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Molecular MRI of Liver Fibrosis by a Peptide Targeted Contrast Agent in an Experimental Mouse Model

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Abstract

Liver fibrosis is a common response to chronic liver injury that is associated with high morbidity and mortality. Clinical utility of conventional and advanced MRI techniques for staging liver fibrosis has yet to be established. Study on molecular MRI of liver fibrosis is limited mainly due to difficulty in searching for a specific biomarker and synthesizing corresponding molecular probe. Liver fibrosis is characterized by an increased amount of extracellular matrix consisting of fibril-forming collagens, and matrix glycoconjugates such as fibronectin, which may serve as a specific molecular target for contrast-enhanced molecular MRI. In this study, liver fibrosis was induced by carbon tetrachloride (CCl₄) intoxication mimicking hepatic fibrosis and cirrhosis after 4 and 8 weeks. The feasibility of CGLIIQKNEC (CLT1) peptide-targeted nanoglobular contrast agent (Gd-P) for detection of liver fibrosis through molecular imaging of fibronectin was investigated at 7 T. Differential enhancements were observed and characterized between normal and fibrotic livers using Gd-P at 0.03 mmol/kg, comparing to non-targeted controls (Gd-CP and Gd-C). For Gd-P injection, both the peak and steady-state R_1 of normal livers was significantly lower than those after 4-week and 8-week of CCl₄ dosing. Liver fibrogenesis with increased amount of fibronectin in the extracellular space in insulted livers were confirmed by histological observations. These results indicate that Gd-P binds to fibrin-fibronectin complexes in livers, demonstrating the possibility of staging and characterizing liver fibrosis by probing the accumulation of fibronectin in fibrotic livers with molecular MRI.

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Keywords

MRI; Liver; Fibronectin; Gadolinium; Molecular Imaging

Introduction

Liver fibrosis is a common response to chronic liver injury which is associated with high morbidity and mortality (1). It may subsequently lead to cirrhosis with its consequences of portal hypertension, hepatocellular carcinoma (HCC) and liver failure (2). Liver fibrosis is characterized by an increased amount of extracellular matrix consisting of fibril-forming collagens, and matrix glycoconjugates such as fibronectin (3–5). Recently, fibronectin isoforms have been identified as a viable biomarker for liver fibrosis (6). Hepatic fibrogenesis is a dynamic process with potential of regression (7). Early diagnosis and quantification of liver fibrosis could facilitate early interventions and treatments, thus prevent its progression to cirrhosis (8). Percutaneous liver biopsy has been considered the standard technique for diagnosis and staging of liver fibrosis (9). However, liver biopsy is invasive in nature with potential complications and its ability to assess fibrosis progression and treatment response longitudinally is limited by the sampling error and interobserver variation (10). Current techniques in assessing liver fibrosis such as routine liver function tests, serological tests of specific serum markers and liver stiffness measurement using noninvasive ultrasound transient elastography are still under investigation (2,11–13). Development of noninvasive and reproducible alternatives for early diagnosis and staging of liver fibrosis, and monitoring disease progression or regression due to treatment effects is therefore essential.

Conventional MRI has been employed to assess liver fibrosis and cirrhosis; however the anatomical analysis has been subjected to interobserver variability and limited in sensitivity and specificity (14,15). Double contrast MRI using both gadolinium chelates and superparamagnetic iron oxides (SPIOs) was suggested to provide synergistic effects in visualizing liver fibrosis directly based on the hepatic texture alterations (16,17). MR elastography has shown promise in assessing liver fibrosis by measuring tissue stiffness (18–20). Alternatively, tagging MRI was proposed to measure the cardiac-induced motion and deformation in the liver to assess liver stiffness (21). Apparent diffusion coefficient (ADC) measured by diffusion-weighted imaging has been shown to decrease in fibrotic and cirrhotic liver as compared with normal liver (22,23). Changes in both ADC and other diffusion properties were observed during the progression of liver fibrosis using diffusion tensor imaging and intravoxel incoherent motion analysis (24) (25). Proton magnetic resonance spectroscopy was also demonstrated to provide a sensitive means to assess liver fibrosis through characterizing metabolic changes without the use of contrast agents (26). More recently, longitudinal and transverse relaxation times, as well as spin-lattice relaxation time in the rotating frame ($T_{1\rho}$), were shown to alter in fibrotic livers (27–29(30). However, the clinical utility of these advanced MRI techniques for staging liver fibrosis has yet to be established.

