and classified it into four characteristic stages. They showed that tumor vascular morphology markedly changed according to tumor growth. By using similar tumor cell lines, our previous work demonstrated that microcirculatory functions, such as tumor blood flow (TBF) and tumor interstitial fluid pressure, also markedly changed according to tumor growth.[17,18]

Here, the question of whether nanoparticles leak from all tumor vessels was investigated. This issue is of interest because if nanoparticles do not extravasate from certain tumor vessels, nanomedicines would not reach tissues supplied by such vessels. In such cases, different drug delivery measures would be required. Although this potential problem is a key point in diagnosis and therapy with nanomedicines, many studies of drug delivery systems published to date have not addressed it.

To examine this question, polymeric micelles labeled with fluorescein isothiocyanate (FITC) were synthesized and injected i.v. into rats with implanted transparent chambers. Polymeric micelles have been studied as a carrier system of mainly hydrophobic anticancer drugs.[19,20] Polymeric micelles are self-assembling nanostructures (diameter range, 10–100 nm) that are typically composed of amphiphilic block copolymers. To date, doxorubicin,[21] taxans,[22,23] a derivative of oxaliplatin,[24] camptothecin,[25] a derivative of camptothecin,[26] and an MRI contrast agent[27] have been encapsulated in polymeric micelles, some of which are now in clinical trials.[6]

The specific purposes of the present study were to identify blood vessels from which polymeric micelles readily extravasated and to clarify characteristics of tumor tissues in which such extravasation occurred. A combination of time-lapse vital microscopy and image analysis was used to analyze differences in extravasation of FITC micelles from tumor vessels at different stages of tumor growth. This analysis permitted discussion of the microcirculatory mechanism of accumulation of polymeric micelles or other nanomedicines in tumor tissues.

MATERIALS AND METHODS

Rats and Tumors

Male Donryu rats (Crj-Donryu; Nippon Charles-River, Yokohama, Japan), 7–8 weeks old and with an average weight of 220–250 g, were used for all experiments. Rats were bred and maintained in accordance with The Law (No. 105) and Notification (No. 6) issued by the Japanese Government. Specifically, they were comfortably maintained in a ventilated, temperature-controlled ($24 \pm 1°C$), specific pathogen-free environment on a bed of wood shavings, with food and water freely available and a 12-h light–dark cycle. Rats that were equipped with transparent chambers for vital microscopic observations (see below) were caged singly (cage volume, 30 cm × 40 cm × 25 cm).

A variant of Yoshida sarcoma, LY80, was used in all experiments. This cell line, which can grow in ascites and in solid form, was maintained by successive i.p. transplantation.

All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments of Tohoku University and were carried out according to the Guidelines for Animal Experiments issued by Tohoku University.

FITC-Labeled Tracers

As a tracer for extravasation of macromolecules into tissues, a new FITC-labeled polymeric micelle was synthesized. First, poly(ethylene glycol)-*b*-poly(β-benzyl-L-aspartate) [PEG_{12}-*b*-$PBLA_{14}$, molecular weight (M_w), 14,900; PEG_{12}, M_w of PEG chain, 12,000; number of BLA units, 14.0] was prepared. PEG_{12}-*b*-$PBLA_{14}$ [500 mg (33.6 μmol)] was dissolved in 3.5 mL of anhydrous dimethyl sulfoxide, and FITC [15.9 mg (40.8 μmol)] (Sigma–Aldrich Japan Co., Tokyo, Japan) was added to the solution.

To confirm the introduction of FITC into PEG_{12}-*b*-$PBLA_{14}$ and the purity of PEG_{12}-*b*-$PBLA_{14}$-FITC, ^{1}H-NMR spectroscopy with a Varian Unity Inova (400 MHz) spectrometer was used with reversed-phase high-performance liquid chromatography. The binding percentage of the terminal portion of the block copolymer with FITC was 67.7%. In principle, FITC is localized at the hydrophobic micellar inner core. Therefore, highly concentrated FITC would be expected to quench fluorescence. To avoid such intramicellar quenching, the optimal molar ratio of PEG_{12}-*b*-$PBLA_{14}$-FITC in micelles was investigated.

The diameter of the FITC micelles was measured by the use of dynamic light scattering. The preliminary experiment confirmed that these micelles had a monophasic half-life *in vivo*.

Another lot of FITC micelles, synthesized by chance in the present study, had a chemical

composition and physical properties that were almost the same [PEG_{12}-*b*-$PBLA_{14}$, M_w, 14,800; M_w of PEG chain, 12,000; number of BLA units, 13.9] as those of the first micelle preparation, but the preparation's half-life was biphasic. It is not yet clear why these two micelle preparations had different *in vivo* behaviors despite having similar structures. A possible reason for this difference is the slight change in the micelle preparation procedure (concentration and scale of preparation). The second micelle system was used only to determine whether the image analysis used had sufficient accuracy to detect two compartments and whether fluorescence intensity measured by the image analysis was quantifiable.

Fluorescein sodium (M_w, 376.3) (Tokyo Chemical Industry Co., Tokyo, Japan) served as the low-molecular-weight tracer. The chemical structure of fluorescein sodium is very similar to that of FITC (M_w, 389.4).

FITC micelles and fluorescein sodium were administered i.v. at doses of 20 and 1 mg/kg, respectively.

Anesthesia

Both pentobarbital sodium salt (Tokyo Kasei Kogyo Co., Tokyo, Japan) and enflurane (Abbott Laboratories, North Chicago, IL) were used for anesthesia. Enflurane is suitable for long experiments in rodents because of its minor irritation of the rat airway, although it cannot be purchased today because newer anesthetics have replaced it. We were able to use enflurane because we had purchased enough in 2007 before a production halt and stocked it at our laboratory. Pentobarbital powder was dissolved in distilled water (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) to give a concentration of 50 mg/mL. The solution was administered i.m., 10 min before the experiment, at a dose of 30 mg/kg, and supplemental doses (15 mg/kg i.m.) were given at 90-min intervals to maintain immobilization. Enflurane concentration was maintained at 1% in the inhaled gas, which was administered at a rate of 1 L/min by means of an anesthetic apparatus for small laboratory animals. Hydration of the animals was maintained during anesthesia by i.m. administration of about 0.1 mL of water every 90 min. We confirmed in a previous experiment that our anesthesia conditions caused little disturbance of the microcirculation.

Implantation of Transparent Chambers and Tumor Transplantation

For direct observation of changes in tumor vessels and measurement of extravasation of FITC-labeled compounds, transparent chambers were implanted, under aseptic conditions, in dorsal skin flaps of rats. Transparent chambers used here were a "sandwich" system,[28] with each chamber consisting of a pair of identical titanium frames containing a circular quartz glass window, 10 mm in diameter and 300 μm thick. In this sandwich system, tumor tissue and normal tissue did not overlap. Therefore, extravasation of FITC-labeled compounds into tumor tissue could be analyzed without interference by normal tissue and vice versa. The implantation method was previously described in detail.[29] For tumor transplantation, a small fragment (approximately 0.1 mm^3) of solid tumor from a donor rat was transplanted onto the normal tissue in a transparent chamber while the chamber was being implanted in the dorsal skin flap.

Classification of Growth Stage of the Tumor and Its Vascular System

The tumor and its vascular system growing in the transparent chamber were divided into four stages according to the classification of Yamaura and Sato.[15] The first stage is the beginning of tumor growth. In this stage, tumor cells freely invade surrounding tissue, and many capillaries begin to proliferate and form rapidly growing granulation tissue. The second stage involves exponential tumor growth. In this stage, to support growth, new tumor vascular networks with active and abundant blood flow form. In the third stage, tumor vascular density reaches a plateau, and the tumor growth rate begins to gradually decrease. In this stage, many so-called giant capillaries,[16] which have extremely thin walls and large lumens, appear. The fourth stage is the period of a rapid decrease in tumor vascular density, and tumor tissue degradation progresses. Tumor vessels in this stage run adjacent to necrotic areas, so tumor cord structure[30] is seen. The first- and second-stage tumor vessels and the third- and fourth-stage tumor vessels were monitored 7–9 and 11–14 days after tumor transplantation, respectively.

In the present study, a necrotic area was defined as a tumor region in a transparent chamber in which circulation completely stopped for more

than 24 h and thus the color of the tissue changed to gray. That such a region was a necrotic area was histologically confirmed (Fig. 1).

