Original Article

Molecular MRI of activated hepatic stellate cells in rats with early stage of liver fibrosis by targeting hepatic integrin α5β1

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Abstract: Objective: To investigate the feasibility of ultrasmall superparamagnetic iron oxide (USPIO) modified by anti-α5β1 integrin monoclonal antibody (mAb) for targeting activated hepatic stellate cells (HSCs) in early stage of hepatic fibrosis rats in vivo. Methods: The early stage hepatic fibrosis model of rats was established by injecting carbon tetrachloride subcutaneously. The anti-α5β1 integrin mAb-USPIO probe was prepared by conjugating anti-α5β1 integrin mAb with USPIO through carbodiimide method. Four normal rats were treated with anti-α5β1 integrin mAb-USPIO as control group. Twelve hepatic fibrosis rats were divided randomly into three groups and then were treated with anti-α5β1 integrin mAb-USPIO, naked USPIO and anti-α5β1 integrin mAb respectively. Abdominal magnetic resonance imaging (MRI) was performed for all rats before and 4 h after administration of the probes. The liver: muscle contrast-to-noise ratio (CNR) of the rats in all 4 groups before and 4 h after probe injection was calculated and analyzed. Liver tissue was subjected to pathologic scoring of fibrosis and location of iron particles of probes in normal and fibrosis rats was also observed. Results: The liver: muscle contrast-to-noise ratio (CNR) of the rats in all 4 groups before and 4 h after probe injection was calculated and analyzed. Results: The liver: muscle CNR 4 h after probes injection in hepatic fibrosis rats treated with anti-α5β1 integrin mAb-USPIO was much higher than that in normal rats treated with anti-α5β1 integrin mAb-USPIO and fibrosis rats treated with naked USPIO and anti-α5β1 integrin mAb respectively. Abdominal magnetic resonance imaging (MRI) was performed for all rats before and 4 h after administration of the probes. The liver: muscle contrast-to-noise ratio (CNR) of the rats in all 4 groups before and 4 h after probe injection was calculated and analyzed. Results: The liver: muscle CNR 4 h after probes injection in hepatic fibrosis rats treated with anti-α5β1 integrin mAb-USPIO was much higher than that in normal rats treated with anti-α5β1 integrin mAb-USPIO and fibrosis rats treated with naked USPIO (P<0.01). Prussian blue staining and transmission electron microscopy demonstrated that the anti-α5β1 integrin mAb-USPIO probe was specifically engulfed by the activated HSCs in fibrosis rats. Conclusion: In vivo molecular MRI of activated HSCs in early stage of hepatic fibrosis rats was feasible by targeting α5β1 integrin with anti-α5β1 integrin mAb-USPIO probe.

Keywords: Molecular imaging, magnetic resonance imaging, liver fibrosis, hepatic stellate cell, integrin α5β1, ultrasmall superparamagnetic iron oxide

Introduction

With the increasing trend of aging population, chronic liver disease tends to be a severe global health problem. Long-lasting chronic liver injury is likely to lead to liver fibrosis which is characterized by different etiologic forms of chronic liver disease such as hepatitis B or C, non-alcoholic steatohepatitis and so on [1]. Liver fibrosis can be treated due to its reversibility to some extent [2]. Only if diagnosed in early stage and measures be taken promptly, liver impairment in hepatic fibrosis is most likely to resume normal structure and function. On the contrary, liver fibrosis may eventually lead to cirrhosis, the final stage of the disease which may result in a series of complications including refractory ascites, digestive tract bleeding, hepatocellular carcinoma, etc. [3]. Pathological diagnosis by percutaneous liver biopsy has been considered as the gold standard for assessing fibrosis progression, but it is an invasive procedure during which patient compliance and potential complications is a great concern [4]. In addition, clinical application of liver biopsy is partly limited by sampling error and interobserver variation [5]. Serum markers of liver fibrosis are relatively unreliable because of the low sensitivity and specificity [6]. Conventional imaging techniques, including US [7], CT [8] and MR imaging [9] have played an important role in evaluating advanced hepatic
fibrosis and cirrhosis. Nevertheless, they are incapable for detecting early stage of liver fibrosis. More recently, transient elastography [10], magnetic resonance elastography (MRE) [11, 12] and magnetic resonance spectroscopy (MRS) [13, 14] have been studied for their role in staging liver fibrosis, but these techniques are still in their infancy for widespread use clinically. Therefore, a novel strategy is in urgent need which is both noninvasive and reliable for the diagnosis of the early stage of liver fibrosis.

