Quantitative Molecular Magnetic Resonance Imaging of Tumor Angiogenesis Using cNGR-Labeled Paramagnetic Quantum Dots

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Abstract

The objective of this study was to develop and apply cyclic Asn-Gly-Arg (cNGR)-labeled paramagnetic quantum dots (cNGR-pQDs) for the noninvasive assessment of tumor angiogenic activity using quantitative in vivo molecular magnetic resonance imaging (MRI). cNGR was previously shown to colocalize with CD13, an aminopeptidase that is highly overexpressed on angiogenic tumor endothelium. Because angiogenesis is important for tumor growth and metastatization, its in vivo detection and quantification may allow objective diagnosis of tumor status and evaluation of treatment response. I.v. injection of cNGR-pQDs in tumor-bearing mice resulted in increased quantitative contrast, comprising increased longitudinal relaxation rate and decreased proton visibility, in the tumor rim but not in tumor core or muscle tissue. This showed that cNGR-pQDs allow in vivo quantification and accurate localization of angiogenic activity. MRI results were validated using ex vivo two-photon laser scanning microscopy (TPLSM), which showed that cNGR-pQDs were primarily located on the surface of tumor endothelial cells and to a lesser extent in the vessel lumen. In contrast, unlabeled pQDs were not or only sparsely detected with both MRI and TPLSM, supporting a high specificity of cNGR-pQDs for angiogenic tumor vasculature. [Cancer Res 2008;68(18):7676–83]

Introduction

Angiogenesis, the formation of new capillaries from existing blood vessels, is key to tumor growth and metastatization by providing proliferating tumor cells with oxygen and nutrients (1, 2). Moreover, angiogenic activity is related to tumor malignancy (3, 4). Noninvasive detection of angiogenic activity is therefore highly relevant for adequate tumor diagnosis. Quantification of angiogenesis may furthermore allow objective monitoring of tumor progression, for instance in response to treatment.

Currently, molecular imaging techniques are being developed that allow direct visualization and characterization of cellular or molecular activation of angiogenesis-related pathways (5). More specifically, molecular imaging uses contrast agents that home to up-regulated biomolecules (e.g., receptors, enzymes) via interaction with high-affinity ligands coupled to the contrast agent. Ideally, this results in an altered signal intensity at the location of these molecules. Of the different imaging modalities, magnetic resonance imaging (MRI) may be the most desirable for molecular imaging due to its excellent spatial resolution and soft tissue contrast. Moreover, molecular MRI potentially allows direct visualization of tumor angiogenic activity with anatomy. However, the inherently low sensitivity of MRI is a problem due to the typically low abundance of up-regulated biomolecules. This can be overcome by large molecular weight constructs carrying a high payload of gadolinium or iron, and multiple targeting ligands to enhance the relaxivity and targeting efficacy, respectively, of the particle (6).

One of the best-defined ligands for molecular imaging of angiogenesis is the cyclic Arg-Gly-Asp (cRGD) peptide, which binds specifically to the αvβ3 integrin (7, 8). However, for the cyclic Asn-Gly-Arg (cNGR) motif, the tumor-homing capability was shown to be 3-fold higher compared with cRGD (9). The clinical applicability of cNGR as a tumor-homing ligand was previously shown by conjugating cNGR to tumor necrosis factor α (TNFa). Compared with unlabeled TNFa, cNGR-TNFα displayed a significantly increased antitumor activity with similar systemic toxicity (10–12).

The vascular address of cNGR is a specific isoform of CD13 (aminopeptidase N), a transmembrane glycoprotein involved in cancer angiogenesis, tumor invasion, and metastasis, which is overexpressed by activated endothelial cells (ECs) of tumor vasculature (9, 13, 14). CD13 is not required for vessel growth during embryonic development and normal adult function, as shown in CD13-null mice (15). In a model of retinal neovascularization, these mice had significantly decreased vessel growth, suggesting that CD13 is important in pathologic neovascularization. In addition, fluorophore-conjugated cNGR allowed detection of the in vivo expression of CD13 in tumors and infarcted myocardium (16, 17). Competition with unconjugated cNGR significantly decreased the fluorescence signal, indicating high specificity of cNGR for CD13 (16, 17).

