Vascular endothelial growth factor (VEGF), released by tumor cells, is an important growth factor in tumor angiogenesis. The humanized monoclonal antibody bevacizumab blocks VEGF-induced tumor angiogenesis by binding, thereby neutralizing VEGF. Our aim was to develop radiolabeled bevacizumab for noninvasive in vivo VEGF visualization and quantification with the single γ-emitting isotope $^{111}$In and the PET isotope $^{89}$Zr.

Methods: Labeling, stability, and binding studies were performed. Nude mice with a human SKOV-3 ovarian tumor xenograft were injected with $^{89}$Zr-bevacizumab, $^{111}$In-bevacizumab, or human $^{89}$Zr-IgG. Human $^{89}$Zr-IgG served as an aspecific control antibody. Small-animal PET and microCT studies were obtained at 24, 72, and 168 h after injection of $^{89}$Zr-bevacizumab and $^{89}$Zr-IgG (3.5 ± 0.5 MBq, 100 ± 6 μg, 0.2 mL [mean ± SD]). Small-animal PET and microCT images were fused to calculate tumor uptake and compared with ex vivo biodistribution at 168 h after injection. $^{89}$Zr- and $^{111}$In-bevacizumab ex vivo biodistribution was compared at 24, 72, and 168 h after injection (2.0 ± 0.5 MBq each, 100 ± 4 μg in total, 0.2 mL).

Results: Labeling efficiencies, radiochemical purity, stability, and binding properties were optimal for the radioimmunoconjugates. Small-animal PET showed uptake in well-perfused organs at 24 h and clear tumor localization from 72 h onward. Tumor uptake determined by quantification of small-animal PET images was higher for $^{89}$Zr-bevacizumab—namely, 7.38 ± 0.016 %ID/g compared with 3.39 ± 1.16 %ID/g (percentage injected dose per gram) for human $^{89}$Zr-IgG ($P = 0.011$) at 168 h and equivalent to ex vivo biodistribution studies. Tracer uptake in other organs was seen primarily in liver and spleen. $^{89}$Zr- and $^{111}$In-bevacizumab biodistribution was comparable. Conclusion: Radiolabeled bevacizumab showed higher uptake compared with radiolabeled human IgG in a human SKOV-3 ovarian tumor xenograft. Noninvasive quantitative small-animal PET was similar to invasive ex vivo biodistribution. Radiolabeled bevacizumab is a new tracer for noninvasive in vivo imaging of VEGF in the tumor microenvironment.

Key Words: VEGF; bevacizumab; small-animal PET; xenograft; in vivo

Several factors are involved in the development and growth of tumors. Angiogenesis, the formation of new blood vessels, is one of these factors. New vasculature allows tumor cells to execute their critical growth by supplying the tumor with nutrients and oxygen, enabling disposal of metabolic waste products and providing a route for metastatic spreading (1,2). An important factor involved in angiogenesis is vascular endothelial growth factor A (hereafter referred to as VEGF) (3). VEGF consists of at least 4 splice variants, containing 121, 165, 189, and 206 amino acids (3). It is produced by tumor cells and induces tumor neovascularization (4–7).

Overexpression of VEGF occurs in many human tumor types, which makes VEGF a rational target for antiangiogenic therapy (8–11). This has led to interest in blocking the signaling of VEGF in human tumors. Antibodies binding to this ligand and its receptors, and chemical molecules that can block the tyrosine kinase function of VEGF receptors, have been developed (12).

The drug that is currently most widely used in the clinic is the humanized monoclonal antibody bevacizumab. It blocks VEGF-induced endothelial cell proliferation, permeability, and survival, and it inhibits human tumor cell line growth in preclinical models (8,13–15). The likely mechanism is that bevacizumab binds to VEGF—both soluble and bound to the extracellular matrix—and thereby prevents VEGF binding to its receptors, blocking the biologic pathways induced after VEGF binding. Bevacizumab is approved for clinical use in metastatic colon carcinoma and non–small cell lung cancer (16,17). Approval for several other tumor types is expected soon.
Despite the success of antiangiogenic therapy, a large percentage of patients does not benefit from this targeted therapy. Currently, it is impossible to predict which patient will benefit from antiangiogenic therapy. Reasons for treatment failure may be that the target for the drug is not present or that the drug may not reach the target. Tumor cells produce VEGF, which can lead to paracrine effects in the microenvironment. VEGF_{121} is freely soluble, whereas VEGF_{165} is secreted, though a significant fraction remains localized to the extracellular matrix, such as VEGF_{189} and VEGF_{206} (18). This will most likely lead to locally high VEGF levels. It is currently impossible to evaluate these local VEGF levels (19). Noninvasive measurement of VEGF in the tumor might give insight to the available target for VEGF-dependent antiangiogenic therapy and thus assist in tumor response prediction. To allow in vivo VEGF imaging, we set up bevacizumab labeling with the relatively new long-lived PET isotope $^{89}$Zr and the widely available single $\gamma$-emitting isotope $^{111}$In (20–23).

