Molecular MR Imaging of Neovascular Progression in the Vx2 Tumor with $\alpha_v\beta_3$ -Targeted Paramagnetic Nanoparticles¹

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Purpose:

To assess the dependence of neovascular molecular magnetic resonance (MR) imaging on relaxivity (r_1) of $\alpha_v\beta_3$ -targeted paramagnetic perfluorocarbon (PFC) nanoparticles and to delineate the temporal-spatial consistency of angiogenesis assessments for individual animals.

Materials and Methods:

Animal protocols were approved by the Washington University Animal Studies Committee. Proton longitudinal and transverse relaxation rates of $\alpha_v\beta_3$ -targeted and nontargeted PFC nanoparticles incorporating gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) bisoleate (BOA) or gadolinium tetraazacyclododecane tetraacetic acid (Gd-DOTA) phosphatidylethanolamine (PE) into the surfactant were measured at 3.0 T. These paramagnetic nanoparticles were compared in 30 New Zealand White rabbits (four to six rabbits per group) 14 days after implantation of a Vx2 tumor. Subsequently, serial MR (3.0 T) neovascular maps were developed 8, 14, and 16 days after tumor implantation by using $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($n = 4$) or nontargeted Gd-DOTA-PE nanoparticles ($n = 4$). Data were analyzed with analysis of variance and nonparametric statistics.

Results:

At 3.0 T, Gd-DTPA-BOA nanoparticles had an ionic r_1 of $10.3 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$ and a particulate r_1 of $927000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$. Gd-DOTA-PE nanoparticles had an ionic r_1 of $13.3 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$ and a particulate r_1 of $1197000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$. Neovascular contrast enhancement in Vx2 tumors (at 14 days) was $5.4\% \pm 1.06$ of the surface volume with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles and $3.0\% \pm 0.3$ with $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles ($P = .03$). MR neovascular contrast maps of tumors 8, 14, and 16 days after implantation revealed temporally consistent and progressive surface enhancement ($1.0\% \pm 0.3$, $4.5\% \pm 0.9$, and $9.3\% \pm 1.4$, respectively; $P = .0008$), with similar time-dependent changes observed among individual animals.

Conclusion:

Temporal-spatial patterns of angiogenesis for individual animals were followed to monitor longitudinal tumor progression. Neovascularity enhancement was dependent on the relaxivity of the targeted agent.

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Folkman (1) hypothesized that the process of angiogenesis could be impaired to inhibit tumor growth, and antiangiogenic therapies were subsequently developed and implemented, including bevacizumab (Avastin; Genentech/Roche, San Francisco, Calif) (2), sorafenib (Nexavar; Bayer, West Haven, Conn), and sunitinib (Sutent; Pfizer, New York, NY). Antiangiogenic therapy may be considered the fourth modality for cancer treatment in addition to surgery, chemotherapy, and radiation therapy (3). Unfortunately, antiangiogenic agents, even in

combination with traditional chemotherapy, have met with variable success, with effectiveness often observed only in certain subgroups of patients (4–6). Clearly, an unmet need persists to risk stratify patients for adjunctive angiogenic therapy and to better manage the timing and dosing of such treatments for maximum benefit (6,7). The ability to assess and possibly predict the efficacy of novel antiangiogenic cancer therapies could greatly increase the benefit-risk ratio for candidate patients.

Current techniques to assess alterations in vascular supply include perfusion-based parameters that correlate with microvessel density at computed tomography (CT) (8,9) and dynamic contrast material-enhanced magnetic resonance (MR) imaging (10). Although microvessel density and dynamic contrast-enhanced MR imaging measurements have been useful prognostic markers for some cancer types, none have shown a clear association with antiangiogenic response (11,12). In particular, neovascularity estimates obtained with blood pool contrast diffusion pharmacokinetics have been shown to differ greatly from results obtained with integrin-targeted paramagnetic nanoparticles (13). Other molecular imaging techniques have proved useful for characterizing animal tumors at the molecular and cellular levels (14), as well as for evaluating the therapeutic efficacy of antiangiogenic agents, including optical imaging (15), positron emission tomography/CT (16,17), contrast-enhanced ultrasoundography (18,19), photoacoustic tomography (20), and MR imaging

(21,22). Of these modalities, MR imaging offers the distinct advantage of high-spatial-resolution imaging without ionizing radiation, as well as the ability to noninvasively probe shallow and deep tumors. Specific and reliable molecular MR imaging of angiogenesis could enable physicians to better select and treat patients with cancer, particularly those who are candidates for adjunctive antiangiogenesis treatment.

