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Lactosamination of liposomes and hepatotropic targeting research

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INTRODUCTION

Site-specific delivery of therapeutic drugs to their target cells is a major s cientific challenge for the pharmaceutical sciences. It offers a number of advantages over conventional drug administration. With drug targeting, high local conc entrations of the drug can be achieved, thus circumventing many unwanted side ef fects. Various carriers have been suggested for the delivery of drugs, including liposomes[1-5]and (neo) glycoproteins[6-8]. The asialoglycopr otein receptor (ASGP-R) has frequently been utilized for targeting drugs to the parenchymal liver cell[6-12]. Liposomes have several advantageous cha racteristics as drug carrier, and particularly, ligand-tacked liposomes achieve a highly effective targeting[13]. Hara *et al* reported that asialofetuin (AF)-tacked liposomes distributed to rat hepatocytes selectively *in vivo*[14], and ASGP-R mediated the uptake of AF-liposomes encapsulating IFN-γ by isolated rat hepatocytes *in vitro* [15]. Lacto saminated huma n serum albumin (L-HSA) is a neoglycoprotein taking number of galactose residue as terminal sugar[6].

In this paper, we studied the preparation and rat hepatocyte uptake of the conjugate of L-HSA and liposomes, and the inhibitory effect of L-HSA-liposomes containing IFN-α on replication of hepatitis B virus (HBV) on 2.2.15 cells line.

MATERIALS AND METHODS

Materials

Sodium cyanoborohydride was purchased from Aldrich, Chemical Co., Milwaukee, WI, USA, α-lactose, phosphatidylcholine (PC), cholesterol (Chol), and N-succini midyl-S-acetylthioacetate (SATA) were obtained from Sigma, St. Louis, MO, U SA. Maleimido-4-(p-phenylbutyryl) phosphatidylethanolamine) (MPB-PE) was pu rch ased from Avanti Polar lipids, Birmingham, AL, USA. Dulbecco's modified eagle medium, penicillin and streptomycin were obtained from GIBCO, Grand Island, NY, USA. MTT was from Boehringer Mannheim, Germany. Fetal calf serum was a product of Hyclone, USA. Other reagents were of analytical grade.

Methods

Lactosamination of human serum albumin Lactosamination was performed according to Schwartz & Gray[16]; human se rum albumin (HSA) 500 mg was allowed to react at 37℃ with 1.0 g α-lactose and 1.0 g NaBH3CN in 20 ml 0.05 mol/L potassium phosphate buffer solution (PB S), pH 8.0. The reaction was continued for 144 h. And the mixture was dialyse d against double distilled water. The lactose content of lactosaminated human serum albumin (L-HSA) was determined by the phenol-sulphuric acid method of Dubois *et al*[17].

Liposomes preparation PC, Chol, and MPB-PE were mixed in a molar ratio of 23:16:1 and dissolved in ether, dried by a rotary evaporator, dissolved in HN-buffer (10 mmol/L HEPES, 135 mmol/L NaCl, pH 6.7) and lyophilized. Phospholipid phosphorus of liposome preparation was measured by phosphate assay after perchloric acid destruction. Conventional liposomes were prepared with PC and Chol in a molar ratio of 23:16.

Coupling of L-HSA to MPB-PE containing liposomes (MPB-liposomes) L-HSA was coupled to MPB-liposomes by a sulfhydryl-maleimide coupling techni que according to Derksen *et al*[18]. Using SATA as a heterobifunctional reagent, free sulfhydryl groups were introduced in L-HSA[19]. After separation of SATA from the protein by Sephadex G-25 gel permeation chromatog raphy, the acetylthioacetate-L-HSA was deacetylated by a freshly prepared solution of 0.5 mol/L hydroxylamine-HCl, 0.5 mol/L HEPES, 25 mmol/L EDTA, pH 7.0. After deacetylation, the thioacetyl-L-HSA was allowed to react with the MPB-PE containing liposomes for 4 hours at room temperature, in a ratio of 3 mg of prote in per mg liposomes. N-ethylmaleimide was added to cap un-reacted sulfhydryl groups. Liposomes were separated from unconjugated protein by Sepharose 4B

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infiltrat i on. The L-HSA- liposomes conjugates were characterized by determining protein a nd phospholipid phosphorus content, lyophilized, and stored at 4℃.

