The Disposition of Serum Proteins as Drug-Carriers in Mice Bearing Sarcoma 180

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The tumor distribution and the disposition of serum proteins, such as albumin, fetuin, transferrin, and IgG, were investigated in mice bearing Sarcoma 180. Serum proteins labeled with fluorescein isothiocyanate (FITC) were administered to the mice. The FITC-labeled proteins acylated with glutaric anhydride were also administered to the mice in order to investigate the effect of chemical modification. The plasma concentration of each glutarylated serum protein was significantly lower, about 15 to 46-fold, in comparison to that of the non-acylated protein at 24 h after administration. The tissue distributions of the glutarylated serum proteins were also decreased compared to those of the non-acylated proteins. Especially, the hepatic distribution of albumin and IgG was significantly reduced with glutarylation. The urinary excretion of albumin and transferrin, and fecal excretion of IgG, were significantly increased with glutarylation. The serum proteins were accumulated effectively in the tumor tissue in mice bearing Sarcoma 180. It was found that the tumor distributions were not impaired by the glutarylation, except involving fetuin. It was suggested, therefore, that the glutarylated serum proteins were valuable for relative tumor-selectivity and might be utilized in a macromolecular carrier system for antitumor drugs.

Keywords tumor targeting; serum protein; drug carrier; acylation; disposition

Site-specific drug delivery has been one of the most actively pursued goals in chemotherapeutics. Drug carriers offer significant promise for the selective delivery of antineoplastic agents.¹⁾ The effectiveness of a variety of drug-macromolecule conjugates has been demonstrated *in vitro* and *in vivo*, e.g., albumins,^{2,3)} antibodies,^{4–6)} glycoproteins,^{7,8)} dextrans,^{9–11)} and synthetic macromolecules such as polylysine.¹²⁾

These macromolecules accumulate in the tumor tissues, which have a vascular network characterized by both enhanced neovasculature permeability and a lack of a lymphatic recovery system.¹³⁾ The physicochemical properties of macromolecular carriers, such as molecular weight and electric charge, influence the biological properties of the macromolecular conjugates.^{14,15)}

We have previously reported the covalent attachment of mitomycin C (MMC) to serum albumin to give MMC-albumin conjugates.²⁾ Remarkable differences were observed in the macromolecular structure, such as an α -helix and a difference in molecular size between the intact and the modified albumin conjugates. The structural differences affected the distribution of the conjugates to the normal tissues.³⁾ These findings led us to inquire whether the glutarylation of the serum proteins might enhance the tumor selectivity of the macromolecular carriers.

In the present paper, the dispositions of such serum proteins as albumin, fetuin, transferrin, and IgG, both in intact and acylated forms, were studied in mice bearing Sarcoma 180 (S180) using a fluorescence-labeling technique.

Materials and Methods

Reagents Bovine serum albumin (BSA), fetuin, transferrin and IgG were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). BSA and fetuin were purified by dialysis or gel filtration on Sephadex G-150 before use. Fluorescein isothiocyanate (FITC) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade or the best quality available.

Preparation of FITC-Labeled Serum Proteins BSA, fetuin, transferrin and IgG were used to prepare FITC-labeled serum proteins (Protein-FITC).

FITC was covalently attached to the serum proteins according to the previous method.¹⁶⁾ FITC (2 mg) dissolved in 0.5 M NaHCO₃-Na₂CO₃ (pH 9.2, 2 ml) was added dropwise to a stirred solution of the serum protein (100 mg protein) in the same buffer (10 ml). The reaction mixture was gently stirred for 4 h, then applied on a column of Sephadex G-25 (50 × 200 mm) developed with 0.9% NaCl. The Protein-FITC was eluted in the void volume, and free FITC was retarded by the gel. Fractions containing Protein-FITC were collected and lyophilized, giving a water-soluble powder. This was further purified by the use of a Sephadex G-150 column (25 × 470 mm), and lyophilized.

