Cyclic RGD Peptide-Labeled Liposomes for Targeting Drug Therapy of Hepatic Fibrosis in Rats

Shi-Lin Du, Hong Pan, Wei-Yue Lu, Jian Wang, Jian Wu, Ji-Yao Wang

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, China (S-L.D., J.W., J-Y.W.); Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai 200032, China (H.P., W-Y.L.); and Department of Internal Medicine, Transplant Research Program, University of California, Davis Medical Center, Sacramento, CA 95817, USA (J.W.)
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Targeting drug delivery to hepatic stellate cells

Corresponding author:
Prof. Ji-Yao Wang
Dept. of Internal Medicine
Division of Gastroenterology & Hepatology
Zhongshan Hospital, Fudan University
180 Fenglin Road
Shanghai, 200032, China
Tel. 86-21-64041990 (Ext. 2117), Fax: 86-21-64432583
Email: jiyao_wang@hotmail.com

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Abbreviations used in the text:
AGA = Arg-Gly-Arg; ALT = alanine aminotransferase; BDL = bile duct ligation; Chol = cholesterol; cAGA = cyclic AGA peptide; cRGD = cyclic RGD peptide; EPC = egg phosphatidylcholine; HSC = hepatic stellate cells; IFN = interferon-α1b; PEG = polyethylene glycol; RGD = Arg-Gly-Asp; RT-PCR = reverse transcriptase polymerase chain reaction; SSL = sterically stable liposomes.
Abstract

Targeting hepatic stellate cells (HSC) has been challenging due to the lack of specific receptors or motifs on the cells. The aim of the present study was to develop a HSC-specific system for improving drug delivery to HSC. **Methods:** The affinity of a cyclic peptide containing Arg-Gly-Asp (cRGD) to collagen type VI receptor on HSC was examined in both *in vitro* and *in vivo* experiments. Sterically stable liposomes (SSL) were modified with this peptide to yield a new carrier, cRGD-SSL. The targeting efficiency of this carrier in delivering interferon-α1b (IFN) was investigated in a rat model of liver fibrosis induced by bile duct ligation (BDL). **Results:** When incubating HSC or hepatocytes with cyclic RGD peptide, the peptide was bound preferentially to activated HSC. Biodistribution study showed that the accumulation of cRGD peptide-labeled liposomes in HSC isolated from BDL rats was 10-fold more than un-labeled SSL. BDL rats receiving injections of IFN-α1b entrapped in cRGD-SSL exhibited significantly reduced extent of liver fibrosis compared to BDL control rats or BDL rats treated with IFN-α1b entrapped in SSL. **Conclusions:** cRGD-SSL is an efficient drug carrier which selectively targets activated HSC, and improves drug therapy for liver fibrosis to a significant extent. This liposomal formulation represents a new means of targeting drug carrier for the treatment of liver fibrosis, and may have potential clinical applications.
Introduction

Liver fibrosis is a chronic disorder characterized by the disposition of a large amount of extracellular matrix (ECM) components, such as collagens. The fibrogenic process is initiated by the concerted action of many cell types and is regulated by many mediators (Friedman, 1999; Li and Friedman, 1999). Viral infection, drug or alcoholic toxicity, and cholestasis lead to damage to hepatocytes, and in turn activate resident nonparenchymal cells, such as Kupffer cells, sinusoidal endothelial cells (SEC) and hepatic stellate cells (HSC) or infiltrating inflammatory cells. HSC are the major cell population contributing to accelerated ECM production (Reeves and Friedman, 2002). Moreover, activated HSC produce an array of mediators that perturb the fibrotic process independently from the activity of Kupffer cells, SEC, and inflammatory cells (Friedman, 1999; Li and Friedman, 1999; Reeves and Friedman, 2002). Thus, HSC are an essential target for the development of therapeutic strategies that are aimed to interfere with the activation of this cell type (Wu and Zern, 2000); selectively targeting this cell type may be beneficial for the treatment of liver fibrosis.

Due to the fact that the relative less HSC exist in the liver, and that there is the lack of specific receptors or motifs on the cell surface, the attempt to target HSC has been a challenging task. Only few studies focusing on targeting HSC have been reported (Beljaars et al., 2002). These include human serum albumin modified with mannose 6-phosphate (Beljaars et al., 1999), or with a cyclic peptide that recognizes the collagen type VI receptor (Beljaars et al., 2000), and a dominant-negative soluble platelet-derived growth factor-β receptor (Borkham-Kamphorst et al., 2004) which inhibits HSC proliferation. However, the
carrying capacity and clinical applicability of these studies are questionable. Therefore, there is a tremendous demand to develop approaches which improve drug therapy for the disorder.

