Targeted In Vivo Labeling of Receptors for Vascular Endothelial Growth Factor
Approach to Identification of Ischemic Tissue

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Background—A method for identifying tissue experiencing hypoxic stress due to atherosclerotic vascular disease would be clinically useful. Vascular endothelial growth factor-121 (VEGF_{121}) is an angiogenic protein secreted in response to hypoxia that binds to VEGF receptors overexpressed by ischemic microvasculature. We tested the hypothesis that VEGF receptors could serve as markers for ischemic tissue and hence provide a target for imaging such tissue with radiolabeled human VEGF_{121}.

Methods and Results—A rabbit model of unilateral hindlimb ischemia was created by femoral artery excision (n=110). Control rabbits (n=5) underwent identical surgery without femoral excision. On postoperative day 10, rabbits were intravenously administered 100 μCi of ¹¹¹In-labeled recombinant human VEGF_{121}, and biodistribution studies and planar imaging were conducted at 3, 24, and 48 hours. On postmortem gamma counting, there was greater accumulation of ¹¹¹In-labeled VEGF_{121} in ischemic than in control tissue (P<0.02). Differential uptake of isotope by ischemic muscle was not seen in rabbits injected with ¹²⁵I-labeled human serum albumin (n=6). Radioactivity imaged in hindlimb regions of interest was significantly higher in ischemic muscle than in sham-operated and contralateral nonoperated hindlimb at 3 hours (P<0.02). Immunohistochemical staining confirmed upregulation of VEGF receptors in ischemic skeletal muscle.

Conclusions—Identification of the ischemic state via targeted radiolabeling of hypoxia-induced angiogenic receptors is possible. This approach could be useful for monitoring the efficacy of revascularization strategies such as therapeutic angiogenesis. (Circulation. 2003;108:97-103.)

Key Words: angiogenesis ■ hypoxia ■ imaging ■ isotopes ■ receptors

The identification of ischemic but viable tissue in the setting of atherosclerotic cardiovascular disease is an important diagnostic challenge that has implications for the selection of revascularization therapy and the assessment of its efficacy. In the arena of coronary artery disease, noninvasive imaging methods for identifying myocardial ischemia are indirect and typically involve assessment of flow heterogeneity, which is not the same as myocardial ischemia, or evaluation of altered metabolism, which is affected by substrate availability.¹ In peripheral vascular disease, angiography provides anatomic definition of vascularity but does not delineate regional ischemic involvement of the skeletal muscle. A method that localizes ischemia on the basis of hypoxia-specific responses of tissue to chronic hypoperfusion could have advantages over existing clinical methods because it might provide a sensitive means for precisely targeting and monitoring local revascularization strategies such as therapeutic angiogenesis.²

Vascular endothelial growth factor-121 (VEGF_{121}) is a non–heparin-binding isofrom of VEGF secreted in response to hypoxic or ischemic conditions.³ VEGF_{121} is a specific and potent inducer of angiogenesis and binds to the tyrosine kinase receptors Flt-1 and KDR. Because they are largely endothelial cell specific and upregulated during hypoxia,⁴ we tested the hypothesis that these receptors could provide a target for an imaging agent designed to detect ischemia. We report here that radiolabeled VEGF_{121} is capable of identifying VEGF receptor localization to ischemic muscle, and we use a rabbit model of surgically induced hindlimb ischemia to demonstrate proof of this principle.
Methods

Preparation of 111In-Labeled VEGF121

VEGF121 was expressed recombinantly in Pichia pastoris, selectively reduced at Cys-116, and lyophilized as described previously. DTPA-maleimide was synthesized from lysine-derived DTPA and β-maleimidopropionic acid (Sigma). Dried VEGF121 was dissolved in 50 mmol/L phosphate buffer containing 0.3 mol/L NaCl (pH 7.0) and incubated with a 3-fold molar excess of DTPA-maleimide. After 2 hours, free DTPA-maleimide was removed by dialysis. The modification of Cys116 with DTPA was verified by mass spectrometry. For labeling with 111InCl3, 5 μg of DTPA-coupled VEGF121 was incubated with 1.1 μCi of 111InCl3, which resulted in 95% to 98% isotope incorporation, yielding a specific activity of ~93 μCi/μg VEGF121.

In vitro studies were performed to assess the stability of 111In-VEGF121 in serum. The radiolabeled VEGF121 was placed in vials of human serum at 37°C for up to 5 hours. Reverse-phase HPLC and a radiometric detector confirmed integrity of the 111In-labeled DTPA-VEGF121 construct for this period of time.