Vital Microscopic Observation

After anesthesia, each rat with a transparent chamber was placed in the right lateral position on a heated (34.5°C) stage (MATS-SFA; Tokai HIT Co., Tokyo, Japan), which was attached to the mechanical stage of the microscope. Tumor microcirculation in the chamber was transilluminated by a 12-V 100-W halogen lamp and was directly observed with a light microscope (Eclipse E800; Nikon Co., Tokyo, Japan), with a 10× ocular (CFI UW; Nikon Co.) and 2–20× objectives (CFI Plan Fluor; Nikon Co.). Microscopic images were recorded by using a closed-circuit video system consisting of a CCD video camera (CS-900; Olympus Co., Tokyo, Japan), TV monitor (PVM-14M4J; Sony Co., Tokyo, Japan), and S-VHS video recorder (SVO-2100; Sony Co.).

Fluorescence Microscopic Analysis of FITC-Labeled Compounds

Figure 2 provides a schematic illustration of the experimental set-up for fluorescence microscopic analysis of FITC-labeled compounds. For intravital fluorescence microscopy, the light source was changed to a high-pressure mercury lamp (C-SHG1; Nikon Co.). Tumor vessels in the chamber were epi-illuminated and photographed through a 420- to 490-nm primary filter, 505-nm dichroic interference mirror, and 520-nm secondary filter. FITC-labeled compounds were injected into rats as a single i.v. bolus. Microscopic images of extravasation of FITC-labeled compounds were photographed with a silicon-intensified video camera (C2400-08; Hamamatsu Photonics, Hamamatsu, Japan) at 15 time points during the experimental period and were recorded with a video recorder. The maximum ultraviolet (UV) ray exposure time of tissues in transparent chambers at each time point was 6 s. Thus, total exposure time for one rat was less than 90 s. The influence of

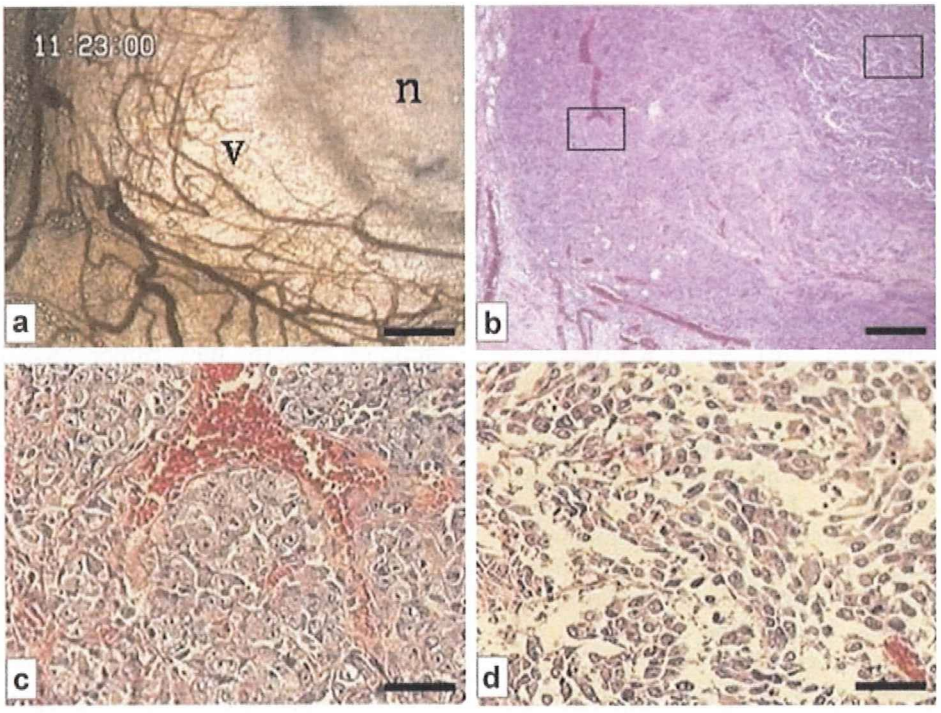

Figure 1. Necrotic area in a tumor observed within a transparent chamber. (a) Vital microscopy. n, necrotic tissue; v, viable tumor tissue. (b) Histology (hematoxylin and eosin). (a) and (b) are the same area. (c) High-power magnification of the left square area in (b). (d) High-power magnification of the right square area in (b). Scale bars represent 250 μm in (a) and (b), and 50 μm in (c) and (d). Histology clearly shows that the gray region [upper right in (a)] with stopped circulation is a necrotic area.

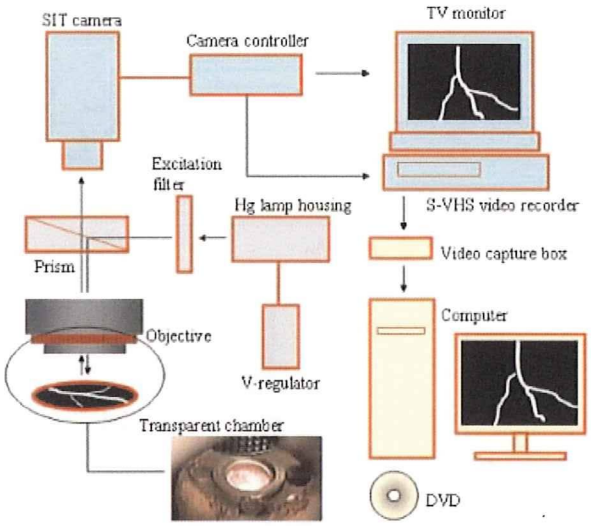

Figure 2. Schematic representation of the vital fluorescence microscopy system. SIT, silicon-intensified target.

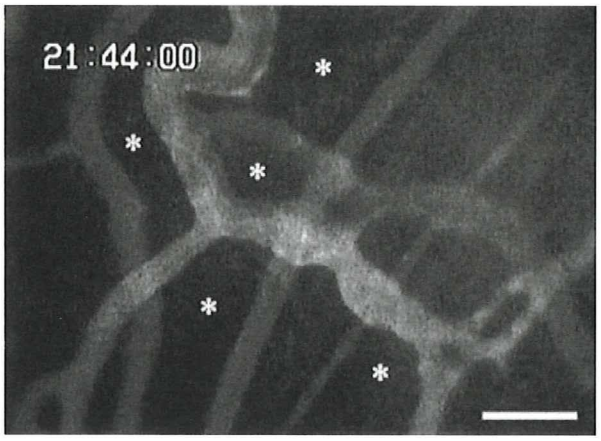

Figure 3. Photograph of extravascular spaces used for measurement of fluorescence intensity, at 4 min after i.v. administration of FITC micelles. Asterisks indicate candidate areas for measurement. Scale bar represents 50 μm.

UV exposure on *in vivo* fluorescence intensity could be ignored because an *in vitro* test without blood circulation showed that the attenuation of fluorescence intensity was less than 10%, even after continuous UV irradiation for 10 min (data not shown). The slight positional change of the observation area that occurred during the long experiment was adjusted every 3–4 h. For precise analysis of the correlation between time after administration of FITC-labeled compounds and fluorescence intensity, a video timer was superimposed on images. Analog images on videotape were converted to digital images by means of a video capture device (PC-MDVD/U2; Buffalo, Inc., Nagoya, Japan). The digital images were recorded on a DVD.

For image analysis, segments of time-lapse images were transferred from the DVD to a computer hard disk (Dimension 9150; Dell Japan, Inc., Kawasaki, Japan). Temporal changes in fluorescence intensity of regions of interest (ROIs) in tissue in the chambers were measured by using NIH Image J (free software). The labeled fluorescent substances circulated in blood vessels only just after i.v. administration (Fig. 3). Therefore, blood vessels and extravascular spaces could easily be distinguished. In the present study, fluorescence intensity in tissues was measured by carefully removing blood vessel areas from the images of the entire tumor tissue. Thus, selected ROIs were distant from the blood vessels. One ROI (10 μm^2) consisted of 100 pixels, and 5–10 ROIs per chamber were randomly selected for analysis.

