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High-Resolution SPECT-CT/MR Molecular Imaging of Angiogenesis in the Vx2 Model

Michal Lijowski, PhD^{*}, Shelton Caruthers, PhD^{*,†}, Grace Hu, MS^{*}, Huiying Zhang, MD^{*}, Michael J. Scott, BS^{*}, Todd Williams, BS^{*}, Todd Erpelding, PhD^{*,‡}, Anne H. Schmieder, MS^{*}, Garry Kiefer, PhD[§], Gyongyi Gulyas, PhD[¶], Phillip S. Athey, PhD[¶], Patrick J. Gaffney, PhD^{||}, Samuel A. Wickline, MD^{*}, and Gregory M. Lanza, MD, PhD

^{*}Washington University Medical School, St Louis, MO

[†]Philips Healthcare, Andover, MA

[‡]Philips Research North America, Briarcliff Manor, NY

[§]Macrocyclics, Dallas, TX

[¶]Dow Chemical Company, Freeport, TX

^{||}St Thomas Hospital, London, UK

Abstract

Background—The use of antiangiogenic therapy in conjunction with traditional chemotherapy is becoming increasingly in cancer management, but the optimal benefit of these targeted pharmaceuticals has been limited to a subset of the population treated. Improved imaging probes that permit sensitive detection and high-resolution characterization of tumor angiogenesis could improve patient risk-benefit stratification.

Objectives—The overarching objective of these experiments was to develop a dual modality $\alpha_v\beta_3$ -targeted nanoparticle molecular imaging agent that affords sensitive nuclear detection in conjunction with high-resolution MR characterization of tumor angiogenesis.

Materials and Methods—In part 1, New Zealand white rabbits (n = 21) bearing 14d Vx2 tumor received either $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles at doses of 11, 22, or 44 MBq/kg, nontargeted ^{99m}Tc nanoparticles at 22 MBq/kg, or $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles (22 MBq/kg) competitively inhibited with unlabeled $\alpha_v\beta_3$ -nanoparticles. All animals were imaged dynamically over 2 hours with a planar camera using a pinhole collimator. In part 2, the effectiveness of $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles in the Vx2 rabbit model was demonstrated using clinical SPECT-CT imaging techniques. Next, MR functionality was incorporated into $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles by inclusion of lipophilic gadolinium chelates into the outer phospholipid layer, and the concept of high sensitivity – high-resolution detection and characterization of tumor angiogenesis was shown using sequential SPECT-CT and MR molecular imaging with 3D neovascular mapping.

Results— $\alpha_v\beta_3$ -Targeted ^{99m}Tc nanoparticles at 22 MBq/kg produced the highest tumor-to-muscle contrast ratio (8.56 ± 0.13 , TMR) versus the 11 MBq/kg (7.32 ± 0.12) and 44 MBq/kg (6.55 ± 0.07) doses, ($P < 0.05$). TMR of nontargeted particles at 22.2 MBq/kg (5.48 ± 0.09) was less ($P < 0.05$) than the equivalent dosage of $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles. Competitively inhibition of ^{99m}Tc $\alpha_v\beta_3$ -integrin-targeted nanoparticles at 22.2 MBq/kg reduced ($P < 0.05$) TMR (5.31 ± 0.06) to the

Reprints: Gregory M. Lanza, MD, PhD, Washington University Medical School, 4320 Forest Park Ave, Cortex, Suite 101, CB 8215, Saint Louis, MO 63108. E-mail: E-mail: greg@cvu.wustl.edu.

S.A.W. and G.M.L. are cofounders Kereos, Inc, St. Louis, MO.

nontargeted control contrast level. Multislice CT imaging could not distinguish the presence of Vx2 tumor implanted in the popliteal fossa from lymph nodes in the same fossa or in the contralateral leg. However, the use of ^{99m}Tc $\alpha_v\beta_3$ -nanoparticles with SPECT-CT produced a clear neovasculature signal from the tumor that was absent in the nonimplanted hind leg. Using $\alpha_v\beta_3$ -targeted ^{99m}Tc -gadolinium nanoparticles, the sensitive detection of the Vx2 tumor was extended to allow MR molecular imaging and 3D mapping of angiogenesis in the small tumor, revealing an asymmetrically distributed, patchy neovasculature along the periphery of the cancer.

Conclusion—Dual modality molecular imaging with $\alpha_v\beta_3$ -targeted ^{99m}Tc -gadolinium nanoparticles can afford highly sensitive and specific localization of tumor angiogenesis, which can be further characterized with high-resolution MR neovascular mapping, which may predict responsiveness to antiangiogenic therapy.

