Monitoring of the Biological Response to Murine Hindlimb Ischemia With $^{64}$Cu-Labeled Vascular Endothelial Growth Factor-121 Positron Emission Tomography

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Background—Vascular endothelial growth factor-121 (VEGF$_{121}$), an angiogenic protein secreted in response to hypoxic stress, binds to VEGF receptors (VEGFRs) overexpressed on vessels of ischemic tissue. The purpose of this study was to evaluate $^{64}$Cu-VEGF$_{121}$ positron emission tomography for noninvasive spatial, temporal, and quantitative monitoring of VEGFR2 expression in a murine model of hindlimb ischemia with and without treadmill exercise training.

Methods and Results—$^{64}$Cu-labeled VEGF$_{121}$ and a VEGF mutant were tested for VEGFR2 binding specificity in cell culture. Mice ($n$/11005 = 58) underwent unilateral ligation of the femoral artery, and postoperative tissue ischemia was assessed with laser Doppler imaging. Longitudinal VEGFR2 expression in exercised and nonexercised mice was quantified with $^{64}$Cu-VEGF$_{121}$ positron emission tomography at postoperative day 8, 15, 22, and 29 and correlated with postmortem $\gamma$-counting. Hindlimbs were excised for immunohistochemistry, Western blotting, and microvessel density measurements. Compared with the VEGF mutant, VEGF$_{121}$ showed specific binding to VEGFR2. Perfusion in ischemic hindlimbs fell to 9% of contralateral hindlimb on postoperative day 1 and recovered to 82% on day 29. $^{64}$Cu-VEGF$_{121}$ uptake in ischemic hindlimbs increased significantly ($P$/11021 < 0.001) from a control level of 0.61 ± 0.17% ID/g (percentage of injected dose per gram) to 1.62 ± 0.35% ID/g at postoperative day 8, gradually decreased over the following 3 weeks (0.59 ± 0.14% ID/g at day 29), and correlated with $\gamma$-counting ($R^2$/11005 = 0.99). Compared with nonexercised mice, $^{64}$Cu-VEGF$_{121}$ uptake was increased significantly ($P$/11349 ≤ 0.0001) in exercised mice (at day 15, 22, and 29) and correlated with VEGFR2 levels as obtained by Western blotting ($R^2$/11005 = 0.76). Ischemic hindlimb tissue stained positively for VEGFR2. In exercised mice, microvessel density was increased significantly ($P$/11021 < 0.001) compared with nonexercised mice.

Conclusions—$^{64}$Cu-VEGF$_{121}$ positron emission tomography allows longitudinal spatial and quantitative monitoring of VEGFR2 expression in murine hindlimb ischemia and indirectly visualizes enhanced angiogenesis stimulated by treadmill exercise training. (Circulation. 2008;117:915-922.)

Key Words: imaging ■ arteriosclerosis ■ exercise ■ angiogenesis ■ tomography ■ peripheral vascular disease ■ growth substances

As the Western world population continues to age, the prevalence of peripheral arterial disease (PAD), which affects ≈12% of adults, is increasing.1 PAD, a manifestation of systemic atherosclerosis, is caused by atherosclerotic stenosis and/or occlusion of lower-extremity arteries, with consecutive muscle tissue ischemia at stress or rest, and it results in 2 major clinical symptoms: intermittent claudication and critical limb ischemia.2 The natural response to muscle tissue ischemia includes the mobilization of circulating cellular elements and the upregulation of angiogenic growth factors with the corresponding binding ligands that together enable development of collateral vasculature.3-5 Novel therapeutic strategies for patients refractory to conventional treatments of PAD (such as exercise training, drug therapy, bypass grafting, or percutaneous interventions) aim to stimulate or augment these physiological adaptive processes by application of angiogenic cytokines and/or gene and cell therapy protocols.2,4 Although such therapeutic strategies have shown encouraging results, with increased vascular collateral growth and improved clinical symptoms in preclinical studies and early nonrandomized clinical trials, they have failed or have provided unsatisfactory responses in

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randomized placebo-controlled clinical trials. These conflicting results may be due to the absence of objective and quantitative measures of the biological response to ischemia to select for patients who would benefit most from a given specific therapeutic approach.8