Liver fibrosis is characterized by an extensive accumulation of the extracellular matrix (ECM) and failure to degrade it [1]. The hepatic stellate cells (HSCs), undergoing the transformation from quiescent to activated phenotype called the myofibroblast-like cells when liver damage occurs, are the main source of ECM materials [15]. Thus, HSCs play a fundamental role in the development of liver fibrosis. Over the past decades, HSCs have been studied not only as a prognostic indicator of progression of liver fibrosis but also as an attractive target for antifibrotic therapies due to their pivotal role in the evolution of liver fibrosis [16, 17]. Integrins are a large family of cell surface receptors and are consisted of α and β subunits, which can interact with other cell adhesion molecules and ECM components, and mediate the cell-cell and cell-matrix adhesions. The integrin-mediated adhesions could promote activation, proliferation, differentiation and survival of the HSCs in the process of liver fibrosis [18]. In addition, the expression of integrin α5β1, the fibronectin receptor, was remarkably increased on activated HSCs stimulated by transforming growth factor-β1 (TGF-β1) in fibrotic livers [19]. Furthermore, integrin α5β1 has been reported in the fibrosis of some other organs and tumor target therapy [20]. However, to the best of our knowledge, there is no correlation study regarding the molecular imaging of liver fibrosis using integrin α5β1 as a target.

In recently years, molecular imaging, which is committed to image cell or molecule in the physiological and pathological process in vivo, has been successfully applied in diagnosing tumor, cardiovascular diseases, etc. and monitoring the effect of treatment with the help of SPECT [21], PET [22], MRI [23] and contrast-enhanced Ultrasoundography [24]. Although, molecular imaging possesses the advantage as novel noninvasive method for certain diseases, the key problem remains in how to find a specific biomarker for molecular probe.

In the present study, we prepared anti-α5β1 integrin mAb-USPIO and explored whether this probe could specifically bind to integrin α5β1 expressed on the surface of the activated HSCs in early stage of liver fibrosis.

Materials and methods

Animals

Sixteen seven-week-old male Sprague-Dawley rats (200-220 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China) and fed on standard laboratory rat chow under 12-hour dark/light cycles. This experiment was performed in accordance with the National Institute of Health Policy on the Care and Use of Laboratory Animals and approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University.

Animal model of liver fibrosis

In order to induce liver fibrosis, twelve normal rats were injected subcutaneously with 3 mL/kg of 40% carbon tetrachloride (CCl4) oil solution twice a week for 3 weeks. Four normal rats treated with only pure olive oil served as a control group. All rats were sacrificed immediately after the final MRI scanning.

Synthesis of the anti-α5β1 integrin mAb-USPIO probe

The anti-α5β1 integrin monoclonal antibody (mAb) was purchased from Bioss Inc (Woburn, MA, USA). USPIO (20 nm, surface: COOH) was purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). The conjugation method of anti-α5β1 integrin mAb to USPIO is based on the carbodiimide/N-hydroxysuccinimide (NHS) activation of the carboxylic acid groups on USPIO surfaces followed by reaction with amino groups of the anti-α5β1 integrin mAb, which leads to a random amide bond formation between them [25] (Figure 1). The specific synthesis procedure was operated by Zoonbio Biotechnology Co. Ltd. (Nanjing, Jiangsu, China), who supplied all chemical reagents and devices needed during conjugation.
Molecular MRI of activated hepatic stellate cells

The twelve fibrosis rats were divided randomly into three groups. One group was given anti-α5β1 integrin mAb-conjugated USPIO while the other two groups and four normal rats were given naked USPIO, anti-α5β1 integrin mAb and anti-α5β1 integrin mAb-USPIO respectively for control purpose. MR imaging was performed on a clinical 1.5-T MR scanner (Philips Achieva System, Holland) three days after the final CCl₄ injection to avoid acute effects, with the rats in prone decubitus and Micro-47 coil wrapped around their abdomen. The rats were scanned using a gradient-echo sequence (FFE T2WI) with an echo time of 12 ms and a repetition time of 544 ms. Additional parameters were as follows: slice thickness, 3 mm; field of view, 60×60 mm²; and matrix, 256×256. MR images were obtained before and 4 h after the injection of molecular probes via femoral vein catheter at an iron dose of 100 μmol/kg body weight.

Image analysis

Regions of interest, excluding the major vessels and artifacts, were drawn on the liver parenchyma and adjacent dorsal muscle on the MR image for measuring the signal intensity (SI). Noise of the background was estimated from the standard deviation (SD) of SI in the air adjacent to the rats. Contrast-to-noise ratio (CNR) was calculated as \( \frac{(SI_{\text{liver}} - SI_{\text{muscle}})}{SD_{\text{air}}} \). The software of ViewForum 4.1 (Philips, Holland) was employed for measuring the MR SI.