Molecular MRI aims to visualize and characterize biological processes at the molecular level in living organisms (31). By coupling molecular activities with MR contrast agents specifically, visualization and monitoring of subcellular processes can be achieved. This technique usually involves specific labeled molecular probes (32). Integrin-targeted ultrasmall SPIOs has been developed to probe the activated hepatic stellate cells in liver fibrosis of rats (33). Study on molecular MRI of liver fibrosis is limited mainly due to difficulty in searching for a specific biomarker and synthesizing corresponding smart molecular probe. Recently, specific binding of a cyclic decapeptide CGLIIQKNEC (CLT1) to the fibronectin-fibrin complexes, formed by clotting of fibrin and fibronectin isoforms including oncofetal fibronectin and an alternatively spliced form of fibronectin (34,35), has been observed in the extracellular matrix of different tumors and tissue lesions (36). CLT1 peptide-targeted nanoglobular MR contrast agent has been investigated for cancer molecular imaging with contrast enhanced MRI (37–39). Specific binding of CLT1 in the tumor tissue after intravenous administration of the agent was also confirmed using fluorescent imaging technique (36,39). The targeted nanoglobular MR contrast agent demonstrated stronger tumor enhancement than a control non-targeted contrast agent at a relatively low dose in a mouse tumor model, leading to potential applications in cancer detection and diagnosis, characterizing tumor angiogenesis, and imaging wounds and atherosclerosis.

Fibrin-fibronectin complexes exist in fibrotic liver due to the cross-linkage between fibrin/fibrinogen and fibronectin (40); therefore, they can serve as a specific molecular target for contrast-enhanced MRI. In this study, we hypothesize that the increased amount of fibrin-fibronectin complexes in fibrotic liver may be probed by CLT1 peptide-targeted nanoglobular contrast agent using contrast-enhanced molecular MRI. We aim to investigate the feasibility to detect and characterize liver fibrosis using CLT1 peptide-targeted nanoglobular contrast agent with dynamic contrast-enhanced MRI (DCE-MRI) in an experimental mouse model.

Methods

All MRI measurements were acquired on a 7 T MRI scanner with a maximum gradient of 360 mT/m (70/16 PharmaScan, Bruker Biospin GmbH, Germany). A 38-mm quadrature resonator was used for RF transmission and receiving. All animal experiments were approved by the local institutional animal ethics committee.

Synthesis and MR Characterization of Contrast Agents

CLT1 peptide-targeted nanoglobular contrast agent (Gd-P) was synthesized as described previously (Fig. 1) (37). In brief, peptide CLT1 with a propargyl group at the N-terminus was synthesized using standard solid-phase peptide synthesis from Fmoc-protected amino acids on a 2-chlorotrityl chloride resin as described previously (41). In brief, generation 3 (G3) poly-L-lysine dendrimers, known as nanoglobules, with an octa(3-aminopropyl)silsesquioxane (OAS) cubic core was synthesized in good yield and purity using solution phase peptide chemistry (42,43). Azido groups were introduced to the nanoglobules by reacting their surface amino groups with 3-[(2-azido-ethylenoxy)-heptao(2-ethylenoxy)]-propionic *N*-hydroxysuccinimide ester (Quanta BioDesign, Powell, OH).

