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Sterically stable liposomes improve the therapeutic effect of hepatic stimulator substance on fulminant hepatic failure in rats

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ABSTRACT

Background and aims: Few drugs have been confirmed to be effective for fulminant hepatic failure (FHF). The purpose of this study was to prepare sterically stable liposomes (SSL) encapsulating hepatic stimulator substance (HSS) and determine their therapeutic effect on FHF.
Methods: HSS were encapsulated into SSL (HSS-SSL). FHF was induced in rats by thioacetamide (TAA) injection (400 mg/kg, three times with a 24-h interval). The agents, including HSS-SSL, SSL, HSS, and sodium chloride (NS), were each injected intravenously 2 h after the second and the third TAA injection.
Results: Freshly prepared HSS-SSL had a mean size of 93.59 nm and the average encapsulation efficiency was 37.20%. HSS encapsulated in SSL showed a longer half life and more potent target to injured livers than free HSS. Twenty-four hours after the third TAA-injection, the survival rate of HSS-SSL-treated rats (80%) was significantly higher than that of rats treated with NS (20%), SSL (25%), or HSS (50%). Histopathologic examination showed that there was the least necrosis and inflammation in the livers of HSS-SSL-treated rats. The incidence of stage 3 or 4 hepatic encephalopathy in HSS-SSL-treated rats was significantly lower than that in rats treated with other agents. The serum pro-inflammatory cytokine levels and hepatic lipid peroxidation levels were both markedly reduced, while hepatocyte proliferative rate was markedly increased after HSS-SSL treatment.
Conclusion: Encapsulation by SSL markedly improved the therapeutic effect of HSS on FHF in rats. Encapsulation by SSL may be an effective approach to enhance the therapeutic potency of drugs for FHF.
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1. Introduction

Fulminant hepatic failure (FHF) is a rare but severe complication of acute hepatitis, which is characterized by massive hepatic necrosis and hepatic encephalopathy (HE) with a very high mortality [1]. The only treatment of proven efficacy for FHF is emergency liver transplantation [2]. Although liver transplantation results in decreased mortality, its availability is often limited by the chronic shortage of donor livers, particularly in developing countries, for the lack of medical resources [2]. Additionally, approximately 40% of patients die while waiting for liver transplantation [3], emphasizing the need to develop new strategies for the management and treatment of FHF. Although a wide variety of medical therapies as well as artificial and bioartificial liver support systems have been used for management of this ominous condition, very few therapies have been evaluated in controlled clinical trials. Unfortunately, the use of such strategies has so far yielded disappointing results [1,2].

Hepatic stimulator substance (HSS) is a heat-stable, alcohol-precipitable extract, first extracted from the cytosol of regenerating adult rat livers and normal livers of weanling rats. HSS has been shown to stimulate liver regeneration when injected intraperitoneally into 34% partially hepatectomized rats [4]. It is a 12–20-kDa polypeptide growth factor, with mitogenic effects on the liver in an organ-specific but species-nonspecific manner [4]. Mao-Hua et al. reported that HSS administration was able to protect the liver of mice against acute failure induced by CCl_4 poisoning, by affecting the stability of biomembranes and cellular enzymatic systems [5]. Additionally, the survival rate in rats with fulminant hepatic failure induced by D-galactosamine was improved after the administration of HSS [6]. Though HSS showed attractive therapeutic potential in several animal models with FHF, it has not shown

Abbreviations: AUC, area under the curve; BrdU, 5-bromo-2′-deoxyuridine; CC, carrying capacity; FHF, fulminant hepatic failure; HE, hepatic encephalopathy; HSS, hepatic stimulator substance; IL, interleukin; MDA, malondialdehyde; CL, serum clearance; NS, sodium chloride; SD, standard deviation; SSL, sterically stable liposomes; TAA, thioacetamide.

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the prospective therapeutic effect for patients with FHF in the clinic [1,2]. HSS has a short half-life and is predominantly eliminated though the renal route. When HSS is administered intravenously, it also rapidly accumulates in other organs and tissues besides liver. As a consequence, sufficient concentrations of HSS could not be accumulated or sustained in hepatocytes, which might result in the insufficient therapeutic effect of HSS in patients with FHF.

For decades, liposomes have been used as versatile delivery systems for biologically active compounds [7]. One evident drawback of 'classical' liposomes is their rapid elimination by cells of the reticuloendothelial system, primarily by liver and spleen [7,8]. An important breakthrough in this respect was the development of long-circulating liposomes, such as liposomes coated with polyethyleneglycol, known as sterically stabilized liposomes (SSL). SSL have a prolonged blood residence time due to the presence of the hydrophilic polymer polyethyleneglycol, which provides steric stabilization. This stabilization inhibits the penetration of plasma proteins that could destabilize the liposomes or could serve as opsonins, resulting in decreased recognition by the reticuloendothelial system [7,9,10]. Another important characteristic of SSL is their localization to infectious targets, which is also the general property of liposomes with a certain size range. In experimental and clinical studies, it has been demonstrated that SSL leave the blood in areas of inflammation as a result of locally increased vascular permeability and endothelial leakage, both developing at the infected site during the progression of the infection [7,10,11]. Thus, these characteristics allow SSL to increase the accumulation amount of biologically active compounds encapsulated in liposomes at infected areas, which may result in the improved efficacy of encapsulated compounds.