The parameter $T_{C_{max}}$, which is the time to reach maximum intensity, was defined for evaluation of extravasation of fluorescein sodium. Washout of FITC-labeled compounds from tissues or blood was evaluated by using the half-life ($t_{1/2}$) of fluorescence intensity.[31]

Measurement of Half-Life of FITC Micelles in Blood

To confirm the quantifiability of FITC-micelle half-life evaluated by fluorescence image analysis, the FITC concentration was measured in samples of rat plasma. FITC micelles with the biphasic half-life were used for this experiment. A permanent cannulation method served for blood collection.[32] An arterial cannula was placed in the thoracic aorta of each rat through the left common carotid artery 1 day before injection of FITC micelles. Samples (0.2 mL) of rat blood were obtained from the cannula at multiple time points (i.e., 10 min and 1, 2, 3, 4, 6, and 8 h) after i.v. injection of the FITC-micelle solutions. To minimize the influence of blood collection, blood sampling was limited to twice for each rat. Before blood samples were collected, the inside of the syringe and needle were washed with heparin to prevent blood clotting. Rat plasma samples were obtained by centrifugation (3000 rpm × 5 min). FITC concentrations in plasma samples were determined by using fluorescence spectroscopy (FP-6500 spectrofluorometer; JASCO Co., Tokyo, Japan). The excitation wavelength was set at 495 nm.

Measurement of TBF and Mean Arterial Blood Pressure (MABP)

To ascertain whether polymeric micelles have any effect on circulation, the effect of micelles on TBF and MABP was determined. TBF was measured by using the hydrogen clearance method, as follows. In brief, inhalation of 9% hydrogen gas in air (at 1 L/min) caused tissues to be saturated with hydrogen, after which blood flow (mL/min/100 g tissue) was calculated from the half-life of the clearance curve obtained. This method was previously described in detail.[33] MABP was measured via a catheter inserted into the right femoral artery. Pressure in the catheter was recorded continuously with a pressure transducer (TNF-R; Spectramed Medical Products, Yishun, Singapore), whose output was fed into an amplifier (6M82; NEC-Sanei Co., Tokyo, Japan). TBF and MABP were measured simultaneously at multiple time points (i.e., 1, 5, 10, and 30 min, 1 h, and every hour thereafter until 6 h) after i.v. micelle administration. In this experiment, polymeric micelles formed with PEG_{12}-*b*-$PBLA_{14}$ block copolymer were used.

Statistics

All results are expressed as means ± SD. The statistical significance of the difference between tumor and normal tissues for extravasation of FITC-labeled compounds was evaluated via repeated-measures ANOVA. The p-values of 0.05 or lower were considered to be significant.

RESULTS

FITC-Micelle Preparation

Figure 4A shows the chemical structure of the PEG_{12}-*b*-$PBLA_{14}$-FITC block copolymer. The binding percentage of the terminal portion of the block copolymer with FITC was 67.7%. When PEG_{12}-*b*-$PBLA_{14}$-FITC and unlabeled PEG_{12}-*b*-$PBLA_{14}$ were mixed at a ratio of 1:3, that is, when 16.9% (67.7% × 0.25 = 16.9%) of block copolymer constituting the micelles was labeled by FITC, the FITC-micelle preparation did not show self-quenching (data not shown).

The diameter of the FITC micelles was 48.2 ± 8.8 nm (the weight-average diameter ± SD). These micelles were accompanied by a very small amount (<2% by weight) of secondary

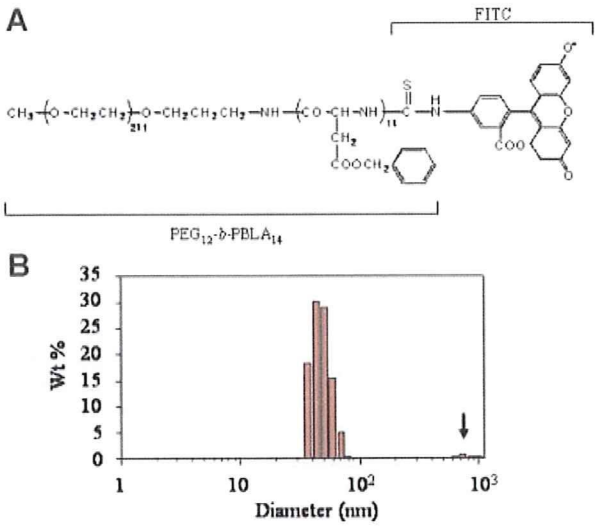

Figure 4. Chemical structure of the PEG_{12}-*b*-$PBLA_{14}$-FITC block copolymer (A), and a weight-average diameter of the dispersed micelles as measured by dynamic light scattering (B). Arrow indicates secondary aggregates.

aggregates with diameters of 208 and 760 nm (Fig. 4B). The basic structure of this micelle system was almost the same as that of the micelle system encapsulating doxorubicin, which was described previously.[21]

Effects of a Polymeric Micelle Preparation on TBF and MABP

Figure 5 shows time changes in TBF and MABP after i.v. administration (40 mg/kg) of a polymeric micelle preparation incorporating PEG_{12}-*b*-$PBLA_{14}$ block copolymer. TBF and MABP did not change significantly during the experimental period of 6 h. A small number of experiments confirmed that the polymer micelles had no influence on MABP and blood flow even at 12 h after administration (data not shown).

Quantifiability of Fluorescence Intensity of FITC Micelles, as Measured by Image Analysis

Figure 6A plots the fluorescence intensity of FITC micelles in an arteriolar vessel as measured by image analysis. Figure 6B shows the FITC concentration in rat plasma samples as measured by fluorescence spectroscopy. Half-life values were calculated from each plot. Both methods produced biphasic washout curves. The half-lives

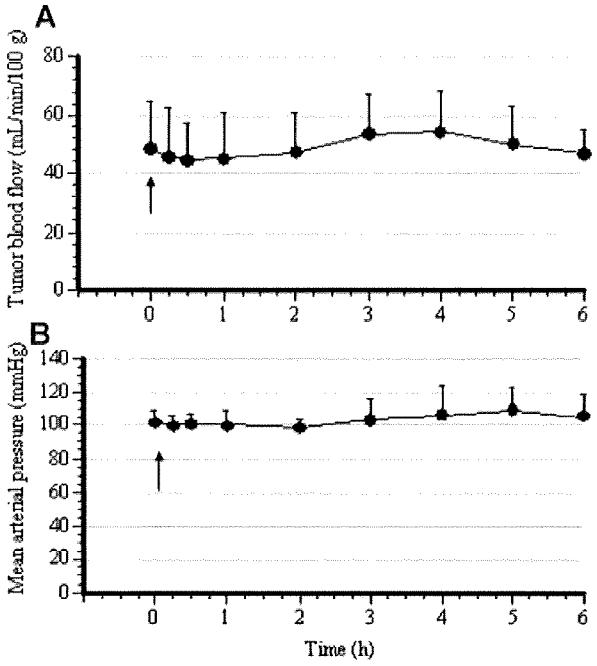

Figure 5. Changes in TBF (A; $n = 15$) and MABP (B; $n = 8$) in rats that received a micelle preparation (40 mg/kg i.v.) incorporating PEG_{12}-*b*-$PBLA_{14}$ block copolymer. The micelle solution was administered at 0 h (arrows). No significant changes were observed during the 6-h experimental period.

of fluorescence intensity in the α- and β-phase were 1.0 and 6.5 h, respectively. The half-lives of FITC concentration in the α- and β-phase were 1.0 and 7.1 h, respectively. The half-life values measured via these two different methods thus corresponded to a considerable degree. This finding indicates that a linear correlation occurred between fluorescence intensity as measured by image analysis and blood concentration of micelles. It was therefore decided that the fluorescence intensity of FITC measured by image analysis could be used as an indicator of micelle concentration.

