High-Resolution Quantitative Computed Tomography Demonstrating Selective Enhancement of Medium-Size Collaterals by Placental Growth Factor-1 in the Mouse Ischemic Hindlimb

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Background—The process of arteriogenesis after occlusion of a major artery is poorly understood. We have used high-resolution microcomputed tomography (μ-CT) imaging to define the arteriogenic response in the mouse model of hindlimb ischemia and to examine the effect of placental growth factor-1 (PlGF-1) on this process.

Methods and Results—After common femoral artery ligation, μ-CT imaging demonstrated formation of collateral vessels originating near the ligation site in the upper limb and connecting to the ischemic calf muscle region. Three-dimensional μ-CT and quantitative image analysis revealed changes in the number of segments and the segmental volume of vessels, ranging from 8 to 160 μm in diameter. The medium-size vessels (48 to 160 μm) comprising 85% of the vascular volume were the major contributor (188%) to the change in vascular volume in response to ischemia. Intramuscular injections of Ad-PlGF-1 significantly increased Sca1 + cells in the circulation, α-actin–stained vessels, and perfusion of the ischemic hindlimb. These effects were predominantly associated with an increase in vascular volume contributed by the medium-size (96 to 144 μm) vessels as determined by μ-CT.

Conclusions—High-resolution μ-CT delineated the formation of medium-size collaterals representing a major vascular change that contributed to the restoration of vascular volume after ischemia. This effect is selectively potentiated by PlGF-1. Such selective enhancement of arteriogenesis by therapeutically administered PlGF-1 demonstrates a desirable biological activity for promoting the growth of functionally relevant vasculature. (Circulation. 2006;113:2445-2453.)

Key Words: angiogenesis ■ gene therapy ■ growth substances ■ ischemia

Clinical Perspective p 2453

In vascular beds with atherosclerotic ischemic disease, collateral formation plays an important role in preserving blood flow and organ function. In the heart, collaterals protect against damage resulting from multiple episodes of ischemia.1 In patients or animals models with cardiovascular risk factors, including hypercholesterolemia, hypertension, diabetes, obesity, and smoking, collateral development is impaired.2-5 The biological bases for the reduced collateral formation are not well understood. Impaired recruitment of inflammatory cells or production of angiogenic factors may attenuate arteriogenesis. For example, reduced angiogenesis in older,5 diabetic,6 and hypercholesterolemic2 animals is associated with reduced expression of vascular endothelial growth factor (VEGF). Recent studies have also shown that circulating progenitor cells that enhance angiogenesis are reduced in patients with cardiovascular risk factors.7,8
contributing to the recovery after therapy is poorly defined. Therefore, to more thoroughly understand the nature of the vasculature responsible for the restoration of blood flow in these settings, we have developed a quantitative, 3D, high-resolution microcomputed tomography (μ-CT) imaging approach. Using μ-CT in a murine hindlimb ischemia model, we now show that ligation of the common femoral artery results in changes in the number and volume of vessels ranging from 8 to 160 μm in diameter, with a 188% increase in new vascular volume attributable to medium-size (48 to 160 μm) vessels.

Placental growth factor-1 (PlGF-1) is a member of the VEGF gene family with unique biological activities. Unlike VEGF, PlGF-1 is not an endothelial cell mitogen but rather promotes smooth muscle cell and macrophage chemotaxis. In particular, PlGF has been suggested to act as an arteriogenic agent. To define the nature of the neovascularization in response to ischemia and the arteriogenic properties of PlGF-1, the new μ-CT imaging approach was used. We found that adenoaviral PlGF-1 gene delivery selectively promoted the vascular volume of medium-size vessels in the ischemic hindlimb.

Methods

Animals
Male BALB/c mice (12 weeks old) from Harlan Sprague-Dawley (Indianapolis, Ind) were used for all studies. Studies were approved by Eli Lilly’s institutional animal care and use committee.

