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Molecular Imaging of Hepatic Stellate Cell Activity by Visualization of Hepatic Integrin $\alpha\nu\beta3$ Expression with SPECT in Rat

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The key factors in the pathogenesis of liver fibrosis are the activation and proliferation of hepatic stellate cells (HSCs), which express integrin $\alpha\nu\beta3$ after activation. This study aimed to explore the potential of $^{99m}$Tc-labeled cyclic arginine-glycine-aspartic acid pentapeptide (cRGD) as a single photon emission computed tomography (SPECT) radiotracer to image hepatic integrin $\alpha\nu\beta3$ expression to reflect HSC activity in fibrotic livers. Rat models of liver fibrosis caused by thioacetamide or carbon tetrachloride (CCl4) treatment were employed to examine the expression and distribution of integrin $\alpha\nu\beta3$ during fibrotic progression or regression. The binding activity of radiolabeled cRGD to integrin $\alpha\nu\beta3$ was assessed in liver sections. SPECT was performed to determine hepatic integrin $\alpha\nu\beta3$ expression in rats with different stages of liver fibrosis. Protein and messenger RNA (mRNA) levels of integrin $\nu$ and $\beta3$ subunits were increased with the progression of liver fibrosis and reduced with its regression. The cell type that expressed the majority of integrin $\alpha\nu\beta3$ in fibrotic livers was found to be activated HSCs. The cRGD binding to activated HSCs displayed a high receptor-coupling affinity and an abundant receptor capacity. Iodine-125 ($^{125I}$)-labeled cRGD bound to fibrotic liver sections and the binding activity was the highest in advanced fibrosis. Intravenously administered carboxyfluorescein-labeled cRGD was accumulated in fibrotic liver, and the accumulation amount was increased with the progression and reduced with the regression of fibrosis. A SPECT imaging study with $^{99m}$Tc-labeled cRGD as a tracer demonstrated that the radioactivity ratio of liver to heart increased progressively along with severity of hepatic fibrosis. Conclusion: Hepatic integrin $\alpha\nu\beta3$ expression in fibrotic liver reflects HSC activity and its imaging using $^{99m}$Tc-labeled cRGD as a SPECT radiotracer may distinguish different stages of liver fibrosis in rats. (HEPATOLOGY 2011;54:1020-1030)

Liver fibrosis and its endstage cirrhosis are major world health problems arising from chronic liver injury by a variety of etiological factors, including hepatitis B, hepatitis C, alcohol, etc.1 The prognosis and management of chronic liver disease often depends on the degree of liver fibrosis.2 To date, liver

Abbreviations:  $\alpha$-HSCs, activated hepatic stellate cells;  $\alpha$-SMA, $\alpha$-smooth muscle actin;  BDL, bile duct ligation;  CCl4, carbon tetrachloride;  cRGD, cyclic arginine-glycine-aspartic acid peptide;  ECM, extracellular matrix;  FAM, carboxyfluorescein;  HSCs, hepatic stellate cells;  $^{125}$I, iodine-125;  $^{125}$I-cRGD, $^{125}$I-labeled cRGD;  LB, liver biopsy;  MRAR, the mean radioactivity ratio of liver to heart;  qHSCs, quiescent hepatic stellate cells;  TAA, thioacetamide;  $^{99m}$Tc, technetium-99m;  $^{99m}$Tc-cRGD $^{99m}$Tc-labeled cRGD;  SPECT, single photon emission computed tomography.

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biopsy (LB) has been the only reliable approach of diagnosing and staging liver fibrosis. However, LB is invasive and may cause life-threatening complications; they are rare but do occur. Furthermore, the accuracy of LB for assessing fibrosis also has been controversial because of sampling errors and intra- and interobserver variability that may lead to over- or understaging of fibrotic severity. In this regard, various noninvasive approaches have been developed to assess liver fibrosis, including ultrasound-based transient elastography (Fibroscan) that evaluates liver fibrosis by measuring liver stiffness, and serum markers of liver fibrosis or more sophisticated algorithms or indices combining the results of panels of markers, such as FibroTest. These approaches not only aid physicians to identify patients with liver fibrosis, but also allow to frequently monitor the disease progression and response to therapeutics in a noninvasive fashion. Nevertheless, they display a lower accuracy in detecting earlier stages of fibrosis, although they are valuable in identifying cirrhosis.

