Growth and regression of vasculature in healthy and diabetic mice after hindlimb ischemia

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Landázuri N, Joseph G, Guldberg RE, Taylor WR. Growth and regression of vasculature in healthy and diabetic mice after hindlimb ischemia. Am J Physiol Regul Integr Comp Physiol 303: R48–R56, 2012.—The formation of vascular networks during embryogenesis and early stages of development encompasses complex and tightly regulated growth of blood vessels, followed by maturation of some vessels, and spatially controlled disconnection and pruning of others. The adult vasculature, while more quiescent, is also capable of adapting to changing physiological conditions by remodeling blood vessels. Numerous studies have focused on understanding key factors that drive vessel growth in the adult in response to ischemic injury. However, little is known about the extent of vessel rarefaction and its potential contribution to the final outcome of vascular recovery. We addressed this topic by characterizing the endogenous phases of vascular repair in a mouse model of hindlimb ischemia. We showed that this process is biphasic. It encompasses an initial rapid phase of vessel growth, followed by a later phase of vessel rarefaction. In healthy mice, this process resulted in partial recovery of perfusion and completely restored the ability of mice to run voluntarily. Given that the ability to revascularize can be compromised by a cardiovascular risk factor such as diabetes, we also examined vascular repair in diabetic mice. We found that paradoxically both the initial growth and subsequent regression of collateral vessels were more pronounced in the setting of diabetes and resulted in impaired recovery of perfusion and impaired functional status. In conclusion, our findings demonstrate that the formation of functional collateral vessels in the hindlimb requires vessel growth and subsequent vessel rarefaction. In the setting of diabetes, the physiological defect was not in the initial formation of vessels but rather in the inability to sustain newly formed vessels.

perfusion; angiogenesis; arteriogenesis

THE FORMATION of the vascular network during embryogenesis encompasses complex and tightly regulated growth of blood vessels, followed by maturation of some vessels and disconnection and pruning of others (14, 24, 26, 27). Vascularization of the retina in neonate mice follows a similar pattern. Pups are born with an immature retinal vasculature. Shortly after birth, vessels rapidly grow and give rise to a dense capillary network. Then, in a temporally and spatially controlled manner, some blood vessels regress while others mature and give rise to a functional and stable vascular network (14, 36). These scenarios indicate that controlled vessel growth and rarefaction are integral parts of vascularization during early stages of development. The adult vasculature, while more quiescent, is also capable of adapting to changing physiological conditions by remodeling blood vessels (9, 11, 29–31). This ability can be compromised in the presence of cardiovascular risk factors such as diabetes (1, 20).

One of the animal models most extensively used to study adult revascularization under physiological and pathological conditions is hindlimb ischemia (HLI). In this model, blood flow through the femoral artery of one leg is interrupted by ligation and/or excision of the artery (8), leading to an endogenous response to revascularize the ischemic tissue. Researchers have been able to identify some of the factors that lead to formation of new vessels and to growth of preexisting vessels, namely 1) the mechanical environment, where increased shear stress leads to collateral growth; 2) upregulation of pro-angiogenic growth factors and cytokines; and 3) mobilization of progenitor and inflammatory cells from the bone marrow, the peripheral circulation, or even the local tissue (5, 7, 15, 16, 23, 31, 32, 35, 37, 39). However, the extent to which vessel rarefaction occurs after the initial phase of vessel growth during vascular recovery in the adult is unknown. Given that regression of newly formed or remodeled vessels can dictate the final outcome of adult vascular repair, characterizing this process is key to designing therapies for ischemic cardiovascular disease.

In this study, we propose to characterize the phases of vascular recovery after an ischemic insult in a mouse model of HLI. Also, we propose to compare how a cardiovascular risk factor may alter this process by comparing the kinetics of recovery between control and diabetic mice. In particular, we based our analysis on three main parameters: morphology of the vasculature measured by microcomputed tomography (micro-CT), recovery of blood flow quantified by LASER Doppler perfusion imaging (LDPI), and functionality of the ischemic limb by voluntary running in a mouse activity wheel system.

