Direct Site-Specific Labeling of the Cys-Tag Moiety in scVEGF with Technetium 99m

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Angiogenesis is a fundamental feature of tumor development, and therefore, the tracers for molecular imaging of specific angiogenic biomarkers are expected to be useful for diagnostics, patient monitoring, and drug development. We have created a new class of imaging agents based on the most important mediator of angiogenesis, vascular endothelial growth factor (VEGF). Our latest version is a single-chain (sc) VEGF protein containing an N-terminal Cys-tag designed for site-specific modification with a variety of imaging and therapeutic moieties. We have recently found that the Cys-tag itself can form a stable chelate with ^{99m}Tc using tin-tricine as an exchange reagent. This self-chelation approach yields a highly stable and fully functional form of radiolabeled scVEGF that can be used as a SPECT tracer for tumor angiogenesis. Also of note is that directly labeled scVEGF has less than one-half the nonspecific renal uptake of ^{99m}Tc-HYNIC-scVEGF. The simple production of scVEGF for direct chelation of ^{99m}Tc makes it a promising molecular imaging agent for the oncology clinic.

INTRODUCTION

Angiogenesis is a fundamental feature of tumor growth, and the onset and/or presence of angiogenesis could be used for diagnostic purposes. On the other hand, since angiogenesis is critical for tumor growth, it is a target of massive drug development efforts. Current antiangiogenic drugs given in combination with chemotherapy or radiotherapy provide positive clinical effects in subsets of patients (*1*−*3*). As antiangiogenic treatments are quite expensive and are not without serious side effects (*4, 5*), there is a great desire for better diagnostic tools to guide the development as well as the efficient and safe use of these new drugs in cancer patients. In particular, clinical development of antiangiogenic drugs and combination therapeutic regimens need imaging tracers that can follow therapeutic targets in angiogenic vasculature (see refs (*6*−*8*) for review). Among such imaging targets, receptors for vascular endothelial growth factor (VEGF) are particularly attractive for several reasons. First, VEGF is the most important regulator of angiogenesis. Its signaling pathway, initiated by binding of VEGF to its cognate receptors VEGFR-1 and VEGFR-2, is the primary target of most anti- and pro-angiogenic therapeutic agents (*9*−*12*). Second, the receptors for VEGF, and most notably, VEGFR-2 (KDR/Flk-1), are selectively overexpressed by the endothelial cells of angiogenic tumor vessels (*9, 13*), providing an opportunity for enhanced tracer accumulation. Finally, VEGF receptors are in direct contact with the microcirculation of a tumor and are also internalized upon VEGF binding, allowing for receptor-mediated accumulation and trapping of VEGF-based imaging agents within target tissue.

To enable VEGF receptor imaging, we have recently developed a novel class of VEGF-based imaging agents (*14*−*17*). We have taken the $VEGF_{121}$ isoform of VEGF-A as the framework with which to engineer a single-chain (sc) fusion VEGF protein containing an N-terminal Cys-tag (*17*). Cys-tag is an α-helical 15-aa long N-terminal fragment of human ribonuclease I, in which arginine in position 4 is substituted for cysteine, providing a unique site for conjugation of a wide variety of payloads to Cys-tagged targeting proteins (*15, 16, 18*−*22*). For imaging in different modalities, we have site-specifically derivatized scVEGF with fluorescent dyes for near-infrared fluorescent (NIRF) imaging and with radionuclide chelators for positron emission tomography (PET) with ^{64}Cu and single photon emission computed tomography (SPECT) with ^{99m}Tc (*17*). Experiments with scVEGF-based tracers revealed VEGF receptor-mediated selective accumulation of such tracers by endothelial cells in tumor and surrounding host vasculature (*17*). Importantly, endogenous VEGF did not prevent enhanced uptake of the tracers in these compartments (*17*).

Although scVEGF-based targeted tracers displayed favorable imaging properties and relatively low uptake in nontargeted organs, we were concerned that making chelator/protein conjugates requires additional production steps that would present a separate set of obstacles for clinical tracer development.

