Specific Targeting of Tumor Angiogenesis by RGD-Conjugated Ultrasmall Superparamagnetic Iron Oxide Particles Using a Clinical 1.5-T Magnetic Resonance Scanner

Chunfu Zhang,1,2 Manfred Jugold,1,2 Eva C. Woenne,1,2 Twan Lammers,3 Bernd Morgenstern, Margareta M. Mueller,1 Hanswalter Zentgraf,4 Michael Bock,2 Michael Eisenhut,5 Wolfhard Semmler,1 and Fabian Kiessling1

1Junior Group Molecular Imaging, 2Department of Medical Physics in Radiology, 3Clinical Cooperation Unit Radiotherapeutic Oncology, 4Tumor and Microenvironment, 5Applied Tumor Virolology, and 6Radiopharmaceutical Chemistry, German Cancer Research Center, Heidelberg, Germany

Abstract

Angiogenesis is essential for the development of malignant tumors and provides important targets for tumor diagnosis and therapy. To noninvasively assess the angiogenic profile of tumors, novel αvβ3 integrin–targeted ultrasmall superparamagnetic iron oxide particles (USPIOs) were designed and their specific uptake by endothelial cells was evaluated in vitro and in vivo. USPIOs were coated with 3-aminopropytrimethoxysilane (APMTMS) and conjugated with Arg-Gly-Asp (RGD) peptides. Accumulation in human umbilical vein endothelial cells (HUVECs) was evaluated using Prussian blue staining, transmission electron microscopy, magnetic resonance (MR) imaging, and inductively coupled plasma mass spectrometry. Uptake of RGD-USPIO by HUVECs was significantly increased when compared with unlabeled USPIO and could be competitively inhibited by addition of unbound RGD. The ability of the RGD-USPIO to noninvasively distinguish tumors with high (HaCaT-ras-A-5RT3) and lower (A431) area fractions of αvβ3 integrin–positive vessels was evaluated using a 1.5-T MR scanner. Indeed, after RGD-USPIO injection, there was a more pronounced decrease in T2 relaxation times in HaCaT-ras-A-5RT3 tumors than in A431 tumors. Furthermore, T2*-weighted images clearly identified the heterogeneous arrangement of vessels with αvβ3 integrins in HaCaT-ras-A-5RT3 tumors by an irregular signal intensity decrease. In contrast, in A431 tumors with predominantly small and uniformly distributed vessels, the signal intensity decreased more homogeneously. In summary, RGD-coupled, APMTMS-coated USPIOs efficiently label αvβ3 integrins expressed on endothelial cells. Furthermore, these molecular MR imaging probes are capable of distinguishing tumors differing in the degree of αvβ3 integrin expression and in their angiogenesis profile even when using a clinical 1.5-T MR scanner. [Cancer Res 2007;67(4):1555–62]

Introduction

Angiogenesis is an essential step for the growth and spread of malignant tumors (1, 2) and its extension correlates with the degree of malignancy. Therefore, the ability to noninvasively detect αvβ3 integrin expression in living subjects would allow a better characterization of tumors and help to identify tumor regions with higher aggressiveness. This might be valuable to improve radiotherapy planning and the monitoring of antiangiogenic and other noninvasive antitumor therapies (9).

For targeting angiogenic vessels, αvβ3 integrin antibodies were created and are currently evaluated in clinical trials as antiangiogenic therapeutics (10, 11). Additionally, αvβ3 integrins can be targeted by small peptides. A suitable short amino acid sequence that binds to the αvβ3 integrin receptor is Arg-Gly-Asp (RGD). Linear (12, 13) and cyclic RGD peptides (14) have been tested to target αvβ3 integrins for different purposes. Cyclic RGD peptides that consist of a ring system flanked by unrelated amino acids were shown to better resist proteolysis and to have a higher affinity to the target than their linear counterparts.

Several diagnostic compounds based on the above-mentioned targeting vectors have been developed and used in positron emission tomography (PET; refs. 15–17), single-photon emission computed tomography (18, 19), optical imaging (20, 21), and ultrasound (22, 23). Using [18F]galacto-RGD, it was shown by PET that the tracer uptake by melanoma xenografts correlated with its αvβ3 integrin expression level. Furthermore, these specific probes were found to be capable of detecting angiogenesis in squamous cell carcinoma xenografts in mice (A431), which expressed αvβ3 integrins only on angiogenic vessels but not on tumor cells (15).