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Molecular imaging is a rapidly expanding field that attempts to noninvasively visualize, characterize, and quantify biological processes at the cellular and subcellular level in living subjects.9 Direct targeted molecular imaging of specific molecular markers of angiogenesis, such as vascular endothelial growth factor (VEGF) and its receptors (VEGFRs), could provide an objective and quantitative measure for individualized monitoring of PAD therapy. VEGFR2 is one of the major regulators of angiogenesis, and activation of the VEGF/VEGFR2 axis triggers multiple downstream signaling networks that result in increased angiogenesis.10

In the present study, we hypothesized that positron emission tomography (PET) imaging would enable noninvasive in vivo spatial, temporal, and quantitative monitoring of angiogenesis with the use of a radiolabeled protein targeted at VEGFR2. To test this hypothesis, we longitudinally monitored VEGFR2 expression levels by 64Cu-VEGF121 PET in murine ischemic hindlimbs over a 4-week period and quantified the effects of proangiogenic treadmill exercise training on both radiotracer uptake and microvessel density (MVD).

Methods

In Vitro Assays and Cell Culture Experiments

Synthesis of VEGF121 and VEGF_mutant

The gene for VEGF121 was amplified from mRNA extracted from human umbilical vascular endothelial cells via polymerase chain reaction and inserted into a pRSF-Duet1 prokaryotic expression vector (Novagen, San Diego, Calif). A VEGF mutant (D63A/E64A/E67A/R82N/I83L/K84S) with decreased affinity for VEGFR2 and, to a lesser extent, for VEGFR1 (henceforth referred to as VEGF_mutant) was obtained by 2 rounds of polymerase chain reaction with 2 pairs of overlapping primers and 2 flanking primers and was cloned into the pRSF-Duet1 vector. For protein expression and purification, the plasmids were transformed into BL21 (DE3) competent cells.

Radiolabeling of VEGF121 and VEGF_mutant

Copper-64 (64Cu; 12.7 hours) was obtained from the University of Wisconsin, Madison, and DOTA (1,4,7,10-tetraazadecacene- N,N',N''-tetraacetic acid) was purchased from Macrocyclics, Inc (Dallas, Texas). VEGF121 and VEGF_mutant were radiolabeled as described previously.6 For 64Cu-DOTA-VEGF121, the radiolabeling yield was 69.7 ± 6.3%, the number of DOTA molecules was 2.2 ± 0.1, and the specific activity was 116.2 ± 10.5 mCi/mg, with a radiochemical purity >98% in the present study. For 64Cu-DOTA-VEGF_mutant, the radiolabeling yield was 53.9 ± 9.1%, the number of DOTA molecules was 0.9 ± 0.2, and the specific activity was 89.8 ± 15.2 mCi/mg, with radiochemical purity >98%.

Binding Assay of VEGF121 and VEGF_mutant

Porcine aortic endothelial (PAE-KDR) cells stably transfected to express human VEGFR2 (KDR) and porcine aortic endothelial control cells (PAEs) not expressing VEGFR1 and VEGFR2 were cultured in Ham’s F-12 medium containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, Mo). With 64Cu-DOTA-VEGF121 as the radioligand, the receptor-binding affinity of VEGF121, DOTA-VEGF121, VEGF_mutant, and DOTA-VEGF_mutant for VEGFR2 was determined as described elsewhere for both PAE-KDR and PAE cells.7 To analyze the binding affinity of VEGF121, DOTA-VEGF121, VEGF_mutant, and DOTA-VEGF_mutant to VEGFR1, soluble VEGFR1D1-D6 (sVEGFR1; Research Diagnostics, Inc, Concord, Mass) was diluted with coating buffer (15 mmol/L NaCO3, 35 mmol/L NaHCO3, pH 9.6) and coated onto 96-well plates (NUNC, Rochester, NY). Serially diluted VEGF121, DOTA-VEGF121, VEGF_mutant, or DOTA-VEGF_mutant was then added to compete with the radioligand 64Cu-VEGF121. The best-fit 50% inhibitory concentration (IC50) values were calculated by fitting the data by nonlinear regression by use of GraphPad Prism 4.0 software (GraphPad Software, Inc, San Diego, Calif). Experiments were performed in triplicate.