The reaction of Protein-FITC with the glutaric anhydride resulted in glutarylated Protein-FITC (G-Protein-FITC).²⁾ To a stirred solution of Protein-FITC (100 mg protein) in 1 M NaHCO₃ (10 ml), glutaric anhydride (400 mg) was added slowly in small portions. The pH of the reaction mixture was maintained within a range of 7.8–8.2 by the addition of 1 M NaOH for 1 h and stirred gently at 4°C for 16 h. Unreacted glutaric anhydride was separated from the mixture by the use of a Sephadex G-25 column. Fractions containing G-Protein-FITC were collected and lyophilized, giving a water-soluble powder. This was further purified by the use of a Sephadex G-150 column and lyophilized.

The fluorescein isothiocarbamyl (FITC) contents of FITC-labeled proteins (Protein-FITC and G-Protein-FITC) were calculated from the absorbance at 493 nm in buffered saline with reference to the absorbance of the sodium fluorescein standard. The protein contents of the FITC-labeled proteins were determined by the method of Lowry *et al.*¹⁷⁾ for each protein standard. The free amino groups of the conjugates were determined according to the method of Habeeb.¹⁸⁾

Size-Exclusion Chromatography An HPLC system (CCPD, Tosoh, Tokyo, Japan) equipped with a column of TSKgel G3000SW (7.5 × 600 mm, Tosoh) was used according to the previous method.²⁾

Animal Experiments Male ddY mice (18–22 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). S180 cells were maintained in ddY mice by weekly intraperitoneal transplantation. S180 cells (1×10^7 cells/mouse) were inoculated into the subcutaneous tissue of the axillary region of ddY mice. Ten to 14 d after the inoculation, the mice bearing S180 were administered the FITC-labeled protein (7 mg protein/mouse) through the tail vein, then housed in metabolic cages. Blood was collected from the *vena cava* under ether anesthesia and the tumor tissue, liver, spleen, kidney, lung, heart, intestine, muscle and brain were excised and weighed. The blood was centrifuged at 10000 g for 30 s to obtain a plasma sample. The excised tissues were homogenized (10%) in 0.1 M NaH₂PO₄-Na₂HPO₄ (pH 7.4). Urine and feces samples were collected at adequate intervals and stored in a freezer until assay. Urine was diluted with 0.1 M NaH₂PO₄-Na₂HPO₄ (pH 7.4) and feces was homogenized with the same buffer.

Analytical Method FITC-labeled proteins in the plasma, homogenates and urine were measured in 2 ml of 0.5 M Tris-HCl buffer (pH 7.5)

containing 0.1% sodium dodecyl sulfate (SDS) using a fluorescence spectrophotometer 650-10S (Hitachi, Ltd., Tokyo, Japan) at 495 nm for excitation and 520 nm for emission.

Results

Synthesis of FITC-Labeled Serum Proteins and Glutarylation The serum proteins used were labeled with FITC, then glutarilated with glutaric anhydride. Protein-FTC and G-Protein-FTC were obtained by the purification of gelfiltration. They showed an absorbance which contains the pattern of a fluorescein between 400 and 600 nm (data not shown). The FTC contents of the proteins were 0.6–1.2 w/w% per protein (Table I). Although the serum proteins were highly glutarilated (71.5–97.1%), the glutarylation did not markedly reduce the FTC content of Protein-FTC.

Tissue Distribution of Albumin-FTC and G-Albumin-FTC Figure 1 shows the tissue distribution of albumin-FTC and G-albumin-FTC in mice bearing S180. After intravenous injection, these were eliminated from the plasma being distributed to the tumor and the normal tissues. Although 10% of the albumin-FTC dose was still retained in the plasma at 24 h after injection, G-albumin-FTC had almost disappeared from the plasma by this time. The plasma concentrations were plotted on a semilogarithmic graph and

were analyzed by a one-compartment open model (Fig. 2). At the initial stage, albumin-FTC and G-albumin-FTC were eliminated from plasma with a half life of 9.75 ± 0.14 h and 5.92 ± 0.48 h, respectively. The elimination rate of G-albumin-FTC was much faster than that of albumin-FTC. This finding suggests that the elimination rate of albumin from the circulation was accelerated by glutarylation.

Tissue Distribution of FITC-Labeled Proteins Figure 3 shows the plasma concentration of the FITC-labeled proteins at 24 h following intravenous injection. The plasma concentration ratios of G-Protein-FTC to Protein-FTC were 1/50 (albumin), 1/15 (fetuin), 1/45 (transferrin), and 1/21 (IgG). Each concentration of G-Protein-FTC tested was significantly lower than that of the Protein-FTC. These

Fig. 2. Plasma Concentration-Time Curves of Albumin-FTC (○) and G-Albumin-FTC (●) after Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice. *a)* $p < 0.05$ compared with the Protein-FTC.