IFN-α is the most common and effective agent for the treatment of viral hepatitis C (Strader et al., 2004). It has been shown that in IFN-α-treated patients, along with inhibition of hepatitis C virus replication and improvement of liver injury, there is significant improvement in the inhibition of progression of hepatic fibrosis (Poynard et al., 2002). In order to investigate whether IFN-α itself has any antifibrotic effects, we previously reported that IFN-α given by subcutaneous administration was effective in reducing gene expression and deposition of collagen type I and III in a rat model of carbon tetrachloride (CCl₄)-induced liver fibrosis (Zhang et al., 1999). However, IFN-α has many adverse effects, such as anemia, flu-like syndrome, etc. Many patients cannot tolerate these side effects, and discontinue the treatment. We hypothesize that using liposome-mediated targeting delivery of IFN-α may improve the therapeutic effects and at the same time reduce its adverse effects. We report here that we have developed a liposomal carrier modulated by the labeling with a cyclic eight amino acid residue peptide containing Arg-Gly-Asp (RGD) and recognizing collagen type VI receptors, which are upregulated in activated HSC of fibrotic livers. Our in vivo experiments demonstrate that the delivery of interferon-α1b (IFN) to HSC with this cyclic RGD peptide-labeled liposomal carrier improved the efficacy of this medication in the treatment of liver fibrosis.
Methods

Chemicals and reagents

Reagents were obtained from following sources: egg phosphatidylcholine (EPC), cholesterol (Chol), methoxy (polyethylene glycol) (2000) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (mPEG2000-DOPE) and N-hydroxysulfosuccinimide-polyoxyethylene-maleimide (NHS-PEG-MAL) from Avanti Polar Lipids, Alabaster, AL; Sephadex G-50 and Sepharose CL-4B from Pharmacia-LKB Ins, Uppsala, Sweden; Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS) from Invitrogen Corp. Carlsbad, CA; cell culture plates from Corning Corp. New York, NY, USA. All other chemicals were of analytical grade.

Cell culture

HSC and hepatocytes used for in vitro experiments were isolated from normal male Wistar rats by two-steps of collagenase digestion as we previously reported (Zhu et al., 1999). HSC purity, as estimated by the autofluorescence of the cells under an ultraviolet-excited fluorescence microscope, was nearly 95%. Cells were seeded on uncoated plastic culture dishes, incubated in DMEM supplemented with 10% FBS (v/v), 100 U/ml penicillin and streptomycin, and maintained at 37°C in a humidified incubator (90% humidity) containing 5% CO₂. Primary rat HSC at a density of $1 \times 10^4$ cells/cm² attained confluence at approximately 6 days as a monolayer of closely apposed polygonal cells. The cells at confluence were used between three and five passages. By that time of culture, HSC were
free of Kupffer cells and SEC.

**Preparation of MAL-PEG-DOPE**

The synthesis of maleimide-PEG-2000-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (MAL-PEG\textsubscript{2000}-DOPE) was conducted as described previously (Allen et al., 1995). DOPE was added to a NHS-PEG-MAL solution at a ratio of 1:0.2 in chloroform containing triethylamine. The reaction mixture was stirred at 25°C for 6 hrs. After thin layer chromatography in CHCl\textsubscript{3}/CH\textsubscript{3}OH/H\textsubscript{2}O (65:25:4) showed disappearance of NHS-PEG-MAL with appearance of a more polar material, the solvent was evaporated. And then 5 ml of acetonitrile was added, and the mixture was kept at 4°C for overnight. After centrifuge at 5000 rpm for 10 min, the upper clear solution was collected and evaporated. The product was dried in vacuum for overnight, and verified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometrical (MALDI-TOF-MS) analysis.

**Synthesis of cyclic RGD peptide and preparation of sterically stable liposomes (SSL)**

The sequence of cyclic RGD peptide (Cys-Gly-Arg-Gly-Asp-Ser-Pro-Lys or C*GRGDSPK*) was selected based on its cell adhesion mediated by collagen VI (Beljaars et al., 2000). We changed the sequence of the reported peptide (C*GRGDSPC*) by replacing cysteine with lysine. The cyclic RGD and AGA (C*GAGASPK*) peptides were synthesized at a purity of 95% as determined by solid-phase synthesis and labeled with fluorescein isothiocynate (FITC). Cyclic RGD peptide was linked via a sulfhydryl group at the cysteine residue to a liposomal formulation, as shown in Fig. 1.