Animals and Treadmill Exercise Protocol

Mouse Model of Hindlimb Ischemia

Animal protocols were approved by the Stanford Institutional Administrative Panel on Laboratory Animal Care. Unilateral hindlimb ischemia in the left leg was introduced in 4-month-old male C57BL/6J mice (n = 58, Jackson Laboratories, Bar Harbor, Me) by ligation of the left femoral artery proximal and distal to the caudal femoral artery. The arterial segment between the ligatures was excised. A sham procedure was performed on the contralateral leg in 10 mice.

In Vivo Assessment of Hindlimb Function and Ischemic Damage

Animals were killed on days 1, 8, 15, 22, and 29 after surgery, and the right hindlimb was removed and excised. A tissue damage score was calculated in each animal, with the temperature of the mice kept constant at 37 ± 0.5°C. Average hindlimb perfusion was expressed as the ratio of ischemic to nonischemic hindlimb by drawing regions of interests over both hindlimbs.

Treadmill Exercise Training

A subgroup of 20 mice were exercised on a rodent treadmill (Exer-3-6; Columbus Instruments, Columbus, Ohio). The treadmill exercise training began 3 days after surgery and was performed 5 times a week. Each training session started with a speed of 9 m/min for 3 minutes and was then increased by 3 m/min every 3 minutes until a maximum speed of 18 m/min was reached. The training was performed until the mice were unable to keep pace (mean training time per day 35 minutes; range 30 to 40 minutes). Five mice were trained for 1 week, 5 for 2 weeks, 5 for 3 weeks, and 5 for 4 weeks.

Small-Animal Imaging Experiments

PET Imaging

PET imaging was performed on a Concorde R4 microPET system (Siemens AG, Malvern, Pa) with the animals maintained under 2% isoflurane anesthesia. One, 4, and 20 hours after intravenous injection of 64Cu-VEGF121 (mean 238 ± 143 mCi; range 217 to 277 mCi) via the tail vein, a 5-minute static scan with an approximate resolution of 2 mm in each axial direction was obtained in all animals. Images were reconstructed with the OSEM (ordered-subsets expectation maximization) algorithm. 64Cu-VEGF121 was administered in an additional 3 nonexercised mice (day 8 after surgery), and PET scanning was performed as
described above. In vivo blocking studies were performed in an additional 3 nonexercised mice (day 8 after surgery). First, 250 μg of rat anti-mouse VEGFR2 monoclonal antibody (Avas 12a1; eBioscience, San Diego, Calif) was injected via tail vein in each of these 3 mice. Sixty minutes after injection of the monoclonal antibodies (to allow for distribution of the antibodies in the tissue), 64Cu-VEGF121 was injected through the tail vein, and PET imaging was performed as described above.

**Image Analysis of PET Images**

PET images were analyzed offline in random order with nonproprietary PET analysis software (AMIDE version 0.8.2; http://amide.sourceforge.net). Regions of interest encompassing both the ischemic and nonischemic muscles of both hindlimbs were drawn. Percentage of injected dose per gram (% ID/g) was calculated. No corrections for partial volume or attenuation were performed.

**Postmortem Analysis**

γ-Well Counting

After PET imaging, the skeletal muscles of the ischemic and nonischemic hindlimbs were excised and weighed in 3 mice. Tissue radioactivity was measured with a γ-counter (Cobra II Auto-Gamma; PerkinElmer, Wellesley, Mass) and was corrected for background, decay time, and tissue weight.

**Biodistribution Studies**

At day 8 after surgery, an additional 12 mice were injected with 19.2 μCi (range 15.8 to 22.9 μCi) of 64Cu-VEGF121 through the tail vein, euthanized, and dissected at 1 hour (n = 4), 4 hours (n = 4), and 20 hours (n = 4) after injection. Blood, hindlimb muscle tissue, and major organs and tissues were collected and weighed wet, and the radioactivity in the tissues was measured with a γ-counter.