The key factors in hepatic fibrogenesis are the activation and proliferation of hepatic stellate cells (HSCs). As a result of sustained or repeated liver injury, HSCs undergo a process of activation and transform into myofibroblast-like cells, which are characterized by α-smooth muscle actin (α-SMA) expression, excessive synthesis of extracellular matrix (ECM) proteins, mainly type I and type III collagen, and an accelerated rate of proliferation. Consequently, activated HSCs (aHSCs) contribute largely to the intrahepatic connective tissue expansion during fibrogenesis. Thus, these cells represent an ideal target for visualization of fibrogenic processes and potential antifibrotic therapies.

Integrins comprise a large family of cell surface receptors, which are composed of two subunits, α and β, and each αβ combination has its own binding specificity and signaling properties. Integrins link the intracellular cytoskeleton with ECM components, thereby playing an important role in cell signaling, cell-to-cell adhesion, apoptosis, and cell-matrix interactions. Among various integrins discovered to date, integrin αvβ3 is the most extensively studied. A common feature of integrins like αvβ3 is that they bind to ECM proteins by way of the three amino acid sequence of arginine-glycine-aspartic acid (RGD). Over the past decade, many radiolabeled cyclic RGD peptides (cRGD) have been developed to be new radiotracers for selectively imaging integrin αvβ3-positive tumors by positron emission tomography (PET) or single photon emission computed tomography (SPECT).

Recently, Patsenker et al. observed that hepatic expression of integrin β3 subunit was markedly upregulated in rats with bile duct ligation (BDL) and correlated with the stage of fibrosis. Additionally, it has been demonstrated that integrin αvβ3 is expressed by HSCs during their activation in vitro and that it promotes HSC proliferation and survival. In the present study we further evaluated hepatic integrin αvβ3 expression and identified the cell type that expressed majority of integrin αvβ3 at different stages of liver fibrosis in rats, then used radiolabeled cRGD as a SPECT radiotracer to image hepatic integrin αvβ3 expression in order to develop a noninvasive approach to monitor HSC activity during fibrotic progression.

### Materials and Methods

**Animals.** Eight-week-old inbred male Sprague-Dawley rats (body weight 200 ± 20 g) were obtained from the Laboratory Animal Research Center of Fudan University (Shanghai, China) and fed standard laboratory rat chow on a 12-hour light/dark cycle with free access to water and food. The study was approved by the Institutional Ethical Committee of Animal Experimentation and all experiments were performed strictly according to governmental and international guidelines on animal experimentation.

**Synthesis and Radiolabeling of cRGD.** cRGD (cyclo[Arg-Gly-Asp-D-Phe-Lys], cRGDfK) was synthesized at the Fudan-Pharmaco Targeting Drug Research Center, Fudan University (Shanghai, China) as described. The synthesis of carboxyfluorescein-labeled cRGD (FAM-cRGD) was carried out by mixing the resin containing the cyclic peptides with preactivated 5-FAM (Sigma-Aldrich, Hong Kong, China) for 3 hours before cleaving.

cRGD was labeled with iodine-125 (125I) by dissolving the peptide (20 µg) into [125I]NaI (37 MBq) (PerkinElmer, Hong Kong, China) in a 1.5-mL polypropylene vial coated with 100 µg of iodogen as described. To label cRGD with technetium-99m (99mTc), 20 µg of cRGD was added to 1.0 mL of Na[99mTcO4] solution (10 µCi) (Shenke Medicinal, Beijing, China) as described. Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed to examine the purity of cRGD and its derivatives. Electrospray ionization mass spectrometry (ESI-MS) analysis was conducted to examine the molecular weight of final products.

**Isolation and Culture of Hepatocyte and HSCs.** HSCs and hepatocytes (HCs) were isolated from rats (450-550 g) by two steps of collagenase...
digestion. Primary rat HSCs cultured for 3 or 7 days after isolation (referred to as day-3 or day-7 HSCs) and primary HC cultured for 24 hours after isolation were used for further experiments. Human umbilical vein endothelial cells (provided by Chinese Academy of Sciences Shanghai Branch) were used as a control.

**Fluorescence Trace of FAM-cRGD in Cells.** Day-3 HSCs, day-7 HSCs, and HCs were first incubated respectively with a solution of 10 μmol/L cRGD, a solution of 10 μmol/L FAM-cRGD, or a mixed solution containing 10 μmol/L FAM-cRGD and 150 μmol/L cRGD for 45 minutes at 37°C in the dark. After incubation, these cells were washed by centrifugation at 4°C. Cell nuclei were stained with 6-diamidino-2-phenylindole (DAPI) (1:2,000) and examined with Zeiss FISH (fluorescent in situ hybridization) Imager system (Axioskop2 and Axiosvert100).