Lipids composed of EPC/Chol/mPEG\textsubscript{2000}-DOPE/MAL-PEG-DOPE in a molar ratio of 2:1:0.1:0.02 were dissolved in chloroform and the solvent was evaporated to form a lipid
film under reduced pressure. The lipid mixture was hydrated in an appropriate buffer and extruded through double layers of polycarbonate membranes in 100 nm open mesh for 15 times using a mini-extruder to obtain a homogeneous liposome suspension (Wu et al., 1998a). For loading IFN, IFN-α1b was dissolved in PBS, and lipid mixture was hydrated in PBS containing IFN. Resulting liposomes were passed through a Sepharose CL-4B column to remove free IFN-α1b. For labeling with cyclic RGD peptide with IFN-loaded liposomes (SSL-IFN), liposome suspension was incubated with cyclic RGD peptide at a molar ratio of 10 to 1 overnight (Blume et al., 1993; Dubey et al., 2004). Unbound cyclic RGD peptide was separated by passing through a Sepharose CL-4B column. For the liposomal formulations used in this study, SSL-IFN refers to liposomes entrapping IFN-α1b; RGD-SSL-IFN refers IFN-α1b encapsulated in SSL labeled with cyclic RGD peptide. The diameter of liposomes was determined by dynamic light scattering (Nicomp 380 ZLS, Particle Sizing Systems, Santa Barbara, CA). The entrapping rate of IFN-α1b was determined after Sepharose CL-4B filtration to separate the lipid fraction and entrapped IFN-α1b, and then using high performance lipid chromatography and enzyme-linked immuno-sorbent assay to measure the content of lipids and IFN-α1b in the RGD-SSL-IFN-α1b, respectively. The entrapping rate was calculated according to the formula: Entrapping rate = Entrapped IFN-α1b / total IFN-α1b in solution × 100%. The carrying capacity (CC) of RGD-SSL was determined according to an equation: CC = W_E/W_LN × 100%. Here W_LN refers to total weight of lipids. W_E refers to the weight of the entrapped substance. The morphology of cyclic RGD peptide-labeled liposomes was examined by a transmission electronic microscope (Hitachi 7000) after being stained with 2% phosphotungstic acid and dried on carbon-coated grids,
and the size of liposomes was verified at the same time.

**Binding of cyclic RGD peptide to HSC**

FITC-conjugated cyclic RGD or AGA peptides were incubated at concentrations of 20-1000 nM with quiescent or activated HSC (1×10^6 cells/well) at either 4°C for 1 hour or 37°C for 1-4 hours. In competition experiments, the cells were pre-incubated with non-FITC-conjugated cyclic RGD peptide (20-100,000 nM) at 4°C or 37°C for 30 min, and then with FITC-conjugated cyclic RGD peptide (200 nM). After the incubation, the cells were washed three times with cold phosphate-buffered saline (PBS, pH 7.4). The FITC-positive cells were counted by flow cytometry, and expressed as percentage of counted cells.

In separate experiments, cells were plated at 2×10^6 cells/well in 24-well plates, and incubated with FITC-conjugated cyclic RGD or AGA peptides at 200 nM for 1 hour. After washing three times with PBS and staining by 4',6-diamidino-2-phenylindole (DAPI), the cells were visualized with a TCS-SP2 laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany), using an ultraviolet laser with emission at 488 nm and 372 nm for scanning. Cells were optically sectioned and digital images were acquired. All instrumental parameters pertaining to fluorescence detection and image analyses were held constant to allow sample comparison.

**Inhibition of HSC proliferation by IFN**

**In vitro** inhibition of HSC proliferation by free IFN or IFN in various liposomal formulations was determined using an MTT proliferation assay. Briefly, HSC at 8×10^5/ml were seeded in 96-well plates and incubated with free IFN (IFN-α1b), SSL-IFN or
RGD-SSL-IFN. After the cells were incubated for 2, 24 or 48 hrs under the treatments, medium was replaced with 50 µl of 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium tetrazolium bromide (MTT) at 10.5 mg/ml in DMEM, and the mixture was incubated at 37°C for another 4 hrs. Acid-isopropanol was added into each well (0.1 ml of 40 mM HCl in isopropanol), and mixed thoroughly until all crystals were dissolved. The plates were read immediately in a Titerk Multiskan PLUS MK II plate reader (Flow Laboratories, Mississauga, ON) using test (570 nm) and reference (650 nm) wavelengths. IC$_{50}$ (the concentration of an inhibitor needed to inhibit ligand binding by 50%) was calculated by nonlinear regression using GraphPad Prism software (GraphPad Software, San Diego, CA).

**Animal model of liver fibrosis induced by bile duct ligation**

Male Wistar rats (250 to 300g) were obtained from the Fudan University Animal Care Center (Shanghai, China). The protocol of animal experiments was approved by the Institutional Ethical Committee of Animal Experimentation, and the experiments were performed strictly according to governmental and international guidelines on animal experimentation. Liver fibrosis was induced in rats by ligation of the common bile duct (CBD) as previously reported (Zhan et al., 2006). The bile duct ligated (BDL) rats were randomly divided into 4 groups: BDL Controls, BDL plus IFN, SSL-IFN or RGD-SSL-IFN groups. One day after completing the CBD ligation procedure, rats were injected with IFN ($5 \times 10^4$ U/rat) in various formulations every other day via the tail vein. After 4 weeks of the treatment, rats were sacrificed, and blood samples were collected for the determination of serum levels of alanine aminotransferase (ALT) and total bilirubin (TB) with routine methods in a clinical laboratory of the hospital. Liver specimens were collected for formalin fixation and for snap
freezing. The frozen tissue was used for the assay for hydroxyproline content and RNA extraction. Liver total RNA was extracted by TRIzol reagent, and quantitated spectrophotometrically. cDNA was generated by reserve transcriptase reaction and liver procollagen type I/III gene expression was semiquantitiated by reserve transcriptase polymerase chain reaction (RT-PCR), using glyceraldehyde phosphate dehydrogenase (GAPDH) as a house-keeping gene control. The primer pairs used are shown in Table 1. The agarose gel images of the PCR products after 28 cycles of amplification were densitometrically analyzed and the average density ratios of the genes of interest over GAPDH were used to reflect the relative gene expression levels (Jiang et al., 2004). Liver total hydroxyproline content was determined spectrophotometrically as reported previously (Zhu et al., 1999).

