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Specific uptake of asialofetuin-labeled liposomes by isolated hepatocytes

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Summary

Interactions between asialofetuin (AF)-labeled liposomes (AF-liposomes) and hepatocytes were investigated in vitro. The hepatocytes were isolated from rat liver by the collagenase perfusion method. Labeling the surface of liposomes with AF enhanced the hepatocyte uptake of liposomes consisting of phosphatidylcholine, phosphatidic acid and cholesterol. The uptake of AF-liposomes (cholesterol-labeled) by hepatocytes decreased on adding AF, and virtual total inhibition was attained with the addition of unlabeled AF-liposomes. The enhancing effect of AF on liposome uptake increased with liposomal cholesterol content. The membrane fluidity of AF-liposomes decreased with increasing cholesterol. From these results, there appears to be the possibility that AF-liposomes are specifically incorporated into hepatocytes through the action of galactose-binding protein.

Introduction

Pharmacologically active agents are effective depending on the means or methods employed to ensure each reaches the target intended. Specific receptors capable of recognizing a sugar with a galactose configuration have been reported to be present on the plasma membranes of hepatocytes (Van Lenten and Ashwell, 1972; Baenziger and Maynard, 1980), and thus used to target agents to hepatocytes (Banno et al., 1983; Spanjer and Scherphof, 1983; Das et al., 1985). Hepatocytes are considered to be suitable cells to which liposomes may be directed as targets, since liver capillaries possess a discontinuous basement membrane and a fenestrated endothelial lining (Wisse et al., 1982). The fenestration has the diameter of approximately 0.1 μm (Wisse et al., 1982) and only small liposomes can traverse the fenestrations (Poste et al., 1984).

Asialofetuin (AF) is a glycoprotein having several triantennary galactose terminated sugar chains (Spiro, 1973). Previously, we reported the disposition of AF (Aramaki et al., 1985) and AF-labeled liposomes (AF-liposomes) in rats (Tsuchiya et al., 1986; Hara et al., 1987). Both of them were rapidly cleared from systemic circulation and taken up by hepatocytes. The disposition of liposomes in a biological system is affected by numerous factors such as size, charge and lipid composition (Juliano and Stamp, 1975; Allen and Everest, 1983;

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Abra et al., 1984; Nagamura et al., 1986). Poste and Papahadjopoulos (1976) found liposomal membrane fluidity to be a determining factor in the uptake of liposomes into cells.

In the present study, an examination was made of the effects of AF labeling and cholesterol content on liposome uptake by hepatocytes in vitro.

Materials and Methods

Materials

Fetuin (F, type III), L-α-phosphatidic acid (PA) and galactose oxidase (type V) were purchased from Sigma (U.S.A.). AF was prepared by splitting the terminal *N*-acetylneuraminic acid (NANA) of F by the method of Spiro (1960); more than 90% of the NANA was removed from F as described previously (Aramaki et al., 1985). Phosphatidyl choline (PC), cholesterol (Chol), 1,6-diphenyl-1,3,5-hexatriene (DPH) and collagenase were from Wako Pure Chemicals (Japan). [^{14}C]Chol (NEC-018), NaB^3H_4 (NEC-086) and Aquasol-2 were obtained from New England Nuclear (U.S.A.). The other reagents were of the best grade available. Male Wistar rats (200–250 g) were purchased from Sizuoka Agricultural Co. (Japan).

Preparation of [^{3}H]Asialofetuin

[^{3}H]AF was prepared by the method of Gahmberg and Hakomori (1973), using NaB^3H_4 and galactose oxidase. The C-6 position of the galactose residue of AF was labeled by tritium; the specific radioactivity was obtained as 17.5 μCi/mg of AF. [^{3}H]AF was dissolved in Krebs-Henseleit buffer (pH 7.4, 0.9 mg/ml) for uptake experiments.