Dendrimeric gadolinium(III)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic monoamide acid (Gd-DOTA monoamide) conjugates, nanoglobular MR contrast agents, were synthesized using standard liquid-phase peptide synthesis chemistry. Gd-P was formed by conjugating CLT1 peptide to G3 nanoglobular contrast agent via “click chemistry”. The characterization and structural confirmation were carried out by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DE-STR spectrometer (PerSeptive BioSystems, Framingham, MA) in linear mode with α -cyano-4-hydroxycinnamic acid as a matrix. Gadolinium(III) content in the contrast agent was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 3100XL, Perkin-Elmer, Shelton, CT). Approximately 3 peptides and 43 Gd-DOTA chelates were conjugated onto the surface of one G3 nanoglobular dendrimer (total 64 amine groups), as determined by amino acid analysis and the sulfur content was measured by ICP-OES.

For comparison, a control peptide KAREC with a propargyl group at the N-terminus was prepared using standard solid-phase peptide synthesis (m/z , $[M+H]^+$: 919.45, observed: 919.63). Note that peptide KAREC has been reported to show no binding to fibrin-fibronectin complexes (36). Similarly, control peptide KAREC conjugated nanoglobular contrast agent (Gd-CP) was synthesized following the procedure of Gd-P synthesis. In this study, peptide-free G3 nanoglobular Gd-DOTA monoamide conjugate was also prepared as the control non-targeting nanoglobular contrast agent (Gd-C).

Longitudinal relaxivity (r_1) and transverse relaxivity (r_2) were determined for Gd-P, Gd-CP and Gd-C suspensions. In brief, randomly uniform suspensions were prepared for nanoglobular contrast agent concentrations of 5 and 10 mM with the addition of saline. They were then placed in separate 2-mL cylindrical phantom tubes for longitudinal relaxation rate (R_1) and transverse relaxation rate (R_2) mapping. R_1 maps of nanoglobular contrast agents were acquired with a series of spin-echo images with varying pulse repetition times (TRs). The parameters were TRs = 40, 80, 160, 320, 640, 1280 ms, echo time (TE) = 8 ms, field of view (FOV) = 30 mm $\times$ 30 mm, acquisition matrix = 128 $\times$ 128, spatial resolution = 0.234 $\times$ 0.234 $\times$ 2 mm³ and number of excitations (NEX) = 1. Similarly, R_2 maps were acquired with multi-echo spin-echo imaging sequences. The parameters were TR = 1500 ms, 8 TEs = 5.5 to 44 ms with 5.5 ms increment, FOV = 30 mm $\times$ 30 mm, acquisition matrix = 128 $\times$ 128, spatial resolution = 0.234 $\times$ 0.234 $\times$ 2 mm³ and NEX = 1.

Liver Fibrosis Model

Male adult C57BL/6N mice (22–25 g; N = 54) were prepared and were divided into fibrosis (N = 36) and normal (N = 18) groups. The overall schedule of the experiment is shown in Fig. 2. Liver fibrosis was induced in fibrosis group by subcutaneous injection of 1:3 mixture of carbon tetrachloride (CCl₄) in olive oil at a dose of 4 μ L/g of body weight twice a week for 8 weeks (24,26,44). Intermittent administration of CCl₄ has been widely used to experimentally induce liver fibrosis in rodents by evoking a marked infiltration of inflammatory cells, thus mimicking the changes in chronic viral hepatitis-associated fibrosis in many ways (45,46). The twice-weekly dosing can induce established hepatic fibrosis and cirrhosis after 4 and 8 weeks of CCl₄ administration, respectively, in rodents (44). This well-

controlled CCl₄-induced liver fibrosis model allows the study of a homogeneous population of liver fibrosis (25,29). DCE-MRI was performed in the CCl₄-insulted animals at 4 and 8 weeks after the start of CCl₄ administration. Normal group was untreated and served as a control.