Despite the aforementioned high tumor-homing capability of cNGR, its potency as a targeting ligand for molecular imaging of tumor angiogenesis is currently unknown. Therefore, the objective of this study was to explore cNGR-labeled paramagnetic quantum dots (cNGR-pQDs) for the noninvasive and selective in vivo detection of tumor neovascularization using quantitative molecular MRI. QDs were chosen as contrast agent scaffolds because of their excellent photophysical properties, i.e., broad excitation, small emission spectra, and limited photobleaching (18, 19). Furthermore, QDs enabled binding of multiple targeting ligands and gadolinium chelates. The bimodal nature of the particle (i.e., paramagnetic and fluorescent) allowed validation of the results with ex vivo two-photon laser scanning microscopy (TPLSM). With
THERAPEUTIC APPLICATIONS OF MRI

Figure 1. Schematic representation of a cNGR-labeled paramagnetic quantum dot. Each particle carries ~10 streptavidin moieties to which 6 cNGR groups and 24 dendritic gadolinium constructs were bound. The total number of gadolinium ions per particle was maximally 192.

MRI of Angiogenesis with cNGR-Labeled Quantum Dots

Materials and Methods

Preparation of cNGR-Labeled Paramagnetic Quantum Dots

NAC-Cys(4MeBzl)-Asn(Xanthyl)-Gly-Arg(Tosyl)-Cys(4MeBzl)-Gly-Gly-Lys(Fmoc)-peptide was synthesized by tBoc solid phase peptide synthesis, as described previously (16, 20). After covalent coupling of biotin-succinimidyl ester (Molecular Probes) to the lysine ε-amino group to obtain biotinylated peptide-resin, the peptide was deprotected and cleaved from the resin using anhydrous hydrogen fluoride for 1 h at 0°C. The cystein side chains due to the generation of 1 disulfide bond (S-S), was synthesized and chromatography (HPLC)-purified (C18 RP-HPLC) and lyophilized. ESI-MS revealed a mass of 1,287.4, corresponding well to the theoretical average mass (1,288.7) of the reduced biotinylated Nac-Cys(4MeBzl)-Asn-Xanthyl-Gly-Arg-Tosyl-Cys(4MeBzl)-Gly-Gly-Lys(biotin)-NH₂ peptide. Oxidative folding of the crude product in 0.1 mol/L Tris (pH 8), 1 mol/L guanidin at 4°C for 16 h yielded the internal disulfide bridged biotin-cyclic NGR, which was high performance liquid chromatography (HPLC)-purified (C18 RP-HPLC) and lyophilized. ESI-MS confirmed a mass decrease of 2, representing the loss of 2 protons from the cystein side chains due to the generation of 1 disulfide bond (S-S). Biotinylated polylysine dendritic wedge, a construct comprising 8 Gd-diacetoneamidinepentaacetic acid (DTPA) moieties, was synthesized and purified similarly (21, 22).

Curnis and colleagues (23) previously showed that cNGR spontaneously converts into isoDGR by asparagine deamidation at slightly basic pH, generating an α5β3-integrin ligand. Using a combination of HPLC and mass spectrometry up to 24 h after dissolving cNGR in water (pH 6.0) and 1 μmol/L borate buffer (pH 8.3; supplemented with 0.05% NaN₃), respectively, it was found that this process did not occur in the time period of the experiments (data not shown).

To prepare bimodal, multivalent contrast agent was prepared as follows. Streptavidin-conjugated QDs [1 μmol/L in borate buffer (pH 8.3), emission at 585 nm] were purchased from Invitrogen. QDs were composed of a CdSe core with a ZnS shell and covered with polyethyleneglycol-2000. Each QD holds ~10 surface-bound streptavidins, allowing 30 biotinylated compounds to bind on average. For each experiment, cNGR-pQDs were prepared freshly at room temperature by sequential mixing of 100 μL QD solution with biotin-cNGR and biotin-poly(lysine) dendritic wedge, both dissolved in HBSS (pH 7.4; Invitrogen), in a molar ratio of 1:6:24 to a total volume of ~120 μL. Samples were mildly vortexed during each preparation step to ensure a homogeneous distribution of biotin-cNGR and biotin-poly(lysine) dendritic wedge over the QD surface. Overall, each QD carried a maximum of 192 Gd ions and 6 cNGR peptides. Unlabeled pQDs carried the same number of Gd constructs but no cNGR. A schematic representation of the cNGR-pQD particle is shown in Fig. 1.

Animal Model

All animal studies were approved by the institutional animal welfare committee. Human colorectal adenocarcinoma cells (1.5 to 3×10⁶; LS174T; American Type Culture Collection CL-188) were s.c. and unilaterally injected on the flank of ~15- to 20-g-old male Swiss nu/nu mice (Charles River). Mice were subjected to the MRI examination when the tumor diameter was >1.0 cm, which was ~16 d after LS174T injection.

For in vivo MRI, mice were anesthetized using 1.5% to 2.0% isoflurane (Abbott Laboratories Ltd) in medical air and were placed prone in a dedicated animal holder with built-in mask for anesthesia gas supply. An infusion line was placed in the jugular vein for contrast agent administration during the MRI experiment. A heating pad was placed over the mice to maintain normothermic conditions. Respiration rate and body temperature were continuously monitored via a balloon sensor and rectal temperature probe, respectively, interfaced to an MR compatible small animal monitoring system (SA Instruments, Inc.).