Over the years, several groups reported that proteins engineered to have an additional cysteine with a free thiol group might be directly radiolabeled with ^{99m}Tc (*23*−*25*). Therefore, we tested whether Cys-tag in scVEGF can be directly radiolabeled with ^{99m}Tc and whether it would lead to a tracer with favorable imaging, biodistribution, and stability profile. We report here that direct labeling of scVEGF with ^{99m}Tc is a highly efficient process, providing a stable tracer with good imaging characteristics and a great reduction in nonspecific uptake, most notably in the kidney, as compared with HYNIC-labeled scVEGF. We expect that the simplicity of direct radiolabeling of the Cys-tag would be beneficial for the development of scVEGF and other protein-based SPECT tracers.

EXPERIMENTAL PROCEDURES

Production of Cys-Tagged scVEGF. The construction, expression, and purification of Cys-tagged scVEGF have been described recently (*17, 19, 22*). The protein was refolded in red-ox buffer containing glutathione, and therefore, in the final preparation of scVEGF the C4 thiol group in the Cys-tag was "protected" in a mixed disulfide bond with glutathione. Prior

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to radiolabeling, the C4 thiol group in scVEGF was "deprotected" by incubating scVEGF with equimolar amounts of DTT at 25 °C for 20 min in 0.1 M Tris−HCl pH 8.0 (*17*). Free SH groups were detected and quantitated by reaction with *N*-(1-pyrene)-maleimide as previously described elsewhere (*26*).

scVEGF Direct Radiolabeling with ^{99m}Tc. Deprotected scVEGF was radiolabeled, using [^{99m}Tc]-tricine as a precursor complex (*27*). Briefly, lyophilized tin-tricine reagent was reconstituted with 0.25 mL of degassed saline to give a final $SnCl_2 \cdot 2H_2O$ concentration of 3 mg/mL and a final tricine concentration of 100 mg/mL (pH 7.1). 2.5−5.0 μL tin-tricine was mixed with 30−50 μg of deprotected scVEGF and 10−20 mCi of ^{99m}Tc-pertechnetate in a final volume of 50−100 μL. After 30 min of incubation at 37 °C, radiolabeled protein, named scVEGF/Tc, was purified by gel-filtration on PD-10 Sephadex-25 columns. Specific activities ranged from 130 to 200 μCi/μg protein. Radiopurity was determined by ITLC using PBS as a solvent, and we observed ^{99m}Tc batch-dependent variations in the range 90−95%. The impurities were migrating with the front and appeared to arise from small and variable amounts of colloidal forms of ^{99m}Tc that were coeluted with scVEGF/Tc from PD-10 column.

Animals and Tumor Models. 5−6 week old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). 4T1*luc* cells were implanted into the left axillary fat pad, 10^4 cells per mouse. The protocol for the animal studies was approved by the Stanford University Institutional Animal Care and Use Committee.

Stability of scVEGF/Tc in Vitro and in Vivo. Testing was done as described in Backer et al. (*17*) for scVEGF labeled with ^{64}Cu. Briefly, Balb/c mice (n = 3) were injected i.v. with scVEGF/Tc (90 μCi/0.5 μg per mouse) and sacrificed 2 min later. Blood was collected and clarified by centrifugation. Plasma samples were incubated at 37 °C. After 15, 30, and 60 min of incubation, 1 μL plasma aliquots were analyzed by ITLC for a rough estimation of protein-bound radioactivity. Stability of scVEGF/Tc was tested by incubation of the probe in PBS at room temperature or at 95 °C with consequent analysis by ITLC and SDS-PAGE.

Structural and functional integrity of scVEGF/Tc recovered from blood was tested by its ability to bind to soluble VEGFR-2 receptor KDR-Fc (R&D Systems, Minneapolis, MN) immobilized on Protein A Sepharose as described previously (*17*). Briefly, Balb/c mice (n = 3) were injected via tail vein with scVEGF/Tc (1.2 mCi, 11 μg protein). At 3, 15, and 45 min after injection of tracer, blood samples were collected via supraorbital bleed, placed in citrate buffer, and clarified by centrifugation. Plasma aliquots containing equal amounts of radioactivity were mixed with Protein A Sepharose, either empty or loaded with KDR-Fc. After 1 h binding at 4 °C, Sepharose beads were spun down, extensively washed with RIPA buffer, separated by reducing SDS-PAGE on 10% gel, and analyzed by Western blotting with VEGF-specific mouse monoclonal antibody (BD Biosciences Pharmingen, San Jose, CA).