TABLE I. FTC Content and Degree Glutarylation of Protein-FTC and G-Protein-FTC

Carrier protein	FTC content ^{a)}		Glutarylation ^{b)}
	w/w%	mol/mol	
Albumin-FTC	1.16	1.97	
G-Albumin-FTC	0.91	1.53	97.1
Fetuin-FTC	0.64	0.72	
G-Fetuin-FTC	0.59	0.67	71.5
Transferrin-FTC	0.56	1.11	
G-Transferrin-FTC	0.57	1.13	72.0
IgG-FTC	0.79	3.03	
G-IgG-FTC	0.60	2.30	90.9

a) FTC content of each carrier protein was analyzed by the method of UV absorption. Protein content was analyzed by the method of Lowry *et al.* Each data represents FTC/protein. *b)* Free amino groups were analyzed by the method of Habeeb.

Fig. 1. Tissue Distributions of Albumin-FTC (A) and G-Albumin-FTC (B) after Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice. ●, plasma; ▲, tumor; ■, liver; ○, kidney; △, spleen.

Fig. 3. Plasma Concentrations of Protein-FTC (□) and G-Protein-FTC (▨) at 24 h Following Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice. *a)* $p < 0.05$ compared with the Protein-FTC.

Fig. 4. Tissue Distributions of Protein-FTC (□) and G-Protein-FTC (▨) at 24h Following Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice. A, albumins; B, fetuins; C, transferrins; D, IgGs. a) $p < 0.05$ compared with the Protein-FTC.

findings indicate that the elimination rates of serum proteins are accelerated by glutarylation.

The normal-tissue distribution of the FITC-labeled proteins is shown in Fig. 4. The concentration of G-Protein-FTC in all the tissues, except transferrin in the liver (Fig. 4C) and IgG in the muscle and brain (Fig. 4D), was lower than those of the Protein-FTC. In the case of FITC-labeled albumins (Fig. 4A), the amounts of distribution in the reticulo-endothelial system, such as in the liver, spleen and lung, were significantly decreased by glutarylation. The liver concentration of IgG-FTC was highest at a 12% dose per gram tissue, which corresponded to a 30% dose, if the liver weight of the mice is taken into consideration. By contrast, G-IgG-FTC was distributed in the liver at only 2.5% of the dose (Fig. 4D). These findings suggest that the distribution of serum proteins in normal tissue is depressed by glutarylation.

Tumor Accumulation of FITC-Labeled Proteins Figure 5 shows the tumor distribution of Protein-FTC and G-Protein-FTC. FITC-labeled proteins were distributed to the tumor at a concentration of 1.5–5.5% of dose/g tissue. The tumor distribution of serum proteins, except fetuin, was not affected by glutarylation. On the basis of the data shown in Figs. 3 and 5, the concentration ratio of tumor to plasma (T/P ratio) was evaluated. The T/P ratio of

IgG-FTC was 2.34 ± 1.16 , which was the highest value involving Protein-FTC (Fig. 6). As seen in Fig. 6, however, the T/P ratios of G-albumin-FTC, G-fetuin-FTC, G-transferrin-FTC and G-IgG-FTC were 26.3 ± 14.6 , 11.5 ± 7.5 , 43.9 ± 23.3 and 61.0 ± 15.2 , respectively. The T/P ratios of the serum proteins were markedly enlarged by glutarylation.

Urinary and Fecal Excretions of FITC-Labeled Proteins

In order to clarify the accelerated elimination of serum proteins from the circulation, urinary and fecal excretions of FITC-labeled proteins were investigated. Table II shows the cumulative urinary and fecal excretion of the serum proteins. It was found that the urinary excretion was significantly increased by glutarylation in albumin and transferrin. The rates of fecal excretions, up to 24h, of transferrin and IgG were also increased by glutarylation.