Preparation and sizing of liposomes

Unilamellar AF-liposomes consisting of PC, PA and Chol in a molar ratio of 6 : 1 : 1, 6 : 1 : 3 and 6 : 1 : 6 were prepared by the detergent dialysis method using 5 mg of AF as described in the previous report (Tsuchiya et al., 1986). By this method, AF is covalently coupled to palmitic acid which serves as a hydrophobic anchor among lipids of liposomal membrane. Control liposomes (N-liposomes) were also prepared without AF. In some experiments, trace amounts of [^{14}C]Chol were incorporated into the liposomal membranes as a marker of the lipid phase. The liposomes were successively extruded through polycarbonate membranes (Nuclepore Co., 1.0, 0.4, 0.2 and 0.1 μm) followed by dialysis through the same membrane (0.1 μm) against 10 mM Tris-HCl buffer (pH 7.4) containing NaCl (0.9%) at 4°C for 3 days. Liposome suspensions were routinely obtained at the concentration of 9.75–11.55 μmol of phospholipid(PL)/ml. Mean liposome diameter was 0.13 μm. The amount of AF effective toward the outer surface of AF-liposomes was determined by a hemoagglutination inhibition assay (Tsuchiya et al., 1986).

Preparation of hepatocyte suspension

Hepatocytes were isolated from rat livers according to the methods of Moldéus et al. (1978), and suspended (1×10^6 cells/ml) in Krebs-Henseleit buffer (pH 7.4). In some experiments, the hepatocytes were suspended in Eagle's medium (Nissui Seiyaku Co., Ltd.) followed by preincubation at 37°C for 1 h.

Hepatocyte uptake

Twenty μl of [^{3}H]AF (18 μg) solution or [^{14}C]Chol labeled AF- or N-liposome (195–231 nmol of PL) suspension was added to 30 ml of the hepatocyte (3×10^7 cells) suspension and then incubated at 37°C for 90 min. The suspension was gently shaken every 10 min and gassed with $O_2 : CO_2$ (95 : 5) throughout incubation. To 1 ml samples of the suspension withdrawn at the time indicated, ice-cold Krebs-Henseleit buffer (5 ml) was added. Following centrifugation at $100 \times g$ for 1 min, the pelleted cells were dispersed in 1% Triton X-100 (0.5 ml) and heated at 58°C to affect solubilization. The lysate was mixed with Aquasol-2 (7 ml) and counted for radioactivity. Inhibition experiments were performed similarly in the presence of unlabeled AF (3 μg/10^6 cells) or AF- or N-liposomes (325–385 nmol of PL/10^6 cells). Hepatocyte viability as estimated by Trypan blue exclusion was retained by more than 80% throughout the experimental period.

Membrane fluidity

Membrane fluidity of the AF-liposomes was estimated by fluorescence polarization using DPH as a fluorescence probe. Fluorescence labeling of AF-liposomes and the measurement of fluorescence polarization were done according to the method reported by Shinitzky and Barenholz (1974). Briefly, a tetrahydrofuran solution of DPH (1 mM) was diluted with phosphate-buffered saline. The DPH dispersion was mixed with equal volume of AF-liposomes (0.09–0.12 μmol of PL/ml) and incubated at 25°C for 1 h. The DPH-labeled AF-liposomes (3 ml) were mixed with 0.5 ml of hepatocyte membrane fraction (3.2 mg of protein/ml) prepared by the method of Hock and Hollemberg (1980). Fluorescence intensities (E_x 430 nm) were measured through a polarizer situated parallel and perpendicular against the vertically polarized exciting beam (E_x 360 nm) at 37°C (Shinitzky and Barenholz, 1974). The fluorescence polarization (P) value was then calculated.

