Magnetic Resonance Visualization of Tumor Angiogenesis by Targeting Neural Cell Adhesion Molecules with the Highly Sensitive Gadolinium-Loaded Apoferritin Probe

Simonetta Geninatti Crich,1 Benedetta Bussolati,2 Lorenzo Tei,1 Cristina Grange,1 Giovanna Esposito,1 Stefania Lanzardo,3 Giovanni Camussi,2 and Silvio Aime1

1Department of Chemistry IFM and Center for Molecular Imaging, University of Torino; 2Department of Internal Medicine and Research Center for Experimental Medicine, University of Torino, Torino, Italy; and 3Department of Clinical and Biological Sciences, University of Torino, Orbassano, Italy

Abstract

Tumor vessel imaging could be useful in identifying angiogenic blood vessels as well as being a potential predictive marker of antiangiogenic treatment response. We recently reported the expression of the neural cell adhesion molecule (NCAM) in the immature and tumor endothelial cell (TEC) lining vessels of human carcinomas. Exploiting an in vivo model of human tumor angiogenesis obtained by implantation of TEC in Matrigel in severe combined immunodeficiency mice, we aimed to image angiogenesis by detecting the expression of NCAM with magnetic resonance imaging. The imaging procedure consisted of (a) targeting NCAMs with a biotinylated derivative of C3d peptide that is known to have high affinity for these epitopes and (b) delivery of a streptavidin/gadolinium (Gd)-loaded apoferritin 1:1 adduct at the biotinylated target sites. The remarkable relaxation enhancement ability of the Gd-loaded apoferritin system allowed the visualization of TEC both in vitro and in vivo when organized in microvessels connected to the mouse vasculature. Gd-loaded apoferritin displayed good in vivo stability and tolerability. The procedure reported herein may be easily extended to the magnetic resonance visualization of other epitopes suitably targeted by proper biotinylated vectors. (Cancer Res 2006; 66(18): 9196-201)

Introduction

Among the available “in vivo” diagnostic modalities, magnetic resonance imaging (MRI) is the most powerful technique thanks to its superb anatomic resolution (<100 μm) and its ability to report on both the structure and function of soft tissues (1). Moreover, the use of contrast agents could further enhance the differences between healthy and diseased tissues (2, 3). Gadolinium (Gd)-based contrast agents are the most used systems in both clinical and experimental settings, however, the commercially available Gd contrast agents are only extracellular agents endowed with a nonspecific biodistribution. The development of new Gd-based contrast agents with high contrast ability and targeting capability is the key step for the set-up of innovative magnetic resonance-molecular imaging (MRMI) protocols (4–7).

In fact, in MRMI procedures, one has to visualize epitopes that are present at very low concentrations (typically in the 50-100 nmol/L range), and therefore, it is necessary to design proper methods to amplify the response upon recognition of the target of interest. In this work, we have applied a targeting procedure aimed at visualizing tumor endothelial cells (TEC) by using the recently described Gd-loaded apoferritin system as an imaging reporter (8). Each Gd-HPDO3A entrapped in the apoferritin cavity displays the highest relaxivity peak reported thus far (~80 mmol/L−1 s−1), and the amplification effect is further enhanced by the presence of 8 to 10 molecules of Gd-HPDO3A per apoferritin unit (8). In order to develop a system of general applicability, the outer surface of the Gd-loaded apoferritin was biotinylated so that it could be delivered to the site of interest by means of a well-established avidin/biotin recognition pathway. Many efforts have already been devoted to develop a MRMI protocol for the visualization of neoformed vessels in the tumor region (9, 10). Angiogenesis has a crucial role in tumor growth, metastasis, and inflammatory diseases; therefore, targeting epitopes hyperexpressed in the neovasculature is of great interest for the early detection of tumors and the follow-up of therapeutic treatments (11, 12). The most investigated systems have dealt with the visualization of integrin receptors (13–18), E-selectin (19, 20), and intercellular cell adhesion molecule-1 (21).

