Cyclic Arg-Gly-Asp Peptide-Labeled Liposomes for Targeting Drug Therapy of Hepatic Fibrosis in Rats

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

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Zhongshan Hospital, Fudan University, Shanghai, China (S.-L.D., J.Wa., J.-Y.W.); Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai, China (H.P., W.-Y.L.); and Department of Internal Medicine, Transplant Research Program, University of California, Davis Medical Center, Sacramento, California (J.Wu)

Received March 9, 2007; accepted May 16, 2007

ABSTRACT

Targeting hepatic stellate cells (HSCs) 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 HSCs. The affinity of a cyclic peptide containing Arg-Gly-Asp (cRGD) to collagen type VI receptor on HSCs was examined in both in vitro and in vivo experiments. Sterically stable liposomes (SSLs) were modified with this peptide to yield a new carrier, cRGD-SSL. The targeting efficiency of this carrier in delivering interferon (IFN)-α1b was investigated in a rat model of liver fibrosis induced by bile duct ligation (BDL). When incubating HSCs or hepatocytes with cyclic RGD peptide, the peptide was bound preferentially to activated HSCs. Biodistribution study showed that the accumulation of cRGD peptide-labeled liposomes in HSCs isolated from BDL rats was 10-fold more than unlabeled SSLs. BDL rats receiving injections of IFN-α1b entrapped in cRGD-SSL exhibited significantly reduced extent of liver fibrosis compared with BDL control rats or BDL rats treated with IFN-α1b entrapped in SSLs. Thus, cRGD-SSL is an efficient drug carrier, which selectively targets activated HSCs 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 it may have potential clinical applications.

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 it 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, they activate resident nonparenchymal cells, such as Kupffer cells, sinusoidal endothelial cells (SECs), and hepatic stellate cells (HSCs) or infiltrating inflammatory cells. HSCs are the major cell population contributing to accelerated ECM production (Reeves and Friedman, 2002). Moreover, activated HSCs produce an array of mediators that perturb the fibrotic process independently from the activity of Kupffer cells, SECs, and inflammatory cells (Friedman, 1999; Li and Friedman, 1999; Reeves and Friedman, 2002). Thus, HSCs 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 there are relatively few HSCs in the liver and that there is a lack of specific receptors or motifs on the cell surface, the attempt to target HSCs has been a challenging task. Only a few studies focusing on targeting...
HSCs have been reported (Beljaars et al., 2002). These include human serum albumin (HSA) modified with mannose 6-phosphate (M6P) (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 that improve drug therapy for the disorder.

Interferon (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). To investigate whether IFN-α itself has any antifibrotic effects, we reported previously 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 and flu-like syndromes. Many patients cannot tolerate these side effects, and they 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 cyclic RGD peptide and recognizing collagen type VI receptors, which are up-regulated in activated HSCs of fibrotic livers. Our in vivo experiments demonstrate that the delivery of INF-α1b to HSCs with this cyclic RGD peptide-labeled liposomal carrier improved the efficacy of this medication in the treatment of liver fibrosis.

Materials and Methods

Chemicals and Reagents. Reagents were obtained from the following sources: egg phosphatidylycholine (EPC), cholesterol (Chol), methoxy-polyethylene glycol [PEG]_{2000}·1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), and N-hydroxysulfosuccinimidepolyoxyethylene-maleimide (NHS-PEG-MAL) were from Avanti Polar Lipids (Alabaster, AL); Sephadex G-50 and Sepharose CL-4B were from Pharmacia-LKB (Uppsala, Sweden); Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, and fetal bovine serum were from Invitrogen (Carlsbad, CA); and cell culture plates were from Corning (New York, NY). All other chemicals were of analytical grade.

Cell Culture. HSCs and hepatocytes used for in vitro experiments were isolated from normal male Wistar rats by two steps of collagenase digestion as we reported previously (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% fetal bovine serum (v/v) and 100 U/ml penicillin and streptomycin, and they were maintained at 37°C in a humidified incubator (90% humidity) containing 5% CO₂. Primary rat HSCs at a density of 1 × 10⁴ cells/cm² attained confluence at approximately 6 days as a monolayer of closely apposed polygonal cells. The cells at confluence were used between passages 3 and 5. By that time of culture, HSCs were free of Kupffer cells and SECs.