Scatchard Analysis of scVEGF/Tc Binding to Cellular VEGFR-2. Binding analysis was performed as described elsewhere for scVEGF-PEG-DOTA conjugate radiolabeled with ^{64}Cu (*17*). Briefly, 293/KDR cells overexpressing VEGFR-2 grown on 12-well plates were shifted to serum-free medium at room temperature for 45 min. Varying amounts of scVEGF/Tc were added to cells in triplicate wells for 90 min, followed by two PBS and one high-salt (0.5 M NaCl in PBS) wash. Cells were lyzed in 1% Triton X-100 containing buffer and assayed for ^{99m}Tc activity in a scintillation well counter (140 keV level, 20% window). Nonspecific binding was determined for the same amounts of scVEGF/Tc supplemented with 100-fold molar excess of nonradiolabeled scVEGF.

Blood Clearance and Biodistribution Studies. For blood clearance and biodistribution studies, 0.1−0.15 mCi of scVEGF/Tc per mouse (n = 4) was injected via the tail vein, and serial ~50 μL blood samples were taken via supraorbital sinus at various time points after injection. Tissue samples taken 1 and 3 h after injection were counted in a gamma counter along with three samples of standard activity (1/100 of injected dose). Results were calculated as the average of the percentage of injected dose (ID) or %ID per gram (g) of tissue ± 1 standard deviation of the mean.

A-SPECT-Imaging and Autoradiography. For SPECT imaging, 1.2 mCi of scVEGF/Tc per mouse (n = 4) was injected via tail vein. SPECT images were obtained 1 h after injection with the following parameters: 360° rotation, 64 steps, 30 s per step, 0.5 mm pinhole aperture, a 64 × 64 image matrix, and a 2.7 cm FOV using a small animal SPECT imaging gamma camera (A-SPECT, LumaGEM, Gamma Medica, Los Angeles). For autoradiography, tumors and pectoralis muscle from the healthy chest side were snap-frozen immediately after imaging, cryosectioned (60 μm thickness), and exposed to a phosphor storage screen for 16 h. The phosphor screen images were read out with a laser digitizer at a resolution of 50 μm per pixel. ROI analysis of radiotracer activity was performed using *ImageQuant TL* software. The maximal cts/pixel of the rim and the average cts/pixel of the center of the tumor were normalized to corresponding contralateral pectoral muscle uptake (minimum cts/pixel).

Tumor uptake analysis was performed using parametric 3-D regions of interest (ROI), interactively placed and sized, but geometric as described previously (*28*). ROIs of the rim, center, and contralateral axillary soft tissue (BKG) were drawn. The sum of the total number of counts adjusted for volume of the rim (upper 95th percentile) and soft tissue BKG (lower fifth percentile) was tabulated from the histograms of the rim and contralateral soft tissue ROI, respectively, for each tumor-bearing animal. The sums from the 34th and 68th percentiles (central portion of histogram) determined from the ROIs of the center of each tumor were also tabulated. The uptake of the rim and center of each tumor was then divided by the contralateral soft tissue uptake and presented as ratios of rim/BKG and center/BKG, respectively.

RESULTS

Direct Radiolabeling of Cys-Tagged scVEGF with ^{99m}Tc. scVEGF is functionally active single chain version of VEGF consisting of two 3−112 amino acid fragments of human $VEGF_{121}$ genetically fused head-to-tail and expressed with N-terminal Cys-tag for site-specific modification (*17*). Since scVEGF was refolded in the red-ox buffer containing glutathione, the thiol group in Cys-tag was "protected" in mixed disulfide bond with glutathione (*17*), and therefore, it was deprotected by incubation with equimolar amounts of DTT. This was immediately followed by radiolabeling with ^{99m}Tc using tin-tricine as a precursor complex. We found that the specific activity of a typical scVEGF/Tc preparation ranged from 130 to 200 μCi/μg protein, which compared favorably to the values obtained from the radiolabeling of scVEGF-HYNIC conjugates with ^{99m}Tc.

