Quantitative microcomputed tomography analysis of collateral vessel development after ischemic injury

Craig L. Duvall,1 W. Robert Taylor,2,3 Daiana Weiss,2 and Robert E. Guldberg4

1Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta 30332; 2Division of Cardiology, Department of Medicine, Emory University, Atlanta 30322; 3Veterans Affairs Medical Center, Atlanta 30303; and 4Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332

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Duvall, Craig L., W. Robert Taylor, Daiana Weiss, and Robert E. Guldberg. Quantitative microcomputed tomography analysis of collateral vessel development after ischemic injury. Am J Physiol Heart Circ Physiol 287: H302–H310, 2004. First published March 11, 2004; 10.1152/ajpheart.00928.2003.—Transgenic mouse models are increasingly being used to investigate the functions of specific growth factors or matrix proteins to design therapeutic strategies for controlling blood vessel growth. However, the available methodologies for evaluating angiogenesis and arteriogenesis in these models are limited by animal size, user subjectivity, the power to visualize the three-dimensional vessel networks, or the capability to employ a vigorous quantitative analysis. In this study, we employed contrast-enhanced microcomputed tomography imaging to assess collateral development after induction of hindlimb ischemia in the mouse. The morphological parameters vessel volume, connectivity, number, thickness, thickness distribution, separation, and degree of anisotropy were evaluated in control and surgery limbs 0, 3, and 14 days postsurgery. Results indicate that the vascular volume of the surgically manipulated limb was reconstituted as early as 3 days after femoral artery excision through development of a series of highly connected, small caliber, closely spaced, and isotropically oriented collateral vessels. Parametric analyses were completed to assess the sensitivity of the calculated morphological parameters to variations in image binarization threshold and voxel size. Images taken at the 36-μm voxel size were found to be optimal for evaluating collateral vessel formation, whereas 8- to 16-μm voxel sizes were needed to resolve smaller vascular structures. This study demonstrates the utility of microcomputed tomography as a robust method for quantitative, three-dimensional analysis of blood vessel networks. Whereas these initial efforts focused on the mouse hindlimb ischemia model, the developed techniques may be applied to a variety of model systems to investigate mechanisms of angiogenesis and arteriogenesis.

angiogenesis; arteriogenesis; hindlimb ischemia; mouse

COLLATERAL VESSEL FORMATION can functionally compensate for obstructive vascular lesions in patients with atherosclerosis. Animal models of peripheral limb ischemia play a vital role in preclinical research efforts that test the efficacy of administering angiogenic factors and other therapeutic agents to stimulate formation of collaterals to compensate for blocked arteries. One such model, the mouse hindlimb ischemia model, has been used extensively in efforts to define the mechanisms involved in postnatal blood vessel formation (9). Whereas the utility of this model is well established, the available methodologies for quantification of collateral vessel development in this setting are not ideal.

In the context of hindlimb ischemia, three different mechanisms may contribute to reconstitution of limb perfusion. Angiogenesis involves sprouting or intussusception of new capillaries from preexisting vessels and is triggered by tissue ischemia. Arteriogenesis occurs when preexisting arterioles dilate and remodel through endothelial and smooth muscle cell expansion to meet increased physiological demands (4, 40). It is generally accepted that this process, which is mediated at least in part by monocytes and macrophages, is triggered by the increased shear stress that occurs in small, interarterial anastomoses after occlusion of a parent artery (22). Finally, there is evidence that vasculogenesis or de novo blood vessel formation by bone marrow-derived endothelial progenitor cells may play a role in postnatal vessel growth, although this remains controversial (5, 48). Collateral vessel growth, primarily via an arteriogenic pathway, is widely believed to be the most efficient means for reconstituting perfusion after arterial occlusion (40).