Preparation of MAL-PEG-DOPE. The synthesis of MAL-PEG-2000-OPE was conducted as described previously (Allen et al., 1998). 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 h. After thin layer chromatography in CHCl₃/CH₃OH/H₂O (65:25:4) showed disappearance of NHS-PEG-MAL with appearance of a more polar material, the solvent was evaporated. Then, 5 ml of acetoniitrile was added, and the mixture was kept at 4°C overnight. After centrifugation at 5000 rpm for 10 min, the upper clear solution was collected and evaporated. The product was dried in vacuum overnight, and it was verified by matrix-assisted laser desorption ionization/time of flight mass spectrometric analysis.

Synthesis of Cyclic RGD Peptide and Preparation of SSLs. 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 they were labeled with fluorescein isothiocyanate (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/methoxy-PEG-2000-OPE/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 15 times using a mini-extruder (Avanti Polar Lipids) to obtain a homogeneous liposome suspension (Wu et al., 1998a). For loading IFN, IFN-α1b was dissolved in phosphate-buffered saline (PBS), and the 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

Fig. 1. Schematic illustration of cRGD-SSLs. The link of cyclic RGD peptide with MAL-PEG-DOPE (A) and its incorporation into the sterically stable liposomes (EPC/Chol/PEG-2000-OPE/MAL-PEG-DOPE) (B). See Materials and Methods for details.
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 SSLs 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 by using high-performance lipid chromatography and enzyme-linked immunosorbent 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 of RGD-SSL was determined according to the equation carrying capacity = W_L / W_N × 100%, where W_L refers to total weight of lipids, and W_N 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; Hitachi, Tokyo, Japan) after being stained with 2% phosphotungstic acid and dried on carbon-coated grids. The size of liposomes was verified at the same time.

**Binding of Cyclic RGD Peptide to HSCs.** FITC-conjugated cyclic RGD or AGA peptides were incubated at concentrations of 20 to 1000 nM with quiescent or activated HSCs (1 × 10⁴ cells/well) at either 4°C for 1 h or 37°C for 4 to 1 h. In competition experiments, the cells were preincubated with non-FITC-conjugated cyclic RGD peptide (20–100,000 nM) at 4 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 ice-cold PBS, pH 7.4. The FITC-positive cells were counted by flow cytometry, and the data are expressed as percentage of counted cells.

In separate experiments, cells were plated at 2 × 10⁶ cells/well in 24-well plates, and they were incubated with FITC-conjugated cyclic RGD or AGA peptides at 200 nM for 1 h. After washing three times with PBS and staining by 4,6-diamidino-2-phenylindole (DAPI), the cells were visualized with a TCS-S2P laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany), using an ultraviolet laser with emission at 488 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 a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) proliferation assay. In brief, HSCs at 8 × 10⁴/ml were seeded in 96-well plates, and then they were incubated with free IFN (IFN-α1b), SSL-IFN, or RGD-SSL-IFN. After the cells were incubated for 2, 24, or 48 h under these treatments, medium was replaced with 50 μl of MTT at 10.5 mg/ml in DMEM, and the mixture was incubated at 37°C for another 4 h. Acid-isoazopropanol 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, Canada) using test (570-nm) and reference (650-nm) wavelengths. The IC₅₀ (the concentration of an inhibitor needed to inhibit ligand binding by 50%) was calculated by nonlinear regression using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