Magnetic resonance (MR) imaging (MRI) is also a highly desirable modality for molecular imaging because it provides not only high spatial resolution but also excellent soft tissue contrast. However, the low sensitivity of MRI to contrast agents often reduces the success of imaging approaches with targeted contrast agents. Thus, particles, polymers, or liposomes loaded with high amounts of gadolinium or superparamagnetic iron oxide particles are usually conjugated to the specific ligands to generate a sufficiently high tissue contrast. Addressing extravascular targets with these conjugates, however, is often problematic due to their limited extravasation and high uptake by the reticuloendothelial system. On the other hand, imaging of angiogenic targets is promising because the targets are presented on the surface of the vessels and thus can directly be addressed from the blood. In this context, T1 contrast agents have been developed to target αvβ3 integrins by MRI. Sipkins et al. (24) showed that paramagnetic...
nanoparticles conjugated with an antibody against \( \alpha_\text{v}\beta_3 \) integrin can successfully detect tumor angiogenesis by MRI in vivo. Also, paramagnetic nanoparticles containing peptidomimetic \( \alpha_\text{v}\beta_3 \) integrin antagonists successfully visualized early angiogenesis induced by VX-2 tumor implants in rabbits (25) and nascent melanoma xenograft (26) in mice using clinical MR scanners. Molecular MRI of angiogenesis has also been shown using RGD-conjugated paramagnetic liposomes that bound to \( \alpha_\text{v}\beta_3 \) integrins expressed on the surface of endothelial cells (27). All of these studies used compounds containing gadolinium chelates to generate a positive MR contrast.

In MRI, a higher sensitivity for specific contrast agents can be achieved with negative contrast agents that decrease the \( T_2^* \) and \( T_2 \) relaxation times of tissues and thus allow to circumvent in part the sensitivity problem of the method. In this context, super-paramagnetic iron oxide particles and ultrasmall superparamagnetic iron oxide particles (USPIOs) that are coated with dextran and its derivatives are most frequently used. Alternatively, USPIO coatings with citric acid (28), polystyrene (29), siloxane (30), and polyethylene glycol (PEG; ref 31) have been suggested. Moreover, alkoxysilanes, such as 3-aminopropyltrimethoxysilane (APTMS), with functional amino groups, have been suggested as coating materials for iron oxide particles (32, 33). We consider APTMS coating to be attractive because it is compatible with many of the materials used in a biological context (34). Additionally, for APTMS modification, a very thin monolayer coating can be realized (35). This is important to keep the total particle diameter small. Usually, small particle sizes show an improved pharmacokinetic behavior with a lower uptake by the reticuloendothelial system, a higher extravasation rate, and a better binding of particles to target after conjugating specific ligands. Nevertheless, thus far, there is no report about the in vivo application of APTMS-coated USPIOs as MRI contrast agents.

Thus, in this study, USPIOs were coated with APTMS and functionalized with RGD peptides to target \( \alpha_\text{v}\beta_3 \) integrins. We show that these particles specifically bind with high affinity to \( \alpha_\text{v}\beta_3 \) integrins and subsequently accumulate in the cytoplasm of endothelial cells. After i.v. injection of RGD-USPIO in mice, s.c. tumor xenografts with high and low density of \( \alpha_\text{v}\beta_3 \) integrin-positive vessels could be faithfully distinguished on \( T_2^* \)-weighted MR images, and differences in RGD-USPIO accumulation were quantified by \( T_2 \) relaxometry.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), if not indicated otherwise. The RGD peptides, cyclo(Arg-Gly-Asp-D-Tyr-Glu), were obtained from Peptides International, Inc. (Louisville, KY).

Synthesis and Characterization of the APTMS-Coated USPIOs

For preparation of USPIO, a modified Massart method (36) was used. A mixture of \( \text{FeCl}_3 \) (20 mL, 1 mol/L) and \( \text{FeCl}_2 \) (20 mL, 0.5 mol/L) in hydrochloric acid (0.2 mol/L) was added dropwise to a mechanically stirred solution of sodium hydroxide (200 mL, 1.5 mol/L) within 5 min under an inert nitrogen atmosphere at 80°C. The mixture was stirred for 20 min and subsequently cooled down to room temperature. The sediment was separated by an external magnetic field, washed thrice with deionized water, and dispersed into 100 mL water (pH 2.0). For coating, USPIOs (1 g) were washed once with methanol (50 mL), thereafter with a mixture of methanol and toluene (50 mL v/v 1:1), and finally with toluene alone (50 mL). Subsequently, the particles were dispersed into toluene (100 mL) and APTMS (0.1 mL, 5.73 mol/L) was added dropwise into the suspension within 5 min. The ferrofluid suspension was transferred into a three-necked flask with N2 flow and refluxed under mechanical stirring for 10 h. The modified particles were magnetically collected and dispersed into methanol by ultrasonication. After washing with methanol, the APTMS-coated USPIO particles were dispersed into water.