**Flow Cytometry Analysis.** To assess the binding characteristics of cRGD on HSCs and HC, day-3 HSCs, day-7 HSCs, and HCs were incubated with 125I-cRGD solutions at different concentrations (100-15,000 pmol/L) in a final volume of 0.5 mL for 3.5 hours at 4°C in the dark. Nonspecific binding was measured in the presence of 100 nmol/L FAM-cRGD, or a mixed solution containing 10 μmol/L FAM-cRGD and 150 μmol/L cRGD for 45 minutes at 37°C. After incubation, these cells were washed by centrifugation at 1,776g for 15 minutes and analyzed by FACS scan flow cytometry (FACSCalibur) with CellQuest software (BD Biosciences, Franklin Lakes, NJ).

In order to assess the binding efficiency of cRGD at different concentrations and different incubation durations to aHSCs, day-7 HSCs were incubated respectively with FAM-cRGD at concentrations of 0.04, 0.2, 1, 5, 25, and 125 μmol/L for 45 minutes, or with 2 μmol/L FAM-cRGD solution for 15, 30, 45, 60, 75, and 90 minutes at 37°C in the dark. After incubation, these cells were washed by centrifugation and analyzed.

**Radioligand Binding Analysis.** Day-7 HSCs were incubated with 125I-cRGD solutions at different concentrations (100-15,000 pmol/L) in a final volume of 0.5 mL at 3.5 hours at 4°C in the dark. Nonspecific binding was measured in the presence of 100 nmol/L cRGD. Radioactivity in cell pellets was determined with a gamma-counter (Wallac 1470-002, PerkinElmer, Finland). Bound ligand was calculated by deduction of the nonspecific radioactivity from the total radioactivity of the ligand. According to the Scatchard plot, the binding constant (Kd) and the maximum binding content (Bmax) of 125I-cRGD were calculated.

**Animal Models of Liver Fibrosis.** In order to induce liver fibrosis, rats were administered thioacetamide (TAA) (0.2 g/kg) intraperitoneally every Tuesday and Friday. Three weeks or 9 weeks after the treatment, treated rats were used for further experiments (referred to as TAA-3w and TAA-9w rats). Rats treated with sodium chloride served as a control group.

**Analyses of Liver Fibrosis.** Liver sections were stained with hematoxylin and eosin (H&E) and Sirius red. Extent of liver fibrosis was staged by an experienced histologist who was blind to the treatment protocol according to the Ishak staging criteria. Fibrosis was categorized as mild fibrosis (Ishak score ≤2) and advanced fibrosis (Ishak score ≥3). For morphometric analysis of liver fibrosis, 10 fields (100×) from each section were randomly selected and recorded. The Sirius red staining (fibrotic) areas were measured using a computer-aided manipulator (KS400, Carl Zeiss Vision, Germany).

In addition, liver hydroxyproline content and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using assay kits (JianCheng, Nanjing, China) according to the manufacturer’s instructions.

**Immunofluorescent Colocalization of Integrin αvβ3 in Various Cell Types of Fibrotic Livers.** Immunofluorescent staining was performed to reveal the colocalization of integrin αvβ3 with α-SMA (aHSCs), albumin (HC), CD31 (vascular endothelial cells), CD68 (macrophages), and CD163 (Kupffer cells) in the liver sections. There is no specific antibody against rat integrin αvβ3 available. To date, the majority of β3 has been shown to bind to αv (αvβ3) or αIIb (αIIbβ3), and the latter is a membrane receptor expressed only in cells of megakaryocytic lineage and some tumor cells. Hence, evaluating positive immunofluorescent staining of the β3 subunit represents the positivity of integrin αvβ3. Primary antibodies against polyclonal anti-β3 integrin (1:200; Chemicon, Billerica, MA), monoclonal anti-SMA (1:400; Chemicon), polyclonal anti-albumin (1:50; AbD Serotec, Oxford, UK), monoclonal anti-CD31 (1:50; AbD Serotec), monoclonal anti-CD68 (1:50; AbD Serotec), and monoclonal anti-CD163 (1:50; AbD Serotec) were used. Secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated IgG (1:200) and Cy3-conjugated IgG (1:200). 0.2% Triton X-100 was used for permeabilization when appropriate. DAPI was used for nuclear counterstaining. Multicolored fluorescent staining of liver sections was analyzed by confocal laser scanning microscopy (Leica Microsystems, Wetzlar, Germany). The fluorescent signals of liver sections were video-digitized and analyzed with a software program that automatically outlined the total stained areas with threshold setting (Photoshop 4.0; Adobe). These
areas were then quantified with NIH Image 1.62 software and the percentage of the merged yellow color region to the total integrin zv/β3-stained green region in each section was calculated. Ten randomly selected amplifying fields (400×) in each section were assessed.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis. The hepatic messenger RNA (mRNA) levels of zv, β3 integrin subunits and z-SMA were quantitated using qRT-PCR analysis as described.26 All PCR primers (Table 1) were designed by Primer Premier 5.0 using published rat gene sequences obtained from the National Center for Biotechnology Information database.