**Animal Model of Liver Fibrosis Induced by Bile Duct Ligation.** Male Wistar rats (250–300 g) 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 as reported previously (Zhan et al., 2006). The bile duct-ligated (BDL) rats were randomly divided into four groups: BDL control, BDL + IFN, SSL-IFN, or RGD-SSL-IFN. One day after completing the common bile duct ligation procedure, rats were injected with IFN (5 × 10⁵ 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 (Invitrogen), and it was quantitated spectrophotometrically. cDNA was generated by reverse transcriptase and DNA 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 polymerase chain reaction 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 ⁹⁹mTc by the reaction between of ⁹⁹mTcO₄⁻ and the lipid diethylenetriaminepentaacetic-1,2-DOP (Avanti Polar Lipids) in SSLs in the presence of SnCl₂ (Ah-kong and Tilcock, 1992), and unlabeled isotope was separated by Sephadex G-50. ⁹⁹mTc-labeled liposomes were injected via tail vein in BDL rats. Four hours after injection, hepatocytes and HSCs 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 liquid scintillation counter (LS 6500; Beckman Coulter, Inc., Fullerton, CA) for the evaluation of the cell type-selective distribution based on cell number. In a separate experiment, ⁹⁹mTc-labeled SSL or RGD-SSL was injected via tail vein in BDL rats. Whole-body scanning was conducted with single photon emission tomography (Philips-IRIX; Skannerudvikling Corp., Veenendaal, The Netherlands).

<table>
<thead>
<tr>
<th>Primer pairs for semi quantitative RT-PCR</th>
<th>Gene of Interest</th>
<th>Sequence</th>
<th>Locus</th>
<th>Size (bp)</th>
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</thead>
<tbody>
<tr>
<td><strong>Procollagen I</strong></td>
<td>Forward Primer:</td>
<td>5'-GGTTGGGAGAGAGCATGAGC-3'</td>
<td>3941–3960</td>
<td>514</td>
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<tr>
<td></td>
<td>Reverse Primer:</td>
<td>5'-TTCCGGGAAAAATTGATCTT-3'</td>
<td>4454–4453</td>
<td>425</td>
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<tr>
<td><strong>Procollagen III</strong></td>
<td>Forward primer</td>
<td>5'-ATGCTGCTTCATAGCTACG-3'</td>
<td>1509–1528</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-TGGGTCTCGAAGGTCCGG-3'</td>
<td>1933–1914</td>
<td>300</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Forward primer</td>
<td>5'-GAGAACACGTCTGCTCTG-3'</td>
<td>856–875</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-GAGGCAATAATGAGGAGAAG-3'</td>
<td>1155–1136</td>
<td>300</td>
</tr>
</tbody>
</table>

bp, base pair.
lands) under anesthesia of ketamine 15 min 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 that of the whole body.

Statistical Analysis. All data are shown in mean values ± S.D. Data were analyzed by analysis of variance 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 HSCs. To determine the affinity of the cyclic RGD peptide with HSCs, quiescent HSCs, activated HSCs, 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 HSCs incubated with cyclic RGD peptide at 37°C had a much higher FITC-positive rate as determined by flow cytometry compared with either quiescent HSCs or hepatocytes, whereas cyclic AGA peptide did not display any affinity to all cell types examined. This suggests that activated HSCs selectively bound to the cyclic RGD peptide. The FITC-positive HSC number was significantly increased when the incubation temperature was elevated from nonpermissive (4°C) to permissive temperature (37°C) (Fig. 3, A and B), suggesting that the endocytosis of cyclic RGD peptide was boosted with the elevation in temperature. Moreover, the endocytosis of the peptide seemed to be concentration-saturated, because a plateau was observed when the peptide reached approximately 500 nM (Fig. 3A). Meanwhile, when HSCs were preincubated with the cyclic RGD peptide at 37°C, the subsequent cell binding of FITC-conjugated cyclic RGD peptide was inhibited in a concentration-dependent manner (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. 4, A and B) 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 conditions, no fluorescence signal was detected within hepatocytes or HSCs when they were cocultured (to simulate in vivo activation of HSCs) in the presence of cyclic AGA peptide. These results were consistent with no binding of cyclic AGA peptide to HSCs, or to hepatocytes with the absence of the receptor (Fig. 4B). In summary, it is conceivable that activated HSCs interacted with cyclic RGD peptide in a receptor-specific manner and that 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 times. As shown in Table 2, the
The proliferation of HSCs was determined with an MTT test in a 96-well plate (Fig. 4). The inhibitory effect of RGD-SSL-IFN was approximately 20-fold higher than SSL-IFN \((p < 0.001)\) at 2 and 24 h, and it remained higher (9-fold) even after 48 h 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 it reached its maximum effect within 2 to 24 h. The inhibitory effect of RGD-SSL-IFN at 3 h of the incubation was 3-fold higher than that seen in SSL-IFN at 48 h \((p < 0.01)\). 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 \(\mu\)M) for RGD-SSL implied that neither cyclic RGD peptide, lipids, nor 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 SSLs 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 electron microscope. 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 transmission electron microscopic examination. The entrapment rate of IFN-\(\alpha\text{-1b}\) in RGD-SSL was 40.2%, and the entrapping capacity was 1 \(\times 10^6\) IFN/\(\mu\)mol lipid as determined by measuring the phosphorus content. After labeling with \(^{99m}\text{Tc}\), both SSL and RGD-SSL were injected intravenously in BDL rats, and the total radioactivity in the liver 15 min 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, \(^{99m}\text{Tc}\)-labeled SSL or RGD-SSL was injected intravenously in rats 4 weeks after BDL. Four hours after the injection, hepatocytes and HSCs were isolated, and the radioactivity in hepatocytes and HSCs was determined. As shown in Fig. 5C, it is evident that unlabeled SSLs were largely distributed in hepatocytes, whereas the distribution of cyclic RGD peptide-labeled SSLs was markedly increased more than 10-fold in vivo-activated HSCs, and it dropped nearly 14-fold in hepatocytes. These findings demonstrated that cyclic RGD peptide-labeled SSLs had a preferential liver uptake, and they were largely distributed in activated HSCs rather than hepatocytes in vivo.