Results

Uptake of AF by hepatocytes

The functional capacity of galactose-binding protein on the isolated hepatocytes was estimated on the basis of [^{3}H]AF. As shown in Fig. 1, the uptake of radioactivity by the hepatocytes was rapid, reaching a constant level by 45 min. The time course of uptake was essentially the same, with or without preincubation. Thus, an experiment without preincubation was carried out in which the acid-soluble radioactivities of the degraded products of [^{3}H]AF appeared in the incubation medium after 30 min (data not shown). In the presence of excess AF, the uptake of radioactivity was diminished to less than 5% at all points examined.

Uptake of AF-liposomes by hepatocytes

The uptake of AF-liposomes consisting of PC, PA and Chol in a molar ratio of 6:1:3 by hepatocytes was examined. When the hepatocytes were incubated with [^{14}C]Chol labeled AF- or N-liposomes, hepatocyte associated radioactivity

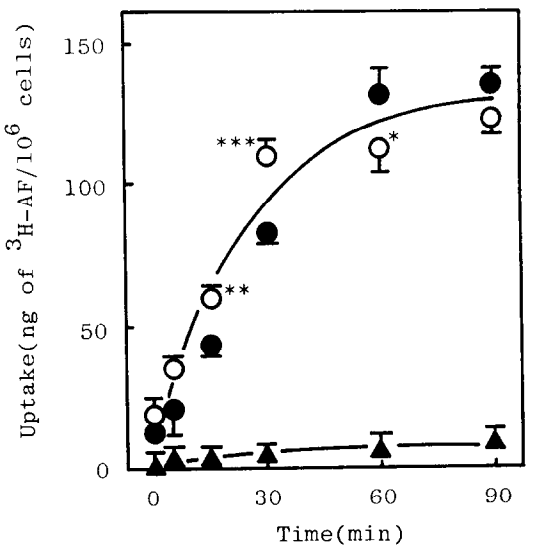

Fig. 1. Uptake of asialofetuin by isolated hepatocytes. Each point presents the mean from three individual experiments ± S.D. ○——○, [^{3}H]AF; ●——●, [^{3}H]AF(preincubated hepatocytes); ▲——▲, [^{3}H]AF + AF(preincubated hepatocytes). Statistical significant differences between with (●) and without preincubation (○); *, $P < 0.05$; * *, $P < 0.01$; * * *, $P < 0.001$. The uptake in the presence of unlabeled AF (▲) are significantly different ($P < 0.001$) from that in the absence (●, ○) at all points examined.

increased with the lapse of time (Fig. 2). The uptake of AF-liposomes was greater than that of N-liposomes. In the presence of AF, it was inhibited by about 50% and almost completely so by unlabeled AF-liposomes throughout these experiments (Fig. 2A). The uptake of N-liposomes, however, was not affected by the presence of AF, though unlabeled N-liposomes, as well as AF-liposomes, caused about a 60% suppression of uptake (Fig. 2B).

Effect of cholesterol content on the uptake of AF-liposomes

The uptake of liposomes containing various amounts of Chol in the lipid layer was investigated as a function of time (Table 1). In this experiment, the amount of AF (117–137) μg/μmol of PL) with effect on the outer surface of AF-liposomes was essentially the same regardless of Chol content. The extent of uptake by hepatocytes varied according to the type of liposomes. In the