Catabolism of Albumin-FTC and G-Albumin-FTC Since the urinary excretion of albumin-FTC was significantly increased by glutarylation, the urine was analyzed by means of high performance size-exclusion chromatography. As shown in Fig. 7, although a small amount of high-molecular fraction was seen in the urine after intravenous injection of G-albumin-FTC, most of the dose administered was excreted as low-molecular compounds. Furthermore, when the urine was treated by trichloroacetic acid (TCA),

Fig. 5. Tumor Concentrations of Protein-FTC (□) and G-Protein-FTC (▨) at 24 h Following Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice. *a)* $p < 0.05$ compared with the Protein-FTC.

Fig. 6. Concentration Ratio of Tumor to Plasma of Protein-FTC (□) and G-Protein-FTC (▨) at 24 h Following Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice. *a)* $p < 0.05$ compared with the Protein-FTC.

$86.7 \pm 1.0\%$ (albumin-FTC) and $70.4 \pm 7.9\%$ (G-albumin-FTC) of the fluorescence intensity of the urine samples were detected in the acid-soluble fraction. These findings indicate that intravenously administered albumin-FTC and G-albumin-FTC were excreted into urine as low molecular compounds.

TCA-treatment of the plasma and tissue homogenates was done in order to compare the catabolism of albumin-FTC and G-albumin-FTC. As shown in Fig. 8, the ratios of the TCA soluble fraction of G-albumin-FTC were significantly higher than those of albumin-FTC.

TABLE II. Urinary and Fecal Excretions of Protein-FTC and G-Protein-FTC within 24 h after Intravenous Injection in Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Carrier protein	Urinary excretion (% of dose)	Fecal excretion (% of dose)
Albumin-FTC	13.1 ± 2.7	23.3 ± 2.5
G-Albumin-FTC	$48.1 \pm 17.0^a)$	25.8 ± 4.5
Fetuin-FTC	26.8 ± 6.3	37.3 ± 6.5
G-Fetuin-FTC	36.2 ± 12.2	46.8 ± 7.6
Transferrin-FTC	19.4 ± 2.1	13.6 ± 10.7
G-Transferrin-FTC	$44.7 \pm 4.2^a)$	$34.2 \pm 6.8^a)$
IgG-FTC	31.5 ± 1.0	20.6 ± 1.0
G-IgG-FTC	38.7 ± 7.4	$30.9 \pm 5.6^a)$

Each value represents the mean value with S.D. of three mice. *a)* $p < 0.05$ compared with the value of non-glutarylated protein.

Fig. 7. Size-Exclusion Chromatograms of Urine Samples Taken for 12 h Following Intravenous Injection of Albumin-FTC and G-Albumin-FTC to Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

The excitation and emission wavelengths were set at 495 and 520 nm, respectively. ---, albumin-FTC; —, G-albumin-FTC; ·····, sodium fluorescein; —, urine after administration of albumin-FTC; —, urine after administration of G-albumin-FTC.

Fig. 8. Ratio of TCA Soluble Fraction in Tissues at 6 h Following Intravenous Injection of Albumin-FTC (□) and G-Albumin-FTC (▨) to Mice Bearing S180, Each Dose Being 7 mg Protein/Mouse

Each point represents the mean value with S.D. of three mice.

Discussion

In this paper, we have demonstrated that such serum proteins as albumin, fetuin, transferrin and IgG, accumulated in tumor tissues. These results are in accordance with a recent series of experimental studies on albumin,¹⁹⁾ α_1 -acid glycoprotein,²⁰⁾ transferrin,²¹⁾ and IgG¹⁹⁾ which suggested a passive leakage of proteins into the extravascular space of a tumor. Generally, macromolecules accumulate in the tumor tissues because the tissues have a vascular network characterized by both enhanced permeability of the neovasculature and a lack of lymphatic recovery system.¹³⁾ These findings led to an investigation of the antitumor drug-macromolecule conjugate system, such as MMC-dextran conjugate,⁹⁻¹¹⁾ MMC-albumin conjugate^{2,3)} and neocarzinostatin-styrene maleic acid copolymers.^{13,19)}

A study of the disposition of cationic MMC-dextran conjugate in mice demonstrated that it accumulated, to a considerable degree, in the liver, while an anionic conjugate showed a slow rate of plasma clearance and accumulated only in very small amounts in the liver.^{14,15)} Cationic macromolecules were adsorbed onto the hepatocyte surface as a result of an electrostatic force.²²⁾ In the previous paper, we demonstrated that the distribution patterns of the MMC-albumin conjugates were partly explicable by a difference in molecular size due to conformational changes such as a decrease in α -helical content.³⁾

