Collateral density, remodeling, and VEGF-A expression differ widely between mouse strains

Dan Chalothorn, Jason A. Clayton, Hua Zhang, Daniel Pomp, and James E. Faber

Department of Cell and Molecular Physiology, University of North Carolina, Chapel Hill, North Carolina

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Chalothorn D, Clayton JA, Zhang H, Pomp D, Faber JE. Collateral density, remodeling and VEGF-A expression differ widely between mouse strains. Physiol Genomics 30: 179–191, 2007. First published April 10, 2007; doi:10.1152/physiolgenomics.00047.2007.—Substantial variability exists in collateral density and ischemia-induced collateral growth among species. To begin to probe the underlying mechanisms, which are unknown, we characterized two mouse strains with marked differences in both parameters. Immediately after femoral artery ligation, collateral and foot perfusion were lower in BALB/c than C57BL/6 (P < 0.05 here and below), suggesting fewer pre-existing collaterals. This was confirmed with angiography and immunohistochemistry (~35% fewer collaterals in the BALB/c’s thigh). Recovery of hindlimb perfusion was attenuated in BALB/c, in association with 54% less collateral remodeling, reduced angiogenesis, greater ischemia, and more impaired hindlimb use. Densities of CD45+ and CD45+ leukocytes around collaterals increased similarly, but TNF-α expression was 50% lower in BALB/c, which may contribute to reduced collateral remodeling. In normal tissues, compared with C57BL/6, BALB/c exhibit an altered arterial branching pattern and, like skeletal muscle above, have 30% fewer collaterals in intestine and, remarkably, almost none in pial circulation, resulting in greatly impaired perfusion after cerebral artery occlusion. Ischemic induction of VEGF-A was attenuated in BALB/c. Analysis of a C57BL/6 × BALB/c recombinant inbred strain dataset identified a quantitative trait locus for VEGF-A mRNA abundance at or near the Vegfa locus that associates with lower expression in BALB/c. This suggests a cis-acting polymorphism in the Vegfa gene in BALB/c could contribute to reduced VEGF-A expression and, in turn, the above deficiencies in this strain. These findings suggest these strains offer a model to investigate genetic determinants of collateral formation and growth in ischemia.

pre-existing collateral network; collateral development and arteriogenesis; cerebral circulation; vascular endothelial growth factor and mouse strains; expression quantitative trait loci

UNDERSTANDING THE ADAPTIVE PROCESSES of collateral formation and growth (arteriogenesis) and capillary sprouting (angiogenesis) in ischemia is important in treating occlusive vascular diseases of the heart, brain, and peripheral limbs. Besides the arterial-capillary-venous and lymphatic circulations, collaterals constitute a “third circulation” comprising vessels with unique properties that are present in most tissues. Collaterals are rare arteriole-to-arteriole anastomoses ~10–30 μm in diameter that interconnect adjacent major arterial trees. Because collaterals lack a significant pressure drop over their length, collateral endothelial cells are normally exposed to little or no flow-dependent shear stress (21). However, critical narrowing or occlusion of the main artery supplying one of the trees causes a pressure drop across the collaterals. This increases flow/shear stress inducing them to outwardly (positively) remodel into large caliber “endogenous bypass vessels.”

Collateral growth has greater potential for restoring oxygen delivery to a tissue after artery occlusion than angiogenesis, which can only improve distribution of flow supplied by collaterals. However, compared with angiogenesis much less is known about mechanisms that mediate arteriogenesis. Moreover, nothing is known about how collaterals develop in normal tissues. Collateral density varies widely among species (21). For example, coronary collaterals are abundant in guinea pigs and sparse in swine, resulting in minimal damage in guinea pigs and severe infarctions in swine following occlusion of a major branch of the left coronary artery (44). Similarly, findings in a recent study (62) suggest that collateral density may also vary widely among humans. Coronary collateral conductance was found to exhibit up to a 10-fold variation in a group of 60 normal human subjects. There is also evidence that collateral remodeling varies among humans with diabetes, dyslipidemia, and hypertension. It is known from animal studies that these diseases impair the remodeling process (2, 50, 58).

Although these differences suggest that variation in collateral number and capacity for remodeling may have a genetic component, there is little data addressing this possibility. Recently, BALB/c mice were reported to exhibit significantly reduced recovery of blood flow after femoral artery ligation compared with C57BL/6 (17, 45). Provocative evidence has also been reported suggesting that BALB/c have fewer pre-existing collaterals (22). Unfortunately, these studies were not able to differentiate potential contributions of differences in capillary density, caliber of pre-existing collaterals, collateral remodeling, or angiogenesis to the impairment in the BALB/c strain. Nevertheless, if these two strains differ significantly in collateral density or collateral remodeling, this would provide a powerful model to investigate genetic determinants of normal collateral development, as well as collateral growth in ischemia. Therefore, the purpose of this study was to characterize in detail the arteriogenic and angiogenic response to ischemia in these strains and to investigate potential mechanisms. In addition to substantial differences in collateral remodeling and angiogenesis, we report that these strains have a profound difference in collateral density in multiple tissues. These differences result in large differences in flow impairment after arterial occlusion. Significant variation exists between the strains in arterial tree structure and VEGF-A expression, suggesting that this morphogen/cytokine may be important in specifying collateral density. In addition, we analyzed a genome-wide microarray expression database generated for a
recombinant inbred set of mice derived from a cross of BALB/c and C57BL/6 mice. Multiple quantitative trait loci were identified on MMU17, near the *Vegfa* locus, that associate with differences in expression in the BALB/c vs. C57BL/6 genomes of VEGF-A and several genes that drive its expression or are driven by downstream VEGF-A signaling.