Investigators have utilized a variety of techniques to evaluate vascular structures in animal models. Histology is commonly employed to analyze capillary or arteriole density, but it is relatively subjective, not truly quantitative, two-dimensional (2-D), and not necessarily representative of vascularity throughout the entire sample. Laser Doppler perfusion imaging has been used to analyze functional blood flow in hindlimbs because it offers semiquantitative data and a measure of functionality (1, 2, 9, 40, 41). However, this technique does not provide anatomic information and is limited by the fact that only the most superficial, cutaneous blood flow is measured. Another popular technique, X-ray microangiography, provides high-resolution 2-D angiograms of the hindlimb vascular anatomy but lacks the ability to employ a quantitative, volumetric analysis (2, 29, 42). Injection of colored or fluorescent microspheres presents a method for longitudinal study of reconstitution of perfusion to ischemic tissues using quantitative spectroscopy or flow cytometry analyses (8, 10, 24, 35, 46). However, the required arterial catheterization or injection of microspheres into the left ventricle or atrium presents a considerable challenge in mice because of their small size. Other imaging modalities such as magnetic resonance angiography and PET serve as viable methods for analyzing vascular function, but the resolution of these methods is typically not sufficient for studies involving small animals.
Microcomputed tomography (micro-CT) has emerged as a promising technology that can overcome several of the challenges associated with evaluation of vascular networks. Recently, vascular imaging procedures have been developed for small animal models based on the use of perfused contrast agents and X-ray micro-CT (7, 12, 19, 23, 27, 33, 38, 43, 47). The goal of these studies has been to visualize 3-D vascular networks in organs (i.e., heart and kidneys) or tumors. The objective of the current study was to use micro-CT vascular imaging methods to examine the time course of collateral formation induced by femoral artery excision in the mouse hindlimb ischemia model. In addition to vessel volume, measures of vascular network morphology and anisotropy were quantified and the effects of varying the imaging parameters voxel size and binarization threshold were analyzed. The developed micro-CT-based techniques represent an innovative and quantitative approach for investigating 3-D vascular responses associated with a broad range of conditions, including vascular injury, tumorigenesis, coronary artery disease, atherosclerosis, formation, skeletal development, and fracture healing. In these settings, micro-CT imaging in combination with an appropriate contrast agent has the potential to overcome the shortcomings of other vascular imaging techniques because it can provide high resolution, volumetric, objective, and highly quantitative analyses.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were between 10 and 11 wk of age. 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. To study collateral formation, we utilized the well-characterized mouse hindlimb ischemia model (9). The animals were anesthetized with intraperitoneal injections of xylazine (10 mg/kg) and ketamine (80 mg/kg). All hair was removed from the surgical site, and the area was thoroughly cleansed with sterile water. A unilateral incision was then made over the right medial thigh of the mouse. The superficial femoral artery and vein were ligated proximal to the branching of the tibial artery, and then a second ligation was performed just proximal to the caudally branching deep femoral artery, and then a second ligation was performed just proximal to the branching of the tibial arteries. The length of the artery and vein was excised between the two ligation points, and the skin was closed with interrupted silk sutures. After awakening, animals were returned to their cages and allowed to ambulate freely. The mice were euthanized postoperatively for microcomputed tomography imaging at 0 (n = 7), 3 (n = 6), and 14 (n = 5) days or histological analysis at 14 days (n = 3).

Imaging specimen preparation. Tissues were prepared in accordance with previously described methods (7). 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 heparin sodium (100 U/ml) at a pressure of ~100 mmHg via a needle inserted into the left ventricle. The specimens were then pressure fixed with 10% neutral buffered formalin. Formalin was flushed from the vessels using heparinized saline, and the vasculature was injected with either a 15% barium sulfate, 2% gelatin suspension or a radiopaque silicone rubber compound containing lead chromate (Microfil MV-122, Flow Tech; Carver, MA). In preliminary studies using barium-gelatin mixtures as the contrast agent, 15% barium sulfate and 2% gelatin were suspended into saline. To prepare the contrast agent for perfusion, the gelatin was added a small amount at a time as the saline was heated on a stir plate. The barium was subsequently added to the solution and the suspension was stirred until any clumps had dispersed. The warmed solution was then perfused into the animal, which was subsequently placed on ice for 1 h to promote gelling of the suspension within the vasculature. Samples were then stored at 4°C in 10% neutral buffered formalin until imaging.