Extravasation of FITC Micelles from the Vessels in Normal Tissue and Advanced Tumor Tissue

Figure 7A and B presents typical vital microscopic differences in extravasation of FITC micelles between normal tissue and advanced tumor tissue. Figure 7C shows time changes in mean fluorescence intensity of FITC micelles in normal subcutaneous tissues (20 areas from four transparent chambers) and advanced tumor tissues (22 areas from four transparent chambers). Almost no leakage of FITC micelles from normal blood vessels was observed, so the fluorescence intensity of normal tissues was only slightly increased compared with the background value (intensity before FITC-micelle administration) during 12 h of observation. In contrast, FITC micelles began to leak promptly from advanced tumor vessels (mainly the latter half of third-stage vessels), and fluorescence intensity reached a plateau in about 1 h. Washout of FITC micelles from tumor tissue was very slow, however. The difference in

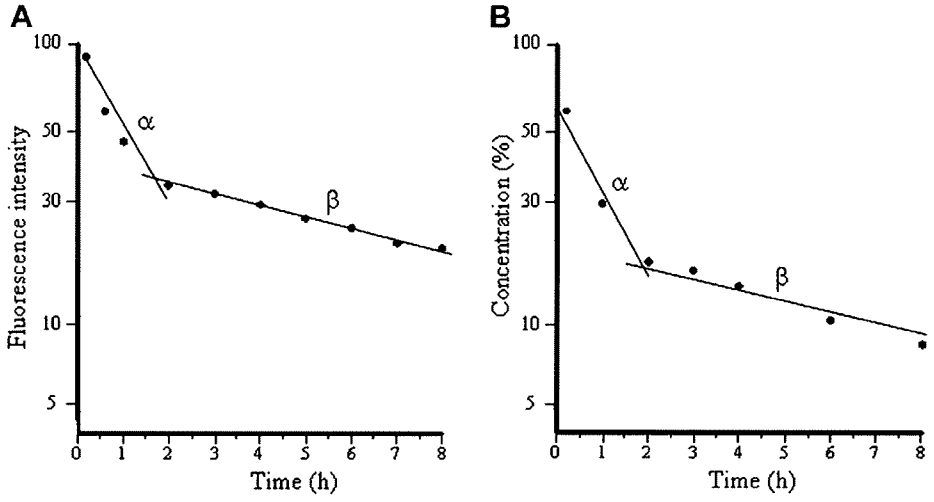

Figure 6. Half-life of FITC micelles in blood as measured by image analysis (A) and spectroscopy (B). Polymeric micelles with a biphasic half-life were used. Half-life values of the α- and β-phase measured by the two different methods showed considerable agreement.

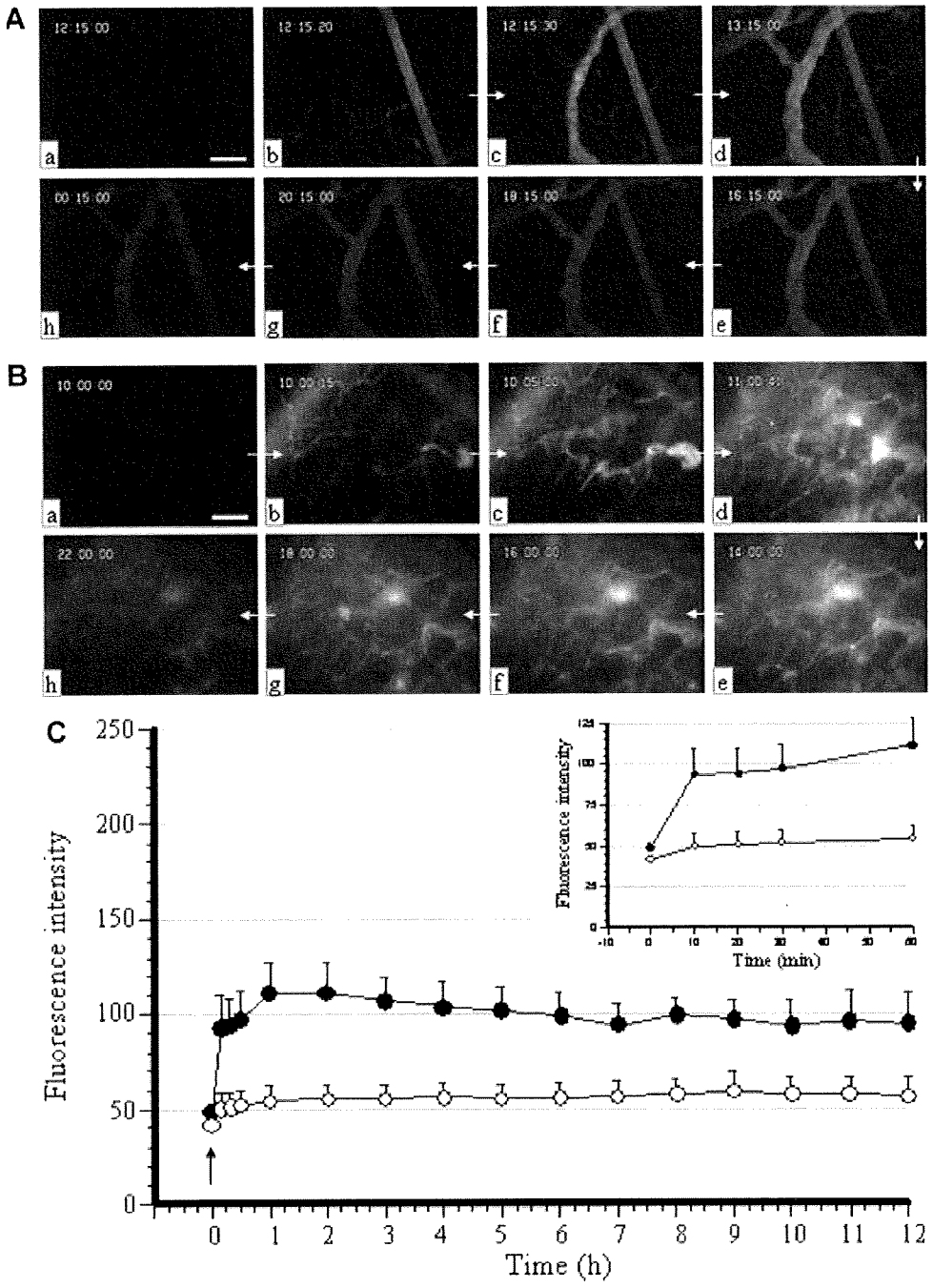

Figure 7. Extravasation of FITC micelles from vessels in normal subcutaneous tissue and advanced tumor tissue. (A) Typical extravasation of FITC micelles from normal tissue. (a) At the start of i.v. FITC micelle (20 mg/kg) administration; (b, c, d, e, f, g, h) 20 s, 30 s, 1 h, 4 h, 6 h, 8 h, and 12 h later, respectively. (B) Typical extravasation of FITC micelles from advanced tumor tissue. (a) At the beginning of FITC-micelle (20 mg/kg) administration; (b, c, d, e, f, g, h) 15 s, 5 min, 1 h, 4 h, 6 h, 8 h, and 12 h later, respectively. Arrows indicate the direction of time; scale bars represent 100 μm. (C) Time changes in fluorescence intensity of FITC micelles in normal tissue (○) ($n = 20$) and advanced tumor tissue (●) ($n = 22$). Small graph indicates the changes in 0–1 h. The micelle solution was administered i.v. at 0 h (arrow). Maximum fluorescence intensity was 255. Extravasation of FITC micelles was markedly high in the advanced tumor.

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fluorescence intensity of FITC micelles in normal tissues and advanced tumor tissues was highly significant ($p < 0.001$).

Figure 8 presents typical vital microscopic changes in extravasation and washout of fluorescein sodium, used as a positive control, after i.v. administration. Fluorescein sodium was evenly and promptly distributed to all tissues, that is, nontumor tissue, tumor tissue, and the interface between nontumor and tumor tissues. Fluorescence intensity reached a maximum at about 3 min ($T_{C_{max}}$, 2.5–3 min) after i.v. administration, and fluorescein sodium was completely washed out from all tissues 150 min later ($t_{1/2}$, 45–55 min). No marked differences in $T_{C_{max}}$ and $t_{1/2}$ occurred among the three different areas.

Extravasation and Retention of FITC Micelles in Tumor Tissue, Including Necrotic Areas

Figure 9A presents typical extravasation of FITC micelles in tumor tissue, including necrotic areas. Figure 9B shows time changes in fluorescence intensity in tissues during the 16 h after i.v. administration of FITC micelles. FITC micelles readily extravasated from fourth-stage tumor vessels near a necrotic area. These FITC micelles first diffused into viable tumor tissue, which received blood from the vessels, and then gradually infiltrated nearby necrotic tissues where they accumulated. Although a concentration gradient occurred between necrotic and viable tissues until several hours after administration of FITC micelles, both tissues showed almost equal FITC-micelle concentrations 9 h later, which indicated that micelles moved from viable tumor tissues to necrotic tissues.

Extravasation and Retention of FITC Micelles in and Around Microtumors

Figure 10A presents typical extravasation of FITC micelles in and around a microtumor, less than 1 mm in diameter. Figure 10B shows the time course of fluorescence intensity in tumor tissue during 24 h after i.v. administration of micelles. Vascular networks in this microtumor were all composed of second-stage tumor vessels, which had an active, high flow rate. Extravasation of micelles from the second-stage tumor vessels was very low and was approximately the same as that from normal vessels. Also, FITC micelles did not accumulate in tumor tissues of this stage. Extravasation of micelles from the vessels of the interface between normal tissue and tumor tissue was very high. In such a microtumor, however, FITC micelles that had extravasated into the interface did not remain there (Fig. 10Aj).