In Vivo MRI Experiments

Before each imaging session, each mouse was anesthetized with isoflurane/air at 3% for induction and 1.5% for maintenance via a nose cone. Femoral vein catheterization was performed with a 1-m long tube (MTV-1, Braintree Scientific Inc., Braintree, MA) connected to a 28-gauge needle. The dead space in the catheter was about 0.2 mL. During imaging, animals were anesthetized with isoflurane/air using 1.0–1.5% via a nose cone with respiratory monitoring. Body temperature was maintained at about 36.5°C by circulating warm water in a heating pad. Each mouse was fixed in a supine position. A reference phantom containing phosphate-buffered saline (PBS) was placed next to each animal. For each imaging session, anatomical images, preinjection T_1 measurements, DCE-MRI, and maximum intensity projection images before and after contrast injection were obtained.

High resolution anatomical T_1 -weighted images were acquired with two-dimensional (2D) fast spin-echo (FSE) sequence using TR = 200 ms, TE = 10 ms, FOV = 30 mm × 30 mm, acquisition matrix = 256 × 256, spatial resolution = 0.117 × 0.117 × 2 mm³, echo train length = 2 and NEX = 10. Preinjection T_1 values were measured with a series of spin-echo images with varying TRs. The parameters were TRs = 125, 250, 500, 1000, 2000, 4000 ms, TE = 8 ms, FOV = 30 mm × 30 mm, acquisition matrix = 128 × 128, spatial resolution = 0.234 × 0.234 × 2 mm³ and NEX = 1. DCE-MRI of mouse liver was then performed with T_1 -weighted 2D fast low-angle shot (FLASH) sequence for 1 hour using TR = 50 ms, TE = 2.5 ms, flip angle (FA) = 80°, FOV = 30 mm × 30 mm, acquisition matrix = 128 × 128, spatial resolution = 0.234 × 0.234 × 2.0 mm³, NEX = 4 and temporal resolution = 20 s. For each imaging session, 0.1 mL of contrast agent (0.03 mmol Gd/kg) was slowly injected (~2 s) into the catheter at a rate of 3 mL/min about 6 min after the start of dynamic scan. A 0.4 mL saline flush (~8 s) was administered immediately thereafter to drive the contrast agent from the catheter into the femoral vein. T_1 -weighted maximum intensity projection images were also acquired using 3D FLASH sequence before and after the injection of the contrast agents. The parameters were TR = 12.5 ms, TE = 2.5 ms, FA = 60°, FOV = 30 mm × 60 mm × 30 mm, acquisition matrix = 128 × 256 × 128, spatial resolution = 0.234 × 0.234 × 0.234 mm³ and NEX = 1.

Data and Statistical Analysis of DCE-MRI

DCE-MRI was analyzed to yield postinjection $R_1(t)$ maps for quantitative measurements. In detail, DCE images were first co-registered using Automated Image Registration (AIR5.2.5) (47). Normalized liver signal intensity was calculated as the ratio of signal intensity of liver to that of the phantom, and was averaged over slices in each animal. Assuming negligible T_2 effects from the injected contrast agents due to the short TE and low dose used, peak R_1 maps were computed on a pixel-by-pixel basis (48) as

$$\frac{S_{pre}}{S(t)} \approx \frac{1 - \exp(TR \cdot R_{1pre})}{1 - \exp(TR \cdot R_1(t))}$$

where S_{pre} is the average intensity of 15 preinjection images and $S(t)$ the intensity of postinjection image with maximum contrast enhancement. Similarly, steady-state R_1 maps were obtained using $S(t)$ as the average intensity of 90 postinjection images at the end of the dynamic scan. To estimate liver peak and steady-state R_1 in each mouse, a large region of interest (ROI) was manually drawn in a homogeneous liver region based on the high resolution FSE images. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests was employed to compare differences in peak and steady-state R_1 values in different groups, with $p < 0.05$ considered as statistically significant.

Histology

After the MR examination following 4 weeks of CCl_4 administration, 6 out of 18 animals were sacrificed for histological examination. Furthermore, 6 out of 18 animals were sacrificed after MR examination following 8 weeks of CCl_4 insult as shown in Fig. 2. Six normal animals were sacrificed as a control. Liver specimens were fixed in formalin, embedded in paraffin, sectioned and examined by light microscopy after standard hematoxylin-eosin (H&E) staining and Masson's trichrome staining for collagen deposition (49,50).

