Biodisposition Characteristics of *N*-Succinyl-chitosan and Glycol-chitosan in Normal and Tumor-bearing Mice

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Two water-soluble chitosan derivatives, *N*-succinyl-chitosan (Suc-chi; average MW 3×10^5) and glycol-chitosan (Gly-chi; average MW 1.5×10^5), were examined concerning their biodisposition characteristics in order to evaluate their possible use as water-soluble drug carriers. Their body distribution and urinary excretion were investigated by i.v. administration of FITC-labeled Suc-chi (FTC-Suc-chi) and FITC-labeled Gly-chi (FTC-Gly-chi) to normal and Sarcoma 180 solid tumor-bearing mice. In normal mice, both polymers showed good retention in blood circulation; especially, FTC-Suc-chi exhibited a long half-life of 51 h, and its distribution to other tissues was very small. FTC-Gly-chi was distributed into the kidney to a relatively high extent. In tumor-bearing mice, FTC-Suc-chi and FTC-Gly-chi were eliminated faster from the blood circulation than in normal mice, that is, with half-lives of 11 and 7 h, respectively. FTC-Suc-chi was less partitioned to the tumor tissue but accumulated more easily into it compared with FTC-Gly-chi. This suggested the enhanced permeability and retention (EPR) effect of Suc-chi and explained the previous result that a water-soluble Suc-chi-mitomycin C conjugate injected intravenously exhibited a good effect against Sarcoma 180 solid tumor. FTC-Gly-chi showed greater distribution to the kidney than in normal mice. Urinary excretion studies indicated the faster excretion of both polymers in tumor-bearing mice. The molecular weight of the products excreted into urine indicated that both polymers should be pretty resistant to the hydrolytic enzyme, lysozyme. Taking toxicities into account, Suc-chi is considered to be available as a drug carrier showing long systemic retention and tumor accumulation.

Key words *N*-succinyl-chitosan; glycol-chitosan; body distribution; urinary excretion; normal mice; tumor-bearing mice

Chitin, chitosan and their derivatives have been investigated as polymeric drug carriers for the optimization of drug delivery^{1–7)} because of their biocompatibility and biodegradability.^{8–13)} Chitin is very biocompatible and slowly biodegradable, but it is rather difficult to modify physically or chemically due to its aqueous insolubility and because only hydroxy groups are reactive. Therefore, chitin is hard to utilize as a soluble drug carrier. Chitosan can be treated more easily due to its good solubility in an acidic aqueous solution and because it possesses amino groups which are very reactive. However, chitosan is generally low soluble in biological pH, which limits the possibility for chitosan to act as a water-soluble carrier. Further, chitosan has been reported to exhibit toxic side effects due to the interaction with tissues or cells.¹⁴⁾ Thus, chitosan is also considered limited for use as a drug carrier. On the other hand, *N*-Succinyl-chitosan (Suc-chi) and glycol-chitosan (Gly-chi) are water-soluble polymers in biological pH. The former is very low toxic,^{15,16)} and it has been utilized as a polymer support for a wound healing patch.^{17,18)} Further, the conjugates of Suc-chi with mitomycin C (MMC) have been developed,^{5,19)} and the conjugate could act effectively under the condition in which the toxic side effect of the polymer could be avoided.^{15,20–22)} Gly-chi was reported to interact with tissues or cells,¹⁴⁾ but the conjugate of Gly-chi with MMC was also recognized to operate effectively.

Suc-chi has been examined as a carrier for insoluble and soluble macromolecular prodrugs of MMC.^{5,19)} The insoluble system exhibited long retention in the body, but the pharmacokinetic properties seemed to be influenced by physical characteristics such as particle size rather than by physicochemical or chemical characteristics of the polymer itself such as molecular weight or electric charge.²³⁾ From the pharmacokinetics of MMC at i.p. administration of the

water-soluble prodrug of MMC with Suc-chi,²¹⁾ the low biodegradability of Suc-chi was suggested. Gly-chi was also proposed to be low biodegradable by a similar investigation.²¹⁾ The water-soluble conjugates of MMC with Suc-chi and Gly-chi showed a good efficacy in the previous work.²¹⁾ These observations suggested that both the polymers may be useful as water-soluble drug carriers, especially in cancer chemotherapy. The pharmacokinetics and biodisposition of the polymers are critical concerning evaluation of the polymer-drug conjugate systems. Namely, molecular weight and electric charge^{24–26)} influence the biodisposition of the polymer-drug conjugates, and the passive targeting based on the enhanced permeability and retention (EPR) effect^{27,28)} can be achieved dependent on such polymer characteristics. However, detailed pharmacokinetics and biodisposition characteristics of Suc-chi and Gly-chi are scarcely known. Therefore, in this study, the biodistribution and excretion characteristics of Suc-chi and Gly-chi have been examined using their fluorescein-labeled forms in normal and tumor-bearing mice.