Mice were randomly selected for injection with either cNGR-labeled or unlabeled pQDs. Seven mice were included for each contrast agent group. Mice were kept inside the magnet during the entire MRI experiment to preserve their position.

MRI Protocol

All MRI experiments were performed on a 7 T Bruker Biospec 70/30 USR MRI system, interfaced to an AVANCE II console (Bruker Biospin GmbH). The BGA12-S mini imaging gradient (maximum gradient strength, 4.7 T/m; maximum duration, 10 ms) was used to create the desired field of view (FOV) and to generate the field of phase. The BGA12-S mini imaging gradient (maximum gradient strength, 4.7 T/m; maximum duration, 10 ms) was used to create the desired field of view (FOV) and to generate the field of phase.

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720 mTm⁻¹; slew rate, 6,000 Tm⁻¹ s⁻¹) and a 3.5-cm inner diameter quadrature volume resonator were used.

Molecular MRI. Tumors were localized using T₂-weighted anatomic images (TR, 4,200 ms; TE, 37.4 ms). Next, precontrast \( R₁ \) values were determined using a series of IR measurements with increasing inversion times (TR, 4,000 ms; TE, 8.4 ms; TI, 500, 1,000, 1,500, 2,000, 2,500, and 3,500 ms; total scan time, 18 min). Subsequently, mice were injected with 120 \( \mu l \) of CNGR-labeled or unlabeled pQDs, followed by a 50 \( \mu l \) saline flush. IR experiments were repeated −30 min postcontrast to ensure adequate contrast agent circulation and a reduced level of intravascular contrast agent. Images were recorded using a field of view (FOV) of 4.0 × 4.0 cm², a 192 × 192 acquisition matrix interpolated to 256 × 256 by means of zero-filling, and a slice thickness of 1.2 mm, resulting in 0.16 × 0.16 × 1.2 mm³-sized voxels. On average, 15 contiguous slices were recorded in multislice mode (range, 11–22 slices; depending on tumor size and orientation). After MRI, mice were euthanized by cervical dislocation.

Competition experiment. Four tumor-bearing mice were randomly selected for a competition experiment of CNGR-pQDs with unconjugated CNGR, i.e., nonbiotinylated, nonparamagnetic, and nonfluorescent. Imaging was performed as described above, except that 352 μg per mouse of unconjugated CNGR, i.e., a 1,000-fold excess compared with QD-bound CNGR, was injected i.v. 10 min after administration of CNGR-pQDs.

Biodistribution. Healthy Swiss mice (Charles River) were injected with either CNGR-pQDs, unlabeled pQDs, or no contrast agent. After −1 h circulation time, mice were sacrificed and whole body \( T₁ \)-weighted spin echo images were recorded (TR, 1,100 ms; TE, 8.5 ms; FOV, 4.0 × 6.0 cm²; matrix, 256 × 512; resolution, 0.16 × 0.12 × 1.2 mm³). Two mice were included per group.

Tissue harvesting. After MRI, tumor, spleen, liver, kidney, hind limb muscle, heart, and lung were excised and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Europe). Next, tissues were snapfrozen in cold 2-methylbutylacet (Acros Organics) for −2 min and subsequently transferred to liquid nitrogen. Tissues were stored at −80°C until TPLSM measurements.

Contrast agent relaxivity. \( T₁ \) relaxivity (\( r₁ \)) was determined by diluting CNGR-pQDs in HBSS in 9 steps to concentrations of 0 to 0.001 mmol/L (corresponding gadolinium concentrations, 0–0.192 mmol/L). The \( R₁ \) of each sample was determined using the IR series as described above. Absolute gadolinium concentrations were measured using Inductively Coupled Plasma Mass Spectrometry. Longitudinal relaxivity was determined by the slope of a linear fit of \( R₁ \) versus gadolinium concentration.

MRI Data Analysis

All data processing was performed in Matlab (The Mathworks), unless stated otherwise. IR images were first spatially coregistered using the mutual information algorithm in the MRIT software package (24) to correct for possible animal motion in the images with different \( T₁ \) contrast, and smoothed with a three dimensional Gaussian kernel with a full-width-at-half-maximum of 0.4 × 0.4 × 3.0 mm³. Regions of interest (ROI) were drawn manually in MRcro (25) to define tumor and muscle tissue. Both \( T₁ \)- and \( T₂ \)-weighted images were used to accurately delineate tumors from surrounding tissue and edema.