Activity and Stability of scVEGF/Tc. We then evaluated whether scVEGF/Tc retained its native affinity for the VEGFR-2 receptor using a radioligand binding assay performed with 293/KDR cells; a cell line that constitutively overexpresses VEGFR-2 (*29*). Scatchard analyses (n = 3) revealed a disassociation constant (K_d) of 1.28 ± 0.12 nM. The measured affinity of scVEGF/Tc for VEGFR-2 was similar to the K_d values reported previously for the scVEGF-PEG-DOTA/^{64}Cu complex (*17*) and iodinated $VEGF_{165}$ (*30*). We then tested the stability of scVEGF/Tc with several assays as outlined below.

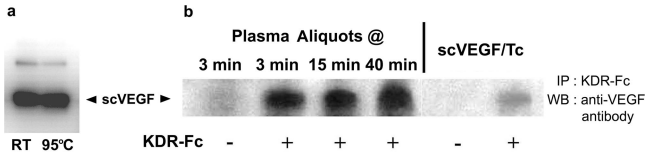

Figure 1. scVEGF/Tc stability in plasma. (A) scVEGF/Tc withstands nonreducing SDS-PAGE conditions even after a short incubation at 95 °C. Autoradiograph of nonreducing SDS-PAGE gel with scVEGF/Tc incubated at room temperature (RT) for 5 h or at 95 °C for 7 min; (B) scVEGF/Tc retains the ability to interact with soluble receptor after at least 40 min in the circulation. Western blot analysis of the SDS-PAGE gel with scVEGF/Tc was collected with KDR-Fc from blood volumes containing similar amounts of radioactivity. Free scVEGF/Tc at the indicated concentrations was similarly collected with KDR-Fc. (See Materials and Methods for experimental details.)

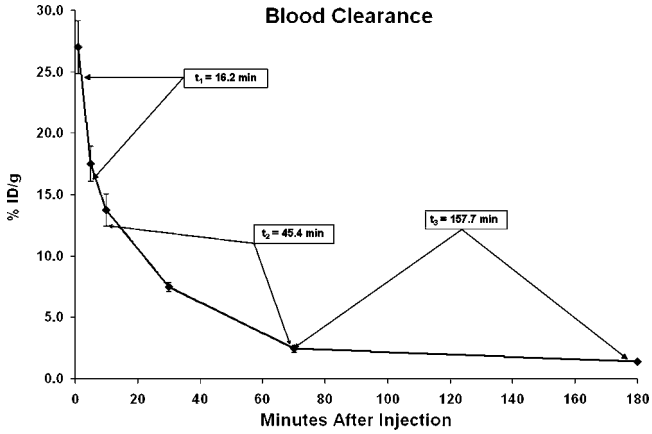

Figure 2. Blood Clearance of scVEGF/Tc. scVEGF/Tc was injected via the tail vein, 0.15 mCi per mouse ($n = 4$), and serial ~50 μL blood samples were taken via supraorbital bleed at various time points after injection. Results were calculated as the average of the percentage of injected dose (ID) per gram (g) of tissue ± standard deviation of the mean. t = half-life (in minutes). %ID/g = percent of injected dose per gram of blood.