Immunohistochemical technique was used to detect and depict fibronectin. In brief, the liver sections were heated in antigen retrieval solution (0.01 M citrate buffer, pH 6.0; Sigma, St. Louis, MO) and then incubated with primary anti-fibronectin antibody (Sigma, St. Louis, MO) diluted to 1:400 with 0.01 M PBS (pH 7.4) overnight at room temperature. After washing with phosphate buffer solution, the sections were incubated with biotinylated secondary antibody and a horseradish peroxidase-streptavidin complex for 1 hour each. Tissue samples then were colorized with 3,3'-diaminobenzidine substrate, counterstained with hematoxylin and mounted (37).

Results

At 7 T, R_1 and R_2 values of the nanoglobular contrast agent at 5 and 10 mM were plotted in Fig. 3. Note that the r_1 relaxivities of Gd-P, Gd-CP and Gd-C were 6.79, 6.15 and 6.25 $\text{mM}^{-1} \text{s}^{-1}$ respectively, while the r_2 relaxivities of Gd-P, Gd-CP and Gd-C were 19.12, 17.71 and 12.17 $\text{mM}^{-1} \text{s}^{-1}$ respectively.

Contrast enhancements were consistently observed in all mice studied for Gd-P, Gd-CP and Gd-C. No apparent adverse effects were observed in those mice during and after imaging sessions. Fig. 4 illustrates the liver images typically observed during Gd-P, Gd-CP and Gd-C injections (0.1 mL of 0.03 mmol/kg) for mice after 4-week CCl_4 dosing. For Gd-P injection, Fig. 4a shows one of the preinjection FLASH T_1 -weighted images, the FLASH T_1 -weighted image with the maximum contrast enhancement at ~4 min postinjection and the FLASH T_1 -weighted image at ~54 min postinjection. Fig. 4b depicts the typical T_1 -weighted signal time courses during Gd-P injection with ROIs indicated in Fig. 4a. Two ROIs were selected from

one homogeneous liver region (LV) and the region covering inferior vena cava (IVC) regions. For Gd-CP injection, Fig. 4c shows one of the preinjection FLASH T_1 -weighted images, the FLASH T_1 -weighted image with the maximum contrast enhancement at ~4 min postinjection and the FLASH T_1 -weighted image at ~54 min postinjection. Similarly, Fig. 4d depicts the typical T_1 -weighted signal time courses during Gd-CP injection with ROIs indicated in Fig. 4c. For Gd-C injection, Fig. 4e shows one of the preinjection FLASH T_1 -weighted images, the FLASH T_1 -weighted image with the maximum contrast enhancement at ~4 min postinjection and the FLASH T_1 -weighted image at ~54 min postinjection. Similarly, Fig. 4f depicts the typical T_1 -weighted signal time courses during Gd-C injection with ROIs indicated in Fig. 4e. Fig. 5 illustrates the corresponding images typically observed during Gd-P, Gd-CP and Gd-C injections (0.1 mL of 0.03mmol/kg) for mice after 8-week CCl₄ dosing, while Fig. 6 illustrates the corresponding images for normal mice.

Strong signal enhancements were observed in liver parenchyma at postinjection of Gd-P, when compared with Gd-CP and Gd-C. Note the similar signal time courses observed in LV and IVC (Figs. 4c and e, 5c and e and 6c and e) for Gd-CP and Gd-C injections. Fig. 7 shows the normalized liver signal intensity time curves with Gd-P ($N = 6$), Gd-CP ($N = 6$) and Gd-C ($N = 6$) injection for (a) normal animals, animals after (b) 4-week and (c) 8-week CCl₄ injection. Gd-P consistently showed higher contrast enhancement than Gd-CP and Gd-C in liver at postinjection steady-state. Furthermore, contrast enhancement by Gd-P persisted during the dynamic scan, whereas enhancements by Gd-CP and Gd-C decreased and vanished rapidly after the maximum enhancement.

