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Note

Uptake characteristics of mannosylated and fucosylated bovine serum albumin in primary cultured rat sinusoidal endothelial cells and Kupffer cells

Yuriko Higuchi[a], Makiya Nishikawa[b], Shigeru Kawakami[a], Fumiyoshi Yamashita[a], Mitsuru Hashida[a,*]

[a] *Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606 8501, Japan*
[b] *Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606 8501, Japan*

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Abstract

The purpose of this study is to delineate uptake characteristics of mannosylated and fucosylated proteins in primary cultured sinusoidal endothelial cells and Kupffer cells. In cultured sinusoidal endothelial cells, uptake of mannosylated and fucosylated bovine serum albumin (BSA) was significantly inhibited by excess mannosylated and fucosylated BSAs but not by galactosylated BSA, suggesting that both glycosylated proteins might be primarily taken up via mannose receptors. In cultured Kupffer cells, uptake of fucosylated BSA was significantly inhibited by excess galactosylated BSA as well as mannosylated and fucosylated BSAs, although that of mannosylated BSA was inhibited only by mannosylated and fucosylated BSAs. This suggests that uptake of fucosylated BSA by Kupffer cells might be mediated by both Kupffer cell lectin (fucose receptor) and mannose receptor. On the other hand, in vivo hepatic uptake of fucosylated BSA was inhibited to a greater extent by $GdCl_3$ pretreatment than that of mannosylated BSA. Based on in vitro and in vivo experiments, it was concluded that fucosylated BSA is more Kupffer cell-selective because it exhibited a lower sinusoidal endothelial cell uptake than mannosylated BSA.

Keywords: Sinusoidal endothelial cells; Kupffer cells; Cell targeting; Drug delivery; Mannosylated BSA; Fucosylated BSA

1. Introduction

Receptor-mediated drug targeting is a promising approach to deliver therapeutic agents selectively to target cells and maximize their efficacy (Takakura

* Corresponding author. Tel.: +81 75 753 4525; fax: +81 75 753 4575.
E-mail address: hashidam@pharm.kyoto-u.ac.jp (M. Hashida).

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and Hashida, 1996). We have demonstrated the usefulness of carbohydrate receptor-mediated drug delivery systems directed at both liver parenchymal (Nishikawa et al., 1995; Kawakami et al., 1998, 2000; Opanasopit et al., 2001a) and non-parenchymal (Ogawara et al., 1999; Opanasopit et al., 2001b) cells. Glycosylation of macromolecular (Nishikawa et al., 1993) and particulate (Kawakami et al., 1998; Ishida et al., 2004) carriers and proteinous drugs themselves (Fujita et al., 1992) with 2-imino-2-methoxyethyl-1-thioglycoside, which we have used so far, has several advantages including the simplicity of its structure, ease of synthesis, broad applicability, and satisfactory targetability.

In a series of investigations, we have demonstrated that mannosylated (Ogawara et al., 1999) and fucosylated (Opanasopit et al., 2001b) bovine serum albumins (BSAs) are efficiently taken up by liver non-parenchymal cells (NPC), mainly composed of sinusoidal endothelial cells and Kupffer cells, after intravenous injection. Similar results have been obtained with mannosylated liposomes (Opanasopit et al., 2001a), and fucosylated liposomes (Kawakami et al., 2000). Inhibition experiments (Opanasopit et al., 2001b) have shown that efficient NPC uptake of mannosylated and fucosylated delivery systems is primarily due to specialized sugar recognition systems.

However, the uptake by NPC via mannose and fucose receptors appears to be complicated. In vitro binding experiments using isolated receptor proteins have shown that the mannose receptor recognizes mannose and fucose but not galactose (Haltiwanger and Hill, 1986; Otter et al., 1992), and that the fucose receptor recognizes fucose and galactose but not mannose (Lehrman et al., 1986). It is also known that the mannose receptor is present on both Kupffer cells (Kuiper et al., 1994) and sinusoidal endothelial cells (Magnusson and Berg, 1993; Otter et al., 1992), and that the fucose receptor is presented on Kupffer cells (Lehrman and Hill, 1986; Haltiwanger et al., 1986). The multiplicities in substrate specificity and cellular localization of the receptors make it difficult to evaluate the contribution of each pathway to the overall in vivo NPC uptake. Further details of the mechanisms involved in NPC uptake are required to develop efficient drug delivery systems that can target sinusoidal endothelial cells and Kupffer cells. In order to obtain such information, it is necessary to elucidate the contribution of mannose receptor and fucose receptor on the uptake of mannosylated and fucosylated BSA using primary cultured Kupffer cells and sinusoidal endothelial cells.