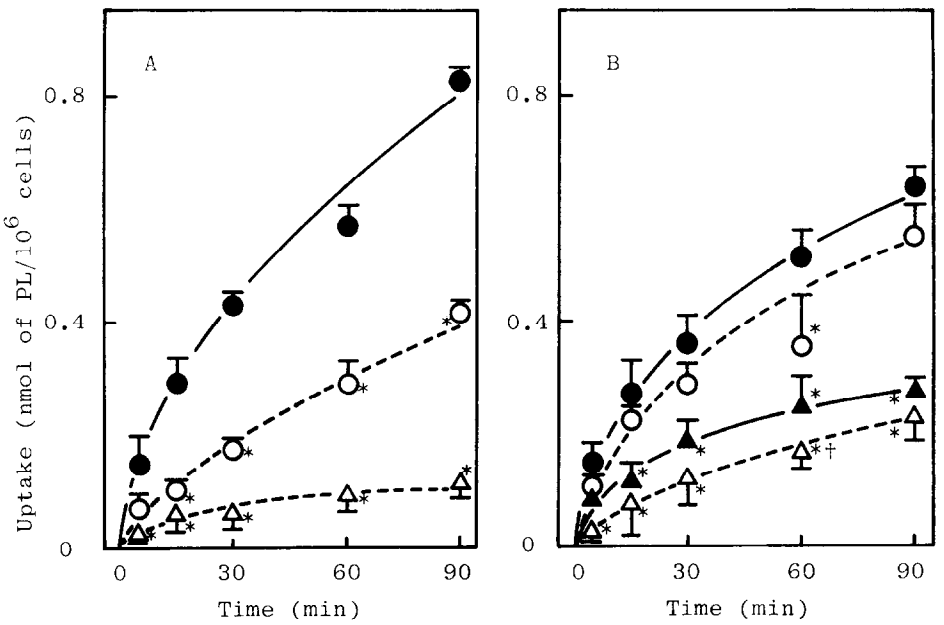

Fig. 2. Uptake of asialofetuin labeled liposomes (A) and control liposomes (B) by isolated hepatocytes (PC : PA : Chol = 6 : 1 : 3). Each point presents the mean from three individual experiments ± S.D. ●——●, [^{14}C]Chol-liposomes; ○——○, [^{14}C]Chol-liposomes + AF; △——△, [^{14}C]Chol-liposomes + AF-liposomes; ▲——▲, [^{14}C]Chol-liposomes + N-liposomes; *, significantly different ($P < 0.05$) when compared to the uptake without treatment (●); †, significantly different ($P < 0.05$) when compared to the uptake in the presence of unlabeled N-liposomes (▲) with that in the presence of unlabeled AF-liposomes (△).

TABLE 1

Effect of cholesterol content on the uptake of AF- and N-liposomes by hepatocytes

Incubation time (min)	AF [a] (3 μmol)	nmol [b] of PL/10^6 cells					
		6 : 1 : 1 [c]		6 : 1 : 3 [c]		6 : 1 : 6 [c]	
		AF-liposome	N-liposome	AF-liposome	N-liposome	AF-liposome	N-liposome
		132 [d]	–	117 [d]	–	137 [d]	–
0	–	0	0	0	0	0	0
	+	0	0	0	0	0	0
5	–	0.08 ± 0.06	0.11 ± 0.06	0.16 ± 0.06	0.13 ± 0.02	0.12 ± 0.01	0.15 ± 0.04
	+	0.01 ± 0.05	0.14 ± 0.06	0.08 ± 0.01	0.09 ± 0.03	0.12 ± 0.04	0.09 ± 0.05
15	–	0.20 ± 0.03	0.11 ± 0.04	0.30 ± 0.06	0.26 ± 0.03 [f]	0.43 ± 0.01 [e,f,g]	0.26 ± 0.05 [f]
	+	0.05 ± 0.01 [h]	0.16 ± 0.03	0.11 ± 0.01 [f,h]	0.23 ± 0.02	0.20 ± 0.03 [f,g,h]	0.16 ± 0.05
30	–	0.23 ± 0.07	0.15 ± 0.01	0.44 ± 0.02 [e,f]	0.36 ± 0.02 [f]	0.56 ± 0.07 [e,f]	0.35 ± 0.04 [f]
	+	0.17 ± 0.06	0.19 ± 0.01 [h]	0.18 ± 0.02 [h]	0.28 ± 0.02 [f,h]	0.23 ± 0.07 [h]	0.24 ± 0.04
60	–	0.51 ± 0.04 [e]	0.32 ± 0.04	0.58 ± 0.02 [e]	0.52 ± 0.02 [f]	0.82 ± 0.02 [e,f,g]	0.51 ± 0.09
	+	0.34 ± 0.08	0.40 ± 0.06	0.29 ± 0.04 [h]	0.34 ± 0.09	0.36 ± 0.09 [h]	0.31 ± 0.03 [h]
90	–	0.62 ± 0.07	0.53 ± 0.04	0.82 ± 0.02 [e,f]	0.65 ± 0.04 [f]	1.01 ± 0.04 [e,f,g]	0.56 ± 0.05
	+	0.58 ± 0.03	0.57 ± 0.06	0.41 ± 0.02 [h]	0.55 ± 0.06	0.43 ± 0.01 [h]	0.41 ± 0.06