MATERIALS AND METHODS

Chemicals *N*-Succinyl-chitosan (Suc-chi; average MW 3×10^5 , succinylation degree 51%) was supplied from Katakura Chikkarin Co., Ltd. (Japan), and glycol-chitosan (Gly-chi; average MW 1.6×10^5 , deacetylation degree 77%) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). Fluorescein and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (U.S.A.). All other chemicals were of a reagent grade.

Fluorescein thiocarbonyl-Suc-chi (FTC-Suc-chi) and fluorescein thiocarbonyl-Gly-chi (FTC-Gly-chi) were prepared as follows by referring to Maeda et al.²⁹⁾ and Tanaka et al.³⁰⁾: Suc-chi (100 mg) was dissolved in a small volume of 0.5 M

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carbonate buffer (pH 8.3). Immediately after FITC (5 mg) was dissolved in a small volume of the same carbonate buffer, the solution was added to the Suc-chi solution. Then, the solution volume was adjusted to 10 ml by the addition of the same carbonate buffer, and the solution was subsequently stirred for 24 h at room temperature. After that, the solution was chromatographed using a Sephadex G-50 gel column (4 cm i.d. $\times$ 50 cm) with 0.5 M carbonate buffer (pH 8.3) as an elution solvent to separate FTC-Suc-chi from unreacted FITC. The fractions of FTC-Suc-chi were collected and dialyzed sufficiently in water using the membrane Seamless Cellulose Tubing (MW cut off 1.2×10^4). FTC-Suc-chi was obtained as a fluffy powder by lyophilization of the dialyzed solution. FTC-Gly-chi was prepared in the same manner as FTC-Suc-chi except that FITC (3 mg) was reacted with 100 mg of Gly-chi. After FTC-Suc-chi, FTC-Gly-chi and FITC were dissolved in the above carbonate buffer, their visible absorption spectra were measured using a DU® 640 spectrophotometer (Beckman, U.S.A.). The FTC content of each FITC-derived polymer was determined by comparison of the visible absorption at the maximum wavelength of around 493 nm between FITC-labeled polymer and FITC.

Animals In all the experiments, male ddY mice (6 weeks old) weighing 26–29 g were purchased from Tokyo Laboratory Animals Co., Ltd., and were used soon after being supplied.

Tumors Sarcoma 180 cells were maintained by weekly transplantation of 1×10^7 of the cells suspended in Hanks' balanced salt solution (0.1 ml) into the peritoneal cavity of each mouse. In producing the solid tumor of Sarcoma 180, 1×10^7 of the cells were inoculated subcutaneously at the axillary region of each mouse.

Biodistribution **Recovery Ratio:** At first, the recovery ratio was determined in normal mice. After being fasted for 24 h, the normal mice were sacrificed, blood was drawn, and tissues, that is, liver, spleen, kidney, lung, heart, small intestine and brain, were excised and washed with phosphate buffered saline, pH 7.4 (PBS). With the exception of blood, after briefly wiping tissues with a filter paper, each whole tissue was weighed and PBS was added by a three-fold volume to its weight, and the mixture was homogenized using a glass homogenizer with a Teflon pestle. Then, 50 μ l of a solution of FTC-Suc-chi or FTC-Gly-chi in PBS with concentrations of 0.1, 1 and 10 mg/ml was added to the homogenate or blood. The mixture was stirred sufficiently and centrifuged at 3000 rpm for 10 min. The fluorescence intensity of the supernatant was measured at 520 nm with the excitation at 495 nm. Fluorescence intensities were measured using an FP-777 spectrofluorometer (Jasco Co., Japan) throughout this study. The blank samples were prepared in the same way except that 50 μ l of PBS alone was added to the homogenate or blood, and their fluorescence was measured under the same condition. The concentration of FTC-Suc-chi or FTC-Gly-chi in each sample was calculated from the net fluorescence intensity obtained by subtracting the fluorescence intensity of the blank from that of each sample based on the calibration curve. The ratio of the observed concentration to the ideal one was obtained as the recovery ratio. As to the recovery ratio in plasma, when x% was transferred from blood into the plasma fraction, the recovery ratio was treated as x%. The recovery ratio of fluorescein from each tissue or plasma was in-

vestigated in a similar way, in which the final samples were fluorometrically measured at 510 nm with the excitation at 493 nm.

