figure 1 for scale); n=6 to 20 per data point. c through e, Quantification of perfusion measured over plantar ROI shows less reduction immediately after ligation (“Post-Op”) and better recovery in VEGFhi/H11001, and opposite effects in VEGFho/H11001. f, Representative Doppler images of the indicated time points (Pre and Post). ANOVA followed by Dunn–Bonferroni t test; *P<0.05, **P<0.01, ***P<0.001 vs wild type; n=6 to 16 per data point. g, Mean arterial pressure (MAP) was measured under anesthetic conditions used for Doppler measurements.
ever, 7 days after ligation, VEGF expression increased in pericollateral skeletal muscle (Figure 5e, right). Costaining with CD45 revealed a small population of recruited leukocytes that also expressed VEGF (Figure 5e, right, and supplemental Figure IV).

**VEGF Genotype Determines the Number of Preexisting Collaterals**

To determine whether VEGF expression influences native collateral density, as suggested by the above findings, we performed x-ray arteriography immediately and 7 days after ligation (the latter to improve detection of smaller collaterals after onset of remodeling). Analogous to Rentrop analysis, we counted vessels crossing a line drawn through the middle of the adductor collateral zone (Figure 6a); this method agrees with other methods of quantifying preexisting collateral capacity in the hindlimb. An apparent VEGF gene–dose relationship was detected, wherein VEGFhi/H1 mice had more and VEGFlo/H1 mice had fewer collaterals than wild-type mice (Figure 6b).

We next sought to determine whether collaterals are present at birth and whether VEGF expression correlates with newborn and adult density in another tissue besides skeletal muscle. We recently reported that differences in collateral density among mouse strains exist across multiple tissues. This includes collaterals of the cerebral cortical circulation, which are confined to the pial (leptomeningeal) membrane and thus readily quantified. We therefore counted pial collaterals in VEGFhi/H1, VEGFlo/H1, and wild-type mice using a polyurethane casting agent (Figure 6c; resolution of x-ray and polyurethane arteriography given in the online data supplement). Interestingly, collaterals were present at birth (P1) (Figure 6d). As in the hindlimb (Figure 6b), an apparent VEGF gene–dose relationship was detected at P1, P21, and 3 months of age, with VEGFhi mice having more and VEGFlo mice having fewer collaterals (Figure 6c and 6d). Wild-type and VEGFhi mice were born with the same number of collaterals, whereas VEGFlo were born with fewer, suggesting VEGF levels specify the density of nascent collaterals formed during embryogenesis. In VEGFhi, pial collateral number remained constant throughout postnatal development. In contrast, wild-type mice experienced a decline in collateral density by P21 that remained unchanged in adults. VEGFlo mice also lost collaterals with age.

To determine whether an increase in collateral density offered protection, we performed middle cerebral artery occlusion (MCAO) in wild-type, VEGFlo/H1, and VEGFhi/H1 mice. Twenty-four hours after MCAO, a relationship between VEGF genotype and infarct volume was evident (Figure 6e and 6f), with VEGFhi mice having 2-fold smaller infarcts. Furthermore, infarct volume negatively correlated with collateral number (Figure 6g). These data suggest that collaterals form during the perinatal period; that VEGF is important in their formation, postnatal maturation, and establishment of the adult collateral density; and that the latter may be an important determinant of the severity of stroke.

**Discussion**

Our results suggest that VEGFR-1 positively regulates angiogenesis through recruitment of leukocytes to the pericollateral region. We also provide the first examination of when the native collateral circulation becomes established, finding
that pial collaterals are already present at birth and increase over the first 3 weeks of life to achieve the density present in the adult. In addition, we identify the first gene, VEGF-A, whose expression impacts the density of preexisting collaterals in healthy tissue.