[a] Uptake experiments were performed with (+) or without (−) AF.
[b] Mean from 3 experiments ± S.D.
[c] Lipid composition (molar ratio of PC, PA and Chol).
[d] Amount of AF (μg/μmol of PL) on the outer surface of liposomes.
[e] Significantly different ($P < 0.05$) when compared to corresponding N-liposomes.
[f] Significantly different ($P < 0.05$) when compared to corresponding liposomes (6 : 1 : 1).
[g] Significantly different ($P < 0.05$) when compared to corresponding liposomes (6 : 1 : 3).
[h] Significantly different ($P < 0.05$) when compared without AF.

TABLE 2

Effect of cholesterol content on the fluidity of AF-liposomes

PC : PA : Chol (molar ratio)	*P* value [a]	
	Without membrane fraction	With membrane fraction
6 : 1 : 1	0.178 ± 0.004	0.190 ± 0.011
6 : 1 : 3	0.244 ± 0.004 ***	0.229 ± 0.016 *
6 : 1 : 6	0.297 ± 0.003 ***,†††	0.273 ± 0.016 **,†

[a] mean from three experiments ± S.D.

Statistically significant difference from 6 : 1 : 1: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistically significant difference from 6 : 1 : 3: † $P < 0.05$, ††† $P < 0.001$.

case of AF-liposomes with the lowest Chol content (PC : PA : Chol = 6 : 1 : 1), the uptake was quite low and about the same as that of N-liposomes; i.e. AF labeling had no effect on liposome uptake. In the case of liposomes consisting of PC, PA and Chol (6 : 1 : 3), AF-liposome uptake significantly exceeded that of N-liposomes. With an increase in Chol content (PC : PA : Chol = 6 : 1 : 6) of AF-liposomes, this uptake became remarkably greater, but hardly any change could be noted in that of N-liposomes. The uptake of AF-liposomes at 90 min was about twice that of N-liposomes. This increased uptake with high Chol content was inhibited upon adding AF, becoming the same as that of N-liposomes not affected by AF.

Liposomal membrane fluidity

The membrane fluidity of AF-liposomes at various Chol contents was determined in the presence or absence of a hepatocyte membrane fraction at 37 °C. As shown in Table 2, the *P* values of AF-liposomes were increased with Chol contents, and this trend was not affected by the addition of the hepatocyte membrane fraction. Thus, AF-liposomes with higher Chol content may have higher solidity resulting from interactions between AF-liposomes and hepatocytes.

Discussion

The large size liposomes are preferentially taken up by Kupffer cells, while those smaller in size are transferred to hepatocytes through the endothelial fenestrations (Poste et al., 1982). We previously reported that the hepatocyte uptake of systematically administered small-sized liposomes (mean diameter, 0.13 μm) was significantly increased by AF labeling on the liposomal surface (Hara et al., 1987). This effect was found to depend on specific interactions between AF on the liposomal surface and galactose-binding protein on the hepatocyte surface.

To investigate the interactions between liposomes and hepatocytes in vitro in the present study, isolated hepatocytes were used. First, the functional ability of the galactose-binding proteins of the isolated hepatocytes was estimated. AF was rapidly taken up by the hepatocytes, and the results from a competitive experiment showed that the uptake occurred specifically through the action of galactose-binding proteins (Fig. 1). The detection of acid-soluble radioactivity in the incubation medium indicates the [^{3}H]AF to be internalized in the hepatocytes and subsequently degraded.