The particles were characterized by transmission electron microscopy (TEM, JEOL-100CX), and the particle size and size distribution were calculated using an image analysis program and by measuring the diameters of at least 300 particles. The surface charge of modified USPIO was determined by measuring the \( z \) potential as a function of pH value of USPIO suspensions using a particle charge detector (PCD 03, Muettec, Herrsching, Germany).

Energy dispersive X-ray analysis, attached to scanning electron micrograph (LEO 1530 VP) and organic elementary analysis (inductively coupled plasma (ICP), IRIS Advantage 1000), were done to investigate whether there was an APTMS coating layer on the surface of the particles.

To determine the relaxivities, the particles were diluted in distilled water in concentrations between 0.001 and 100 \( \mu \text{g/mL} \). \( T_1 \) and \( T_2 \) relaxation times of the suspensions were determined using 1.5-T whole-body MR scanner (Siemens Symphony, Erlangen, Germany) in combination with a custom-made radiofrequency coil for excitation and signal reception (37). For the determination of the \( T_2 \) relaxation times, a Carr-Purcell-Meiboom-Gill sequence was used [repetition time (TR), 2,000 ms; echo time (TE) range, 30–960 ms; 32 echoes; field of view (FOV), 134 \( \times \) 67 mm; matrix, 128 \( \times \) 64; slice thickness, 10 mm; bandwidth, 40; number of excitations, 3]. \( T_1 \) relaxation times were obtained using a saturation recovery turbo FLASH sequence with different inversion times (TR, 7,160 ms; TE, 1.67 ms; inversion time, 90–4,000 ms; FOV, 200 \( \times \) 150 mm; matrix, 52 \( \times \) 128; slice thickness, 2 mm). \( T_1 \) and \( T_2 \) relaxation rates were plotted against the iron concentrations in the particle dilutions. The relaxivities were determined by a linear fit.

Coupling of RGD Peptides to APTMS-USPIO

1-Ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (0.4 mg) and \( N \)-hydroxysuccinimide (0.6 mg) were added to 1 mL 2-(N-morpholino)-ethane sulfonic acid buffer (0.1 mol/L, pH 6). Two hundred micrograms of the RGD peptide cyclo(Arg-Gly-Asp-D-Tyr-Glu) were added to 300 \( \mu \text{L} \) of the mixture and incubated for 15 min at room temperature. Ten milligrams of USPIO were dispersed into the solution and incubated for 60 min at room temperature. Then, USPIOs were separated with a magnet and washed thrice with PBS.

In vitro Analysis of (RGD-)USPIO Uptake in Human Umbilical Vein Endothelial Cells

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from Promo Cell GmbH (Heidelberg, Germany). HUVECs were incubated with 5 mL culture medium containing RGD-USPIO or plain USPIO at an iron concentration of 0.03 \( \mu \text{mol/mL} \) for different time intervals (10 min, 20 min, 1 h, and 3 h). Competition experiments were done by adding the free ligand to the RGD-USPIO in a ratio of 10,000:1. After incubation, the culture medium was removed. The adherent cells were washed thrice with PBS (0.1 mol/L, pH 7.4), trypsinized, and centrifuged for 5 min at 2,000 \( \times \) g. The number of cells was determined using a Neubauer counting chamber. A total of 7 \( \times \) 10\(^5\) cells were embedded in gelatin (200 \( \mu \text{L} \), 10%) at 37°C for MRI measurement and the subsequent determination of the iron content by ICP-mass spectroscopy (ICP-MS).

Prussian blue staining. For Prussian blue staining, the cells were cultivated for 24 h in 24-well plates on glass coverslips. HUVECs were washed thrice with PBS and subsequently fixed with methanol and acetone (–20°C). For Prussian blue staining, the fixed cells were incubated with 10% potassium ferrocyanide for 5 min and 10% potassium ferrocyanide in 20% hydrochloric acid for 30 min, and counterstained with nuclear fast red.