Few studies have addressed whether endogenous VEGF mediates collateral growth. Supportive evidence exists in hindlimb13,14 and heart15 ligation models, where systemic administration of sFlt1/sFlk1 adenovirus,13 VEGF receptor inhibitor,14 or neutralizing antibody15 impaired recovery of perfusion and angiographic collateral growth. Also, Matsunaga, Chilian, and colleagues have shown that inhibition of endothelial nitric oxide synthase with L-NAME (N\textsubscript{G}-L-arginine methyl ester), which also interferes with VEGF signaling, reduced ischemic coronary collateral growth but did not affect angiogenesis.28 Our findings that VEGFR-1 mice show reduced recovery of plantar and adductor perfusion, greater ischemia, reduced pericollateral leukocyte, and reduced collateral enlargement, but normal angiogenesis, suggest that VEGF acting through VEGFR-1 mediates collateral remodeling. This is in contrast to the role of VEGFR-1 as a VEGF inhibitor during development.4,18 It is difficult to measure VEGFR-1 signaling because of its weak kinase activity.17 However, studies using the VEGFR-1–specific agonists, placenta growth factor and VEGF-B, and kinase-dead mutants find that VEGFR-1 possesses signaling functions in the adult.4,18 Our data add collateral growth to these functions. They also agree with evidence by Pipp et al that mice lacking placenta growth factor have impaired recovery from hindlimb ischemia.16 In addition, our findings of fewer leukocytes circulating and residing around remodeling collaterals, which suggest reduced leukocyte mobilization and transmigration, are consistent with their known role in arteriogenesis.16 These results are congruent with the expression of VEGFR-1 on leukocytes18 and its proposed role in the bone marrow niche,29 in homing,30 and chemotaxis.31 No deficits were evident in VEGFR-2 mice. However, given the strong kinase activity of VEGFR-2, a single allele may specify sufficient receptors to mediate VEGFR-2–dependent ischemic angiogenesis, as well as any potential contribution of this receptor to arteriogenesis. This could explain why angiogenesis in the ischemic gastrocnemius was not impaired in VEGFR-2 mice. Conditional deletion of VEGFR-2 and VEGFR-1 will be required to confirm that VEGFR-2 does not participate in collateral growth and to determine whether the modest inhibition we observed in the VEGFR-1 reflects only partial reduction in receptor density.

Native collaterals are small in diameter and few in number. In addition, current antibodies cannot distinguish among VEGF isoforms and have limited resolution to detect secreted ligand. Thus, it is not known whether VEGF isoforms increase around remodeling collaterals to contribute arteriogenesis. Deindl et al23 did not detect increased VEGF in rabbit collaterals or in whole quadriceps muscle 3 days after femoral ligation. In contrast, we found that mRNAs for high-molecular-weight isoforms of VEGF increased in the adductor collateral zone of wild-type mice 36 hours after ligation. Because VEGF transcripts agree with protein ex-
pression,\textsuperscript{32} this discrepancy may be attributable to differences in duration of ligation, tissue analyzed, or species studied. We assayed a 5-mm-wide midsection of the adductor that contains hindlimb collaterals, including those in the gracilis muscles that we measured histologically. In contrast to the adductor, only VEGF120 was upregulated in the gastrocnemius. This differential expression where collaterals are remodeling versus where capillaries are sprouting, is consistent with the different physical properties and functions of VEGF isoforms. In the calf, free diffusion of VEGF120 would promote a wide area of angiogenesis. In addition, Hattori et al have shown that VEGF120 released into the circulation from ischemic tissue aids mobilization of leukocytes from bone marrow.\textsuperscript{29} In contrast, collateral growth in the thigh is presumed to require temporal and spatial release of proteases, cytokines, and growth factors. Elaboration of the heparin-binding VEGF164 and VEGF188 isoforms around collaterals may establish VEGF gradients that stimulate diapedesis and proliferation of leukocytes, proliferation of endothelial cells, and migration of smooth muscle cells. Consistent with this hypothesis, immunofluorescence revealed VEGF upregulated in pericollateral skeletal muscle and leukocytes in addition to collateral endothelium and smooth muscle. It will be important in future studies to determine the cell source(s), stimulus for VEGF release, and isoform expression profile.