Next, the recovery ratios of FTC-Suc-chi and FTC-Gly-chi were investigated to Sarcoma 180 solid tumor-bearing mice. Namely, at 7 d after subcutaneous inoculation, the mice were fasted for 24 h, then blood was drawn, and tissues, that is, liver, kidney, spleen and tumor, were excised. The subsequent procedure was the same as that in the normal mice.

Biodistribution in Normal Mice: After the mice were fasted for 24 h, FTC-Suc-chi or FTC-Gly-chi was injected into the tail vein of the normal mice at the dose of 50 mg/kg (0.1 ml saline solution). The mice were sacrificed at 1, 8 and 24 h after injection, blood was drawn, and tissues, that is, liver, spleen, kidney, lung, heart, small intestine and brain, were excised. The blood volume and tissue weight were measured. Then, as in the recovery study, with the exception of blood, after briefly wiping tissues with a filter paper, each whole tissue was weighed and PBS was added by a volume three-fold to its weight and the mixture was homogenized using a glass homogenizer with a Teflon pestle. The homogenate and blood were centrifuged at 3000 rpm for 10 min. The supernatant was measured fluorometrically (Ex. 495 nm; Em. 520 nm). The net fluorescence intensity was calculated by subtracting the fluorescence intensity of the blank from that of each sample. The concentration was determined from the net fluorescence intensity based on the calibration curve. The distributed amount in the tissue was calculated from the concentration and the tissue weight. Fluorescein was investigated in terms of the biodistribution after intravenous injection at the dose of 0.5 mg/kg (0.1 ml saline solution). The concentration and amount of fluorescein in the tissue and blood was determined similarly to that of FTC-Suc-chi and FTC-Gly-chi. Each concentration and amount were complemented by the recovery ratio.

Biodistribution in Tumor-bearing Mice: At 7 d after subcutaneous inoculation, FTC-Suc-chi and FTC-Gly-chi were injected at 50 mg/kg into the tail vein of the tumor-bearing mice having been undergoing fast for 24 h. The mice were sacrificed at 1, 8 and 24 h after injection, blood was drawn, and tissues, that is, liver, spleen, kidney and tumor, were excised and washed by phosphate buffered saline, pH 7.4 (PBS). Then, they were treated in the same way as in normal mice and the concentration in each tissue and blood was determined in the same way. The amount distributed in the tissue was also calculated in the same way as in normal mice. Each concentration and amount were complemented by the recovery ratio.

Urinary Excretion The study of urinary excretion was executed separately from the distribution study. 1) Excretion in normal mice: After the mice were fasted for 24 h, FTC-Suc-chi or FTC-Gly-chi was injected into the tail vein of the normal mice at the dose of 50 mg/kg (0.1 ml saline solution), or fluorescein was injected in the same way at the dose of 0.5 mg/kg (0.1 ml saline solution). After that, urine was collected from each mouse for 24 h, and the volume was measured. Urine was filtered out using a membrane filter (0.45 μ m pore diameter). The filtered sample was appropriately diluted with PBS and measured fluorometrically under the same condition as in the distribution study. After the blank test, the concentration of contained FTC-Suc-chi,

Fig. 1. Chemical Structures of FTC-Suc-chi (A), FTC-Gly-chi (B) and Fluorescein (C)

FTC-Gly-chi or fluorescein was determined from the net fluorescence intensity using a calibration curve, similarly to the distribution study. The total excreted amount in urine was calculated from the concentration and the urine volume.

2) Excretion in tumor-bearing mice: At 7 d after subcutaneous inoculation, FTC-Suc-chi and FTC-Gly-chi were injected at 50 mg/kg into the tail vein of the tumor-bearing mice having been undergoing fast for 24 h. Like normal mice, urine was collected from each mouse for 24 h after injection, then the volume was measured. The concentration and amount of FTC-Suc-chi and FTC-Gly-chi in urine were determined in the same way as in normal mice. Further, the molecular weight of FTC-Suc-chi and FTC-Gly-chi in the urine sample was checked by gel-chromatography and compared with that before injection. Chromatography was executed with an LC-6AD (Shimadzu, Japan) using 50 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl, when FTC-Suc-chi and FTC-Gly-chi were detected by an RF-10AXL fluorometric detector (Shimadzu; Ex. 495 nm, Em. 520 nm), and molecular weight markers (blue dextran: MW 2×10^6 , catalase: MW 2.32×10^5 , ovalbumin: MW 4.3×10^4 , cytarabine: MW 243) were checked spectrophotometrically with an SPD-10AV UV-VIS detector (Shimadzu; 280 nm).