Keywords

angiogenesis; nanoparticle; neoplasia; SPECT-CT; MRI

Molecular imaging strives to noninvasively detect and characterize nascent pathology based upon the unique presentation of biomarkers that differentiate abnormal target cells from normal counterparts. The detection of occult biochemical epitopes, particularly in cancer, requires high sensitivity probes using low-resolution scanning techniques, but quantitative, high-resolution imaging is needed to clearly identify and characterize the soft tissue pathology. From a clinical perspective, nuclear imaging offers the highest sensitivity for detecting scant biomarkers within substantial anatomic regions, but these techniques provide poor resolution of minute pathologies when compared with magnetic resonance imaging (MRI), for example. Conversely, MRI offers extraordinary high-resolution molecular imaging of small, soft tissue pathology with the emerging cadre of nanoparticle agents,^{1–4} but such imaging requires prior knowledge of the tumor location for coil selection and placement.

Angiogenesis, defined as the formation of new blood vessels from pre-existing vasculature, is a central feature of many pathologies, including cancer, diabetic retinopathy, and inflammatory diseases,⁵ which has garnered significant interest from the molecular imaging community. In particular, $\alpha_v\beta_3$ -integrin, a heterodimeric transmembrane glycoprotein, mediates cellular adhesion to several extracellular matrix protein ligands through a specific Arg-Gly-Asp (RGD)-binding site.^{6,7} Although it is not essential for angiogenesis,⁸ the differential up-regulation of $\alpha_v\beta_3$ -integrin on proliferating versus quiescent endothelial cells is frequently used as a neovascular biomarker and as an attractive target for molecular imaging and tumor antiangiogenesis treatments.^{1–4,9–16} Unfortunately, $\alpha_v\beta_3$ -integrin is not pathognomonic for angiogenic endothelium^{17,18} and is expressed by a broad array of cell types including macrophages,¹⁹ platelets²⁰ lymphocytes,²⁰ smooth muscle cells,²¹ and tumor cells.^{22–25}

Numerous radiolabeled $\alpha_v\beta_3$ -integrin or vitronectin antagonists, including antibodies, peptides, peptidomimetics, and disintegrins, have been explored as tumor vasculature-targeting agents.^{26–35} Although these agents can be exquisitely specific for $\alpha_v\beta_3$ -integrin, their penetration beyond the circulation allows binding to a cadre of nonendothelial sources. PFC nanoparticles (250-nm diameter) are constrained by size to the vasculature, which is expected for agents greater than 120 Kda or 100 nm unless vascular integrity is significantly disrupted.^{36,37}

We have previously reported specific high-resolution MR molecular imaging of neovascular-rich pathology using $\alpha_v\beta_3$ -paramagnetic nanoparticles (NP) in a variety of in vivo studies,^{3, 4,16,38,39} and recently reported the utility of ^{111}In $\alpha_v\beta_3$ nanoparticles to provide a high sensitivity, low-resolution signal from the tumor neovasculature.⁴⁰ In the present study, we explored the more clinically relevant and challenging opportunity of combining ^{99m}Tc imaging and MRI to provide high sensitivity detection with high-resolution 3D neovasculature

mapping. The first objective of this study was to develop and characterize $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles in vivo with respect to sensitivity, specificity, and dose. The second objective was to demonstrate enhancement of $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles, used for high resolution MR molecular imaging, with ^{99m}Tc functionality that could sensitively localize occult pathology for high resolution characterization, which was predictive of responsiveness to antiangiogenic therapy in animal models.^{16,39}

Materials and Methods

Preparation of Nanoparticles

The emulsified perfluorooctylbromide (PFOB) nanoparticles, prepared as reported earlier,^{4,16,40} contained 20% (vol/vol) of PFOB (Exflur Corp., Round Rock, TX), 2% (wt/vol) of a surfactant, and deionized water for the balance. The surfactant comixture of the integrin-targeted ^{99m}Tc nanoparticles included 3 mole% bis-pyridyl-lysine-caproyl-phosphatidylethanolamine (Dow Chemical, Freeport, TX; Fig. 1), 0.1 mole% vitronectin antagonist (US Patent No. 6,322,770)^{35,41,42} complexed to PEG₂₀₀₀-phosphatidylethanolamine (Kereos, Inc., St. Louis), and high purity egg phosphatidylcholine (Avanti Polar Lipids, Inc., Alabaster, AL) for balance. The surfactant comixture of the integrin-targeted particles ^{99m}Tc -gadolinium nanoparticles included 30 mole% gadolinium diethylenetriamine-pentaacetic acid-bis-oleate (IQsynthesis, St. Louis, MO) as an equimolar substitution for the lecithin.