Confirmation of Specific Binding Activity of 111In-labeled VEGF121

Three different types of studies were performed in duplicate to determine the effect of the DTPA modification at Cys116 on the specific binding of VEGF121. First, we used a competition assay that can measure small changes in specific binding of VEGF121 to a soluble recombinant KDR-Fc receptor through displacement of biotinylated VEGF121 tracer. With DTPA-VEGF121 labeled with 111In, this assay demonstrated some loss of binding activity (IC50 16.1 ng/mL relative to nonmodified VEGF121 (IC50 8.4 ng/mL). Second, in a human umbilical vein endothelial cell proliferation assay, standard VEGF121 was more mitogenic (ED50 6.8 ng/mL) than the DTPA-modified VEGF121 (ED50 16.3 ng/mL). Third, to calculate the Kd of our modified VEGF121, KDR-coated wells prepared as in the competitive receptor binding assay described above were exposed to incremental amounts of 111In-labeled VEGF121, and washed, and bound protein was desorbed and gamma counted (Packard Inc). On the basis of the weight of 111In-labeled VEGF121 (28 329 Da, which was confirmed by mass spectrometry) and the specific activity of the 111In-labeled protein, the cpm data were converted to protein concentration, and bound/free versus bound protein values were plotted. By Scatchard analysis, the Kd for 111In-labeled VEGF121 was 1.3×10-10 mol/L, which is within the range of previously reported Kd values (3×10-10 to 3×10-12 mol/L) for 111In-labeled VEGF in a variety of cultured cell systems. Thus, although the competitive receptor binding and proliferation studies above suggested some loss of activity of VEGF121, consequent to the DTPA modification, our construct appeared to retain substantial specific binding.

Surgical Preparation

The research protocol conformed to the American Heart Association guidelines for animal research and was approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Thirty-two New Zealand White rabbits (weight 3.2±0.1 kg) were anesthetized with ketamine (50 mg/kg IM) and xylazine (5 mg/kg IM). Persistent hindlimb ischemia was induced surgically as described previously. Briefly, unilateral ischemia on either the right or left hindlimb was created by ligation of the distal external iliac artery and excision of the common and superficial femoral arteries. Control rabbits underwent sham surgery. All animals were allowed to recover and were administered buprenorphine (0.04 mg/kg) analgesia postoperatively. The contralateral, nonoperated hindlimb was used as an internal control.

Biodistribution Studies

Biodistribution was measured after rabbits were killed at 3, 24, or 48 hours. Samples of skeletal muscle, lung, heart, kidney, liver, and spleen were washed and weighed, and radioactivity was measured with a gamma counter.

Immunohistochemical Quantification of Receptor Expression

In 7 rabbits (4 ischemic, 3 sham), skeletal muscle was harvested from both hindlimbs on day 10 and prepared for staining. Immunohistochemistry was performed with primary antibody against Flt-1, KDR, or nonspecific IgG followed by detection with the ABC staining system (Santa Cruz Biotechnology). Flt-1 and KDR expression were quantified with IPLab software (Scionalytics), in which operator-defined positive staining was quantified as a percent of the total microscopic field.

Scintigraphic Imaging

Planar imaging was performed in the anterior view with an Orbiter gamma camera (Siemens Inc) equipped with a medium-energy pinhole collimator. The scintigrams (256×256 matrix) were obtained during a 5-minute acquisition period (245-keV photopeak with 15% windows) at 3 hours and during 10-minute acquisitions at 24 and 48 hours after radioisotope injection. Regions of interest were drawn over each hindlimb and the adjacent background, and background-subtracted count activity was calculated for each hindlimb.

Blood Clearance

Venous blood samples were collected serially up to 3 hours after isotopic injection, and radioactivity was measured in a gamma counter. To allow for decay and measurement of tissue activity as a percent of the dose administered, samples of the injected dose (ID) were counted simultaneously. Blood activity (%ID/gram of blood) was plotted against time after injection, and the data were fit to a biexponential function.

Experimental Protocol

Rabbits underwent femoral artery excision (n=14) or sham surgery (n=5) and were allowed to recover. On postoperative day 10, the animals were anesthetized and administered 100 μCi of 111In-labeled VEGF121 through an ear vein. Twelve animals (9 excised, 3 sham) were killed at 3, 24, or 48 hours after radioisotope injection solely for biodistribution studies. In 5 rabbits (3 excised at each time point; n=3 sham at 3 hours), Seven animals (5 excised, 2 sham) underwent scintigraphic imaging at these same time points, and a subgroup of the 7 was killed after each imaging session for biodistribution studies.