RESULTS AND DISCUSSION

Chemical Properties and Recovery Ratios FTC-Suc-chi and FTC-Gly-chi (Fig. 1) were obtained as orange-colored powder. Although more FITC added to Suc-chi than to Gly-chi, the FTC content of the product was lower in FTC-Suc-chi than FTC-Gly-chi; that is, FTC-Suc-chi and FTC-Gly-chi showed FTC contents of 0.75 and 1.78%, respectively. Their yield and FTC content were reproducible. Since FITC reacts easily with amino groups, Gly-chi possessing amino groups at a higher density was considered to combine better with FITC. It might also be one of the reasons for the higher introduction of FTC to the polymer that a Gly-chi aqueous solution was less viscous than a Suc-chi aqueous solution.

The recovery ratios of FTC-Suc-chi, FTC-Gly-chi and fluorescein were obtained as the mean values of a few experiments, and the result is shown in the following: Recovery ratio of FTC-Suc-chi: 78% (plasma), 100% (liver), 101% (spleen), 87% (kidney), 86% (lung), 103% (heart), 25% (intestine), 102% (brain) and 86% (tumor); recovery ratio of FTC-Gly-chi: 57% (plasma), 55% (liver), 82% (spleen), 43% (kidney), 49% (lung), 65% (heart), 18% (intestine), 62% (brain) and 89% (tumor); recovery ratio of fluorescein: 64% (plasma), 68% (liver), 79% (spleen), 66% (kidney), 74% (lung), 78% (heart), 93% (intestine) and 70% (brain). The result of fluorescein was obtained in normal mice. The recovery ratios in plasma, liver, kidney and spleen were not different between normal and tumor-bearing mice; therefore, the same values were used for both normal and tumor-bearing mice. The recovery ratios in the lung, heart, small intestine and brain were obtained in normal mice. FTC-Gly-chi showed a lower recovery than FTC-Suc-chi in all the tissues except in the tumor. FTC-Suc-chi showed fairly high recovery except from the small intestine, while FTC-Gly-chi exhibited a moderate recovery ratio except from the small intestine. These properties may be derived from their charged characteristics. Generally, macromolecules with a positive charge are more adsorptive to the tissues than negatively charged macromolecules.³¹⁻³⁴ Therefore, FTC-Gly-chi, being positively charged, is considered more adsorptive to the tissues than FTC-Suc-chi which is negatively charged. Fluorescein was recovered at more than 60% in every tissue, though it was not checked in the tumor. Throughout this study, all the results of concentration and amount in plasma and the tissues were corrected with those recovery ratios.

Biodistribution in Normal Mice Figure 2 shows the concentration of FTC-Suc-chi, FTC-Gly-chi and fluorescein distributed at 1, 8 and 24 h after injection. The concentration was expressed as the percent of the dose per 1 ml of plasma or 1 g of tissue. FTC-Suc-chi was maintained at a high level in blood circulation over 24 h and was distributed very little in other tissues. It showed long systemic retention, that is, more than 40% of the dose/ml remained at 24 h after injection.

Fig. 2. Distributed Concentration of FTC-Suc-chi (A), FTC-Gly-chi (B) and Fluorescein (C) in Various Tissues at 1 h (□), 8 h (▨) and 24 h (■) after i.v. Injection in Normal Mice

Results are expressed as the mean $\pm$ S.D. ($n=4$). A: The level was significantly higher in plasma than in each tissue at 1 and 8 h after injection ($p<0.001$) and at 24 h after injection ($p<0.01$). B: The level was significantly higher in plasma than in each tissue at 1 h after injection ($p<0.01$) and than in every tissue except the kidney at 8 and 24 h after injection ($p<0.01$). The level was significantly lower in plasma than in the kidney at 24 h after injection ($p<0.05$). The level was significantly higher in the kidney than in other tissues ($p<0.01$).

tion. The plasma level was significantly higher than that in each tissue for 24 h. FTC-Gly-chi also showed systemic retention to a certain extent. FTC-Gly-chi was distributed into the kidney to a fair extent, 17% of the dose/g at 24 h, and the kidney level was rather significantly higher than the plasma level, 13% of the dose/ml ($p<0.05$). FTC-Gly-chi was observed to be distributed mainly in the blood and kidney. The water-soluble FITC-labeled chitosan previously examined had been found to be excreted quickly from the body.³⁵⁾ The present result suggests that FTC-Suc-chi and FTC-Gly-chi exhibit a different property; they are relatively stable in the normal body. The body distribution of free fluorescein was very low in plasma and every tissue: It was less than 2% of the dose/g or ml at 1, 8 and 24 h after injection. Fluorescein was considered to be only barely distributed in tissues, and it was recognized to be excreted easily into urine, like many other water-soluble small molecules. This supports the idea that the distribution of FTC-Suc-chi and FTC-Gly-chi to the tissues may be little influenced by the fluorescein moiety. The distribution amount was also calculated from the concentration and the tissue weight or volume, but the data were not shown because the distribution tendency was reflected by the concentration.