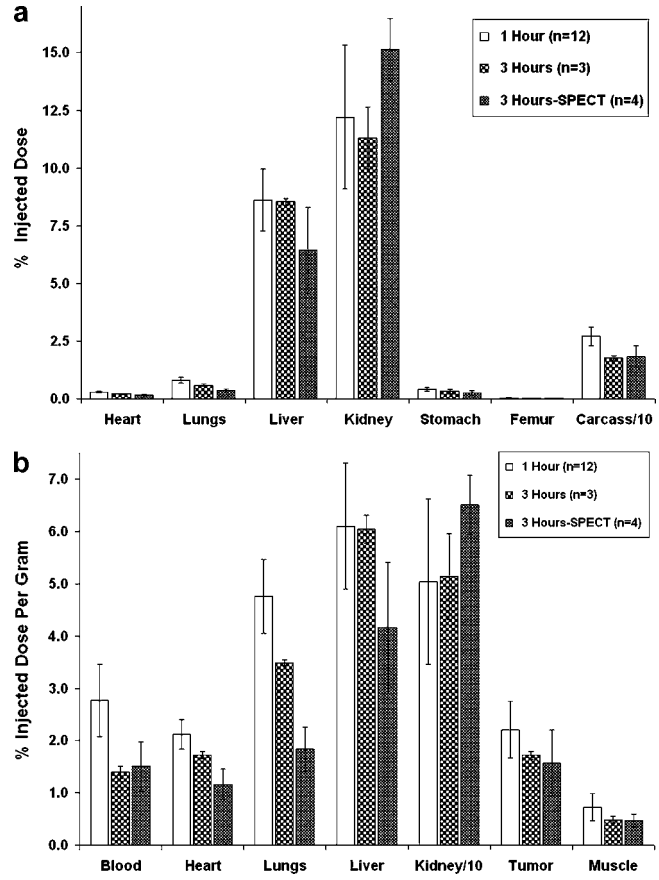

Figure 3. Biodistribution assay at 1 and 3 h after Injection of Tracer. scVEGF/Tc was injected as described in the legend to Figure 2. Biodistribution analysis was done 1 and 3 h postinjection. Three Hours-SPECT, mice received a 10-fold increased dose of tracer (1.2 mCi of radioactivity) and underwent SPECT imaging before sacrifice at 3 h. Standard deviations of the mean values are shown.

Table 1. Average Uptakes (%ID/g @ 1 h) of scVEGF-Based SPECT Tracers

tracer	kidney (%ID/g)	liver (%ID/g)
scVEGF/Tc ($n = 12$)	50.4 ± 15.8	6.1 ± 1.2
scVEGF-HYNIC/^{99m}Tc (*17*) ($n = 12$)	117.1 ± 14.0	5.9 ± 3.0
scVEGF-PEG-DOTA/^{99m}Tc (*17*) ($n = 4$)	28.0 ± 3.3	4.6 ± 1.0

The stability of scVEGF/Tc was initially tested in PBS at room temperature, and ITLC analysis indicated that over 90% of radioactivity remained bound to protein for at least 5 h. Then, we performed a 5 min incubation on an additional set of samples at 95 °C and found that at least 50% of radioactivity comigrated with scVEGF on the nonreducing SDS-PAGE gel (Figure 1a). scVEGF/Tc stability in the circulation of normal Balb/c mice was also tested. Since stability of scVEGF/Tc in vivo is dependent on the dissociation of ^{99m}Tc from protein as well as the integrity of scVEGF itself, we tested both using approaches described previously for scVEGF-HYNIC/^{99m}Tc and scVEGF-PEG-DOTA/^{64}Cu complexes (*17*). Citrated plasma was first obtained at 3, 15, and 40 min postinjection of tracer, and scVEGF/Tc complexes were then precipitated from the plasma aliquots containing the same amounts of radioactivity with a soluble form of VEGFR-2 receptor, KDR-Fc. Since binding to KDR-Fc requires structural integrity of scVEGF, we reasoned that this procedure should only select functionally active scVEGF/Tc complexes. Western blot analysis of the samples revealed that similar amounts of scVEGF were collected from each plasma sample, indicating that circulating scVEGF/Tc remained stable and capable of being a VEGF receptor for at least 40 min (Figure 1b). In this respect, the stability of scVEGF/Tc was similar to that reported for scVEGF-HYNIC/^{99m}Tc.

Blood Clearance and Biodistribution of scVEGF/Tc. scVEGF/Tc cleared rapidly from the circulation, with >95% tracer cleared within 1 h (Figure 2) after tail vein injection of tracer. The clearance rate could be best characterized by three separate exponential decay functions consisting of a rapid component in the first 10−15 min followed by a slower intermediate (20−70 min) and a prolonged terminal (80−180 min) component. The decrease in heart and lung uptake (Figure 3) paralleled the decrease in blood activity, while the tracer uptake of the remaining organs was essentially unchanged between 1 and 3 h. As summarized in Table 1, uptake in kidney obtained with scVEGF/Tc tracer was approximately one-half that seen with scVEGF-HYNIC/^{99m}Tc (*17*). We also prepared scVEGF-PEG-DOTA/^{99m}Tc tracer, based on previously described scVEGF-PEG-DOTA conjugate that proved to be efficient in decreasing nonspecific renal uptake (*17*) and found that pegylated tracer displayed kidney uptake only marginally below that of scVEGF/Tc (Table 1). Interestingly, the tumor uptake of scVEGF/Tc ranged from 1.7 to 2.5%ID/g with a relatively constant ratio of tumor/muscle uptake of 3.1 to 3.5 at 1 and 3 h, respectively.