Liver distribution of RGD-SSL or SSL in BDL rats, and HSC and hepatocyte distribution in BDL rats

Both SSL and RGD-SSL formulations were labeled by $^{99m}$Tc by the reaction between of $^{99m}$TcO$_4^-$ and the lipid, DTPA-DOPE (diethylenetriaminepentaacetic-1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine acid anhydride) (Avanti Polar Lipids) in SSL in the presence of SnCl$_2$ (Ahkong and Tilcock, 1992), and unlabeled isotope was separated by Sephadex G-50. $^{99m}$Tc-labeled liposomes were injected via tail vein in BDL rats. Four hours after injection, hepatocytes and HSC were isolated from the same rats as described above (Wu et al., 1996; Zhu et al., 1999). The radioactivity in each cell type was determined by a scintillation counter for the evaluation of the cell type-selective distribution based on cell number. In a separate experiment, $^{99m}$Tc-labeled SSL or RGD-SSL were injected via tail vein
in BDL rats. Whole body scanning was conducted with single photon emission tomography (SPET, Philips-IRIX, Veenendaal, the Netherlands) under anesthesia of ketamine 15 minutes after the injection. The radioactivity in each organ was estimated by calculating the radioactivity of the image area of individual organ, expressed by a ratio of radioactivity in the organ area/over the whole body.

Statistical analysis

All data are shown in mean values ± SD. Data were analyzed by ANOVA test plus multiple comparisons between two given groups with Newman-Keuls test. \( p < 0.05 \) was considered statistically significant.
Results

Selective binding of cyclic RGD peptide to HSC

To determine the affinity of the cyclic RGD peptide with HSC, quiescent HSC, activated HSC, hepatocytes and Cos-7 cells were incubated with FITC-conjugated cyclic RGD or AGA peptides. The cells with cellular internalization of FITC-conjugated peptides were considered as positive binding. As shown in Fig. 2, activated HSC incubated with cyclic RGD peptide at 37°C had a much higher FITC-positive rate as determined by flow cytometry when compared to either quiescent HSC or hepatocytes; whereas cyclic AGA peptide did not display any affinity to all cell types examined. This suggests that activated HSC selectively bound to the cyclic RGD peptide. The FITC-positive HSC number was significantly increased when the incubation temperature was elevated from non-permissive (4°C) to permissive temperatures (37°C) (Fig. 3A, B), suggesting that the endocytosis of cyclic RGD peptide was boosted with the elevation in temperature. Moreover, the endocytosis of the peptide appeared to be concentration-saturated, because a plateau was observed when the peptide reached approximately 500 nM (Fig. 3A). Meanwhile, when HSC were pre-incubated with the cyclic RGD peptide at 37°C, the subsequent cell binding of FITC-conjugated cyclic RGD peptide was inhibited in a concentration-dependent fashion (Fig. 3C), but the inhibitory concentration of non-FITC conjugated cyclic RGD peptide was higher than we expected.

The affinity of cyclic RGD peptide to HSC was further evaluated under a fluorescence microscope (Fig. 4A, 4B) and a laser-scanning confocal microscope (Fig. 4C). One hour after incubation, FITC-conjugated cyclic RGD peptide was mainly distributed on the cell surface and in the cytoplasm. Under the same condition no fluorescence signal was detected within
hepatocytes or HSC when they were co-cultured (to simulate in vivo activation of HSC) in the presence of cyclic AGA peptide. These results were consistent with unable binding of cyclic AGA peptide to HSC, or to hepatocytes with the absence of the receptor (Fig. 4B). In summary, it is conceivable that activated HSC interacted with cyclic RGD peptide in a receptor-specific manner and the interaction was mainly governed by a receptor-mediated endocytotic process.

Inhibition of HSC proliferation by IFN in various liposomal formulations

Inhibitory effects of free IFN, empty RGD-SSL, SSL-IFN, RGD-SSL-IFN, or IFN plus SSL on HSC proliferation were evaluated by incubating with these agents for various time points. As shown in Table 2, the inhibitory effect of RGD-SSL-IFN was approximately 20-fold higher than SSL-IFN (p<0.001) at 2 and 24 hours, and it remained higher (9-fold) even after 48 hours of the incubation (p<0.001), when the release and uptake of IFN from the unlabeled liposomes would be expected to be high. The RGD-labeled formulation exerted the inhibition, and reached its maximum effect within 2~24 hrs. The inhibitory effect of RGD-SSL-IFN at 3 hours of the incubation was 3-fold higher than that seen in SSL-IFN at 48 hours (p<0.01). At all time points, SSL-IFN was significantly less effective than free IFN in the inhibition of HSC proliferation (p<0.001). A high IC$_{50}$ (1000 µM) for RGD-SSL implied that neither cyclic RGD peptide nor the lipids or the combination of the two resulted in any inhibition on HSC proliferation. Moreover, IC$_{50}$ for free IFN is similar to free IFN plus SSL (p>0.05), which suggests that SSL did not affect the inhibition of IFN on HSC proliferation in vitro.
Morphology and liver distribution of cyclic RGD-labeled liposomes