In the present study, we describe the development and the in vivo evaluation of $^{89}$Zr-bevacizumab and $^{111}$In-bevacizumab in a tumor-bearing mouse model and compare noninvasively obtained in vivo $^{89}$Zr-bevacizumab and $^{89}$Zr-IgG imaging quantification data with ex vivo biodistribution data.

**MATERIALS AND METHODS**

**Conjugation and $^{89}$Zr Labeling of Bevacizumab and Control IgG**

Bevacizumab (Avastin, 25 mg/mL; Roche) and human IgG (Immunoglobuline I.V.; Sanquin) conjugation and labeling were performed as described previously by Verel et al. (22). Human IgG served as an aspecific control antibody. Briefly, bevacizumab and IgG were purified from other excipients by ultrafiltration (Vivaspin-2; Sartorius) and diluted in water for injection to 5 mg/mL. Hereafter, conjugation of purified bevacizumab and IgG was achieved using N-succinyl-desferrioxamine B-tetrafluorphenol (N-sucDF-TFP; VU Medical Center). The ester and the antibodies (bevacizumab and human IgG) were conjugated at room temperature for 30 min at pH 9.5–9.7 (0.1 mol/L Na$_2$CO$_3$; Befa) with 1.5–2.5 chelate groups per antibody molecule. After conjugation, the mixture was set to pH 4.2–4.4 (0.1 mol/L H$_2$SO$_4$; University Groningen), and stored at $-20^\circ$C.

The stability of $^{89}$Zr-bevacizumab, $^{89}$Zr-IgG, and $^{111}$In-bevacizumab was determined by storing the final product (1 mg, 50 MBq) at $2-8^\circ$C and in 1 mL ammonium acetate (50 mmol/L; UMC Groningen), pH 5.5, to eliminate the excess unconjugated ITC-DTPA. Purified conjugated bevacizumab was diluted (5 mg/mL) in ammonium acetate and stored at $-20^\circ$C. Labeling was performed with 1 mg of purified ITC-DTPA-bevacizumab, which was allowed to react with 50 MBq of $^{111}$InCl$_3$ (370 MBq/mL, >1.85 GBq/µg $^{111}$In, 99.9% radionuclidic purity; Tyco Health Care) for 1 h at room temperature. Glassware, materials, and solutions used for the conjugation and labeling procedures were sterilized, pyrogen-free, and metal-free.

**Quality Control of $^{89}$Zr-Bevacizumab, $^{111}$In-Bevacizumab, and $^{89}$Zr-IgG**

The radiochemical purity of the radiolabeled antibodies was determined by size-exclusion high-performance liquid chromatography (SE-HPLC), trichloroacetic acid protein precipitation (TCA) ($^{89}$Zr-antibodies), and instant thin-layer chromatography (ITLC) ($^{111}$In-bevacizumab) to differentiate the labeled product from aggregates and unlabeled $^{89}$Zr and $^{111}$In.

The HPLC system used consisted of a Waters 1500 series manual injector with a 20-µL injection loop (Rhodyne 7725i injector; Milford), a Waters 1525 binary HPLC pump, a Waters 2487 dual-wavelength absorbance detector, and an in-line radioactive detector made of a sodium iodide crystal coupled to a multichannel analyzer (Ortec). Chromatograms were analyzed using the Breeze software (Waters). A Superox 200 30/300 GL size-exclusion column (Amersham Biosciences) was used. The mobile phase consisted of phosphate-buffered saline ([PBS] 140 mmol/L NaCl, 9 mmol/L Na$_2$HPO$_4$, 1.3 mmol/L NaH$_2$PO$_4$; pH = 7.4; UMC Groningen) containing 10% methanol and 23 g/L NaCl. The flow was 0.75 mL/min and the UV-detector wavelengths were set to 220 and 280 nm. The column performance was tested using a reference Bio-Rad gel-filtration standard. The retention time of bevacizumab was 16.7 min. TCA precipitation was performed by mixing equal amounts of water for injection, 20% trichloroacetic acid, and 3 µL $^{89}$Zr-bevacizumab reaction solution. The mixture was centrifuged and the radiochemical purity was determined by separation of the protein fraction and supernatant. The radioactivity in the fractions was measured by an LKB-1282-Compu-gamma-system (LKB Wallac).