We have recently described the isolation and characterization of renal tumor–derived endothelial cells (22). In vivo in severe combined immunodeficiency (SCID) mice, TEC organize within Matrigel in a network of small vessels connected with the mouse vasculature, providing an in vivo model of human tumor angiogenesis. TEC, but not normal endothelial cells, expressed both in vitro and in vivo, the neural cell adhesion molecule (NCAM), an adhesion molecule of the immunoglobulin superfamily (23) largely expressed by embryonic tissue. NCAM is transiently re-expressed during angiogenic endothelial cell differentiation and organization, and is stably expressed by TECs (24).

As a targeting vector for TEC, we used a specific NCAM-binding peptide, C3d (25–28). Therefore, using this peptide coupled to the Gd-loaded apoferritin by the biotin/streptavidin system, we have constructed a molecular imaging probe that we have tested on TEC in vitro and on TEC-formed vessels in vivo in SCID mice.

Materials and Methods

Gd-HPDO3A (Prohance) was kindly provided by Bracco S.p.A. Apoferritin (Milan, Italy) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The longitudinal water proton relaxation rate was measured on a Stelar Spinmaster spectrometer (Stelar, Mede, Italy).

Gd-loaded apoferritin preparation. The loading of paramagnetic chelates in the apoferritin cavity was carried out as described previously (8). Briefly, the dissociation of the apoferritin into its subunits was done by...
lowering the pH of the protein solution (to pH 2; $1 \times 10^{-5}$ mol/L) using 1 mol/L of HCl and maintaining this low pH for about 15 minutes. Afterwards, the Gd-HPD03A complex was added to the solution at a 0.1 mol/L concentration, and successively, the pH was adjusted to 7.4 using 1 mol/L of NaOH. The resulting solution was stirred at room temperature for ~2 hours and then dialyzed against HEPES buffer saline [HEPES 5 mmol/L, NaCl 0.1 mol/L (pH 7.4)] to remove Gd-HPD03A molecules not trapped inside the protein shell. At the end, the solution was filtered with a 0.2 µm membrane filter.

**Biotinylation of the Gd-loaded apoferritin.** The biotinylation of Gd-loaded apoferritin was carried out using NHS-LC-BIOTIN [N-(4-vinylbenzyl)-aminocaproic acid N-succinimidyl ester] according to standard protein modification protocols (29). Briefly, 1.6 µmol of NHS-LC-BIOTIN previously dissolved in DMF were added to a Gd-loaded 8 µmol/L apoferritin solution (10 mL). Afterwards, the solution was stirred for 30 minutes at room temperature and then dialyzed against HEPES buffer saline to remove the unbound NHS-LC-BIOTIN reagent. After dialysis, the ratio of biotin/apoferritin was of 5 ± 1 biotin residues per apoferritin molecule as determined with the HABA colorimetric assay (29). Finally, the biotinylated Gd-loaded apoferritin (Gd-Apo-Bio) solution was concentrated to ~80 to 120 µmol/L using Vivaspin 500 centrifugal filters with a membrane pore size of $M_r$ 30,000.

The final protein concentration of each sample was determined by the Bradford method (30) using bovine serum albumin as the standard. The residual Gd-HPD03A concentrations ranging from 0.8 to 1.2 mmol/L were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Element-2; Thermo-Einnigan, Rodano, Milan, Italy). A single ICP-MS measurement represents the average of three replicates from the same sample (previously digested in concentrated nitric acid).

**Synthesis of C3d-Bio NCAM mimetic peptide.** The synthesis of the biotinylated peptide ligand of the NCAM Ig1 module (C3d-Bio) was accomplished as described previously (24). Briefly, C3d-Bio (Scheme) was synthesized using the standard Fmoc strategy with a Fmoc-Lys (Fmoc)-OH sequence (TR/TE/NEX = 200/3.3/8; FOV, 1.2 cm; one slice, 1 mm; in-plane resolution, 94 × 94 µm).