Deindl et al\textsuperscript{23} also did not detect increased Hif-1\textalpha in the adductor, although differences in species, time after ligation, and tissue sampled could be important. In contrast, we found an $\approx$2-fold increase in Hif-1\textalpha mRNA (and no increase in Hif-1\textbeta) in the adductor collateral zone. Jiang, Semenza, and colleagues have shown that Hif-1\textalpha levels are regulated by tissue oxygen over a broad range.\textsuperscript{33} Thus, oxygen may decline sufficiently, although not to ischemic levels, to increase transcription and stabilization of Hif-1\textalpha transcripts. In addition, it is possible that other factors surrounding remodeling collaterals that regulate Hif-1\textalpha, such as reactive oxygen species, nitric oxide, growth factors, and increased shear stress itself\textsuperscript{44} may contribute to the increase in Hif-1\textalpha.

Consistent with deficient recovery of flow after femoral ligation in VEGFR-1\textsuperscript{1/2} mice, a direct relationship was evident between VEGF genotype and recovery among VEGF\textsuperscript{hi}, wild-type, and VEGF\textsuperscript{lo} mice. Similar to VEGFR-1\textsuperscript{1/2}, VEGF\textsuperscript{lo} had worse ischemic appearance scores and blunted recovery of planar perfusion compared to wild type. In addition, capillary-to-muscle fiber number decreased by day 21 in VEGF\textsuperscript{lo} as a result of greater muscle atrophy. In contrast, VEGF\textsuperscript{lo} mice showed enhanced recovery of perfusion and little ischemia. The role of VEGF in the development and maintenance of striated muscle fiber size, as demonstrated by Bryan, D’Amore, and colleagues,\textsuperscript{28} could contribute to the differences in atrophy and appearance scores. The variation in capillary-to-fiber ratio at day 21 that we observed in the hypomorphs, which is consistent with the role of VEGF in ischemic angiogenesis, could also contribute to the differences in recovery of hindlimb perfusion and ischemic scores, although collateral remodeling is the primary determinant of recovery of hindlimb perfusion.

VEGF\textsuperscript{lo} mice exhibited attenuated adductor perfusion and impaired collateral remodeling like that seen in the VEGFR-1\textsuperscript{1/2} mice. In contrast, collateral remodeling and adductor perfusion were not enhanced in VEGF\textsuperscript{hi}. Although this appears discordant, several considerations are important. Immediately after ligation, perfusion decreased more in VEGF\textsuperscript{lo} and less in VEGF\textsuperscript{hi}. The increase in shear stress in collaterals immediately after ligation favors inhibition of collateral smooth muscle tone. Moreover, studies by Yang, Laughlin, and Terjung have documented that ischemia and low pressure cause myogenic and metabolic inhibition of tone in the vasculature below the point of ligation.\textsuperscript{25} These considerations suggest that the differences in perfusion immediately after ligation arise from anatomic differences in preexisting collateral number, collateral lumen size, and/or, to a lesser degree, capillary density. Baseline capillary density was not different among VEGF\textsuperscript{lo}, VEGF\textsuperscript{hi}, and wild type, and collateral lumen diameter was comparable in the latter 2 groups, whereas it was smaller in VEGF\textsuperscript{lo} mice. Collectively, these data suggest that density of native collaterals varies directly with level of VEGF expression. In VEGF\textsuperscript{lo} mice, a greater number of collaterals in parallel favors lower flow in individual collaterals and thus smaller baseline diameters, which is what we observed. Shear stress is the proximate stimulus of arteriogenesis.\textsuperscript{1,2} A greater number of collaterals favors less increase in shear in individual collaterals, which is consistent with our finding that remodeling was not greater in VEGF\textsuperscript{lo} mice than in wild type despite increased VEGF expression. In VEGF\textsuperscript{lo} mice with fewer collaterals, the expected higher shear forces may not be able to overcome the deficit in VEGF expression. This could explain the reduced collateral remodeling and impaired perfusion that we observed.