TEM. Cells grown on glass coverslips were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.2) at 4°C overnight. Slides were successively stained with 2% osmium tetroxide and 0.5% uranyl acetate and processed for ultrathin sectioning. Micrographs were taken with a Zeiss EM-10 A electron microscope at 80 kV. The magnification indicator was routinely controlled by the use of a grating replica.
Viability of the cells. Viability of the cells cultured under different incubation conditions was evaluated using trypan blue (Trypan Blue Stain, Invitrogen Corporation, Grand Island, NY) and terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining (Roche Diagnostics, Indianapolis, IN). The percentage of nonviable cells was determined by counting trypan blue–positive and trypan blue–negative cells in a Neubauer chamber. TUNEL staining was done for cells grown on glass cover slides according to manufacturer’s instructions. One hundred cells in one vision field and a total of five vision fields were analyzed at a ×40 magnification.

Determination of the intracellular iron content. The amount of iron present in the cells was determined by high-resolution sector field ICP-MS (Element 2, Thermo Electron, Bremen, Germany) equipped with an auto sampler (ASX-100, CETAC, Omaha, NE). Data were acquired in medium resolution (4,000) using rhodium (5 ppb) as internal standard. A PFA spray chamber equipped with a PFA 100 microflow nebulizer (CETAC) was used for sample introduction. The instrument was calibrated via infusion of a 1 ng/mL multielement standard solution (Merk, Darmstadt, Germany).

Digestion of cells was done in a closed vessel microwave reaction system (CEM, Kamp-Lintfort, Germany) after addition of 53% nitric acid (60%) at 3 bar and 600 W for 50 min. Samples were diluted 1:200 in H2O. Calibration was linear between 2 and 150 ng/mL iron (r > 0.999). Iron content was expressed in picogram of iron per cell.

MRI and T2 relaxometry of HUVECs in gelatin. T2 MRI relaxometry of HUVECs in gelatin was done using a multiecho spin echo pulse sequence (TR, 1,200 ms; TE range, 7–350 ms; 32 echoes; FOV, 150 mm; matrix, 128 × 39; slice thickness, 5 mm; voxel size, 1.6 × 1.6 × 5.1 mm3). T2 relaxation times were calculated by a linear fit of the logarithmic region-of-interest signal amplitudes versus TE.

Morphologic MRI was done using a T2-weighted gradient echo sequence (TR, 600 ms; TE, 14 ms; average, 3; FOV, 180 × 73 mm; matrix, 256; slice thickness, 1.5 mm; flip angle, 90°), a T2-weighted turbo spin echo sequence (TR, 1,070 ms; TE, 14 ms; average, 3; FOV, 180 × 73 mm; matrix, 256; slice thickness, 2 mm), and a T2*- weighted gradient echo sequence (TR, 213 ms; TE, 10.9 ms, average, 15; FOV, 130 × 56.9 mm; matrix, 256; slice thickness, 1.5 mm; flip angle, 10°).

Targeting of αvβ3 Integrins In vivo
All experiments were approved by the governmental review committee on animal care. Human squamous cell carcinoma xenografts were induced by s.c. injection of 2 × 106 HaCat-ras-A-5RT3 cells (38, 39) or 5 × 106 A431 cells (15) in the lower thigh of nude mice. MR experiments were done after the tumors were grown for 4 weeks. Eight mice with HaCat-ras-A-5RT3 tumors and six with A431 tumors were injected with RGD-USPIO at the dose of 900 μmol Fe/kg. The same numbers of control animals with HaCat-ras-A-5RT3 and A431 tumors were injected with plain USPIO at the same dose.

Animals were imaged according to the following protocol:

1. T1-weighted gradient echo sequence (TR, 600 ms; TE, 14 ms; FOV, 180 × 73 mm; matrix, 256; slice thickness, 1.5 mm; flip angle, 90°).
2. T2-weighted turbo spin echo sequence (TR, 1,070 ms; TE, 14 ms; FOV, 180 × 73 mm; matrix, 256; slice thickness, 2 mm).
3. T2*-weighted gradient echo sequence (TR, 213 ms; TE, 10.9 ms; FOV, 130 × 56.9 mm; matrix, 256; slice thickness, 1.5 mm; flip angle, 10°).
4. T2 relaxation times in tumor, blood, liver, and muscle before and after particle injection were determined using a multiecho spin echo pulse sequence (TR, 1,330 ms; TE range, 8.3–265.5 ms; 32 echoes; average, 5; FOV, 120 × 34.4 mm; matrix, 33 × 128; slice thickness, 2 mm; voxel size, 1.3 × 0.9 × 2 mm3; flip angle, 180°). Three slices were positioned: one at the tumor at maximum extensions, one at the liver, and one at the kidney level.