A-SPECT-imaging and Autoradiographic Studies with scVEGF/Tc. SPECT imaging revealed marked tumor uptake as compared with soft tissue (Figure 4) in a fashion similar to our previously described forms of radiolabeled scVEGF (*14, 16, 17*). The uptake of tracer was particularly high in the tumor

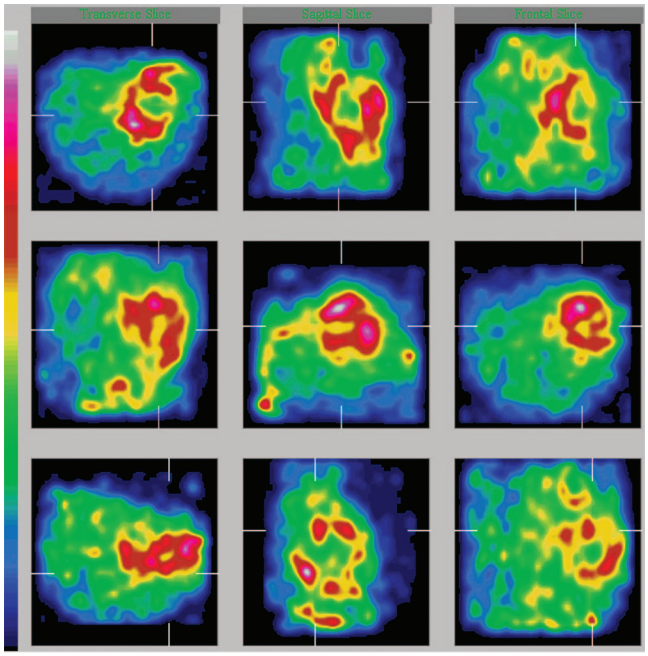

Figure 4. SPECT imaging with scVEGF/Tc. Representative transverse, sagittal, and frontal images of three 4T1 tumor-bearing mice are shown. Note the intense rim uptake of tracer at the tumor periphery that at ROI analysis was 7 to 10 times greater than soft tissue uptake of the contralateral axilla (see Figure 5). The central portions of tumor also had 3- to 4-fold greater uptake of tracer as compared with soft tissue background. Tumors weighed between 0.3 and 0.5 g (8 to 10 mm in diameter).

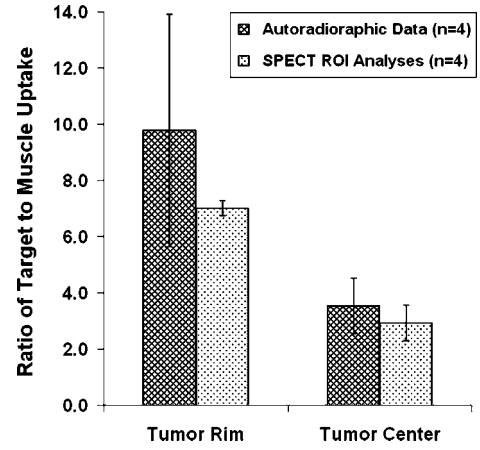

Figure 5. ROI analysis of SPECT and autoradiographic data. Multiple three-dimensional ROIs were placed on the rim and central portions of tumor from each animal that underwent SPECT imaging. Soft tissue background uptake was taken from an ROI placed in the contralateral axilla (muscle) expressed as unity. A similar type of ROI analysis was performed of histologic sections of these same tumors seen with autoradiography. The contralateral pectoralis major muscle was used for the calculation of the soft tissue background uptake of tracer. Standard deviation of mean values for the angiogenic rim and central portions of tumor are shown for both SPECT and autoradiographic ROI analyses.

periphery, in the so-called "angiogenic rim", where VEGF receptors are usually expressed at high levels. In addition, histological examination of tumor sections revealed visibly functional tumor tissue in the central portions of tumor, thereby excluding the possibility that low tracer uptake in these areas might be due to necrosis. ROI analyses of tumor and its margins seen at SPECT imaging demonstrated a rim uptake 7 to 10 times greater than muscle as well as a central tumor to muscle uptake ratio of 3 to 4 (Figure 5). Similar ratios were observed with autoradiography.