The $\alpha_v\beta_3$ integrin, a heterodimeric transmembrane glycoprotein, is differentially upregulated in proliferating versus quiescent endothelial cells. Although numerous cell types, including endothelial cells (23), macrophages (24), platelets (25), lymphocytes (25), smooth muscle cells (26), and tumor cells (27), express $\alpha_v\beta_3$ integrin, steric constraint of perfluorocarbon (PFC) nanoparticles within the vasculature precludes substantial interaction with nonendothelial integrin expression.

Advances in Knowledge

- A neovascular index, calculated as the percentage of contrast-enhanced surface volume relative to the total surface volume, was used as a metric of neovascularity in the Vx2 rabbit tumor model and showed progression of MR imaging–detectable neovascularity of $1.0\% \pm 0.3$, $4.5\% \pm 0.9$, and $9.3\% \pm 1.4$ on postimplant days 8, 14, and 16, respectively; in comparison, the neovascular index for the nontargeted control was less than 1.5% throughout the study.
- MR signal intensity enhancement of the neovascular activation was better with the higher-relaxivity gadolinium tetraazacyclododecane tetraacetic acid phosphatidylethanolamine nanoparticles (ionic $r_1 = 10.3 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$; particulate $r_1 = 927000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$), which positioned gadolinium beyond the lipid surface, than with gadolinium diethylenetriamine pentaacetic acid bisoleate nanoparticles (ionic $r_1 = 13.3 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$; particulate $r_1 = 1197000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$), which located the metal near the water-particle interface.
- Neovascular contrast maps revealed a consistent spatial progression over time for individual animals as well as groups of animals treated similarly.

Implication for Patient Care

- Neovascular mapping and indexes may provide sensitive metrics with which to detect and characterize vascular expansion in nascent proliferating solid tumors, which could be useful to risk stratify antiangiogenesis treatment in patients with cancer or to follow therapeutic responses longitudinally.

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Abbreviations:

BOA = bisoleate
Gd-DOTA = gadolinium tetraazacyclododecane tetraacetic acid
Gd-DTPA = gadolinium diethylenetriamine pentaacetic acid
PE = phosphatidylethanolamine
PFC = perfluorocarbon
3D = three-dimensional

Author contributions:

Guarantors of integrity of entire study, A.H.S., J.S.A., G.M.L.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; manuscript final version approval, all authors; literature research, A.H.S., P.M.W., S.A.W., G.M.L.; experimental studies, A.H.S., P.M.W., T.A.W., J.S.A., G.H., H.Z., S.D.C., G.M.L.; statistical analysis, A.H.S., P.M.W., G.M.L.; and manuscript editing, A.H.S., P.M.W., S.D.C., S.A.W., G.M.L.

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Conflicts of interest are listed at the end of this article.

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This greatly enhances neovascular targeting specificity (28,29). Our laboratory has shown that vascular-constrained $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles provide robust signal amplification (30,31) and simultaneous antiangiogenic drug delivery capability in cancer models (21,32). We have reported that vascular constrained $\alpha_v\beta_3$ -targeted nanoparticles bind to the intraluminal expressed $\alpha_v\beta_3$ integrin up-regulated in proliferating nonpolarized endothelial cells ($\alpha_v\beta_3^+$, PECAM⁺, Tie-2⁻) versus the quiescent endothelial cells of maturing vessels ($\alpha_v\beta_3^-$, PECAM⁺, Tie-2⁺) (33). This neovascular expansion offers a sensitive microanatomic biomarker of the tumor growth front.

Given the relative insensitivity of MR imaging, very high relaxivity contrast agents are required to image biomarkers expressed at relatively low concentrations. We reported on molecular MR imaging in rabbits and rodents with vascular-constrained $\alpha_v\beta_3$ -targeted PFC nanoparticles incorporating a lipid-modified gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) bisoleate (BOA) chelate (30,31). Subsequently, we demonstrated an improved paramagnetic PFC particle with use of the lipophilic chelate Gd-DTPA phosphatidylethanolamine (PE) (34), which repositioned the metal chelate further into the aqueous medium.

Typical preclinical animal molecular imaging studies compare targeted tumor signal with a nontargeted control counterpart on a group basis, but in the clinic, molecular imaging agents will be required to provide quantitative serial assessments within the same individual whether for pretreatment stratification or to assess therapeutic response. Effective characterization of tumor neovascular expansion will require high-relaxivity contrast agents that provide quantitative and reproducible imaging over time. The overall objective of this research was to assess the dependence of neovascular molecular MR imaging on relaxivity (r_1) of $\alpha_v\beta_3$ -targeted paramagnetic PFC nanoparticles and to delineate the temporal-spatial consistency

of angiogenesis assessments for individual animals.

Materials and Methods

The nanotechnology intellectual property presented herein is owned by Washington University and Barnes-Jewish Hospital, St Louis, Mo, and was sublicensed to Kereos (St Louis, Mo). G.M.L. and S.A.W. are scientific co-founders of Kereos and retain a very small equity position.