PIGF Adenoviral Vector
Adenoviral human-PIGF-1 (Ad-PIGF-1) was generated by cloning of 2 restriction fragments of the PIGF-1 gene. The p-shuttle transfer vector was linearized with Pmel, and the resulting fragment was cotransformed with use of the pAdEasy-1 vector (Stratagene, La Jolla, Calif) into Escherichia coli BJ5183. On homologous recombination in the bacteria, plasmid was generated. After characterization, the recombinant adenoaviral vector was purified and linearized with restriction enzyme PacI. The linearized DNA was transfected into HEK293 cells to obtain viral plaques, which were then amplified and purified (Viraquest Inc, North Liberty, Iowa).

PIGF Bioassay
The activity of the PIGF-1 generated from the adenoviral construct was determined in cells expressing human VEGFR1 (flt-1) and VEGFR2 (KDR). The PIGF-induced signal was 3- to 4-fold higher in cells transfected with both flt-1 and KDR than with flt-1 alone; therefore, the former were used for routine assay. Cells were cotransfected with the luciferase reporter gene containing transcriptional activator Ets-like protein (ELK), which is activated on phosphorylation by mitogen-activated protein kinase (Stratagene, La Jolla, Calif). HEK293 cells were transfected with flt-1, KDR, and ELK-luciferase plasmids with use of the Mirus LT-1 transfection system. After 18 hours, cells were treated with Ad-PIGF-1 and incubated for another 24 hours. Luciferase activity, an indication of PIGF-1 response, was determined with a luminometer (La Roche Diagnostics, Indianapolis, Ind). PIGF-1 protein was determined by ELISA (R&D Systems, Minneapolis, Minn). Recombinant human PIGF-1 expressed in E coli was obtained from R&D Systems; mammalian cell–derived human PIGF-1 was prepared at Eli Lilly by cloning and expression in Chinese hamster ovary cells.

In Vivo Expression of PIGF
Control vector or Ad-PIGF (25 μL total) was administered into the calf muscle in 3 separate injections. After 3 days, mice were euthanized, and the calf muscles were frozen in LN₂ and stored at −80°C. Total RNA was prepared by use of the Trizol method (Invitrogen, Carlsbad, Calif). The PIGF-1 mRNA was amplified with a human PIGF-1–specific probe and primers in a real-time polymerase chain reaction (ABI 7900 sequence detection system). Levels of human PIGF-1 mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase.

Progenitor Cell Mobilization
Ad-PIGF-1 was administered to the calf muscle of nonligated mice. After 3 days, the mice were anesthetized with isoflurane, and blood samples were collected by cardiac puncture. Human granulocyte colony stimulating factor (G-CSF; R&D Systems, Minneapolis, Minn) was administered subcutaneously daily (200 μg per mouse). On day 5, blood was collected by cardiac puncture. Red blood cells were lysed by mixing 300 μL of blood with 5 mL of the lysis buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). After 10 minutes, cells were pelleted by centrifugation and resuspended in 400 μL of phosphate-buffered saline containing 0.1% bovine serum albumin. Aliquots (100 μL) of the cell suspension were then labeled with PE-antimouse Scal antibodies (BD Biosciences, San Jose, Calif). Similarly, staining with other markers (c-kit, CD45, CD11b, KDR, and flt-1) was performed with antibodies from BD Biosciences. Cells were washed, fixed with 0.5% formaldehyde, and analyzed by fluorescence-activated cell sorting. Peripheral blood cells were analyzed with use of a Hemavet 850 hematology system (Drew-Scientific Inc, Ramsy, Minn).

Hindlimb Ischemia Model
Mice were anesthetized with a mixture of 1.5% isoflurane in O₂ (1.5 L/min). On day 1, both the iliac artery (and the vein proximal to the pudendopelvicagastic vein (and the femoral artery (and vein) were ligated with a 6-0 silk suture. After ligation, mice received 3 injections (total, 25 μL) of saline, control vector, or Ad-PIGF-1 in the adductor muscle of the left hindlimb at 3 sites near the ligation. The first site was on the ventral surface, the second on the dorsal surface (femoral biceps muscle) at the same height, and the third in the adductor muscle below. Preligation and postligation laser Doppler scans were taken to ensure consistency of ligation. The hindlimbs were then scanned weekly. At the end of 4 weeks, the mice were prepared for μ-CT and immunohistochemistry.