Tissue distribution of L-HSA-liposomes L-HSA-liposomes were labled with ^{131}I by Iodogen's method. Male Sprague-Dawley rats weighing 200-250 g were anaesthetized by intraperitoneal injection of 20-25 mg sodium pentobarbital. Radiolabled liposomes were injected via the penile vein and the abdomen was opened. At the end of the experiment, liver lobules and other tissues were removed and weighed. Radi oactivity was determined by γ-counter. And the results were registered as cpm per gram tissue.

Inhibitory effect of L-HSA-liposomes encapsulating interferon-α on HBV replication To assess the effects of L-HSA- liposomes en c apsulating interferon-α on HBV replication, 2.2.15 cells were plated at a dens ity of 2×10^5 per 17 mm culture dish and pre-incubated in Dulbecco's modified eagle medium containing 10% fetal calf serum for 24 h. After being washe d with phosphate -buffered saline, they were cultured at 37℃ for 12 d, with fresh medium changed every 3 d supplemented with L-HSA-liposomes encapsulating interferon-α (LL-IFN), conventional liposomes encapsulating interferon-α (CL-IFN), and free interferon-α (IFN) at an appropriate concentr ation. The culture medium was collected every 3 days and the cells were used in subsequent experiments. The viability of cells was examined spectrophotometrically by the MTT methods, and the cytotoxicity of the compounds was also monitored by the MTT as say.

RESULTS

Synthesis and characterization of L-HSA-liposomes

Having reacted with lactose, the HSA and lactose mixture was dialysed against water for 3 days. There was no lactose in the final dialysis solution, suggesting the L-HSA had been purified completely. Each HSA molecule was modified with about 17 molecules of lactose. When L-HSA was coupled to MPB-liposomes, the amount of L-HSA that could be coupled to 1 μmol MPB-liposomes was 538.7 μg. A number of about 107 000 L-HSA molecules per liposome particle were calculated, assuming that the molecular weight of L-HSA was 7 600 and the average diameter of MPB-liposomes was 400 nm. The liposomes conjugate was stored at 4℃ for at least 8 weeks and filtered by Sepharose 4B. The infiltration figure showed as a single absorbing peak, suggesting that the liposomal conjugate was stable at 4℃.

Tissue distribution of L-HSA-liposomes

Forty minutes after i.v. injection of ^{131}I labled L-HSA-liposomes, r adioactivity in liver was higher than the other organs ($P <= 7.84 \times 10^{-6}$) (Table 1). Spleen uptake of L-HSA-liposomes was less than half of that of liver, and the uptake by kidney, heart, and lung was 1/4 to 1/9.

When L-HSA was pre-injected, the liver uptake decreased significantly, showing no statistic difference with spleen ($P = 0.38$) (Table 2). However, ^{131}I labled L-HSA-liposomes uptake was also observed in the other organs.

Table 1 Tissue distribution of ^{131}I-labeled L-HSA-liposomes in rat

Rat No.	Tissue distribution *in vivo* (cpm per gram tissue)				
	Liver	Spleen[a]	Kidney[b]	Heart[c]	Lung[d]
1	31777.2	15394.2	7912.1	4427.5	9568.5
2	30476.1	15464.1	5424.5	3180.8	6044.1
3	30013.1	14041.4	7932.3	3777.5	5886.1
4	36099.3	10817.5	6372.3	3722.7	8717.3
5	36479.8	17882.6	7228.6	3885.5	7695.8
Average	32969.1	14719.9	6974.1	3798.8	7582.4

^{131}I labeled L-HSA-liposomes were injected into rats. Radioactivity in different tissues was determined 40 min after injection as described in *Mate rials and Methods*.
Compared with liver: [a]$P = 7.84 \times 10^{-6}$; [b]$P = 1.06 \times 10^{-7}$; [c]$P = 2.98 \times 10^{-8}$; [d]$P = 2.1 \times 10^{-7}$