For the in vitro binding experiments, TEC were isolated from specimens of clear cell-type renal cell carcinomas using anti-CD105 antibodies coupled to magnetic beads, by magnetic cell sorting using the MACS system (Miltenyi Biotec), as previously described (22). TEC cell lines were established renal carcinomas and maintained in culture in EBM complete medium supplemented with epidermal growth factor (10 ng/mL), hydrocortisone (1 mg/mL), bovine brain extract (all from Cambrex Bioscience, East Rutherford, NJ), and 10% FCS. TEC were previously characterized as endothelial cells by morphology, positive staining for VWF antigen, CD105, CD146, vascular endothelial-cadherin, and negative staining for cytokeratin and desmin.

**Tumor-derived endothelial cells.** TEC were isolated from specimens of renal cell-type renal cell carcinomas using anti-CD105 antibodies coupled to magnetic beads, by magnetic cell sorting using the MACS system (Miltenyi Biotec), as previously described (22). TEC cell lines were established renal carcinomas and maintained in culture in EBM complete medium supplemented with epidermal growth factor (10 ng/mL), hydrocortisone (1 mg/mL), bovine brain extract (all from Cambrex Bioscience, East Rutherford, NJ), and 10% FCS. TEC were previously characterized as endothelial cells by morphology, positive staining for VWF antigen, CD105, CD146, vascular endothelial-cadherin, and negative staining for cytokeratin and desmin.

**Binding experiments.** For the in vitro binding experiments, TEC were harvested using a nonenzymatic dissociation solution (Sigma), washed with PBS, counted in a microcylinder chamber, and resuspended in DMEM (2 × 10^6 in 250 µL of DMEM). C3d-Bio peptide, streptavidin, and Gd-loaded/apoferritin were sequentially added to TEC at a molar ratio of 20:1:1 (at 20°C), with a 20-minute time interval between the addition of each component. After each binding step, the cells were washed thrice with 5 mL of ice-cold PBS. The amount of Gd bound to cells was measured by ICP-MS. The extent of aspecific binding of streptavidin/biotinylated Gd-loaded apoferritin was assessed by repeating the above experiments without the addition of the targeting peptide. MR images were acquired at 7 T on glass capillaries containing cell pellets placed in an agar phantom.

**In vivo model of human tumor angiogenesis.** For the in vivo studies, TEC were implanted s.c. into SCID mice (Charles River) within growth factor–reduced Matrigel, as previously described (22). Cells were harvested using nonenzymatic dissociation solution (Sigma), washed with PBS, and resuspended in DMEM (1 × 10^6 in 250 µL DMEM). Cells were chilled on ice, added to 250 µL of Matrigel at 4°C and injected s.c. into the left posterior side of SCID mice via a 26-gauge needle using a 1 mL syringe. On day 6, previously reported as the requested time for connection between mice and human microvessels, mice were processed for MRI experiments.

**MRI.** MR images were acquired on a Bruker Avance 300 spectrometer (7 T) equipped with a Micro 2.5 microimaging probe. The image of cell pellets was obtained using a standard TR/TE/NEX = 250/53.5/8; FOV, 1.2 cm; one slice, 1 mm; in-plane resolution, 94 × 94 µm).