Laser Doppler Blood Flow
Flow images of the foot were acquired with a Moor LDI infrared laser Doppler imager (Moor Instruments Ltd, Wilmington, Del). Skin temperature was maintained at 36.5°C to 37.5°C. Scanning was performed before and immediately after surgery and on days 7, 14, 21, and 28. Mice were positioned ventrally with their rear legs outstretched to allow scanning of the entire plantar surface. The data were analyzed with a Moor LDI image processor V3.09 and reported as left/right foot flow after background subtraction.

Immunohistochemistry
The hindlimbs were fixed in 3% paraformaldehyde (EMS, Fort Washington, Pa) for 24 hours and preserved in 70% ethanol. A 4-mm segment of the calf muscle medial to the left femur was embedded in paraffin. Ten serial sections (5 μm thick) were cut and mounted. Sections were treated 3 times sequentially with xylene, 100% ethanol, and 95% ethanol/water. Antigen retrieval was performed by sequential incubation at 96°C and at room temperature for 15 minutes. The sections were washed with water, TBST (10 mmol/L Tris-HCl, pH 7.4; 130 mmol/L NaCl; and 0.1% Tween-20), and 3% H₂O₂. After protein blocking, the sections were washed and then incubated with either α-actin monoclonal antibody (Dako, Carpinteria, Calif) or isoelectin B4 (Vector Labs, Burlingame, Calif). Histology images at ×200 magnification were captured with a digital camera. Images were quantified by ImagePro v4.5.1.29 analysis software (Media Cybernetics, Silver Spring, Md). Data were expressed as the mean±SEM and compared by ANOVA.
Three-Dimensional High-Resolution \(\mu\)-CT

Mice were injected with heparin (100 U) 5 minutes before euthanasia with isoflurane. The vasculature was flushed with saline containing papaverine (4 mg/L) and adenosine (1 g/L) through a needle inserted into the left ventricle. The inferior vena cava was cut to allow outflow of the perfusate. For fixation, 3.7% paraformaldehyde was perfused for 5 minutes at 100 mm Hg pressure. The descending aorta was then cannulated, and 0.7 mL of contrast agent (50% bismuth [Sigma-Aldrich, St. Louis, Mo], 5% gelatin) was injected. The vasculature was imaged by \(\mu\)-CT (GE Healthcare, Waukesha, Wis) at a detector pixel size of 8 \(\mu\)m; x-ray tube voltage, 80 kV; x-ray tube current, 80 \(\mu\)A; detector bin mode, 1x1; and 500 view angles. The field of view was 2 cm in the axial direction and 1.04 cm in the slice. Reconstruction at 8 \(\mu\)m of 500 slices or at 16 \(\mu\)m of 250 slices was performed with a cone-beam algorithm. Bone and preexisting vessels were used as anatomic references. A 2D maximum-intensity projection image was generated with the use of Microview Software (GE Healthcare). A threshold was chosen on the basis of visual 3D vessels reconstruction. Numerals indicate preexisting vessels: common femoral artery (1), superficial caudal epigastric artery (2), great saphenous artery (3), and popliteal artery (4). Asterisks in B indicate the site of ligation. A high-resolution 2D reconstruction at an 8-\(\mu\)m z-plane slice (C) and x-plane (D) depicts connecting sites and major medium-size collaterals (arrows) in the ligated leg. Bar indicates 200 \(\mu\)m in A, B, and D and 100 \(\mu\)m in C.

Figure 1. High-resolution \(\mu\)-CT analysis of hindlimb vasculature. Two-dimensional \(\mu\)-CT images of a normal hindlimb (A) and 4 weeks after femoral artery ligation (B) were obtained with an 8-\(\mu\)m scan and a 16-\(\mu\)m reconstruction. Numerals indicate preexisting vessels: common femoral artery (1), superficial caudal epigastric artery (2), great saphenous artery (3), and popliteal artery (4). Asterisks in B indicate the site of ligation. A high-resolution 2D reconstruction at an 8-\(\mu\)m z-plane slice (C) and x-plane (D) depicts connecting sites and major medium-size collaterals (arrows) in the ligated leg. Bar indicates 200 \(\mu\)m in A, B, and D and 100 \(\mu\)m in C.