Table 2 Tissue distribution of ^{131}I labeled L-HSA-liposomes in rat, after L-HSA pre-injection

Rat No.	Tissue distribution *in vivo* (cpm per gram tissue)				
	Liver	Spleen[a]	Kidney[b]	Heart[c]	Lung[d]
1	34682.0	35187.6	5535.7	2474.2	6368.0
2	20587.4	19791.1	4541.8	2277.2	8507.7
3	24288.8	27297.1	4564.4	2089.5	7280.7
4	33733.1	17637.5	5560.3	2698.3	12561.1
5	25600.9	18994.5	6999.2	3227.0	11087.1
Average	27778.4	23781.6	7699.1	2553.2	8679.4

Fifteen min after L-HAS pre-injection, ^{131}I labeled L-HSA-liposomes were injected into rats. Radioactivity in different tissues was determined 40 min af ter injection as described in Materials and methods.
Compared with liver: [a]$P = 0.38$; [b]$P = 4.35 \times 10^{-5}$; [c]$P = 1.66 \times 10^{-5}$; [d]$P = 2.52 \times 10^{-4}$

Table 3 Inhibitory effect on HBeAg expression in 2.2.15 cells of di fferent types of interferons

Treatment	Inhibitory effect on HBeAg expression (%)			
	3 days	6 days	9 days	12 days
Liposomes	0	1.8	0	0
L-HSA-Liposomes	0	0	0	0
IFN 0.08 MU[a]	0	4.7	12.7	2.3
CL-IFN 0.08 MU[b]	0	0	22.8	5.8
LL-IFN 0.08 MU	44.8	60.7	43.5	45.5
LL-IFN 0.04 MU	6.8	47.4	28.6	12.1
LL-IFN 0.02 MU	0	24.6	23.8	4.6
LL-IFN 0.01 MU	0	4.8	15.4	5.2

IFN: interferon; CL-IFN: interferon entraped in conventional liposomes; LL-IFN : interferon entraped in L-HSA-liposomes.
Compared with LL-IFN 0.08 MU: [a]$P = 0.003$, [b]$P = 0.015$

Anti-HBV activity of L-HSA-liposomes encapsulating interferon-α

Table 3 shows the antiviral activity of L-HSA-liposomes encapsulating interferon-α tested against HBV *in vitro*. The liposomes and L-HSA-l iposomes did not show any anti-

HBV effect. However, when entraped in L-HSA-liposomes, int erferon was 3 to 5 times more effective against HBV replication in 2.2.15 cells than CL-IFN and IFN (*P* value was 0.015 and 0.003, respectively). To achieve similar antiviral effects, the dose of LL-IFN was only 1/4, 1/8 of CL-IFN and IFN, respectively. The treatments did not appear to be cytotoxic to 2.2.15 cell s at the concentrations used in the anti-HBV assay.

DISCUSSION

Worldwide, HBV infection is the main cause of chronic liver disease. HBV carrie r s are at risk for chronic hepatitis, cirrhosis, and hepatocyte carcinoma. Previo us studies have indicated the correlation of HBVX gene to hepatocyte carcinoma[20]. An effective treatment for HBV infection is therefore urgently needed. Interferon-α, nucleotide analogy, Chinese medicinal herbs, and other i mmunological approaches have shown promising results in a subset of patients treated for prolonged periods[21-24]. But overall response rates have been unsatisfactory. Selective drug delivery to the parenchymal liver cell by drug conjugation to a carrier would improve curative effect, reduce therapeutic dosage and side effects of drugs. In this report we describe the preparation of a liposomal drug carrier system, which could target the therapeutic drug to liver via the asialoglycoprotein receptor. Lactose was coupled to human serum albumin by reductive lactosamination in the presence of sodium cyanoborohydride. Then L- HSA was covalently coupled with liposomes using the heterobifuntional reagent N-Succinimidyl-S-acetylthioacetate. This is a well established method for coupli ng proteins to liposomes, based on the reaction of thiolated proteins and liposo mal maleimido-4-(*p*-phenylbutyryl) phosphatidylethanolamine[10]. T hio lation of L-HSA, in which about 17 of the free ε-amino groups of lysine were derivatized with lactose, still allowed the introduction of several sulfhydryl molecules in this protein. This indicates that SATA is suitable for coupling deri vatized protein to liposomes.