Mice (n = 6) were treated i.v. with C3d-Bio or with the vehicle alone (n = 4), followed by the administration of biotinylated Gd-loaded apoferritin (Gd-Apo-Bio) and streptavidin. In selected experiments, streptavidin was replaced with FITC-labeled streptavidin (Sigma). The amount of the C3d-Bio (20 µmol/kg) administered to the first group of mice was 20 times higher than the amount of streptavidin (1 µmol/kg) and Gd-Apo-Bio (1 µmol/kg) in order to pursue the highest binding to the target molecules on tumor cells. To allow the removal of the unbound peptide from the circulation, the administration of streptavidin and Gd-Apo-Bio was done ~45 minutes after the C3d-Bio injection. Gd-loaded apoferritin and streptavidin were administered together on the assumption that, being present in a 1:1 molar ratio, each streptavidin molecule still has three binding sites available for anchoring to the biotin residues on C3d-Bio pretargeted to the tumor cells. Fat-suppressed T1-weighted spin echo MR images (TR/TE/NEX = 250/3.2/6; FOV, 2.8 cm; one slice, 1 mm; in-plane resolution, 220 × 220 µm) were recorded before, 10 minutes, 5, 24, and 48 hours after contrast administration. Fat suppression was done by applying a presaturation pulse (90 degrees BW = 1,400 Hz) at the absorption frequency of fat (~1,100 Hz from water). The mean signal intensity (SI) enhancement was evaluated on the regions of interest which were manually drawn around the tumor. The SI measured on each image was normalized using a standard Gd solution. Hyperintense regions were defined including all pixels with a SI increased by >3 SD of the precontrast tumor. For statistical analysis, one-way Student’s t test was used. Probability values <0.05 were considered statistically different.

**Histology of dissected plugs.** Five hours after treatment, mice were sacrificed, and tumor endothelial plugs were recovered and processed for histology and immunofluorescence. Typically, the overlying skin was cut away exposing the tumor. After fixation, the tissue was sectioned (20 µm) in a cryostat and stained with antibodies directed to vWF antigen, CD105, and CD146. Sections were counterstained with hematoxylin.

**Scheme.** Schematic representation of the adduct formed by C3d-Bio, streptavidin, and biotinylated Gd-loaded apoferritin (Gd-Apo-Bio). The sequence of the dendrimeric C3d-Bio NCAM mimetic peptide is outlined.

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**Molecular Imaging of Tumor Angiogenesis**

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avidin and biotinylated Gd-loaded apoferritin. As shown in Fig. 1A, the MRI visualization of the C3d-Bio binding to TEC using streptavidin linked to FITC was amplified by the use of an anti-FITC polyclonal antibody (Molecular Probes, Leiden, the Netherlands), followed by FITC-labeled anti-rabbit goat antibodies (Sigma). The nature of the vessels formed by TEC was evidenced by positive immunofluorescence staining for human HLA class I antigen using anti-HLA antibodies (BioLegend, San Diego, CA).

Results

Preparation and characterization of the biotinylated Gd-loaded apoferritin. Apoferritin (Scheme), loaded with about 8 to 10 molecules of the commercially available Gd-HPDO3A, was prepared according to the method previously reported (8). The paramagnetic complex, inside the protein cavity, shows an outstanding high millimolar relaxivity of \( \sim 70 \pm 10 \text{ mmol/L}^{-1} \text{s}^{-1} \) at 20 MHz and 25°C, which is \( \sim 20 \) times higher than that observed in pure water. It has been recently shown that a large contribution to the outstanding relaxivity of this system arises from the interaction of the paramagnetic complex with the exchangeable protons (and likely the hydration water) on the protein surface of the inner cavity of apoferritin (31). For the purpose of developing a targeting procedure based on the well known biotin/avidin recognition pathway, Gd-loaded apoferritin was biotinylated using the commercially available NHS-LC-BIOTIN reagent. The protein possesses several \(-\text{NH}_2\) groups available for conjugation with the NHS-LC-BIOTIN. After dialysis, the ratio of biotin/apoferritin was 5 \(\pm\) 1 biotin residues per apoferritin molecule as determined by the HABA colorimetric assay (29). The relaxivity of Gd-HPDO3A inside the cavity did not change after the conjugation reaction.

“*In vitro*” MR imaging of tumor-derived endothelial cells. It is well established that NCAM molecules are the targets for the C3d peptide (25–28). The latter peptide is easily biotinylated in solid phase at the four-terminal NH\(_2\) by the standard Biotin/HATU/DIPEA method to obtain C3d-Bio in good yield.