Paramagnetic Nanoparticle Synthesis

Paramagnetic perfluorocarbon nanoparticles were prepared as previously reported (21,30). The emulsions were composed of 20% (vol/vol) of perfluorooctyl bromide (Elf Atochem, Philadelphia, Pa), 2% (wt/vol) of a surfactant commixture, 1.7% (wt/vol) glycerin, and tartaric acid buffer (7.5 mmol/L; pH, 5.5) representing the balance. The surfactant commixture consisted of 68 mol% lecithin (E80; Lipoid, Ludwigshafen, Germany), 0.1 mg/mL of an $\alpha_v\beta_3$ integrin antagonist conjugated to PEG₂₀₀₀-PE (Kereos, St Louis, Mo), and 0.1 mg/mL of a paramagnetic lipophilic chelate—either gadolinium tetraazacyclododecanetetraacetic acid

(Gd-DOTA) PE (Kereos) or Gd-DTPA-BOA (Gateway Chemical Technologies, St Louis, Mo) (Fig 1). This was combined with perfluorooctyl bromide, buffer, and glycerin, and the mixture was emulsified (Microfluidics, Newton, Mass). Nontargeted nanoparticles excluded the $\alpha_v\beta_3$ integrin antagonist, which was replaced with equimolar PE. The mean nominal particle size ($\pm$ standard deviation) obtained by means of dynamic light scattering (Brookhaven Instruments, Holtsville, NY) was 220 nm $\pm$ 80, with a polydispersity index of less than 0.2.

The $\alpha_v\beta_3$ integrin antagonist was a quinalone nonpeptide developed by Bristol-Myers Squibb Medical Imaging (Billerica, Mass; U.S. patent 6511 648 and related patents) and produced by Kereos (St Louis, Mo). It was initially reported and characterized as the indium 111 (¹¹¹In)-DOTA conjugate RP478 and cyan 5.5 homolog TA145 (35). PFC nanoparticles present approximately 300 ligands per particle, with an IC₅₀ of 50 pmol/L for the Mn²⁺-activated $\alpha_v\beta_3$ integrin (Kereos, unpublished data, 2005) (32). Homing specificity of the vascular-constrained agent has been demonstrated through in vivo competition studies in preclinical cancer models (30,31) and biochemically

Figure 1

Figure 1: Chemical structures of two paramagnetic Gd³⁺ chelates that can be incorporated onto nanoparticle surface. Top: Gd-DTPA-BOA is our first-generation linear chelate. Bottom: Gd-DOTA-PE is an improved design, with a macrocyclic structure that increases relaxivity and stability of agent.

defined in the Matrigel plug (BD Biosciences, San Jose, Calif) implanted into *Rag1^{tm1Mom}* Tg (Tie-2-lacZ)182-Sato mice (33). Pharmacokinetic and biodistribution studies of ^{111}In $\alpha_v\beta_3$ -targeted nanoparticles were previously reported (28).

Neutron activation analysis was used to determine the Gd^{3+} contents (University of Missouri Research Reactor Center, Columbia, Mo). The number of Gd^{3+} chelates per nanoparticle was calculated from the ratio of Gd^{3+} and nanoparticles in the emulsion.

In Vitro Relaxivity Measurements

Gd-DTPA-BOA and Gd-DOTA-PE nanoparticles were diluted 1:10, 1.5:10, 2:10, 2.5:10, and 3:10 in distilled, deionized water. Proton longitudinal and transverse relaxation rates (r_1 and r_2 , respectively) were measured at 3.0 T (Achieva; Philips Medical Systems, Andover, Mass) with a mixed spin-echo, inversion-recovery imaging sequence (36,37). The "mixed" sequence generated a series of images with different amounts of T1 and T2 weighting. T1 was calculated from the real component of images collected with an inversion-recovery MR imaging pulse sequence (six inversion times ranging from 50 to 1500 msec for Gd-DTPA-BOA and from 5 to 200 msec for Gd-DOTA-PE). T2 was calculated from spin-echo images with various echo times. The common imaging parameters for both pulse sequences were as follows: repetition time msec/echo time msec of 1000/5, four signals acquired, 128×128 matrix, 7×7 -cm field of view, 90° flip angle, and 5-mm-thick sections. The r_1 and r_2 relaxivities (in $\text{L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$) were calculated from the slope of longitudinal and transverse relaxation rates versus Gd^{3+} (ie, ionic relaxivity) or nanoparticle (ie, particulate relaxivity) concentrations.

Rabbit Vx2 Tumor Studies

All animal protocols were approved by the Washington University Animal Studies Committee.