Western Blot Assay. The hepatic protein amount of rat zv, β3 integrin subunits and z-SMA was determined by western blot analysis as described.18

Historadioautographic Visualization. The liver sections of TAA-treated or control rats (n = 8 per group) were used to visualize 125I-cRGD binding to livers as described.27 In brief, the liver sections were incubated in Tris-HCl buffer containing 100 pmol/L 125I-cRGD at 4°C for 24 hours. At the same time, the parallel sections were incubated in the buffer mixed with 100 pmol/L 125I-cRGD and 5 μmol/L cRGD to verify whether the excess cRGD would block the binding of 125I-cRGD in liver sections. After incubation, radioautographic films (Amersham, Buckinghamshire, UK) were exposed to labeled sections. After exposure and developing, the films were scanned with an automatic imaging analyzer and the relative absorbance of hepatic historadioautography was measured. The grayscale of films represented the binding amount of 125I-cRGD in liver sections.

SPECT Imaging. SPECT imaging was performed in control rats and TAA-treated rats (n = 3 per group). Each animal was administered 6 μCi of ⁹⁹mTc-cRGD by way of the penile vein. Animals were placed supine on a SPECT meter (Philips IRIX, Best, Netherlands). Anterior images were acquired 15, 30, and 45 minutes after the injection and stored digitally. Then a computer-aided manipulator discriminated the region of interest in the liver and heart and the radioactivity ratio (counts/pixel) of liver to heart was calculated.

Results

Organ Distribution Studies. A tracer dose (6 μCi) of 125I-cRGD was intravenously administrated to control rats and TAA-treated rats (n = 3 per group). Additionally, 6 μCi 125I-cRGD was also administered simultaneously with excessive unlabeled cRGD (500-fold high dosage of 125I-cRGD) (n = 3 per group). Blood samples were collected by heart puncture 45 minutes after dosage and the organs and tissues were collected, washed in saline, and weighed. Subsequently, radioactivity in the samples was determined by a gamma-counter. The total radioactivity per organ was calculated and corrected for the blood-derived radioactivity. The organ accumulation of 125I-cRGD was calculated as a percentage of the injected dose per gram of wet tissue mass (%ID/g).

Statistical Analysis. All collected data were expressed as mean ± standard deviation (SD). Comparisons between groups were achieved by one-way analysis of variance tests (ANOVA) followed by post-hoc tests with SPSS 11.5 statistical software (Chicago, IL) and P < 0.05 was considered statistically significant.