Precontrast and postcontrast \( R₁ \) values were determined on a voxel-by-voxel basis by nonlinear curve fitting of the IR signal intensity function (26):

\[
S = S₀(1 - 2e^{-T₁R₁} + e^{-T₂R₂})
\]

using the Levenberg-Marquardt optimization algorithm. \( S₀ \) is a scaling factor including proton density, excitation pulse profile, echo time, and preamplifier gain.

The detection limit for changes in \( R₁ \) (>Δ\( R₁ \) = \( R₁ \)post - \( R₁ \)pre) was determined with a Monte Carlo simulation using Eq. 1, as \( R₁ \) relaxation rates, and representative noise levels as derived from the in vivo experiments. A voxel was considered significantly enhanced when \( Δ\( R₁ \) > 1.96 \) (i.e., 95% confidence interval) times higher than the detection limit of 0.005 s⁻¹. We defined the Quantitative Contrast derived from the \( Δ\( R₁ \) \) measurements (QC\( R₁ \)) as the product of the mean \( Δ\( R₁ \) \) and the percentage of significantly enhanced voxels for each tissue type, i.e., tumor and core, and muscle tissue. QC\( R₁ \) indicates both the level and spatial extent of contrast agent binding. Changes in \( S₀ \) (\( Δ\( S₀ \) = \( S₀ \)post - \( S₀ \)pre) were also evaluated, and the Quantitative Contrast from \( S₀ \) (QC\( S₀ \)) was defined analogously to QC\( R₁ \) to yield a quantity that reflects proton visibility (27).

Tumor rim/core analysis. To investigate the differences between tumor rim, i.e., the region with the highest expected angiogenic activity, and core, the tumor rim was first defined as a 1-mm thick peripheral zone with the strongest \( R₁ \) enhancement, in accordance with the approach taken by others (28, 29). Using this thickness, the difference between CNGR-labeled and unlabeled pQDs was maximal (Fig. 3C). The rim comprised 29.0% ± 5.8% and 31.6% ± 3.5% of all tumor voxels for mice injected with CNGR-labeled and unlabeled pQDs, respectively. The tumor core was defined as the difference between whole tumor and tumor rim ROIs. Second, a contour was drawn to calculate the number of voxels with a significantly increased \( Δ\( R₁ \) \) as a function of the distance to the tumor rim. As an empirical measure of spatial heterogeneity in angiogenic tumor activity, the half-value-depth was defined as the distance from the rim at which the percentage of enhanced voxels has decreased by 50% compared with its value at zero distance, i.e., the rim. The half-value-depth was calculated by fitting the group-averaged data presented in Fig. 3C with a monoeponential decay function.

Biodistribution. ROIs defining the spleen, liver, kidney, heart, lung, and aorta were drawn manually in MRcro. Signal intensities were averaged over the entire tissue and normalized to hind limb muscle.

Statistical analysis. Statistical analysis of paired samples was performed using a nonparametric Wilcoxon signed-rank test in SPSS 14.0 (SPSS).

As both QC\( R₁ \) and QC\( S₀ \) represent contrast agent presence, QC\( R₁ \) and QC\( S₀ \) were combined to a summary value according to O’Brien and Lauter (30, 31), which is more sensitive to contrast effects than the individual measures. Therefore, QC\( R₁ \) and QC\( S₀ \) were first standardized by \( z = [QC - mean(QC)/sd(QC)] \). Subsequently, the absolute values of \( z_{QC,R₁} \) and \( z_{QC,S₀} \) were averaged per animal. The resulting summary measure was tested using a nonparametric Mann-Whitney \( U \) test. \( p < 0.05 \) was considered statistically significant.

TPLSM Data Acquisition

Tissue samples were thawed and washed with HBSS to remove OCT compound. Except for the spleen and liver, tissues were incubated with 25-fold diluted CD31-FITC (0.5 mg/mL; BD Biosciences PharMingen) to fluorescently label ECs. Next, tissues were embedded in 2% agarose gel (Invitrogen), with their rim upwards. For measurements in the tumor core, tumors were cut transversally to resemble the slice orientation of the MRI measurements.

TPLSM imaging was performed using a Nikon Eclipse E600FN upright microscope, incorporated in the Bio-Rad Radiance 2100MP imaging system and operated by Lasersharp2000 V6.0 (Bio-Rad). Tissue samples were excited by the Tsunami Tесhapphire laser (Spectra-Physics), which was pumped by a Millennia Vu 5 W pump laser (Spectra-Physics) and mode locked at 800 nm, with a 82.5 MHz repetition rate and 100 fs pulse width. Tissues were observed through a water dipping 60× fluor objective with a 1.00 numerical aperture (Nikon). Photomultiplier tubes (PMTs 9108R02 and 9136B05; Electron Tubes Limited) were used to acquire fluorescence photons in three spectral regions: 420 to 470 nm (autofluorescence), 520 to 560 nm (FITC) and 570 to 600 nm (QD). Each PMT was tuned for minimal bleed through of the fluorescent markers to adjacent PMTs. Images, color coded blue, green, and red, respectively, were subsequently merged into a single image. The in-plane pixel dwell time was 11.8 μs, which, together with a 2-fold Kalman averaging, resulted in an imaging speed of 0.16 Hz. The FOV was 179 × 179 μm² with a matrix size of 512 × 512, resulting in 0.35 × 0.35 μm² sized pixels.