METHODS

Animals. Male 129 mice were purchased from Charles River Laboratories (Wilmington, MA), and male C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were fed a standard chow diet ad libitum and had free access to water. All protocols were approved by the Institutional Animal Care and Use Committee of Emory University and done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Hindlimb ischemia model. At 9 to 13 wk of age, the animals were anesthetized with intraperitoneal injections of xylazine (10 mg/kg) and ketamine (80 mg/kg). A unilateral incision was made over the left
medial thigh of the mouse. The superficial femoral artery and vein were ligated proximal to the caudally branching deep femoral artery and proximal to the branching of the tibial arteries. The portion of the artery and vein between the ligation points was excised. The skin was closed with interrupted silk sutures.

Mouse model of diabetes. Eight-week-old C57Bl6/J mice received a daily intraperitoneal injection of 60 mg/kg of streptozotocin for 5 consecutive days after overnight fasting. Glucose levels were measured 3 to 4 wk after induction of diabetes (immediately before hindlimb ischemia) and every other week thereafter. Mice with glucose levels above 300 mg/dl were considered diabetic.

Measurement of morphological parameters by micro-CT. After the animals were euthanized, the thoracic cavity was opened and the inferior vena cava was severed. The vasculature was flushed with 0.9% normal saline containing 4 mg/ml papaverine at a pressure of 100 mmHg via a needle inserted into the left ventricle. The excess papaverine was flushed with saline and the vasculature fixed with 10% neutral buffered Formalin. Formalin was flushed from the vessels using saline. The vasculature was injected with undiluted Microfil (MV-122, Flow Tech; Carver, MA) as previously described (12). Samples were stored at 4°C overnight for contrast agent polymerization. Mouse legs (not including the feet) were dissected from the specimens and soaked in 10% neutral buffered Formalin to ensure complete tissue fixation. Tissues were subsequently treated for 48 h in Cal Ex II (Fisher Scientific; Pittsburgh, PA) and then stored in 10% neutral buffered Formalin. The hindlimb vasculature was imaged with a micro-CT imaging system (CT 40, Scanco Medical; Bassersdorf, Switzerland). The scanner was set to a voltage of 55 kVp and a current of 145 μA. Resolution was set to medium, and the limbs were scanned at a 30-μm voxel size. A threshold of 110 was chosen based on visual interpretation of thresholded two-dimensional tomograms. Surgery and control legs were evaluated individually to quantify the three-dimensional histomorphometric values vessel volume, connectivity, number, thickness distribution, and spacing. The results were reported as ratios of surgery to control leg for each animal to account for potential changes in vessel volume after injection of microfil.

Measurements of perfusion by LASER doppler perfusion imaging. LDPI with a LASER of 810 nm (MoorLDI, Moor Instruments, Wilmington, DE) was used to evaluate the perfusion in the ischemic and nonischemic legs after surgery. The distance between the source of the laser and the leg was 21 cm, the scan speed was 4 ms/pixel, and the resolution was 256 × 256 pixels. Mean perfusion was estimated in the foot and in the ischemic portion of the leg, extending from the thigh to the ankle. The nonischemic legs and feet were used as controls. The results for mean perfusion were reported as ratios of the surgery to nonsurgery leg or foot for each animal.

Voluntary running wheel activity system. Immediately after HLI, animals were individually housed in cages with a single activity wheel (Lafayette Instrument, Lafayette, IN). The animals had free access to an activity wheel. The distance run in the wheels was recorded daily.

Quantification of microvasculature by lectin staining. Eight and twenty-three days after HLI, paraffin sections of hindlimb tissue were stained with FITC fluorescein-labeled GSL I-isolectin B4 (Vector Laboratories, Burlingame, CA). Sections were imaged by fluorescent microscopy using a ×20 objective. The number of microvessels from five animals per group and four fields of view per hindlimb was counted. The results for density of microvessels were reported as ratios of the surgery to nonsurgery limb.

Data analysis. The data were summarized as means ± SE. Statistical analysis was performed using Prism software Version 4.01. A t-test was used to compare the means between two groups. A two-way analysis of variance (ANOVA) was used to compare measurements of various parameters over time. The Bonferroni comparison test was used to conduct pairwise comparisons between means. To compare the microvasculature density, a general linear model using day, diabetic versus nondiabetic and surgery leg versus nonsurgery leg as factor variables was used. The least square means was calculated and
Fig. 2. Quantification of morphological parameters based on microCT scans of the surgery leg (■) and the nonsurgery leg (●) in 129 mice. For each parameter, a ratio between the surgery and the nonsurgery leg was calculated (△). The dotted lines indicate a ratio of 1. The quantified parameters were the following: vascular volume normalized for tissue volume (A), density (B), connectivity (C), spacing (D). Each point represents the mean ± SE, n = 6 to 8.
the significance of the interactions was assessed. Differences at $P < 0.5$ were considered statistically significant.