Mammalian liver contains a unique asialoglycoprotein receptor responsible for the rapid serum clearance and lysosomal catabolism of desialylated glycoproteins bearing terminal, non-reducing galactose residues. Previous studies demonst rated that, for bovine serum albumin, at least 13 lactosyl groups were needed for high affinity recognition by the ASGP-R[25]. In our study, about 107 000 molecules L-HSA perliposome were coupled, and each HSA molecule was modifi ed with 17 lactose molecules, hence the liposomal conjugate had a high affinity to ASGP-R. The *in vivo* study indicated that the liver uptake of liposomal c onjugate was higher than other organs, which was about 2 times higher than that of spleen, and 4 to 9 times higher than kidney, heart, and lung. L-HSA pre-injection almost prevented the selective liver delivery of liposomal conjugate, suggesting that the galactose-specific nature of hepatic uptake. Modified with 17 lactose molecules, L-HSA was capable of inhibiting the hepatic uptake of liposomal conjugate, owing to the high affinity for the hepatic galactose-recognizin greceptor. Another study also showed that liposomes modified with several galatose residues had a similar liver targeting[26].

2.2.15 cell is a stable expression system of transfetced HBV DNA, which could produce HBV particle, and is often taken as a cell model for screening anti-hepatitis B virus drugs[27,28]. Interferon had the inhibitory effect on HBV replication in this system[29]. Wu *et al*[30] proved that this system contained ASGP-R. *In vitro*, interferon-α entraped in liposomal conjugate had a higher activity against HBV free than interferon and than interf eron entrapped in conventional liposomes. To achieve similar antivirus effects, the doses of LL-IFN capable of inhibiting virus growth were 4 to 8 times less than t hat of CL-IFN and IFN.

In conclusion, the results obtained in this study indicated that the conjugate of lactosaminated human serum albumin and liposomes achieved good liver targeting , and allowed the development of a potent liver-targeting drug carrier system.

REFERENCES

1 Dumont S, Muller CD, Schuber F, Bartholeyns J. Antitumoral pr operties and reduced toxicity of LPS targeted to macrophages via normal or mannosylated liposomes. *Anticancer Res*, 1990;18: 155-160
2 Xu YK, Qiao DF, Liu XY, Zhang JN. The experimental study of iopromide loading liposomes: a potential CT contrast medium for liver reticuloendothe lial system. *Zhongguo Yixue Yingxiang Jishu*, 1998;14:732-734
3 Zhuo C, Wang XG, Liu YH, Yu DG, Yu Y. Levo-praziquantal liposome and praziquantel liposome therapy of schistosomiasis in mice. *Zhongguo Renshoug onghuanbing Zazhi*, 1997;13:31-35
4 Xu YK, Zhang JN, Qiao DF, Liu XY, Huang QL. The experimental study on the preparation of diatrizoate liposomes and their application for liver targeting contr ast enhancement. *Zhongguo Yixue Yingxiangxue Zazhi*, 1999;7:122-123
5 Guo QL, Ding QL, Zhu JB, Zheng WS. The effect of doxorubicin prolip osome on toxicity of liver and heart in rats and experimental tumour. *Zhongguo Bingli Shengli Zazhi*, 1999;15:537-540
6 Jansen RW, Kruijt JK, Van Berkel TJC, Meijer DKF. Coupling of the a ntiviral drug Ara-AMP to lactosaminated albumin leads to specific uptake in rat and human hapatocytes. *Hepatology*, 1993; 18:146-152
7 Zhong S, Wen SM, Zhang DF, Wang QL, Wang SQ, Ren H. Sequencing of PCR amplif ied HBV DNA prec and cregions in the 2. 2.15 cells and antiviral action by targ eted antisense oligonucleotide directed against the sequence. *World J Gastroen tero*, 1998; 4:434-436
8 Xie Q, Guo Q, Zhou XQ, Gu RY. Effect of adenine arabinoside monoph osphate coupled to lactosaminated human serum albumin on duck hepatitis B virus. *Shijie Huaren Xiaohua Zazhi*, 1999;7:125-126
9 Fiume L, Stefano GD, Busi C, Mattioli A, Gervasi GB, Bertini M. Hepatotropic conjugate of adenine arabinoside monophosphate with lactosaminated poly-L-lysine. *J Hepatol*, 1997;26:253-259
10 Zhang ZR, He Q. Study on liver targeting and hepatocytes permeable valac iclovir polybutylcyanoacrylate nanoparticles. *World J Gastroentero*, 1999;5:330-333
11 Zhang ZR, He Q, Liao GT, Bai SH. Study on the anticarcinogenic effect and acute toxicity of liver targeting mitoxantrone nanoparticles. *World J Gastroentero*, 1999;5:511-514
12 He XX, Wang JL. Present status and expectation of gene therapy for hepat ocyte carcinoma. *Shijie Huaren Xiaohua Zazhi*, 1998; 6:158
13 Huang A, Kennel SJ, Huang L. Interactions of immunoliposomes with target cells. *J Biol Chem*, 1983;258:14034-14040
14 Hara T, Aramaki Y, Tsuchiya S, Hosoi K, Okada A. Specific incorporation of asialofetuin-labeled liposomes into hepatocytes through the