**MATERIALS AND METHODS**

**Hindlimb ischemia model.** We purchased 11- to 12-wk-old male mice (C57BL/6 and BALB/c) from Charles River Laboratory, (Wilmington, MA), Taconic Farms (Germantown, NY), and The Jackson Laboratory (Bar Harbor, ME) to minimize any potential effect on results of vendor-specific genetic differences between the strains. No differences were detected among mice of the same strain from the three vendors. The femoral artery was ligated proximal to the genu artery and distal to the origin of both the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) as described previously (5). All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

**Laser Doppler perfusion imaging.** C57BL/6 (26.9 ± 0.3 g, n = 10) and BALB/c (26.0 ± 0.4 g, n = 10) were used. Hindlimb perfusion was assessed noninvasively in the ventral adductor thigh region and plantar foot before, immediately after, and 3, 7, 14 and 21 days after femoral ligation by scanning laser-Doppler [model LD12-IR modified for high resolution (5); Moor Instruments, Wilmington, DE]. The hindquarters were placed in a heated positioning mold during scanning to minimize variation in positioning during scanning. Doppler perfusion of the ventral adductor and plantar foot was assessed within anatomically defined regions of interest (ROIs; High Resolution, version 5.0, Moor Instruments). The ROI for the adductor was drawn to obtain perfusion within the collateral zone [CZ, collateral-containing region between the lateral caudal femoral artery (LCFA) and saphenous artery (SA)] (Fig. 1A). The ROI for the plantar foot consisted of the hind paw margins (Fig. 1B). Procedures for animal preparation, scanning, and ROI construction have been de-

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Fig. 1. BALB/c have lower perfusion immediately after femoral artery ligation and impaired recovery compared with C57BL/6. Laser Doppler scanning in adductor thigh (A) and plantar foot (B). A: perfusion in anterior and posterior gracilis muscle collaterals becomes evident immediately after ligation. Reduced perfusion in saphenous artery distribution and plantar foot (B) immediately after ligation is dependent on conductance of pre-existing collaterals. Improvement of perfusion with time is dependent on collateral growth and angiogenesis. Quantification of perfusion within anatomically defined region of interest (outlined in 1st panels of A and B) in adductor (C) and foot (D). Adductor perfusion was sustained in C57BL/6 immediately after ligation but dropped in BALB/c and thereafter increased more slowly than C57BL/6. Plantar perfusion dropped more in BALB/c immediately after ligation and increased less with time. Two-way ANOVA followed by Dunn-Bonferroni corrected t-test; **, *** P < 0.05, <0.01, <0.001 vs. C57BL/6. Values are means ± SE for n number of mice per strain in Fig. 1 (n = 10) and other figures and in text, unless indicated otherwise. Data given here, in other figures, and in text were obtained by observers blinded to strain.
scribed in detail previously (5). All ROIs were drawn by an investigator blinded to animal genotype (and for other analyses, below, where possible). The average velocity in a ROI was normalized to the area of the ROI. This was done because ROI construction differed for the same animal on different imaging days as a result of unavoidable variation in animal positioning during scanning.

**Histological analysis.** Tissue preparation, histological procedures, and measurements have been detailed previously (5). Lumen diameters of collaterals in the anterior and posterior gracilis muscles were measured in the collateral midzone, angiogenesis was assessed in the adductor (semimembranosus and caudal femoralis) and calf (gastrocnemius), and α-smooth muscle actin-positive (α-SMA⁺) vessel density was determined in the CZ of the semimembranosus muscle in the adductor. Leukocyte and T-helper cell densities were determined around pre-existing and remodeled collaterals in the gracilis muscles. Positive cells were labeled with a rat anti-mouse CD45 antibody (pan-leukocyte marker, Ly-1; 1:200; BD Biosciences, Pharmingen, Boston, MA) or rat anti-mouse CD4 antibody (T-helper, GK1.5; sc-13573; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated mouse anti-α-SMA antibody (1:100, Vectastain ABC), and diaminobenzidine. Sections were lightly counterstained with Mayer’s hematoxylin solution. Cells with a blue nucleus surrounded by a brown reaction product on their surface were counted. Counts were normalized to area (~260 × 340 μm) within the field of view.

**In vivo assessment of ischemia and function in the hindlimb.** At 3, 7, 14, and 21 days after femoral artery ligation, animals were individually inspected for foot appearance score [index of ischemia: 0, normal; 1–5, cyanosis or loss of nail(s), where the score is dependent on the number of nails affected; 6–10, partial or complete atrophy of digit(s), where the score reflects number of digits affected; 11, partial atrophy of forefoot]. Hindlimb use scores [index of muscle function] were obtained (53): 0, normal; 1, no foot flexion; 2, no plantar flexion; 3, dragging foot.

**Postmortem angiography and analysis of tree structure in skeletal muscle.** In a separate set of experiments, mice were perfusion-cleared, heparinized, maximally dilated (10 mg/ml adenosine and 4 mg/ml papaverine, freshly prepared), fixed with 4% paraformaldehyde (PFA) at 100 mmHg, and placed in a positioning mold, as described in detail previously (4). The vasculature was injected with barium sulfate to provide X-ray arteriograms, as described previously. Films were digitized, and a Rentrop-like line analysis was performed by counting detectable arteries intersecting: 1) a vertical line beginning at the midpoint between the proximal and distal ligations of the femoral artery and extending through the CZ to the posterior edge of the thigh (index of pre-existing collateral number in the acutely ligated limb and remodeled collaterals in the day 7 and 21 ligated limbs), 2) a vertical line beginning at the medial epicondyle of the femur and extending to the edge of the calf (index of conductance of pre-existing collateral network), 3) a vertical line extending from the lesser trochanter of the femur to the posterior edge of the thigh of the acutely ligated leg (index of second-order arteries branching laterally from the profundus artery), and 4) a horizontal line connecting the iliac crest to the lateral edge of the abdominal wall (estimate of second-order arteries branching caudally from the superior epigastric artery). In ROI in the thigh and calf, pixel intensity of barium in each artery (index of amount of arterial filling) was determined in an anatomically defined area (area intensity) with ImageJ (National Institutes of Health). The area-intensity values from the chronically ligated limb were normalized to the acutely ligated limb.