For studies using the silicone rubber injection agent, the manufacturer’s protocol was followed, with the exception that no diluent was used to maximize the lead chromate content. Samples were stored at 4°C overnight for contrast agent polymerization. Mouse hindlimbs were dissected from the specimens and soaked for 4 days in 10% neutral buffered formalin to ensure complete tissue fixation. Tissues were subsequently treated for 48 h in a formic acid based solution, Cal Ex II (Fisher Scientific; Pittsburgh, PA), to decalcify the bone and facilitate image thresholding of the hindlimb vasculature from the surrounding tissues. Samples were rinsed thoroughly, soaked for 1 h in water, and then stored at 4°C in 10% neutral buffered formalin until micro-CT imaging.

Micro-CT imaging. Hindlimb vasculature was imaged using a high-resolution (8–36 μm isotropic voxel size) micro-CT imaging system (μCT 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, which created a 1,024 × 1,024 pixel image matrix. Serial tomograms were reconstructed from raw data using a cone beam filtered backprojection algorithm adapted from Feldkamp et al. (11). Noise was removed using a low-pass Gaussian filter (σ = 1.2, support = 2). The tomograms were globally thresholded based on X-ray attenuation and used to render binarized 3-D images of the hindlimb vascular network segmented (i.e., partitioned) from the surrounding tissues. Histomorphometric analysis based on direct distance transform methods was subsequently applied to the 3-D images to quantify parameters of vascular network morphology and anisotropy (17, 18).

Micro-CT parametric analysis. One representative control (left) hindlimb was chosen at random to test the effects of resolution and threshold on the histomorphometric analysis of vessel volume, connectivity, number, thickness, separation, and degree of anisotropy. These parameters are standard for the analysis of trabecular bone microstructure but have not been determined previously for microvascular networks (17). In this study, these measures were taken in a volume of interest (VOI) defined as the upper hindlimb, extending from the proximal femoral artery ligation point distally to the lower ligation point.

Vessel volume was computed based on the voxel size and the number of segmented voxels in the 3-D image after application of the binarization threshold. Connectivity was determined using the method of Odgaard and Gundersen (32), which is based on the Euler characteristic and is free from assumptions on the 3-D structure of the segmented object. Here, connectivity is defined as the maximal number of branches that can be broken within a structure before it is divided into two separate parts. Vessel number, thickness, thickness distribution, and separation were calculated using a model-independent method for assessing thickness in 3-D images (18). This technique defines a local thickness at every point (voxel) in the VOI as the diameter of the largest sphere that both contains the point (not
works could only be generated at low thresholds that did not remove bone from the image. To eliminate the confounding effects bone imaging would have on the morphological analyses, we attempted to use higher thresholds to segment the vasculature only. However, this significantly diminished image quality (Fig. 1). Conversely, a closer inspection showed that due to the high radiodensity of barium sulfate, the segmented object often appeared artifically large when lower threshold values were used. Further investigation also demonstrated that the barium sulfate settled within the vessels after removing it from 4°C to place it within the micro-CT scanner, resulting in incomplete vascular filling and an inhomogeneous coefficient of attenuation within the vessels.

After gaining insight into some of the disadvantages of using barium sulfate in a gel suspension, we began to employ a silicone rubber contrast agent, which provides X-ray attenuation due to its lead chromate content. This compound polymerizes into a silicone rubber solid ~20 min after it is catalyzed by addition of a curing agent. Compared with preliminary studies using barium sulfate, the polymerized compound lowered the risk of displacing the contrast when manipulating the tissues to prepare for imaging. This compound also allowed us to maintain a more homogeneous mixture and

Histological analysis. Mice were euthanized, cleared using heparinized 0.9% normal saline, and perfusion fixed with the use of 10% neutral buffered formalin. The adductor muscles were then excised from both the occluded and control limbs of each mouse. These tissues were processed and paraffin embedded for histological analysis. Sections (5 μm thick) were cut and immunostained with a mouse monoclonal smooth muscle α-actin antibody (Sigma, St. Louis, MO). To count arterioles using light microscopy, the primary antibody was detected using an avidin-biotin-alkaline phosphatase method from a commercially available kit (Vectorstain ABC-AP, Vector Laboratories; Burlingame, CA). Sections were counterstained with hematoxylin, and the number of positively stained vessels was counted from two transverse tissue sections taken 150 μm apart at ~5 mm from the proximal insertion of the adductor. Arteriole densities were counted as the number of vessels per square millimeter and reported as ratio between the surgery and control limb for each specimen.