Quantitative Image Analysis

Image-Pro Plus 5.0.1 (Media Cybernetics, Silver Spring, Md) was used for image analysis and quantification of the number, length, and volume of vessels. The algorithm was built within the AutoPro programming language (Visual Basic as program engine) inside Image-Pro Plus. The 3D imaging data were analyzed on each of the 2D planes that made up the 3D volume. On each plane, the “spots” of vasculature were counted by width and area. The total number of spots at each width across all planes corresponds to the vessel length at that width. The total area at each width corresponds to the vascular volume for that vessel size. The minimum width was used in the 3D analysis.

Statistical Analysis

Statistical significance was determined by ANOVA from SAS (version 8.2) and JMP (version 5.1) programs (SAS Institute, Cary, NC). With small sample sizes and unequal variances among groups, the averaged ranked data of each variable were used in the ANOVA. Logarithmic transformation was applied to vessel number and vessel volume whose data distributions were skewed. For time-dependent or segment-dependent effects on relative blood flow or vessel number and vessel volume from animals treated independently, a repeated-measures ANOVA was applied. A repeated-measures test by considering a split-plot experiment with correlated whole plots was applied when the animals were not treated independently, and the multiple segments were measured. Significance was declared when \(P<0.05\). Results are expressed as mean±SEM.

The authors have full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Arteriogenesis in the Ischemic Hindlimb

To more precisely define the nature of arteriogenesis in the murine hindlimb ischemia model, we used a \(\mu\)-CT–based method. To this end, a 3D \(\mu\)-CT image analysis system was developed to quantify the changes in the number as well as volume of the vessel segments of different sizes. Figure 1 is a high-resolution \(\mu\)-CT image of normal leg vasculature (A) and 4 weeks after femoral artery ligation (B). The major vessels of the normal leg principally contributing to the blood supply are designated. The medium and small vessels appear straight, with little or no evidence of tortuosity. In contrast, 4 weeks after ligation, several distinct vessels of \(\approx8\) to 120 \(\mu\)m, not previously visible, become highly prominent (Figure 1B and 1C, arrows). These vessels originated from the regions near or above the ligation site and connected all the way to the lower limb. The 2D \(\mu\)-CT images clearly show primary collateral development in response to ischemia. To define the extent of the vasculature, high-resolution scanning with 8-\(\mu\)m reconstruction enabled visualization of vessels in the 8- to 300-\(\mu\)m range. A single slice of the z-position shows the range of vessel size detectable by \(\mu\)-CT imaging, as depicted by the white spots in Figure 1C and the connecting sites with the large artery in the lower limb (Figure 1D).

Image analysis of the z-slices enabled determination of the number of vessels ranging from 8 to 250 \(\mu\)m in size at various points along the leg. The vessel diameter determination by \(\mu\)-CT was validated by using wires of specified diameters shown in Figure 2. The actual size, the number of \(\mu\)-CT pixels, and the estimated size after image analysis are shown in the table within Figure 2. These data confirm that the size estimation by \(\mu\)-CT and image analysis are highly accurate, providing vessel size values within 10% of the actual size.

Figure 3 shows the representative longitudinal z-position slices of the upper and lower limbs from control and ligated animals at their matched sites. Bone and preexisting vessels were
used as anatomic references to closely match the sections from the 2 groups. These representative sections selected from different sites provided visual evidence indicating that femoral artery ligation resulted in an increased number of vessels. We then used 500 serial z-position slices representing 4000 μm of tissue length to determine the total number and the volume of vessel segments within the indicated size range. Although changes in the number as well as volume of vessels ranging from 8 to 160 μm in diameter occurred in response to ischemia. For determination of the contribution by different size vessels to total vessel number and volume, we arbitrarily categorized the vessels into 2 groups of small (8 to 40 μm) and medium (48 to 160 μm) size. Vessel segments >160 μm were few, which included the preexisting large vessels, and therefore were not included in the quantification. The Table shows that even though 62% of vessel segments (20 574, versus 32 981 in the ligated leg) were small, they only contributed to 15% (165 396 versus 1 067 526 mm3) of the vascular volume. The major proportion (86%) of the vascular volume (902 131 versus 1 067 526 mm3) was attributed to the medium-size vessels. These data suggest that measurement of vessel volume is a more relevant parameter than is capillary number obtained by histology. Ligation resulted in an 86% and a 128% increase in the number of small and medium-size vessel segments, respectively. More important, the major change in vessel volume (188%) was contributed by medium-size vessels. These data show that the increase in vessel volume due to the formation of medium-size collaterals was a major contributing factor to recovery after femoral artery ligation. Because μ-CT is a contrast perfusion–based method, the ability to discriminate veins and arteries remains a limitation. Because angiogenesis and arteriogenesis are the major responses after ischemia, despite the limitation, the method has proven highly effective in discerning the vascular anatomy and quantifying and discriminating vascular changes previously not achieved.