Fig. 8 summarizes the peak and steady-state R_1 measurements for all animals studied. For Gd-P injection, the peak R_1 of normal animals ($0.11 \pm 0.03 \text{ s}^{-1}$) was significantly lower than those after 4-week of CCl₄ dosing ($0.26 \pm 0.12 \text{ s}^{-1}$; $p < 0.05$) and 8-week of CCl₄ dosing ($0.32 \pm 0.10 \text{ s}^{-1}$; $p < 0.01$). The steady-state R_1 of normal animals ($0.07 \pm 0.04 \text{ s}^{-1}$) was also significantly lower than those after 4-week of CCl₄ dosing ($0.16 \pm 0.07 \text{ s}^{-1}$; $p < 0.05$) and 8-week of CCl₄ dosing ($0.19 \pm 0.03 \text{ s}^{-1}$; $p < 0.01$). No significant difference in peak and steady-state R_1 was observed among different groups of animals for Gd-CP and Gd-C injections.

Fig. 9 shows the preinjection and 1-hour postinjection T_1 -weighted maximum intensity projection images of mice after 8-week CCl₄ dosing for Gd-P, Gd-CP and Gd-C. Compared to other organs, strong contrast enhancement was observed in the urinary bladder one hour after injection of all contrast agents.

Fig. 10 shows typical H&E staining and Masson's trichrome staining for normal liver and livers at 4 weeks and 8 weeks after CCl₄ insult. Collagen deposition was stained as blue by Masson's trichrome staining in fibrotic livers. Compared with normal liver (Fig. 10a), collagen deposition and intracellular fat vacuoles were consistently observed in livers with CCl₄ insult (Figs. 10b and c). Similar histological findings were observed in all liver samples collected, and they were largely consistent with those from the earlier studies of CCl₄-induced liver fibrosis in rodent models (51). The histological observations of collagen deposition in the liver samples collected confirmed the liver fibrogenesis in the animals studied. Fig. 10 also shows typical immunohistochemical staining of fibronectin for normal

liver and livers at 4 weeks and 8 weeks after CCl₄ insult. Compared with the normal liver (Fig. 10a), the insulted liver showed increased amount of fibronectin in the extracellular space as revealed by brown deposits after staining with an anti-fibronectin antibody (Figs. 10b and c). These histological findings were consistent with those observed in previous hepatic fibrosis immunohistochemical study (25,29,40,52).

Discussions

In this study, CLT1 peptide-targeted nanoglobular contrast agent was successfully demonstrated for the first time in detecting liver fibrosis through molecular MRI. Peptide-targeted nanoglobular contrast agent (Gd-P) and non-targeted controls (Gd-CP and Gd-C) were examined for their contrast enhancement effects by dynamic liver imaging at 7 T. The increased binding of CLT1 peptide-targeted nanoglobular contrast agent to the increased fibrin-fibronectin complexes in fibrotic liver resulted in strong and prolonged enhancement. Peak and steady-state R_1 induced by Gd-P (0.1 mL of 0.03 mmol/kg) for fibrotic livers were higher than that of normal livers, while values of peak and steady-state R_1 upon Gd-CP and Gd-C injections were comparable in all animals. These results indicated that Gd-P could be used as a fibrin-fibronectin targeting MR contrast agent to detect and characterize liver fibrosis at early phase.