Experiment 1: in vivo chelate comparison.—Twenty male New Zealand

White rabbits (mean weight, 2 kg) were anesthetized with intramuscular ketamine and xylazine, and a 2–3-mm³ Vx2 tumor fragment (National Cancer Institute Tumor Repository, Frederick, Md) was implanted into the popliteal fossa (38–40). On day 14, MR imaging was performed to compare neovascularity assessed with Gd-DOTA-PE or Gd-DTPA-BOA PFC nanoparticles by using a three-dimensional (3D) gradient-echo, fat-suppressed, black blood, T1-weighted sequence (42/5.7, 45° flip angle, in-plane resolution of 250×250 μm , 500- μm -thick sections, 67 sections, two signals acquired, total imaging time of 25 minutes) with an eight-element coil. Rabbits were sedated with ketamine and xylazine, and anesthesia was maintained with 1.0% isoflurane in oxygen. Following baseline acquisition of tumor MR images, rabbits received intravenous administration of 1 mL/kg (0.03 nmol particles per kilogram) of $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles with either Gd-DTPA-BOA ($n = 4$) or Gd-DOTA-PE ($n = 6$) or nontargeted nanoparticles with either Gd-DTPA-BOA ($n = 4$) or Gd-DOTA-PE ($n = 6$). Contrast-enhanced MR images were acquired 3 hours after injection at 3.0 T (Philips Medical Systems, Andover, Mass) by using the same MR technique. Blood samples were collected 3 hours after injection, and the Gd^{3+} content was measured. After MR imaging, anesthetized animals were euthanized and tumors excised for histologic examination.

An additional cohort of six rabbits implanted 16 days previously with Vx2 tumor was used to demonstrate competitive inhibition. Rabbits received $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($n = 4$) or $\alpha_v\beta_3$ -targeted PFC-PE nanoparticles (no gadolinium, 3 mL/kg) with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles (1 mL/kg) ($n = 2$). MR imaging was performed as described earlier.

Experiment 1: ex vivo microscopic imaging.—Two rabbits bearing 16-day Vx2 tumors ($n = 2$) were euthanized, and tumors were excised and frozen in optimal cutting temperature compound (Sakura Finetek, Torrance, Calif) for

cryosectioning. Frozen sections (8 μm thick) were stained with hematoxylin-eosin or $\alpha_v\beta_3$ integrin (LM609; Millipore, Billerica, Mass) and studied with a microscope (model BX61; Olympus, Tokyo, Japan) to corroborate the regional tumor distribution of angiogenesis.

Experiment 2: MR spatial mapping of neovascular progression.—The improved Gd-DOTA-PE nanoparticle, as determined in experiment 1, was selected for imaging tumor neovascular progression over 8 days. Eight male New Zealand White rabbits bearing Vx2 tumors (as detailed earlier) were imaged before and after administration of $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($n = 4$) or nontargeted Gd-DOTA-PE nanoparticles ($n = 4$) on days 8, 14, and 16 after implantation. MR images were acquired by using T1-weighted gradient-echo pulse sequences as presented for experiment 1. Tumor volumes were obtained on days 8, 14, and 16 by using a T2-weighted 3D turbo spin-echo sequence (2000/250, 400×520 - μm in-plane resolution, 2-mm-thick sections, 32 sections, two signals acquired).

Image Analysis

In each experiment, the signal intensities of the MR images were analyzed with software (Matlab; MathWorks, Natick, Mass). Signal intensities at dynamic T1-weighted imaging were normalized to a 5-mL syringe containing a 0.02 mg/mL gadopentetate dimeglumine in water reference (1:20000 Magnevist; Bayer Healthcare, Montville, NJ) that was included within each imaging field of view. For each animal, a region of interest was manually placed around the tumor edge on each baseline section and the standard deviation of the average tumor signal intensity calculated (Fig E1 [online]). Voxels on contrast-enhanced MR images were defined as enhanced when the signal intensity exceeded the mean tumor signal intensity at baseline by more than 3 standard deviations. The tumor region of interest was objectively partitioned into a rim and core region by using an automated erosion method developed in Matlab. The core region was set to

approximately half of the tumor volume. The neovascular area of enhancement was calculated, as well as the relative distribution of enhancing voxels in the periphery versus the core of the tumor. High-spatial-resolution 3D tumor reconstructions were created in Matlab to characterize the surface distribution of enhancing neovascular voxels in each treatment group. The overall 3D structure of the tumor was displayed as a mesh surface plot by using isosurface rendering and a smoothing filter. A surface plot of the enhancing voxels was reconstructed similarly and overlaid onto the tumor volume.