Morphology of cyclic RGD-labeled liposomes was examined by a transmission electronic microscope (EM). As shown in Fig. 5A, the liposomes are unilamellar, round and regular in size. The average size of liposomes determined by laser-based light scattering was 101±17.7 nm, which was further verified by EM examination. The entrapping rate of IFN-α1b in RGD-SSL was 40.2%, and the entrapping capacity was 1×10^4 U IFN/µmol lipid as determined by measuring the phosphorus content. After labeling with 99mTc, both SSL and RGD-SSL were injected intravenously in BDL rats, and the total radioactivity in the liver 15 minutes after the injection was higher in animals receiving RGD-SSL than those receiving SSL injection (Fig. 5B). There was no significant difference in liposomal radioactivity accumulation between these two formulations in other organs. In separate experiments, 99mTc-labeled SSL or RGD-SSL were injected intravenously in rats four weeks after BDL. Four hours after the injection, hepatocytes and HSC were isolated, the radioactivity in hepatocytes and HSC was determined. As shown in Fig. 5C, it is evident that unlabeled SSL were largely distributed in hepatocytes, whereas, the distribution of cyclic RGD peptide-labeled SSL was markedly increased more than 10-fold in in vivo activated HSC, and dropped nearly 14-fold in hepatocytes. These findings demonstrated that cyclic RGD peptide-labeled SSL had a preferential liver uptake, and were largely distributed in activated HSC rather than hepatocytes in vivo.

Anti-fibrotic effects of IFN encapsulated in RGD-SSL in BDL rats

As shown in Fig. 6, the BDL resulted in obvious cholestasis and enlarged micro bile ducts with accumulation of bile, as well as the marked accumulation of connective tissue and
inflammatory infiltration in the portal triads (Fig. 6A, B). Administration of free IFN-α1b (Fig. 6B) did not significantly alter the extent of liver connective tissue deposition of BDL rats when compared to BDL controls (Fig. 6A). Intravenously administering SSL-IFN markedly reduced the extent of hepatic fibrosis and cholestasis. Of note, BDL rats receiving RGD-SSL-IFN (Fig. 6D) displayed the least deposition of connective tissue in the liver when compared with other BDL groups (Fig. 6A, B, C). Reduced serum ALT and total bilirubin levels in BDL + SSL-IFN, and further reduction in the BDL + RGD-SSL-IFN group, indicated that SSL or RGD-SSL were non-toxic to the liver, and that IFN-α1b is beneficial to the hepatocyte damage caused by cholestasis as a result of the BDL (Fig. 7A). The liver hydroxyproline quantitation shown in Fig. 7B verified the histopathological findings.

The mRNA levels of procollagen type I/III in BDL (Fig. 8) were significantly higher than sham-operated controls. Consistent with histology, intravenous administration of SSL-IFN significantly reduced the procollagen type I/III mRNA levels in these BDL rats. The amount of liver procollagen type I/III mRNA in RGD-SSL-IFN-treated animals was much lower than BDL controls and the SSL-IFN-treated group, and almost reached the levels in the sham-operated group. Thus, RGD-SSL-IFN appeared to be the most effective in suppressing the development of liver fibrosis by inhibiting procollagen type I/III mRNA expression when compared to IFN encapsulated in SSL without cyclic RGD peptide labeling.
Discussion

In the present study, we employed a cyclic RGD peptide, which has been previously shown to be specific in the recognition of collagen VI receptors on HSC, to label a sterically stable liposome formulation. The cyclic RGD peptide-labeled liposomes were used to entrap IFN-α1b, and the efficiency of targeting drug delivery of the liposomes was evaluated in both in vitro and in vivo. Our findings showed that the cyclic RGD peptide was coupled to the activated HSC in a time and dose-dependent fashion, and much more cyclic RGD peptide-labeled liposomes were up-taken by the liver and distributed in activated HSC of BDL rats after intravenous injection when compared to those without cyclic RGD peptide-labeling. Furthermore, IFN-α1b delivered with cyclic RGD peptide-labeled liposomes exhibited better anti-fibrotic efficacy than that in un-labeled liposomes. To our knowledge, this is the first successful attempt, which employs liposomes as a carrier for targeting drug delivery for improving anti-fibrotic therapy in a rat model of liver fibrosis, and as such, it represents a proof of the principle for drug delivery and targeting strategies.