The histological observations revealed the collagen deposition and thus confirmed liver fibrogenesis in the animals studied. For all the animals studied, the higher contrast enhancement of Gd-P compared to Gd-CP and Gd-C in the liver at postinjection steady-state indicated the specific binding of Gd-P to the liver, leading to strong and prolonged contrast enhancement. Note that the existence of fibronectin in normal liver due to its function of producing plasma fibronectin (40). In contrast, non-targeting effects of Gd-CP and Gd-C were confirmed by its vanishing enhancement after the maximum enhancement for all animals. These results were consistent with literatures using the same contrast agents in mouse cancer models (37,39). Both the peak and steady-state R_1 after Gd-P were higher in fibrotic livers than in normal livers, likely due to the increased amount of accumulated Gd-P in consequence of raised quantity of fibronectin in fibrotic livers (Figs. 7b and c). The abundant presence of fibronectin in fibrotic livers allowed the specific binding of Gd-P in fibrotic livers. From the quantitative peak and steady-state R_1 analysis, fibrotic livers could be readily distinguished from normal livers following injection of Gd-P. It showed the feasibility of early diagnosis of liver fibrosis following injection of CLT1 peptide-targeted nanoglobular contrast agent through molecular imaging of fibronectin.

The relaxivities r_1 and r_2 of the nanoglobular contrast agents increased with increasing sizes. The relaxivities of Gd-P and Gd-CP were slightly higher than that of Gd-C. The relaxivities of the nanoglobular contrast agents (37,39) were similar to those of other reported dendrimeric Gd-DOTA-monoamide conjugates ($r_1/r_2 \approx 11/13 \text{ mM}^{-1} \text{ s}^{-1}$) (53,54). Dynamic light scattering analysis showed that the nanoglobular contrast agents had a particle size of 5.8 – 6.2 nm. Considering the renal filtration threshold of 8.0 nm (55), the small sizes of the nanoglobular contrast agents allow rapid excretion after injection (37). The T_1 -weighted maximum intensity projection images (Fig. 9) also confirmed that the unbound nanoglobular

contrast agents were gradually excreted through renal filtration and accumulated in the urinary bladder.

The G3 dendrimer synthesized exhibits a globular morphology and relatively compact and rigid three-dimensional structure. The high functionality for conjugation is suitable for preparation of targeted contrast agents with high peptide to gadolinium chelate ratios. The contrast agent dosage used in current study (0.03 mmol/kg) was lower than that used in clinical contrast-enhanced MRI (0.1 mmol/kg) (56). However, its large size and high number of conjugated CLT1 peptides resulted in slow excretion and might limit its future clinical application (37). By optimizing the dosage, peptide to gadolinium chelate ratios and size, it is possible to enhance the sensitivity and specificity for future clinical applications.

Study on molecular MRI of liver fibrosis is limited so far. Recently, integrin-targeted ultrasmall SPIOs has been developed to probe the activated hepatic stellate cells in liver fibrosis of rats (33). In liver fibrosis, activated hepatic stellate cells would engulf integrin-targeted ultrasmall SPIOs for negative signal enhancements. However, ultrasmall SPIOs could also be taken up by Kupffer cells in both normal and injured livers; it may limit future clinical applications. In this study, we presented an alternative approach to target molecular probes in liver fibrosis for positive signal enhancements through identifying fibrin-fibronectin complexes as biomarkers in fibrotic liver. Molecular MRI is therefore feasible if one can identify specific molecular probes in visualizing and monitoring of subcellular processes.

One potential limitation of the current study is the difference in the development and progression of liver fibrosis in the CCl₄-intoxication fibrosis model when compared with those in humans due to different etiology of the disease. In particular, the repetitive dosing of CCl₄ in animals can induce fibrosis development within weeks, while the development of fibrosis in patients with liver diseases is more gradual and may take several months or years depending on the underlying causes. Despite different etiology, the underlying pathophysiologic processes of fibrogenesis (e.g. inflammatory cell infiltration, cell necrosis, fibroblast proliferation, pronounced extracellular matrix/collagen deposition, and potential progression to cirrhosis) are largely similar (57,58). In addition, the current histological finding of collagen deposition and increased fibronectin in the experimental fibrosis model was in accordance with those reported in patients with liver fibrosis (3–5), suggesting that the targeting capability of Gd-P over fibrotic livers in clinical settings would be similar.