Statistical Analysis

Quantitative MR signal intensity data were analyzed by using analysis of variance (type III sum of squares) and least-square means comparisons to adjust for unequal subclass numbers when the results of the overall *F* test were significant ($P < .05$). These results were corroborated by means of nonparametric (Wilcoxon) analysis by using a Kruskal-Wallis test statistic; $P < .05$ was considered to indicate a significant difference (SAS Institute, Cary, NC). Slightly unequal replication numbers in experiment 1 resulted from additional animal and nanoparticle formulation availability. In the cohort of rabbits used for competitive inhibition testing, two animals were removed from the competition group: One pilot animal received a 1:1 competition dose mixture, which was modified to 3:1 for the other animals, and the tumor of a second animal was atypically small or slow growing. For the repeated measure analysis over time, a significant treatment (ie, targeted vs nontargeted nanoparticles) $\times$ time (ie, days 8, 14, or 16) interaction was determined; therefore, comparisons over days were performed within each nanoparticle treatment group. Data are reported as means $\pm$ standard errors of the mean. Exact probabilities of differences in the least-square means are reported. Box plots show the means, medians, and 10th,

Figure 2: Unprocessed two-dimensional MR images of rabbit Vx2 tumor obtained, A, at baseline, B, 3 hours after injection of $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles, and, C, 3 hours after injection of nontargeted Gd-DOTA-PE nanoparticles. Outline of tumor periphery is clearly seen on targeted contrast image, along with variation in peripheral enhancement.

25th, 75th, and 90th percentiles for treatment group comparisons.

Results

Each paramagnetic nanoparticle formulation contained a similar number of Gd^{3+} complexes per particle ($\sim 90,000$) on the basis of the average particle size of 220 nm. The ionic r_1 relaxivity at 3.0 T of the prototype Gd-DTPA-BOA nanoparticles was very high ($10.3 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$), which is similar to that in previous studies (34); however, Gd-DOTA-PE nanoparticles had a 30% higher ionic relaxivity at 3.0 T ($13.3 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$). Particulate r_1 relaxivities were determined to be $1260,000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$ for Gd-DOTA-PE nanoparticles and $927,000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$ for Gd-DTPA-BOA nanoparticles. Gd-DTPA-BOA nanoparticles had an ionic r_2 of $11.4 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$ and a particulate r_2 of $1,026,000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$, whereas Gd-DOTA-PE nanoparticles had an ionic r_2 of $15.5 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$ and a particulate r_2 of $1,395,000 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{sec}^{-1}$.

Molecular MR imaging with $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles in New Zealand White rabbits implanted 14 days previously with Vx2 tumors revealed prominent neovascularity along segments of the tumor periphery as well as stimulated neovascular

expansion on the adjacent popliteal lymph node surface, the popliteal artery wall, and other proximate small vessels in the associated fascia, as previously observed (31). The brightened tumor periphery was easily discerned on two-dimensional axial MR images for those animals receiving the integrin-targeted paramagnetic nanoparticles, but the tumor border was poorly discernible in rabbits given the nontargeted paramagnetic nanoparticles (Fig 2). There was a lower level of noticeable signal enhancement in nontargeted rabbit tumors, which we attribute to nonspecific local retention, consistent with findings in previous studies (31). This signal was consistently less prominent than the signal from targeted tumors.

The tumor neovascular index obtained for the $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticle group was about twice the level of tumor peripheral volume enhancement when compared with animals receiving $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles ($5.4\% \pm 1.06$ vs $3.0\% \pm 0.3$, respectively; $P = .03$; Fig 3). The neovascular index obtained for $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles ($3.0\% \pm 0.3$) did not differ from that for the nontargeted Gd-DOTA-PE nanoparticle ($1.5\% \pm 0.2$, $P = .15$) or nontargeted Gd-DTPA-BOA nanoparticle ($1.4\% \pm 0.3$, $P = .14$) control groups in this experiment. A typical difference

Figure 3

Figure 3: Box plots compare neovascular index with Gd-DOTA-PE or Gd-DTPA-BOA nanoparticles 14 days after Vx2 tumor implantation. Box plots show means (black square), medians (line within box), and 10th, 25th, 75th, and 90th percentiles (bottom bar, bottom of box, top of box, and top marker, respectively). Enhanced tumor volume was significantly larger (*) with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles compared with $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles ($P = .03$) and both nontargeted groups ($P < .0009$ for analysis of variance with all four treatments). Use of $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles also numerically increased the area of contrast enhancement compared with nontargeted Gd-DTPA-BOA nanoparticles, but the difference was not significant given the degree of replication ($P = .15$).

in the spatial distribution and confluency of contrast-enhanced voxels between the two targeted nanoparticle formulations was visually apparent in the 3D neovascular reconstructions of two tumors of similar size and age (Fig 4). In a separate cohort of rabbits implanted with Vx2 tumor, the neovascular index of rabbits given $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles was $6.9\% \pm 0.7$ and was greater than that in the competition group that received a 3:1 mixture of $\alpha_v\beta_3$ -targeted PFC nanoparticles (ie, no gadolinium) and $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($3.0\% \pm 1.0$, $P = .03$), further supporting the specificity of the molecular imaging technique as previously reported (30,31) (Fig E2 [online]). Background blood pool levels of Gd^{3+} at MR imaging (ie, 3 hours) were nondetectable and were low analytically,