We found that VEGF expression determines collateral density in a second tissue: the pial circulation. An additional intriguing finding was that VEGF\textsuperscript{lo} mice were born with fewer pial collaterals compared to wild type and VEGF\textsuperscript{hi}. These data suggest VEGF levels contribute to collateral formation in the embryo. We previously hypothesized that collaterals form during arterial tree formation and that reduced VEGF results in reduced collateral formation.\textsuperscript{19} Studies by the laboratories of Bautch, Shima, and Tomanek have shown that VEGF is important in branching morphogenesis,\textsuperscript{35–37} including formation of coronary artery stems by VEGF-B, which activates VEGFR-1.\textsuperscript{37} Our data support the concept that collateral density and vascular branching are intimately related. In addition, collateral density was reduced by postnatal day 21 in wild-type and VEGF\textsuperscript{lo} mice, whereas density was maintained in VEGF\textsuperscript{hi}. This is consistent with the role of VEGF in stabilizing nascent blood vessels.\textsuperscript{4,5,18}

We reported that native collateral density and VEGF expression differ strongly in 2 mouse strains.\textsuperscript{19} Inducible VEGF expression and collateral density in hindlimb, cerebral cortex, and intestine were high in C57BL/6 mice and low in BALB/c. This association led us to hypothesize that VEGF is a determinant of native collateral formation. Our present studies using mice with a single targeted genetic difference, ie, altered VEGF expression,\textsuperscript{8,9,22} provide strong support for this hypothesis. We also suggest that genetic polymorphisms and environmental factors that reduce VEGF during embryonic or perinatal periods could reduce collateral formation,
resulting in increased severity of stroke, myocardial infarction, and peripheral artery disease. How VEGF affects collateral formation and stabilization are intriguing questions for future study. Our data suggest that murine pial collaterals form before birth and mature within a narrow 3-week period. In the embryo, early vascular patterning involves genetically determined events and morphogenetic factors including VEGF, whereas during later stages when flow becomes important, excess vessels are thought to be pruned, leaving “hemodynamically favored” channels. It is possible that the role of VEGF in collateral formation involves its known role in vascular patterning and/or hemodynamic patterns produced by local vasodilator actions of VEGF.

Other molecules in the VEGF signaling pathway may also contribute to collateral formation. Resar et al recently identified a HIF-1α polymorphism that correlated with the presence of coronary collaterals in patients. The polymorphism results in an amino acid substitution that confers increased HIF-1 activity. However, patients with the substituted allele did not have visible collaterals, which is at variance with our findings. Among mechanisms proposed by the authors to explain the apparent discrepancy between higher HIF-1 activity and lack of visible collaterals, we propose an additional hypothesis. Because clinical angiography only detects large vessels, collaterals in a person with a small native density would be more likely to experience larger increases in shear and thus enlarge to detectable diameters during progression of coronary artery disease. In contrast, collaterals in a person with high density would be expected to experience smaller increases in shear, resulting in less outward remodeling during disease progression and absence of angiographic detection.

In conclusion, our results suggest that VEGFR-1 mediates collateral growth in ischemic disease by mobilizing leukocytes and recruiting them to the pericollateral space. In addition, we show that collaterals form before birth and stabilize at their adult density by the third postnatal week. Lastly, we identify VEGF as the first molecule specifying preexisting collateral density in normal tissue. We propose a model whereby collaterals form in a VEGF-dependent manner during embryogenesis. Further, these nascent collaterals require adequate VEGF during a critical period after birth to stabilize and mature sufficiently to achieve their full adult density. Further studies will be required to define when and how VEGF and other molecules contribute to collateral formation, as well as the downstream effectors that stabilize and maintain collaterals in tissues. An interesting recent study by Wustmann, Seiler, and colleagues reported that coronary collateral flow in healthy patients varies widely. Moreover, several VEGF polymorphisms linked to altered expression have been described. Thus, it will be important to examine whether polymorphisms affecting the expression of VEGF and other genes are associated with altered collateral density. An understanding of pathways that specify collateral formation in normal tissues may lead to therapies to induce formation of new collaterals in patients with ischemic disease.