The surfactant comixtures were dissolved in chloroform, evaporated under reduced pressure, and dried in 50°C vacuum overnight into a lipid film. The lipids were resuspended in minimal distilled deionized water, coarse blended with perfluorooctylbromide (PFOB), diluted to 20% PFOB (wt/vol) with water, and emulsified in a M110S fluidizer (Microfluidics, Newton, MA) at 20,000 psi for 4 minutes. $\alpha_v\beta_3$ -Targeted particles were measured with a Brookhaven dynamic light scattering system (Brookhaven Instruments Corp, Holtsville, NY) at 37°C and were typically 270-nm diameter with a polydispersity index of 0.2. ^{99m}Tc was coupled to the bis-pyridyl-lysine after emulsification through a tricarbonyl precursor as described below.^{43,44}

Homing Ligands

The $\alpha_v\beta_3$ -integrin antagonist is a quinalone nonpeptide developed by Bristol-Myers Squibb Medical Imaging (US patent 6,511,648 and related patents), which was initially reported and characterized as the ^{111}In -DOTA conjugate RP478 and cyan 5.5 homologue TA145.^{35,39} The specificity of the $\alpha_v\beta_3$ -ligand mirrors that of LM609 as assessed by staining and flow cytometry and has a 15-fold preference for the Mn^{2+} activated receptor (21 nmol/L).³⁵ The IC_{50} for $\alpha_n\beta_5$, $\alpha_5\beta_1$, and GP IIb/IIIa was determined to be $> 10 \mu\text{M}$ (BMSMI, Billerica, MA; unpublished data). Perfluorocarbon nanoparticles present approximately 300 ligands/particle with an IC_{50} of 50 pM for the Mn^{2+} activated $\alpha_v\beta_3$ -integrin (Kereos, Inc.; unpublished data). The bioactivity of the $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles was confirmed and monitored using an in vitro vitronectin cell adhesion assay.^{4,39}

Preparation of ^{99m}Tc -Tricarbonyl Precursor and ^{99m}Tc Nanoparticles

Specifically, sodium borohydride NaBH_4 (0.53 M), sodium carbonate (0.14 M), and sodium tartrate (0.24 M) in 660- μL deionized water were admixed in a glass serum vial. The vial was purged with carbon monoxide for 20 minutes, then 2368 MBq of sodium pertechnetate $^{99m}\text{TcO}_4^-$ was added, and the contents heated at 100°C for 20 minutes. After equilibration to atmospheric pressure, the reaction mixture was adjusted to pH 7 with a 1:3 mixture of 0.1 M phosphate buffer (pH 7.4): 1 M HCl, and purity was determined by HPLC as described below. The reaction mixture was combined with 50- to 100- μL nanoparticles in water bath for 30 minutes at 40°C. The nanoparticle radiolabeling yield was greater than 90%

as determined by TLC developed with 0.1M sodium acetate (pH 5.18):methanol:water (20:100:200), which achieved approximately 6 atoms of ^{99m}Tc per nanoparticle.

The formation of $\text{fac-}[^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$ was confirmed by reversed-phase HPLC system (Waters Corporation, Milford, MA) and a Wizard 2480 gamma counter (PerkinElmer, Waltham, MA) for detection. HPLC conditions included: Waters SymmetryShield RP8 3.5 μm , 4.6×250 mm, reversed-phase column and a mobile phase gradient of 0.05 M triethylammonium phosphate (TEAP) pH 2.68, and methanol (MeOH). The applied gradient was: (a) 0 to 3 minutes 100% TEAP; 3 to 6 minutes, from 100% to 75% TEAP; 6 to 9 minutes from 75% to 66% TEAP, and (b) 34% to 100% MeOH from 9 to 20 minutes, 100% MeOH from 20 to 27 minutes, 100% MeOH to 100% TEAP from 27 to 30 minutes. The flow rate was 1 mL/min at ambient temperature.

Stability of the ^{99m}Tc was assessed in triplicate by coincubating 10 μL of ^{99m}Tc nanoparticles in 200 μL of fresh rabbit plasma at 37°C for 2 hours. CleanAscite HCl (300 μL) was added to the reaction mixture to precipitate the nanoparticles over 30 minutes. The supernatant was separated by low speed centrifugation for 30 minutes and counted for radioactivity in a gamma well counter. The radiolabeled nanoparticles retained 97% of the ^{99m}Tc after 2 hours in plasma, which is consistent with previous reports for labeling annexin with Tc(I)-carbonyl complex.

45

Vx-2 Rabbit Tumor Model

Male New Zealand white rabbits (~2 kg) were anesthetized with ketamine-xylazine IM and implanted with a 2- to 3-mm³ Vx-2 carcinoma tumor (DCTD Tumor Repository, National Cancer Institute, Frederick, MA) into the popliteal fossa at a depth of ~0.5 cm. After 12 days, rabbits were anesthetized with 1% to 2% of Isoflurane, intubated, and ventilated. Intravenous access was placed in a marginal ear vein of each rabbit for injection of the radiolabeled nanoparticles. Animals were monitored physiologically in accordance with a protocol approved by the Animal Studies Committee at Washington University Medical School.