**Determination of collateral number and tree structure in the pial and intestinal circulations.** A separate set of animals was cannulated via the descending abdominal aorta, heparinized, perfusion-cleared, and maximally dilated (10 mg/ml adenosine and 4 mg/ml papaverine). Animals, maintained at 37°C, were then cannulated in the ascending abdominal aorta and perfused with a mixture of 45% (wt/vol) barium sulfate, 12% (wt/vol) gelatin, and 1 mg/ml fluorescein isothiocyanate dextran as above (FITC-dextran-180, T-1287; Sigma-Aldrich). This formulation of FITC-dextran-barium gel results in only a dilute amount of FITC-dextran crossing the capillaries, giving a faint image of venules (Figs. 4, A and C, and 5D). Animals were then chilled on ice. The vasculature was imaged with a Leica MZ16FA microscope (Leica Microsystems, Bannockburn, IL). The number of collaterals connecting the middle cerebral and anterior cerebral arterial (ACA) circulations of both hemispheres was counted. Pial collateral diameter was determined from images focused in the plane of the collaterals (ImageJ). The relative areas supplied by the anterior, middle, and posterior artery branches were determined from images of the dorsally visible cortical area overlain by each cerebral artery tree (this flat field does not take into account the effect of the curvature of the brain).

In animals filled with the FITC-dextran-barium gel solution, images of the intestinal circulation were acquired after pinning the intestine out. The number of collaterals interconnecting second-order arteries in the intestine was determined.

**Functional assessment of middle cerebral artery collaterals.** In a separate group, mice were perfusion-fixed, cleared, and vasodilated as described above. The vasculature was perfusion-fixed with 2% PFA, then demagnified to provide a more thorough view of the vasculature, and the ascending thoracic aorta was ligated. The pial circulation was then exposed, and the left and right middle cerebral artery (MCA) trunks were resected after ligation. A solution of Evans blue dye (4 mg/ml) and albumin (10 mg/ml) in 100 mmol/l sodium phosphate buffer (pH 7.4) was infused into the aorta at 60 mmHg of constant pressure. The time required to fill the second-order branches of the MCA was recorded using digital video microscopy.

**Expression of TNF-α and VEGF-A.** Adductor and gastrocnemius were harvested, frozen in liquid nitrogen, and stored at ~80°C. Tissue was powdered with a tissue pulverizer cooled in liquid nitrogen and homogenized in TRIZol Reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted according to the manufacturer’s protocol. Genomic DNA was removed using a TURBO DNA-free Kit (Ambion, Austin, TX). cDNA was prepared from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen). An equal amount of total RNA was included as a no-RT control for each tissue sample.

Samples for real-time PCR included cDNAs (1:50) in triplicate and the respective no-RT control, along with a no-template control for each gene analyzed. Real-time PCR for the expression of TNF-α was performed on a Mx3000P Real-Time PCR machine (Stratagene, La Jolla, CA) using the Absolute QPCR mix (ABGene, Rochester, NY). TNF-α forward primer was 5’-CTGCTTACATGAACTGGGTG-3’ and TNF-α reverse primer was 5’-GTTCTGGCCCATAGACTGATG-3’. The probe for TNF-α detection was 5’-FAM-ATGAGAGTTCCCAAATGTGCCCTTCTC-TAMRA-3’. A GAPDH primer/probe (#4308313; Applied Biosystems, Foster City, CA) was used as an internal control for all samples. The PCR cycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s.

Real-time PCR for the expression of VEGF-A isoforms was performed on the Rotor-Gene 3000 (Corbett Life Science, Sydney Australia) using SYBR Green chemistry (SYBR Green JumpStart Taq ReadyMix, S4438, Sigma-Aldrich). Murine VEGF-A was amplified using a forward primer common to all VEGF-A isoforms (exon3F) and reverse primers specific to each isoform. Cyclophilin A was used as a control. Primers were designed using VectorNTI (Invitrogen, Carlsbad, CA). The following oligonucleotides were purchased from Invitrogen: VEGF-A common forward primer, VEGF-A-exon3F (5’-ATCTTCAAGCCGTCTGGTGTCG-3’), VEGF-A isoform specific reverse primers: VEGF-A-120R (5’-TTGGCTTGTCACATTTCGATTCTCTG-3’), VEGF-A-164R (5’-CAGGGCTCAAGTATTGTCTCTG-3’), VEGF-A-188R (5’-ATCTTCAAGCCGTCTGGTGTCG-3’), cyclophilin A forward primer (5’-CAGAGCCTACTGTGAGCT-3’), and cyclophilin A reverse primer (5’-TTGTCTTTTGAACCTTGTCTGCA-3’). The PCR cycling parameters were 95°C for 5 min, 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 25 s. Fluorescence
acquisition was performed at the end of each cycle. Specificity of the PCR was verified as a single band visualized with 4% agarose gel electrophoresis and melt curve analysis.