Biodistribution in Tumor-bearing Mice In the tumor-bearing mice, FTC-Suc-chi and FTC-Gly-chi were found to be eliminated faster from systemic circulation than in normal mice. This may be due to a physiological or histological change in the body based on the presence of a tumor. In fact, white blood cells were found to be extensively increased in number in tumor-bearing mice. Further, the inoculation of leukemic cells was reported to increase the level of lysozyme in the kidney and urine of mice,³⁶⁾ and the inoculation of Sarcoma 180 was also reported to raise the lysozyme activity of murine kidney.³⁷⁾ In Sarcoma 180 solid tumor-bearing mice, the distribution of FTC-Suc-chi and FTC-Gly-chi to the tumor and the tissues found important in normal mice, *i.e.*, liver, kidney and spleen, was investigated. The distribution is shown in Fig. 3. As to FTC-Suc-chi, the blood level was more than 40% of the dose/ml at 1 h, but it decreased to almost a quarter of that value at 24 h. This level was similar to

those of bovine serum albumin (BSA) (MW 6.6×10^4) and carboxymethyl-dextran (MW 6.4×10^4).²⁵⁾ A certain amount was distributed in the liver and tumor, but it was less than 10% of the dose/g at each observation time. BSA and carboxymethyl-dextran are reported to increase in these tissues,²⁵⁾ but the distributed FTC-Suc-chi in the liver decreased gradually according to its decline in the blood level. The distribution of FTC-Suc-chi to tumor increased gradually. This indicates that FTC-Suc-chi can accumulate gradually in the tumor. The plasma level of FTC-Suc-chi was significantly higher than the level in each tissue during 24 h, except in the tumor at 24 h after injection. At 24 h after injection, the level of FTC-Suc-chi was not significantly different between plasma and tumor ($p>0.05$). This suggests that FTC-Suc-chi accumulates passively to the solid tumor, probably because of the enhanced permeability and retention (EPR) effect in the tumor tissue. The result reported previously, that the conjugate of MMC with Suc-chi exhibited a relatively good suppression of Sarcoma 180 solid tumor,²¹⁾ may be due to such accumulation, that is, passive targeting.

As to FTC-Gly-chi, its decrease from blood circulation was faster in tumor-bearing mice than in normal mice, and its distribution to the kidney appeared to be greater than in normal mice. At each observation time, the level of FTC-Gly-chi in the kidney was significantly higher than that in other tissues. Further, at 8 and 24 h after injection, the level of FTC-Gly-chi in the kidney was significantly higher than that in plasma. Especially, more than 50% of the dose/g was observed in kidney at 8 h. Distribution to the tumor was recognized at 1 h to some extent, but the distribution seemed to be lowered after that. This probably indicates that some FTC-Gly-chi should be adsorbed by the tumor site and consequently decrease from the tumor according to the decline in blood concentration. This slight accumulation may be related to the weaker effect of the conjugate of MMC with Gly-chi reported previously.²¹⁾ Distribution to the spleen and liver was small. Such a distribution property of FTC-Gly-chi seemed to be fairly different from those of other cationic macromolecules. Namely, other cationic macromolecules such as cationic ϵ -amino-caproic acid-dextran, diethy-

Fig. 3. Distributed Concentration of FTC-Suc-chi (A) and FTC-Gly-chi (B) in Several Tissues at 1 h (□), 8 h (▨) and 24 h (■) after i.v. Injection in Sarcoma 180 Solid Tumor-bearing Mice

Results are expressed as the mean $\pm$ S.D. ($n=5$). A: The level was significantly higher in plasma than in each tissue at 1 and 8 h after injection ($p<0.001$) and than in each tissue except the tumor at 24 h after injection ($p<0.01$). B: The level was significantly higher in plasma than in each tissue at 1 h after injection ($p<0.001$) and than in each tissue except the kidney at 8 and 24 h after injection ($p<0.01$). The level was significantly higher in the kidney than in other tissues ($p<0.01$).

