A Peptide Targeted Contrast Agent Specific to Fibrin-Fibronectin Complexes for Cancer Molecular Imaging with MRI

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A peptide targeted contrast agent, CLT1-(Gd-DTPA), was synthesized for molecular imaging of fibronectin−fibrin complexes in tumor tissue with magnetic resonance imaging (MRI). The T_1 and T_2 relaxivities of CLT1-(Gd-DTPA) were 4.22 and 4.45 mM^{-1} s^{-1} at 3 T, respectively. The targeted contrast agent specifically bound to tumor tissue and resulted in significant tumor contrast enhancement at a dose of 0.1 mmol Gd/kg for at least 60 min in mice bearing HT-29 human colon carcinoma xenografts as shown in dynamic MR images. In contrast, a control nontargeted contrast agent, Gd(DTPA-BMA), was cleared rapidly with little tumor enhancement 60 min postinjection. Tumor enhancement with CLT1-(Gd-DTPA) was significantly reduced after coinjection with a 3-fold excess of free CLT1 peptide. The preliminary study has shown that CLT1-(Gd-DTPA) can specifically bind to the fibrin−fibronectin complexes in tumor tissues, resulting in significant tumor enhancement. The targeted contrast agent has a potential for cancer molecular imaging with MRI.

Magnetic resonance imaging (MRI) is a powerful imaging modality for morphological and functional imaging. MRI provides anatomical images of soft tissues with high spatial resolution and is effective for noninvasive imaging of physiological properties, e.g., diffusion, perfusion, and vascularity, of the tissues of interest (*1*). However, MRI has not been effectively used for molecular imaging because of its low sensitivity. The direct conjugation of targeting agents, e.g., peptides, antibodies, and proteins, to clinical MRI contrast agents, e.g., Gd(III) chelates, could not deliver a sufficient amount of contrast agents for MRI to effectively detect the molecular targets expressed on the surface of cells of interest (*2, 3*). Consequently, a large number of Gd(III) chelates have been incorporated into various targeted nanoparticles for MR molecular imaging to increase local concentration of contrast agents (*4*−*8*). Unfortunately, the size of these nanoparticles is much larger than the renal filtration threshold (ca. 4.5 nm), and they cannot be readily excreted from the body. Long-term tissue retention of high-dose Gd(III) based contrast agents may also cause toxic side effects such as systemic nephrogenic fibrosis (*9, 10*).

The limitations of MRI for molecular imaging can be overcome by proper selection of molecular biomarkers and using agents that can be readily excreted (*11*). Stable Gd(III) chelates have been proven safe in patients except those with intermediate and late-stage renal diseases. Since the amount of biomarkers expressed on the cell surface may not be sufficient for molecular imaging with MRI, other biomarkers with high local expression can be used for MR molecular imaging. It has been reported that tumor stroma have a unique meshwork of clotted plasma proteins that are not present in normal tissues (*12, 13*). The presence of the fibrin in tumor meshwork is known to associate with increased microvessel permeability in neoplastic tissues (*12*), and fibronectin in tumor stroma is also associated with tumor angiogenesis (*13*). The clotted plasma proteins might be attractive targets for molecular imaging with MRI. Recently, Ruoslahti and colleagues have identified peptides, including CLT1 and CLT2 peptides, which can specifically bind to the fibronectin−fibrin clots in various tumor tissues with little nonspecific binding to normal tissues (*14*). The incorporation of such peptides to Gd(III) chelates may result in targeted contrast agents for specific tumor imaging with MRI. As compared to nanoparticulate targeted contrast agents, the small molecular targeted contrast agents can rapidly diffuse into tumor tissue and bind to tumor meshwork. The unbound contrast agents can be rapidly excreted from the body via renal filtration. Consequently, significant signal enhancement of molecular targets can be visualized by MRI with minimal nonspecific background enhancement.