Expression and Biological Activity of Ad-PIGF-1
Having established the μ-CT features of developing collaterals in the mouse hindlimb, we then used this model to define the biological effects of PIGF-1. Ad-PIGF-1 was first characterized for expression of biologically active PIGF-1 in vitro and in vivo. Transduction of the Ad-PIGF-1 construct into HEK293 cells produced a dose-dependent increase in PIGF-1 expression (Figure 5A). Adenoviral vector–generated PIGF-1 was biologically active, as demonstrated by activation of flt-1 receptor (Figure 5B). On the basis of the determination of PIGF-1 protein by ELISA and of its biological activity by flt-1 receptor activation, the specific activity of Ad-PIGF-1 was comparable to that of recombinant PIGF-1 produced in E coli (R&D Systems) or mammalian cells generated at Eli Lilly and Co.

Determination of in vivo PIGF-1 expression from the adenoviral construct after administration into the calf muscle showed a dose-dependent expression of PIGF-1 mRNA in muscle (Figure 5C) and the appearance of low-level PIGF-1 in plasma. A 10⁶ plaque-forming-unit (pfu) dose of Ad-PIGF-1 produced 608±288 pg/mL (n=6) of PIGF-1 in plasma.

Table 1. Vessel Size Calibration

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Figure 2. Validation of μ-CT vessel size determination. Wires of different diameters were imaged by μ-CT as in the legend to Figure 1. Quantitative image analysis of the pixel size with an 8-μm reconstruction at the same threshold as used for imaging the vessels yielded the indicated estimated diameter.

Figure 3. Three-dimensional μ-CT quantitative image analysis of the vasculature. A, Three-dimensional μ-CT isosurface image of control and 4 weeks after ligation, reconstructed at 16 μm. Arrows and lines indicate the axial slices (z-slices; 1250 cross-sections from thigh to calf region) that were used for quantitative analysis. B, Representative z-slices of upper and lower limb regions. White spots show vessels and sizes within a single section. Regions of the normal and ligated leg were matched to bone (arrow) and preexisting vessels as reference points.
plasma after 3 days of injection, which became undetectable by day 7. Similar expression of PIGF-1 mRNA was observed when Ad-PIGF was administered to the adductor muscle. Because of the convenience of quantification of local expression, we routinely used calf muscle to test the in vivo expression of PlGF-1.

To demonstrate the in vivo biological activity of PlGF-1, we studied the effect of Ad-PlGF on the mobilization of progenitor cells. We found that PlGF-1 selectively mobilized Sca1\(^+\) cells into the circulation. On day 3 after Ad-PlGF-1 administration, the number of circulating Sca1\(^+\) cells was 2.5-fold higher than in controls (Figure 5D). The cells exhibiting other markers, including c-kit, CD45, CD11b, KDR, and flt-1, were not altered. Complete blood counts showed that unlike G-CSF, PlGF-1 treatment did not affect circulating leukocytes, lymphocytes, or monocytes (Figure 6). These results demonstrated that Ad-PlGF-1 gene delivery resulted in a dose-dependent expression of biologically active PIGF-1 in vitro and in vivo.

**Improvement in Blood Flow and Arteriogenesis by Ad-PIGF-1**

To study the effect of PIGF-1 on arteriogenesis, the gene construct was injected into the adductor muscle after femoral artery ligation. 