To exclude the possibility of nonspecific retention of VEGF121, additional rabbits underwent femoral artery excision and were injected intravenously with 111In-labeled human serum albumin (111In-HSA, 10 μCi) on postoperative day 10. Animals were killed 30 minutes after injection, and postmortem gamma well counting of hindlimb muscle was performed.

Statistical Analysis

Data are expressed as mean±SD. Within-subject differences (ischemic versus nonischemic contralateral hindlimb) were evaluated with paired t testing, and between-subject differences (sham versus ischemic or nonischemic hindlimb) were evaluated with unpaired t testing. Statistical significance was defined as P<0.05 (2-tailed).

Results

Scintillation Well Counting

Figure 1 summarizes scintillation well counts in the muscles of the thigh (A and B) and entire hindlimb (C and D). At 3 hours (Figure 1, A and C), uptake of 111In-labeled VEGF121 by ischemic muscle was significantly higher than by contralateral nonischemic muscle and sham-operated muscle, and there was no difference in 111In-labeled VEGF121 accumulation between sham-operated and nonoperated muscle. The
significant difference between ischemic and nonischemic thigh muscle uptake of 111 In-labeled VEGF121 persisted at 24 and 48 hours after isotope injection (Figure 1B). There was no difference in 125I-HSA retention between ischemic (0.007±0.002%ID/g) and nonischemic (0.007±0.001%ID/g) muscle (P=0.69) in the 6 animals killed 30 minutes after injection of 125I-HSA.

Scintigraphic Findings
Figure 2A depicts a rabbit with right hindlimb ischemia imaged 3 hours after administration of 111In-labeled VEGF121. Despite decreased flow, there was clearer delineation of the ischemic hindlimb (arrows) than in the contralateral hindlimb because of greater radiolabel uptake. In the sham-operated rabbit, the contour of the hindlimb was ill defined, and visually, uptake of the radiolabel in both hindlimbs appeared symmetric (Figure 2B). There were more scintigraphic image counts in the ischemic hindlimbs (370±130 cpm, n=5) than in the nonischemic, contralateral hindlimbs (280±50 cpm, P=0.04). In the control group, there was no difference in image counts between sham-operated muscle (310±10 cpm, n=2) and the contralateral muscle (280±40 cpm, P=0.6).

Immunohistochemistry
There was strong staining for Flt-1 and KDR receptors in ischemic muscle (Figure 3, A and B), particularly in the microvasculature, which was not seen in nonischemic muscle (Figure 3, D and E) or when the ischemic muscle was stained with nonspecific IgG (Figure 3C). Quantitative evaluation of KDR and Flt-1 receptor expression demonstrated a significantly greater area of VEGF receptor staining in ischemic muscle than in sham-operated and nonoperated controls (Figure 3F).

Blood Clearance and Biodistribution of VEGF121
Blood clearance followed a biexponential curve consistent with a 2-compartment model, with a mean T1/2 of 26±8 minutes (Figure 4A). There was a rapid clearance phase (T1/2α of 2±0 minutes) followed by a slower component (T1/2β of 900±108 minutes). The biodistribution of 111In-labeled VEGF121 in nontarget organs (%ID/g) is summarized in Figure 4B. On a whole-organ level, accumulation (%ID/g) in the liver (44±8%), kidney (14±2%), and lungs (10±4%) accounted for two thirds of the injected dose at 3 hours after injection.

Discussion
The major finding of the present study was that an approach to identifying ischemic tissue using angiogenic receptor label-

Figure 1. 111In activity determined by gamma counter in deep muscles of thigh (A, B) and entire hindlimb (C, D), and hindlimb muscle-to-blood ratios (E, F) at 3 hours (A, C, E) and up to 48 hours (B, D, F) after tracer injection. Control indicates contralateral nonischemic hindlimb in rabbits with unilateral hindlimb ischemia; solid bars, group with unilateral hindlimb ischemia; and open bars, group with sham-operated hindlimb.

Figure 2. Planar image after 10 days of right hindlimb ischemia (A) or sham surgery (B), taken 3 hours after 111In-labeled VEGF121 injection. There is greater isotope accumulation in ischemic hindlimb (A, arrows) than in nonischemic hindlimb and no difference in tracer accumulation between sham-operated and contralateral hindlimb (B).
ing by the naturally occurring ligand of the receptor is possible and that such a method may ultimately permit scintigraphic imaging of hypoxic tissue.