Planar Imaging of Neovasculature in the Vx2 Rabbit Tumor Model

Twenty-one rabbits implanted with Vx-2 tumors were randomized into 5 treatment groups to assess the tumor-to-muscle ratio (TMR) contrast response.

1. 11 MBq/kg $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles (0.1 pmol NP/kg; n = 5)
2. 22 MBq/kg $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles (0.2 pmol NP/kg; n = 4)
3. 44 MBq/kg $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles (0.4 pmol NP/kg; n = 4)
4. 22 MBq/kg nontargeted ^{99m}Tc nanoparticles (0.2 pmol NP/kg; n = 4)
5. 22 MBq/kg $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles (0.2 pmol NP/kg) coadministered with 20-fold excess of unlabeled $\alpha_v\beta_3$ -targeted nanoparticles for competitive inhibition (n = 4).

Total injection volume (0.3 mL/kg; ~60 pmol/kg) was preserved for groups 1 to 4 with inclusion of control nanoparticles (ie, nontargeted, unlabeled, no gadolinium).

Rabbits were positioned 3 cm directly below a high-energy pinhole collimator (3-mm aperture) and imaged with a clinical Genesys single-head, gamma camera (Philips Healthcare, Andover, MA). The ^{99m}Tc images were acquired every 15 minutes for 2 hours beginning with administration of contrast media using a 20% window centered at 140 keV and a resolution of $128 \times 128 \times 16$. Image stacks were exported in DICOM format to a Linux workstation and processed with ImageJ software (<http://rsb.info.nih.gov/ij/>). Regions-of-interest (ROI) of

comparable size were manually placed around the tumor signal, muscle, and background regions to determine average pixel activity.

Demonstration of SPECT-CT/MRI Neovascular Imaging in the Vx2 Tumor Model

Imaging of tumor neovasculature with $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles was illustrated in the Vx2 rabbit tumor model using a clinical precedence SPECT-CT 16-slice scanner (Philips Healthcare). An anesthetized, male New Zealand white rabbit (~2 kg) implanted with the Vx2 was positioned prone, feet first on the table. The animal was administered 11 MBq/kg of ^{99m}Tc $\alpha_v\beta_3$ -nanoparticles [12 μL of radiolabeled nanoparticles (0.1 pmol/kg)] in 0.6 mL of nontargeted control emulsion excipient (120 pmol/kg), which was the lowest dosage of ^{99m}Tc found to allow high sensitivity screening for occult pathology. Thirty minutes postinjection, 2 overlapping rectangular CT and SPECT regions were selected to register and attenuation correct the SPECT images (FOV 350 mm; matrix 512×512 ; CT slice thickness 3.3 mm). Multislice CT settings were 250 mA/slice at 120 keV. SPECT image acquisition consisted of 64, 30-second projections (matrix 128×128 pixels) using low-energy, high resolution collimators with a 2.19 zoom and a $27.3 \text{ cm} \times 27.3 \text{ cm}$ mask.

Reconstruction of the SPECT volume from tomographic projections was performed on the JetStream Workspace 2.5.1 workstation (Philips Healthcare) with Auto-SPECT Plus 3.0 software package using a 3D ordered subset expectation maximization reconstruction algorithm, Astonish (Philips Healthcare), which included CT attenuation map, scatter, and radioisotope decay correction. Coregistration of CT and SPECT reconstructed image sets were performed using Syntegra (version 2.3.1) package on JetStream Workspace.

After establishing the effectiveness of $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles in the Vx-2 model, the lipophilic ^{99m}Tc and gadolinium chelates were incorporated together into a single nanoparticle construct for sequential imaging using a clinical precedence 16-slice scanner and an Achieva 3T medical scanner (Philips Healthcare). An anesthetized male New Zealand white rabbit (~2 kg) with a 12d Vx2 popliteal tumor was positioned on the table in a Styrofoam cradle. Six multimodal markers (IZI Medical, Baltimore, MD) each containing 0.02 mL of ^{99m}Tc (~3.7 MBq) and an aliquot of gadolinium DTPA in water were placed on the towel wrap surrounding the animal. $\alpha_v\beta_3$ -Targeted ^{99m}Tc -gadolinium nanoparticles (12 $\mu\text{L/kg}$; 11 MBq/kg, 0.1 pmol NP/kg) in 0.6 mL of nontargeted (120 pmol/kg, no gadolinium, no ^{99m}Tc , no homing ligand) were administered and image acquisition with reconstruction was performed 30 minutes later.