**Immunohistochemistry**

Frozen tissue slices (5 μm) of the muscle tissue were incubated with rat anti-mouse VEGFR2 antibody (DC101; ImClone Systems, Inc, New York, NY) and visualized with FITC-conjugated donkey anti-rat secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa). For VEGFR1 staining, the slices were incubated with rabbit anti-mouse VEGFR1 antibody and visualized with Cy3-conjugated donkey anti-rabbit secondary antibody (1:50, Laboratory Vision, Fremont, Calif). Slices were also stained for CD31 to localize VEGFR2 expression to muscle vessels or for VEGFR1 staining, the slices were incubated with rabbit anti-mouse VEGFR1 antibody and visualized with FITC-conjugated donkey anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories). To identify the nuclei, all slices were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

On CD31-stained slices, 7 random, nonoverlapping views of the muscle tissue slices of both exercised and nonexercised mice were selected for MVD analysis. The number of vessels counted was divided by the field of view to yield the MVD, expressed as vessels/mm².

**Western Blotting**

Muscle tissue protein was extracted with a tissue protein extraction buffer (T-PER; Pierce Biotechnology, Inc, Rockford, Ill). Forty micrograms of protein from each muscle sample was separated by running the sample on a NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, Calif). The protein was transferred to a nitrocellulose membrane (Invitrogen), and the membrane was then incubated with a rabbit anti-mouse VEGFR2 primary antibody (1:1000; Upstate USA Inc, Charlotteville, VA) and with a horseradish peroxidase–conjugated anti-rabbit secondary antibody (1:5000; GE Healthcare, Piscataway, NJ). As an internal loading control, the same membrane was incubated with anti-α-tubulin antibody. Relative VEGFR2 expression levels were quantified on Western blots with ImageJ software (version 1.32; National Institutes of Health, Bethesda, Md) after densitometric scanning of the exposed films.

![Graphs demonstrate binding assays of VEGF121 and VEGFMutant binding to VEGFR2 (top) and VEGFR1 (bottom). VEGFMutant has decreased affinity to VEGFR2 (IC50 = 10 μmol/L) compared with wild-type VEGF121 (IC50 = 2.9 nmol/L) and has decreased affinity, although to a lesser extent, to VEGFR1 (IC50 = 12 nmol/L). Measurements were done in triplicate.](image-url)
VEGFR1 (28 nmol/L), respectively, were slightly reduced compared with free VEGFMutant. No significant binding was noted of VEGF121, DOTA-VEGF121, VEGFMutant, and DOTA-VEGFMutant to negative control cells (PAEs).

**Laser Doppler Imaging and Clinical Assessment Confirm Ischemia After Femoral Artery Ligation and Hindlimb Blood Flow Recovery Over 4 Weeks**

To confirm successful induction of tissue ischemia after femoral artery ligation, laser Doppler imaging was performed, and mice were evaluated clinically by use of the ambulatory impairment score and the tissue damage score.

Average hindlimb tissue perfusion ratio decreased from 1.0 before surgery to 0.09 at day 1 after surgery, gradually recovered over the following 4 weeks, and eventually reached 0.82 at day 29 after surgery. On serial clinical assessment, a reduced functional use of the ischemic hindlimb was noted at day 1 after surgery (mean ambulatory impairment score 1.6). Functional use of hindlimb improved by day 8 and recovered to normal levels by days 22 and 29, respectively (Table). The mean clinical tissue damage score increased from 1.0 before surgery to 2.6 at day 1 and decreased to near-normal levels at day 29 after surgery (1.2; Table). Clinical ambulatory impairment and tissue damage scores were not significantly different ($P>0.35$) in exercised versus nonexercised mice at any time point after surgery.

**PET Imaging Shows Significantly Higher $^{64}$Cu-VEGF$_{121}$ Uptake in Ischemic Hindlimbs Than in Nonischemic Hindlimbs**

$^{64}$Cu-VEGF$_{121}$ uptake in ischemic hindlimbs was significantly higher ($P<0.001$) than in contralateral nonischemic hindlimbs on PET imaging at days 8, 15, and 22 after surgery (Figure 2). In ischemic hindlimbs, VEGF$_{121}$ uptake was highest at day 8 after surgery (mean 1.62% ID/g; range, 1.23% ID/g to 2.4% ID/g) and decreased thereafter. Twenty-nine days after femoral artery ligation, VEGF$_{121}$ uptake in ischemic hindlimbs (mean 0.59% ID/g; range, 0.46% ID/g to 0.76% ID/g) was not significantly different ($P=0.1$) from radiotracer uptake in the contralateral nonischemic hindlimbs (mean 0.61% ID/g; range 0.40% ID/g to 0.64% ID/g). In vivo $^{64}$Cu-VEGF$_{121}$ uptake correlated well with ex vivo $^{64}$Cu $\gamma$-well counting of the excised muscle tissues ($R^2=0.99$, $P=0.02$). No statistically significant difference ($P>0.33$) existed in radiotracer uptake in ischemic hindlimbs when scans were acquired at day 29 after surgery.
Treadmill Exercise Training Increases 64Cu-VEGF121 Uptake in Ischemic Hindlimbs