We previously showed the specificity and the dose-dependent binding of the C3d-Bio peptide to TEC (and not to normal endothelial cells) by cyt fluorimetric analysis (24). Here, we investigate the MRI visualization of the C3d-Bio binding to TEC using streptavidin and biotinylated Gd-loaded apoferritin. As shown in Fig. 1A, the amount of Gd bound to cell membranes in the presence of C3d-Bio is significantly higher than that found in the aspecific binding experiments, at any concentration of Gd-loaded apoferritin added to the incubation medium. \(T_1\)-weighted spin-echo image (Fig. 1B) showed that TEC incubated with the three components (C3d-Bio, streptavidin, and biotinylated Gd-loaded apoferritin) seem to be hyperintense with respect to the control cells. The maintenance of the Gd-Apo-Bio integrity upon binding to the cell membrane has been assessed by measuring the \(1/T_1\) nuclear magnetic resonance dispersion (NMRD) profile over a range of Larmor frequencies. In fact, Gd-loaded apoferritin has a profile characterized by a relaxivity hump at \(\sim 35\) MHz, typical of slowly moving systems, whereas the relaxivity of free Gd-HPDO3A, at frequencies \(>5\) MHz, is constant at any value of the applied field. On the basis of the \(1/T_1\) data acquired from 0.23 to 2.0 T, one can see that the relaxivity of the Gd-loaded apoferritin bound to TEC displays the same bell-shaped behavior detected in water (Fig. 2). On the contrary, at the expected value of \(\sim 5\) mmol/L\(^{-1}\) \text{s}^{-1}, the relaxivity of Gd-HPDO3A added to the untreated cellular pellet is constant over all the magnetic field ranges tested. Because the relaxivity peak shown in Fig. 2 occurs only when the Gd-HPDO3A complex is internalized.
T1-weighted multislice multiecho MR images were recorded before, the administration of streptavidin and Gd-Apo-Bio. Fat-suppressed C3d-Bio (or with the vehicle alone) followed, after 45 minutes, by connected with the murine vasculature. Mice were treated i.v. with implantation), when human tumor vessels were shown to be TEC organized in capillary structures, as previously described (22). All the experiments were done at this time point (6 days after study was carried out on SCID mice grafted with TEC. After 6 days, tumor vessels by selectively targeting a TEC surface marker, a MRI in vivo capability of the Gd-loaded apoferritin to visualize human in the apoferritin cavity, we can conclude that the Gd-loaded apoferitin remains intact after binding to the cell membrane.

**“In vivo” MRI of TEC-formed vessels.** In order to assess the in vivo capability of the Gd-loaded apoferritin to visualize human tumor vessels by selectively targeting a TEC surface marker, a MRI study was carried out on SCID mice grafted with TEC. After 6 days, TEC organized in capillary structures, as previously described (22). All the experiments were done at this time point (6 days after implantation), when human tumor vessels were shown to be connected with the murine vasculature. Mice were treated i.v. with C3d-Bio (or with the vehicle alone) followed, after 45 minutes, by the administration of streptavidin and Gd-Apo-Bio. Fat-suppressed T1-weighted multislice multiecho MR images were recorded before, 10 minutes, 5, 24, and 48 hours after the contrast administration. In Fig. 3A, the tumor SI measured on two different groups of mice were compared 5 and 24 hours after the administration of the imaging probe. Marked SI enhancement (>30%) was measured in the region of interest corresponding to the whole tumor 5 hours after the injection of Gd-Apo-Bio pretargeted with the C3d-Bio peptide. On the contrary, no significant difference with the pretreatment image (3-5%) was measured after the injection of Gd-Apo-Bio alone, indicating that the nonspecific probe accumulation was below the detection limit. After 24 hours, the SI enhancement decreases to 20%, but the difference between the two groups of animals remains significant ($P = 0.0034$). Figure 3 shows the T1-weighted MR image of the tumor region obtained 5 hours after the administration of the streptavidin/Gd-Apo-Bio constructs in the presence and in the absence of the pretargeting with C3d-Bio. In Fig. 3C, the red-colored pixels are those showing a SI increased by >3 SD of the precontrast tumor. These enhanced pixels represent ~24% of the total tumor pixels in the first group of mice, whereas in the second group, they represent only 1%.