Fluorescence threshold was set and the cycle at which each sample crossed the threshold (Ct) was recorded in triplicate and averaged. mRNA was quantified using the $2^{-\Delta\Delta Ct}$ mathematical model, where $\Delta Ct = Ct_{\text{BALB/c}} - Ct_{\text{C57BL/6}} (\Delta Ct = Ct_{\text{Gene of Interest}} - Ct_{\text{Housekeeping gene}})$.

Expression quantitative trait loci mapping. Interval mapping was performed using WebQTL (GeneNetwork; http://genenetwork.org) (60) on an expression database for hippocampus mRNA (Hippocampus consortium CXB M430v2). This database was generated from a recombinant inbred set of 13 genetically distinct strains derived from the F2 cross of BALB/c and C57BL/6 mice (CXB, 7, 14, 61) that has been genetically mapped in fine detail (MIT markers; 52). Expression levels of genes of interest obtained by microarray analysis (Affymetrix, see Supplemental Table S1 for probe sets) of the 13 strains were mapped. Logarithm of odds (LOD) scores of $\geq 3.0$ were taken as suggesting a genetic linkage between expression level and the marker locus for the two progenitor strain genotypes. A cis-acting expression quantitative trait locus (eQTL) was defined as an LOD $\geq 3.0$ that mapped within $\sim 10$ cm of the gene’s physical locus (13). Allele effects of each eQTL were obtained to estimate the phenotype of the gene of interest for the two progenitor strains.

**RESULTS**

BALB/c mice have reduced hindlimb perfusion immediately after femoral artery ligation and impaired recovery over time. The hindlimb has a small population of collaterals in the middle of the thigh (CZ). These interconnect several distal arterioles of the profunda (deep femoral artery) and LCFA with the popliteal and SA tissues. We used noninvasive high-resolution infrared laser Doppler imaging ($\sim 2$ mm sampling depth) (5) to measure perfusion in the CZ and plantar foot of C57BL/6 and BALB/c mice after femoral artery ligation (Fig. 1). Doppler perfusion in the CZ before ligation reflects primarily capillary perfusion supplied either by the SA or LCFA, since collaterals in normal tissues exhibit little or no net flow (21). In contrast, perfusion immediately after ligation reflects both capillary flow and collateral perfusion induced by the pressure drop caused by ligation. Perfusion in the CZ dropped immediately after ligation in BALB/c but was sustained in C57BL/6 (Fig. 1C), suggesting fewer or narrower collaterals in BALB/c mice. The contribution to the perfusion signal from the collaterals is affected by their volume flow, diameter, and velocity. As collateral diameter remodels outward, collateral velocity may vary inversely, depending on volume flow. Since collateral diameter (dilated, fixed) was determined at the end of the study by histology (day 21, discussed below), only inferences can be made regarding how the factors determining adductor

1. The online version of this article contains supplemental material.
we next asked if diameter of collaterals in the CZ of the adductor, angiogenesis in gastrocnemius, TNF-α expression, and recovery of hindlimb appearance and use after femoral ligation. A: diameter (histomorphometry) of collaterals present in the “collateral midzone” of the anterior and posterior gracilis before (baseline) and 21 days after ligation. Values for both collaterals were similar, thus data were averaged. Paired t-test; ***P < 0.001 vs. C57BL/6; n = 10. Collateral diameter increased less in BALB/c. Percentage change in collateral lumen diameter from baseline to day 21 is significant between strains (C57BL/6 \( \uparrow 143 \pm 24\% \) and BALB/c \( \uparrow 69.5 \pm 13.7\% \)). Paired t-test; \( P = 0.02 \) vs. C57BL/6. B: increase in capillary density in gastrocnemius \( 7 (n = 10) \) and 21 days \( (n = 5–9) \) after ligation was less in BALB/c. Nonparametric paired t-test; **P < 0.01 vs. baseline. Paired t-test analysis of the percentage change in capillary density at day 7 from baseline was significant between strains \( (49 \pm 23\% \) for C57BL/6 vs. 12 \( \pm 10\% \) for BALB/c, \( P = 0.05 \)). At day 21, percentage increase in capillary density was not different \( (86 \pm 42\% \) for C57BL/6 vs. 29 \( \pm 32\% \) for BALB/c). C and D: density of CD45+ leukocytes and CD4+ T cells around collaterals in anterior and posterior gracilis increased similarly in both strains. Paired t-test; *, **, ***P < 0.05, <0.01, <0.001 vs. baseline; \( n = 10 \). E: fold increase in TNF-α mRNA in adductor 36 h after ligation. Unpaired t-test vs. BALB/c; \( n = 3 \) for both strains. F: foot appearance score (index of ischemia): 0, normal; 1–5, cyanosis or loss of nail(s), where the score is dependent on number of nails affected; 6–10, partial or complete atrophy of digit(s), where the score reflects number of digits affected; 11, partial atrophy of forefoot; \( n = 20 \). G: hindlimb use score (index of muscle function): 0, normal; 1, no toe flexion; 2: no plantar flexion; 3, dragging foot; \( n = 10 \). Foot appearance was worse and foot use more impaired in BALB/c. Nonparametric Mann-Whitney U-test; **, ***P < 0.01, 0.001 vs. C57BL/6. See Supplemental Figs. S1 and S2 for additional data.
impaired. Indeed, the ligated hindlimb of BALB/c had greater tissue damage and loss of use (Fig. 2, F and G). Consistent with this, skeletal muscle fiber size tended to atrophy more by day 21 in BALB/c adductor (−15 ± 8% in BALB/c vs. −5 ± 4% in C57BL/6, n = 10) and gastrocnemius (−61 ± 12% vs. −42 ± 10%, n = 6–7, P = 0.09).