action of galactose-binding protein. *Biopharm Drug Dispos*, 1987; 8:327-339
15 Hara T, Ishihara H, Aramaki Y, Tsuchiya S. Specific uptake of asialofetuin labeled liposomes by isolated hepatocytes. *Int J Pharm*, 1988;42:69-75
16 Schwartz BA, Gray GR. Proteins containing reductively aminated disaccharides. Synthesis and chemical characterization. *Arch Biochem Biophys*, 1977;181:542-549
17 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Biochem*, 1956;28:350-356
18 Derksen JTP, Scherphof GL. An improved method for the covalent coupling of protein and liposomes. *Biochem Biophys Acta*, 1985;814:151-155
19 Duncan RJS, Weston PD, Wrigglesworth R. A new reagent which may be used to introduce sulfhydryl groups into protein, and its use in the preparation of conjugates for immunoassay. *Anal Biochem*, 1983;132:68-73
20 Wang XZ, Tao QM. Correlation of HBV X gene and hepatocyte carcinoma. *S hijie Huaren Xiaohua Zazhi*, 1999;7:1063-1064
21 Zhu Y, Wang YL, Shi L. Clinical analysis of the efficacy of interferon alpha treatment of hepatitis. *World J Gastroentero*, 1998;4 (Suppl 2):85
22 Shi JJ, Miao F, Liu FL. Therapeutic effect of medicinal herbs and western drugs on hepatitis B virus. *World J Gastroentero*, 1998;4(Suppl 2):61
23 Geroge KK Lau. Immunological approaches to the breakdown of hepatitis B viral persistence. *World J Gastroentero*, 1998;4(Suppl 2):32
24 Xu KC, Wei BH, Yao XX, Zhang WD. Present TCM-WM therapy for chronic hep atitis B. *Shijie Huaren Xiaohua Zazhi*, 1999;7: 970-974
25 Vera DR, Krohn KA, Stadelnik RC, Scheibe PO. Kinetic sensitivity of are cept or binding radiopharmaceutical: technetium 99m galactosyl-neoglycoalbumin. *J Nucl Med*, 1984;25:779-787
26 He W, Yang JX, Yang J, Yin WH. The research on liver targeting delivery system of cantharidin polyphase liposome modified by strengthened targeting material. *Zhongguo Yaokedaxue Xuebao*, 1998;29:413-417
27 Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA*, 1987;84:1005-1009
28 Ueda K, Tsurimoto T, Nagahata T, Chisaka O, Matsubara K. An *in vitro* system for screening anti hepatitis B virus drugs. *Virology*, 1989;169:213-216
29 Hayashi Y, Koike K. Interferon inhibits hepatitis B virus replication in as table expression system of transfected viral DNA. *J Hepatol*, 1989;63:2936-2940
30 Wu GY, Wu GH. Receptor mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem*, 1987;262:4429-4433

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