To qualitatively assess the ability of micro-CT to resolve vascular structures using different image resolutions, ~1-mm-thick transverse cross sections of tissue were cut from the upper hindlimb of contrast perfused specimens. These samples were then sequentially treated in 25, 50, 75, 95%, and absolute ethanol for 24 h each, followed by 24 h in methyl salicylate (Sigma) for tissue clearing. The cleared tissues were first transilluminated and photographed using a high-resolution digital camera. These same sections were then scanned using 8, 16, and 36 μm voxel sizes to qualitatively evaluate micro-CT in rendering the localized vascular structures.

Statistical analysis. All data are presented as means ± SE. Statistical analyses were performed with Minitab software. Tukey’s method was used for post hoc analyses, and ANOVA was used to model the effect of time postsurgery on all response variables. P < 0.05 was interpreted as significant in all analyses.

RESULTS

Evolution of micro-CT imaging and hindlimb ischemia model surgery techniques. In preliminary studies, perfusion of barium sulfate and gelatin suspensions was used to enhance X-ray attenuation of the vasculature. With the use of this method, high-resolution images of the hindlimb vascular networks could only be generated at low thresholds that did not

Fig. 1. Microcomputed tomography (micro-CT) angiograms demonstrating the progression of our hindlimb ischemia model specimen preparation and surgical techniques. A and B: images from preliminary studies using a 15% barium, 2% gelatin suspension as a contrast agent for vascular imaging. To remove the bone tissue from B, use of a high threshold results in loss of small blood vessel filling and an inhomogeneous coefficient of attenuation within the vessels. C: day 0 postsurgery control showed that our original surgical procedures ligated the femoral artery too distally to preclude blood flow in arteries that can supply blood to the lower leg. D: another day 0 postsurgery control demonstrates optimization of the surgical protocol by shifting the proximal ligation point. The dashed boxes represent the volume of interest considered in the control and surgery limbs for all morphological analyses.

AJP-Heart Circ Physiol • VOL 287 • JULY 2004 • www.ajpheart.org
hence X-ray attenuation within the vessels independent of the environment within the scanner during imaging.

Although the contrast agent formulation was chosen to maximize contrast of the perfused vasculature, image segmentation of vascular structures from bone remained a challenge. To circumvent this problem, specimens were placed in a decalcification solution for 48 h. After this treatment, a lower threshold could be used to segment the vasculature from the surrounding tissues, and superior quality images were produced (Fig. 1, C and D). Improved imaging techniques also led to optimization of the surgery protocol. Figure 1C illustrates that in pilot studies, the upper ligation point was too distal to prevent perfusion of major arteries branching from the femoral artery that supply the lower limb. After this observation, shifting the ligation point proximally to preclude the branching of the deep femoral artery allowed us to create a more robust model of peripheral limb ischemia (Fig. 1D).

Effects of voxel size and binarization threshold on quantification of vascular network morphological parameters. Isotropic voxel size is a main determinant of the ability to resolve small objects on micro-CT scans and thus affects image quality. While smaller voxel sizes result in longer scan times and much larger data sets, it can be seen in Fig. 2A that they also afford the opportunity to resolve smaller caliber vessels that cannot be visualized with larger voxel sizes. When evaluating the VOI defined in Fig. 1D for a representative control limb, it was evident that voxel resolution affects the different morphological parameters to varied degrees (Fig. 2B). Vascular volume decreased as voxel size increased due to the contribution of smaller vessels that could only be resolved when smaller voxel sizes were used. Vessel connectivity, the most drastically affected parameter, decreased markedly on larger voxel size scans that did not resolve the well-connected networks of small arterioles. In agreement with this conclusion, there was a discernable decrease in vessel number and an evident increase in both average vessel thickness and vessel separation at larger voxel sizes. Degree of anisotropy, one of the more mildly affected parameters, increased with larger voxel sizes, indicating that the smaller vessel networks resolved at smaller voxel sizes were more randomly oriented than the primary arteries.