Immunohistochemistry. For immunohistochemical diagnosis and assessment of tumor tissue and its vascularization, tumors were dissected, embedded in Tissue-Tek (Sacura, Zoeterwoude, the Netherlands), and frozen in liquid nitrogen vapor. Five-micrometer sections were fixed first in methanol for 10 min, then in acetone for 5 min at −20°C, and air dried for 30 min. The expression of αvβ3 integrins on mouse vessels was investigated by immunofluorescence staining using a primary hamster anti-mouse CD61 antibody (BD Biosciences, San Jose, CA) and a Cy2-conjugated secondary antibody against hamster IgG. Countering staining of endothelial cells was done using a rat anti-mouse CD31 antibody (BD Biosciences) in combination with a Cy3-labeled anti-rat IgG secondary antibody (Jackson Immunoresearch, Inc., Baltimore, MD) and with Hoechst nuclear staining (Invitrogen, Paisley, United Kingdom).

Quantitative analysis of positive area of CD31 and αvβ3 integrin–positive vessels was done using the AnalySIS Software (Soft Imaging System GmbH, Muenster, Germany). In this context, at least three randomly selected vision fields of each tumor were analyzed.

Presence of αvβ3 integrins on tumor cells was excluded by staining human CD51/CD61 with a primary mouse anti-human CD51/CD61 antibody (BD Biosciences) and a Texas red–labeled anti-mouse IgG secondary antibody (Jackson Immunoresearch Lab).

Statistical evaluation. Statistical analysis was conducted using a Wilcoxon rank sum test. P < 0.05 was considered significant.

Results

The mean size of the particles as determined by TEM was 10 ± 3 nm (5% < 7 nm, 25% < 8 nm, median 9 nm, 75% < 10 nm, 95% < 15 nm), and energy dispersive X-ray measurement indicated silicon surface deposition of 11.9 weight %. The contents of C and N were 1.3 ± 0.4 weight %, respectively. Surface charge of the particles was positive with isoelectric point at pH 9.6. T2 and T1 relaxivities of the particles were r2 = 134 s−1 (mmol/L)−1 and r1 = 1 s−1 × (mmol/L)−1, respectively.

USPIO and RGD-USPIO uptake in HUVECs. The uptake of USPIO, RGD-USPIO, and RGD-USPIO plus free RGD after blocking the target by addition of free RGD peptides was assessed histologically using Prussian blue staining (Fig. 1). After 10 min, a strong uptake of RGD-USPIO was observed, whereas there was no significant uptake for plain particles. Blocking the αvβ3 integrin receptor with free RGD effectively reduced the amount of blue granules in the cytoplasm of HUVECs, which indicates that the accumulation of these particles was specifically mediated by its αvβ3 integrin binding. After 3-h incubation, however, the differences in particle uptake between all conditions were less prominent.

TEM gave insight into the subcellular localization of the particles in HUVECs after incubation with USPIO and RGD-USPIO for 10 min (Fig. 2). Although large amounts of RGD-USPIO were internalized and accumulated in the cellular endosome, most of the plain USPIOs were deposited outside the cells and some of them were attached to the cell membrane.

Additionally, quantification of the cellular iron content by ICP-MS spectrometry and the measurement of T2 relaxation times of HUVECs in gelatin by MR relaxometry proved the higher uptake of RGD-USPIO compared with the plain USPIO and the RGD-USPIO after blocking the αvβ3 integrin. After 10-min incubation, the mean cellular iron content in HUVECs incubated with plain USPIO was 0.11 ± 0.001 μg Fe per cell, whereas it increased to 0.25 ± 0.002 μg Fe per cell for cells incubated with RGD-USPIO. After blocking the αvβ3 receptor by adding the free ligand, the mean cellular iron content of RGD-USPIO–incubated cells decreased to 0.18 ± 0.002 μg Fe per cell (Fig. 3A). In line with the histologic findings, the difference in particle uptake by HUVECs for USPIO, RGD-USPIO, and RGD-USPIO plus free ligand was less prominent after 3 h (USPIO, 0.54 ± 0.002 μg/cell; RGD-USPIO, 0.70 ± 0.009 μg/cell; and RGD-USPIO + RGD, 0.60 ± 0.004 μg/cell).

These changes were also reflected in the T2 relaxation times. After 10-min incubation, mean T2 relaxation time was 176.20 ± 0.84 ms for HUVECs incubated with USPIO and significantly lower for the
cells that were incubated with RGD-USPIO (79.90 ± 8.04 ms). After blocking the binding site with free RGD and subsequent incubation with RGD-USPIO, the T2 relaxation times of HUVEC suspensions only decreased to 99.20 ± 10.03 ms (Fig. 3B). Again, after 3-h incubation, there was no longer a significant difference in T2 relaxation times between HUVECs in gelatin incubated with USPIO (66.80 ± 4.49 ms), RGD-USPIO (68 ± 3.67 ms), and RGD-USPIO in the presence of free RGD (57.2 ± 7.19 ms), respectively.