The peptide sequence we used to label the liposome formulation was previously employed to label human serum albumin (HSA) (Beljaars et al., 2000). We modified the sequence by replacing cysteine with lysine, and the modified peptide was easily conjugated to the liposomal formulation via a sulfhydryl group in the cysteine residue. The modified cyclic RGD peptide tends to form a more stable cyclic peptide than the original one, which was cyclized by forming an instable disulfo bond (–S-S–) between two neighboring cysteine residues. In contrast, our new cyclic peptide forms a cycle with a peptide bond (–NH-CO–)
between the lysine and cysteine residues. The peptide bond is much more stable than the disulfo bond due to less possibility to be oxygenized. Although HSA can be used to deliver agents that react with it, the list of agents which react with HSA, but without changes in their chemical and pharmacological features is very short. Thus, developing drug carriers that possess much more delivering capacity and are clinically applicable is a critical step towards selective drug delivery for the treatment of liver fibrosis. Based on our experience in targeting drug and gene delivery in the treatment of liver injury (Wu et al., 1998a; Liu et al., 2003; Wu et al., 2004) and in the establishment of liposomal formulations (Pan et al., 2006), we established a new formulation of sterically stable liposomes, which are approximately 100 nm in diameter, and contain PEG as a spacer for the cyclic peptide linkage. The entrapping rate for IFN-α1b was very high, and the liposome size is in the range of the fenestrae of SEC for easily crossing through the fenestrae to reach the Disse space. The cyclic RGD peptide-labeled liposomes are long-circulating, which is a crucial property for in vivo drug delivery (Lee and Low, 1994). Moreover, cyclic RGD peptide labeling significantly enhanced the biodistribution of the liposomes in the fibrotic liver of BDL rats, resulted in a more than 10-fold increase of the liposomal accumulation in activated HSC, and improved the antifibrotic efficacy of IFN-α1b in treated animals. Thus, we have successfully established a new formulation of sterically stable liposomes which are clinically applicable and readily for being conjugated by molecules, such as peptides, for tissue- or cell type-specific recognition or delivery.

Targeting HSC has been much more difficult than hepatocytes and Kupffer cells due to no specific motifs or receptors existing on the cell surface, relatively less cell number and
residence of HSC in the Disse space side of SEC (Beljaars et al., 2002; Wu et al., 2002).
Ideally, targeting therapeutics should have a high degree of site preference, i.e., they should be specifically delivered to their target site(s) in order to achieve a high level of therapeutic efficacy and a low level of adverse effects, because many potential anti-fibrotic agents have a wide range of effects on many other organs or cell types (Wu and Zern, 2000; Yen et al., 2006). One means that has been utilized to increase the selectivity of antifibrotic agents is to be encapsulated in liposomes, which have been approved by the Food and Drug Administration for drug delivery in chemotherapy, such as doxorubicin (DOXIL®). Further improvements in the targeting effects of anti-fibrotic drugs might be achieved by coupling ligands selective for the target cells to the liposome surface. However, a previously confirmed targeting approach, incorporation of mannose-6-phosphate (M6P-HSA)-modified human albumin into liposomes, did not show any improvement in anti-inflammatory and anti-fibrotic effects of bioactive lipid dilinoleoylphosphatidylcholine (DLPC) in a BDL model of rat hepatic fibrosis, and the inflammatory responses were even worse in animals receiving M6P-HSA-liposome containing DLPC than those receiving DLPC-liposomes without M6P-HSA incorporation (Adrian et al., 2007). We are not clear why the conflicting results were obtained. One possible explanation is the different model systems that were employed. In that work, the liposomes with DLPC were administered only once, the BDL rats were evaluated one day after the therapeutics. Our animals were injected every other day for four weeks, thus providing a more optimal anti-fibrotic regimen.

Small molecules, e.g., peptides, carbohydrates or antibody fragments, may ultimately be useful targeting ligands. Small peptides have the advantage of being chemically defined,
able to be manufactured in large quantities and high purity without biological contaminants. They can be selected for specific targets with "one-bead one-compound" combinatorial libraries (Aina et al., 2005). The cyclic peptide C*GRGDSPC* has been shown to specifically inhibit the attachment of collagen type VI to cells (Marcelino and McDevitt, 1995). We linked the cyclic RGD peptide to the distal end of PEG, and obtained a stable drug delivery system, sterically stable liposomes (SSL). The in vitro experiment with rat HSC showed that IFN-α1b entrapped in the RGD-labeled liposomes exerted much greater effects in inhibiting HSC proliferation than that in non-labeled formulation. The mechanism underlying IFN-α-induced inhibition of HSC proliferation is associated with its effects on promoting an apoptotic process of activated HSC (Saile et al., 2003). IFN-α has been found to promote gene expression of antioxidant enzymes, such as cupper/zinc superoxide dismutase and glutathione peroxidase in hepatocytes and HSC, to enhance antioxidative defense capability, and in turn, improving hepatocellular function and inhibiting HSC activation (Lu et al., 2002). The biodistribution of cyclic RGD peptide-labeled liposomes showed the preferential liver uptake and much increased HSC accumulation in the comparison with unlabeled liposomes.