This dearth of mechanistic information prompted us to investigate the uptake characteristics of mannosylated and fucosylated BSAs in rat Kupffer cells and sinusoidal endothelial cells. After both cell types were isolated from rat liver and cultivated, the cellular uptake characteristics of mannosylated and fucosylated BSAs were evaluated in the presence of inhibitors. In order to clarify the contribution of these cell types in vivo, we examined the hepatic uptake of mannosylated and fucosylated BSAs after intravenous administration of $GdCl_3$, a compound that down-regulates the function of Kupffer cells, to mice (Hardonk et al., 1992).

2. Materials and methods

2.1. Chemicals

BSA, collagenase (type I), dexamethasone, vascular endothelial growth factor (VEGF) and Minimum essential medium eagle Joklik modification for suspension culture were purchased from Sigma Chemicals Inc. (St. Louis, MO, USA). D-Mannose, D-galactose, L-fucose, and chloramine-T were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Indium-111 chloride [^{111}In] was kindly supplied by Nihon Medi-Physics Co. Ltd. (Nishinomiya, Japan). Sodium iodide-125 [^{125}I] was purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). RPMI1640 and Eagle's MEM 'Nissui' were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Diethylenetriaminepentaacetic acid (DTPA) anhydride was purchased from Dojindo Laboratory (Kumamoto, Japan). Rat tail collagen type I, insulin-transferin-selenious acid (ITS+ Premix), and human recombinant fibronectin were purchased from Becton Dickinson (Franklin Lakes, N.J., USA). All other chemicals were of the highest grade available.

2.2. Animals

Male ddY mice (5 weeks old) and male Wistar rats (200–250 g) were purchased from Shizuoka Agricul-

tural Co-operate Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by US National Institutes of Health and with the Guidelines of the Kyoto University Animal Experimentation Ethics Committee.

2.3. The synthesis of BSA derivatives with sugar moieties

Coupling of mannose and galactose moieties to BSA was carried out according to the method of Lee et al. (1976). Briefly, cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-D-mannoside was treated with 0.01 M sodium methoxide at room temperature for 24 h, and a syrup of 2-imino-2-methoxyethyl-1-thio-D-mannoside (IME-thiomannoside) was obtained after evaporation of the solvent. A quantity of the resultant syrup was added to BSA (100 mg) in 10 ml 50 mM borate buffer (pH 9.0). Coupling of fucose moieties to BSA was carried out by the method of Lee et al. (1976) with minor modifications (Kawakami et al., 2000). The molecular weight of the glycosylated proteins was determined by SDS-PAGE and the number of sugar residues was determined by the anthrone-sulfuric acid method using galactose, mannose and fucose as standards (Table 1).

2.4. Radiolabeling with indium-111 and iodine-125

^{111}In-labeled BSA was prepared using a bifunctional chelating agent, DTPA anhydride (Hnatowich et al., 1982). ^{125}I-labeling was performed by the chloramine-T method (Hunter and Greenwood, 1962).

2.5. Cell isolation and culture of sinusoidal endothelial cells and Kupffer cells

Liver sinusoidal endothelial cells and Kupffer cells from rat liver were isolated according to the method of Nagelkerke et al. (1983) with some modification. Endothelial cells were plated at 3.0×10^5 cells/cm^2 onto 24-well plates coated with rat tail collagen type I and human recombinant fibronectin, and cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 5 μg/ml vascular endothelial growth factor (VEGF), 1% (v/v) ITS+ Premix, 10 mg/ml amphotericin B, 10 μM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM/ml L-glutamine for 3–4 days. Kupffer cells were cultured on 24-well plates (2.5×10^5 cells/cm^2) in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/ml L-glutamine, and 10 mg/ml amphotericin B for 24 h. Cells were cultured in a 5% CO_2–95% air incubator thermostatically controlled at 37 °C. The purity of the isolated endothelial cells and Kupffer cells was checked by factor VIII immunostaining and uptake by 4.5 μm latex beads, respectively.