**Figure 4.** Effect of femoral artery ligation on vessel number and volume. For 3D analysis, a block of 700 x 1000 x 500 \(\mu m\) was selected. Five hundred slices at 8-\(\mu m\) reconstruction of the \(\mu\)-CT images from 3 different animals were subjected to 3D image analysis by Image-Pro 5.0.1 software. The number of vessel segments (A) and the volume of vessel segments (B) from 8 to 160 \(\mu m\) were then quantified for control (open bar) and ligated (solid bar) hindlimbs. *Statistical significance at \(P<0.05\).

**Figure 5.** Ad-PIGF-1 expression and biological activity. A, In vitro expression was determined by adding the indicated concentrations of Ad-PIGF-1 to HEK293 cells in 6-well plates. After 24 hours, the culture medium was removed, and PIGF-1 concentrations were determined by ELISA. Measurements were performed in triplicate on 3 different wells from control and treatment groups. B, In vitro activity of Ad-PlGF-1 was determined in HEK 293 cells cotransfected with flt-1, KDR, and ELK-luciferase reporter genes. flt-1 selective reporter gene activation is shown here. C, In vivo expression of PlGF-1 was determined by quantitative reverse transcription–polymerase chain reaction 3 days after Ad-PIGF-1 or empty vector administration into the calf muscle (\(n=4\)). D, Ad-PIGF-1 (10\(^6\)) or vector was injected into the calf muscle (\(n=3\) or 4). After 3 days, blood cells were collected, labeled with Sca1\(^+\) antibodies, and subjected to fluorescence-activated cell sorting analysis. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.
artery ligation. Laser Doppler measurements showed a time-dependent recovery of tissue perfusion, reaching 52±5% of preischemia blood flow after 4 weeks. Control vector did not produce any significant change in the laser Doppler perfusion measurement (53±4%). Administration of 10^8 pfu of Ad-PIGF-1 resulted in an overall significant improvement in blood flow (67±7%; Figure 7A). Determination of capillary density by isolectin staining and arterial vessel density by α-actin staining showed that total capillary density in vector- and Ad-PIGF-1–treated limbs was similar (Figure 7B and 7C), whereas Ad-PIGF-1 produced a 60% (P<0.05) increase in α-actin–stained vessels (Figure 7B and 7D).

High-resolution, 3D μ-CT image analysis was further used to examine the effect of Ad-PIGF-1 on the vascular response to ischemia. Ad-PIGF-1 was administered into the ischemic adductor muscle after femoral artery ligation. A, Blood flow was determined by an infrared laser Doppler method. The data are expressed as percentage of flow in the left ischemic versus normal right foot (n=6 or 7). At the end of 4 weeks, limbs were excised and fixed in formalin. Thin sections were stained with α-actin from control (B1 and Ad-PIGF-1–treated (B2) mice and with isolectin (B3 and B4, control and Ad-PIGF, respectively). Image magnification was ×200. Quantification of capillary density by isolectin staining in 3 different sections of the lower limb from each animal (n=8) is shown in C. Similarly, α-actin–stained vessels >30-μm diameter in three sections from the lower limb are shown in D.

**Figure 6.** Effect of PIGF-1 on circulating blood cells. Blood samples collected from animals treated with G-CSF for 5 days (top) or 3 days after a single injection of Ad-PIGF-1 (bottom) were analyzed for different cell populations with the Hemavet 850 hematology system. Actual cell numbers in a milliliter of blood are shown here. WBC indicates white blood cell.
to ischemia. Ad-PIGF-1 doses were selected on the basis of the initial studies demonstrating a minimum, nonspecific effect on Sca1⁺ cell mobilization. Control vector 5 × 10⁸ pfu induced mobilization of bone marrow cells and a nonspecific inflammatory response in the tissue. Therefore, doses < 10⁸ pfu were used in these studies. The vessel number and segmental volume were determined in a block of 700×900×500-μm tissue from control and Ad-PIGF-1–treated animals. (Note that a larger tissue block [700×1000×500 μm] was used in Figure 4, which resulted in a larger number of total vessels and volume compared with those in Figure 8.) Determination of vessel number and segmental volume after 4 weeks of treatment showed that PIGF-1 did not significantly alter the number or the volume