**Antifibrotic Effects of IFN Encapsulated in RGD-SSL in BDL Rats.** As shown in Fig. 6, the BDL resulted in obvious cholestasis and enlarged microbile ducts with accumulation of bile, as well as the marked accumulation of connective tissue and inflammatory infiltration in the portal triads (Fig. 6, A and B). Administration of free IFN-\(\alpha\text{-1b}\) (Fig. 6B) did not significantly alter the extent of liver connective tissue and inflammatory infiltration in the portal triads (Fig. 6, A and B). Administration of free IFN-\(\alpha\text{-1b}\) (Fig. 6B) did not significantly alter the extent of liver connective tissue and inflammatory infiltration in the portal triads (Fig. 6, A and B).

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**Table 2**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Time</th>
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<tbody>
<tr>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>RGD-SSL ((\mu)M phospholipids)</td>
<td>2.46 ± 0.93</td>
</tr>
<tr>
<td>IFN</td>
<td>&gt;200**</td>
</tr>
<tr>
<td>SSL-IFN</td>
<td>161 ± 13.8**</td>
</tr>
<tr>
<td>RGD-SSL-IFN</td>
<td>7.99 ± 4.33**</td>
</tr>
<tr>
<td>IFN + SSL</td>
<td>2.28 ± 1.14</td>
</tr>
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</table>

IFN + SSL, free IFN mixed with SSLs without encapsulation.

**p < 0.01** compared with SSL-IFN.

**p < 0.01** compared with free IFN or RGD-SSL-IFN.
tissue deposition of BDL rats compared with BDL controls (Fig. 6A). Intravenously administering SSL-IFN markedly reduced the extent of hepatic fibrosis and cholestasis. It is noteworthy that BDL rats receiving RGD-SSL-IFN (Fig. 6D) displayed the least deposition of connective tissue in the liver compared with other BDL groups (Fig. 6, A–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 nontoxic 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 in 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 in BDL controls and in the SSL-IFN-treated group, and it almost reached the levels in the sham-operated group. Thus, RGD-SSL-IFN seemed to be the most effective in suppressing the development of liver fibrosis by inhibiting procollagen type I/III mRNA expression compared with IFN encapsulated in SSL without cyclic RGD peptide labeling.

**Discussion**

In the present study, we used a cyclic RGD peptide, which has been previously shown to be specific in the recognition of collagen VI receptors on HSCs, 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 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 manner, and many more cyclic RGD peptide-labeled liposomes were taken up by the liver and distributed in activated HSCs of BDL rats after intravenous injection compared with those without cyclic RGD peptide labeling. Furthermore, IFN-α1b delivered with cyclic RGD peptide-labeled liposomes exhibited better antifibrotic efficacy than that in unlabeled liposomes. To our knowledge, this is the first successful attempt that uses liposomes as a carrier for targeting drug delivery for improving antifibrotic 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 used previously to label 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 peptide, 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 that 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 that are clinically applicable is a critical step toward 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, 2004; Liu et al., 2003) 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 which 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 SECs for easily crossing through the fenestrae to reach the Disse
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 HSCs, and improved the antifibrotic efficacy of IFN.