**BALB/c mice have fewer collaterals in the hindlimb.** Since baseline collateral diameter and capillary density are similar in both strains, the greater drop in perfusion in the CZ and foot immediately after ligation in BALB/c suggests they have fewer collaterals. In support of this, we detected fewer vessels in the CZ of BALB/c immediately after ligation, by X-ray angiography (performed with contrast material that does not cross capillaries) followed by a Rentrop-like line analysis (“baseline” Fig. 3, A and B). Consistent with this, the number of calf arteries that filled during a constant interval of contrast infusion immediately after ligation in BALB/c (Fig. 3 C) was sevenfold lower in BALB/c (Fig. 3 B). BALB/c evidenced a smaller increase in vessel number detected in the CZ at day 7 (Fig. 3 B), which is predicted from the smaller number before ligation. Similar results for collateral number and conductance before and 7 days after ligation were obtained by an ROI angiogram analysis (Supplemental Fig. S3, A and B). Both mouse strains showed a decline in vessel number 21 days after ligation (Fig. 3 B). This has been ascribed to a renarrowing of a portion of the collateral population that is “permitted” by the persistence of several greatly enlarged collaterals which are sufficient to supply downstream tissue needs at rest (40). To confirm the angiographic data (Fig. 3 B), we counted the number of α-SMA⁺ arterioles (41–100 μm diameter range) in CZ of semimembranosus before and after ligation. Number increased less in BALB/c. Unpaired t-test; P < 0.01 vs. C57BL/6; n = 10.

Reduced pre-existing collateral density in BALB/c mice generalizes to other tissues. To determine if the lower collateral density in skeletal muscle of BALB/c extends to other tissues, we examined small intestine and brain using a fluores-
cent vascular contrast material that does not cross capillaries. BALB/c had 30% fewer collaterals than C57BL/6 in the jejunum (Fig. 4, A and B). And remarkably, BALB/c were almost devoid of pial collaterals between the MCA and ACA (Fig. 4C). A total of five MCA-ACA collaterals were found in only three of nine BALB/c studied. In contrast, collaterals were abundant in all 10 C57BL/6 examined (175 collaterals total, 15-fold more than in BALB/c) (Fig. 4D). The diameter of MCA-ACA collaterals in BALB/c was also ~30% smaller (Fig. 4F). Although not quantified, collaterals between the MCA and ACA were almost devoid of pial collaterals between the MCA and ACA (Fig. 4, BALB/c had 30% fewer collaterals than C57BL/6 in the cent vascular contrast material that does not cross capillaries. Abundant in C57BL/6 yet virtually absent in BALB/c (Fig. 4, H11011). C57BL/6 (Fig. 4, H11006), which is dependent on collaterals connecting the MCA to the vascular contrast media infused into the aorta to reach the distal material (28) that filled all vessels including capillaries. This further confirms that intracerebral collaterals are not present in BALB/c mice.

Differences in pial collateral density or diameter among mouse strains have not been reported previously. However, BALB/c have recently been shown to suffer lethal strokes within several days after MCA occlusion, whereas C57BL/6 tolerate the procedure (1, 46). The reason for this difference, which could be from vascular and/or nonvascular factors, was not examined. Therefore, we compared the time required for vascular contrast media infused into the aorta to reach the distal MCA tree after acute occlusion of the main MCA. This time, which is dependent on collaterals connecting the MCA to the ACA and PCA, was about five times longer in BALB/c than C57BL/6 (Fig. 4F). Besides differences in collateral density, the cerebral volume supplied by the MCA (i.e., the size of the MCA “tree”) also impacts the area of perfusion deficit and severity of stroke after MCA occlusion (34). Thus, we measured the cortical area overlap by each cerebral artery tree to determine if the MCA distribution was larger in BALB/c. No differences were found between strains (Fig. 4G). Taken together, these data suggest that deficiency of pial collaterals in BALB/c is central to their heightened susceptibility to lethal stroke.

Arterial tree structure differs between BALB/c and C57BL/6 mice. We noticed during angiographic analyses that the number of distal arterial branches appeared to differ between BALB/c and C57BL/6. Although it is not known if collaterals develop during embryogenesis or after birth, it is possible that their formation is linked to or induced by factors that specify branching characteristics as arterial trees form in the embryo and expand in size postnatally. Therefore, we measured the number of second-order arteries branching laterally from the right profunda and superior epigastric arteries (line 1 and 3, Fig. 5A). BALB/c have fewer branches (white bars, Fig. 5, B and C). Average diameter of the epigastric artery, measured at proximal, middle, and distal points, was similar (C57BL/6 = 177 ± 4 μm, n = 7; BALB/c = 170 ± 4 μm, n = 9). We also measured the number of second-order branches extending from the profunda 7 days after ligation (line 2, Fig. 5A). No changes occurred (Fig. 5B), which strengthens the assumption that arteries detected by angiography in the adductor CZ (Fig. 3B) represent primarily pre-existing collaterals (23). The higher resolution of fluorescent angiography permitted a more detailed analysis of branching in the pial circulation. Similar to the virtual absence of pial collaterals in BALB/c (above), intra-tree arteriole-to-arteriole anastomoses between adjacent branches within the MCA or ACA trees were abundant in C57BL/6 but absent in BALB/c (Fig. 5D). The reduced second-order branches in skeletal and pial beds, absence of anastomoses, and additional differences in the pia (i.e., branching angles and numbers of arterioles at more distal branch orders (see legend of Supplemental Fig. S3) demonstrate that the arterial tree structure in skeletal muscle and pia differs between BALB/c and C57BL/6.