In further support of the above data, MRI signal intensity (SI) of HUVECs in gelatin incubated with RGD-USPIO for 10 min was significantly decreased compared with those that were incubated with plain USPIO. After blocking the binding site on αvβ3 integrins with free RGD, the subsequent incubation with RGD-USPIO caused only a little decrease in SI of HUVECs in gelatin (Fig. 4).

Trypan blue staining did not indicate a reduced viability of HUVECs after incubation with USPIO, RGD-USPIO, and RGD-USPIO plus free RGD at an iron concentration of 0.03 μmol/mL for different time intervals. Even after 3-h incubation, only 5.3 ± 2.7% (USPIO), 6.1 ± 3.4% (RGD-USPIO), and 5.7 ± 3.4% (RGD-USPIO + RGD) dead cells were found, which did not differ from the percentage of nonviable control cells (7.5 ± 3.3% dead cells).

Accordingly, compared with the control cells (4.0 ± 7.4% positive cells), the percentages of apoptotic cells as indicated by TUNEL staining were not increased after incubation with USPIO.
A branched network of strong SI decrease was found at the border and in certain central parts of the tumor, which is consistent with the heterogeneous pattern of angiogenesis that was described in these tumors (40). In contrast, SI changes in A431 tumors were less pronounced and more homogeneous, and only few spots with strong SI decrease could be observed at the tumor periphery (Fig. 5A).

SI decrease in HaCaT-ras-A-5RT3 tumors that were injected with plain USPIO was weaker and differed in its distribution pattern with changes occurring predominantly at the lateral tumor borders as small spots. Almost no accumulation of plain USPIO could be detected in A431 tumors.

To verify the MR data histologically, the presence of αvβ3 integrins on tumor vessels and tumor cells was investigated. By using the anti-human αvβ3 integrin antibody, no positive staining was observed in the tumor cells of both models. To visualize and quantify αvβ3 integrins expressed by the murine tumor vessels and the tumor stroma, the anti-mouse-αvβ3 integrin antibody was used.

In line with the MR results, in HaCaT-ras-A-5RT3 tumors, significantly (P < 0.01) higher area fractions of CD31-positive (mean ± SD, 1.9 ± 1.1%) and αvβ3 integrin–positive vessels (mean ± SD, 1.0 ± 0.5%) were found than in A431 tumors (mean CD31/αvβ3 integrin area fraction ± SD, 0.9 ± 0.4%/0.3 ± 0.1%).

On fluorescence images, HaCaT-ras-A-5RT3 tumors showed a highly heterogeneous vascularization with large, intensively branched vascular networks but also low vascularized areas with only few small vessels. As described previously (41), discontinuous and heterogeneous αvβ3 integrin expression was found on endothelial cells and in perivascular stromal cells (Fig. 6). In contrast, A431 tumors showed a homogeneous vascularization with predominantly small vessels of <20 μm diameter. These differences in the vascular phenotypes with focal intense vascularized areas showing high levels of αvβ3 integrins in HaCaT-ras-A5RT3 tumors and with the more homogeneous vascularization in A431 tumors plausibly explain the different SI change pattern seen on the T2*-weighted MR images.

Furthermore, the change of T2 relaxation times in liver, kidney, muscle, and blood were analyzed 6 h after particle injection. Changes of T2 relaxation times in all analyzed organs were not significantly different between animals that received RGD-USPIO or plain particles (Fig. 5C). The most pronounced decrease in T2 relaxation time was found in the liver, indicating a strong but unspecific USPIO uptake. No significant differences of T2 relaxation

### Figure 3

A. results from ICP-MS measurement of the mean cellular iron contents after incubation of HUVECs with plain USPIO, RGD-USPIO, and RGD-USPIO competed with free RGD (competition) for different time intervals (iron concentration, 0.03 μmol/mL of growth medium). B. T2 relaxation times of HUVECs in gelatin incubated with plain USPIO, RGD-USPIO, and RGD-USPIO plus free RGD for different time intervals. Cells were incubated with particles in a dose of 0.03 μmol Fe/mL of growth medium. After 10 and 20 min, T2 relaxation times of cell suspensions after incubation with RGD-USPIOs were significantly shorter as the T2 relaxation times of HUVEC suspensions that were incubated with plain USPIO or RGD-USPIO in the presence of the free ligand. After 3-h incubation, the T2 relaxation times of cell suspensions became not significantly different. *, P < 0.05; **, P < 0.01.