Selection of an image threshold defines the cutoff grayscale (attenuation) value that partitions the image voxels into either the background or part of the segmented object. During reconstruction of image projections, a grayscale value that represents the capacity of the material within that space to attenuate X-rays is assigned to each voxel. If this coefficient is higher than the assigned threshold, the voxel remains in the 3-D image as part of the segmented object. Selection of an optimal threshold value is made especially challenging by attempting to account for partial volume effects. At lower thresholds, smaller vessels that are apparent on the 2-D tomograms will remain as part of the segmented object, but larger vessels will appear artifically large because the partially filled voxels surrounding them will remain in the image. Conversely, to gain the most accurate image of the larger vessels, a higher threshold must be used, and smaller vessels are often omitted from the 3-D rendering (Fig. 3A). Therefore, there is a tradeoff that exists, and when global thresholding is used, careful attention must be paid in choosing the best possible threshold.

The ability to define an appropriate threshold for binarizing computed tomography data sets is an important issue to resolve before completing a morphological analysis of the rendered 3-D image. Alteration of the threshold value had an observable effect on the morphometric parameters of the vascular tree, but this effect was not as substantial as that seen from varying voxel size (Fig. 3B). Vascular volume, connectivity, and vessel number all decreased with increasing threshold with, as before in the resolution test, connectivity being the most strongly affected parameter. Increasing the threshold also resulted in higher average thickness, separation, and degree of anisotropy of the vessel network. As with increasing voxel size, these trends result from removing smaller, partial voxel vessels from the segmented volume.

Comparison of control and surgically manipulated hindlimb blood vessel morphology. To obtain a more quantitative assessment of the forming collateral network, we evaluated the potential utility of several morphological parameters. As described in MATERIALS AND METHODS, vessel volume, connectivity, number, thickness, separation, and degree of anisotropy were determined for both the control and surgically manipulated limbs of all experimental animals (Fig. 4). In acute
preparations (day 0 postsurgery), vascular volume was significantly reduced in experimental limbs compared with control limbs immediately after femoral artery excision. However, as early as 3 days postsurgery, collateral vessels formed and the reconstituted vascular volume of the surgery legs was significantly higher than day 0 preparations. In fact, at both 3 and 14 days postsurgery, the vascular volume was completely recovered and was not different from control limbs. Connectivity of the vascular network in the surgery limbs was significantly less than in controls at day 0, but it increased rapidly postsurgery. While there was a trend toward increased connectivity at day 3, at day 14, a more developed collateral system was formed, resulting in a connectivity value that was significantly higher than both day 0 surgery limbs and the contralateral control limbs. As the collateral network grew and a series of small, densely packed vessels augmented blood flow to the hindlimb, the 3- and 14-day postsurgery hindlimbs had significantly increased vessel number compared with day 0 surgery limbs or control limbs. Average vessel thickness was decreased significantly after surgery and remained significantly lower at 3 and 14 days than in the intact control limbs, which contained the large, conduit femoral artery. There was a nonsignificant trend toward increased vessel separation in acutely prepared samples, but as the collateral network developed, at 3 and 14 days postsurgery, vessel separation became significantly less than in control or day 0 specimens. Finally, surgery limb degree of anisotropy was significantly reduced compared with controls at all time points, indicating that the new collateral network was more isotropic than the original intact vessel network. These data indicate that vascular volume, vessel network connectivity, vessel number, and vessel separation may be particularly useful parameters for quantifying adaptive vascular changes after surgery in the hindlimb ischemia model.

As an additional method of analysis, histograms were compiled to show the blood vessel size frequency distribution in the control and experimental limbs 0, 3, and 14 days postsurgery (Fig. 5). Control limbs represent the normal anatomic distribution of blood vessel sizes in nonmanipulated limbs. Immediately after surgery (day 0), the ischemic limb had reduced perfusion to all vessel sizes. Relative to control limbs, 3- and 14-day postsurgery legs showed an increase in small, collateral-sized vessels and a decrease in the larger, conduit vessel.
diameters (14-day data not shown). In addition, a visual representation of the blood vessel size distribution was produced by mapping a color-coded scale to the object surface (Fig. 6).