Fig. 4. Analytical Models for the Distribution of FTC-Suc-chi and FTC-Gly-chi

(A), model by partition and accumulation; (B), model by accumulation and outflow; (C), model by partition and accumulation and outflow. Each parameter is as follows: C_p , plasma concentration; V_1 , plasma volume; C_a , partitioned concentration in the tissue; V_2 , tissue weight; k_1 , accumulation rate constant from the partitioned fraction; k_2 , accumulation rate constant from the plasma fraction; Q , accumulated amount (not including partition); k_3 , accumulation (inflow) rate constant; k_e , outflow rate constant; k_o , rate constant related to accumulation from both the partitioned fraction and the plasma one.

laminoethyl-dextran (DEAE-dextran) and hexamethylenediamine-introduced BSA (cationized-BSA)^{24–26} decrease more quickly in plasma than FTC-Gly-chi, and their accumulations to liver^{24–26} are much greater than that of FTC-Gly-chi. The distribution amount was also calculated from the concentration and the tissue weight or volume, but the data were not shown because the distribution tendency was reflected by the concentration.

Thus, the distribution mode was found to be different between FTC-Suc-chi and FTC-Gly-chi. Also, they showed specific distributions when compared with other polymers. FTC-Gly-chi showed extensive distribution to the kidney in tumor-bearing mice. FTC-Suc-chi seemed to accumulate gradually in the tumor, but FTC-Gly-chi was decreased from the tumor together with a decline in plasma concentration. The tumor accumulation degrees of FTC-Suc-chi and FTC-Gly-chi were not much different from those of other macromolecules.^{24,25} A distribution study over a longer time course may be needed in order to evaluate the accumulation or EPR effect of these polymers in more detail.

Analysis of Biodistribution Most tissues showed a gradual decrease in FTC-Suc-chi and FTC-Gly-chi, while some exhibited gradual accumulation. Such distribution could be explained by considering the partition between the

tissue and blood and the accumulation (not containing a partition) and outflow from the tissue. Thus, three analytical models were proposed, as shown in Fig. 4. In each model, a decline in blood concentration with a first order rate constant (k_e), partition coefficient (K) and first order rate constant (k_1 , k_2 , k_3 ; k_i , k_o) representing accumulation or outflow were supposed. The elimination profiles and rate constants for plasma concentration are described in Fig. 5 and Table 1, respectively.

The following equations, 1, 2 and 3, can be obtained from the models, A, B and C, respectively.

$$C_{tis}(t) = (Q + C_a V_2) / V_2 = (C_0 k_e / k_e) \times (1 - \exp(-k_e t)) + K C_0 \exp(-k_e t) \quad (1)$$

$$C_{tis}(t) = Q / V_2 = (C_0 k_i / (k_o - k_e)) \times (\exp(-k_e t) - \exp(-k_o t)) \quad (2)$$

$$C_{tis}(t) = (Q + C_a V_2) / V_2 = (C_0 k_e / (k_3 - k_e)) \times (\exp(-k_e t) - \exp(-k_3 t)) + K C_0 \exp(-k_e t) \quad (3)$$

where $C_{tis}(t)$ means the calculated tissue distributed concentration, and all other parameters are the same as those expressed in Fig. 4. Then, curve fitting was executed using the non-linear least squares program, MULTI.³⁸ According to the curve fitting, Eq. 1 gave good and consistent profiles to

the observations, except for the kidney distribution of FTC-Gly-chi in tumor-bearing mice. The distribution of FTC-Gly-chi to kidney in tumor-bearing mice was difficult to express consistently by Eq. 1, that is, by partition and linear accumulation. The distribution of FTC-Gly-chi to kidney in tumor-bearing mice was recognized to be expressed well and consistently by Eq. 2 or Eq. 3; but although Eq. 3 gave a very good and consistent curve, more observed points will be required for detailed fitting.

The results by analysis are shown in Tables 2 and 3 for normal and tumor-bearing mice, respectively. In normal mice, FTC-Suc-chi showed a small partition to each of the tissues. Further, it was found to accumulate in the small intestine to a fair extent, whereas accumulation was observed very little in other tissues. FTC-Gly-chi exhibited a high par-

Fig. 5. Semi-logarithmic Plots for Plasma Concentration-time Profiles

○, FTC-Suc-chi, normal mice ($n=4$); △, FTC-Gly-chi, normal mice ($n=4$); ●, FTC-Suc-chi, tumor-bearing mice ($n=5$); ▲, FTC-Gly-chi, tumor-bearing mice ($n=5$). Results are expressed as the mean $\pm$ S.D. Each correlation coefficient (r) in curve fitting by first order kinetics: ○, 0.93; △, 0.98; ●, 1.00; ▲, 0.99.