After the positive SPECT-CT scan, the rabbit received the remainder of the usual MR dosage of $\alpha_v\beta_3$ -targeted gadolinium nanoparticles (1.0 mL/kg; 200 pmol NP/kg). Two hours later, the rabbit was placed inside an 8-element SENSE knee coil (used for “whole body” imaging) and imaged in the MRI scanner at 3.0 T using a 3D, T_1 -weighted, fat suppressed gradient echo sequence ($250 \times 250 \text{ mm}$, 500-mm thick slices, $\text{TR/TE} = 39/5.1$ millisecond, 65-degree flip angle, 11 minutes scan time) and a 4.7-cm diameter circular surface coil placed over the tumor.

The pixel intensities from T_1 -weighted images were analyzed with MATLAB software (MathWorks, Inc., Natick, MA). Using the average Vx2 tumor signal and standard deviation determined in a previous cohort of rabbits,¹⁶ pixels with signal intensity at 2 hours greater than 3 standard deviations over the reference value were selected as enhanced. The spatial distribution of selected pixels was characterized using high-resolution 3-dimensional images reconstructed with MATLAB software. The overall 3D structure of the tumor was displayed by creating a mesh surface plot of the stacks of tumor ROIs using isosurface rendering and a smoothing filter. A surface plot of the enhancing voxels was reconstructed similarly and overlaid in blue onto the tumor volume.^{16,39}

Histology

After imaging, all animals were euthanized, and tumors were excised and quickly frozen in OCT for routine histopathology to confirm tumor pathology. In addition, fluorescence microscopy was used to assess the physiologic distribution of the integrin-targeted nanoparticles with respect to the tumor vasculature in a satellite animal. A tumor-bearing rabbit was anesthetized as described above and administered the $\alpha_v\beta_3$ -targeted rhodamine-labeled nanoparticles (1 mL/kg; 200 pmol NP/kg) followed 2 hours later by 500- μ L FITC-Lycopersicon esculentum lectin (Vector Laboratories, Burlingame, CA), a general stain for vascular endothelium, which circulated for 10 additional minutes. Next, the rabbit vasculature was then extensively perfused with saline until clear to remove unbound label, and the tumor was excised, frozen in OCT, sectioned (8 μ m), and counterstained with DAPI. Microscopic images were obtained with an Olympus BX61 microscope, F-view II B&W CCD Camera and analyzed with Olympus MicroSuite software (Olympus America Inc., Center Valley, PA).

Statistical Analysis

Data were analyzed using general linear models, which included analysis of variance (www.r-project.org) and Student *t* test (GSL packages, www.gnu.org/software/gsl) and SAS, Inc. (Cary, NC). Mean separations invoked the LSD method ($P < 0.05$). Data are presented as the mean $\pm$ standard error of the mean unless otherwise stated.

Results

In Vivo Targeting of Vx-2 Tumor With $\alpha_v\beta_3$ -Targeted ^{99m}Tc Nanoparticles

Nuclear signal from the tumor neovasculature dynamically acquired over the first 2 hours after injection of $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles increased monotonically with escalating dosages (Fig. 2). Increased Vx2 tumor signal (TMR) was rapidly and immediately recognized with the lowest ^{99m}Tc $\alpha_v\beta_3$ nanoparticles studied (7.32 ± 0.12). Doubling the dosage of $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles from 11 MBq/kg to 22 MBq/kg increased ($P < 0.05$) TMR (8.56 ± 0.13) over the 2-hour study interval but by only 17%. A second doubling of the dose to 44 MBq/kg resulted in poorer ($P < 0.05$) TMR than appreciated with the 22 MBq/kg (6.55 ± 0.07 ; Fig. 3). The response of ^{99m}Tc nanoparticles signal from the tumor neovasculature likely followed a sigmoid response function, but the number of titration levels was too few for precise modeling. The TMR of nontargeted ^{99m}Tc nanoparticles at 22 MBq/kg (5.54 ± 0.47) was lower ($P < 0.05$) than the response with $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles given at the same dose. In vivo competitive inhibition of $\alpha_v\beta_3$ -targeted ^{99m}Tc -targeted nanoparticles (22 MBq/kg) with nonlabeled $\alpha_v\beta_3$ -nanoparticles yielded a TMR (5.31 ± 0.06) equivalent to the nontargeted ^{99m}Tc nanoparticle ($P > 0.05$).

Neovascular Imaging With $\alpha_v\beta_3$ -Targeted ^{99m}Tc -Gadolinium Nanoparticles in the Vx2 Tumor Model

The concept of tumor neovascular imaging using a clinical scanner was demonstrated using a the minimal effective dosage (11 MBq/kg) of $\alpha_v\beta_3$ -targeted ^{99m}Tc -targeted nanoparticles. Figure 4 presents 2-dimensional tomographic CT images (panels A-C) of the rabbit hindquarters revealing a nodular mass or lymph node within both popliteal fossa, which cannot be discriminated visually in the images. The attenuation- and decay-corrected SPECT images in Figure 4 (panels D-F) below show a high-contrast signal associated with an ~ 1 -cm tissue mass located in the superior right aspect of the popliteal fossa that is distinguished from the adjacent and contralateral lymph nodes. Although physiologic sources of angiogenesis in this young male rabbit (testis and bone marrow) and nanoparticle clearance (bone marrow) of ^{99m}Tc signal were appreciated bilaterally, the asymmetric pathologic neovascular signal was easily discerned.