TPLSM Data Analysis

Data were analyzed with Image-Pro Plus 6.0 (MediaCybernetics) and ImageJ 1.35 (NIH). Image quality was improved by convolution with a 1.05 × 1.05 μm² Gaussian filter. Spatial distribution of pQDs was classified into four groups: intravascular, intracellular, colocalized with the EC membrane, or extravasated to the interstitium.
Results

In vivo targeting of activated tumor endothelium. The ability of cNGR to target angiogenic tumor ECs was evaluated in tumor-bearing nude mice by injecting them with cNGR-labeled or unlabeled pQDs. Tumor volumes of cNGR and control groups did not differ on MR images (mean $\pm$ SD, 1.0 $\pm$ 0.7 cm$^3$ and 1.0 $\pm$ 0.6 cm$^3$, respectively).

For both cNGR-labeled and unlabeled pQDs, changes in $R_1$ ($\Delta R_1$) were spatially heterogeneous throughout the tumor and were most pronounced at the tumor rim for cNGR-pQDs. Although an $R_1$ increase in the tumor rim was also observed for unlabeled pQDs, the average response was 3-fold lower when compared with cNGR-pQDs, indicating a high specificity of cNGR for angiogenic tumor endothelium. This is further supported by the low changes in $R_1$ found in muscle tissue. Changes in $R_1$ were most pronounced at the tumor rim for cNGR-pQDs. Although an $R_1$ increase in the tumor rim was also observed for unlabeled pQDs, the average response was 3-fold lower when compared with cNGR-pQDs, indicating a high specificity of cNGR for angiogenic tumor endothelium.

Figure 2. $T_2$-weighted anatomic images with color overlay of $\Delta R_1$ (A) and $\Delta S_0$ (B) for tumor (T) and muscle (M) tissue of mice injected with cNGR-labeled or unlabeled pQDs ($n = 7$ for both groups). Changes in $R_1$, were most pronounced at the tumor rim for cNGR-pQDs. Although an $R_1$ increase in the tumor rim was also observed for unlabeled pQDs, the average response was 3-fold lower when compared with cNGR-pQDs, indicating a high specificity of cNGR for angiogenic tumor endothelium. This is further supported by the low changes in $R_1$ found in muscle tissue. Changes in $S_0$ (B) coregistered almost completely with changes in $R_1$ (A). Representative TPLSM images of tumor (C) and muscle tissue (D) showing pQD signal (red) and EC-specific cCD31-FITC (green). cNGR-pQDs accurately colocalized with tumor ECs, indicating binding of the contrast agent to the tumor endothelium (C). cNGR-pQDs were also detected in muscle tissue with TPLSM (Fig. D, arrows), although to a much lesser extent than in tumor tissue. cNGR-pQDs did not display any colocalization with muscle ECs and were only found intraluminally. Unlabeled pQDs were not or only sparsely detected in both tumor and muscle tissue. Bar, 50 $\mu$m.

Figure 3. Spatial distribution of angiogenic activity. A and B, Quantitative Contrast as derived from changes in $R_1$ ($QCR_1$; A) and from changes in $S_0$ ($QCS_0$; B) for tumor rim, tumor core, and hind limb muscle tissue. Data are shown for cNGR-pQDs ($n = 7$), unlabeled pQDs ($n = 7$), and the competition experiment of cNGR-pQDs with excess unconjugated cNGR ($n = 4$). C, percentage of enhanced voxels at a certain distance versus distance from the tumor rim for cNGR-labeled and unlabeled pQDs. Although enhanced voxels were mostly found at the tumor rim for both contrast agents, more than twice as many rim voxels were enhanced for cNGR-pQDs than for unlabeled pQDs. In the tumor core, similar values were found for both cNGR-labeled and unlabeled pQDs. Columns, median; bars, SE. *, $P < 0.05$; #, $P < 0.05$ for the O’Brien-Läuter summary measure of $QCR_1$ and $QCS_0$ (see text).
often with tumor ECs than unlabeled pQDs (Fig. 2). cNGR-labeled and unlabeled pQDs were also found in the vessel lumen, albeit that cNGR-pQDs were approximately three times more prevalent than unlabeled pQDs. Both contrast agents were only sparsely found to have extravasated into the tumor interstitium. Although cNGR was previously reported to be an internalizing peptide (32), cNGR-pQDs were not detected inside ECs with TPLSM.