RESULTS

To obtain a morphological assessment of the time course of collateral development after induction of HLI, at various days after surgery we prepared the legs from 129 mice for scanning by microCT (Fig. 1). We assessed the following quantitative parameters provided by these scans: vascular volume normalized for tissue volume, connectivity, mean vessel density (i.e., number), spacing, and vessel thickness distribution. Vascular volume, density, and connectivity were lower in the surgery leg than in the nonsurgery leg immediately after surgery. At day 7, their values peaked and exceeded those in the nonsurgery leg. The ratios of surgery to control leg reached 1.25 ± 0.08 for volume, 1.13 ± 0.02 for density, and 2 ± 0.34 for connectivity, and then slowly declined over several weeks. Forty days post-HLI, the ratios for density and volume were 1, whereas the ratio for connectivity was 1.5 (Fig. 2, A–C). Consistent with these findings, we observed that vascular spacing decreased in the surgery leg over 7 days postsurgery and then increased slowly (Fig. 2D). While analyzing the distribution of vessels of various thicknesses as a function of time, we observed that vessels of small diameter substantially developed only in the surgery leg (Fig. 3, A–D). The volume occupied by vessels of diameter smaller than 330 μm was up to 1.5 times higher in the surgery leg compared with the control leg. In contrast, the volume occupied by vessels of diameter larger than 420 μm remained lower in the surgery at all time points (Fig. 3, C and D).

Taken together, our results showed that vascular volume, density, and connectivity in the surgery leg exceeded or were equal to these same parameters in the nonsurgery leg. Interestingly, we noticed that interconnected collaterals extensively developed for 1 wk after surgery but were gradually reduced at later time points.

To assess the extent to which each of these two phases of collateral remodeling, namely, growth and rarefaction, contributed to recovery of perfusion, we scanned both limbs by LDPI. We first focused on the area we analyzed by micro-CT; that is, the region extending from the ankle to the thigh. We observed that perfusion increased until day 7 post-HLI and then reached a plateau at a ratio between 0.5 and 0.6 (Fig. 4, A and C). This indicated that perfusion to the surgery leg increased during the phase of vascular growth but remained unchanged during the phase of vascular pruning.

While most studies quantify the number of vessels in the areas we used for micro-CT analysis (thigh or calf), LDPI is usually targeted to the feet. When measuring the perfusion ratio between the feet, we found that it increased linearly with time for 3 wk and then reached a plateau at a ratio of 0.5 on day 21 (Fig. 4, B and C), thus demonstrating that distal perfusion continued to increase during growth and pruning of blood vessels in the proximal area.

Finally, to evaluate the extent to which vascular remodeling and recovery of perfusion related to functional recovery of

![Fig. 3. Thickness distribution of developing blood vessels after hindlimb ischemia (HLI) surgery in 129 mice. Based on micro-CT scans, the thickness distribution of blood vessels was assessed at various days after HLI in the surgery leg (A) and the control leg (B). The vascular volume including only vessels of a given diameter (C, D) was calculated as a ratio of surgery leg to the nonsurgery leg. Each point represents the mean + SE, n = 6 to 8.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00002.2012)
limb usage, we placed the animals in a voluntary running wheel activity system the day of HLI and recorded the distance mice run on a daily basis. We found that within 4 wk after HLI, mice could run the same distance as control mice (Fig. 4D).

To determine whether these findings could be generalized to mouse strains other than 129, we conducted a similar experiment using C57Bl6/J mice. In addition, to assess how a cardiovascular risk factor would alter vessel remodeling and recovery of perfusion, we also used diabetic mice on a C57Bl6/J background. Our micro-CT results indicated that the biphasic pattern of vascular remodeling in control and diabetic mice was similar to that observed in 129 mice. The volume of blood vessels in the surgery leg rapidly increased during the first week after HLI, reached values higher than those in the control leg, and then decreased (Fig. 5A). Interestingly, we observed that diabetic mice demonstrated a robust increase in parameters vascularity after HLI. We found that the vascular volume of size-delineated vessels and microvessel density were higher at day 8 in the diabetic mice (Fig. 5, B–F). However, we also observed a striking reduction in vascular volume and microvessel density in diabetic mice at 23 days post-HLI, suggesting that in the diabetic mice, the newly formed vasculature was not sustained (Fig. 5, A–F). Taken together, these data indicated that small vessels in diabetics paradoxically underwent more pronounced growth at early time points after induction of ischemia and then subsequently did not sustain this higher level of vascularization with resultant pruning of the neovasculature.