At all time points after surgery, average 64Cu-VEGF121 uptake in ischemic hindlimbs was higher in exercised versus nonexercised ischemic hindlimbs. The ANOVA found significant main effects of treadmill exercise training (exercised mice had higher uptake values than nonexercised mice, P<0.0001) and time point after surgery (later time points had lower uptake values than earlier time points after surgery, P<0.001; Figure 4).

Immunohistochemistry Shows Higher Amounts of VEGFR2 in Ischemic Hindlimbs Than in Nonischemic Hindlimbs

Immunohistochemical staining for VEGFR2 in ischemic muscle tissue was strong, and staining in nonischemic control muscle tissue was almost nonexistent at day 8 after surgery (Figure 5A). At day 29 after surgery, VEGFR2 expression levels were reduced but still higher than in control muscle tissue. Costaining of VEGFR2 and CD31 was unsuccessful; however, visual examination of CD31 and VEGFR2 staining of slices of the same hindlimb muscle tissue suggested colocalization of VEGFR2 and CD31 on muscle vessels (Figure 5B). Low VEGFR1 expression levels were present in both ischemic and nonischemic control muscle tissues (Figure 5A).

MVD Is Increased in Ischemic Hindlimb Muscle Tissue of Exercised Compared With Nonexercised Mice

In ischemic muscle tissue, MVD increased after treadmill training (Figure 5C). At days 8 and 29, respectively, MVD was 179.4±26.2 and 127.8±12.0 vessels/mm2 in nonexercised mice and increased significantly (P<0.001) to 245.7±25.4 and 178.1±18.3 vessels/mm2 in exercised mice. In nonischemic control muscle tissue, MVD was 131.3±21.1 vessels/mm2 in nonexercised mice and did not increase significantly (P=0.7) in exercised mice (136.4±19.1 vessels/mm2).

Western Blotting Shows Higher VEGFR2 Protein Expression in Exercised Than in Nonexercised Mice

The amount of VEGFR2 protein was higher in ischemic than in nonischemic muscle tissue and was increased after treadmill exercise training at all time points after surgery (Figure 6). A positive correlation existed (R2=0.76, P<0.001) between relative VEGFR2 expression levels as measured by
Western blotting and $^{64}$Cu-VEGF$_{121}$ uptake as assessed by PET imaging at different time points after surgery.

**Discussion**

In this study, we demonstrated that expression levels of VEGFR2 can be monitored in murine hindlimb ischemia by PET imaging with $^{64}$Cu-labeled VEGF$_{121}$. In addition, we showed that increased VEGF expression levels correlate with increased MVD, a measure of angiogenesis, which can be monitored by $^{64}$Cu-VEGF$_{121}$ PET after treadmill exercise training.

We first created a mutant VEGF protein (VEGF$_{Mutant}$) and showed in cell culture that the mean affinity of VEGF$_{Mutant}$ for VEGFR2 (PAE-KDR) was 3400-fold lower than with wild-type VEGF$_{121}$. Cells that lack expression of VEGFR2 (PAE cells) served as control for cell culture experiments and showed no binding affinity to either VEGF$_{121}$ or VEGF$_{Mutant}$. After DOTA labeling, only a minimal difference was noted in VEGFR2 binding affinity compared with free VEGF$_{121}$, which indicates that the lysine residues or N-terminal amine groups used for DOTA conjugation may not be located at the VEGFR2 binding domain of VEGF$_{121}$. Taken together, these in vitro results suggest binding specificity between recombinant human VEGF$_{121}$ and its ligand, VEGFR2 (KDR). Murine VEGFR2 (flk-1) shows $\approx$85% homology with human VEGFR2 (KDR) and plays a role in mouse physiology analogous to the role of KDR in humans. In fact, VEGFR2 is very often referred to as KDR/flk-1, which reflects the close analogy between the 2 VEGFR2 homologs. Therefore, we reasoned that human $^{64}$Cu-VEGF$_{121}$ could be used to noninvasively and quantitatively image VEGFR2 expression levels in vivo in a murine hindlimb model of ischemia in the present study.