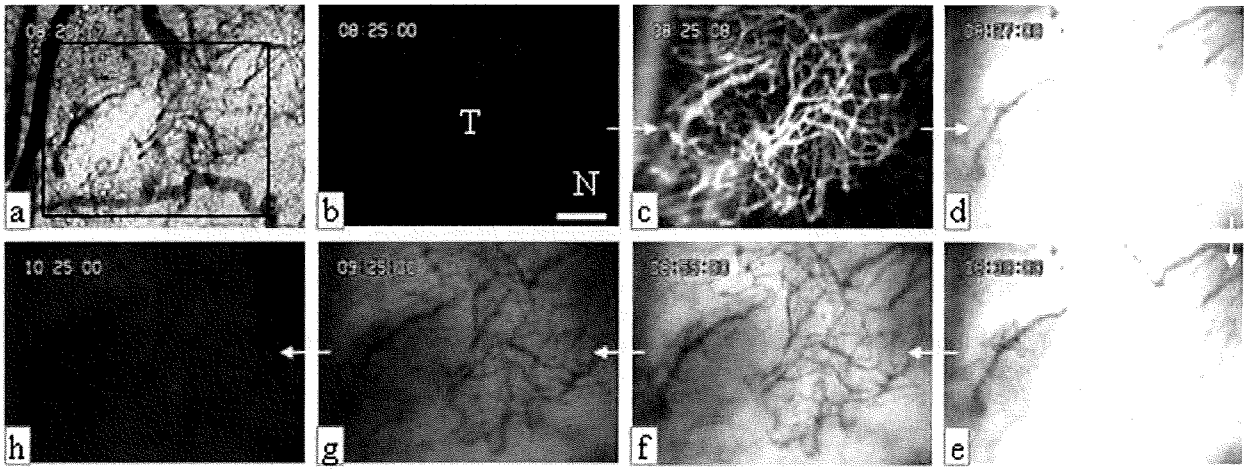

Figure 8. Extravasation of fluorescein sodium from vessels in normal and tumor tissues and washout from those tissues. Fluorescein sodium (1 mg/kg) was administered i.v. (a) Vital microscopy. The black frame is the observation area. (b) At the start of micelle administration; (c) 8 s later; (d) 2 min later; (e) 5 min later; (f) 30 min later; (g) 1 h later; (h) 2 h later. T, tumor; N, normal tissue. Arrows indicate the direction of time; scale bar represents 200 μm. No marked differences in extravasation and washout between normal and tumor tissues were found.

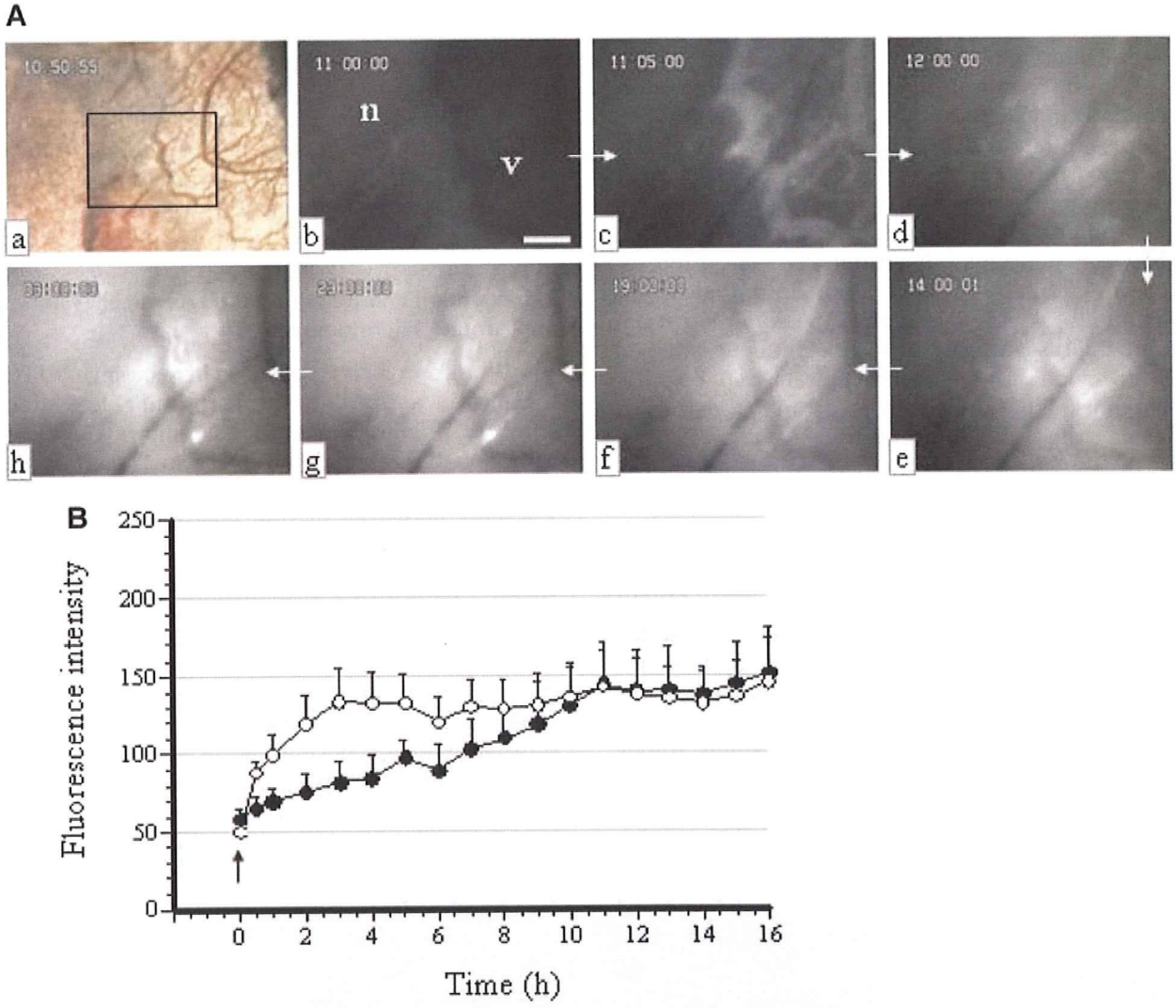

Figure 9. Extravasation and retention of FITC micelles in tumor tissue, including necrotic areas. The FITC-micelle preparation (20 mg/kg) was administered i.v. (A) Typical extravasation of FITC micelles in tumor tissue including necrotic areas. (a) Vital microscopy. The black frame is the observation area. (b) At the start of micelle administration; (c) 5 min later; (d) 1 h later; (e) 3 h later; (f) 8 h later; (g) 12 h later; (h) 16 h later. n, Necrotic tissue; v, viable tumor tissue. Arrows indicate the direction of time; scale bar represents 100 μm. (B) Time changes in fluorescence intensity of FITC micelles in viable tumor tissue (○) ($n = 5$) and necrotic tumor tissue (●) ($n = 5$). The micelle solution was administered i.v. at 0 h (arrow). Maximum fluorescence intensity was 255. Note that micelles moved from viable tumor tissues to necrotic tissues and stayed there.

DISCUSSION

The present study used an FITC-labeled tracer to investigate the *in vivo* behavior of polymeric micelles. To analyze the *in vivo* behavior of such macromolecules with a long half-life in blood, it is important to perform prolonged monitoring. Although some studies have shown extravasation of fluorescent macromolecules by means of intravital fluorescence microscopy,[34–36] the monitoring time in many cases was 2 h or less. In our study reported here, determination of optimal anesthesia conditions permitted lengthy *in vivo* observations, so that marked variations in extravasation and intratumor retention of these micelles according to tumor growth stage could be demonstrated.