In the present study, we prepared SSL encapsulating HSS (HSS-SSL) and determined the therapeutic effect of HSS-SSL on FHF in rats induced by thioacetamide (TAA) poisoning.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: HSS was provided by Sailuojin Pharmaceutic Company (Weihai, Shangdong, China), which was extracted and purified from the fresh livers of healthy Anthony pigs with a purity of above 98%; egg phosphatidylcholine, cholesterol, and methoxy-polyethylene glycol$_{2000}$-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine were from Avanti Polar Lipids (Alabaster, AL); epidermal growth factor (EGF) was from Promega (Beijing, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5-bromo-2′-deoxyuridine (BrdU) and 1,1,3,3-tetraethoxypropane were from Sigma-Aldrich (Shanghai, China); Dulbecco's modified Eagle's medium and fetal bovine serum were from Invitrogen (Carlsbad, CA); iodogen was from Pierce (Rockford, IL); [^{125}I]NaI was from PerkinElmer (Hong Kong, China). TAA and all other chemicals were of analytical grade and from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. 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 maintained on standard laboratory rat chow on a 12-h light/dark cycle with free access to water and food. All studies were approved by the Institutional Ethical Committee of Animal Experimentation, and all experiments were performed strictly according to governmental and international guidelines on animal experimentation.

2.3. Preparation and properties of HSS-SSL

2.3.1. Preparation of HSS-SSL

Liposomes were prepared as previously described [12]. In brief, lipids composed of egg phosphatidylcholine/cholesterol/methoxy-polyethylene glycol$_{2000}$-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine at a molar ratio of 2:1:0.1 were dissolved in chloroform and the solvent was evaporated to form a lipid film under reduced pressure. The lipid mixture was hydrated in phosphate buffer saline (pH 7.4) and sequentially extruded 15 times through a double layer of polycarbonate membrane (Whatman, Kent, UK) in 400-, 200-, 100-, and 50-nm open mesh using a mini-extruder (Avanti Polar Lipids) to obtain a homogeneous liposome suspension (SSL). To prepare HSS-SSL, the lipid mixture was hydrated in phosphate buffer saline containing HSS and extruded. The resulting liposomes were passed through a Sepharose CL-4B column (Pharmacia-LKB, Uppsala, Sweden) to remove unencapsulated HSS. The prepared liposomes were stored at 4 °C for further experiments.

2.3.2. Properties of HSS-SSL

The morphology of HSS-SSL 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 and polydispersity index of the resultant liposomes were determined using dynamic light scattering (Nicomp 380 ZLS; Particle Sizing Systems, Santa Barbara, CA). After passing through the Sepharose CL-4B column, the concentration of unencapsulated HSS was measured with an ultraviolet spectrophotometer and the encapsulation efficiency of HSS was calculated by deduction of the unencapsulated dosage from the total dosage of HSS as previously described [13]. The lipid concentration was determined by Bartlett analysis. Then the carrying capacity (CC) of SSL was determined according to an equation: $CC = W_E/W_{LN} \times 100\%$. Here W_{LN} refers to total weight of lipids and W_E refers to the weight of the entrapped substance.

The encapsulation efficiency, size, and polydispersity index of HSS-SSL were respectively measured at days 1, 7, and 30 after preparation to evaluate the stability of the resultant liposomes. These properties were also compared between HSS-SSL and SSL. All measurements were repeated three times.

2.3.3. Effect of HSS-SSL on hepatocyte proliferation in vitro

Primary rat hepatocytes were isolated by a two-step collagenase perfusion [14] and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and antibiotics for 12 h. To evaluate the effect of HSS-SSL and HSS on hepatocyte proliferation, hepatocytes (4000/well) in 96-well plates were respectively incubated with HSS-SSL, HSS or SSL for 24 h. The concentration range of HSS in the medium was 2–1 500 ng/mL, and the concentration of SSL was the same as that of HSS-SSL. HSS alone did not affect the growth of primary cultured hepatocytes to a great extent, but allowed them to be significantly stimulated by EGF and other hepatic mitogenic factors [15]. Therefore, 10 nmol/L EGF was added to the medium at the same time. After incubated for 24 h, MTT assay was performed as described [16]. The absorbance rate of each well optical density (OD value) was measured at 595 nm by a spectrophotometer and the cell proliferation rate was calculated as (average OD value of wells with administered agent/average OD value of control wells) × 100. The assays were repeated thrice.

2.3.4. Pharmacokinetics and organ biodistribution of HSS-SSL in rats with liver injury

HSS was labeled with iodine-125 (^{125}I) using the iodogen method. Briefly, HSS was labeled with iodine-125 (^{125}I) by

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