Extending these results, $\alpha_v\beta_3$ -targeted ^{99m}Tc -gadolinium nanoparticles were synthesized and administered IV to rabbits bearing nascent Vx2 adenocarcinoma to further demonstrate the principle of high sensitivity nuclear detection of neovasculature combined with high resolution MR molecular imaging and 3D neovascular mapping. SPECT-CT images obtained 30 minutes after injection of $\alpha_v\beta_3$ -targeted ^{99m}Tc -gadolinium nanoparticles (Fig. 5) were analogous to those previously shown in Figure 4. After nuclear imaging, the remaining MR dosage was injected, and T1w, fat-suppressed, gradient echo images were obtained 2 hours later. Slight misalignment of the SPECT-CT and MR images occurred, which reflected error in registering the true centers of high-resolution gadolinium MR signal and the lower-resolution ^{99m}Tc signal obtained from the peripheral fiducial markers. 3D mapping of the contrast-enhanced pixels revealed an asymmetrically distributed, patchy pattern along the periphery of the tumor that was consistent with previous MR results in this model.¹⁶

Histology

Vx2 tumors were excised from the popliteal fossa to confirm their pathology and angiogenic features, which proved to be consistent with previous reports from our laboratory.^{3,4,16,40} In general the Vx-2 tumors were typically round and between 0.6 cm and 1.5 cm or less in their greatest dimension. In contrast, $\alpha_v\beta_3$ -integrin-targeted rhodamine nanoparticles were spatially distributed in the tumor periphery, constrained to the vasculature, and colocalized with endothelial FITC-lectin contrast (Fig. 6), consistent with earlier studies in animal tumor models.^{16,39,40} Within the tumor core, prominent fluorescent signal from the microvessel-bound lectin but not the $\alpha_v\beta_3$ -targeted rhodamine nanoparticles was observed, which corroborated the pattern of $\alpha_v\beta_3$ -targeted paramagnetic nanoparticle enhancement observed in the MR reconstructed images.

Discussion

The concept of molecular imaging in cancer implies that tumor-related pathognomonic biomarkers are detected with high sensitivity and characterized with high resolution, which we have demonstrated previously with MR molecular imaging in this and related models.^{3,4} However, the localization of occult tumors or metastases within large tissue volumes favor high-sensitivity imaging techniques, but nuclear imaging offers relatively low voxel resolution, which precludes pathologic characterization based on microscopic anatomic detail. In this manuscript, we have proposed and demonstrated a dual modality (nuclear/MR) molecular imaging nanomedicine agent that offers highly sensitive and specific localization of angiogenesis and high-resolution 3D mapping of neovasculature patterns.

^{99m}Tc $\alpha_v\beta_3$ -nanoparticles were administered in titrated doses from 11 to 44 MBq/kg (0.1 to 0.4 picomoles NP/kg) yielding excellent radiocontrast signal at the tumor site. However, at the 44 MBq/kg dose, the increased tumor signal was accompanied by a greater increase in muscle (ie, blood pool) signal, which reduced TMR. Although too few titration points were for precise mathematical modeling, the incremental changes in tumor signal to the doubling doses likely followed a sigmoid relationship.

The specificity of the $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles was demonstrated in vivo by competitive inhibition, which was greatly diminished the targeted neovascular signal to the level of the nontargeted control group consistent with previous findings using ^{111}In $\alpha_v\beta_3$ -nanoparticles.⁴⁰ The potential clinical utility of $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles was evaluated with a state-of-the-art commercial SPECT-CT scanner using the lowest effective ^{99m}Tc dosage (11 MBq/kg). The benefit of targeted nuclear agent to detect and distinguish the small Vx2 tumor in the popliteal fossa from physiological sites of angiogenesis versus multislice CT alone was clearly shown.

