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Tissue Distribution of Sialoglycopeptide-Bearing Liposomes in Rats

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The effect of sialoglycopeptide (GP) derived from fetuin on the tissue distribution of [^{3}H]inulin-containing small unilamellar vesicles (SUV) was examined in rats. As the amount of GP covalently bound to the SUV was increased, the liver uptake of GP–SUV decreased, while their level in the blood increased. No significant difference in the [^{3}H]inulin levels in heart, kidney, lung and spleen was observed after administration of control or GP–SUVs. These results suggest that surface modification of SUV with fetuin GP selectively alters their hepatic uptake. This may be a useful technique for designing liposomes with a long circulation time.

Keywords——tissue distribution; liposome; sialoglycopeptide; fetuin; surface modification; liver uptake

Previously, we demonstrated that modifying the surface of small unilamellar vesicles (SUV) with sialoglycopeptide (GP) derived from fetuin inhibited their clearance from the circulation of rats.[2)] However, the relationship between the clearance of GP–SUV and its uptake by hepatic tissue was not examined.

Recently, Allen and Chonn[3)] have shown that gangliosides and sphingomyelin dramatically diminish the rate and extent of uptake of liposomes by macrophages *in vivo*. The role of cell surface carbohydrates, in particular the surface sialic acid residues in cellular antirecognition phenomena is widely appreciated.[4-6)] Therefore, a series of experiments were conducted to determine the effect of GP-modification on the tissue distribution of intravenously administered SUV. Our results show that the liver uptake of SUV can be significantly inhibited by the GP-modification of the liposomal membrane.

Materials and Methods

Materials——Egg yolk phosphatidylcholine (PC), synthetic dipalmitoyl phosphatidylethanolamine (PE), *N*-acetylneuramic acid (NeuNAc), cholesterol (CH), and fetuin were obtained from Sigma Chemical Co. (St. Louis, MO). Pronase and PL HQ-Auto 15 were purchased from Kaken Seiyaku Co. (Tokyo, Japan) and Nissui Seiyaku Co. (Tokyo, Japan), respectively. RBC Sialic Acid Test Reagents were obtained from Kyokuto Pharmaceutical Industry Inc. (Tokyo, Japan). [^{3}H]Inulin (308 μCi/mg) was obtained from New England Nuclear (Boston, MA). All other reagents were commercial products of high purity and all operations utilized freshly redistilled water.

Preparation of GP–Liposomes Containing [^{3}H]Inulin——Glycopeptide was prepared from calf serum fetuin as previously described.[2)] Multilamellar vesicles (MLV) were prepared from 20 μmol of PC, 10 μmol of CH and 5 μmol of PE. After evaporation of the solvent $CHCl_3$, the dry lipid film was dispersed with 1 ml of phosphate-buffered saline (PBS) containing 30 μCi of [^{3}H]inulin. The suspension was then sonicated in a probe-type sonicator (Tomy Seiko, UR-200R) for a total of 60 min (2 s sonication with 2 s cooling periods) at 0°C. The SUV formed was centrifuged at 3000 rpm to remove metal powder, which was derived from the tip of the probe during the sonication, then gel-filtered on a Sephadex 4B column (control SUV). The filtrate was transferred to a test tube and was mixed with 1—4 μmol of GP contained in 0.2 ml of PBS. Glutaraldehyde (100 μl of 25% solution) was added slowly to the SUV suspension and the mixture was incubated at room temperature. After 30 min, 71 μl of 50% ethanolamine was added and the mixture

was allowed to stand for an additional 1 h. Uncoupled ethanolamine, glutaraldehyde and untrapped [^{3}H]inulin were removed by gel chromatography on a Sepharose 4B column (1.2 × 30 cm). Thus, two types of GP–SUV (GP/PE = 0.015 and 0.022, molar ratio) were prepared.

Estimation of Sialic Acid and Lipid——Liposomes were lysed by 2.5% Triton X-100 and the liposome-bound GP content was estimated by determination of sialic acid using the method of Jourdian *et al.*[7] Three mol of sialic acid was considered to be equivalent to 1 mol of GP, assuming that GP has three sialic acid residues. Phospholipid concentration in vesicle preparations was measured by enzymatic choline determination using a PL HQ-Auto 15 Kit.