The findings in the present study demonstrate that cyclic RGD peptide-labeled liposomes are specifically recognized by activated HSC. IFN exerts better pharmacological actions, such as inhibiting their proliferation, reducing ECM synthesis and suppressing fibrogenic TGF-β release when it was delivered with targeting liposomes. The recognition of cyclic RGD peptide-labeled liposomes by activated HSC is critical for its preferential localization in HSC, because liposomes without cell-specific labeling after being uptaken by
the liver are normally cleaned up by Kupffer cells (Wu et al., 1998a; Wu et al., 1998b). The intravenous injection of IFN in various formulations did not cause any elevation of serum ALT levels, but displayed significant benefits in improving the extent of liver injury and fibrosis as evidenced by reduced serum ALT and total bilirubin levels, decreased liver hydroxyproline content, less connective tissue deposition in liver histology, and reduced mRNA levels of procollagen type I and III genes in the groups of BDL + SSL-IFN and BDL + RGD-SSL-IFN, and further reduction in the BDL + RGD-SSL-IFN group. Although the reduction in the BDL + RGD-SSL-IFN group was not as robust as we expected, it is statistically significant, and proves the usefulness of the targeting strategy. This may be due to the fact that liposomes and entrapped IFN-α are taken up by cells via endocytosis, and endocytosed IFN-α may undergo lysosomal or endosomal degradation. Anti-fibrotic agents that can be encapsulated in liposomes for site-selected delivery have a large spectrum (Wu and Zern, 2000). Many of these agents are not degradable in endosomes or lysosomes, and are expected to exhibit a much better improvement. Taken together, it appeared that IFN encapsulated in RGD-SSL further improved its antifibrotic activity which involves the inhibition of procollagen type I gene expression at both steady-state and activated levels of HSC (Inagaki et al., 2003), as well as promotion of an apoptotic process of activated HSC (Saile et al., 2003), which is an important step towards the cease or reverse of the fibrogenic process (Canbay et al., 2004).

In conclusion, the present study demonstrates that cyclic RGD peptide-labeled liposomes are selectively up-taken by activated HSC via receptor-mediated endocytosis, and that interferon-α1b encapsulated in the sterically stable liposome formulation displayed better
suppression on HSC proliferation \textit{in vitro}. The cyclic RGD peptide labeling markedly increased the liposomal accumulation in activated HSC \textit{in vivo}, and improved IFN’s efficacy to a significant extent in blocking the fibrogenesis in a rat model of bile duct ligation. Thus, the cyclic RGD peptide-labeled sterically stable liposomes represent a new means of targeting drug carrier for the treatment of liver fibrosis, and may have potential clinical applications.
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Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. *Hepatology* **43**:435-443.


Footnotes

Both Drs. Ji-Yao Wang and Jian Wu share the equal corresponding authorship. Dr. Jian Wu’s contact information: Department of Internal Medicine, Transplant Research Program, UC Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento, CA 95817, CA. Tel. 916-734-8044, Fax: 916-734-8097. Email: jdwu@ucdavis.edu. The study was supported in part by a grant from the National Nature Science Foundation (#30270595), P. R. China to Ji-Yao Wang. Dr. Jian Wu is supported by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) (DK069939).
Legends for Figures

**Fig. 1** A schematic illustration of cyclic RGD peptide-labeled sterically stable liposomes (cRGD-SSL). The link of cyclic RGD peptide with MAL-PEG-DOP️ (A), and incorporation into the sterically stable liposomes (EPC/Chol/PEG2000-DOP️/MAL-PEG-DOP️) (B). See the text in the section of Methods for details.

**Fig. 2** Binding of cyclic RGD peptide to activated HSC. The binding of cyclic RGD peptide at 200 nM to quiescent and activated rat HSC was determined at 37°C for 4 hrs. Cyclic AGA peptide was used as a control. Both cyclic peptides were conjugated with FITC. The FITC-positive cells were counted by flow cytometry and expressed as percentage of counted cells. **p<0.01 compared with quiescent HSC (n=5).**

**Fig. 3** The binding of cyclic RGD peptide to HSC in a dose- and temperature-dependent manner. A. Binding of activated HSC with FITC-conjugated cyclic RGD peptide in different concentrations at 4°C or 37°C for 4 hrs. FITC-conjugated cyclic AGA peptide was used as a control. FITC-positive cells were counted by flow cytometry after the incubation, and expressed as percentage of counted cells. B. Binding of activated HSC with cyclic RGD peptide at different time points at 4°C or 37°C. The levels of RGD peptide binding to HSC peaked at 4 hrs, and sustained till 8 hrs. C. Competitive binding of unlabeled cyclic RGD peptide with HSC (37°C, 4 hrs). HSC was pre-incubated with increasing concentration of un-conjugated cyclic RGD peptide at 37°C, and the binding of FITC-conjugated cyclic RGD peptide to HSC was determined in the presence of non-FITC-conjugated cyclic RGD peptide.
cyclic RGD peptide. N=5 in all groups of each panel.