The glutarylation affected the urinary excretion of albumin and transferrin. Over the first 24 h after administration, $48.1 \pm 17.0\%$ of G-albumin-FTC was recovered in the urine, while the amount of albumin-FTC excreted in the urine was only $13.1 \pm 2.7\%$ of the dose (Table II). It is difficult to discuss the relationship between the plasma concentration at 24 h and urinary excretion up to 24 h. In the cases of albumin and transferrin, the amount excreted in the urine was significantly increased by glutarylation ($p < 0.05$). Although the increases in the amount of urinary excretion of fetuin or IgG were not statistically significant, glutarylation also tended to increase their urinary excretion. Size-exclusion chromatographic analysis demonstrated clearly that most of the fluorescence intensity in the urine after injection of FITC-labeled albumins was attributed to a low-molecular compound. On the other hand, the quantity of fecal excretions of transferrin and IgG up to 24 h was increased by glutarylation (Table II). This may be caused by the increased biliary excretion of those two serum proteins.

The stability of albumin-FTC and G-albumin-FTC was studied *in vitro*. They were incubated for 30–240 min in mice plasma, which was then deproteinized by TCA-treatment. There was no detection of the TCA-soluble fraction of either (data not shown). This suggested that the proteins would be stable in plasma.

Catabolism of albumin occurs in the lysosomes of tissue, for example, in the liver, kidney and intestine.²³⁾ Thus, the tissue obtained after the injection of albumins was deproteinized by TCA-treatment. The ratio of TCA soluble fraction of G-albumin-FTC was higher than that of albumin-FTC (Fig. 8). This finding suggested that G-albumin-FTC was catabolized more to the low-molecular compound than was albumin-FTC. Since the thiocarbamyl bond between protein and FITC was highly stable, no free FITC was detected in the urine (Fig. 7). The accelerated

excretion of the low-molecular compound might be partially due to the reduced overall stability of the peptide linkage.

In the previous work we synthesized two kinds of drug-protein conjugates, *i.e.*, MMC-albumin conjugate (BSA-MMC) and its glutarylated counterpart (G-BSA-MMC). The tissue concentration of the conjugated MMC was determined by HPLC. Both the conjugates accumulated mainly in the tumor tissues 24 h after a single intravenous injection. G-BSA-MMC was distributed to the lung and the heart to some extent, and trace amounts of the conjugate were detected in the plasma. BSA-MMC accumulated not only in the tumor tissue but also in such tissues as the liver spleen and lung. Significant amounts of BSA-MMC remained in circulation even after 24 h. The concentration ratios of the tumor against the plasma were 62 and 2.8 for G-BSA-MMC and BSA-MMC, respectively. The same phenomena were observed in the distribution of the FITC-labeled proteins. The exact mechanism has not been elucidated so far, but enhanced permeability of the neovasculature and possible lymphatic drainage of the tumor tissue might explain the effect of acylated proteins.

The use of monoclonal antibodies for the radioimmuno-detection of malignant tumors holds great promise for the future, but there are still significant problems that must be solved before the procedure will become clinically useful. One problem is how to diminish accumulation in the liver.²⁴⁾ Although the plasma clearance of IgG-FTC was markedly increased by glutarylation, its passive tumor accumulation was not impaired. If the binding of monoclonal antibodies to the target tumor cells was highly specific and rapid, glutarylation would diminish their distribution to the liver, maintaining the potential for radioimmunodetection. Accordingly, the emphasis of future research should be on monoclonal antibodies which can be cleared from the liver by glutarylation.

Furthermore, glutarylation of a protein before the conjugation of drugs not only prevents protein polymerization, but also increases the extent of conjugation of the drugs.²⁾ The present study has shown that glutarylation diminishes the normal-tissue distribution of carrier proteins, leading to relatively high tumor-tissues accumulation. Therefore, glutarylation is anticipated to be useful in the preparation of macromolecular carriers of antitumor drugs. Further experiments are in progress to evaluate the effect of the other acylations on the disposition of serum proteins as macromolecular carriers.

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