The SI enhancements measured on the liver, kidneys, and muscle upon administration of the streptavidin/Gd-Apo-Bio construct show that the elimination of the Gd-loaded apoferritin occurs largely through the liver (Fig. 4). The i.v. injection of the same dose of Gd-HPDO3A showed that the liver SI remains unchanged (data not shown) as expected for this neutral and highly hydrophilic contrast agent whose excretion occurs only through the kidneys (32). This observation is further evidence of the maintenance of a Gd complex inside the apoferritin cavity, which then follows the protein elimination pathways through liver uptake.

The in vivo binding of C3d-Bio NCAM mimetic peptide at the TEC neoformed vessels was confirmed by immunofluorescence following in vivo administration of FITC-labeled streptavidin/Gd-Apo-Bio. As shown in Fig. 5, the C3d-Bio peptide was detected by the FITC-labeled streptavidin/Gd-Apo-Bio complex as fluorescent dots underlining the human neoformed vessels. The normal murine renal endothelium (Fig. 5C), as well as the endothelium of skeletal muscles (data not shown) used as controls, were negative.

**Discussion**

An important application in molecular imaging is the detection of neoangiogenesis in tumors. For this purpose, the development of highly efficient MRI contrast agents has to be combined with the use of specific vectors able to carry the imaging reporter to the target site. In this work, NCAM was chosen as a selective marker of TECs as it is not expressed by normal quiescent endothelial cells in the apoferritin cavity, we can conclude that the Gd-loaded apoferritin remains intact after binding to the cell membrane.

Figure 3. A, MRI SI enhancement of tumors in SCID mice grafted with TEC treated with Gd-loaded apoferritin targeted and nontargeted to NCAM. MR T1-weighted images of tumors done before (B) and 5 hours after (C and D) the administration of C3d-Bio/streptavidin/Gd-loaded apoferritin (20:1:1), which corresponds to a Gd dose of 0.01 mmol/kg. The tumors are outlined in blue, red highlights the areas where the signal is 3 SD more intense than the precontrast tumor intensity (D). Marked SI enhancement (>30%) was measured in the region of interest corresponding to the whole tumor 5 hours after the injection of Gd-Apo-Bio pretargeted with the C3d-Bio peptide.

Figure 4. A, time course of MRI SI enhancement of liver (●), kidneys (●), and muscle (●) measured on tumor-bearing mice after Gd-loaded apoferritin administration. T1-weighted MR images of mouse liver acquired before (B) and after (C) 5 hours after the administration of Gd-loaded apoferritin. The data represent the cumulative values from the two groups of mice, i.e., treated and untreated with C3d-Bio. These images show that the elimination of the imaging probe occurs largely through the liver.
The nature of the vessels is indicated by the expression of NCAM. In principle, the same procedure can be extended to a biotinylated peptide that binds to the selective surface molecule NCAM. In conclusion, Gd-loaded apoferritin displays enough sensitivity to allow the MRI visualization of human tumor–derived endothelial cells transplanted into mice and targeted with a biotinylated peptide that binds to the selective surface molecule NCAM. In principle, the same procedure can be extended to a number of biotinylated targeting vectors, i.e., peptides and peptido mimetics, oligosaccharides, as well as larger macromolecules such as proteins and antibodies. Gd-loaded apoferritin recalls, to same extent, properties widely exploited with liposomes and other vesicle-like systems but with the advantage of having a smaller size (12-15 nm), thus improving its accessibility to targets outside the bloodstream. As apoferritin can be loaded with other molecules, one may envisage a more extensive use for this carrier system in several molecular imaging protocols. This approach can be exploited for the detection of tumor angiogenesis, for the evaluation of the effect of antiangiogenic therapy, as well as coupling the vector with drugs, for direct imaging, and quantification of drug delivery.

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