Targeting HSCs has been much more difficult than hepatocytes and Kupffer cells due to no specific motifs or receptors existing on the cell surface, relatively fewer cell numbers, and residence of HSCs in the Disse space side of SECs (Beljaars et al., 2002; Wu et al., 2002). Ideally, targeting therapeutics should have a high degree of site preference, i.e., they...
HSCs (Saile et al., 2003). IFN— with its effects on promoting an apoptotic process of activated HSCs, because liposomes without cell-specific labeling after uptake and much increased HSC accumulation in comparison with unlabeled liposomes. The findings in the present study demonstrate that cyclic RGD peptide-labeled liposomes are selectively taken up by activated HSCs via receptor-mediated endocytosis and that interferon-α1b encapsulated in the sterically stable liposome formulation displayed better suppression on HSC proliferation in vitro. The cyclic RGD peptide labeling markedly increased the liposomal accumulation in activated HSCs in vivo, and it improved the efficiency of IFN 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 they may have potential clinical applications.

Acknowledgments

The synthesis of cyclic peptide was kindly conducted by Dr. Wuyuan Lu (Division of Basic Science, Institute of Human Virology, University of Maryland Biotechnology Institute, Baltimore, MD).

References


should be specifically delivered to their target site(s) to achieve a high level of therapeutic efficacy and a low level of adverse effects, because many potential antifibrotic 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 used 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. Further improvements in the targeting effects of antifibrotic drugs might be achieved by coupling ligands selective for the target cells to the liposome surface. However, a previously confirmed targeting approach, incorporation of M6P-HSA-modified human albumin into liposomes, did not show any improvement in anti-inflammatory and antifibrotic 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-liposomes containing DLPC than in 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 used. In that work, the liposomes with DLPC were administered only once; the BDL rats were evaluated 1 day after the therapeutics. Our animals were injected every other day for 4 weeks, thus providing a more optimal antifibrotic 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, and they are also able to be manufactured in large quantities and at 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 we obtained a stable drug delivery system, SSLs. The in vitro experiment with rat HSCs showed that IFN-α1b entrapped in the RGD-labeled liposomes exerted much greater effects in inhibiting HSC proliferation than that in nonlabeled formulation. The mechanism underlying IFN-α-induced inhibition of HSC proliferation is associated with its effects on promoting an apoptotic process of activated HSCs (Saile et al., 2003). IFN-α has been found to promote gene expression of antioxidant enzymes, such as copper/zinc superoxide dismutase and glutathione peroxidase in hepatocytes and HSCs, to enhance antioxidative defense capability, and in turn, to improve hepatocellular function and inhibit 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 comparison with unlabeled liposomes.

The intravenous injection of IFN in various formulations did not cause any elevation of serum ALT levels, but it 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, lessened 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 it 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. Antifibrotic 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 they are expected to exhibit a much better improvement. Taken together, it seemed 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 HSCs (Inagaki et al., 2003), as well as promotion of an apoptotic process of activated HSCs (Saile et al., 2003), which is an important step toward cessation or reversal of the fibrogenic process (Canbay et al., 2004).

In conclusion, the present study demonstrates that cyclic RGD peptide-labeled liposomes are selectively taken up by activated HSCs via receptor-mediated endocytosis and that interferon-α1b encapsulated in the sterically stable liposome formulation displayed better suppression on HSC proliferation in vitro. The cyclic RGD peptide labeling markedly increased the liposomal accumulation in activated HSCs in vivo, and it improved the efficiency of IFN 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 they may have potential clinical applications.


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**Address correspondence to:** Dr. Ji-Yao Wang, Department of Internal Medicine, Division of Gastroenterology and Hepatology, Zhongshan Hospital, Fudan University, 180 Fenglin Rd., Shanghai 200032, China. E-mail: jiyaowang@hotmail.com