Histopathological examinations and detection of iron nanoparticles in the HSCs in liver of rats

The rats were sacrificed with deep anesthesia (3.6% chloral hydrate, 1 ml/100 g body weight, intraperitoneally). The liver collected from the rats after MRI scanning was divided into several lumps according to the examinations introduced below. The histopathological examinations were performed with Hematoxylin-eosin and Masson’s trichrome staining using standard staining protocol to score the amount of liver disease according to the method of
Molecular MRI of activated hepatic stellate cells

Ishak fibrosis score [26]. Prussian blue staining and transmission electron microscopy (TEM, Hitachi H-600, Tokyo, Japan) examination were performed according to standard procedures for detecting iron particles of the probe in the liver of rats. A board certified liver pathologist reviewed all slides blindly.

Statistical analysis

All collected data were expressed as mean ± SD. Statistical analysis was carried out with SPSS 17.0 statistical software (Chicago, IL, USA). The CNR before and 4 h after administration of the probes inside each group was compared using paired-samples t-test. Comparisons of the CNR between groups were fulfilled by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test. P<0.05 was considered statistically significant.

Results

Characterization of animal model

After treatment with 40% CCl₄ in rats for 3 weeks, early stage of liver fibrosis was established: five rats with an Ishak score of 1 (Figure 2B) and seven rats with an Ishak score of 2 (Figure 2D). No detectable fibrosis in the control rats was found (Ishak score 0, Figure 2A, 2C).

Molecular MR imaging of activated HSCs in early stage of liver fibrosis

The SI of liver of four groups of rats was similar on unenhanced MR images (Figure 3A-D). After injection of contrast agents, signal decrease of entire liver was apparently visualized in the group of control rats treated with α5β1-USPIO (E) and fibrosis rats treated with USPIO (F). More significant reduction of the SI of the liver is observed in the group of fibrosis rats treated with α5β1-USPIO (G). There is no obvious change of the SI of liver in the group of fibrosis rats treated with anti-α5β1 integrin mAb (H). CR and FR are short for control rats and fibrosis rats respectively.

Table 1. The liver: muscle CNR of four groups of rats comparing pre and post administration of probes by paired-samples t-test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-injection</th>
<th>Post-injection</th>
<th>t value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR α5β1-USPIO</td>
<td>45.37±1.87</td>
<td>175.51±12.49</td>
<td>26.65</td>
<td>0</td>
</tr>
<tr>
<td>FR α5β1-USPIO</td>
<td>44.63±1.61</td>
<td>255.74±18.3</td>
<td>30.32</td>
<td>0</td>
</tr>
<tr>
<td>FR USPIO</td>
<td>44.56±2.08</td>
<td>157.96±13.72</td>
<td>20.38</td>
<td>0</td>
</tr>
<tr>
<td>FR α5β1</td>
<td>44.82±1.39</td>
<td>45.46±8.24</td>
<td>1.45</td>
<td>0.24</td>
</tr>
</tbody>
</table>

CR and FR are short for control rats and fibrosis rats respectively.

Figure 3. MR imaging of liver in control rats and fibrosis rats before and 4 h after injection of specific probes. The signal intensity (SI) of liver in all four groups is similar before injection of probes (A-D). Four hours after injection of probes, signal decrease of entire liver was apparently visualized in the group of control rats treated with α5β1-USPIO (E) and fibrosis rats treated with USPIO (F). More significant reduction of the SI of the liver is observed in the group of fibrosis rats treated with α5β1-USPIO (G). There is no obvious change of the SI of liver in the group of fibrosis rats treated with anti-α5β1 integrin mAb (H). CR and FR are short for control rats and fibrosis rats respectively.
The liver: muscle CNR of four groups of rats was shown in Table 1. There were significant differences between the liver: muscle CNR before injection and that 4 h after injection in three other groups of rats (P<0.01) except the group of fibrosis rats treated with anti-α5β1 integrin mAb (P=0.24). The liver: muscle CNR 4 h after injection of probes had no significant statistic difference between control rats treated with α5β1-USPIO and fibrosis rats treated with USPIO (P=0.094), while there were significant differences between all the other groups (P<0.01) when they were multi-compared with each other by the SNK method (Figure 4).

Distribution of iron nanoparticles of probes in the liver

By Prussian blue staining, the location of iron nanoparticles of probes in the control rats (Figure 5A) and fibrosis rats (Figure 5B) treated with USPIO was similar, mainly situated in the blood sinusoid in the liver in consistent with the location of the Kupffer cells. The iron nanoparticles in the fibrosis rats treated with α5β1-USPIO (Figure 5C) were found distributing more intensively in the perisinusoidal space of Disse in accord with where HSCs was located. TEM proved that there was massive iron nanoparticles in the lysosomes inside the HSCs in the fibrosis rats treated with α5β1-USPIO (Figure 6A). In contrast, the lysosomes, containing iron nanoparticles, were not found in the HSCs in the control rats (Figure 6B) and fibrosis rats treated with USPIO (Figure 6C).