Figure 8. Ad-PIGF-1 promotes formation of medium-size vessels. Four weeks after femoral artery ligation, ligated legs were scanned by μ-CT imaging. Representative 3D (A, B) and 2D z-section (C, D) images of control vector (A, C) and Ad-PIGF-1–treated (B, D) groups are shown here. Bar indicates 100 μm. Three-dimensional image analysis performed on a block of 700×900×500 μm shows vessel number (E) and vessel volume (F) from saline (open bar), vector alone (hatched bar), and Ad-PIGF-1 (solid bar) groups. Data shown are mean ± SEM.

*P < 0.05, **P < 0.01, n = 6 or 7 mice/group. Control and ligated hindlimbs were scanned and reconstructed at 8-μm resolution. The vessel segments in each category (8 to 40 μm and 48 to 160 μm) were pooled, and the percentage change in response to ligation was determined from the total vessel segments and vessel segment volume. Data shown are mean ± SEM. *P < 0.05, n = 7 mice/group.
of small vessels (Figure 8). However, Ad-PIGF-1 treatment produced a significant ($P < 0.05$) increase in the volume of vessel segments in the 96- to 136-µm-diameter range. These effects of Ad-PIGF-1 were dose dependent. For example, relative to vector control, the increase in vascular volume in vessel size ranging from 104 to 128 µm was 58% ($P < 0.05$) at $10^7$, 29% ($P < 0.05$) at $5 \times 10^7$, and 0.2% (not significant) at $10^9$ pfu Ad-PIGF dose. These data show that PIGF-1 gene delivery produced a significant and selective increase in vascular volume contributed by medium-size collaterals.

**Discussion**

Collateral development plays an important role in the restoration of distal blood flow and tissue salvage in a number of physiological and clinical settings. Deficiency in collateral development has been well documented in patients and animals with cardiovascular risk factors. At the present time, the nature and mechanism of collateral development are not well characterized. In large measure, this stems from our inability to visualize and quantify changes in the entire vascular tree in an organ. Indeed, commonly used histological and laser Doppler techniques are limited to semiquantitative vessel counts within a limited area and measurement of superficial blood flow, respectively, whereas x-ray angiography is limited by its spatial resolution.

In this study, we used a high-resolution, 3D µ-CT imaging technique to investigate the nature of vascular development and the effect of PIGF-1 gene delivery in a mouse model of hindlimb ischemia. Our principle findings are that arteries in the 48- to 160-µm range accounted for the predominant change and the principle component responsible for the restoration of arterial perfusion. Importantly, PIGF-1 selectively enhanced growth of arterial vessels that overlapped with this size class.

Delineation of a specific size of arterial vessel that appears to play a key role in the restoration of arterial perfusion is an unexpected and important finding. Because the number of vessels required to restore functional perfusion in the capillary category would be very high, the development of few larger collaterals would be a physiologically beneficial mechanism. This is also supported by the x-ray angiographic findings in large animals. Using µ-CT, we have clearly established that the increase in vessel volume results predominantly from the generation of new medium-size vessels (48 to 160 µm) that are likely to be associated with the change in blood flow and tissue perfusion. Because the majority of the change in vessel volume in response to ischemia occurred in medium-size vessels, our studies are the first to demonstrate that measurement of vessel volume rather than the commonly used histological determination of capillary density is a more relevant parameter.

In a recent study, Duvall et al. described a µ-CT analysis of vascular recovery after hindlimb ischemia in mice. Our studies have further extended the methodology and present new findings with regard to collateral development after ischemia, quantification of arteriogenesis, and the effect of therapy. Using an 8-µm voxel resolution, appropriate selection of tissue sections, and threshold settings, we were able to use a bismuth contrast agent without requiring bone decalcification. Use of high-resolution imaging and an advanced image analysis program has provided highly accurate quantification of vessel number and segmental volume. In an earlier preliminary study, we reported changes in vessel number in the mouse thigh region in response to PR-39. Here we show that determination of vessel volume at each thickness provides a more accurate picture of the vascular changes that enabled the detection of differences between control and PIGF-1–treated groups and the delineation of the exact vessel size modified by therapy.