VEGF-A expression is reduced in BALB/c mice. Recent studies are beginning to identify factors, specifically VEGF-A isoforms, which are important in specification of vascular branching pattern at different stages of embryogenesis (8, 26, 42, 56). It is possible that formation of collaterals and thus collateral density may also be determined during this specification and be dependent on VEGF-A. VEGF-A is paramount in capillary sprouting during ischemia (16), which was strongly reduced in BALB/c gastrocnemius. Thus, we investigated whether BALB/c are deficient in VEGF-A expression. Expression of VEGF-A isoforms was determined in the tissue samples of adductor CZ and gastrocnemius before and 36 h after femoral ligation using quantitative real-time PCR. After ligation, VEGF-A in the adductor CZ did not increase in either strain, which is in agreement with absence of ischemia (12) and angiogenesis therein (Supplemental Fig. S1B). VEGF-A in gastrocnemius before ligation trended lower in BALB/c, and VEGF-A-164 and −188 increased 3- and 18-fold more in ischemic gastrocnemius of C57BL/6 than BALB/c (Fig. 6). These data suggest that impaired VEGF-A expression may underlie the deficient angiogenesis and reduced collateral density in BALB/c mice.

Gene polymorphisms in BALB/c mice may underlie their low expression of VEGF-A. Expression levels of VEGF-A and TNF-α were markedly lower in gastrocnemius of BALB/c mice after femoral artery ligation, even though ischemia was greater than in C57BL/6. Basal expression of VEGF-A also trended lower in BALB/c. VEGF-A is capable of increasing tissue levels of TNF-α directly (35, 63) and also indirectly through leukocyte recruitment. Moreover, TNF-α is important in collateral remodeling (25). Recent evidence implicates VEGF-A in embryonic vascular branching morphogenesis (8, 26, 42, 43). These findings suggest that, compared with C57BL/6 mice, BALB/c may have reduced VEGF-A transcription (and/or altered splicing and transcript stability) to contribute to their altered branching pattern and lower collateral density, reduced TNF-α expression and collateral remodeling, and impaired angiogenesis. To begin to explore this possibility, we conducted a bioinformatic analysis to test for presence of eQTL associated with variation in mRNA abundance for VEGF-A. We also mapped eQTL for genes “upstream” of VEGF-A that regulate its expression or that reside within its downstream signaling pathway. We used a hippocampal mRNA microarray dataset that was generated from a recombinant inbred set of 13 genetically distinct strains derived from the F2 cross of C57BL/6 and BALB/c mice (CB7, 14, 61). Interval mapping (Fig. 7, Supplemental Table S1) identified a putative eQTL (LOD ≥3, unless stated otherwise) for VEGF-A expression within ~10 cM (“cis”) (13) of the Vegfa.
Fig. 4. BALB/c have fewer collaterals in intestinal and pial circulations. A: fluorescent arteriogram of small intestine (jejunum). B: fewer collaterals in BALB/c. Paired t-test; *P < 0.05 vs. C57BL/6; n = 7 C57BL/6 and n = 9 BALB/c. C: fluorescent arteriogram of pial circulation; faint vessels in background are venules containing dilute fluorescein. Branches of anterior cerebral artery (ACA) emanate from midsagittal sulcus. Branches of middle cerebral arteries (MCA) extend from lateral margins. Branches from posterior cerebral arteries emanate from transverse sulcus. Red arrowheads denote collaterals between ACA and MCA trees. Note absence of collaterals in BALB/c. Number (D) and diameter (E) of collaterals connecting distal MCA and ACA were less in BALB/c. Paired t-test; *, ***, P < 0.05, <0.001 vs. C57BL/6; C57BL/6 (n = 10) had 175 collaterals total; BALB/c (n = 9) had 5 collaterals total. F: BALB/c required ~5-fold more time to fill 2nd-order branches of MCA after MCA ligation. Paired t-test, **P < 0.01; n = 5 for both strains. G: percentages of area supplied by cerebral arteries were similar in the 2 strains.
gene on chromosome 17 (MMU17, microarray probe sets did not distinguish among VEGF-A isoforms). This putative cis-acting eQTL in the BALB/c allele is associated with reduced VEGF-A expression relative to C57BL/6. This intriguing finding suggests that BALB/c may have a polymorphism in or near Vegfa that results in the reduced expression that we detected. eQTL for HIF-1α (LOD 2.8) and AP-2, key transcription factors for VEGF-A (39), were also detected that overlap with the MMU17 VEGF-A cis-eQTL (Fig. 7, Supplemental Table S1) and associate with reduced expression in BALB/c.

An eQTL for the VEGF-A-activating morphogen, sonic hedgehog (Shh), was identified that associates with increased expression in BALB/c and overlaps the MMU17 Vegfa eQTL (Fig. 7, Supplemental Table S1). Shh induces, through downstream VEGF-A signaling, induction of Ephrin-B2, which specifies the arterial endothelial cell phenotype during embryonic vascular morphogenesis (30). Thus, this association of increased Shh expression in BALB/c with the cis-acting inhibitory eQTL at MMU17 Vegfa may represent a compensatory response. Interval mapping of Ephrin-B2 identified an eQTL that overlaps with the MMU17 Vegfa eQTL and is associated with lower expression in BALB/c, which may result from reduced expression of VEGF-A. SHP-1 and SHP-2 are cytoplasmic protein tyrosine phosphatases that negatively modulate VEGF-A signaling by dephosphorylating VEGF receptor-2 (Flk-1) (27), which is the receptor that mediates VEGF-A-induced angiogenesis (51). Recently, knockdown of SHP-1 by siRNA was shown to augment angiogenesis in ischemic hindlimb (55). We therefore investigated SHP-1 and SHP-2. An eQTL that overlaps the MMU17 Vegfa eQTL is present for SHP-2 but not SHP-1 and is associated with increased expression in BALB/c (Fig. 7, Supplemental Table S1). Thus besides reduced VEGF expression, BALB/c may also have impaired Flk-1 signaling. The angiogenic factor FGF-2, which is known to induce expression of VEGF-A (48), was also examined.

