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Non-invasively differentiating extent of liver fibrosis by visualizing hepatic integrin αvβ3 expression with an MRI modality in mice

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ABSTRACT

Aims: To explore the potential of a dendrimer nanoprobe labeled with cyclic arginine-glycine-aspartic acid pentapeptide (cRGDyK) as a magnetic resonance imaging (MRI) tracer to non-invasively differentiate the extent of liver fibrosis.
Methods: Synthetic dendrimer nanoprobes were labeled with cRGDyK (Den-RGD) to form a formulation of hepatic stellate cell (HSC)-specific MRI tracer. An MRI modality was employed to visualize hepatic Den-RGD deposition in a mouse model of liver fibrosis caused by thioacetamide treatment.
Results: Den-RGD bound to activated HSCs via integrin αvβ3 receptors. The labeling of nanoprobes with cRGDyK increased their affinity to and accelerated their uptake by activated HSCs. Most of intravenously administrated Den-RGD nanoprobes deposited in the fibrotic areas, and the deposited amount was paralleled with the severity of liver fibrosis. Majority of cells taking-up Den-RGD was found to be activated HSCs in fibrotic livers. An MRI modality using Den-RGD as a tracer demonstrated that the relative hepatic T1-weighed MR signal value was increased in parallel with the severity of liver fibrosis.
Conclusion: The extent of Den-RGD deposition reflects integrin αvβ3 expression in activated HSCs, and Den-RGD appears to be a useful formulation of MRI tracer and may non-invasively and quantitatively assess the extent of liver fibrosis.

1. Introduction

Chronic liver disease (CLD) represents a substantial public health problem with mortality attributable to cirrhosis of around 800,000 deaths per year worldwide [1]. Prognosis and management of CLD largely depend on the severity and progression of liver fibrosis [1]. Hence, the assessment of liver fibrosis is pivotal for the management of patients with CLD. Liver biopsy has traditionally been considered as the gold standard for determining the severity of hepatic fibrosis. However, it is an invasive procedure, with a risk of rare but potentially life-threatening complications, and is also prone to sampling errors [2]. Additionally, for the treatment of CLD, one of the main therapeutic goals is to reverse hepatic fibrosis [3–5]. To monitor the dynamic feature of fibrotic progression and regression, one needs to quantitatively assess the severity of

Abbreviations: CLD, Chronic liver diseases; ECM, extracellular matrix; HSCs, hepatic stellate cells; RGD, arginine-glycine-aspartic acid; cRGDyK, cyclo[Arg-Gly-Asp-(D)Tyr-Lys]; MRI, magnetic resonance imaging; Den-RGD, the targeted nanoprobe labeled with cRGDyK; Den-PEG, the control nanoprobe without cRGDyK labeling; Mal, maleimide; NHS, N-hydroxysuccinimidyl; PEG, polyethylene glycol; DOTA, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; Gd, gadolinium; DAPI, 6-diamidino-2-phenylindole; TAA, thioacetamide; ROI, region of interest; ΔT1, the average value of the relative hepatic signal intensity; ALT, alanine aminotransferase.

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fibrotic deposition. Nevertheless, the quantitation of hepatic fibrosis in biopsied specimens with technologies such as morphometric analysis remains semi-quantitative [1,4,5]. Moreover, it is unpractical to repeatedly perform a liver biopsy procedure during a short-term follow-up due to the invasive nature of liver biopsy when the potential anti-fibrotic effect needs to be evaluated before and after a medication was administrated for several times [4,5]. Thus, it is essential to develop non-invasive methods that are sensitive enough to visualize minor changes of fibrosis. Unfortunately, none of the current non-invasive approaches has reached this degree of sensitivity [4–7].

Liver fibrosis is a result of excessive extracellular matrix (ECM) deposition in response to chronic inflammatory injury, which is determined by a dynamic balance between ECM production and degradation. Myofibroblasts, representing a spectrum of ECM-producing cells that mainly derive from hepatic stellate cells (HSCs) and portal fibroblasts, are the major producers of ECM and are considered to be the most critical cellular effectors of liver fibrosis [3].

Integrins are a large family of heterodimeric cell surface receptors composed of non-covalently linked α- and β-subunits, which act as mechanoreceptors by relaying the signals between ECM and cells, or between different cells [8]. It has been found that integrin αvβ3 is expressed by HSCs during their activation *in vitro*, and promotes HSCs proliferation and survival [9]. More importantly, it has been observed that hepatic expression of integrin αvβ3 is markedly up-regulated in rats with liver fibrosis, and positively correlated to the stages of fibrotic progression [10,11].

Many integrins recognize a common motif in their ligands. One of best characterized motifs is the arginine-glycine-aspartic acid (RGD) sequence. The integrin-binding activity of adhesion ligands can be reproduced by short synthetic peptides containing this motif [12]. In the previous studies, it has been demonstrated that a synthesized cyclic RGD pentapeptide (cyclo [Arg-Gly-Asp-(D)Tyr-Lys], cRGDyK) binds to both purified and membrane-bound integrin αvβ3 receptor on activated HSCs with a high affinity in a reversible fashion [13].