Size Distribution of Liposomes——The size distribution of [^{3}H]inulin-containing SUVs was estimated from that of cold SUVs, which were prepared by using the same procedure described above except for replacement of [^{3}H]inulin with inulin. The size distribution of cold liposomes was measured in an Autosizer, model 700 (Mulvern, England).

***In Vivo* Experiments**——Male Wistar rats weighing 200 ± 20 g were used in all experiments. The rats were anesthetized with ether and fixed on a board. The left femoral vein and the left and right femoral arteries were cannulated with heparinized polyethylene tubing (Hibiki, Size 3). The penis was ligated and the urinary bladder was cannulated with two polyethlene tubes which were joined in parallel. Urine was collected for 3 h and at the end of the sampling period the bladder was washed with PBS and the washings were put together with the urine. Control or GP–SUV (0.3 ml, 1 μmol of PC) was administered *via* the femoral vein cannula at the rate of 0.6 ml per min. Blood samples (*ca.* 200 μl) were withdrawn by needle puncture from the jugular vein at set time intervals after administration and collected in heparinized tubes. At 30 min or 3 h after the administration, the rat was exsanguinated through the two femoral artery cannulas. At the same time about 20 ml of PBS was infused through the femoral vein to flush out remaining blood. Then the heart, liver, kidney, lung and spleen were quickly excised, rinsed with an ice-cold saline solution and weighed.

Determination of Radioactivity in Blood and Tissues——Each 200 μl blood sample was added to 1.5 ml of a Soluene-350/2-propanol mixture (Soluene-350 : 2-propanol = 1 : 1). Then 0.5 ml of 30% H_2O_2 was added and the mixture incubated at 40°C for 1 h. The decolorized solution was neutralized with 10% CH_3COOH and added to 10 ml of Scintisol EX-H. The mixture was left to stand in the dark overnight, and the radioactivity of samples (*CA*) was measured in a scintillation counter (Aloka, LSC-673). Tissue samples (0.2 g) were cut into fine pieces and dissolved in 1 ml of Soluene-350 at 50°C overnight. To these solutions, 0.5 ml of 2-propanol was added. Further procedures were the same as those described for blood samples. Urine samples were diluted with PBS to a final volume of 3 ml, then 60 μl of diluted sample was added to 10 ml of Scintisol EX-H. Total radioactivity in the circulation was expressed as a percentage of the injected radioactivity.

$$\% \text{ of dose} = V_d \cdot CA/DA \times 100 \quad (1)$$

DA is the injected radioactivity of [^{3}H]inulin and V_d is the apparent volume of distribution, which was estimated from Eq. 2.

$$V_d = DA/CA\ (t=0) \quad (2)$$

The zero-time intercept obtained by extrapolation of the initial linear portion of the percentage of dose *versus* time plot to $t=0$ is $CA\ (t=0)$. Radioactivities in heart, liver, kidney, lung, spleen and urine were calculated and expressed as percent of the dose.

Results and Discussion

The diameter of control SUV was 72.8 ± 25 nm (mean ± S.D.) and the equivalent normal weight distribution peak was at 42 nm. The diameter of GP–SUV (GP/PE = 0.015) was 77.6 ± 25 nm (mean ± S.D.) and the equivalent normal weight distribution peak was at 52 nm. The GP–SUV (GP/PE = 0.022) had the diameter of 72.0 ± 24 nm (mean ± S.D.) and the equivalent normal weight distribution peak at 43 nm. Thus, SUV with various amounts of GP were prepared without changing the lipid composition and size distribution. It is considered that the administered [^{3}H]inulin-containing control or GP–SUV had essentially the same size distribution as the cold control or GP–SUV.