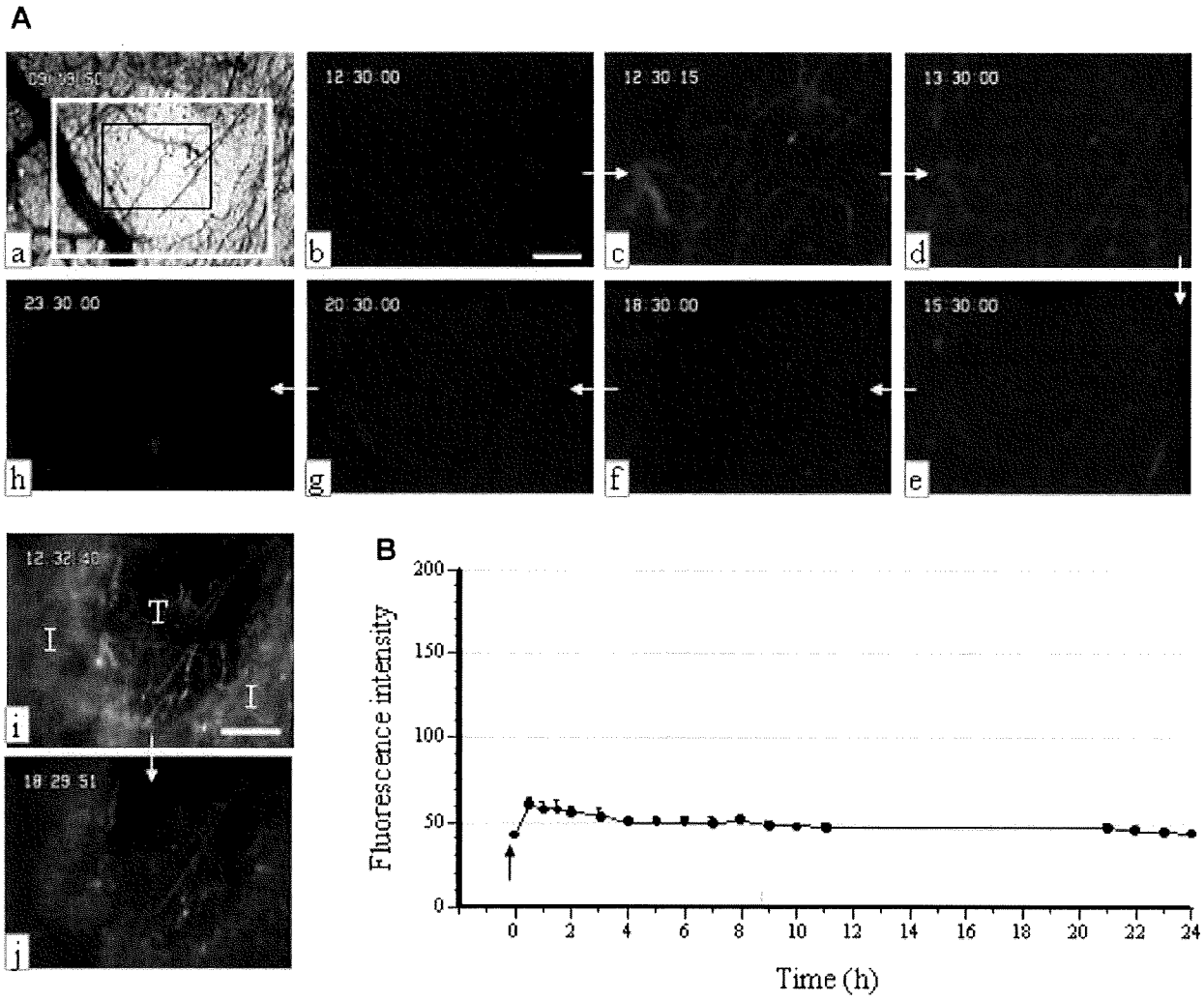

Figure 10. Behavior of FITC micelles in and around microscopic tumor foci. The FITC-micelle solution (20 mg/kg) was administered i.v. (A) Vital microscopy. (a) The black frame is the observation area of (b)–(h) and the white frame is the area of (i) and (j). (b) At the start of micelle administration; (c) 15 s later; (d) 1 h later; (e) 3 h later; (f) 6 h later; (g) 8 h later; (h) 11 h later. (i) 160 s later (low magnification). T, microscopic tumor; I, interface between normal tissue and tumor tissue. (j) 6 h later (low magnification). Arrows indicate the direction of time; scale bars represent 100 and 300 μm in (b) and (i), respectively. (B) Time changes in fluorescence intensity of FITC micelles in tumor tissue ($n = 8$). The micelle solution was administered i.v. at 0 h (arrow). Maximum fluorescence intensity was 255. Note that extravasation of FITC micelles was negligible in the microscopic tumor.

The half-life calculated by means of image analysis agreed well with that determined by fluorescence spectroscopy. Blood flow stabilization is essential for the calculation of the half-life of substances in a tissue. If tissue blood flow changes in an irregular fashion, individual time points plotted for the half-life would not lie in a logarithmic line. Although some macromolecules disturb tumor microcirculation and change TBF in such a way (data not shown), polymeric micelles used in the present study did not directly influence TBF and MABP. It was therefore concluded that fluorescence intensity measured by our image analysis method accurately reflected micelle concentration.

With regard to extravasation of low-molecular-weight compounds from vessels, normal tissue and tumor tissue exhibited no marked differences.

Fluorescein sodium showed almost equal extravasation into normal tissue and tumor tissue and almost equal washout from those tissues. However, although leakage of polymeric micelles from normal vessels was slight, it occurred readily from vessels at the interface between normal tissue and tumor tissue (i.e., the area consisting of granulation tissue caused by cancer cells infiltrating normal tissues) and from tumor vessels at the interface between tumor tissue and necrotic areas.

Yamaura and Matsuzawa[37] examined the permeability of tumor vessels to Brilliant Blue 6B (Pontamine sky blue), which is an albumin-binding dye. They reported almost no increase in permeability in second-stage vessels and the first half of third-stage vessels, whereas permeability to the dye was greatly increased in first-stage vessels and was abnormally high in fourth-stage vessels in and around necrotic tumor tissue. Our present results agreed with their observations, because almost all vessels at the interface between normal and tumor tissues were first-stage vessels, and many vessels at the interface between tumor tissue and necrotic areas corresponded to fourth-stage tumor vessels.

Advanced tumors usually include areas of necrosis. In such tumors, polymeric micelles extravasated from tumor vessels (mainly fourth-stage vessels) that were adjacent to these necrotic areas gradually infiltrated necrotic areas and remained there for a long time. This phenomenon is clearly the enhanced permeability and retention (EPR) effect described by Maeda and Matsumura.[2–6] It is believed that the main driving force of this gradual movement of micelles is circulatory movement of interstitial fluid, because an interstitial fluid flow rate of 2–3 mL/min/100 g was detected even in tumor tissue in which TBF was completely stopped (Hori et al., unpublished work).

Polymeric micelle accumulation in necrotic tumor areas is believed to result in part from weak intratumor drainage, as Maeda et al.[2] emphasized. Although it has been thought that tumors do not have lymph vessels, recent immunohistological studies revealed many lymphatic endothelial cells in tumors.[38] However, evidence strongly suggests that the drainage function of the lymphatic system is quite weak.[39,40] Intratumor lymphatic endothelial cells may construct functionally incomplete lymph vessels. Clearance of polymeric micelles once delivered to the tissue would thereby be markedly suppressed. Another of our experiments showed that when circulation was functional, as in an irradiated tumor, polymeric micelles did not accumulate in necrotic tumor areas (data not shown). This observation also supports Maeda's concept. In view of the result that FITC concentration in necrotic tissue rose above that in blood more than 10 h after i.v. administration of FITC micelles, micelles may be absorbed by such tissues. This question warrants further investigation. However, regardless of the reasons, abundant polymeric micelles clearly accumulate in necrotic tumor tissue with impaired circulatory function.

If anticancer drug-incorporating nanoparticles in necrotic tumor tissue continue to slowly release the anticancer drug, viable cancer cells near this tissue would be exposed to the drug for a long time. Enhanced cancer therapeutic efficacy of nanomedicines, which has been reported,[4,6] may be a function, in part, of this mechanism.

In contrast to an advanced tumor, a microscopic tumor less than 2–3 mm in diameter had no necrotic area, and the circulatory function of its microvascular network was extremely active. The present study found almost no accumulation of polymeric micelles in such microtumors. The primary reason was the extremely low extravasation of micelles from vessels in microscopic tumors (i.e., second-stage tumor vessels) compared with that from vessels in advanced tumors. In fact, almost no difference in extravasation of polymeric micelles from vessels was seen between second-stage tumor vessels and normal blood vessels. A similar tendency was also found for FITC albumin (data not shown).