Table 1. Systemic Elimination Parameters for FTC-Suc-chi and FTC-Gly-chi in Normal and Tumor-bearing Mice^{a)}

Mice	Material	C_0 (% of dose/ml)	k_e (h^{-1})	$t_{1/2}$ (h)
Normal	FTC-Suc-chi	59.3	0.0137	50.6
	FTC-Gly-chi	42.3	0.0498	13.9
Tumor-bearing	FTC-Suc-chi	42.6	0.0635	10.9
	FTC-Gly-chi	42.9	0.0955	7.26

^{a)} Systemic elimination was approximated by first order kinetics as shown in Fig. 5: C_0 , plasma concentration at $t=0$; k_e , elimination rate constant; $t_{1/2}$, plasma half-life.

Table 2. Estimated Parameters for the Distribution to Tissues in Normal Mice^{a)}

Material	Parameter ^{b)}	Tissue						
		Liver	Spleen	Kidney	Lung	Heart	Intestine	Brain
FTC-Suc-chi	K	0.0502	0.0517	0.0847	0.0695	0.0397	0.0270	0.0122
	k_a (h^{-1})	0.000617	0.000903	0	0	0.000557	0.00290	0
FTC-Gly-chi	K	0.0798	0.0297	0.491	0.0697	0.0369	0.122	0.0153
	k_a (h^{-1})	0	0.000260	0.0169	0.000257	0	0.00268	0.0000413

^{a)} The estimation was executed using the equation obtained from the kinetic model A in Fig. 4, in which 0.0137 and 0.0498 (h^{-1}) were used as the values of k_e for FTC-Suc-chi and FTC-Gly-chi, respectively (Table 1). ^{b)} Each parameter corresponds to one shown in Fig. 4. The value of k_a to be 0 means that the k_a was fixed to 0 because it was converged to a value less than 0.

tition to the kidney and intestine, and an accumulation rate was also found in the kidney and intestine to some extent. In other tissues, the accumulation was very little.

In tumor-bearing mice, the elimination rate of both injected polymers from the blood circulation was higher than in normal mice (Table 1). FTC-Suc-chi was distributed to each tissue to a small extent, but a fair accumulation rate was observed at tumor. FTC-Gly-chi was distributed to a high extent in the kidney. It entered the kidney at the initial stage and was later found to decrease in the kidney after 8 h; therefore, it was analyzed by model B or C, when the accumulation rate and outflow rate were obtained as considerable values. FTC-Gly-chi exhibited a higher partition to the tumor but a lower accumulation rate constant in the tumor than did FTC-Suc-chi. Consequently, FTC-Suc-chi and FTC-Gly-chi were recognized to act as a long circulating material; especially, FTC-Suc-chi showed a long residence in blood circulation with little distribution to the tissues in both normal and tumor-bearing mice. FTC-Gly-chi was well distributed to the kidney in both normal and tumor-bearing mice.

Urinary Excretion The total amounts of FTC-Suc-chi and FTC-Gly-chi excreted in urine through 24 h after injection are described in Fig. 6. Fluorescein itself was excreted by more than 50% of the dose at 24 h after i.v. injection in normal mice (data not shown). Considering its very slight distribution to the tissues, fluorescein itself was considered to be excreted easily into urine. This also supported that the elimination of FTC-Suc-chi and FTC-Gly-chi to urine was little influenced by the fluorescein moiety. Both injected

Table 3. Estimated Parameters for the Distribution to Tissues in Tumor-bearing Mice^{a)}

Material	Model ^{b)}	Parameter ^{c)}	Tissue			
			Liver	Spleen	Kidney	Tumor
FTC-Suc-chi	A	K	0.0751	0.0241	0.131	0.0662
		k_a (h^{-1})	0.00231	0.00498	0.00118	0.0121
FTC-Gly-chi	A	K	0.0947	0.0524	—	0.197
		k_a (h^{-1})	0.00259	0.00141	—	0.00577
	B	k_i (h^{-1})	—	—	0.839	—
		k_o (h^{-1})	—	—	0.348	—
	C	K	—	—	0.474	—
		k_a (h^{-1})	—	—	0.333	—
		k_3 (h^{-1})	—	—	0.140	—

^{a)} The estimation was executed using the equation obtained from the kinetic model A except in the case of the kidney in FTC-Gly-chi administration. In the kidney for FTC-Gly-chi, the kinetic model B was adapted, and the result from model C was also described. The k_e values for FTC-Suc-chi and FTC-Gly-chi were 0.0635 and 0.0955 (h^{-1}), respectively (Table 1). ^{b)} The models are described in Fig. 4. ^{c)} Each parameter corresponds to one shown in Fig. 4.