Further evidence for the specificity of cNGR was provided by $\Delta R_1$ in hind limb muscle. Here, average $\Delta R_1$ upon administration of cNGR-pQDs was considerably lower than in the tumor and ranged up to 0.05 s$^{-1}$. TPLSM did not display colocalization of cNGR-pQD with ECs of muscle vasculature. However, the incidence of cNGR-pQDs in the muscle vascular lumen was almost 2-fold higher than for unlabeled pQDs (Fig. 2D).

$S_0$-effect. For both cNGR-labeled and unlabeled pQDs, changes in the scaling factor $S_0$ colocalized strongly with $\Delta R_1$ (Fig. 2B). The $S_0$-effect is likely caused by field inhomogeneities ($T_2$-effect) in the vicinity of the contrast agent, induced by the magnetic properties of QDs (33) and the dense gadolinium concentration on the particle. Analogous to iron oxide particles, such properties result in locally reduced transverse relaxation times $T_2$ and $T_2^*$, a shift in local resonance frequency and a broader water resonance line, which is reflected by a decrease in $S_0$, i.e., a reduced proton visibility (27, 34). Therefore, $\Delta R_1$ and $\Delta S_0$ both represent contrast agent presence.

Spatial heterogeneity. To explore the absolute differences between tumor rim, tumor core, and muscle, QC$_{R1}$ and QC$_{S0}$ were determined for each tissue type for cNGR-labeled and unlabeled pQDs (Fig. 3A and B). Administration of cNGR-pQDs resulted in an ~50-fold increase in QC$_{R1}$ in the angiogenic rim compared with tumor core or muscle tissue. For unlabeled pQDs, significant differences were also found between tumor rim and core, and tumor rim and muscle tissue, although the net increase in QC$_{R1}$ was lower than for cNGR-pQDs. The decreases in $S_0$ showed the same trend as the increases in $R_1$ (cf. Fig. 3A and B).

For each of the three tissue types, no significant differences in QC$_{S0}$ or QC$_{R1}$ were found between cNGR-labeled and unlabeled pQDs. Because $\Delta R_1$ and $\Delta S_0$ were shown to accurately colocalize (Fig. 2), QC$_{R1}$ data were combined with QC$_{S0}$ to a summary measure as described above. This resulted in a statistically significant difference between cNGR-labeled and unlabeled pQDs for the tumor rim only (Fig. 3A and B).

To further investigate the spatial distribution of angiogenic activity in the tumor, the percentage of significantly enhanced voxels was calculated as a function of the distance to the tumor rim (Fig. 3C). Although the highest signal increase was found at the tumor rim for both contrast agents, more than twice as many rim voxels were enhanced for cNGR-pQDs than for unlabeled pQDs. In the tumor core, similar enhancements were found for both contrast agents. These findings qualitatively concur with previous findings showing that angiogenic activity is most pronounced at the tumor rim for this tumor model (7, 35).

Subsequently, half-value-depths were calculated for both cNGR-labeled and unlabeled pQDs. High values indicate a more homogeneous distribution of enhanced voxels over the entire tumor and thus a low spatial heterogeneity, whereas low values indicate a high spatial variation. For cNGR-labeled and unlabeled pQDs, the half-value-depths were 0.6 and 1.1 mm, respectively, indicating a stronger contrast between tumor rim and core for cNGR-pQDs, which suggests that cNGR-pQDs allow a better differentiation between tumor rim and core than unlabeled pQDs.

**Competition experiment.** I.v. injection of a 1,000-fold excess of unconjugated cNGR 10 min after administration of cNGR-pQDs resulted in a statistically significant decrease in QC$_{R1}$ and QC$_{S0}$ for the tumor rim (Fig. 3A and B). With TPLSM, cNGR-pQDs were barely detected in the tumor rim, which confirmed the MRI results (data not shown). These results therefore indicate that binding of cNGR-pQDs to tumor ECs is specific, reversible, and can be competed with unconjugated cNGR.

**Biodistribution.** Figure 4 shows the relative MRI signal intensities for the blood and major organs recorded ~1 hour after the administration of cNGR-pQDs, unlabeled pQDs, or no contrast agent. No differences were found between cNGR-labeled and unlabeled pQDs. Both contrast agents accumulated mainly in the spleen, liver, and kidneys (Fig. 4), which was confirmed by TPLSM and corresponds to previous findings (36). Due to the i.v. administration, pQDs were also expected to accumulate in the lung. However, MRI has only limited signal sensitivity in the lung due to the inherently low signal intensity and air-tissue interfaces. With TPLSM, pQDs could be clearly detected in the lung (Fig. 4), although microscopic imaging was hampered by tissue movement.