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Material and Methods

Unilateral hindlimb ischemia. Twelve-to-16 week-old VEGFR-1\(^{+/−}\), VEGFR-2\(^{+/−}\), VEGF\(^{hi/+}\) and VEGF\(^{lo/+}\) mice\(^1\text{-}^4\) (kindly provided by J Rossant and A Nagy), maintained on the CD-1 background and genotyped by X-gal staining of tail biopsies, were compared to wild-type littermate controls. Animals were randomized and procedures and analyses were conducted blindly. Femoral artery ligation was performed as described.\(^5\text{-}^6\) Briefly, mice were anesthetized with 1.25% isoflurane/O\(_2\) and the hindlimbs depilated. Temperature was maintained at 37.0±0.5\(^\circ\)C. The right femoral artery was exposed through a 2mm incision and ligated with two 7-0 ligatures placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The artery was transected between the sutures and separated by 1-2 mm. The wound was irrigated with sterile saline and closed, and cefazolin (50mg/kg, im), furazolidone (topical) and pentazocine (10mg/kg, im) were administered. Procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Laser Doppler perfusion imaging. As detailed previously,\(^5\text{-}^6\) under 1.125% isoflurane/O\(_2\) anesthesia and 37±0.5\(^\circ\)C, non-invasive perfusion imaging of the adductor thigh region and plantar foot of both limbs was performed before, immediately after, and at 3, 7, 14, and 21 days after femoral ligation. Regions of interest were drawn to anatomical landmarks.
**Assessment of ischemia.** An “appearance score” after ligation was obtained, where 0=normal, 1=cyanosis or loss of nail(s), 2=partial or complete atrophy of digit(s), 3=partial atrophy of fore-foot.6

**Leukocyte counts.** Three days after femoral ligation blood was collected under anesthesia by retro-orbital puncture. Leukocyte counts were performed with a Heska’s Animal Blood Counter.

**Histology.** As detailed previously5, 21 days after ligation, animals were pressure-perfused transcardially (100mmHg) with phosphate-buffered saline (PBS, pH7.4) containing 20mmol/L adenosine, 10^{-4}mol/L papaverine and 10U/ml heparin, followed by pressure-perfused fixation with 4% paraformaldehyde in 100mmol/L sodium phosphate (PFA; pH7.4) for 15min. The hindquarters was post-fixed while shaking for 48h in PFA, with a solution change at 24h. The adductor muscles (medial thigh) below the femur were excised as a ~5mm square block extending ~1mm medial of the “mid-zone” of the gracilis muscle collaterals that interconnect the lateral caudal femoral artery and saphenous artery trees, to the lateral portion of the thigh. A ~5mm square block of the calf was excised beginning at the origin of the Achilles tendon and extending toward the knee. Blocks were rinsed in water, placed in 70% ethanol for 48h with a solution change at 24h and embedded in paraffin. Sections were 5µm cross-sections perpendicular to the long axis of muscle fibers, capillaries and direction of the gracilis collaterals, beginning on the medial side for the adductor and on the knee-side for the calf. After removal of the first 200µm of the full block face, sections were arranged 6 per
slide, separated by 250µm. Lumen diameter of collaterals in the anterior and posterior gracilis muscles was determined from sections of their mid-zone stained with cyano-Massons trichrome. For all histochemical analyses, results reported for each animal were averages of values from two sections separated by 250µm, from digitized images analyzed with ImageJ (NIH).