The clearances of the control and two types of GP–SUV are shown in Fig. 1. A reduced clearance of GP–SUV was associated with increasing amounts of GP-modification. The pattern of the elimination of GP–SUV was consistent with previous observations.[2] The effect of the GP-modification on the 3 h tissue distribution of liposomes is shown in Table I. As the amount of GP-modification was increased, the uptake of liposomes by the liver decreased significantly and the blood level increased. There was no significant difference in [^{3}H]inulin levels in heart, kidney, lung and spleen between control and GP–SUV. On the other hand the

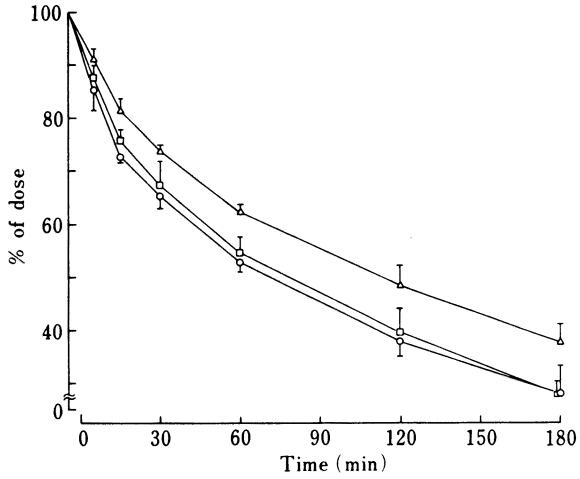

Fig. 1. Clearance of GP–SUV from the Circulation of Rats

Rats were injected intravenously with control SUV (○), GP–SUV (□; GP/PE = 0.015) and GP–SUV (△; GP/PE = 0.022). Each animal received 0.3 ml of [^{3}H]-inulin-containing liposome suspension (1 μmol of PC). Total radioactivity in the circulation was measured at various time intervals and the results are expressed as percentage of the injected dose according to Eq. 1. Each point represents the average of four animals ± S.E.

TABLE I. Tissue Distribution of SUV Carrying Various Amounts of Sialoglycopeptide[a)]

Tissue	Control (% of dose)		GP–SUV (GP/PE = 0.015) (% of dose)		GP–SUV (GP/PE = 0.022) (% of dose)	
	Observed	Estimated[b)]	Observed	Estimated[b)]	Observed	Estimated[b)]
Blood	28.0 ± 3.99	32.6	27.7 ± 2.58	36.6	37.8 ± 3.57	61.7
Heart	3.8 ± 0.34	4.4	2.2 ± 0.09	2.9	3.4 ± 0.59	5.6
Liver	42.6 ± 1.61	49.6	33.2 ± 1.99[c)]	43.9	17.0 ± 0.80[d)]	27.7
Kidney	10.1 ± 1.33	11.8	5.0 ± 0.57[c)]	6.6	7.0 ± 1.19	11.4
Lung	2.4 ± 0.25	2.8	1.6 ± 0.20	2.1	1.9 ± 0.47	3.1
Spleen	1.9 ± 0.08	2.2	2.7 ± 0.24[c)]	3.6	1.6 ± 0.09	2.6
Urine	14.1 ± 5.49	—	24.3 ± 2.33	—	38.7 ± 1.16	—

Results are expressed as percentage of injected dose of liposomal radioactivity in each organ (mean ± S.E., $n = 4$). *a*) Rats were sacrificed at 3 h after the administration. *b*) Values were estimated from observed values according to Eq. 3. *c*) $p < 0.05$. *d*) $p < 0.01$: significantly different from the control.

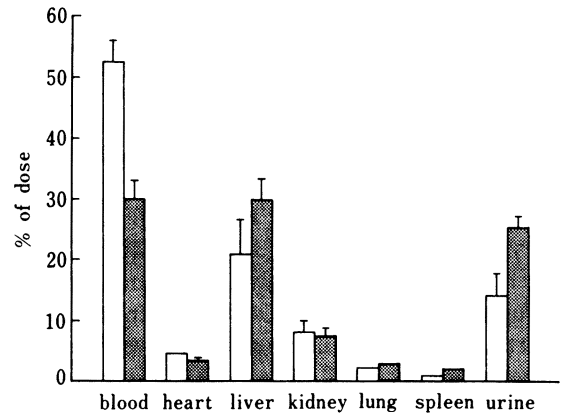

Fig. 2. Time Course of Tissue Distribution of GP–SUV

Rats were injected intravenously with GP–SUV (GP/PE = 0.01, 1 μmol of PC). At 30 min or 3 h after the administration rats were sacrificed and radioactivity in each organ was measured. The results are expressed as percentage of the injected dose (□, 30 min; ▨, 3 h). Each bar represents the average of four animals ± S.E.