2.6. Uptake of ^{125}I-glycosylated BSA in cultured liver sinusoidal endothelial cells and Kupffer cells

Prior to the uptake experiments, cells were equilibrated with serum-free RPMI medium containing 10 mM 2-[4-(2-hydoroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2% BSA at 37 °C for 20–30 min. Then, ^{125}I-glycosylated BSA (100,000 cpm/well) was added to the medium, followed by incubation at 37 °C for 2 h. In some groups, ^{125}I-glycosylated BSA was administered, together with different concentrations of mannosylated, fucosylated or galactosylated BSA. After 2 h incubation, the medium

Table 1
Physicochemical characteristics of synthesized glycosylated BSAs

Compounds	Molecular weight (Da)	Number of sugar residues (mol/mol BSA)
Mannosylated BSA (Man-BSA)	70800	36
Fucosylated BSA (Fuc-BSA)	70800	41
Galactosylated BSA (Gal-BSA)	68000	42

The number of molecular weight of glycosylated BSAs were estimated by SDS-PAGE. The number of sugar residue was determined by the anthron-sulfic method.

was collected, mixed with 45% trichloroacetic acid (TCA), and centrifuged to separate metabolites from intact glycosylated BSA. The radioactivity associated with metabolites in the supernatant was measured using a well-type NaI-scintillation counter (ARC-500; Aloka, Tokyo, Japan). In addition, the cells were washed three times with ice-cold phosphate-buffered saline and lysed with 0.25 ml of 0.3 M NaOH containing 0.1% Triton X-100. An aliquot of the lysate was taken for the determination of ^{125}I radioactivity and protein content. The radioactivity was counted by the scintillation counter while the protein content was measured by the modified Lowry method (Lowry et al., 1951) using BSA as a standard. The result of in vitro uptake experiment was expressed as total uptake amount of count of metabolites in the supernatant and of cell lysate.

2.7. In vivo hepatic disposition in mice pretreated with $GdCl_3$

An amount of 30 mg/kg $GdCl_3$ was intravenously injected into mice to down-regulate the functions of Kupffer cells (Hardonk et al., 1992). Twenty-four hours later, ^{111}In-mannosylated, ^{111}In-fucosylated or ^{111}In-galactosylated BSA (0.1 mg/kg) was administered by intravenous injection. At 5 min post injection, mice were killed under anesthesia and the liver was excised and plasma was collected. The radioactivity associated with these samples was determined in a well-type NaI-scintillation counter (ARC-500; Aloka, Tokyo, Japan).

3. Results

3.1. Uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA by sinusoidal endothelial cells and Kupffer cells

Fig. 1 shows the uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA in cultured sinusoidal endothelial cells. Without inhibitors, uptake of mannosylated BSA by sinusoidal endothelial cell was 1.4-fold higher than that of fucosylated BSA. In the presence of 50 mg/ml mannosylated or fucosylated BSA, the uptake of ^{125}I-mannosylated BSA was significantly inhibited (Fig. 1A). However, the uptake of ^{125}I-mannosylated BSA was not inhibited by the same concentration of galactosylated BSA (Fig. 1A). Similar inhibition patterns were observed with ^{125}I-fucosylated BSA (Fig. 1B).

Fig. 2 shows the uptake characteristics of ^{125}I-mannosylated and ^{125}I-fucosylated BSA in cultured Kupffer cells. In the absence of inhibitors, ^{125}I-mannosylated and ^{125}I-fucosylated were similar with respect to the degree of uptake by Kupffer cells, and their uptake was significantly greater than that by cultured endothelial cells. The uptake of ^{125}I-mannosylated BSA was inhibited by mannosylated and fucosylated BSAs, but not by galactosylated BSA, similar to that observed in cultured endothelial cells. In contrast, the uptake of ^{125}I-fucosylated BSA was inhibited by galactosylated BSA, as well as mannosylated and fucosylated BSAs. Thus, fucosylated BSA exhibits different uptake characteristics from mannosylated BSA in Kupffer cells.

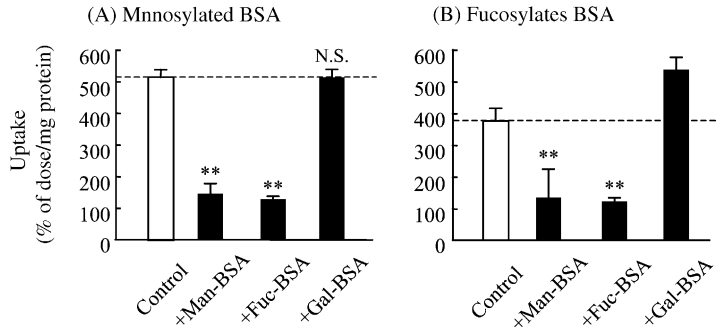

Fig. 1. The uptake of ^{125}I-mannosylated BSA (A), and ^{125}I-fucosylated BSA (B) by cultured endothelial cells. Cells were incubated for 2 h with or without an excess of other forms of glycosylated BSA (50 μg/ml). Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's t-test (**, $P < 0.01$; N.S., not significant).

