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Interaction of targeted liposomes with primary cultured hepatic stellate cells: Involvement of multiple receptor systems

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***Background/Aims*: In designing a versatile liposomal drug carrier to hepatic stellate cells (HSC), the interaction of mannose 6-phosphate human serum albumin (M6P-HSA) liposomes with cultured cells was studied.**

***Methods*: M6P-HSA was covalently coupled to the liposomal surface and the uptake and binding of ^{3}H-labelled M6P-HSA liposomes by primary rat HSC and liver endothelial cells was determined. The targeting ability of M6P-HSA liposomes to HSC was tested in bile duct ligated rats using immunohistochemical methods.**

***Results*: The association of M6P-HSA liposomes with HSC was 4-fold higher than of control liposomes. An excess of M6P-HSA inhibited this association by 58%, indicating M6P receptor specificity. The scavenger receptor competitor polyinosinic acid abolished association of M6P-HSA liposomes with HSC. M6P-HSA liposomes also amply associated with endothelial cells, which abundantly express scavenger receptors. Endocytosis of M6P-HSA liposomes by HSC was temperature dependent and could be inhibited by monensin. In the fibrotic liver M6P-HSA liposomes co-localised with HSC.**

***Conclusions*: Coupling of M6P-HSA to liposomes strongly increases the in vitro uptake of these liposomes by HSC and endothelial cells. Both the mannose 6-phosphate receptor and the scavenger receptors are involved in the uptake process. M6P-HSA liposomes are potential drug carriers to HSC in the fibrotic liver.**

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***Keywords*: Hepatic stellate cells; Liposomes; M6P/IGF II receptor; Liver fibrosis; Scavenger receptor**

1. Introduction

In the healthy liver, hepatic stellate cells (HSC) express a quiescent phenotype and are responsible for storage of vitamin A [1]. During liver fibrosis, HSC become activated and transform into proliferating fibroblast-like cells, which produce large amounts of collagen [2]. Since HSC are identified as key players in the development of fibrosis, they are attractive targets for antifibrotic therapy.

Human serum albumins (HSA) modified with sugar moieties or cyclic peptides that recognise receptors on HSC were shown to selectively accumulate in HSC in a rat model of liver fibrosis. In particular, mannose 6-phosphate groups attached to HSA (M6P-HSA) exerted specificity for the mannose 6-phosphate/insulin like growth factor II (M6P/IGF II) receptors present on HSC [3–5]. The expression of these receptors is increased during HSC transformation from the quiescent into the activated phenotype [6,7]. In the fibrotic liver M6P/IGF II receptors facilitate the activation of transforming growth factor β (TGF-β) [8,9] which is a potent cytokine that stimulates the production of collagen by HSC. Binding of latent TGF-β to the M6P/IGF II receptors mediated by two M6P groups on a

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short peptide enables plasmin-mediated cleavage of the peptide and generation of active TGF-β [8,9].

Intracellular M6P receptors are responsible for sorting of lysosomal enzymes [10,11]. Approximately 10–20% of the total number of M6P/IGF II receptors is present on the surface of the cells [12] where they bind diverse M6P- and non M6P-containing ligands such as TGF-β, proliferin, IGF II, retinoic acid and urokinase-type plasminogen activator receptor [13].

In the present study, we coupled M6P-HSA to the surface of liposomes as a homing ligand to HSC. Liposomes have a high capacity for encapsulation of various drugs, but so far this drug delivery system had not been specifically targeted to HSC in fibrotic livers. We performed in vitro studies in primary cultures of HSC and in vivo experiments using a rat model of liver fibrosis to determine whether M6P-HSA modified liposomes specifically associate with HSC. Furthermore, the mechanism of this interaction was characterised. Since modification of HSA by M6P groups introduces negative charges to the protein, we also examined the interaction of M6P-HSA liposomes with primary cultures of liver endothelial cells (LEC) that express scavenger receptors (ScR) [14].

It is conceivable that ScR also play a role in the association of M6P-HSA liposomes with LEC and HSC.

2. Materials and methods

2.1. Materials

Cholesterol (Chol), *N*-succinimidyl-*S*-acetylthioacetate (SATA), cis-aconitic anhydride, monensin and polyinosinic acid (poly I) were from Sigma (St Louis MO, USA). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(maleimidophenyl)butyramide] (MPB-PE) were purchased from Avanti Polar Lipids (Alabaster AL, USA). [^{3}H]cholesteryloleyl ether (^{3}H-COE) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Human serum albumin fraction V was from the Central Laboratory of the Red Cross (Amsterdam, The Netherlands). Dulbecco's modified Eagles Medium (DMEM), RPMI-1640, L-glutamine were obtained from Invitrogen (Paisely, Scotland, UK), foetal calf serum (FCS) from BioWhittaker Europe (Verviers, Belgium), penicillin, streptomycin from Sigma. All other chemicals were analytical grade or the best grade available.