Various kinds of tumor cells secrete VEGF/VPE.[9–11] Therefore, VEGF tends to be regarded as the most important permeability factor in tumors. However, in view of the present results—that polymeric micelles readily extravasated from first-stage tumor vessels, which are affected by inflammation, and from fourth-stage tumor vessels, which are undergoing degradation—not only VEGF/VPF but also classical inflammation-related permeability factors such as histamine, serotonin, bradykinin, and prostaglandin E_2 may play a relatively important role in extravasation of polymeric micelles.

Taken together, our findings suggest that microscopic metastatic foci may escape from the attack of nanomedicines that encapsulate an anticancer drug.

In conclusion, polymeric micelles can be selectively delivered to tumor tissues. However, to

enhance the therapeutic effect of cancer chemotherapy with the use of nanomedicines, including anticancer drug-incorporating micelles, a means that can deliver sufficient nanomedicine to microtumors and ensure its presence there for a long period must be developed. Such a novel therapeutic strategy against microtumors will be described in a separate article.

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Research Paper

Accelerated Blood Clearance Was Not Induced for a Gadolinium-Containing PEG-poly(L-lysine)-Based Polymeric Micelle in Mice

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Purpose. Accelerated blood clearance (ABC) is induced by repeated injections of PEGylated liposomes. In this study, the ABC was investigated for a gadolinium-containing PEG-poly(L-lysine)-based polymeric micelle (Gd-micelle) and PEGylated liposome (Gd-liposome) in mice.
Materials and Methods. Effects of the first injection of Gd-micelle on the tissue distribution of the second dose of Gd-micelle were studied. Additionally, effects of the first injection of Gd-micelle, Gd-liposome, empty liposome, polyethyleneglycol ($PEG_{500,000}$), and PEG-lipid on the distribution of the second dose of the Gd-liposome were evaluated.
Results. Results indicated that the tissue distribution of the second injection of the Gd-micelle at a dose of 33, 5, or 2μmol Gd/kg was not affected by the first injection of the Gd-micelle at different doses and time intervals or of the empty PEGylated liposome 7 days before. ABC of Gd-liposome at a dose of 2.3μmol Gd/kg (corresponding to 10 μmol lipids/kg) was observed when the empty PEGylated liposome or Gd-liposome, but not the Gd-micelle, $PEG_{500,000}$ or PEG-lipid, was pre-administered.
Conclusions. The hydrophobic core of the micelle or lipid bilayer of PEGylated liposome has a major effect on this phenomenon. These studies have significant implications for the evaluation of PEG-poly (L-lysine)-based micellar formulation of Gd-based contrast agents.

KEY WORDS: accelerated blood clearance; gadolinium; PEGylated liposome; polyethylene glycol (PEG); polymeric micelle.

INTRODUCTION

Long-circulating liposomes with surface-modified polyethyleneglycol (PEG) are often used as carriers of therapeutic agents, since they avoid capture by the reticuloendothelial system (RES) and can extend the systemic circulation time of agents, thereby improving drug delivery (1,2). It was hypothesized that PEG on the surface of liposomes forms a water shell, resulting in decreased adsorption of opsonins and subsequent phagocytosis by cells of the RES (3,4). However, PEGylated liposomes are known to lose their long-circulating property with multiple dosing. Recently, it has been reported that the first dose of PEGylated liposomes injected intravenously caused a loss of the long-circulating property and extensive accumulation in the liver at the second dose injected several days later in mice, rats, rabbit, and rhesus monkeys (5–11), a phenomenon known as accelerated blood clearance (ABC). Besides PEGylated liposomes, other nanocarriers, such as nanoparticles containing PEG, also produced this phenomenon (12). Therefore, ABC would have a significant impact on the application of long-circulating liposomes and nanoparticles with multiple administrations. In clinical applications of liposomal carriers, Gabizon *et al.* reported a reduced clearance of doxorubicin-containing PEGylated liposome in the repeated injections. This opposite behavior to the ABC phenomenon resulted from toxic activity of the encapsulated doxorubicin against the RES (13). Presently, the ABC phenomenon is not a problem in a cancer chemotherapy by the use of a PEG-liposomal carrier, whereas the ABC phenomenon in human clinics must be important for less toxic drug or gene delivery applications of the PEGylated liposomes.

To date, studies of ABC have focused mainly on PEGylated liposomes. Many factors can affect the extent to which ABC is induced by PEGylated liposomes. First of all, the dose of lipid plays an important role, with ABC enhanced at lower concentrations of lipid (6,7,12). Second, ABC occurs in a time-dependent manner (5,7). The time interval between the first and second doses is a key factor. Third, when the amount of PEGylated lipid in the first injection was ≤5 mol%, the second dose of PEGylated liposomes was eliminated more quickly from plasma than liposomes containing >10 mol% PEGylated lipid injected as a first dose (7,8). In addition, the ABC phenomenon

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was reported to be independent of liposomal size, surface charge, and PEG molecular weight (5,7,8).

During the past decade, polymeric micelles, supramolecular assemblies of block copolymers, have demonstrated their utility in drug delivery systems and are currently recognized as promising nanocarriers for enhancing the efficacy of drugs and genes (14–16). Since ABC has a considerable impact on the multiple drug administration, it is necessary to study whether the phenomenon is induced by repeated injections of polymeric micelles. Gadolinium (Gd)-based contrast agents are widely used in magnetic resonance imaging (MRI) to improve the conspicuity of lesions or visualization of blood vessels (17). However, these agents are rapidly cleared from the circulation. To overcome this problem, nanocarriers, such as liposomes and polymeric micelles, are used to encapsulate the agents so as to prolong their circulation and allow them to accumulate in tumors for diagnosis (18–20). If polymeric micelles containing a diagnostic agent cause the ABC phenomenon, then circulation time will be reduced after a second dose and the accuracy of the diagnosis will be affected. Furthermore, polymeric micelles containing MRI agents or drugs administered during diagnosis and treatment will lose some of their drug efficacy because of the accelerated clearance. Hence, it is of great importance to know whether the ABC phenomenon can be induced by polymeric micelles or not. Recently, the accelerated clearance of [^{3}H]-labeled PEGylated liposomes was observed in mice pre-administered with an empty polymeric micelle composed of poly (ethylene glycol)-*b*-poly (β-benzyl L-aspartate) (PEG-PBLA) 50 nm in diameter (16).

In this study, we first investigated whether the ABC effect was caused by repeated injections of a polymeric micelle encapsulating Gd-DOTA (Gd-micelle) and of a PEGylated liposome encapsulating Gd-DTPA (Gd-liposome) as a positive control. Concentrations of Gd ions were measured for this investigation. Furthermore, we examined the effect of a PEG homopolymer on the tissue distribution of Gd-liposomes.

MATERIALS AND METHODS

Materials

Magnevist® (Gd-DTPA) was purchased from Bayer Schering Pharma (Berlin, Germany). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (mPEG$_{2000}$-DSPE), hydrogenated soy bean phosphatidylcholine (HSPC), and egg phosphatidylcholine (EPC) were purchased from the NOF Corporation (Tokyo, Japan). Cholesterol and polyethylene glycol 500,000 (PEG$_{500,000}$) were of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

Animals

Four-week-old female ddY mice were purchased from Sankyo Lab Service Corp. (Tokyo, Japan). All care and handling of animals were performed with the approval of the Animal and Ethics Review Committee of Hoshi University and of Principles of Laboratory Animal Care (NIH #publication 85-23, revised in 1985).

Preparation of the Gd-micelle

Synthesis of a chelate moiety-binding block copolymer was performed as reported in our previous paper (19). Briefly, a poly(ethylene glycol)-*b*-poly(L-lysine) block copolymer (PEG-P(Lys)) was prepared through acid hydrolysis of a poly(ethylene glycol)-*b*-poly[ε-(benzyloxycarbonyl)-L-lysine] (PEG-P(Lys(Z)) block copolymer (Fig. 1). We synthesized PEG-P(Lys(Z) with polymerization of a Lys(Z) *N*-carboxy anhydride monomer from PEG-NH_2 (molecular weight of PEG-NH_2 = 5,200). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (*N*-hydroxysuccinimide ester) was fully conjugated to lysine residues of PEG-P(Lys).