Figure 4

Figure 4: Three-dimensional reconstructions of neovascular signal enhancement by paramagnetic nanoparticles 14 days after implantation of Vx2 tumor. Whole tumor volume of interest is shown in gray, and enhancing voxels from neovasculture are shown in blue. Images were obtained with, A, $\alpha_v\beta_3$ -targeted Gd-DOTA-PE and, B, $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA paramagnetic nanoparticles. These 3D maps of MR signal enhancement show tumor neovascular morphology and structured patterns of enhancement, which was most evident with the higher-relaxivity Gd-DOTA-PE agent (A) and not fully recognized on the two-dimensional sections on their own. The two tumors were of similar volume (near the mean for both groups).

$24.1 \mu\text{mol/L} \pm 3.9$ ($\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles) versus $31.4 \mu\text{mol/L} \pm 6.7$ ($\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles, $P = .38$). Both nontargeted chelate agents produced low angiogenesis enhancement secondary to nonspecific entrapment ($1.4\% \pm 0.3$).

Three-dimensional reconstructions illustrated the spatial distribution of the neovascular contrast enhancement along the proliferating tumor growth front (Fig 4). Tumor surface enhancement with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles showed numerous small confluent regions of neovascularity along with many voxels heterogeneously dispersed within the tumor periphery (Fig 4, A). With use of the lower-relaxivity $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles, some confluent regions of voxel enhancement were again detected, but the sparser regions of angiogenic neovessel expansion were less prominent (Fig 4, B). Minimal neovascular enhancement was noted with nontargeted contrast agents.

Dominant $\alpha_v\beta_3$ integrin distribution along the peripheral border of the Vx2 tumor was corroborated by using specific immunostaining (LM609) of the adhesion molecule in excised

tumors. Examination of a cohort of tumors revealed predominant but heterogeneous expression of $\alpha_v\beta_3$ integrin in the tumor capsule and peripheral parenchyma (Fig 5), with virtually no vascular expression detected in the more necrotic core of the lesion, consistent with MR findings of this study and previous reports in preclinical cancer models (31,32).

Tumor volume increased progressively on days 8, 14, and 16, with no difference at any time point noted between the $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($0.33 \text{ cm}^3 \pm 0.1$, $1.18 \text{ cm}^3 \pm 0.26$, and $1.85 \text{ cm}^3 \pm 0.5$, respectively) and nontargeted Gd-DOTA-PE nanoparticles ($0.28 \text{ cm}^3 \pm 0.05$, $1.95 \text{ cm}^3 \pm 0.4$, and $2.37 \text{ cm}^3 \pm 0.4$, respectively). At 8 days after implantation, a minimal level of Vx2 peripheral neovasculture was detected ($P = .56$) with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($1.0\% \pm 0.3$) and nontargeted Gd-DOTA-PE nanoparticles ($0.7\% \pm 0.2$) (Fig 6, Fig E3 [online]). By day 14, however, 3D MR imaging showed a neovascular expansion pattern, with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles ($4.5\% \pm 0.9$) asymmetrically distributed on the tumor periphery

Figure 5: Images obtained with light microscopy of Vx2 peripheral tissue region illustrate dense $\alpha_v\beta_3$ integrin expression predominantly associated with microvasculature in tumor capsule and peripheral parenchyma. *A*, Photomicrograph (hematoxylin-eosin [H & E] stain; original magnification, $\times 4$) of Vx2 tumor shows peripheral capsule and central parenchyma. Box indicates region of tissue depicted at higher power in *B–D*. *B*, Photomicrograph (hematoxylin-eosin H & E stain; original magnification, $\times 10$) of region indicated by box in *A* shows outer loose and inner denser connective tissue of tumor capsule and tumor parenchyma, which is a typical mixture of cancer cells and inflammatory infiltrate. *C*, Photomicrograph (LM609 immunostain; original magnification, $\times 10$) shows dense $\alpha_v\beta_3$ integrin expression within tumor capsule from region indicated by box in *A*. Note that dense purple staining of integrin is predominantly associated with capsular microvasculature. *D*, Magnification (original magnification, $\times 20$) of image in *C* helps better delineate $\alpha_v\beta_3$ integrin neovascular expression. Blue arrows = analogous regions of immunostained integrin.

but not within the core. The neovascular areas of enhancement for tumors exposed to the nontargeted Gd-DOTA-PE nanoparticles on day 14 ($1.5\% \pm 0.2$) were similar to the 8-day result in magnitude and voxel pattern ($P = .08$). At 16 days, tumors re-exposed to $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles revealed a further increase in neovascular surface area ($9.3\% \pm 1.4$, $P = .0002$), whereas the neovascular signal obtained with nontargeted Gd-DOTA-PE nanoparticles remained unchanged from day 14 ($1.4\% \pm 0.2$, $P = .88$).