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**Figure 4.** Biodistribution of cNGR-labeled and unlabeled pQDs ($n = 2$ for each group). A, postmortem $T_1$-weighted MRI results. Signal intensities were normalized to hind limb muscle signal and subsequently averaged. No CA, no contrast agent administration ($n = 2$). Both cNGR-labeled and unlabeled pQDs were found to accumulate mainly in the spleen, liver, and kidneys. Columns, median; bars, SE. B, representative TPLSM images of spleen, liver, lung, and kidney. Because a similar biodistribution was found for cNGR-labeled and unlabeled pQDs, no differentiation was made for the TPLSM results. Red, pQDs; green, αCD31-FITC; blue, autofluorescence; bar, 50 μm.
caused by heating of the sample during excitation, resulting in expansion of air in the pulmonary alveoli.

**Contrast agent relaxivity.** The ionic $T_1$ relaxivity of cNGR-pQDs, i.e., per Gd ion, was $7.1 \pm 0.4$ mmol/L·s$^{-1}$ at 7 T and $20^\circ$C, which lies in the expected range for macromolecular contrast agents and is in correspondence with previously reported values for Annexin A5 conjugated pQDs (37).

**Discussion**

**Current findings.** In this study, the ability of cNGR-labeled paramagnetic QDs to visualize and quantify angiogenic activity in LS174T tumors was evaluated using two complementary imaging modalities: in vivo MRI and ex vivo TPLSM. To our knowledge, this study shows the first results of the application of cNGR for molecular MRI. First, cNGR-pQDs were found to have a 3-fold higher quantitative MRI contrast in the tumor rim, i.e., the tumor region with the highest angiogenic activity, compared with unlabeled pQDs. Second, cNGR-pQDs were barely detectable in muscle tissue, indicating a high specificity of cNGR-pQDs for angiogenic vessels. Third, ex vivo TPLSM showed colocalization of cNGR-pQDs, but not unlabeled pQDs, with ECs in tumor vasculature. Fourth, cNGR-pQDs allowed a more accurate assessment of the rim-core heterogeneity in tumor angiogenic activity. Fifth, the competition experiment indicated that the binding of cNGR-pQDs to tumor ECs is specific, reversible, and can be competed with excess unconjugated cNGR. Finally, no qualitative differences in biodistribution were found between cNGR-labeled and unlabeled pQDs with MRI and TPLSM. For a full quantitative biodistribution assessment, Positron Emission Tomography and unlabeled pQDs with MRI and TPLSM. For a full quantitative biodistribution assessment, Positron Emission Tomography would be better suited because the local relaxivity of the MRI contrast agent, which is required to convert $R_1$ to concentrations, is unknown. Taken together, the presented results designate cNGR as an effective ligand for discriminating between quiescent and activated endothelium and for quantifying the extent of tumor angiogenic activity.

In correspondence with other studies (7, 35), our results indicate that angiogenic activity is highest at the tumor rim, which was reflected by a high QC$_{R1}$ and a strongly negative QC$_{C0}$ for cNGR-pQDs. This was supported by the statistically significant difference between cNGR-labeled and unlabeled pQDs found using the summary value. Unlabeled pQDs also showed differences between tumor rim versus core and muscle, albeit smaller than for the cNGR-pQDs. This is likely due to the intrinsically higher vascular density of the tumor rim and corresponding blood pool fraction, resulting in a larger amount of circulating contrast agent compared with tumor core or muscle tissue (35). Additionally, heterogeneous blood flow and long wash-in and wash-out times of macromolecular contrast agents, which was previously described for dendritic agents (38), may have contributed to a prolonged retention of unlabeled pQDs in the tumor peripheral vasculature.

**Methodologic considerations.** Nonlinear fitting of the IR signal over a range of inversion times provided a sensitive and reliable method for detecting $R_1$ changes induced by contrast agent binding. Compared with signal intensity measurements, it is relatively independent of technical settings, e.g., repetition time, echo time, and flip angle, thereby allowing objective comparison between different subjects, both spatially and temporally. A disadvantage of the quantitative approach is the lengthy acquisition time because an adequate number of data points is required for accurate fitting of the IR curve. Although prolonged precontrast and postcontrast acquisition of a single image at a fixed inversion time may also allow accurate detection of changes in signal intensity, this will not provide quantitative information on $R_1$ and $S_0$. In our quantitative approach, coregistration of these variables ensured increased sensitivity to detect differences between cNGR-labeled and unlabeled pQDs and is therefore preferred over acquisition of a single image.