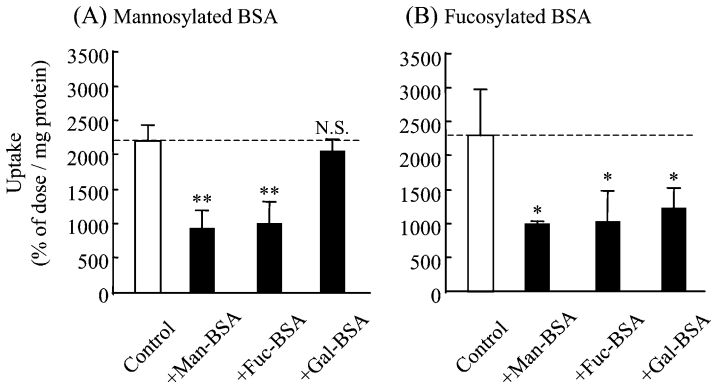

Fig. 2. The uptake of ^{125}I-mannosylated BSA (A), and ^{125}I-fucosylated BSA (B) by cultured Kupffer cells. Cells were incubated for 2 h with or without an excess of other forms of glycosylated BSA (50 μg/ml). Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$; N.S., not significant).

3.2. *Liver accumulation of ^{111}In-mannosylated and ^{111}In-fucosylated BSA with or without $GdCl_3$ pretreatment*

^{111}In-labeled BSA derivatives were used in the in vivo distribution studies, since ^{111}In is trapped in the cell interior after degradation so that the uptake rate can be evaluated precisely (Hnatowich et al., 1982). Fig. 3 shows the plasma concentration and hepatic uptake of ^{111}In-mannosylated and ^{111}In-fucosylated BSAs 5 min after intravenous injection. When mice were pretreated with $GdCl_3$, the plasma clearance and hepatic uptake of these proteins significantly decreased, and the inhibition effect was greater with fucosylated BSA than mannosylated BSA. Since $GdCl_3$ completely inhibits binding of mannosylated and fucosylated BSA to Kupffer cells without adversely affecting either hepatocytes or sinusoidal endothelial cells (Hardonk et al., 1992), it appears that the contribution of Kupffer cells to uptake by whole liver is greater in the case of fucosylated BSA.

4. Discussion

Haltiwanger and Hill (1986) isolated macrophage mannose receptors from alveolar macrophages, and showed that these receptors recognize mannose, fucose, and *N*-acetylglucosamine but not galactose. Otter et al. (1992) found that mannose receptors

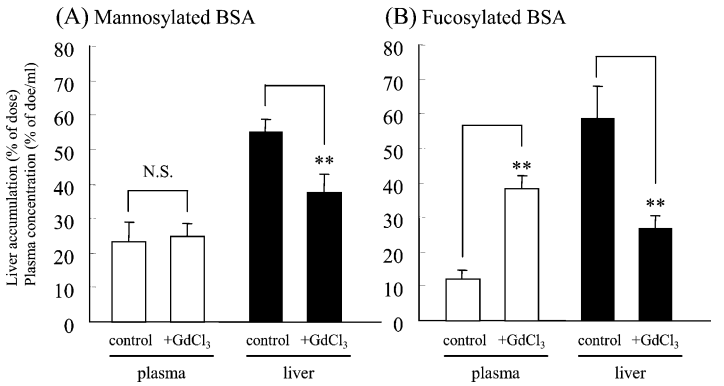

Fig. 3. Liver accumulation (■) and plasma concentration (□) of ^{111}In-mannosylated BSA (A), and ^{111}In-fucosylated BSA (B) (0.1 mg/kg) with or without pretreatment of $GdCl_3$ (30 mg/kg) at 5 min after intravenous injection into mice. $GdCl_3$ was injected 24 h before the experiments. Results are expressed as means + S.D. ($n = 3$). Statistical analysis was performed by Student's *t*-test (**, $P < 0.01$).

exhibiting the same substrate specificity are expressed in liver NPC. Magnusson and Berg (1993) reported that mannose receptors are expressed on isolated sinusoidal endothelial cells in binding experiments using an isolated sinusoidal endothelial cell suspension and ovalbumin, a mannose-terminated glycoprotein. In the present study, mannosylated and fucosylated BSAs were taken up efficiently by liver sinusoidal endothelial cells in primary culture, where the uptake was significantly inhibited by mannosylated and fucosylated BSAs but not by galactosylated BSA (Fig. 1). Therefore, it is likely that uptake of mannosylated and fucosylated BSAs by cultured sinusoidal endothelial cells is mediated primarily by mannose receptors.