ITLC was performed on silicone gel strips using 0.15 mol/L citrate buffer (pH 6.0) as the mobile phase. Radioactivity was determined by an ITLC scanner (VCS-101; Veenstra Instruments).

**In Vitro Evaluation of Radiolabeled Compounds**

The stability of $^{89}$Zr-bevacizumab, $^{89}$Zr-IgG, and $^{111}$In-bevacizumab was determined by storing the final product (1 mg, 50 MBq) in 1 mL ammonium acetate (50 mmol/L) at 4°C and in 1 mL human serum (UMC Groningen) at 37°C for 7 d. SE-HPLC, TCA, and ITLC procedures were performed 24, 72, and 168 h after labeling.

The binding properties for VEGF of $^{89}$Zr-bevacizumab, $^{111}$In-bevacizumab, and $^{89}$Zr-IgG were evaluated using a VEGF-coated enzyme-linked immunosorbent assay, comparable with the method described by Collingridge et al. (25). Briefly, recombinant human VEGF$_{165}$ (catalog no. 293-VE/CF; R&D systems) was used as the
target antigen and coated to Nunc-ImmuNo BreakA part plates (NUNC). Recombinant human VEGF165 was diluted in PBS to a concentration of 5 μg/mL. The solution was adjusted to pH 9.2–9.5 (50 mmol/L Na2CO3). Fifty microliters were added to the wells, incubated overnight at 4°C, and then blocked with 1% human serum albumin (HSA; Sanquin) in PBS. After blocking, the plates were washed with 0.1% polysorbate 80 (Sigma-Aldrich) in PBS. 89Zr-Bevacizumab, 111In-bevacizumab, and 89Zr-IgG solutions were diluted in PBS (concentrations, 1.0–1.0 × 106 ng/mL), added to the wells, and incubated for 2 h. Subsequently, the reaction solution was collected from the wells in 2 wash steps. Both the sustained radioactivity stacked to the antigen-coated wells and the collected reaction solution containing unbound radiolabeled antibody were measured by an LKB-1282-Compu-gamma-system. The percentage of binding was calculated as the fraction of radioactivity stacked to antigen-coated wells divided by the total amount of radioactivity added.

Cell Culture and Animal Studies

The human ovarian cancer cell line SKOV-3 was cultured in Dulbecco’s modified Eagle medium (DMEM; UMC Groningen) with 4.5 g/mL glucose and 10% fetal calf serum. SKOV-3 cells were chosen because of identified high VEGF production in vivo (26). Before animals were inoculated, the SKOV-3 cells were harvested by trypsinization and resuspended in culture medium and Matrigel (BD Bioscience). In vivo imaging and biodistribution experiments were conducted using male athymic mice (HSD; Athymic nude-nu) obtained from Harlan Nederland. At 8 wk of age, the mice were injected subcutaneously with 1 × 106 SKOV-3 cells mixed equally with 0.1 mL Matrigel. Animals were used for in vivo studies when the tumor measured between 6 and 8 mm in diameter. Mice were sacrificed at 24 h after injection in the animal, at which time the tumors were excised, rinsed for residual blood, and weighed. Samples and primed standards were counted for radioactivity in a calibrated well-type LKB-1282-Compu-gamma-system and corrected for physical decay. Biodistribution samples were measured using dual-isotope counting programs for 89Zr and 111In, enabling comparison between 89Zr-bevacizumab and 111In-bevacizumab distribution. Tissue activity is expressed as the %ID/g.

All animal experiments were approved by the Animal Experiment Committee of the University of Groningen.

Immunohistochemistry

Frozen-embedded tumors were stained with antibodies against mouse CD31 (PEGAM-1; BD Pharmingen) and human IgG (Dako). Anti-CD31 staining was performed to determine the microvessel density in the tumors of mice that received 89Zr-bevacizumab or 89Zr-IgG. Microvessel density was scored in 3 areas, defined as hot spot areas with the maximum number of microvessels, by using a calibrated grid. Anthuman IgG staining was performed to obtain more detailed information about the localization of the tracers. The slides were examined at ×200 magnification.
demonstrating almost equal binding characteristics of radiolabeled and unlabeled bevacizumab. These results ensure adequate VEGF binding by $^{89}$Zr- and $^{111}$In-bevacizumab.