Among several growth factor receptor pathways, VEGF and its different isoforms and receptors have been identified as the major regulators of angiogenesis. VEGF is secreted in response to ischemic or hypoxic conditions, and levels of VEGF protein are significantly elevated in skeletal muscles as the major regulators of angiogenesis. Activation of VEGFR2 tyrosine cascade mediates the majority of downstream effects of VEGF in angiogenesis, including microvascular permeability, endothelial cell proliferation, migration, and survival. After exercise training or electrical stimulation of muscle tissue, the unique role of VEGFR2 in enhancing capillary growth has been further underlined in several studies. After stimulation of muscle activity in rats, increased protein levels of both VEGF and VEGFR2 were seen, whereas expression levels of VEGFR1 protein were only present at increased protein levels of VEGFR2 after stimulated muscle activity. The results of the present study confirm these findings. In the present study, VEGFR2 expression was substantially upregulated in ischemic hindlimb
tissue and further increased after treadmill training, as assessed by both immunohistochemistry and Western blotting. In contrast, VEGFR1 expression was not increased in ischemic hindlimb tissue of either nonexercised or exercised mice in the present study. In addition, MVD was substantially elevated in exercised compared with nonexercised mice. Therefore, $^{64}$Cu-VEGF$_{121}$ uptake in ischemic hindlimb tissue in the present study primarily reflects binding of VEGF$_{121}$ to VEGFR2 and not to VEGFR1; $^{64}$Cu-VEGF$_{121}$ uptake also indicates the presence of angiogenesis in hindlimb ischemia. The specificity of $^{64}$Cu-VEGF$_{121}$ PET to monitor VEGFR2 but not VEGFR1 expression levels is also corroborated by the observation that VEGF$_{121}$mice, which had substantial binding affinity to VEGFR1 in cell culture experiments, did not accumulate substantially in ischemic hindlimb muscle tissue in vivo.

Several studies using hindlimb ischemia models have addressed the potential of radionuclide imaging approaches for visualization and quantification of molecular markers of angiogenesis in ischemia. Uptregulation of $\alpha$,$\beta_3$-integrin 3 days after creation of hindlimb ischemia in mice has been demonstrated by scintigraphic imaging of radioiodine-labeled RGD $^{[125]}$I-(RGDyIK). In another study, expression levels of $\alpha$,$\beta_3$-integrin were obtained over a 2-week period after induction of hindlimb ischemia in mice by use of a $\gamma$-camera and a $^{99m}$Tc-labeled chelate-peptide conjugate containing an RGD motif. Experience with radionuclide imaging of VEGFR2 expression, however, remains very limited. Lu et al $^{24}$ used $^{111}$In-labeled recombinant human VEGF$_{121}$ and a $\gamma$-camera for visualization of VEGFR2 expression over a 2-day period in a rabbit model of hindlimb ischemia. A subtle increase in scintigraphic image counts in the ischemic hindlimb (mean 370 cpm) could be detected compared with nonischemic control (mean 280 cpm) and sham-operated (mean 310 cpm) hindlimbs in that study. We significantly add to that study, first, by tracking expression levels of VEGFR2 over a 4-week period rather than a 2-day period only, and second, by investigating angiogenesis by PET rather than $\gamma$-camera imaging. PET is increasingly being used for cancer and cardiovascular imaging clinically and holds significant advantages over $\gamma$-camera imaging in that the sensitivity for detecting molecular probes is up to 2 orders of magnitude higher than that for $\gamma$-camera imaging. This may explain the much higher relative radiotracer uptake values in ischemic versus nonischemic hindlimbs in the present study compared with the values obtained with $\gamma$-camera imaging in the study by Lu et al. $^{24}$ The present study is unique in that we addressed for the first time the effects of stimulated angiogenesis on PET tracer uptake in a murine hindlimb ischemia model using the clinically relevant and widely used therapeutic approach of treadmill exercise training. In exercised mice, $^{64}$Cu-VEGF$_{121}$ uptake was increased substantially compared with nonexercised mice and reached statistically significant differences 2 to 4 weeks after initiation of exercise training. This increase in radiotracer uptake on PET imaging correlated well with VEGFR2 expression levels in mice after treadmill exercise training as measured semiquantitatively by Western blotting and was paralleled by an increase of MVD. In contrast, we did not observe a significant difference between exercised and nonexercised mice by the 2 clinical assessment scores used in the present study. These findings suggest that $^{64}$Cu-VEGF$_{121}$ PET imaging may provide a more sensitive means to objectively quantify the kinetics of VEGF121 expression and the presence of angiogenesis than is possible with clinical evaluation scores. Therefore, this novel approach may be used as an imaging surrogate end point for studying various therapeutic approaches for PAD in preclinical studies and ultimately perhaps also in clinical studies. However, this hypothesis needs to be tested in further studies both in large animals and in humans. Further studies are also warranted to address whether the use of $\alpha$,$\beta_3$-integrin, VEGFR2, or the combination of those 2 or other angiogenesis molecular markers may represent the most optimal imaging targets for monitoring angiogenesis in ischemia, both in animals and in humans.