Comparison of micro-CT and histological analysis. To confirm our micro-CT data using a previously used technique, we performed arteriole density analyses after smooth muscle α-actin staining. Because it is difficult to directly validate the micro-CT calculations, we compared the ratio of the surgery and control limb vessel number as determined by micro-CT with a similar ratio for the histologically determined arteriole density values. There was excellent agreement between these two methods at 14 days postsurgery, at which point the ratio of vessel number in the surgical and control limbs was determined to be $1.26 \pm 0.07$ using micro-CT and $1.28 \pm 0.28$ using histology (Fig. 7A).

To further evaluate the accuracy of micro-CT in depicting localized vascular structures, a small, 1-mm section of tissue from a contrast-filled hindlimb was cleared and photographed yielding a vivid and precise representation of the vasculature. A qualitative comparison of this photograph and 8-, 16-, and 36-μm voxel size micro-CT images of the same specimen illustrates that micro-CT has the capability to accurately depict the smallest vessels when high-resolution scans are used, but only larger arterioles and arteries can be effectively imaged using a 36-μm voxel size (Fig. 7B).

DISCUSSION

Small animal models have been utilized to study the mechanisms of angiogenesis and collateral vessel growth (3, 9, 13, 34). Insights gained from these models have played a role in the design of therapeutic strategies for clinical trials aimed at recovering vascular function in patients with peripheral artery disease (6, 20, 21, 25, 26, 30, 36, 37). Despite advances in analysis of these models, no current method offers an optimal analysis of blood vessel microarchitecture and function. The current study demonstrates that micro-CT imaging combined with the use of perfused contrast agents and bone decalcification provides a robust methodology for evaluation of vascular networks. Specifically, micro-CT is advantageous because it provides high-resolution, quantitative, 3-D, and objective data analysis. This was evident in our validation study using immunohistochemistry, which found micro-CT to offer a highly accurate, less variable, and less time-consuming alternative for quantitative measure of collateral development. Micro-CT techniques also offer flexibility to the user in defining the VOI to be evaluated. A global analysis of the entire surgery and contralateral control hindlimb vascular anatomy can be evaluated for comparative purposes, or if desired, the collateral vessels that form in the thigh can be digitally isolated for morphological evaluation as was done in this study. Alternatively, the functional reconstitution of the vascular network can be assayed by defining the VOI as the distal portion of the hindlimb to quantify perfusion to the large conduit vessels in the lower portion of the leg. Thus contrast-enhanced micro-CT imaging provides broad applicability to model systems that...
require a vigorous and highly quantitative evaluation of vascular structure or growth.

We examined vascular anatomy in the thigh of surgically manipulated and control hindlimbs of wild-type mice 0, 3, and 14 days after femoral artery ligation and excision. The blood vessel networks were evaluated within this VOI for vessel volume, connectivity, number, thickness, thickness distribution, separation, and degree of anisotropy. These lumped assessments of the vascular morphology led to the conclusion that removal of the large, conduit femoral artery stimulates reconstitution of vascular volume through a highly connected network of closely spaced, small vessels. Alternatively, histological assessments of the vascular volume, connectivity, number, thickness, thickness distribution, separation, and degree of anisotropy. These lumped assessments of the vascular morphology led to the conclusion that removal of the large, conduit femoral artery stimulates reconstitution of vascular volume through a highly connected network of closely spaced, small vessels.

Fig. 7. A: photomicrographs of muscle cross sections from 14-day postsurgery control and experimental limb adductors identifying arterioles using a smooth muscle α-actin antibody and counterstained with hematoxylin. Quantification shows excellent agreement between the histological and micro-CT determinations of the surgery to control limb vessel number ratio. B: qualitative assessment of micro-CT vascular imaging using different voxel sizes. A representative 1-mm-thick transverse tissue section was taken from a contrast-filled hindlimb in the region shown. This tissue specimen was cleared using methyl salicylate and imaged with high-resolution digital photography and micro-CT at voxel sizes of 8, 16, and 36 μm. These images illustrate that as micro-CT voxel size is increased, small vessels are no longer clearly depicted, whereas large arterioles and arteries are still accurately resolved.