### Figure 4

A. Cellular iron uptake of HaCaT-ras-A-5RT3 and A431 tumors elucidated differences in the distribution pattern of RGD-USPIO. In HaCaT-ras-A-5RT3 tumors, a branched network of strong SI decrease was found at the border and in certain central parts of the tumor, which is consistent with the heterogeneous pattern of angiogenesis that was described in these tumors (40). In contrast, SI changes in A431 tumors were less pronounced and more homogeneous, and only few spots with strong SI decrease could be observed at the tumor periphery (Fig. 5A).

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times before and 6 h after particle injection were found in the blood, indicating that most of the particles were already cleared at that time. Also, no significant changes of T2 relaxation times were found in muscle and kidney.

**Discussion**

In this study, RGD peptide–conjugated, APTMS-coated USPIOs were developed and their ability to specifically bind to the αvβ3 integrin on endothelial cells was investigated in *vitro* and *in vivo*. In contrast to most previous molecular MRI approaches using dextran-coated particles, the USPIOs used in this study were coated with alkoxysilane. This coating provides the significant advantage that the presence of amine groups on the particles surface can easily be used to covalently attach specific ligands (42) and that the coating layer, which does not contribute to the MR signal, can be kept very thin.

Although MR-contrast agents that were previously used to detect angiogenesis cause a positive T1 contrast (24–27), USPIOs generate a reduction of the T2 relaxation time, which is enhanced by susceptibility effects that can clearly exceed the voxel. This leads to an increased sensitivity of MRI to these contrast agents compared with gadolinium-containing ones.

Targeting αvβ3 integrins can be achieved by the use of linear or cyclic peptides containing the RGD sequence. We used the cyclic RGD peptide because it provides a 30-fold higher stability compared with the linear one (43). The cyclic RGD peptides were conjugated to the particles by condensing carboxylic groups on glutamic acid with aminogroups of the APTMS coating layer. However, it cannot be excluded that

Figure 5. A, T2*-weighted MR images of nude mice bearing s.c. HaCaT-ras-A-5RT3 (top row) and A431 tumors (bottom row) before and 6 h after i.v. injection of RGD-USPIO and USPIO, respectively. In HaCaT-ras-A-5RT3 tumors, focal areas with strong and heterogeneous SI decrease are observed in the tumor center and periphery after injection of RGD-USPIO (arrows). Only few spots with high susceptibility are found at the margin of the control tumor after injection of USPIO. SI changes in the A431 tumor are much less pronounced and more homogeneous, and focal areas with strong SI decrease are only found at the tumor margins (arrows). No signal changes are observed in control tumors by visual inspection. B, change of T2 relaxation times in HaCaT-ras-A-5RT3 (n = 6) and A431 (n = 6) tumors after injection of RGD-USPIO and USPIO, respectively. Absolute T2 relaxation times before/after injection with RGD-USPIO were 124.44 ± 55.09/94.09 ± 17.12 in HaCaT-ras-A-5RT3 and 105.66 ± 10.67/90.16 ± 7.84 in A431 tumors. The values for injection with USPIO were 95.97 ± 16.97/93.74 ± 15.86 (HaCaT-ras-A-5RT3) and 106.47 ± 13.16/101.46 ± 13.28 (A431 tumors), respectively. *, P < 0.05. C, change of T2 relaxation times in blood, liver, kidney, and muscle of A431 tumor-bearing mice after injection of RGD-USPIO (n = 6) and USPIO (n = 6), respectively. Absolute T2 relaxation times (±SD) before/after injection of RGD-USPIO were 101.8 ± 17.58/101.76 ± 17.77 in blood, 53.06 ± 3.81/31.56 ± 5.54 in liver, 90.53 ± 10.05/90.14 ± 8.60 in kidney, and 51.33 ± 8.88/50.40 ± 5.11 in muscle. For USPIO, the values were 77.26 ± 15.73/79.28 ± 10.90 in blood, 56.66 ± 7.90/37.33 ± 4.43 in liver, 82.26 ± 17.37/70.90 ± 17.36 in kidney, and 50.40 ± 5.68/46.04 ± 3.78 in muscle. (Note that the means of individual T2 relaxation time changes shown are a little different from values resulting from subtracting the mean organ T2 relaxation times of all animals before and after particle injection.) Columns, mean; bars, SD.
some of the RGD peptides were bound to the USPIOs via the aspartic acid at the active binding site, which would have reduced their target specificity. Despite this potential limitation, specific binding of our RGD-USPIO to HUVECs was shown and could be antagonized by the addition of unbound ligands. In this context, the highest difference in the uptake of USPIO, RGD-USPIO, and RGD-USPIO plus free RGD by HUVECs was found at early time points, which points to a receptor-mediated endocytosis mechanism. After 3-h incubation, the difference in the uptake of RGD-coupled and plain USPIO by HUVECs was less pronounced. This can be explained by the fact that after the αvβ3 integrins were saturated, phagocytosis became the predominant mechanism of particle uptake.