Discussion

To the best of our knowledge, this is the first report of the molecular MR imaging of activated HSCs in rats with early stage of liver fibrosis by targeting hepatic integrin α5β1 in vivo. The histopathological examinations demonstrated that all the fibrosis rats were in the early stage of liver fibrosis according to the criteria of Ishak. We observed that decrease of SI of the entire liver was the most pronounced by treated with anti-α5β1 integrin mAb-USPIO than with naked USPIO in hepatic fibrosis rats and with anti-α5β1 integrin mAb-USPIO in control rats. Furthermore, the activated HSCs in early stage of liver fibrosis could engulf iron nanoparticles of the probe specifically. These findings indicate that molecular targeting imaging of activated HSCs in liver fibrosis can be realized in vivo using MRI via a specific probe.

MRI has played an important role in the diagnosis and staging of liver fibrosis because of its non-invasive, non-ionizing radiation and high spatial resolution. Conventional MRI [9, 27] and double-contrast MRI [28] have been widely applied to evaluate liver fibrosis, but the sensitivity and specificity remain low. Functional MRI, including MRE [29], MRS [30], diffusion tensor imaging (DTI) [31] and perfusion weighted MR imaging (PWI) [32], has shown promising role in detecting fibrosis. However, the parameters used in these methods are non-specific
Molecular MRI of activated hepatic stellate cells

for liver fibrosis and are easily affected by factors such as inflammation, ascites, and liver perfusion. The molecular MRI may be complementary to these above-mentioned MRI techniques and in fact has been tentatively applied in assessing liver fibrosis [33]. Unfortunately, the research on molecular MRI of liver fibrosis is scant up till now. The main reason is that it is difficult to find the biomarker of liver fibrosis and then to synthesize the corresponding targeted probe.

In our study, we prepared anti-α5β1 integrin mAb-conjugated USPIO as the specific probe to target the activated HSCs in rats with early stage of liver fibrosis by combining integrin α5β1 on the surface of the activated HSCs. We then performed MRI for all rats by applying anti-α5β1 integrin mAb-conjugated USPIO, naked USPIO and anti-α5β1 integrin mAb to fibrosis rats as well as applying anti-α5β1 integrin mAb-conjugated USPIO to normal rats. We found that the decrease of SI of the entire liver was similar in normal rats treated with anti-α5β1 integrin mAb-conjugated USPIO (Figure 3E) and fibrosis rats treated with naked USPIO (Figure 3F); significant reduction was observed in the group of fibrosis rats treated with anti-α5β1 integrin mAb-USPIO (Figure 3G); but there was no obvious change in the group of fibrosis rats treated with anti-α5β1 integrin mAb (Figure 3H). We speculate that the iron particles which could cause the decrease of T2 SI of liver could be only devoured by the Kupffer cells in both normal rats treated with anti-α5β1 integrin mAb-USPIO (Figure 5A) and fibrosis rats treated with USPIO (Figure 5B). However, the activated HSCs could specifically engulf the iron particles in fibrosis rats treated with anti-α5β1 integrin mAb-conjugated USPIO by taking anti-α5β1 integrin mAb as the ligand for integrin α5β1 receptor. So the iron particles could...
be taken up not only by the Kupffer cells (Figure 5C) but also mostly by the activated HSCs (Figure 6A) in fibrosis rats. As a result, there was more increase of liver: muscle CNR in hepatic fibrosis rats group treated with anti-α5β1 integrin mAb-conjugated USPIO after injection of probes than that in normal rats treated with anti-α5β1 integrin mAb-USPIO and fibrosis rats treated with naked USPIO (Table 1).

Our study has several limitations. Firstly, there may be some differences in rat liver fibrosis model induced by CCl₄ compared with human hepatic fibrosis caused by different etiology. Most notably, repeated administration of CCl₄ in rats will induce liver fibrosis within a few weeks, whereas patients with liver disease may take several years in the development of liver fibrosis. Secondly, we did not illustrate the correlation between the amount of iron particles engulfed by activated HSCs and stages of liver fibrosis. So, the relationship between amount of iron particles uptake by activated HSCs and the related reduction of signal intensity of the liver in different pathological stages of liver fibrosis needs to be further studied. Thirdly, we did not take any anti-fibrotic modalities for liver fibrosis, thus the efficacy of this specific MR molecular probe in monitoring of therapeutic effects in fibrosis cannot be evaluated.

In conclusion, our study has shown that it is feasible to achieve the specific targeted imaging of activated HSCs in early stage of liver fibrosis in vivo using the specific molecular probe targeting integrin α5β1. This method can be used in diagnosing liver fibrosis in early stage, however, its role in monitoring antifibrogenetic therapeutic effects remains to be further studied.

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Disclosure of conflict of interest

None.

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References

Molecular MRI of activated hepatic stellate cells