Identification of Ischemic Tissue via Labeling of VEGF Receptors

VEGF is a fundamental mediator of hypoxia-induced angiogenesis. Of the various receptors for VEGF that have been described to date, Flt-1 and KDR have been most fully characterized and are tyrosine kinase receptors expressed by endothelial cells. Numerous studies have shown that the genes for Flt-1 and KDR are directly or indirectly responsive to hypoxia. For example, conditions such as acute and chronic lung hypoxia or myocardial infarction are associated with a dramatic increase in mRNA for VEGF receptors. Hypoxic cultured endothelial cells exhibit a 13-fold increase in KDR receptors. Thus, VEGF receptor expression is a marker of a hypoxic state.

Given these considerations, we hypothesized that detection of VEGF receptors would ultimately enable identification of tissue experiencing hypoxic stress. We chose the rabbit hindlimb model to establish proof of this principle because of its well-characterized natural history. We used gamma counting of tissue specimens as the standard of truth for evaluating our approach of injecting radiolabeled VEGF into this animal model.

Postmortem gamma counting demonstrated significantly greater uptake of In-labeled VEGF by ischemic muscle than by contralateral nonischemic and sham-operated muscle. That In-labeled VEGF retention was a receptor-mediated phenomenon rather than a nonspecific sign of increased vascular permeability secondary to ischemia was substantiated by the low retention of radiolabeled albumin in the ischemic hindlimb. Quantitative immunohistochemistry demonstrated VEGF receptor upregulation by the ischemic skeletal muscle, thus further substantiating the basis for enhanced In-labeled VEGF uptake by this tissue.

Our scintigraphic image data provide preliminary demonstration of a potential clinical approach to identifying ischemic muscle by labeling angiogenic receptors. The images suggested enhanced uptake of In-labeled VEGF by ischemic skeletal muscle relative to nonischemic muscle. Furthermore, uptake by ischemic muscle was also greater than that of sham-operated muscle, which confirms that our findings were
The present study is unique in several ways. Unlike previous studies, we targeted hypoxia-induced microvascular receptors. Furthermore, we used the receptors’ naturally occurring ligand, human VEGF121, as the imaging probe. We chose VEGF121 as the targeting ligand for several reasons. First, among the VEGF isoforms, VEGF121 is unique in lacking the coding sequences for exons 6 and 7, which confer high affinity for heparin. Given its lack of heparin binding, we theorized that VEGF121 would be an ideal ligand because of its exclusive binding to the hypoxia-inducible Flt-1 and KDR receptors. Second, we anticipated that the lower molecular weight of VEGF121 relative to antibodies would result in a more rapid blood clearance time and thus favorable pharmacokinetics for nuclear imaging. Third, methods exist for the large-scale production of recombinant human VEGF121, not only providing suitable quantities for imaging purposes but eliminating the immunogenicity of animal-derived antibodies.

Unlike our VEGF121 radioligand, other investigational radionuclide methods for imaging hypoxia use a non–receptor-mediated approach based on cytoplasmic retention of tracers by hypoxic cells. The most commonly studied of these methods uses 2-nitroimidazole as the targeting moiety. Although studies have suggested retention of these radiocompounds by ischemic myocardium, their use has been limited by extensive hepatic uptake, low cardiac specificity, and low target-to-background uptake of tracer. For example, only 0.008% of an intracoronary injection of a 99mTc-labeled nitroimidazole was retained per gram of ischemic porcine myocardium, which is lower than the ischemic skeletal muscle retention of our intravenously injected VEGF121. This may be due in part to the threshold requirement of extremely low oxygen tensions before these compounds are retained.

Comparison With Previous Studies

Methods for targeted nuclear imaging of cardiovascular disease have generally taken one of two approaches. One approach has been to radiolabel antibodies directed against disease-specific epitopes. Radiolabeled monoclonal antibodies against myosin and major histocompatibility complex II antigens have been used to detect myocardial infarction and acute cardiac transplant rejection, respectively. Atherosclerotic lesions in rabbits have been identified with radiolabeled antibodies or their Fab’ fragments that bind to oxidized LDL or proliferating smooth muscle. Although antibodies confer specificity for the target, their use is limited by immunogenicity and high molecular weight, which prolongs circulation time and leads to sustained background blood activity.