We also excluded early toxic effects of USPIO, RGD-USPIO, and RGD-USPIO plus free RGD on HUVECs that might interfere with the phagocytosis of the particles. This aspect was particularly important for the competition experiments, in which reduced cell viability after addition of the free ligand would have mimicked a successful receptor blockade.

After administration of RGD-USPIO in mice, we could successfully show that SI changes seen on T2*-weighted images were characteristic for each tumor model. Although application of RGD-USPIO caused a heterogeneous decrease in SI in HaCaT-ras-A-5RT3 tumors, SI changes in A431 tumors were less pronounced and more homogeneous. These results are in line with the vascularization type and the expression pattern of αvβ3 integrins of the tumors, which were seen histologically. MR images of tumor-bearing mice that were injected with plain USPIO showed a low degree of background signal change, which can be attributed to particles that were extravasated from tumor vessels and scattered in the interstitial space. However, this signal change remained insignificant compared with the specific RGD-mediated signal.

Additionally, HaCaT-ras-A-5RT3 tumors, which had a higher amount of αvβ3 integrin–positive vessels, showed a more pronounced decrease in T2 relaxation time than A431 tumors. The fact that the change of relaxation time between both models failed statistical significance may be explained by the heterogeneous angiogenesis and αvβ3 integrin expression in HaCaT-ras-A-5RT3 tumors. In this context, it might be interesting to investigate whether there is an association between the expression of αvβ3 integrins and the heterogeneous growth of HaCaT-ras-A-5RT3 tumors and thus their different volumes at the day of examination. However, this question cannot be answered in our preliminary study on few animals. Furthermore, it has to be considered that in vivo targeting by MRI cannot be as accurate as immunohistology because the sensitivity for the marker is lower. Furthermore, due to extravasation and interstitial accumulation of the MR probes and their unspecific uptake by phagocytizing cells, the background signal is higher. The delivery of the contrast agent to the target is another obstacle, which, however, may be less relevant for imaging angiogenesis in which targets are located on endothelial cells and thus directly accessible from the bloodstream.

As for most other USPIOs, the uptake of our particles by liver and reticuloendothelial system is high (44) and even exceeded the uptake in A431 tumors. This may make localization and characterization of angiogenic lesions in these organs difficult and may complicate clinical translation. High unspecific uptake by the liver and the reticuloendothelial system can be reduced by additional PEG coating (45), which, however, can also reduce the

Figure 6. Triple-stained immunofluorescence images of HaCaT-ras-A-5RT3 (A–C) and A431 tumors (D–F). A and D, CD31 stainings (red); B and E, αvβ3 integrin stainings (green). C and F, overlays of the images with additional Hoechst nuclear staining (blue). Both tumors show CD31-positive vessels with heterogeneous colocalization of CD61. Additional αvβ3 integrin staining can be found in the perivascular stroma, which is more pronounced in the HaCaT-ras-A-5RT3 (C) than in the A431 tumors (F). Also, the differences in the vascular phenotypes are clearly mirrored. Although HaCaT-ras-A-5RT3 tumors show large, branched vessels with pronounced stroma components, A431 tumors develop many small vessels of capillary sizes. Bar, 200 μm.
target affinity of the probes. Thus, the improvement of the pharmacokinetic properties of our particles while maintaining their specificity is one important aim of our ongoing research.

Nevertheless, our findings indicate that it is possible to non-invasively characterize the degree of angiogenesis in tumors with RGD-specific, APTMS-coated USPIOs even when using a clinical 1.5-T MR scanner. Thus, molecular imaging of $\alpha_v\beta_3$ integrins using clinical hardware can noninvasively provide insights into the molecular profile of tumor vessels and may have potential to monitor changes in angiogenesis during tumor therapy.

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**References**

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Chunfu Zhang, Manfred Jugold, Eva C. Woenne, et al.


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