**Fig. 4** Affinity of FITC-conjugated cyclic RGD peptide with HSC under a confocal microscope. Micrographs are the representatives of FITC-conjugated cyclic RGD peptide under a laser-scanning confocal microscope. **A.** Activated HSC with positive FITC fluorescent signal in the cytoplasm; **B.** HSC were co-cultured with hepatocytes in order to simulate *in vivo* HSC activation. FITC-conjugated cyclic RGD peptide fluorescent signals were localized exclusively within activated HSC, but not in hepatocytes. Activated HSC are morphologically stellate-like, with many projections. Their cytoplasm was stained in green and nucleus in blue by counter-staining with DAPI. Hepatocytes are relatively smaller in this image and only positive for DAPI staining in nuclei. **C.** Activated HSC. HSC with internalized FITC-conjugated cyclic RGD peptide showed green fluorescence within the cytoplasm with DAPI counter-staining in the nucleus.

**Fig. 5** Microscopic morphology and *in vivo* distribution of RGD-labeled sterically stable liposomes (RGD-SSL). **A.** The RGD-SSL were stained with phosphotungstic acid, and examined under a transmission electronic microscope. The bar indicates the liposome size (30,000×). **B.** Radioactivity in each organ was determined 15 minutes after intravenous injection of ⁹⁹m⁹Tc-RGD-SSL or ⁹⁹m⁹Tc-SSL. The percentage of the radioactivity in each organ was calculated based on the total whole body radioactivity (n=6) after correction with time. **C.** Distribution of cyclic RGD peptide-labeled SSL or un-labeled SSL in HSC or hepatocytes from BDL rats. Radioactivity of HSC or hepatocytes 4 hours after intravenous injection of either ⁹⁹m⁹Tc-SSL or
99mTc-RGD-SSL in BDL rats was determined and was expressed as percentage of radioactivity (dpm) in cells/total liver radioactivity after correction with time. ** p<0.01 compared to un-labeled SSL, and n=3.

**Fig. 6** Representative images of liver histology treated with IFN in different liposomal formulations in BDL rats. Liver fibrosis was induced by bile duct ligation (BDL). Liver sections were stained with H&E. BDL rats were randomly divided into 4 groups: BDL controls, BDL plus IFN, SSL-IFN or RGD-SSL-IFN. One day after the BDL procedure, rats were injected with IFN, SSL-IFN or RGD-SSL-IFN via tail vein in every other day for 4 weeks. A. BDL control, B. BDL + IFN, C. BDL + SSL-IFN, D. BDL + RGD-SSL-IFN (100X). Brown dots in Fig. 6A and 6B are the accumulated bile in micro bile ducts. Hepatocellular damage is obvious, thick fibrotic septa are seen, and there were infiltrated inflammatory cells in the portal triads in Fig. 6A and 6B. However, there was less hepatocellular injury, inflammatory infiltration, and thinner fibrotic septa infiltration in both Fig. 6C and 6D compared to A and B, and further improvements in liver injury and fibrosis in 6D is evident compared to 6C.

**Fig. 7** Serum ALT, total bilirubin (TB) levels, and liver hydroxyproline content. Serum ALT and TB levels were determined after the animals were sacrificed by a routine method (A). Liver content of hydroxyproline was determined spectrophotometrically (B). * p < 0.05 compared to Sham-operated group. Δ p<0.05 compared to BDL controls. ◇ p <0.05 compared to BDL + SSL-IFN. N=5 in all groups in both panels except the sham-operated group (n=7).

**Fig. 8** Procollagen type I/III mRNA levels in BDL rats treated with IFN in different
formulations. The levels of type I/III procollagen mRNA was determined by semiquantitative RT-PCR using GAPDH as a house-keeping gene control (A). The PCR product agarose gel images were analyzed by densitometry (B). * p < 0.05 compared to Sham-operated group. ∆ p<0.05 compared to BDL controls. ◊ p <0.05 compared to BDL + SSL-IFN. N=5 in all groups in both panels except the sham-operated group (n=7).
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Table 2  Inhibitory effect of interferon-α1b in different formulations on HSC proliferation in vitro

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<td>SSL-IFN</td>
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<td>3.35±1.20**</td>
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<td>1.01±0.45</td>
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The proliferation of HSC was determined with a MTT test in a 96-well plate (n=5 in each group). Data are expressed as IC_{50} (U/ml of IFN, unless otherwise stated) and were extrapolated from dose-response curves. IFN + SSL = free IFN mixed with SSL without encapsulation. ** p < 0.01 compared to SSL-IFN. _\text{AA} p <0.01 compared to free IFN or RGD-SSL-IFN.
Cyclic RGD peptide-SH + \( \text{PEG-C-NH-DOPE} \) → Cyclic RGD peptide-S-\( \text{PEG-C-NH-DOPE} \)

**Fig. 1**
Fig. 2
Fig. 3

A.

B.

C.
Fig. 4
Fig. 5
Fig. 7
Fig. 8