Biotinylated *Griffonia Simplicitoflia* isoelectin-1-B₄ (1:100, Vector Labs) plus Alexa Fluor 488 conjugate of streptavidin (1:50 Molecular Probes) were used to determine capillary density and capillary-to-muscle fiber number in the gastrocnemius muscle 21 days after ligation. Capillaries were identified as lectin-positive vessels with diameters <7µm counted in coronal sections of the gastrocnemius muscle within a delineated area. For muscle atrophy, average muscle fiber area was determined for the fascicles within the same area. Boundaries of fibers and fascicles were outlined by autofluorescence during imaging. Capillary density was reported as average density per square micrometer and as capillary number–to–muscle fiber number ratio. Capillary density, ratio and fiber area were obtained from two digitized 20X fields of view (~434 µm X 330µm) within two sections each of the medial and lateral heads of the gastrocnemius of the ligated and non-ligated leg and averaged (ImageJ, NIH). Leukocyte density around gracilis collaterals was determined with CD45 (BD Pharmingen) immunohistochemistry.⁵, ⁶

For VEGF localization, adductor sections (6 per slide) from VEGF²/² (n=5) were analyzed for β-galactosidase and CD45. β-galactosidase was detected with rabbit anti-β-galactosidase antibody (ab616, Abcam, 1:100) followed by Cy5-conjugated donkey anti-rabbit antibody (Jackson Immuno Research, 1:500) Leukocytes were detected with
rat anti-CD45 antibody (clone 30-F11, BD Pharmingen, 1:100) followed by FITC-conjugated donkey anti-rat antibody (Jackson Immuno Research, 1:500).

**Post-mortem arteriography.** As detailed previously, animals were maximally dilated and perfusion-fixed 7 days after ligation when the contralateral femoral artery was also ligated. A high-viscosity barium sulfate solution (85% w/v) with impaired capillary transit was infused after ligation of the ankles to minimize arterial-venous shunting, followed by X-ray angiography. A Rentrop-like line analysis was performed on digitized films by counting vessels intersecting a line drawn perpendicular to the femur beginning at a point distal to the femoral ligation that approximated the midpoint of the gracilis collaterals. Cerebral pial arteriography was obtained in separate groups following maximal dilation and perfusion-fixation. After removal of the dorsal calvarium, high-viscosity polyurethane (60% resin in 2-butanone with blue dye) that is unable to cross capillaries was infused via the thoracic aorta during stereoscopic imaging, followed by topical and the whole-brain fixation in 2% PFA overnight at 4°C. Brains were imaged and collaterals interconnecting the middle and anterior cerebral artery trees were counted. Collateral density is defined in skeletal muscle as the number of arteries crossing the Rentrop-like line drawn through the collateral zone in the thigh below the femur (**Fig. 6a,b**). In the cerebral cortical circulation, it is the number of collaterals interconnecting the middle and anterior cerebral arteries (**Fig. 6c,d**).

Parameters for hindlimb x-ray arteriography were as follows: Faxitron MX-20, 5x geometric magnification, MIN-R EV Kodak film and MIN-R cassettes, a lead sheet shelf with a window cut to the dimensions of a mouse was placed in the Faxitron between the
cathode and cassette to reduce x-ray scatter, 26kV/6.8 sec. These parameters yield a phantom resolution of ~35µm. Absence of venule filling plus low background (Fig. 6a) indicates that the contrast material does not appreciably cross the capillary bed. Although hindlimb collaterals smaller than 35µm diameter could not be detected by X-ray angiography, this was not the case with pial arteriography. Therein we used a polyurethane casting material (Fig. 6b) whose viscosity was adjusted to assure filling of pre-capillary vessels and collaterals down to ~8µm in diameter, with little capillary transit. Since light microscopy was used for pial arteriography, resolution was not limited as in x-ray angiography. We saw the same relationship for collateral number in VEGF^{hi/+}, VEGF^{lo/+} and wildtype mice in the skeletal muscle and cerebral circulations (Fig 6). Thus, although we cannot be certain that the same direct relationship exists between VEGF expression and relative collateral number among these strains in hindlimb collaterals smaller than 35µm, this uncertainty does not exist for the pial collateral population where all collaterals could be readily counted and where the relationship obtained in the hindlimb was also found.