To investigate how these differences in vascular remodeling affected recovery of perfusion, we scanned the feet of the mice at various times after HLI. The perfusion ratio was equivalent between control and diabetic mice up to 14 days post-HLI, but control mice demonstrated significantly improved recovery over the diabetic mice beginning at day 23 post-HLI (Fig. 6, A and B). These findings suggested that excessive pruning of vessels hindered distal recovery of perfusion.

Finally, we assessed the distance that mice run on a daily basis after HLI. The distance that control mice run increased over time and reached a plateau level 9 to 11 days post-HLI, when the distance run by mice with and without HLI was similar (Fig. 6C). In contrast, the ability of diabetic mice to run did not improve over the same period of time (Fig. 6D).

**DISCUSSION**

In the present study, we showed that the endogenous process of vascular repair after an ischemic insult in the adult mouse is biphasic. It encompasses an initial phase of vessel growth followed by a later phase of vessel rarefaction. In healthy 129 and C57Bl6/J mice, this process resulted in partial recovery of perfusion and completely restored the ability of mice to run voluntarily. In diabetic mice, these two phases were more pronounced than in healthy mice, with a paradoxical exaggerated increase in vascularity at early time points followed by an enhanced vascular rarefaction at later time points. These discrepant responses resulted in impaired recovery of perfusion,
as measured by LDPI, and impaired functionality of the ischemic limb, as measured by running distances.

The initial phase of rapid vessel growth started shortly after induction of HLI. Interconnected blood vessels developed by day 7. Total vascular volume, mean density, and mean connectivity in the surgery leg peaked and exceeded the values from the control leg. This phase appears to temporally coincide with the upregulation of angiogenic and arteriogenic key factors reported in the literature and with the mobilization of cells to the site of injury (8, 19, 28, 33).

During the second phase, which lasted several weeks, vessels gradually regressed. By day 40 postsurgery, vascular volume and density in the surgery leg had decreased and were similar in both legs. Connectivity still remained higher in the surgery limb but maintained a decreasing trend. This general process recapitulates other scenarios of vascular development, including early postnatal adaptations to the environment and the control of arterial branching in embryogenesis. Postnatal vessel maturation and rarefaction are tightly regulated by signaling molecules that participate in both arteriogenesis and

Fig. 5. Quantification of morphological parameters of the vasculature in control and diabetic C57Bl6J mice. At various days after HLI, the total vascular volume (normalized for tissue volume) was measured based on micro-CT scans, and the density of microvessels (<30 μm) was quantified by histology. The vascular volume ratio between the surgery and the nonsurgery leg was calculated based on micro-CT parameters (A). The vascular volume including only vessels of a given diameter was calculated as a ratio of surgery leg to the nonsurgery leg (B–E). The dotted lines indicate a ratio of 1. *Statistically significant differences between control and diabetic mice (P < 0.05), n = 5–8. The density of microvessels was calculated as the ratio of the surgery leg to the nonsurgery leg (F). *Statistically significant differences between surgery and nonsurgery legs (P < 0.05). Each point represents the means ± SE.
angiogenesis. For example, upregulation of vascular endothelial growth factor sustains immature vessels and facilitates vessel maturation, whereas downregulation of vascular endothelial growth factor results in the selective pruning of immature vessels that have not yet acquired a periendothelial coating (3, 4). This type of signaling to prune vessels responds to the surrounding environment, in particular, to the metabolic requirements of the tissue. For example, infants treated with hyperoxia can suffer from retinopathy of prematurity, because their retinal vessels regress to match lower oxygen requirements (2, 4, 22). Another factor that determines the fate of vessels is hemodynamics. In fact, high shear stress prevents apoptosis of endothelial cells, thus inhibiting regression of highly perfused vessels (10). Also, during embryogenesis, the flow velocity difference between a branch and the main artery, as well as the branching angle, appear to regulate the rate of disconnection of side branches. This disconnection ultimately permits progression of blood flow into more distal areas (27).