Here, we report a targeted contrast agent, a conjugate of a cyclic decapeptide CGLIIQKNEC (CLT1) and Gd-DTPA, for MR molecular imaging of fibrin−fibronectin complexes in tumor tissues. The peptide CLT1 was first synthesized using standard solid-phase peptide synthesis from Fmoc-protected amino acids (*15*) on a 2-chlorotrityl chloride resin. At the end of the peptide synthesis, an excess of DTPA dianhydride in DMSO was reacted with the peptide on the beads at room temperature for 4 h to conjugate DTPA at the N-terminal of the peptide. The resin was completely washed with water, DMF, dichloromethane, and methanol three times each. The CLT1−DTPA was then removed from the resin using TFA solution (TFA 94%, 1,2-ethanedithiol 2.5%, triisobutylsilane 2.5%, water 1%). The product was exposed to air for about 2 h to allow the formation of disulfide bonds for the cyclic peptide and then purified using preparative HPLC with a C18 column. CLT1−(Gd-DTPA) was finally prepared by complexation of CLT1−DTPA with $Gd(OAc)_3$ at pH 6. Excess $Gd(OAc)_3$ was removed by precipitation at pH 11. The final product was purified by preparative HPLC. The mass (*m*/*z*, M + H^+) of CLT1−(Gd-DTPA) was 1650.75 (calcd 1650.2) as determined by MALDI-

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10.1021/bc800211r CCC: $40.75 © 2008 American Chemical Society
Published on Web 12/03/2008

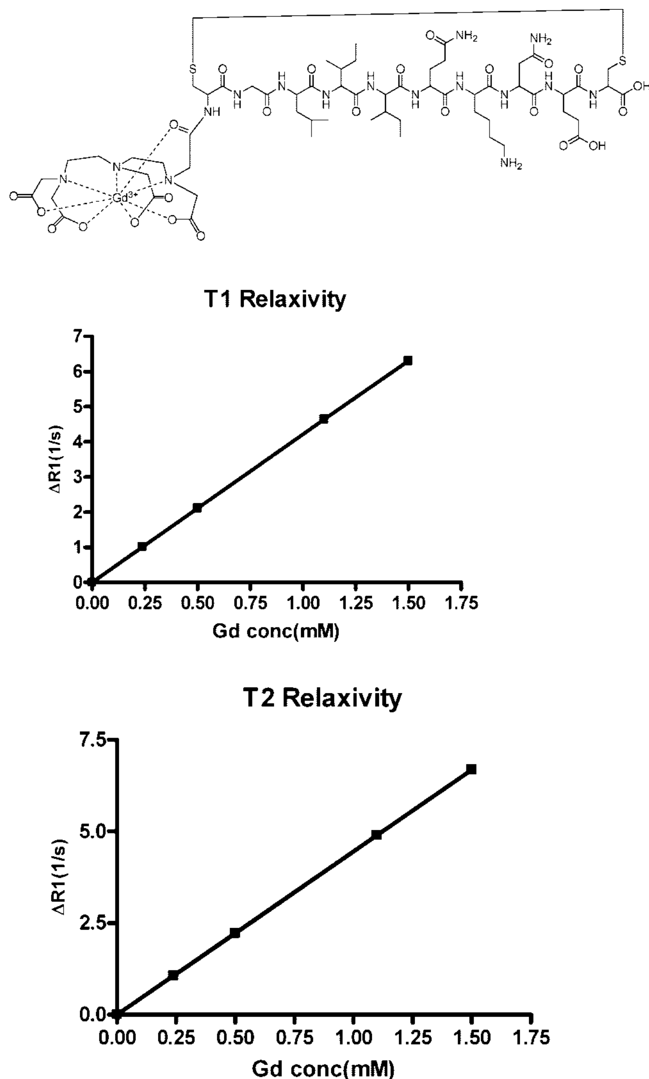

Figure 1. Chemical structure of CLT1−(Gd-DTPA) and plots of $1/T_1$ and $1/T_2$ versus the concentration of the contrast agent.

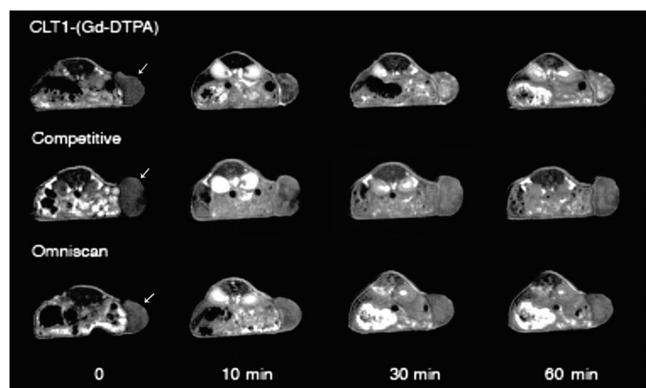

Figure 2. T_1-weighted 2D spin−echo images of mice bearing HT-29 xenografts before contrast and at 10, 30, and 60 min postinjection of CLT1−(Gd-DTPA), Omniscan and competitive mixture. Arrow points to the tumor tissue.