As indicated earlier, remarkably, the population of vessels carrying the bulk of arterial blood was precisely the population enhanced by PIGF-1. This observation implies that specific biological signals that distinguish this population of vessels exist and that selective therapy may conceivably be developed to augment their growth. We used adenoviral PIGF-1 gene delivery to achieve sustained expression of PIGF-1. Ad-PIGF-1 was first rigorously characterized with regard to its expression and biological activity, which established a dose-dependent expression of PIGF-1 in vitro and in vivo. Adenovirally derived PIGF-1 was biologically active, as demonstrated by flt-1 activation and mobilization of Sca1+ cells.

We observed that PIGF-1 selectively increased Sca1+ cells in the circulation without affecting other cell types. This contrasts with G-CSF, which, while mediating mobilization of the Sca1+ population, also mobilizes leukocytes and monocytes. Because nonspecific mobilization of inflammatory cells may be undesirable for therapy, selective mobilization of Sca1+ cells may be superior for inducing progenitor cell mobilization. Previous studies have demonstrated expression of flt-1 in monocytes and its role in arteriogenesis. The purpose of monitoring bone marrow cells in our studies was to use it as a pharmacodynamic biomarker for in vivo PIGF-1 activity. Whether these cells also play a role in arteriogenesis is unknown.

While inducing growth of medium-size arteries, PIGF-1 did not affect small vessels, including arterioles and capillaries. In this regard, our µ-CT data are consistent with the lack of a PIGF-1 effect on capillary density reported previously. The effect of PIGF-1 in promoting medium-size, stable, and perfusing vessels may be clinically important for the treatment of ischemic coronary and peripheral vascular diseases. Although the clinical efficacy of PIGF-1 remains to be tested, the quantitative 3D µ-CT imaging data strongly support that PIGF-1 represents an important therapeutic molecule to promote large conducting vessels in patients with ischemic coronary and peripheral arterial diseases.

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**Disclosures**

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**References**

For patients with severe occlusive arterial disease, a major medical need is the restoration of blood flow. Percutaneous transluminal angioplasty and drug-eluting stents provide highly successful device-based interventions in some of these patients. Therapeutic induction of collaterals is an attractive alternative approach that may be effective for a large number of patients. However, to date, pharmacological stimulation of therapeutic angiogenesis has not been achieved in humans. In part, this failure may be due to the use of agents that predominantly induce angiogenesis (capillary growth) rather than arteriogenesis.

CLINICAL PERSPECTIVE

For patients with severe occlusive arterial disease, a major medical need is the restoration of blood flow. Percutaneous transluminal angioplasty and drug-eluting stents provide highly successful device-based interventions in some of these patients. Therapeutic induction of collaterals is an attractive alternative approach that may be effective for a large number of patients. However, to date, pharmacological stimulation of therapeutic angiogenesis has not been achieved in humans. In part, this failure may be due to the use of agents that predominantly induce angiogenesis (capillary growth) rather than arteriogenesis (arterial/collateral growth). The data presented in the present study have 3 potential clinical implications: (1) Use of µ-CT for quantification of arterial vessels in the mouse model provides a direct translation of preclinical data to clinical studies. (2) We have shown that in response to ischemia, a major change occurred in vascular volume through the expansion of medium-size collaterals. This response largely contributed to the rapid restoration of flow and function. Therefore, measurement of vascular volume is a more relevant parameter than is superficial blood flow by laser Doppler methods or histological capillary density not relevant to clinical efficacy. (3) The present study has revealed a unique effect of placental growth factor-1 on medium-size arteries relevant to therapy in humans. Furthermore, a selective increase of progenitor cells in the circulation by placental growth factor suggests its potential use for pharmacological enhancement of progenitor cells that are deficient in patients with cardiovascular risk factors, including diabetes and dyslipidemia.
High-Resolution Quantitative Computed Tomography Demonstrating Selective Enhancement of Medium-Size Collaterals by Placental Growth Factor-1 in the Mouse Ischemic Hindlimb

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