Antiangiogenic therapy has gained wide clinical usage in conjunction with traditional chemotherapy for an increasing number solid tumors, but the optimal benefits of these expensive therapies accrue only to a small portion of the patients treated, whereas all are exposed to the risk of adverse effects, including hypertension, renal damage, thrombosis, and gastrointestinal perforation.^{46,47} Imaging techniques such as MRI with delayed contrast enhancement,^{48,49} PET/CT imaging with ¹⁸F fluorine deoxyglucose (FDG),⁵⁰ and more recently ultrasound^{51,52} have shown promise for staging or monitoring tumor response to cancer or antivascular treatments. Some agents, termed “theranostics,” provide simultaneous diagnostic and therapeutic functionality, where the imaging not only confirms therapy delivery but also predicts treatment response in animals.^{53–55}

However, pretreatment risk stratification of aggressive tumors from more benign or inflammatory lesions with specific, noninvasive diagnostic imaging of the tumor neovasculature has only recently been reported for in conjunction with antiangiogenic treatment.^{16,39} In these studies, consistent with our previous reports^{3,4} and the immunofluorescence studies presented in this report reveal that MR signal enhancement of angiogenesis was essentially confined to the tumor periphery and consisted of merged regions of neovasculature interspersed with a sparser, reticular pattern of angiogenic positive voxels. In the syngeneic Vx2 rabbit tumor model, neovascularity on day 16 post implantation accounted for 7.2% of the tumor rim volume in control rabbits, which was associated with the cancer growth front. In Vx2 rabbits receiving $\alpha_v\beta_3$ -targeted nanoparticles containing fumagillin (30 $\mu\text{g/kg}$), a specific inhibitor of endothelial cell proliferation, the neovascular pattern occupied less surface volume (2.8%; $P < 0.05$) tumor size was reduced ($470 \text{ mm}^3 \pm 120 \text{ mm}^3$) when compared with the 3 control groups (average $1143 \text{ mm}^3 \pm 166 \text{ mm}^3$; $P < 0.05$). In contradistinction to the aggressive angiogenic phenotype observed in the syngeneic Vx2 tumor model, the neovasculature of the MDA-MB-435 xenograft nude mouse model was very sparse ($<2\%$ of surface volume). Antiangiogenic treatment with $\alpha_5\beta_1(\alpha_v\beta_3)$ -targeted fumagillin nanoparticles further reduced the neovasculature to negligible levels, confirmed by histology, without impacting tumor size.³⁹ The effectiveness of targeted antiangiogenic therapies logically requires adequate expression of the neovascular biomarker and a dependence of the tumor on angiogenesis for rapid development.

In the present study, $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticle signal permitted sensitive detection of neovascular tumor pathology with SPECT-CT, but the extent and pattern of angiogenesis as a function of tumor surface could not be achieved with the nuclear images. Incorporation of ^{99m}Tc functionality into the $\alpha_v\beta_3$ -targeted gadolinium nanoparticles provided a highly detectable nuclear signal that could be focused upon with MR neovascular characterization. MR molecular imaging with 3D neovascular mapping using the dual modality approach demonstrated a coherent, asymmetric, patchy pattern of angiogenesis along the outer aspects of the tumor mass, consistent with an angiogenic phenotype responsive to targeted fumagillin.¹⁶

In pilot studies leading to the SPECT-CT/MRI results reported, initial treatment with $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles (ie, without gadolinium) was found to markedly impair follow-on MR molecular imaging of neovasculature with $\alpha_v\beta_3$ -targeted gadolinium nanoparticles. Moreover, $\alpha_v\beta_3$ -targeted ^{99m}Tc-gadolinium nanoparticles at the 22 MBq/kg dose provide excellent nuclear but inadequate MR neovascular contrast. However, administration of $\alpha_v\beta_3$ -targeted gadolinium nanoparticles after SPECT-CT provided excellent MR detection of tumor angiogenesis and facilitated robust 3D characterization.

Limitations

In previous studies, the sensitivity and specificity of $\alpha_v\beta_3$ -targeted gadolinium nanoparticles has been demonstrated in vivo in cancer models,^{3,4,16,39} which included nontargeted and

competitive inhibition groups as performed dynamically and reported for $\alpha_v\beta_3$ -targeted ^{99m}Tc nanoparticles in this study. In this report, we have demonstrated the concept of high sensitivity high-resolution imaging using SPECT-CT/MRI but did not repeat the full compliment control groups given this previous body of MR results. In addition, although the amount of supplemental $\alpha_v\beta_3$ -targeted gadolinium nanoparticles administered was chosen to reconstitute the usual dosage applied in previous studies, this level has not carefully titrated with the lipophilic gadolinium DTPA chelate used in this study or improved versions previously reported.^{56,57}