Theoretically, the measured longitudinal relaxation rates and contrast agent relaxivity allow estimation of local contrast agent concentration and might be used to gain insight in the density of the molecular target. However, the conditions under which the relaxivity was determined differ strongly from the in vivo situation. Not only is the relaxivity affected by the chemical environment of the particle, i.e., aqueous buffer versus blood plasma (39), specific binding to vascular endothelium reduces its tumbling rate, thereby increasing the relaxivity. Taken together, this would lead to an overestimation of the local concentration in vivo. Unfortunately, accurate measurements of in vivo relaxivity are currently unavailable.

**Contrast agent.** Quantum dots were chosen as scaffold to enable bimodal, i.e., MRI and TPLSM, visualization of tumor angiogenic activity, which is an essential step in the characterization and validation of cNGR as a targeting ligand. Furthermore, streptavidin-coated QDs provide a suitable and versatile research scaffold to identify and test other potential targeting ligands. In addition, QDs show minimal extravasation, both from healthy and hyperpermeable tumor vasculature, which is beneficial for EC targeting. However, QDs may pose serious health limitations due to the potential release of toxic cadmium ions. Although this can be prevented by effective shielding of the core (40, 41), QDs are not cleared from the body and accumulate in spleen, liver, and kidneys. Cadmium-based QDs will therefore not be approved for clinical application. Recently developed nontoxic and renally excretable QDs may provide a potential solution for this problem (42, 43). Nevertheless, once a robust MRI method has been accepted in clinical practice, validation with luminescent particles is no longer necessary and clinically more suitable particles may be applied.

The magnetic and semiconductive properties of QDs give increase to field inhomogeneities when placed inside a magnetic field (33), which likely result in a local decrease of the transverse relaxation times $T_2$ and $T_2^*$ in addition, $T_2$ contrast becomes more effective at high field strength, whereas $T_1$ contrast decreases. Using a standard multislice multiecho spin echo sequence, an average $R_2$ increase of $5.7$ s$^{-1}$ upon cNGR-pQD injection was detected in the tumor rim at a mean tumor precontrast $R_2$ of $\sim 27$ s$^{-1}$. However, the percentage of significantly enhanced voxels in the tumor rim was only 4%, which is considerably lower than the 42% found for $R_1$. This shows that $T_2$ changes did not interfere with the effects on $T_1$ and $S_0$. Consequently, the $T_1$ and $S_0$ quantification was more sensitive in discriminating between angiogenic activity in the tumor rim, tumor core, and muscle tissue than $T_2$.

**Clinical perspectives.** Regarding the potential clinical applicability, quantitative molecular MRI with a suitable contrast agent has a number of advantages over the currently used immunohistochemical methods to quantify tumor angiogenic activity. First, molecular MRI is noninvasive and does not interfere with tissue integrity. Second, it can probe the entire tumor, whereas

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* Unpublished observations.
immunohistochemistry requires biopsies at one or multiple selected locations. Third, it allows co-localization of angiogenic activity with local anatomy. Fourth, tumor status or therapeutic response may be objectively monitored over time due to the absolute quantification methodology. Finally, molecular MRI allows direct detection of activated endothelium in functional vasculature, whereas immunohistochemistry measures both perfused and nonperfused vessels.

With respect to the applied tumor model, a human colorectal adenocarcinoma, MRI is clinically important for local T-staging of rectal cancer and for the identification of tumors close to or invading the mesorectal fascia (44). On diagnostic $T_1$-weighted images, however, it remains difficult to differentiate between fibrotic tissue and viable tumor cells. Molecular MRI of angiogenesis may facilitate this demarcation because only viable tumor cells induce angiogenesis, which may be visualized upon administration of the targeted contrast agent.

Besides the availability of suitable contrast agents, clinical implementation of quantitative molecular MRI requires rapid imaging techniques. Possible sequences that allow fast quantification of relaxation times are Look-Locker (45), IR-true-FISP (46), and the recently described QAPTEST (47). However, these methods are relatively sensitive to subject movement and field inhomogeneities, although the Look-Locker method was recently modified to allow in vivo $T_1$-mapping of the heart (48). Thus, the development of fast quantification of relaxation rates seems to support future clinical application of quantitative molecular MRI.

In summary, we have shown that cNGR-labeled paramagnetic quantum dots are suitable for the noninvasive visualization and quantification of tumor angiogenic activity using in vivo molecular MRI. These results provide a promising basis for further developments in contrast agent design and synthesis, data acquisition, and postprocessing techniques, which may be valuable for future clinical applications to pathologies in which abnormal vessel growth plays a pivotal role.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

MRI of Angiogenesis with cNGR-Labeled Quantum Dots


Quantitative Molecular Magnetic Resonance Imaging of Tumor Angiogenesis Using cNGR-Labeled Paramagnetic Quantum Dots

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