Serial 3D neovascular maps illustrated the ability of the $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles to help identify the progressive changes in the neovascular phenotype between days 8 and 16 after implantation. The spatial

organization of neovessels was predominantly confined to the tumor periphery, where densely coalesced regions of neovascularity were interspersed with a sparse distribution of angiogenic-positive voxels (Fig 7). Importantly, neovascular progression observed 2 days apart (day 16) showed “filled-in” voxel patterns initially recognized on day 14 but now expanded with new regions of neovascular contrast enhancement. In comparison, tumors exposed to nontargeted Gd-DOTA-PE nanoparticles had consistently sparse noncoherent distributions of enhanced voxels that varied with reexamination from day 8 to day 16.

Because patients are treated as individuals rather than in groups, the individual neovascular responses of

rabbit Vx2 tumors receiving targeted and nontargeted nanoparticles within the 16-day interval were reported. As observed in Figure 6, *C*, the peripheral volume enhancement with nontargeted Gd-DOTA-PE nanoparticles was very low and essentially unchanged despite progressive increases in tumor volume over the experimental window. In contradistinction, the peripheral neovascular index for each rabbit treated with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles increased between days 8 and 14; for three of the four animals, angiogenesis increased markedly between days 14 and 16. In one animal, the neovascular index decreased between days 14 and 16, and this animal was also noted to have a slight decrease in tumor volume; the other three animals had stable or increased tumor size. Plotting neovascular index versus tumor volume for individual animals showed a clear increase in neovascularity as the Vx2 tumor increased in size among animals treated with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles, whereas in control animals receiving nontargeted Gd-DOTA-PE nanoparticles there was no relationship between MR imaging-detected tumor neovascularity and lesion volume (Fig E4 [online]). In general, a very high level of consistency for neovascular expansion within individual rabbits was observed over time, whether measured as an integrative neovascular index or portrayed as a spatially defined 3D reconstruction.

Discussion

In our study, we demonstrated the robust spatial consistency of 3D MR imaging neovascular contrast enhancement obtained over time within treatment groups, as well as in individual animals with $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles. We showed that the magnitude of T1 contrast relaxivity at MR imaging has a significant effect on the sensitivity in the assessment of neovascularity, which is reflected in the timing, extent, and pattern of enhancement measured with molecular MR imaging. Tumor angiogenesis is microscopically observed in Vx2 tumors

less than 8 days after implantation, but molecular MR imaging has much lower sensitivity. At 8 days, neovessel contrast enhancement with $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles was manifest as randomly distributed points that were indistinguishable from nonspecific nontargeted control particles. By day 14, confluent voxels of neovascular enhancement were detected, and these were reproduced with clear evidence of further progression on an individual animal basis 2 days later. The marked increase in the MR neovascular index estimates between days 14 and 16 likely relates to the expected exponential increase in branching neovessel expansion occurring in the rapid tumor growth phase. In addition, with time the number of neovessels expressing $\alpha_v\beta_3$ integrin within a voxel sufficiently increases to overcome partial volume dilution and the three standard deviation threshold imposed by our postprocessing approach. To afford sensitive tumor neovascular characterization as early as possible in the natural course of tumor progression, $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles with the highest particulate relaxivity will be preferred, if not essential.

Our study also introduced a new paramagnetic lipophilic chelate, Gd-DOTA-PE, and demonstrated its improved relaxivity over Gd-DTPA-BOA, which was previously reported in preclinical cancer and atherosclerosis studies (30,31,41–43). With Gd-DOTA-PE, a 100% improvement in neovascular imaging was measured in vivo, highlighting the importance of maximizing the particulate relaxivity. Previously, Morawski et al (44) demonstrated through modeling that $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles could be detected at less than 100 pmol/L per voxel with a contrast-to-noise ratio of 5 at 3.0 T, whereas $\alpha_v\beta_3$ -targeted Gd-DTPA-PE nanoparticles increased the diagnostic sensitivity to approximately 50 pmol/L per voxel. In our study, a similar improvement in T1 relaxivity was sought. However, the association of gadolinium bound to linear chelates with nephrogenic systemic fibrosis in patients with severe renal

Figure 6

Figure 6: Neovascular index (A, C) and tumor volume (B, D) over time in four rabbits bearing Vx2 tumors. Rabbits were imaged before and after administration of paramagnetic nanoparticles (NP) at 8, 14, and 16 days after implantation. The improved-relaxivity Gd-DOTA-PE nanoparticles revealed increasing areas of contrast enhancement from 8 to 14 days. Contrast enhancement even increased during the short time from 14 to 16 days. The neovascular index in one rabbit (+) decreased over 14–16-day period and was associated with an unexpected decrease in tumor volume. The level of contrast enhancement associated with nontargeted Gd-DOTA-PE nanoparticles remained at the same minimal level even as tumor continued to grow.

disease encouraged utilization of a macrocyclic DOTA to minimize potential transmetallation.