The successful labeling of perfluorocarbon nanoparticles in this report was accomplished by incorporating a lipophilic bis-pyridyl-lysine conjugate into the surfactant in combination with ^{99m}Tc coupling through a tricarbonyl precursor. However, several lipophilic chelates were synthesized and evaluated for radiolabeling perfluorocarbon nanoparticles with limited success. These included 6-hydrazinonicotinic-phosphatidylethanolamine (HYNIC-PE), diethylenetriamine pentaacetate-caproyl-phosphatidylethanolamine (DTPA-cap-PE), Gly-Gly-Gly-caproyl-phosphatidyl-ethanolamine (TriGly-cap-PE), Gly-Gly-Gly-Asp-caproyl-phosphatidyl-ethanolamine (triGly-Asp-cap-PE), N2S2-phosphatidylethanolamine (N2S2-PE), and N2S2-NH₂-phosphatidylethanolamine (N2S2-NH₂-PE). Although most of the ^{99m}Tc chelates provided strong binding alone in buffer, incorporation of these lipophilic chelates into the nanoparticle surfactant resulted in no or low radiolabeling efficiency. For those chelates, stannous tartrate reduction of ^{99m}Tc was used in conjunction with a tricine intermediate “shuttle” step to minimize the formation of $^{99m}\text{TcO}_2$ during metalation. Proper controls were employed to avoid misinterpretation of $^{99m}\text{TcO}_2$ precipitates for radiolabeled nanoparticles when performing chromatography to evaluate coupling efficiency.

Conclusion

In summary, we have reported the development of a dual-modality (nuclear/MR) molecular imaging nanoparticle that permits high-sensitivity nuclear detection of small tumors in large tissue volumes and focused, high-resolution MR characterization of tumor neovasculature. Given the increasing use, expense, and risks associated with current anti-VEGF-related therapies, we suggest that this nanomedicine approach, if translated to the clinic, could help identify the subset of patients with occult tumors who are more likely to benefit from specific antiangiogenesis therapy.

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Figure 1. Bis-pyridyl-lysine-caproyl-phosphatidylethanolamine was custom synthesized and incorporated into the phospholipid surfactant at 3 mole%.

Figure 2.
 ^{99m}Tc images of the Vx2 tumor neovascularity from the popliteal fossa obtained with pinhole collimation 15 minutes after intravenous ear vein injection of 11 MBq/kg (A), 22 MBq/kg (B), or 44 MBq/kg (C) dosages.

Figure 3.

All figures present tumor-to-muscle ratio of counts versus time post injection. A, 11 MBq/kg versus 22 MBq/kg dosages. B, 22 MBq/kg versus 44 MBq/kg dosages. C, Nontargeted 22 MBq/kg versus targeted 22 MBq/kg. D, Competitive inhibition of targeted 22 MBq/kg with nonlabeled, integrin-targeted nanoparticles.

Figure 4.

A–C, Axial, sagittal, and coronal reconstructions from tomographic CT images of the rabbit hindquarters revealed the leg, bones, and a nodular mass within the popliteal fossa. Note the tissue within the popliteal fossa (yellow arrow heads) cannot be discriminated as tumor or lymph node because relatively prominent lymph nodes are always associated with this region. D–F, In combination with the attenuation corrected SPECT images, the presence of neovascular signal from ^{99m}Tc $\alpha_v\beta_3$ -targeted nanoparticle signal (blue arrow heads) associated with an ~1-cm tissue mass located superior to the lymph node proper is readily appreciated and distinguished. Other regions of increased nuclear signal are associated with growing bone (green arrow heads) and testis (red arrow heads), which are all appreciated bilaterally. The pelvic signal (brown circle) reflects the clearance of ^{99m}Tc into the bladder. The combination of high-sensitivity molecular imaging in conjunction with high-resolution CT imaging readily facilitates the discrimination of pathologic from physiologic sources of signal.

Figure 5.

A, SPECT-CT image of nascent Vx2 adenocarcinoma in rabbit popliteal fossa after 11 MBq/kg dosage, $\alpha_v\beta_3$ -targeted ^{99m}Tc -gadolinium nanoparticles given via ear vein superimposed on the MR image obtained at 2 hours. B, T1w, fat-suppressed, gradient echo image of Vx2 adenocarcinoma of rabbit shown in panel A 2 hours after administration of remaining MR dosage, revealing the asymmetric, peripheral neovascular signal enhancement previously reported for this model. A slight misalignment of the SPECT-CT and MR images occurred, which reflected error in registering the true centers of high-resolution gadolinium MR signal and the low-resolution ^{99m}Tc signal from the peripheral fiducial markers. C, MR 3D neovascular map of Vx2 adenocarcinoma of rabbit in panel A and B, illustrating heterogeneity of neovasculature in tumor periphery.

Figure 6.

A, High-power (40 $\times$) fluorescent image shows colocalization of rhodamine-labeled $\alpha_v\beta_3$ -targeted nanoparticles (shown in B, 40 $\times$) with FITC-labeled lectin, a vascular endothelial marker (shown in C, 40 $\times$) obtained in tumor periphery, illustrating the vascular constraint of nanoparticles in the tumor vasculature. D, Although FITC-labeled vasculature was prominent within the central partially necrotic core of the tumor, integrin-targeted rhodamine nanoparticles were rarely observed (40 $\times$).