TOF mass spectrometry. The T_1 and T_2 relaxivities of CLT1−(Gd-DTPA) were determined on a Siemens Trio 3T scanner at room temperature using an inversion recovery (IR) prepared turbo spin−echo (TSE) imaging pulse sequence and a turbo spin−echo imaging sequence with turbo factor 3 (*16*), respectively. The T_1 and T_2 relaxivities of CLT1−(Gd-DTPA) were 4.22 and 4.45 mM^{-1} s^{-1} at 3 T. Figure 1 shows the structure of CLT1−(Gd-DTPA) and the plots of the T_1 and T_2 water proton relaxation rates at various concentrations of the contrast agent at 3 T.

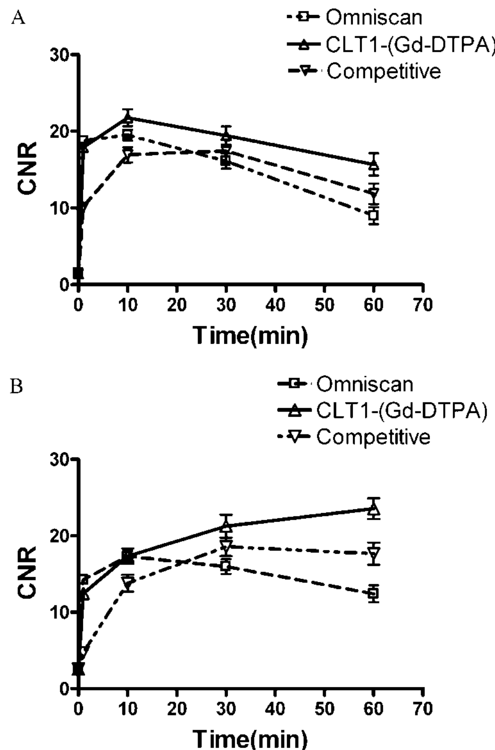

Figure 3. Plots of CNR versus time in tumor periphery (A) and inner area (B) before contrast and at 1, 10, 30, and 60 min postinjection of CLT1−(Gd-DTPA), Omniscan, and competitive mixture.

In vivo molecular imaging of fibrin−fibronectin complexes in tumor with the targeted contrast agent and MRI was evaluated in female athymic nu/nu mice bearing HT-29 human colon carcinoma xenografts with a T_1-weighted 2D spin−echo sequence. A clinical contrast agent, Gd(DTPA-BMA), was used as a control. Competitive targeting of free CLT1 peptide to CLT1−(Gd-DTPA) was also studied with coinjection of CLT1−(Gd-DTPA) and a 3-fold excess of free CLT1 peptide. A group of 3 mice was used for each contrast agent. The mice were anesthetized by intramuscular administration of a mixture of ketamine (45 mg/kg) and xylazine (6 mg/kg) for MRI. The CLT1−(Gd-DTPA) and Gd(DTPA-BMA) was intravenously injected at a dose of 0.1 mmol/kg. Contrast-enhanced MR images were acquired before and after injection at 1, 10, 30, and 60 min on a Siemens Trio 3T scanner using a human wrist coil. High-resolution 3D images were acquired with a 3D FLASH sequence with 25° flip angle, TR/TE = 7.8/2.7 ms, slice thickness 0.5 mm, field of view (FOV) 120 mm, voxel size 0.5 × 0.5 × 0.5 mm^3. T_1-weighted 2D axial tumor images were acquired with a 2D spin−echo sequence with TR 400 ms, TE 10 ms, 90° tip angle, and FOV 50 mm. Mice were sacrificed 24 h postinjection, and tumor tissues were removed and fixed with 3% paraformaldehyde and embedded in paraffin. Tissue sections (4 μm thickness) were incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity and boiled in antigen retrieval solution for 15 min in microwave. The sections were then incubated with primary antibody overnight and secondary antibody the next day for visualization using the ABC kit (Santa Cruz Biotechnology).