The rapid arteriogenic response detected represents a particularly interesting observation. Previous researchers (15, 16, 22, 40) have suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis, and it has also been suggested that arteriogenesis is temporally dissociated from angiogenesis. However, based on the type of information sought and sample throughput considerations, scanning at larger voxel resolutions can be more effective for some applications. For example, when using the mouse hindlimb ischemia model, we were interested in gaining a global perspective of collateral growth in the upper thigh as is commonly done in 2-D by investigators who utilize X-ray microangiography. We believe that a voxel size of 36 μm is the best choice for this application because it allows us to preferentially focus our analysis on the larger, arteriole-sized vessels, which are the best indicators of ischemic limb recovery. However, as shown here through voxel size sensitivity analysis and assessment of different micro-CT voxel sizes for resolving local vascular anatomy in cleared tissue sections, higher resolution scans are necessary to image smaller vascular structures. For example, a voxel size of 8 μm or smaller may...
be required for studies whose aim is to measure angiogenesis or small blood vessel structure within a confined area (i.e., tumor growth or fracture healing models). When smaller voxel sizes are used, the major disadvantage is the increased scan time, along with the increased computational time and complexity required for analyses of a much larger data set.

Another possible drawback of the techniques used in this study is that they do not allow for longitudinal analyses at different time points within the same animal. Because this is a postmortem analysis, the number of animals required for completion of a time course study is increased compared with methods such as laser Doppler perfusion imaging, which offers the ability to acquire multiple scans on the same living animal at different time points after surgery. The recent commercial availability of high speed in vivo micro-CT scanners that provide maintenance of animal anesthesia within the scanning system may remedy this limitation. However, before using these systems, one must consider the possible adverse biological effects that may result from repeated exposure of the animals to anesthetics and relatively high doses of X-ray radiation that would occur in a sequential study. In addition, the increased image quality afforded by decalcifying specimens will not be feasible in the in vivo systems. Other potential challenges presented by this technology are development of nontoxic contrast agents that persist in the bloodstream long enough for image acquisition and gating methods to account for heartbeat and respiratory movements during the scan.

Previous studies that involved micro-CT imaging of the vasculature predominantly have used either barium sulfate-based contrast agents (27, 31, 44) or polymerizing compounds containing lead chromate (7, 12, 19, 23, 33, 38, 43). As shown in our preliminary studies, choice of the ideal contrast agent can be a strong determinant of image quality for some applications. We found the polymerizing agent to be far superior for the hindlimb ischemia model, but it is our recommendation that both contrast agents can potentially be useful depending upon the application. In this study, the silicon polymer was shown to be ideal for imaging collateral development compared with barium sulfate because it allowed us to circumvent problems such as settling and lack of homogeneity within the vasculature, clumping during injection, and difficulty with perfusion due to high viscosity. However, the presence of bone, a similarly attenuating material, required decalcification when global thresholding was used to segment the vasculature from surrounding tissues. The ability to mix barium sulfate to higher concentrations allows the possibility to achieve a stronger attenuating contrast agent and, therefore, allows use of a higher binarization threshold. As a result, barium sulfate can provide sufficient image thresholding of large arterial vessels even without bone decalcification, but high viscosity may limit the ability to perfuse small arterioles and the venous circulation.

Micro-CT has several superior features compared with other techniques for blood vessel morphological evaluation, but ultimately, the ideal strategy for microvascular analysis may include multiple methods that allow for both anatomic and functional measures. The micro-CT vascular imaging methodology has the flexibility and potential to be adapted to other applications where vascular structures must be analyzed in a rigorous and quantitative fashion (e.g., fracture healing, tumor angiogenesis, coronary collateral growth, etc.). The most significant limitation of micro-CT is that it is a strictly anatomic assessment. While inferences regarding physiological function can be deduced from micro-CT images, its strength is in the quantitative anatomic information it provides. Therefore, combining micro-CT with a more powerful method for functional analysis such as laser Doppler perfusion imaging, magnetic resonance angiography, or micro-PET may provide the most comprehensive evaluation of vascular network structure and function.

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