DISCUSSION

We report here that the Cys-tag in scVEGF can be directly radiolabeled with ^{99m}Tc in a site-specific fashion, yielding a tracer suitable for SPECT imaging. Over the years, several groups reported that proteins engineered to have an additional cysteine with a free thiol group could be directly labeled with ^{99m}Tc (although with different exchange agents such as tin-glucoheptonate) without modifying the protein with chelators such as HYNIC, DTPA, or DOTA (*23*−*25*). As these investigators found, the preparation of a tracer via direct site-specific radiolabeling is far less complicated than a two-step process that requires the synthesis of protein/chelator conjugate prior to radiolabeling. Direct site-specific radiolabeling of scVEGF produces a tracer with a high specific activity, high in vivo stability, rapid blood clearance, and favorable imaging characteristics similar to those of previously described scVEGF-HYNIC/^{99m}Tc (*17*). Furthermore, given the simplicity and short production time of directly labeled scVEGF, we suggest that the Cys-tag might be suitable for direct radiolabeling with ^{94}Tc for PET imaging at facilities equipped with on-site cyclotrons ($t_{1/2}$ of ^{94}Tc = 52.5 min). Unexpectedly, scVEGF/Tc displayed a superior biodistribution, with an approximately 2-fold decrease in kidney uptake of tracer, as compared to scVEGF-HYNIC/^{99m}Tc. This of course is a distinct advantage with respect to the further development of scVEGF as a new radiopharmaceutical for SPECT imaging.

In addition to the simplicity in preparation, scVEGF/Tc has several other potential advantages over several VEGF-based tracers tested by other groups for the imaging of VEGF receptors in ischemic and tumor angiogenic vasculature in humans, mice, and rabbits (*31*−*38*). These tracers were prepared by random ^{123}I-iodination of VEGF$_{121}$ and VEGF$_{165}$ (*31*−*34*), by random conjugation of ^{64}Cu chelator to a wild-type and mutant VEGF$_{121}$ (*35, 36*), by site-specific conjugation of ^{111}I chelator to Cys-116 in VEGF$_{121}$ (*37*), and by constructing transferrin-VEGF$_{165}$ fusion protein for exchange loading with ^{111}I (*38*). As our scVEGF/Tc, other tracers selectively accumulated in the areas of angiogenic vasculature with tumor uptake in the range from 1% to 7%ID/g (*31*−*34*) and reaching 15%ID/g for very small tumors (*36*). As expected, the random modification of VEGF resulted in nonuniform preparations of tracers with a significant decrease in functional activity (see, for example, refs ,*36*), as compared with unmodified protein. The decreased functional activity of radiolabeled VEGF means the loss of the ability to selectively bind to and specifically image VEGF receptors, which compromises the whole idea of molecular targeted imaging. Of note, we have previously used random modification of scVEGF with biotin to create an inactive protein control to study the specificity of scVEGF binding in vivo (*17*). Another concern with all but one (*37*) of the previously described VEGF-based tracers is the high liver uptake of these constructs (more than 30%ID/g in refs (*37, 38*) and more than 15% in refs *35, 36*).

In summary, we have found that the Cys-tag of scVEGF can be used to site-specifically attach ^{99m}Tc without the need for a bifunctional chelator such as HYNIC. The labeling process is both fast and efficient and has no demonstrable effect on the native binding affinity of scVEGF to VEGFR-2. The tracer also is stable in vivo with rapid blood clearance and has half the renal uptake of ^{99m}Tc-HYNIC-scVEGF. scVEGF/Tc also displays excellent SPECT imaging characteristics and is able to define both central tumor uptake and its angiogenic rim in a fashion similar to that observed at autoradiography. The ease of labeling and the simplicity of production make scVEGF/Tc

a promising tracer for the oncology clinic in the near future. Furthermore, considering that direct radiolabeling described here depends on Cys-tag, rather than on the nature of Cys-tagged protein, we expect that this strategy might be used as a standardized approach to the development of protein-based SPECT tracers.

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