**Animal Studies**

microPET and microCT fusion enabled excellent tumor visualization and quantification (Fig. 1). microPET images showed uptake in well-perfused organs and plasma at 24 h after injection, which declined at later time points. Increasing tumor-to-blood ratio resulted in clear tumor localization of $^{89}$Zr-bevacizumab from 72 h onward after injection (Fig. 2). In addition, uptake of $^{89}$Zr-bevacizumab was predominantly seen in liver and spleen. Quantitative measurement of $^{89}$Zr-bevacizumab in the tumor by microPET was significantly higher compared with human $^{89}$Zr-IgG—namely, $7.38 \pm 2.06$ %ID/g compared with $3.39 \pm 1.16$ %ID/g ($P = 0.011$) at 168 h after injection (Fig. 3). Similar results were seen in biodistribution experiments when tumors were excised and counted in a $\gamma$-counter: Tumor uptake was $6.82 \pm 1.80$ %ID/g for $^{89}$Zr-bevacizumab compared with $2.87 \pm 0.48$ %ID/g for human $^{89}$Zr-IgG ($P = 0.006$) (Fig. 3). Beside tumor uptake, ex vivo biodistribution experiments showed an equal organ distribution pattern for $^{89}$Zr-bevacizumab and $^{89}$Zr-IgG (Fig. 4), except for kidney uptake, which was significantly higher for $^{89}$Zr-IgG compared with $^{89}$Zr-bevacizumab ($P = 0.004$).

The ex vivo biodistribution for $^{89}$Zr-bevacizumab and $^{111}$In-bevacizumab was similar at 24, 72, and 168 h after injection (Table 1), demonstrating that both tracers could be used for VEGF imaging.

**Immunohistochemistry**

There was no difference in microvessel density or blood vessel distribution pattern between the tumors of the $^{89}$Zr-bevacizumab– and the $^{89}$Zr-IgG–injected mice. Antihuman IgG staining was seen primarily in the blood vessels of the tumor (Fig. 5).

**DISCUSSION**

$^{89}$Zr and $^{111}$In labeling of bevacizumab resulted in high labeling efficiencies and adequate preservation of VEGF-binding properties. Tumor uptake of $^{89}$Zr-bevacizumab and $^{111}$In-bevacizumab were both significantly higher compared with that of the control $^{89}$Zr-IgG. Tumor uptake of $^{89}$Zr-bevacizumab and $^{111}$In-bevacizumab was already high at 24 h after injection, increasing in time with clear tumor visualization from 72 h onward after injection. These results demonstrate that radiolabeled bevacizumab is a potential new tracer for noninvasive imaging of VEGF in the microenvironment of the tumor.

Two human anti-VEGF antibodies, VG76e and HumMV833, have been used previously for noninvasive
VEGF imaging (25, 27). 125I- and 124I-labeled VG76e—an IgG1 mouse monoclonal anti-VEGF antibody that recognizes the 121, 165, and 189 isoforms of human VEGF-A—showed specific tumor targeting in a human fibrosarcoma xenograft mouse model. Maximum uptake was seen after 24 h, declining at 48 h after injection. These results are in contrast to our results, in which radiolabeled bevacizumab did not show tumor clearance up to 168 h after injection, which increases the available time for imaging. Jayson et al. (27) used 124I-HuMV833, a humanized monoclonal IgG4κ antibody that binds VEGF121 and VEGF165, to perform PET studies on patients with various progressive solid tumors. Tumor uptake of 124I-HuMV833 was highly variable between and within patients. For example, there was high uptake of 124I-HuMV833 in an ovarian tumor and low uptake in a poorly vascularized metastasis from colon cancer. These differences may represent the variation in available target for the antibody between tumor types and lesions, which could have implications for anti-VEGF therapy (27). The monoclonal antibody bevacizumab that we used in this study, with high binding affinity to all VEGF-A isoforms and proven clinical effectiveness, is more attractive and accessible for this approach. Another point of application in the evaluation of anti-VEGF therapy could be VEGF-receptor imaging. Cai et al. described the development of VEGF-receptor imaging with 64Cu-DOTA-VEGF121 (DOTA is 1,4,7,10-tetraazacyclododecane-N,N', N"-tetraacetic acid) (28). They showed that VEGF-receptor imaging with 64Cu-DOTA-VEGF121 corresponded with immunohistochemical analysis in a human glioblastoma-bearing mouse model. At this point, the correlation between levels of VEGF expression and VEGF-receptor expression is not elucidated. Therefore, in vivo VEGF-receptor imaging with 64Cu-DOTA-VEGF121 might facilitate the evaluation of VEGF-receptor expression, whereas radiolabeled bevacizumab could be used to evaluate VEGF levels. Both techniques could be complementary in the clinical evaluation of antiangiogenic therapy.