Table 1. Primer Sequences Designed for Quantitative Real-Time PCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
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<tbody>
<tr>
<td>zv</td>
<td>GCTGACCAAGAGAGAAGAATC</td>
<td>ACACCCCAAGTGTAAACACATCTTC</td>
</tr>
<tr>
<td>β3</td>
<td>CTGAGGACACGTCGAGAAATG</td>
<td>TCTCAGACTCCCAACAGCTTCTTCTT</td>
</tr>
<tr>
<td>z-SMA</td>
<td>GCTGCCATCTGGCTCTTCATC</td>
<td>GGGAGCTTCTGACATC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGDCAGCCAGAGAAATC</td>
<td>CCAGTGACCTTCGGTCAG</td>
</tr>
</tbody>
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of advanced fibrosis. As shown in Figs. 2, 3, the positive staining of integrin αvβ3 was mainly overlapped with α-SMA staining (Fig. 2B). The percentage of overlapped areas (yellow) of integrin αvβ3 staining (green) with α-SMA positive-staining (red) was as high as 69.77 ± 8.41%, which was markedly higher than the overlapped staining with albumin (3.70 ± 1.69%, Fig. 2A), CD31 (17.67 ± 5.20%, Fig. 2C), CD68...
(8.20 ± 0.69%, Fig. 3A), and CD163 (2.10 ± 0.90%, Fig. 3B) \((P < 0.05\) for all comparisons, Fig. 3C). Because \(\alpha\)-SMA is thought to be the marker of aHSCs, cardinal cells expressing integrin \(\alpha v \beta 3\) in the liver sinusoid areas with advanced fibrosis are considered aHSCs. In livers with mild fibrosis, cardinal cells
expressing integrin \(\alpha v\beta 3\) were also found to be \(\alpha HSCs\) (data not shown). Therefore, the findings confirm that the majority of integrin \(\alpha v\beta 3\) is expressed in \(\alpha HSCs\), and much less \(\alpha v\beta 3\) is expressed in parenchymal cells and other nonparenchymal cells.

**Binding Characteristics of cRGD with HSCs In Vitro.** Day-3 HSCs displayed a quiescent phenotype (qHSCs), and were negative for \(\alpha\)-SMA staining. After being cultured for 7 days, HSCs transformed into an activated cell type (aHSCs) and were positive for \(\alpha\)-SMA staining (data not shown). The cRGD binding features were characterized as follows.

At first, the binding of FAM-cRGD to qHSCs, aHSCs, and HC was assessed. FAM-cRGD was uptaken by aHSCs, not by qHSCs or HC (Fig. 4A). Fluorescent intensity of qHSCs incubated with 10 \(\mu\)mol/L unlabeled cRGD was higher than that of aHSCs \((P < 0.05)\), which indicated that there was higher fluorescent background in qHSCs. However, after being incubated with 10 \(\mu\)mol/L of FAM-cRGD for 45 minutes, the fluorescent intensity of qHSCs did not increase. In contrast, the fluorescent intensity of aHSCs increased up to nearly 3-fold compared to qHSCs. When aHSCs were incubated with the mixed solution containing FAM-cRGD and excess cRGD for 45 minutes, the increase in fluorescent intensity was abrogated in aHSCs (Fig. 4B). There was no marked change in fluorescent intensity of HC after culture with FAM-cRGD.

Second, when aHSCs were incubated with FAM-cRGD in a series of increasing concentrations for 45 minutes their fluorescent intensity was accordingly increased to 1.0 to 11.1-fold. In addition, when aHSCs were incubated with 2 \(\mu\)mol/L of FAM-cRGD for 15 to 90 minutes a 1.3 to 4.5-fold increase in fluorescent intensity was noted accordingly (Fig. 4C).

Lastly, \(125^I\)-cRGD was used to further assess the binding characteristics of cRGD with aHSCs. According to the Scatchard plot, the Kd was \(4.808 \times 10^{-9}\) mol/L and Bmax was \(2.112 \times 10^{-10}\) mol/L, which indicated that the binding of synthetic cRGD to aHSCs displayed a high receptor-coupling affinity and that there was an abundant receptor capacity in aHSCs (Fig. 4D).

**Historadioautographic Visualization of \(125^I\)-cRGD in Liver Sections.** Hepatic radioautographic visualization of \(125^I\)-cRGD was determined. The hepatic relative densitometry of exposed films from fibrotic rats was significantly higher than that of control rats \((P < 0.05)\) and was the highest in rats with advanced fibrosis \((P < 0.05)\). When the hepatic sections were incubated with the mixed solution of \(125^I\)-cRGD and excess cRGD, the relative hepatic absorbance in control rat liver did not change, whereas it was significantly reduced in fibrotic rats \((P < 0.05)\) (Fig. 5).

**SPECT Imaging of \(99^mTc\)-Labeled cRGD In Vivo.** We further assessed hepatic expression of integrin \(\alpha v\beta 3\) in TAA-treated rats and control rats with SPECT imaging. \(99^mTc\)-labeled cRGD was used as a SPECT imaging tracer. After intravenous administration, \(99^mTc\)-labeled cRGD was gradually distributed to organs and tissues. The mean radioactivity ratio of liver to heart (referred to as MRAR) in fibrotic rats and control rats gradually increased over time. Thirty minutes after intravenous administration, MRAR in rats with advanced fibrosis was higher than that in control rats and rats with mild fibrosis \((P < 0.05)\), but there was no significant difference between rats with mild fibrosis and control rats \((P = 0.17)\). Forty-five minutes after intravenous administration, MRAR in fibrotic rats was significantly higher than that in control rats, and the highest was seen in rats with advanced fibrosis \((P < 0.05)\) (Fig. 6).