polymers were excreted into urine significantly faster in tumor-bearing mice than in normal mice. This result was consistent with their elimination from the blood circulation. FTC-Suc-chi and FTC-Gly-chi were excreted by 6% and 19% of the doses at 24 h in normal mice, respectively. However, the excreted amounts of FTC-Suc-chi and FTC-Gly-chi were near 27% and 50% of the doses at 24 h in tumor-bearing mice, respectively. A higher lysozyme activity has already been shown in tumor-bearing mice,^{36,37)} and this is considered to be one of the reasons for this faster excretion. Further, enhanced excretion is considered to be caused by physiological or histological changes due to the presence of a tumor. The difference in molecular weight, electric charge and biodegradability is considered to cause the difference in the excretion rate of the polymers. FTC-Suc-chi seems to be less excreted.

FTC-Suc-chi and FTC-Gly-chi excreted in urine were checked in terms of molecular weight by gel-chromatography. The results are shown in Fig. 7. Non-treated FTC-Suc-chi showed a sharp peak at the narrow range of more than a few hundred thousand. The FTC-Suc-chi excreted into urine gave a broad peak at the molecular weight range of more than 1×10^5 to less than 1×10^4 . Non-treated FTC-Gly-chi spread from the middle size to a smaller size. The urinary ex-

creted FTC-Gly-chi showed a slight shift to a lower molecular size, but a fairly similar elution profile was obtained. Since Gly-chi has a polymerization degree of 800, it is strange that non-treated FTC-Gly-chi is eluted after ovalbumine (MW 4.3×10^4). Probably, the adsorption of FTC-Gly-chi with the column stationary phase occurred. Both the urinary excreted products gave no similar peak, and no elution peak was observed in the range of a low molecular weight. This indicates that the cleavage of the FTC moiety from FITC-labeled polymers is not feasible. Therefore, the products excreted into urine are considered to be either FITC-labeled polymers or fragments caused by the breakdown of the polymer backbone. Glomerular filtration is generally said to be possible for molecules with a molecular weight of less than $4-5 \times 10^4$. However, according to literature, some molecules with a MW of 10^5 can be excreted into urine, and the excretion degree seems to depend on the kind of polymer.^{24,39)} Therefore, elution of a polymer with a higher molecular weight suggests the feasibility for Suc-chi to exhibit easier urinary excretion. Further, the excretion might be enhanced by physiological or histological changes in the kidney in the case of tumor-bearing mice. The elution profiles of the excreted products indicated that neither polymer underwent extensive degradation in the body or urine.

In this study, Suc-chi (average MW 3×10^5) and Gly-chi (average MW 1.5×10^5) were examined in terms of their biodistribution and urinary excretion using their FITC-labeled forms. The effect of molecular weight on biodistribution or urinary excretion has not been checked. However, since both polymers have a much higher molecular weight than the glomerular filtration limit (MW $4-5 \times 10^4$), the results tell us the characteristics of Suc-chi and Gly-chi as drug carriers. Suc-chi is characterized as being retained for a long period in blood circulation in normal and tumor-bearing mice, and it can accumulate to the tumor site, probably due to the EPR effect. Gly-chi can be well distributed to kidney. However, since Gly-chi has been reported to have a problem in blood compatibility,¹⁴⁾ its utility may be limited. On the other hand, Suc-chi has been found to be slightly toxic and is utilized as a component of a wound-protecting material; therefore, Suc-chi is suggested to be a possible polymeric carrier, being maintained long in the blood circulation and targeted passively to the tumor. Research on water-soluble

Fig. 6. Urinary Excretion through 24 h after i.v. Injection in Normal (□) and Tumor-bearing Mice (■)

Results are expressed as the mean $\pm$ S.D. ($n=4$ for normal mice, $n=5$ for tumor-bearing mice). Urinary excretion was significantly more in tumor-bearing mice than in normal mice ($p<0.001$). In both normal and tumor-bearing mice, FTC-Gly-chi was excreted significantly more than FTC-Suc-chi ($p<0.001$).

Fig. 7. Gel-chromatograms of FTC-Suc-chi (A) and FTC-Gly-chi (B) Excreted into Urine through 24 h after Injection

—, urine after injection; ---, polymer itself (not containing urine); ·····, urine when not injected. The arrays (a—d) show the elution peaks of markers: a, blue dextran 2000; b, catalase; c, ovalbumin; d, cytarabine.

antitumor drug-Suc-chi conjugate systems is now being executed in detail.

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