**Quantitative RT-PCR.** The hindlimbs were perfused 36h after unilateral femoral artery ligation with RNA-later (Ambion) via that abdominal aorta under deep anesthesia. 5mm thick sections of proximal calf and the adductor centered on the collateral mid-zone and perpendicular to the femur were excised and stored in RNA-later at -80ºC. Tissue was pulverized under liquid nitrogen. Total RNA was extracted (RNeasy Fibrous Tissue mini kit, Qiagen) according to the manufacturer. Genomic DNA was removed by on-column DNase treatment (Qiagen). Total RNA was reverse-transcribed (Verso
cDNA kit, Abgene). Real-time PCR was performed with triplicate cDNA samples diluted 1:100 using SYBR Green chemistry (Sigma) on a Rotor-Gene 3000 (Corbett Life Science). Data were analyzed according to the Pfaffl method.6

**Arterial pressure measurement.** Arterial pressure was determined in VEGF$^{hi/+}$ and VEGF$^{lo/+}$ mice via the acutely catheterized femoral artery. Measurements were obtained with a pressure transducer at 37°C rectal temperature and under isoflurane anesthesia at the level used during laser Doppler perfusion measurements.

**Middle cerebral artery occlusion.** The right middle cerebral artery trunk was exposed at its position midway between the zygomatic arch and the pinna of the ear and occluded by micro-cautery. 24 hours later, the mouse received an overdose of ketamine (100 mg/kg ip) and xylazine (15 mg/kg ip). The brain was removed, sliced into 1 mm coronal sections, and incubated in a PBS solution containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 min. Sections were washed in PBS, fixed in 10% formalin and imaged (MZ16FA, Leica). Infarct volume was calculated as the sum of the cortical volume devoid of TTC in each section, and expressed as a percent of total right cortex volume.

**Statistics.** All data are reported as means ± SEM. Significance ($P<0.05$) was determined by two-way ANOVA followed by Dunn-Bonferonni Corrected $t$-tests. One-way ANOVA was used for comparisons between VEGF genotypes (VEGF$^{hi/+}$, VEGF$^{lo/+}$,
wild-type), followed by paired or unpaired \( t \)-tests. Capillary number-to-muscle fiber ratios and appearance scores were subjected to Mann Whitney U tests.
**Supplemental Table**

**Online Table I.** Whole blood leukocyte counts (10³/µl) are reduced in VEGFR-1⁺/- but not VEGFR-2⁺/- mice.

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Supplemental Figure Legends

**Online Figure I.** Capillary area density (a-d) and muscle fiber area (e-h) for VEGFR-1+/− (a,e), VEGFR-2+/− (b,f), VEGF<sup>hi/+</sup> (c,g), and VEGF<sup>lo/+</sup> (d,h) mice before (open bars) and 21 days after femoral ligation (black bars). Capillary area density increased in wild-type mice, with a similar trend in VEGFR-1+/− mice (a). Muscle fiber area declined similarly in wild-type and VEGFR<sup>+/−</sup> mice (e). Together, the data suggest that VEGFR-1 haplo-insufficiency does not affect angiogenesis or muscle atrophy. Capillary area density tended to increase more (b) and muscle fiber area tended to decrease more (f) in VEGFR-2+/− mice. The higher capillary area density and smaller muscle fiber area resulted in an increase in capillary-to-muscle fiber number ratio (Fig. 2f), which suggests that heterozygosity does not impair angiogenesis. Capillary area density increases slightly less in VEGF<sup>hi/+</sup>, consistent with a less ischemia in the hindlimb (c). Interestingly, VEGF<sup>hi/+</sup> muscle fiber area was greater at baseline and at 21 days (g). The difference in muscle fiber area may indicate altered skeletal muscle physiology from chronic VEGF overexpression. Capillary area density was not different in VEGF<sup>lo/+</sup> mice, which is incongruent with increased ischemia (d). However, there is greater atrophy of skeletal muscle, which is consistent with increased ischemia in VEGF<sup>lo/+</sup> mice (h). Together, these data explain the slight decrease in capillary-to-muscle fiber number ratio (Fig. 5d).