The following limitations of the study need to be addressed. Hindlimb ischemia was created in healthy mice in the present study. Collateral vessel creation and thus expression of molecular markers of angiogenesis depend on the mouse strain and may vary in older, atherosclerotic, and hypercholesterolemic mice. Therefore, future studies are warranted to test the utility of $^{64}$Cu-VEGF$_{121}$ PET imaging of VEGFR2 expression in animal models that better reflect the vascular status of older, atherosclerotic, and probably hypercholesterolemic patients with PAD. Furthermore, it has been shown that hindlimb perfusion in C57BL/6J mice, which were used in the present study, recovers relatively quickly (within 4 weeks) after femoral artery ligation compared with other mouse strains. Therefore, our mouse model may reflect chronic PAD only during a limited time interval after surgical creation of ischemia. Thus, the kinetics of VEGFR2 expression levels as determined in the present study cannot be extrapolated directly to other animal models of hindlimb ischemia or to patients with PAD. Finally, although a recent study has shown only small estimated radiation-absorbed doses of $^{64}$Cu-VEGF$_{121}$ in both rats and humans, a radiotracer with a shorter half-life than $^{64}$Cu for labeling VEGF$_{121}$ would be desirable in future translatable studies in humans. This is further supported by our finding that radiotracer uptake in ischemic hindlimbs was not significantly different at 1 hour, 4 hours, and 20 hours after intravenous administration, corroborating the use of a radiotracer with a short half-life such as $^{19}$F ($t_{1/2}=109.7$ minutes) or $^{68}$Ga ($t_{1/2}=68$ minutes).

In conclusion, the results of the present study suggest that PET imaging with $^{64}$Cu-labeled VEGF$_{121}$ allows noninvasive spatial visualization and quantification of VEGFR2 expression in a murine model of hindlimb ischemia. Modulation of VEGFR2 expression levels by treatment with treadmill exercise training in mice can be measured and correlated to increased MVD in angiogenesis by $^{64}$Cu-VEGF$_{121}$ PET imaging.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Angiogenesis, the recruitment of new vessels, is part of the adaptive processes in ischemia and involves the interaction of various angiogenic factors. One of the major regulators of angiogenesis is vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR2). VEGFR2 is an endothelium-specific receptor tyrosine kinase that is overexpressed in ischemic tissues and that triggers multiple signaling networks that result in endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability. By radiolabeling the natural ligand of VEGFR2, the isoform VEGF121, using copper-64 (64Cu), we found that temporal and spatial expression levels of VEGFR2 can be tracked by positron emission tomography (PET) in a chronic hindlimb ischemia model in mice. In mice that underwent treadmill exercise training over several weeks, VEGFR2 expression levels were increased compared with nonexercised mice and could be monitored noninvasively by 64Cu-labeled VEGF121 PET imaging in vivo. With PET imaging being increasingly used for cardiovascular applications in clinics, our proof-of-concept study suggests that molecular imaging with PET-based approaches may be valuable for noninvasive monitoring of angiogenesis in patients with peripheral arterial disease. In particular, in patients with peripheral arterial disease and refractory symptoms who are undergoing novel therapeutic approaches such as gene- and cell-based therapies, quantitative PET imaging may be helpful in patient stratification and decision making in future clinical studies.
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