Fig. 5. Arterial tree structure of BALB/c has fewer 2nd-order arteries in skeletal muscle and lacks intratree arteriole-to-arteriole anastomoses in pial circulation. A: X-ray arteriogram of hindlimb. B: number of detectable 2nd-order arteries branching laterally from profundus artery that intersect line (A) drawn from lesser trochanter to thigh edge on acutely ligated side (baseline, line “1”) or 7 or 21 days after ligation (line “2”). C: number of detectable 2nd-order arteries branching caudally from superior epigastric artery that intersect line “3” (A) drawn from iliac crest to abdominal wall. BALB/c have fewer 2nd-order arteries (i.e., branches form profundus and superior epigastric arteries). Paired t-test; *P < 0.05 vs. C57BL/6; n = 10 C57BL/6 and n = 9 BALB/c. D: intra-tree arteriole-to-arteriole anastomoses between 3rd-order arteries extending from 2nd-order branches of MCA and ACA of pial circulation (yellow arrowheads, anastomoses; red arrowheads, collaterals shown in Fig. 4).
Interestingly, an eQTL (LOD 2.8) that overlaps the MMU17 Vegfa eQTL is present for FGF-2 and is associated with increased expression in BALB/c, which could be compensatory for low VEGF-A expression. These colocalized linkages for expression of multiple genes that either drive or are driven by VEGF-A suggest that regulation of VEGF-A expression and its downstream signaling may differ systematically between the C57BL/6 and BALB/c strains and may be caused in part by a single cis-acting QTL at the Vegfa locus itself.

**DISCUSSION**

Collateral density in normal tissues and collateral growth in ischemic disease are known to vary widely among species and human subjects (62). Genetic variation has long been suspected to contribute to these differences, but this possibility has not been examined. Identification of differences among inbred mouse strains in developmental, physiological, and pathological processes provides a first step toward investigating genetic contributions. To this end, we describe pronounced differences in BALB/c vs. C57BL/6 mice in angiogenesis, collateral density and remodeling, and VEGF-A expression. We also report preliminary yet provocative data showing evidence for a putative eQTL near the Vegfa gene that may account for the variation in mRNA abundance for VEGF-A (in cis) and for several additional genes (in trans) both upstream and downstream of VEGF-A signaling in the CXB recombinant inbred strain set.

BALB/c mice evidenced reduced angiogenesis and collateral growth in the hindlimb after femoral artery ligation. This was associated with lower expression of VEGF-A and TNF-α, cytokines important in capillary sprouting and collateral remodeling in ischemia, respectively. Compared with C57BL/6 mice, BALB/c also had significantly lower density of pre-existing collaterals in skeletal muscle and intestine and, most notably, almost complete absence of collaterals in the pial circulation. The lower density in skeletal muscle was recently reported (22), but extension to other tissues or underlying mechanisms was not examined. We found that the deficiency in collateral density in BALB/c mice resulted in greater ischemic damage in skeletal muscle after femoral artery ligation and impaired perfusion of the cortex after MCA occlusion. This latter finding provides an attractive hypothesis to explain the recent observation that BALB/c suffer lethal stroke within 3 days after MCA occlusion, whereas C57BL/6 mice are spared (1, 46). However, results in a recent study (36) appear at variance with our hypothesis that the difference in size of cerebral infarction between the strains resides in their marked difference in collateral abundance. Majid et al. (36) did, in fact, observe a threefold larger infarct volume in BALB/c than C57BL/6 mice after MCA occlusion, which is in agreement with our results and those of others (1, 46). However, they measured a smaller difference in perfusion between the strains than predicted from our results. This likely reflects the fact that perfusion was measured in a restricted region with a Doppler flow probe that overlaid a small craniotomy. In addition, this arrangement simultaneously sampled dural flow, which is not supplied by the MCA.

Substantial heterogeneity has been reported in in vitro assays of hypoxic stimulation of VEGF-A expression by monocytes from patients with coronary artery disease (47). Likewise,
significant variation in VEGF expression in human myoblasts has been linked to haplotypes in the VEGF-A gene (41). Other studies also suggest genetic variability in factors controlling VEGF expression (29, 54). Given these findings and the differences in VEGF-A expression that we observed, we conducted eQTL mapping of VEGF-A and associated genes using a database generated for the CXB recombinant inbred set of mice. Evidence for a cis-eQTL near the Vegfa locus, associated with low VEGF-A expression in BALB/c, may represent polymorphisms in the Vegfa promoter, enhancer, or other regulatory elements that result in the reduced VEGF-A expression we observed in BALB/c relative to C57BL/6. We also identified eQTL for the key VEGF-A transcription factors, HIF-1α and AP-2 (39, 49), that overlap the MMU17 cis-Vegfa eQTL. These eQTL are associated with reduced expression in BALB/c. These findings suggest possible cis- and trans-acting mechanisms that could underlie reduced VEGF-A expression in BALB/c mice (Fig. 8).