**Online Figure II.** VEGFR-1+/− and VEGF<sup>lo/+</sup> mice have attenuated, and VEGFR-2+/− and VEGF<sup>hi/+</sup> mice have normal perfusion over the adductor collateral zone. (a-c) Representative laser Doppler images of the ventral adductor with ROI outlined.
Collateral perfusion is blunted in VEGFR-1+/− suggesting a positive role for VEGFR-1 in mediating arteriogenesis (d). Impaired collateral perfusion is likely due to a decrease in collateral remodeling (Fig. 2e) as a result of a decrease in leukocyte recruitment (Fig. 2f) and leukocyte mobilization (Online Table I). Adductor perfusion is unchanged in VEGFR-2+/− mice, suggesting that VEGFR-2 is not required for collateral remodeling or, despite heterozygosity, receptor density and/or signaling is sufficient to mediate normal arteriogenesis (e). In VEGF^{hi/+} mice, collateral perfusion is not different from wild-type (f) while adductor perfusion is decreased in VEGF^{lo/+} (g). Inhibition of adductor perfusion in VEGF^{lo/+} is consistent with attenuation of perfusion in VEGFR-1+/− and suggests a model whereby sufficient VEGF levels are required to adequately recruit VEGFR-1-containing leukocytes to the growing collateral. However, collateral perfusion from VEGF^{hi/+} would seem to contradict that model. This could reflect that VEGF expression exceeding wild-type levels may not be able to further augment collateral remodeling. Consistent with this, no further augmentation of collateral lumen diameter was observed in VEGF^{hi/+} mice (Fig. 5c). Alternatively, the greater number of native collaterals in VEGF^{hi/+} mice (Fig. 6) favors reduced flow and shear in any individual collateral. A decrease in the shear stimulus, combined with an increase in VEGF production, may result in a similar amount of remodeling compared to wild-type mice.

**Online Figure III.** Quantitative RT-PCR of non-ischemic calf skeletal muscle confirms genotype of VEGF^{hi/+} and VEGF^{lo/+} mice (a). VEGF expression in normal skeletal muscle has not been previously reported in these adult mouse models. VEGF^{hi/+} mice have 2-fold greater levels of the major VEGF isoforms while there is a 25-
75% decrease in them in VEGF\textsuperscript{lo/+} mice. Non-ischemic adductor HIF subunit expression is similar in all genotypes demonstrating no VEGF-dependent alteration in HIF expression. Data show expression in non-ischemic muscle of VEGF\textsuperscript{hi/+} or VEGF\textsuperscript{lo/+} relative to non-ischemic wild-type muscle. Data are normalized to 18S rRNA. Non-parametric \textit{t}-Test vs. non-ligated; \textit{n}=5 mice per data-point.

**Online Figure IV.** Immunofluorescence of VEGF\textsuperscript{hi/+} mice reveal increased \(\beta\)-galactosidase staining around remodeling collateral artery. \(\beta\)-galactosidase expression recapitulates VEGF expression\textsuperscript{3}. Non-collateral arteriole (\textit{a-c}), gracilis collateral at day 0 (\textit{d-f}) and day 7 post-ligation (\textit{g-i}). Immunostaining for \(\beta\)-galactosidase (\textit{a,d,g}), CD45 (\textit{b,e,h}), and merged images (\textit{c,f,i}).
Online Figure III

(a) Bar charts showing fold change for VEGF120, VEGF164, and VEGF188 in different conditions. The p-values are indicated for each condition:
- VEGF120: p = 0.01
- VEGF164: p = 0.02
- VEGF188: p = 0.001

(b) Bar charts showing expression levels of Hif-1α, Hif-1β, and Hif-2α in different conditions.
References