In the present study, we have shown that microPET and CT imaging, using 89Zr-bevacizumab, not only allowed imaging up to 168 h after injection but also enabled quantitative measurement of the tracer in the tumor. Tumor uptake values as assessed by noninvasive microPET quantification of 89Zr-bevacizumab were identical to uptake values as assessed by γ-counting of excised tumors, demonstrating the potential role of PET quantification beside in vivo visualization. The biodistribution of bevacizumab was investigated earlier by Lin et al. in rabbits with 125I-labeled bevacizumab; however, the uptake of bevacizumab was only investigated in normal tissues (29). The results were similar to
our findings, with high uptake in heart and blood after 24 h, decreasing in time, and high uptake in liver and spleen at later time points. Our study additionally demonstrated that the tumor is a major compartment, besides plasma, liver, and spleen, in bevacizumab distribution.

We used 100 μg of $^{89}$Zr-bevacizumab for imaging and biodistribution studies. Smaller doses of bevacizumab could result in accelerated and increased clearance of $^{89}$Zr- and $^{111}$In-bevacizumab in the absence of IgG production by the nude mice (30). As a control, we used $^{89}$Zr-labeled human IgG to provide evidence that $^{89}$Zr-bevacizumab uptake is due to specific targeting in the tumor and is not caused by blood perfusion and passive diffusion within the tumor. Another frequently used approach to show specificity is blocking with an excess of the unlabeled agent (25). However, the use of an excess of unlabeled bevacizumab (up to 10-fold) could induce functional changes and, in this way, change the perfusion and uptake of radiolabeled bevacizumab in the tumor at later time points (31). Vascular changes after a single infusion of bevacizumab have been described earlier. Willett et al. showed a decreased microvessel density in rectal cancer patients already at 12 d after a single infusion of bevacizumab (31). To exclude that these effects were present in our study—due to 100 μg of radiolabeled bevacizumab—the microvessel density was determined in the tumors of the $^{89}$Zr-bevacizumab- and $^{89}$Zr-IgG–injected mice. There was no difference in microvessel density between the tumors of the 2 groups. These findings substantiate the result that high uptake of radiolabeled bevacizumab is due to specific targeting and not a result of vascular changes.

To obtain more detailed information with regard to the localization of radiolabeled bevacizumab (humanized IgG1 antibody) in the tumor, we performed antihuman IgG staining. Antihuman IgG was predominantly present in the localization of radiolabeled bevacizumab. Antihuman IgG staining was predominantly present in the blood vessels of the tumors. Cell-surface binding of antihuman IgG was not clearly evident. These results suggested that radiolabeled bevacizumab binds primarily to VEGF present in the blood vessels of the tumor. Binding to VEGF localized in the extracellular matrix of tumor cells could not be demonstrated.

In the present study, we used 2 isotopes for the development of radiolabeled bevacizumab. The PET isotope $^{89}$Zr has good characteristics for antibody imaging. The relatively long half-life of 3.27 d makes imaging possible—matching the pharmacokinetics of bevacizumab (half-life = 17–21 d)—combined with the advantages of high-resolution PET imaging. Borjesson et al. described the first clinical application of $^{89}$Zr, using labeled U36, an anti-CD44v6 antibody.
to detect lymph node metastases in head and neck cancer patients (23). 89Zr will be commercially available soon. 89Zr might give a relatively high radiation exposure to patients, due to the high-energy, high-abundance γ-ray (909 keV, 99.9%) and low-abundance β+ (22%) emitted during the decay of 89Zr (32). However, the clinical dose for adequate 89Zr-bevacizumab PET has yet to be established. Increased sensitivity of new-generation PET cameras could further reduce the radiation exposure. As an alternative for PET, we also developed 111In-labeled bevacizumab for SPECT, as we described previously for trastuzumab, a HER-2 neu antibody (20). The ex vivo biodistribution results for 111In-bevacizumab were similar to those of 89Zr-bevacizumab. Both 111In-bevacizumab and 89Zr-bevacizumab can be used clinically for noninvasive in vivo VEGF imaging, although the feasibility of 111In-bevacizumab for in vivo quantification was not established by this study.

CONCLUSION

This study illustrates the potential of 89Zr-bevacizumab and 111In-bevacizumab as a specific VEGF tracer, with excellent in vivo quantitative measurement of 89Zr-bevacizumab. Noninvasive measurement of VEGF levels in the tumor could provide essential information in the evaluation of antiangiogenic therapy. This could lead to better understanding and patient-tailored therapy.

REFERENCES

In Vivo VEGF Imaging with Radiolabeled Bevacizumab in a Human Ovarian Tumor Xenograft


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