2.2. Animals

Specified pathogen free (SPF) male Wistar and Wag/Rij rats (Harlan, Horst, The Netherlands) were housed under standard laboratory conditions with free access to food and water. The local committee for care and use of laboratory animals approved the presented study.

2.3. Preparation of modified HSA

HSA was modified either with mannose 6-phosphate moieties [4] or with cis-aconitic anhydride yielding cis-aconitylated HSA (AcoHSA) [15] as described before.

2.4. Preparation of liposomes

Liposomes were composed of POPC, CHOL, MPB-PE in molar ratio of 23:16:1, labelled with a trace amount of ^{3}H-COE, a non-degradable bilayer marker and prepared as described before [16]. The phospholipid phosphorus content of each liposome preparation was assessed by phosphate assay [17]. The lipid concentration was calculated taking into account the amount of cholesterol in the preparation of liposomes. Size and size distribution were determined by dynamic laser light scattering with a Nicomp submicron particle analyzer (NICOMP 380 ZLS, Santa Barbara, CA, USA). The average diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. M6P-HSA and AcoHSA were subsequently coupled to liposomes by the SATA method described before [15]. The phospholipid phosphorus, protein content [18] and particle size of both M6P-HSA and AcoHSA liposomes were determined. Control liposomes were prepared similarly but they were not coupled with protein. Liposomes were stored under argon at 4 °C and used within three weeks after preparation.

2.5. Isolation of HSC and LEC

HSC were isolated from livers of male Wistar rats (550–600 g) (Harlan) as described before [19]. Isolated HSC were cultured in DMEM containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. One day before the experiments, HSC were trypsinized and seeded in 24 wells plates (Costar).

LEC were isolated from livers of male Wag/Rij rats (200–250 g) (Harlan) as described before [16]. LEC were cultured in RPMI-1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml endothelial cell growth factor (Boehringer, Mannheim, Germany) for 2 days in 24 wells collagen-coated plates (Greiner) before being used in the experiments.

2.6. Association of M6P-HSA liposomes and AcoHSA liposomes with HSC and LEC

To study association of M6P-HSA liposomes and AcoHSA liposomes in cultures of HSC or LEC, the cells were pre-incubated for 1 h with FCS-free medium. Next, 3 h incubation with ^{3}H-COE-labelled liposomes (160 nmol/ml, at 37 °C unless stated otherwise) in culture medium without FCS was performed. When appropriate, other agents were added to the incubation mixture as indicated. Incubation was stopped by placing the culture plate on ice and cells were washed with ice-cold PBS pH 7.4. Subsequently, cells were lysed using 0.4 M NaOH. Cell associated radioactivity was determined by liquid scintillation counting of the lysed cell suspension and radioactivity was normalized for protein content measured according to Lowry [20].

2.7. Detection of M6P/IGF II receptor expression by the cells

2.7.1. RNA isolation and reverse-transcription polymerase chain reaction

Total RNA was isolated from HSC and LEC using Absolutely RNA Microprep kit (Stratagene, Amsterdam, The Netherlands) according to the protocol of the manufacturer. Subsequently, synthesis of first strand cDNA from total cellular RNA was performed with SuperScript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and 40 units RNaseOut (Invitrogen) in a volume of 20 μl containing 250 ng random primers (Promega). One microliter of cDNA (10 ng/μl) was used for polymerase chain reaction in a total volume of 30 μl containing 1 unit Taq polymerase (*T. aquaticus*; Amersham Bioscienses, Roosendaal, The Netherlands) and 10 μM primers. Primers for M6P/IGF II rat receptor were as follows: forward 5′-GTGTCCTCTGGGTGTGGACT-3′, reverse 5′-CTCCTCCTTGCTGACCTTTG-3′ (Sigma Genosys). GAPDH (forward 5′-CGCTGGTGCTGAGTATGTCG -3′, reverse 5′- CTGTGGTCAT-GAGCCCTTCC-3′) (Sigma) was used as house keeping gene. Thirty cycles of reaction for M6P/IGF II receptor primers at 94 °C for 5 min, 94 °C for 30 s, 57 °C for 45 s, 72 °C for 45 s and 72 °C for 7 min, and 26 cycles for

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