Conclusions

In this study, CLT1 peptide-targeted nanoglobular contrast agent was successfully demonstrated for the first time in detecting liver fibrosis through molecular imaging of fibronectin at 7 T. Considerable contrast enhancements were observed and characterized in normal and fibrotic livers using CLT1 peptide-targeted nanoglobular contrast agent at a relative low dose, when compared to non-targeted control. Differential enhancements were demonstrated between normal and fibrotic livers following CLT1 peptide-targeted nanoglobular contrast agent injection. These results indicated that Gd-P binds to fibrin-fibronectin complexes in the fibrotic livers, demonstrating the possibility of staging and

characterizing liver fibrosis by probing the accumulation of fibronectin in fibrotic livers with molecular MRI.

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FIG. 1.
Synthetic illustration of peptide CLT1-targeted nanoglobular contrast agents.

FIG. 2. Schedule of carbon tetrachloride (CCl_4) twice-weekly administration for induction of liver fibrosis in male adult C57BL/6N mice, DCE-MRI and histology.

FIG. 3.

R_1 and R_2 values of the nanoglobular contrast agent suspensions at 5 and 10 mM at 7 T. Error bars represent standard deviation.

FIG. 4.

Corresponding images from the mouse liver after 4-week CCl₄ dosing. During Gd-P injection (0.1 mL of 0.03 mmol/kg over ~2 s): (a) preinjection FLASH T₁-weighted image, the FLASH T₁-weighted image with the maximum contrast enhancement at ~4 min postinjection and the FLASH T₁-weighted image at ~54 min postinjection. (b) T₁-weighted signal time courses in different regions during Gd-P injection. Corresponding images during Gd-CP and Gd-C injections were depicted in (c) to (f), respectively. ROIs were selected from one homogeneous liver region (LV) and the region covering inferior vena cava (IVC) regions for time course measurements, as shown in (a), (c) and (e).

FIG. 5.

Corresponding images from the mouse liver after 8-week CCl_4 dosing. During Gd-P injection: (a) preinjection FLASH T_1 -weighted image, the FLASH T_1 -weighted image with the maximum contrast enhancement at ~4 min postinjection and the FLASH T_1 -weighted image at ~54 min postinjection. (b) T_1 -weighted signal time courses in different regions during Gd-P injection. Corresponding images during Gd-CP and Gd-C injections were depicted in (c) to (f), respectively. ROIs used for time course measurements are shown in (a), (c) and (e).

FIG. 6.

Corresponding images from the normal mouse. During Gd-P injection: (a) preinjection FLASH T_1 -weighted image, the FLASH T_1 -weighted image with the maximum contrast enhancement at ~4 min postinjection and the FLASH T_1 -weighted image at ~54 min postinjection. (b) T_1 -weighted signal time courses in different regions during Gd-P injection. Corresponding images during Gd-CP and Gd-C injections were depicted in (c) to (f), respectively. ROIs used for time course measurements are shown in (a), (c) and (e).

FIG. 7. Normalized liver signal intensity time curves with Gd-P, Gd-CP and Gd-C injection for (a) normal animals, and animals after (b) 4-week and (c) 8-week CCl₄ insult. Error bars represent standard deviation.

FIG. 8.

In vivo measurements of peak and steady-state R_1 for all animals studied. One-way ANOVA with Tukey's multiple comparison test was performed with * for $p < 0.05$, ** for $p < 0.01$ and n.s. for insignificance.

FIG. 9.

T_1 -weighted maximum intensity projection images for mice following 8-week CCl_4 dosing before and 1 hour after the injection of Gd-P, Gd-CP and Gd-C.

FIG. 10.

Typical H&E staining (200 $\times$; left column), Masson's trichrome staining (200 $\times$; middle column) and immunohistochemical staining of fibronectin (200 $\times$; right column) for (a) normal liver, and livers subjected to (b) 4-week and (c) 8-week CCl₄ twice-weekly administration. Fat vacuoles (blue arrows) and collagen deposition (green arrows) were observed in the insulted livers with H&E and Masson's trichrome staining respectively. Immunohistochemical staining of fibronectin shows a higher amount of extracellular fibronectin (red arrows) in the insulted livers as brown deposits.