Figure 2 shows the representative axial T_1-weighted 2D spin−echo images of the tumor tissues of the mice bearing HT-29 tumor xenografts before and after injection of the contrast agents. Significant enhancement was observed in tumor tissues for CLT1-(Gd-DTPA) 10 min postinjection, and strong en-

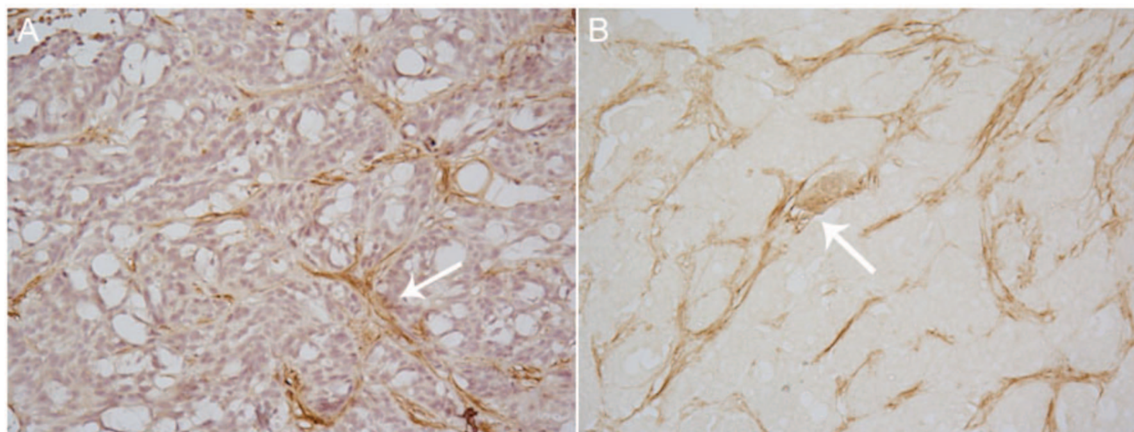

Figure 4. Staining of fibronectin in HT-29 tumor tissues with hemoxylin (A) or without hemoxylin (B) (40× magnification). Arrows point to the stained fibronectin (brown color) in the EES space, and blue in A indicates nuclus stained with hemoxylin.

hancement was visible in the tumor tissues at 60 min after injection. For the competitive study, the tumor enhancement with CLT1−(Gd-DTPA) was reduced after the coinjection of free peptide, which indicates that the free peptide recognizes the same binding site within tumor tissue as CLT1−(Gd-DTPA). The control agent Gd(DTPA-BMA) did not show a strong tumor enhancement as compared to CLT1−(Gd-DTPA).

MR signal intensity was also measured, and the contrast to noise ratio (CNR) in the tumor tissues was calculated as CNR $= (SI_{tissue} - SI_{muscle})/SD_{noise}$. Statistical analysis was perform using two-way ANOVA. Figure 3 shows the CNR of the tumor tissue in tumor periphery and inner areas before and at various time points after injecting the contrast agents. CLT1−(Gd-DTPA) showed higher CNR at 10 min after injection in the tumor periphery area and decreased gradually. At the same time, CNR in tumor inner area increased gradually during the period of 60 min postinjection. The CNR in both tumor periphery and inner tissue with Gd(DTPA-BMA) decreased rapidly after it reached the maximum values at 1 min postinjection. The CLT1−(Gd-DTPA) showed significantly higher CNR than the control Gd(DTPA-BMA) in tumor periphery since 10 min postinjection ($p < 0.05$) and in the tumor inner area since 30 min postinjection ($p < 0.05$). The results indicate binding and retention of the targeted contrast agent to tumor tissue. The presence of free peptide resulted in significant reduction of CNR in both tumor periphery and inner tumor tissue ($p < 0.05$) except at the point of 30 min postinjection. The result indicated that the presence of free peptide inhibited the binding of the targeted contrast agent to its target, resulting in decreased contrast enhancement in tumor tissue.