It was reported that the amount of uptake of galactose ligands by the hepatocyte at 4 °C was increased to several-fold by a preincubation at 37 °C (Weigel, 1980). But in our experiment at 37 °C, preincubation had no effect on the uptake of AF by hepatocytes. Since recycling of galactose receptors rapidly occurs at 37 °C (Schwartz et al., 1982), the amount of AF accumulating in a cell becomes much greater than that which binds to the surface of a cell.

Hepatocyte uptake of AF-liposomes (PC : PA : Chol = 6 : 1 : 3) significantly exceeded that of N-liposomes (Fig. 2); this result is in agreement with the results of our previous in vivo experiment on liver (Hara et al., 1987). Since AF-liposome uptake was reduced to half or virtually failed to occur by AF or unlabeled AF-liposomes (Fig. 2A), respectively, about 50% of the uptake of AF-liposomes must result from specific interactions brought about through the action of galactose-binding protein. The uptake of N-liposomes was not affected by AF but was equally inhibited by both AF- and N-liposomes (Fig. 2B). Hepatocytes may possibly take up [^{14}C]Chol in N-liposome membrane through nonspecific interactions such as adsorption, fusion or exchange.

The mechanism for the receptor-mediated uptake of low density lipoprotein (Goldstein et al., 1979), epidermal growth factor (Carpenter and Cohen, 1976) and glycoproteins (Stahl et al., 1978) by various kinds of cells is adsorptive endocytosis in all cases. This mechanism also appears applicable to the uptake of AF-liposomes by hepatocytes. Solid liposomes labeled with IgG have been reported readily phagocytosed into human neutrophils by F_c receptors on the cell surface, but fluid liposomes not to be phagocytosed (Munn and Parce, 1982). Chol is known as a "fluidity buffer" which decreases the fluidity of the liposomal lipid bilayer above the phase transition temperature (Papahadjopoulos et al., 1973). AF-liposome uptake by hepatocytes according to Chol content was investigated (Table 1), and found to depend on it, but that of N-liposomes to remain the same independent of this content. There may be the possibility that the effect of Chol to increase liposomal uptake can be exerted only through specific interactions with the receptors. Such a possibility finds support from the observations that the phagocytic uptake of lactosylceramide labeled liposomes by hepatocytes depends on their lipid composition (Banno et al., 1986).

In liposomes of limited Chol content, suppression of the effect of AF may in some way be related to the lateral diffusion of AF on the surface of the liposomal bilayer. Munn and Parce (1982) reported that IgG, labeled on solid liposomes, binds sequentially to F_c receptors of neutrophils without movement of IgG on the liposomal bilayer, and that the membrane of the neutrophil then forms coated pits for the occurrence of phagocytosis. The lateral diffusion of IgG on fluid liposomes is sufficiently rapid, and sequential binding of receptors to IgG results in IgG clustering at the point of initial attachment, thus, phagocytosis cannot occur (Munn and Parce, 1982). The membrane fluidity is frequently determined by a fluorescence polarization using DPH. This fluorescence probe is solubilized in gel or liquid crystalline phase of membrane and has anisotropic revolution depending on the viscosity constant of membrane. The P value indicates the lipid structural order parameter or the reciprocal of lipid fluidity (Van Blitterswijk et al., 1981). In our experiment on interactions between AF-liposomes and hepatocytes, the membrane fluidity of the former decreased (Table 2) and uptake increased with liposomal Chol content (Table 1).

On the basis of data presented above, liposome uptake by hepatocytes is concluded to be facilitated by AF labeling and that this can be controlled by the liposomal membrane fluidity. The possibility of using AF-liposomes as carriers of drugs into hepatocytes was also indicated in vitro.

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