level of radioactivity in the urine was increased with increasing amount of GP-modification. The tissue distribution of liposomal radioactivity at 30 min and 3 h after the administration of GP–SUV (GP/PE = 0.01; equivalent normal weight diameter, 101.3 nm) is shown in Fig. 2. The ^{3}H level in liver reached 20% by 30 min post-injection, and about 14% was present in urine and 8% in the kidney. The major fraction (52%) was found in the blood. By 3 h, the blood

level of radioactivity had decreased to 30%, while the ^3H level in liver had increased by 9% and that in the urine by 11%.

When free inulin was administered intravenously in rats, it was cleared rapidly by the kidney and excreted into urine. Abra *et al.* reported that the half-life for free inulin averaged 15 min with approximately 0.6% of the dose remaining in blood, 2.5% in the liver, and 0.15% in the spleen at 1 h post-injection.[8] Therefore, free [^3H]inulin in this study probably made no significant contribution to the measurement of GP–liposomal tissue distribution. As the lipid composition and the size distribution of GP–SUV were essentially the same as the control SUV, the difference in the tissue distribution can be substantially accounted for by the amount of GP-modification. Earlier, we reported that the latency of SUV in pooled plasma was reduced by GP-modification.[2] The increase of ^3H level in urine associated with the increase of GP-modification may be due to the leakage of [^3H]inulin through the liposomal membrane. In contrast, the liver uptake was decreased with an increase of GP-modification. It is conceivable that the decreased liver uptake over 3 h may have been due to the leakage of [^3H]inulin from the GP-modified liposomes. To examine this possiblility, estimated tissue levels were calculated from Eq. 2 assuming no leakage of [^3H]inulin from liposomes, and the results are shown in Table I. If the leaked [^3H]inulin is recovered completely in urine, the following equation may be written for estimated tissue levels.

$$x' = x/(100-u) \times 100 \quad (3)$$

Where x' and x are estimated and observed percentage of dose in each tissue, respectively and u is observed urinary percentage of dose. The estimated ^3H levels in liver were 27.7, 43.9 and 49.6% at GP/PE equal to 0.022, 0.015 and 0 (control), respectively (Table I). Thus, the liver uptake of liposomal inulin was decreased significantly, even when the leakage of marker by GP-modification is taken into consideration. In addition, a greater fraction of the dose remained at 3 h after the administration of GP-liposomes (GP/PE = 0.022) compared to when control liposomes were administered.

In the present study it was shown that the clearance from the circulation and liver uptake of GP–SUV were significantly influenced by a small change of the GP/PE ratio. The change of this ratio from 0.015 to 0.022 corresponded to an increase of sialic acid residues of 0.1 μmol, since GP has three sialic acid residues. The studies reported by Allen and Chonn[3] on the liver uptake of monosialylganglioside (G_{M1})-modified large unilamellar liposomes (LUV) have also indicated that the liver uptake of LUV was decreased by about 10% with an increase of G_{M1} content which was comparable with the increase of sialic acid residues in our experiment. The disposition of GP–SUV seems to be greatly affected by the amount of GP-modification.

It is generally accepted that MLV are mainly taken up by the Kupffer cells, while SUV (smaller than 100 nm) are taken up by parenchymal cells.[9] It has also been reported that the phagocytosis of liposomes by macrophages was diminished by the binding of sialoglycoprotein to the liposome surface,[10] and that the phagocytic reaction of human PMN cells was markedly suppressed when the sialoglycoprotein of human erythrocytes was incorporated into liposomes.[11] However, it is unclear which uptake (by Kupffer cells of parenchymal cells) was inhibited by GP-modification of SUV. Further studies are under way to investigate the distribution of GP-liposomes within the liver cell fraction.

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References and Notes

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