The plasma proteins fibrin(ogen) and fibronectin play a prominent role in hemostasis and wound healing. Fibrinogen is activated to form an insoluble fibrin clot following vascular injury. The clot also serves as a provisional matrix for adhesion and migration of cells or proteins including fibronectin, which is incorporated into the fibrin clot upon fibrin polymerization. The presence of the fibrin and fibronectin in tumor meshwork has also been reported in the literature due to the leakiness of tumor vasculature (*16, 17*). CLT1 peptide was reported to specifically bind the fibrin−fibronectin complexes in tumor tissues (*14*). We further carried out immunohistochemical study to verify the presence of fibronectin in the HT-29 tumor tissues after MR imaging. Figure 4 shows the immunostaining of fibronectin in HT-29 tumor tissues with (Figure 4A) or without (Figure 4B) nucleus staining. The histochemical staining clearly indicated the existence of fibronectin in the extracellular spaces of tumor tissues.

It is a challenge for MRI to detect in vivo molecular targets with molecular imaging because of its low sensitivity. We have shown here that MRI can be effective for molecular imaging if suitable molecular targets are identified. The targeted contrast agent CLT1-(Gd-DTPA) had little nonspecific binding and accumulation in the normal tissue despite that the peptide was hydrophobic. It bound to its molecular target in tumor tissue, resulting in significant and prolonged tumor enhancement. The result was consistent to what was originally reported by Ruoslahti et al. in other tumor models, which further confirmed that CLT1 peptide had a high specificity to fibrin−fibronectin complexes in tumor stroma (*14*). Because of the high content of the fibrin−fibronectin complexes in the tumor stroma, the peptide was able to deliver a sufficient amount of Gd-DTPA chelates to the molecular targets to generate detectable signal enhancement for MRI. Since the contrast agent was a low molecular weight chelate with a small size, it was able to be cleared rapidly from the blood circulation and normal tissues, resulting in strong CNR in the tumor tissue.

MR molecular imaging of fibrin−fibronectin complexes in tumor tissue with CLT1−(Gd-DTPA) also has the potential to characterize tumor angiogenesis. It has been reported that the presence of fibrin in the tumor meshwork is associated with increased microvessel permeability in neoplastic tissues (*12*), and fibronectin in tumor stroma is also associated with tumor angiogenesis (*13*). The result suggested that CLT1−(Gd-DTPA) was able to detect the presence of fibrin−fibronectin complexes in the angiogenic tumor tissues. The correlation of fibrin−fibronectin complexes to tumor angiogenesis may provide an effective method for tumor angiogenesis imaging with MRI and CLT1−(Gd-DTPA). Accurate assessment of tumor angiogenesis is critical for tumor grading, assessment of tumor response to anticancer therapies, particularly antiangiogenesis therapies, and patient management.

Fibrin−fibronectin complexes are also present in the plasma clots of wounds and other pathologic tissues with leaky blood vessels such as atherosclerotic plaques. Ruoslahti and colleagues showed that fluorescein-labeled CLT peptides specifically bound to the injured blood vessel wall, not the intact arteries and tissues (*14*). It suggests that MRI with CLT1−(Gd-DTPA) may have broad applications, including molecular imaging of cancer, angiogenesis, vascular integrity, and assessment of wound healing, anthrosclerosis, and tumor response to antiangiogenesis therapies. As compared to other targeting moieties, including proteins, antibodies, and their fragments, the peptide targeted Gd(III) chelate is advantageous for MR molecular imaging due to its excellent tissue permeability and rapid clearance from nonspecific tissue or organs.

In summary, a targeted contrast agent CLT1−(Gd-DTPA) specific to fibrin−fibronectin complexes was prepared and preliminarily evaluated in an animal tumor model for effective

cancer MR molecular imaging. The targeted contrast agent had minimal nonspecific binding and accumulation in normal tissues. CLT1 peptide was able to deliver a sufficient amount of Gd-DTPA chelates to its molecular target for effective molecular imaging with MRI in tumor. CLT1−(Gd-DTPA) is a promising targeted contrast agent for MR molecular imaging of fibrin−fibronectin complexes. It has a great potential for cancer detection and diagnosis, characterization of tumor angiogenesis, and imaging wounds and atherosclerosis.

ACKNOWLEDGMENT

This research was supported in part by the NIH R01 CA097465. We greatly appreciate Dr. Yongen Sun and Ms. Melody Johnson for their technical assistance in animal handling and MRI data acquisition.

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BC800211R