Among the multiple imaging modalities used in clinic, magnetic resonance imaging (MRI) shows the highest spatial resolution to soft tissues (up to 200 μm) and is especially useful to distinguish small lesions. In the present study, we labeled a well-characterized dendrimer with cRGDyK to formulate an integrin αvβ3-targeted nanoprobe. With this novel nanoprobe as a tracer, we successfully developed a non-invasive MRI modality to quantitatively assess the extent of liver fibrosis by imaging hepatic integrin αvβ3 expression in mice.

2. Materials and methods

2.1. Materials

All organic solvents were analytical grade from Aladdin Reagent (Shanghai, China) unless otherwise specified. Fmoc-Gly-2-cl-Trt resin was purchased from Applied Biosystems (Carlsbad, California, USA). Polyamidoamine (PAMAM) G5 dendrimer (77.35 mg/mL in methyl alcohol, containing 128 primary amino groups, MW: 28,826 Da) was purchased from Dendritech Inc (Midland, MI, USA). Rhodamine succinimide ester was purchased from Thermofisher scientific (NY, USA). Activated polyethylene glycol (PEG) derivatives, Malemide-PEG^{2k}-NHS ester and PEG^{2K}-NHS ester were purchased from JenKem Technology Co. Ltd (Beijing, China). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-NHS ester was purchased from Macrocyclics (TX, USA). $Gd_2(CO3)_3$, DNase I, Nycodenz, 6-diamidino-2-phenylindole (DAPI) and Bolton-Hunter reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagenase NB4 (standard grade) was purchased from SERVA (Heidelberg, Germany). Pronase E was purchased from Roche (Basel, Switzerland). Isoflurane (AERRANE) was purchased from Baxter Healthcare Corporation (New Providence, NJ, USA). Fetal bovine serum, penicillin and streptomycin, high glucose DMEM, Alexa Flour 488-labeled donkey anti-mouse secondary antibody, Alexa Flour 488-labeled rat anti-mouse secondary antibody, Alexa Fluor 488-labeled goat anti-rat secondary antibody, Alexa Flour 488-labeled donkey anti-goat secondary antibody and Alexa Flour 488-labeled goat anti-rabbit secondary antibody were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit anti-mouse α-SMA antibody, rat anti-mouse CD68 antibody and rat anti-mouse CD31 antibody were purchased from Abcam (MA, USA). Rabbit anti-mouse integrin β3 antibody was purchased from Epitomics (Burlingame, CA, USA). Goat anti-mouse CD163 antibody was purchased from SANTA CRUZ Biotechnology INC. (CA, USA).

2.2. Experimental animals

C57BL/6J mice were purchased from the Department of Experimental Animals, Fudan University, and housed in a specific pathogen-free facility. The experimental design and procedures were approved by the Institutional Ethical Committee of Animal Experimentation, and all experiments were performed strictly according to governmental and international guidelines on animal experimentation.

2.3. Synthesis, formulations and characterization of nanoprobes

The targeted dendrimer nanoprobe labeled with cRGDyK (referred to as Den-RGD) and the control dendrimer nanoprobe without cRGDyK labeling (referred to as Den-PEG) were prepared as described previously [13,14]. Briefly, cRGDyK was firstly synthesized by an Fmoc-protected solid-phase peptide synthetic method. Then, dendrimers in the fifth generation (PAMAM G5 dendrimer) were chosen as the platform of the nanoprobe preparation, given that this dendrimer (diameter: 7 nm) possesses a prolonged blood circulation time compared to low generation dendrimers and is excreted from both renal and hepatobiliary systems [15]. In Den-RGD, the cRGDyK peptide was labeled onto the dendrimer surface through a flexible PEG linkage. Both Den-RGD and Den-PEG were modified with rhodamine fluorophores and paramagnetic Gd-DOTA chelators. The synthesis processes were illustrated in Fig. 1.

The characteristics of nanoprobes were measured as previously described [13,14]. In brief, the molar ratio between dendrimer, PEG, cRGDyK and DOTA was quantified by measuring the proton integration of dendrimer (3.3–2.2 ppm), PEG (3.7 ppm, O-CH_2), cRGDyK (7.2–6.7 ppm) and DOTA (3.3–2.2 ppm) in the ^{1}H NMR spectrum. The labeling proportion of rhodamine was determined by measuring the absorbance of rhodamine (ε_{552} = 60,000 M^{-1} cm^{-1}) according to Lambert-Beer's law: A = lg (1/T) = Kbc (A: absorbance, T: transmission factor, c: concentration, b: thickness of the absorber) by UV-2401PC UV–vis Recording spectrophotometer (SHIMADZU, Kyoto, Japan). The molecular weight of nanoprobes was determined by MALDI-TOF MS, which was conducted using an AB SCIEX TOF/TOF™ 5800 mass spectrometer (Applied Biosystems, CA, USA). The hydrodynamic radius of nanoprobes and unmodified G5 dendrimer were determined by a Malvern Zetasizer (Malvern Instruments Inc., Southborough, MA) and dynamic light scattering instrument (DLS) at room temperature. In order to determine the surface charges of nanoprobe, the instrument was calibrated with the standard solution with a Zeta potential of −50 mV. The Gd^{3+} concentration of nanoprobe was determined by a Hitachi P-4010 (Tokyo, Japan) ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) system with RF power at 1100 W and nebulizer gas flow at 0.9 L/min. The longitudinal relaxivity of the nanoprobes and the commercial available MR contrast agent Gd^{3+}-DOTA were determined on a