**Organ Distribution of \(125^I\)-cRGD.** The biodistribution of cRGD was studied in control rats and TAA-treated rats \((n = 3\) per group) at 45 minutes after \(125^I\)-cRGD administration. \(125^I\)-cRGD was mainly present in the kidneys and the livers of control rats and TAA-treated rats, and little accumulated in the spleen, heart, lungs, and muscles. The accumulation amount of \(125^I\)-cRGD in the livers of fibrotic rats was higher than that in control rats \((P < 0.05)\), but there was no significant difference between rats with mild fibrosis and those with advanced fibrosis. In the kidneys of rats with advanced fibrosis, the accumulation amount of \(125^I\)-cRGD was lower than that in the other two groups \((P < 0.05)\). There was no significant difference in the accumulation amount in other organs and tissues between treated and nontreated rats (Fig. 7A).

After \(125^I\)-cRGD was injected simultaneously with excess unlabeled cRGD, the hepatic accumulation amount of \(125^I\)-cRGD was reduced in rats with mild fibrosis \((P = 0.059)\) and in rats with advanced fibrosis \((P = 0.013)\). There was no significant change in the liver of control rats and in other organs and tissues of three groups (Fig. 7B).

**Discussion**

For the past several years, many high-affinity integrin \(\alpha v\beta 3\) antagonists (RGD-containing cyclic peptides and nonpeptide RGD mimetics) have been proposed as targeting biomolecule carriers to deliver the diagnostic "probes" into the integrin \(\alpha v\beta 3\)-positive tumors.
In this study, we confirmed that integrin αvβ3 expression in fibrotic livers of rats treated with TAA was significantly increased compared to that in the normal livers, and was the most significantly increased in advanced fibrosis. We also determined the hepatic integrin αvβ3 expression in fibrotic rats induced by BDL (data not shown), which was similar to those reported by Patsenker et al. The pathogenesis of liver fibrosis induced by TAA treatment and BDL treatment is different. The former represents as entire lobular fibrosis, whereas the latter as secondary cholestatic fibrosis. The pathologic feature of liver fibrosis caused by chronic CC14 intoxication is similar to TAA, and the findings in the Supporting Information showed a significant increase in hepatic integrin αvβ3 expression after 8 weeks of CC14 intoxication. The findings in three models of rat liver fibrosis...
consistently demonstrated that hepatic integrin \( \alpha \nu \beta 3 \) expression is increased along with the development and progression of liver fibrosis. In addition, when liver fibrosis regressed, the hepatic expression level of integrin \( \alpha \nu \beta 3 \) was reduced, which was documented in the rat model induced by CCl4 treatment (Supporting Fig. 1). Thus, these findings provide convincing evidence that hepatic integrin \( \alpha \nu \beta 3 \) expression correlated well with the degree of liver fibrosis. In the present study, serum ALT and AST levels, which were used to reflect hepatic inflammation, were not correlated with hepatic integrin \( \alpha \nu \beta 3 \) expression in models of liver fibrosis induced by either TAA or CCl4 treatment.

In addition to HSCs and some tumor cells, integrin \( \alpha \nu \beta 3 \) was reported to be expressed in endothelial cells and inflammatory cells, especially monocytes and macrophages.\(^{28-30}\) In the present study we demonstrated that positive integrin \( \alpha \nu \beta 3 \) staining in fibrotic livers was essentially overlapped with positive \( \alpha \)-SMA staining, an indicator of aHSCs. By comparing the percentage of overlapped integrin \( \alpha \nu \beta 3 \) staining with markers of various cell types in the liver, integrin \( \alpha \nu \beta 3 \) expressed in parenchymal cells and other nonparenchymal cells was shown to be significantly lower than that in \( \alpha \)-SMA-positive cells, which was as high as \( \approx 70\% \). Thus, we conclude that the major cell type expressing integrin \( \alpha \nu \beta 3 \) in fibrotic livers is aHSCs. Hepatic \( \alpha \)-SMA expression was found to be increased or reduced with the progression or regression of fibrosis, which correlates well with the degree of liver fibrosis and expression of integrin \( \alpha \nu \beta 3 \). In this context, it is convincing that the visualization of hepatic integrin \( \alpha \nu \beta 3 \) expression reflects the activity of aHSCs, which represent an ideal target for monitoring fibrogenic process.