In our model, growth of vessels during the first week postsurgery coincided with an increase in perfusion. Perfusion continued to increase for an additional week and then remained unchanged while vessels regressed. This suggests that pruning was selective for inefficient or nonfunctional vessels. As recognized by others, rarefaction of the unnecessary vasculature can decrease the tortuosity of the vessels, as well as the resistance to blood flow, thereby enhancing perfusion (32, 34). This biphasic behavior affected vessels of all sizes, yet the volume occupied by vessels of diameter smaller than 330 μm remained higher in the surgery leg than in the control leg, while the volume of vessels larger than 420 μm remained higher in the control leg (Fig. 3). Most likely, the transient thickening of large, mature vessels increased the blood flow to the small branching collaterals, thus temporarily stimulating them to remodel and grow. Of importance, we observed this biphasic behavior in two strains of mice that have been shown to differ in their response to ischemia, namely 129 and C57Bl6/J (18), suggesting that growth and regression of the vasculature could be generalized to various genetic backgrounds.

In healthy mice, this endogenous vascular repair process resulted in only 50% to 60% recovery in perfusion. The persistent alteration of vascular morphology, with increased distribution of vascular volume to smaller vessels, may help explain the continued perfusion deficit. In addition, we should note that femoral artery occlusion can lead to partial atrophy of the affected lower leg (6), thereby decreasing its blood flow requirements compared with the control leg. Interestingly, mice completely recovered their ability to run voluntarily, 8,000 to 10,000 meters per day, when recovery of perfusion was around 25% for C57Bl6/J mice and 55% for 129 mice. This indicates that incomplete perfusion fulfills the metabolic requirements for intense physical activity.
In the setting of diabetes, we found that the ischemic limb only recovered 25% of perfusion and that the animals could only run 13% the distance that mice without HLI run. Vascular remodeling in these mice also encompassed growth and rarefaction of vessels. However, both phases were more pronounced in diabetics compared with nondiabetic mice. Previous research reports indicate that diabetics have a reduced ability to grow collaterals (1, 20). Our results raise the possibility that diabetics are actually capable of growing vessels, yet the extent of vessel rarefaction limits sustainable vessel growth. We were able to identify this paradoxical phenomenon, which to our knowledge has not been reported in the literature, through robust quantitative analysis of three-dimensional morphological parameters provided by microCT. As we obtained micro-CT images with a 30-μm voxel resolution, our analysis included collaterals and large blood vessels, but excluded capillaries, thereby providing a different perspective for vascular remodeling than that obtained with conventional histological methods. To compare these findings with those obtained by histology, we quantified the microvascular density in paraffin sections on days 8 and 23 post-HLI. Interestingly, we observed rarefaction at the level of the microvasculature in the setting of diabetes but not in nondiabetic mice.

Perspectives and Significance

The findings from this study emphasize the contribution of vessel rarefaction to vascular repair in the adult. Our results also point to the prospect that poor revascularization in the presence of a cardiovascular risk factor, such as diabetes, can be in part due to excessive vessel regression. Most current preclinical therapies for vascular repair consist of delivering a therapeutic agent shortly after HLI and have had limited success (13, 15, 21, 25, 32–34, 38). Also, some of these therapies have led to an initial acceleration of revascularization but not to a long-term benefit (17). Thus our results raise the possibility that the disparity observed between promising preclinical angiogenic therapies and clinical outcomes in humans may be related to the point in time when results are evaluated. Moreover, our findings highlight the importance of designing therapies to sustain arteriogenesis, as opposed to angiogenesis alone.

Based on our observations, we propose that therapies should not only focus on the early stage of vessel growth but also on the second stage of vessel rarefaction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.L. and G.J. performed experiments; N.L. and W.R.T. edited and revised manuscript; N.L., R.E.G., and W.R.T. interpreted results of experiments; N.L. prepared figures; N.L. drafted manuscript; N.L., R.E.G., and W.R.T. conceived and designed of research; N.L. and G.J. performed experiments; N.L. analyzed data; N.L. and J.M. contributed analytic tools. To compare these findings with those obtained by histology, we quantified the microvascular density in paraffin sections on days 8 and 23 post-HLI. Interestingly, we observed rarefaction at the level of the microvasculature in the setting of diabetes but not in nondiabetic mice.

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