A second approach has used radiolabeled peptides or small nonpeptide ligands. Atherosclerotic lesions in rabbits have been targeted with radiolabeled purine analogs, cholesterol ester analogs, or oligopeptide fragments of apolipoprotein B. Recently, 99mTc-labeled annexin-V was used to detect cardiac allograft rejection and apoptosis. Unlike antibodies, these smaller molecules have shorter circulation times and could allow better target-to-blood activity ratios to be achieved soon after injection. The identification and/or synthesis of peptides with high target specificity and binding strength, however, remains a significant challenge.

![Figure 4. Blood clearance (A) and biodistribution (B) of 111In-labeled VEGF121 in nontarget organs at 3 (n=10), 24 (n=5), and 48 (n=4) hours after injection.](image)

Study Limitations

As with all tracers, the uptake of 111In-labeled VEGF121 depends to some extent on flow to the target tissue. We chose the rabbit hindlimb model in part because the mass of hindlimb muscle in the rabbit (unlike cardiac tissue) allowed us to achieve acceptable count statistics. In this model, however, collateralization is relatively sparse, and although we did not directly measure flow, previous studies confirm significant hypoperfusion of tissue under the conditions specified in the present experiments. To decrease the effect of low flow on isotope delivery, we waited 3 hours before gamma counting to allow greater time for binding.

Increased 111In-labeled VEGF121 activity in the ischemic hindlimb may have reflected nonspecific retention of the isotope due to prolonged tracer washout in the setting of low flow. The persistence of differential uptake of 111In-labeled VEGF121 by ischemic muscle up to 48 hours and the comparably low uptake of 123I-HSA in ischemic and nonischemic hindlimb argue against these data being an epiphenomenon of delayed tracer washout, however.

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to be a limitation when regions remote from the liver, such as the lower extremities, are imaged, its effect on the quality of myocardial imaging remains to be studied. As was the case with early-generation cardiac imaging agents, radiochemical modifications of our VEGF construct could alter its biodistribution characteristics to reduce non–target-organ uptake.

Although statistically significant, the differences in Inu111-labeled VEGF121 uptake between ischemic and nonischemic hindlimb on the scintigrams were subtle. Although this may suggest only slight VEGF receptor upregulation during ischemia, previous studies in a variety of endothelial cell lines have demonstrated up to 250,000 receptors per hypoxic cell, a density that should be acceptable for targeted imaging. A decrease in specific binding affinity resulting from the DTPA modification of VEGF121 may have blunted the differences in radioligand uptake between ischemic and normal muscle despite receptor overexpression. However, altered binding affinity is unlikely to have abolished such differences, because in our in vitro studies demonstrated retention of substantial specific binding of our radioligand.

Technical factors may have contributed to the subtle differences in ischemic muscle versus normal muscle uptake in our images compared with the gamma-counting data. We used a pinhole collimator because of the small animal size, which may have decreased sensitivity. A higher dose of Inu111 might have improved the images, although our dose was similar to that used in other biodistribution studies, and the 5- to 10-fold higher absolute count rate (cpm) in the skeletal muscle relative to background indicates that we had adequate statistics for gamma well counting. In future clinical application, an isotope with a shorter half-life, such as 99mTc, might permit injection of higher doses, although the radiochemistry of 99mTc complexation, which typically requires high temperatures that could damage the structure of VEGF121, would require further study. Despite the subtlety of the present scintigraphic findings, however, this study does establish proof of the principle of angiogenic receptor labeling and sets the stage for further studies in this arena incorporating the considerations described above.

**Clinical Implications**

Targeted VEGF receptor imaging may be incremental to standard clinical assessments of flow or perfusion by providing information on the degree of hypoxic stress experienced by living tissue. Although the present data most specifically pertain to skeletal muscle ischemia due to peripheral vascular disease, this targeted approach could be helpful in the arena of coronary syndromes in identifying ischemic myocardium, which also upregulates VEGF receptors. Identification of VEGF receptor expression by hypoxic tissue can be useful for the implementation and evaluation of therapeutic angiogenesis. Indeed, the optimal method for evaluating the therapeutic response to angiogenic interventions, particularly in the case of interventions targeted to the heart, has been debated. An in vivo imaging method to objectively quantify the cellular response of ischemic tissue to angiogenic interventions could be a useful approach for detecting biological responses to angiogenic therapy. Such an approach could be used to localize regions of skeletal muscle or myocardium that are most hypoxic and at greatest risk for necrosis, thus guiding the selection of sites for local injection of angiogenic treatments, and serial receptor imaging may permit evaluation of the therapeutic effects of such angiogenic strategies.

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