Cultured Kupffer cells and sinusoidal endothelial cells exhibited similar inhibition profiles for mannosylated BSA, but not for fucosylated BSA: the uptake of fucosylated BSA was significantly inhibited by galactosylated BSA in Kupffer cells (Fig. 2B). Lehrman and co-workers isolated fucose receptors (Kupffer cell lectin) that are uniquely expressed in Kupffer cells by an immunohistological method (Lehrman and Hill, 1986), and demonstrated that binding of fucosylated BSA to these receptors can be inhibited by excess fucose or galactose, but not by mannose (Lehrman et al., 1986). In the present study, the uptake of fucosylated BSA was inhibited by galactosylated BSA (Fig. 2B), suggesting that fucose receptors might, at least in part, be responsible for the uptake of fucosylated BSA. In addition, mannose receptors also appear to be responsible for the uptake of fucosylated BSA, since the uptake of fucosylated BSA is inhibited by mannosylated BSA (Fig. 2B).

The present result for fucosylated BSA in Kupffer cells differed from that of Sarkar et al. (1996), who demonstrated that the uptake of fucose–human serum albumin (HSA) by isolated Kupffer cells was not inhibited by galactose–HSA. This difference might be due to the method of fucosylation: Sarkar et al. (1996) coupled *p*-aminophenyl-α-L-fucopyranoside and albumin with carbodiimide whereas we coupled albumin with 2-imino-2-methoxyethyl-1-thio-L-fucoside under weak alkaline conditions. Thus, it is likely that the difference in the structure at the 1-position of fucose might affect recognition of fucosylated albumin by Kupffer cell lectin that interacts with galactose. Glycosylation with 2-imino-2-methoxyethyl-1-thioglycoside is also characteristic in that the net charge of the proteins is unchanged since an imine is formed at the amino group modified with sugar. Jansen et al. (1991) demonstrated that para-aminophenyl mannose-terminated HSA was taken up by NPC via scavenger receptors, presumably due to an increase in negativity in accordance with sugar modification of the amino groups. However, they also showed that the liver uptake of mannosylated HSA synthesized using 2-imino-2-methoxyethyl-1-thiomannoside was not inhibited by formaldehyde-treated albumin, a typical scavenger receptor ligand (Jansen et al., 1991). Thus, the chosen method of sugar modification could play an important role in the design of glycosylated proteins.

We previously found that the cellular uptake of fucosylated BSA at 1 h post intravenous injection was 1.1, 6.2, and 21% of dose/10^8 cells for liver parenchymal cells, sinusoidal endothelial cells, and Kupffer cells, respectively (Opanasopit et al., 2001b), while that of mannosylated BSA was 0.841, 13.4, and 10.4% of dose/10^8 cells (Ogawara et al., 1999). To confirm the contribution of each cell type to the total hepatic uptake from a different point of view, we investigated the in vivo distribution of mannosylated and fucosylated BSAs in mice pretreated with $GdCl_3$ (Fig. 3). It is known that $GdCl_3$ completely inhibits the binding of mannosylated and fucosylated BSA to Kupffer cells without adversely affecting either hepatocytes or sinusoidal endothelial cells (Hardonk et al., 1992). The reduction in the hepatic uptake produced by $GdCl_3$ pretreatment was greater for fucosylated BSA than mannosylated BSA (Fig. 3). This finding corresponded to previous results that fucosylated BSA was taken up more selectively by Kupffer cells than mannosylated BSA (Opanasopit et al., 2001b; Ogawara et al., 1999). The in vitro uptake experiment (Figs. 1 and 2) showed that the uptake by cultured sinusoidal endothelial cells was greater for mannosylated BSA than fucosylated BSA whereas the uptake by cultured Kupffer cells was similar for both proteins. Thus, the higher Kupffer cell-selectivity of fucosylated BSA might be due to lower uptake by the sinusoidal endothelium. When the data obtained following $GdCl_3$ pretreatment were compared with the data obtained from cell fractionation studies, account was taken of the fact that the number of sinusoidal endothelial cells was approximately twice that of Kupffer cells (Blomhoff et al., 1982; Pertoft and Smedsrod, 1986).

5. Concluding remarks

Different carbohydrate receptors with relatively broad substrate specificity are present in different hepatic cell types. Mannosylated BSA appears to be taken up via mannose receptors regardless of sinusoidal endothelial cells and Kupffer cells. However, it is likely that the uptake of fucosylated BSA by Kupffer cells is mediated by not only mannose receptors but fucose receptors while its uptake by sinusoidal endothelial cells is mediated primarily by mannose receptors. Moreover, fucosylated BSA is more Kupffer cell-selective because it exhibited a lower endothelial cell uptake than mannosylated BSA. This is potentially useful information for the development of carbohydrate receptor-mediated delivery strategies for biologically active proteins.

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