After culturing with FAM-cRGD, aHSCs, but not qHSCs or HC, took up FAM-cRGD, and the uptake rate was partially inhibited by excess unlabeled cRGD. These findings indicate that the synthetic cRGD, which specifically binds to integrin \( \alpha \nu \beta 3 \) receptors, was taken up largely by aHSCs. In addition, the binding of FAM-cRGD to aHSCs was increased along with prolonged culture duration and with an increased concentration of FAM-cRGD, which implies that the...
binding was time-dependent and concentration-dependent. Our radioligand binding assay further demonstrated that the binding of synthetic cRGD to aHSCs displayed a high receptor-coupling affinity and an abundant receptor capacity. After incubation with $^{125}$I-cRGD, there was more $^{125}$I-cRGD accumulation in fresh hepatic sections from fibrotic rats than those from the control rats, and the sections from rats with advanced fibrosis had the highest coupling activity. Excess unlabeled cRGD did not block the accumulation of $^{125}$I-cRGD in the sections from the normal control rats, but significantly reduced the accumulation in the sections from the fibrotic rats. More important, after intravenous injection FAM-cRGD was found to accumulate in fibrotic livers and the accumulation amount was increased with the progression of liver fibrosis and reduced with the regression of liver fibrosis (Supporting Fig. 2). Taken together, these results provide convincing evidence that visualizing hepatic expression of integrin $\alpha_v\beta_3$ could distinguish HSC activity in different stages of liver fibrosis.

In the organ distribution study of $^{125}$I-cRGD, we found that the predominant excretion pathway of synthetic cRGD was through kidneys and hepatobiliary routes in both control and fibrotic rats, and that there was minimal accumulation in other organs 45 minutes after intravenous administration, indicating that the in vivo retention of radionuclide was minimal. The hepatic accumulation of $^{125}$I-cRGD in rats with liver fibrosis was higher than that in control rats and administration of excessive unlabeled cRGD reduced $^{125}$I-cRGD accumulation in fibrotic livers, especially in advanced fibrosis, which indicated that the accumulation of $^{125}$I-cRGD in fibrotic liver was interfered by competing for the receptors with unlabeled cRGD.

Given the fact that synthesized cRGD was mainly excreted through the renal and hepatobiliary routes, the image of $^{99m}$Tc-labeled cRGD in livers by SPECT modality would inevitably be interfered by the shadow of the kidneys, especially the right kidney neighboring the liver. In normal rats, the highest basal expression of $\beta_3$ integrin mRNA was found in liver and less was found in heart. In our biodistribution study, accumulation of $^{125}$I-cRGD in the heart did not change markedly in fibrotic rats compared to control rats. Therefore, the accumulation of cRGD in the heart was relatively invariant in fibrotic rats in comparison to control rats, and MRAR could be considered a valuable index to reflect the relative binding amount of $^{99m}$Tc-labeled cRGD in the liver. MRAR was significantly increased in rats with liver fibrosis compared to that in control rats. It was the highest in the rats with advanced fibrosis, whereas the biodistribution study showed that there was the least $^{125}$I-cRGD accumulation in the kidneys from these rats with advanced fibrosis. Furthermore, in the organ distribution study it was evident that there was no significant difference in $^{125}$I-cRGD accumulation in kidneys between the rats with mild fibrosis and the control rats, whereas MRAR in the mild fibrosis was significantly higher than that in normal livers. Based on these findings, we overcame the disturbance of renal visualization to hepatic visualization in imaging integrin $\alpha_v\beta_3$ expression with SPECT imaging by comparing MRAR in rats, although further studies are needed to further improve this imaging modality.

In conclusion, the findings in the present study demonstrate that enhanced expression of integrin $\alpha_v\beta_3$ in fibrotic liver reflects the activity of activated HSCs and that the expression levels correlate with fibrotic progression and regression. SPECT imaging using
99mTc-labeled cRGD as a radiotracer may noninvasively distinguish different stages of liver fibrosis, which implicates a potential value in monitoring HSC

References