We have previously demonstrated the effectiveness of $\alpha_v\beta_3$ -targeted Gd-DTPA-BOA nanoparticles to target, delivery therapy to, and measure neovascular drug responses in cancer and atherosclerosis animal models (21,32). For example, following antiangiogenesis therapy with fumagillin in hyperlipidemic rabbits, molecular MR imaging was used to monitor and quantify the recrudescence of neovasculture (43). In our study, before and after treatment comparisons were reported as group averages. However, molecular imaging must enable diagnosis, stratification, and reassessment of treatment responses within individuals for clinical translation.

In our present study, we demonstrated consistent time-dependent expansion of the neovascular index over time on an individual basis with $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles; this finding was not observed with the nontargeted paramagnetic nanoparticles. Interestingly, three of four rabbits given $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles had an increase in neovasculture index and tumor volume between 8, 14, and 16 days after implantation. In one animal, the neovascular index decreased between days 14 and 16 and there was a modest decrease in tumor volume. We have previously reported (31) that Vx2 tumor implants rejected by the host, confirmed with histologic examination, have a strong T2-weighted signal owing to edema and inflammation but no T1 neovascular MR signal after the

Figure 7

Figure 7: Neovascular maps show contrast-enhanced voxels over time. Increase in significantly enhanced tumor voxels is clearly apparent on 3D reconstructions of MR signal enhancement derived from paramagnetic nanoparticles. Tumor volume is outlined in gray, and contrast-enhanced pixels are shown in blue. Maps illustrate that MR imaging detection of neovessels markedly increased between days 8 and 14, with continued spatial progression noted on day 16. Arrows indicate examples of consistent enhancement patterns over time. The tumor of rabbit that received nontargeted paramagnetic nanoparticles shows little enhancement despite continued tumor growth and progression of neovessels (as detected at histologic examination).

administration of $\alpha_v\beta_3$ -targeted paramagnetic nanoparticles. From those previous results, we speculated that this decrease observed in tumor neovascular index may be an early indicator of a decrease in tumor viability. Similarly, we hypothesized that neovascular imaging could not only help recognize diminished tumor viability owing to treatment or rejection but could also provide an early indication of inadequate cancer therapy with local malignant recrudescence.

Kiessling et al (45) previously investigated arginine-glycine-aspartic acid-labeled ultrasmall superparamagnetic iron oxide and conjectured and showed that the propensity of particles to extravasate owing to their small size might elicit untoward biologic effects that have been previously unrecognized with

vascular constrained particles, such as those reported herein (250 nm) and those reported previously by our laboratory (30,31) and by others (46,47). In our study, individual tumor growth was unaffected by the repeated transient intravascular blockade of the neovascular integrin receptor by the targeted nanoparticles, which was consistent with a similar lack of biologic effects reported with this diagnostic agent when applied in preclinical models of atherosclerosis (43,48) and rheumatoid arthritis (49,50). The dose of particles used in our study was small (1.0 mL/kg), which would project to 0.33 mL/kg in humans on the basis of U.S. Food and Drug Administration apometric conversion recommendations for oncology (<http://www.accessdata.fda.gov/scripts/cder/onctools/animal-query.cfm>).

The total gadolinium dosage level of $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles administered to rabbits in our study was 0.02 mmol Gd³⁺ per kilogram, with the human projected level estimated at 0.006 mmol Gd³⁺ per kilogram.

Our study had limitations, particularly with regard to quantitative histologic examination. Although immunohistochemistry effectively corroborated the peripheral versus core distribution of neovascular contrast enhancement measured with MR imaging, unfortunately quantitative comparisons between the MR imaging and microscopic findings were precluded owing to competitive inhibition of the LM609 antibody, which was used to stain rabbit $\alpha_v\beta_3$ integrin, by the higher-avidity $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles. This is further complicated by the use of repeated molecular MR imaging in the same animal. However, in our study as in several others, in vivo competitive imaging corroborated the specificity of targeting to the $\alpha_v\beta_3$ integrin by using the quinolone peptidomimetic used in these nanoparticle formulations.

In conclusion, high-spatial-resolution molecular MR imaging with improved-relaxivity $\alpha_v\beta_3$ -targeted Gd-DOTA-PE nanoparticles provided quantitative, temporal, and spatially resolved assessments of tumor neovascularity, which may enable judicious patient selection for antiangiogenic therapy and aid in treatment monitoring.

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