We also found eQTL overlapping with the MMU17 cis-acting Vegfa eQTL for ephrin-B2 and angioptietin-1, which are in VEGF-A’s downstream signaling pathway and are important for differentiation of endothelial cell, vascular growth, and remodeling (20, 59), that were associated with reduced expression in BALB/c, relative to C57BL/6; moreover, an MMU17-overlapping eQTL associated with increased expression of the phosphatase, SHP-2, in BALB/c was also identified, which could further impair VEGF-A downstream signaling through decreased phosphorylation of VEGF receptor 2 (Flk-1) (27). These findings suggest possible deficits in factors within VEGF-A’s downstream signaling pathways that could contribute to the differences in pre-existing vascular morphology and adaptive angiogenesis and collateral growth in ischemia exhibited by BALB/c mice (Fig. 8).

The power of eQTL mapping in the small (13-line) CXB recombinant inbred line (RIL) set is very limited. Because of this, we have only focused our analysis on the region of MMU17 where Vegfa is harbored to examine the hypothesis, analyzed by a single statistical test, that eQTL exist at this locus affecting VEGF-A expression. After finding evidence for a potential cis-eQTL associated with low VEGF-A expression in BALB/c, we then tested a second specific hypothesis, i.e., that this eQTL also associates with variation in mRNA abundance for genes involved in VEGF-A signaling. Our results showing a putative cis-eQTL for Vegfa that also has trans-acting effects on several transcripts related to VEGF-A signaling provide provocative hypotheses for future study. In particular, the possibility that one or more eQTL may underlie the low VEGF-A expression in BALB/c mice needs to be confirmed through sequencing and subsequent studies. Furthermore, studies are underway to measure collateral density in the CXB RIL, to test the hypothesis that a QTL for collateral density overlaps the MMU17 Vegfa eQTL. Additional power could be achieved by employing the recombinant inbred cross strategy for CXB as proposed by Zou et al. (64) or by using a large F2 cross between C57BL/6 and BALB/c for QTL and eQTL mapping. Future studies also need to evaluate eQTL using mRNA derived from vascular sources during formation of collaterals in normal tissues and collateral remodeling in ischemia and to confirm expression differences with real-time RT-PCR. Finally, sequencing of VEGF-A from BALB/c and comparison to the existing C57BL/6 genomic sequence may identify polymorphisms with potential effects on transcription.

The above mapping differences in BALB/c and C57BL/6 mice lend support to evidence from previous studies suggesting a role for VEGF-A signaling in collateral growth in ischemia (6, 31, 57). Moreover, they provide insights regarding genetic factors that may underlie the altered arterial branching pattern and large deficit, especially in the pial circulation, that we observed in density of pre-existing collaterals in multiple tissues of BALB/c mice. This association suggests that determinants of branching pattern or outgrowth of the arterial tree during development differ between these two strains and may underlie differences in collateral formation. Little is known about such determinants; however, VEGF-A has recently been implicated in branching pattern (8, 26, 42, 43, 56). While only a single VEGF-A isoform is required for embryonic capillary formation (32, 33, 38), evidence suggests that the final vessel architecture is specified by gradients established by expression of VEGF-A isoforms (42). We hypothesize that collaterals form during the specification of arterial tree structure and that altered VEGF-A expression/gradients in BALB/c mice result in reduced formation of collaterals. Other studies underway within our group support this possibility. We have found that pial collaterals form during the late embryonic-to-early postnatal period and reach the adult density by 3 wk after birth in C57BL/6 mice (3). By comparison, BALB/c have formed 50% fewer collaterals by birth, and their diameters are smaller. Most interestingly, these collaterals are lost by 3 wk after birth, suggesting they may fail to mature. In addition, we have direct support that VEGF-A is important in collateral formation. Mice with targeted mutations of the VEGF-A gene resulting in either increased (37) or decreased (11) VEGF-A expression...
have marked increases and decreases, respectively, in collateral density in multiple tissues (9, 15). Consistent with this finding, the mutants also have smaller vs. greater perfusion deficits, respectively, immediately after femoral artery ligation and better vs. worse recovery of perfusion and hindlimb use thereafter.

While nothing is known regarding how collaterals form in normal tissues, our above (3, 9, 15) and present findings suggest a model for perinatal collateral formation (Fig. 8). Accordingly, we postulate that insufficient VEGF-A expression in BALB/c compared with C57BL/6 mice, arising from the above gene polymorphisms in HIF-1α and AP-2, together with reduced downstream induction by VEGF-A of Ephrin-B2 and Ang-1, contributes to reduced formation of arteriole-to-arteriole cross-connections (collaterals) between adjacent arterioles. Interestingly, we also identified a single eQTL for Ang-2 (MMU7, 132–140 Mb, LOD 4.4, data not shown) associated with increased expression in BALB/c. It is well known that newly formed vessels fail to mature and are pruned away in the context of low VEGF-A, low Ang-1, and high Ang-2 activity (20). This is consistent with our observation that the reduced number of collaterals that do form in the BALB/c embryonic pial circulation failure to mature and are pruned away by P21 (3). It is important to note that hemodynamic factors and other genes are undoubtedly also involved in collateral formation and maturation.

The striking difference in collateral density between BALB/c and C57BL/6 mice, together with the availability of the CXB RIL strain set, underscores their potential as a model to uncover the signaling mechanisms that direct collateral development in normal tissues and collateral remodeling in ischemia. Determination of these mechanisms will likely provide insight into the genetic mechanisms underlying the marked variability in collateral density and remodeling among species and humans. Such information could lead to novel strategies to induce formation of new collaterals and enhance remodeling of existing collaterals in patients with ischemic heart disease, stroke, and peripheral vascular disease.

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