The composition of PEG-P(Lys-DOTA) was determined by means of ^{1}H-NMR spectroscopy in D_2O under alkali conditions (pH>10). $GdCl_3 \cdot 6H_2O$ was added to PEG-P(Lys-DOTA) at pH 6.0 to 6.5 for 3 hr at 50°C. Gd content was determined using inductively coupled plasma (ICP) (SPS7800, SII Nano Technology Inc., Tokyo, Japan). We obtained the block copolymer as PEG-P(Lys-DOTA-Gd) (Gd content = 7.7 wt%, the number average of Gd is 8.2). The block copolymer formed a polymeric micelle spontaneously in an aqueous solution (Gd-micelle). The size and zeta-potential of the Gd-micelle diluted with saline for three independent preparations was 84.5±6.0 nm and −1.70±0.80 mV, respectively, at 25°C as determined by dynamic light scattering (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan).

Preparation of the Empty Liposome and Gd-liposome

First, an empty liposome, which induced the ABC phenomenon, was prepared by the lipid film hydration method as described previously (21). Briefly, a mixture of HSPC, cholesterol, and mPEG$_{2000}$-DSPE in a molar ratio of 1.85:1.0:0.15 was dissolved in chloroform. The solution was

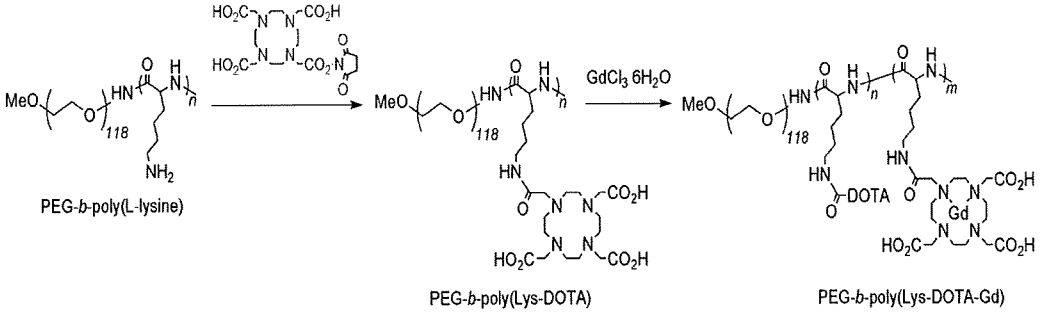

Fig. 1. Synthesis of PEG-P(Lys-DOTA-Gd).

evaporated dry to form the lipid film. Then, the liposome was produced by hydration of the lipid film with saline, followed by size reduction with sonication. The size and zeta-potential of the liposome diluted with saline were 178.5 nm and −22.1 mV, respectively.

Two kinds of Gd-liposomes were prepared because Gd-DTPA content was dependent on the preparation methods. One kind of Gd-liposome was prepared by an ethanol injection method (GdL-E). In brief, a mixture of EPC, cholesterol, and $mPEG_{2000}$-DSPE in a molar ratio of 2.15:0.88:0.15 was dissolved in ethanol and then hydrated with Gd-DTPA at 50°C. The resulting liposomes were sonicated for 10 min, then subjected to exhaustive dialysis against phosphate-buffered saline (PBS, 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) with a dialysis membrane having a 2,000 molecular-weight cutoff for 24 hr. The size and zeta-potential of the liposome were 150.1±18.8 nm and −0.94±6.78 mV, respectively, for three independent preparations. As a control of GdL-E, an empty liposome not including Gd-DTPA (empty GdL-E) was prepared by the same method as the Gd-liposome (GdL-E), except that saline was used to hydrate the ethanol solution of lipid. The particle size of empty GdL-E was 139.5 nm. Another kind of Gd-liposome was prepared by reverse phase evaporation (GdL-R) to encapsulate a larger amount of Gd-DTPA. The lipid was the same as GdL-E described above and dissolved in 4 mL of chloroform and 2 mL of diethyl ether. Gd-DTPA was added to the lipid solution. The mixture was sonicated to form an emulsion, which was evaporated to produce the liposome. Finally, the resulting liposome was sized at 60°C on an extruder (Avanti Polar Lipids, Inc., AL, USA) with three passes through a 0.4 μm Nuclepore membrane (Waterman, Maidstone, UK) and five passes through a 0.2 μm Nuclepore membrane, followed by exhaustive dialysis as described above. The particle size and zeta-potential of the liposome were 140.9± 13.5 nm and −2.52±5.18 mV, respectively, for three independent preparations. The phospholipid concentration of the liposome including HSPC or EPC was measured with the Phospholipids C-test Wako (Wako Pure Chemical Industries, Ltd.). GdL-E contained 2.26 μmol Gd per 10 μmol lipids, and GdL-R contained 2.29 μmol Gd per 5 μmol lipids.

Release Studies of Gd-micelle and Gd-liposomes

The release of Gd–DTPA from Gd–liposome (GdL–E or GdL–R) and Gd from Gd–micelle was evaluated by dialysis method using a Spectrapor 6 tubing with molecular weight cut-off of 1,000 Da (Spectrum Laboratories Inc., Tokyo, Japan). Briefly, the sample of Gd-micelle containing 1.2 mM Gd and Gd-liposomes of GdL-E containing 0.96 mM Gd-DTPA or GdL-R containing 0.96 mM Gd-DTPA (1 mL) were dialyzed against PBS (pH 7.4, 200 mL) at 37°C. At the indicated time points (10 min, 1, 3, 6, 24 h), 1 mL aliquots of the medium were withdrawn, and the same volume of fresh medium was added. The Gd concentration was analyzed by ICP. The accumulative release of Gd or Gd-DTPA released from the Gd-micelle or Gd-liposome, respectively was expressed as a percentage of the released Gd or Gd-DTPA and plotted as a function of time.

Pharmacokinetics and Tissue Distribution of the Gd-micelle and Gd-liposome

For pharmacokinetics study, the mice were intravenously injected with the Gd-micelle at a dose of 33 μmol Gd/kg (67.3 mg polymer/kg) or the Gd-liposomes including GdL-E at a dose of 6.75 μmol Gd/kg and 10 μmol lipids/kg and GdL-R at 2.65 μmol Gd/kg and 5 μmol lipids/kg. About 30 to 100 μL of blood were taken from a tail vein with a quantitative capillary at 10 min, 1 h, 3 h, 6 h, and 24 h after the injection. The Gd-micelle or the Gd-liposome was injected into a lower part of a tail vein, and blood sample was taken at a certain time point described above from an upper part of the tail vein at the other side of the injected vein. Therefore, this experiment was free from the sample pollution problem. The blood samples were added to saline and centrifuged at 3,000 rpm for 15 min, and the supernatant was used to measure Gd content by ICP. The elimination half-life ($T_{1/2}$) was calculated based on a single compartment model. For the tissue distribution of Gd-micelles and Gd-liposomes study, the second dose of Gd-micelles or Gd-liposomes was injected intravenously through the tail vein at a certain time interval after the first injection. Samples of blood were taken from the hepatic portal vein 6 h after the second injection, and tissues of liver, spleen, and kidney were removed at the same time. The plasma and blood volume were calculated as 0.0488 mL/g body weight for plasma and 0.0778 mL/g body weight for blood, respectively (19).

Measurement of Gd Content

For the quantitative determination of Gd content, blood samples were centrifuged at 3,000 rpm for 15 min, and then plasma was taken out and diluted with 0.1% HNO_3 for ICP. Tissue samples of the liver, spleen, and kidney were digested with a mixture of 98% H_2SO_4 and 62% HNO_3 (1:2, *v:v*) and then subjected to ICP.

Statistical Analysis

The statistical analysis was performed with the Dunnett's multiple comparison test. The level of significance was set at $p<0.05$ or $p<0.01$.

RESULTS

Release Behavior of Gd-micelle and Gd-liposomes

Gd or Gd-DTPA release behavior from Gd-micelle or Gd-liposomes was studied by the dialysis method. As shown in Fig. 2, only 0.2% of Gd leaked from the Gd-micelle at 37°C in PBS (pH 7.4) for 24 h. On the other hand, 4.8% of entrapped Gd-DTPA leaked from the Gd-liposome prepared by reverse phase evaporation method (GdL-R) and 22.4% for 24 h from the Gd-liposome prepared by ethanol injection method (GdL-E). Hence, it is obvious that Gd-micelle has hardly release behavior of Gd, and GdL-R showed much slower release than GdL-E. The results indicated that the leakage of Gd or Gd-DTPA from nanocarriers was greatly affected by the preparation methods.

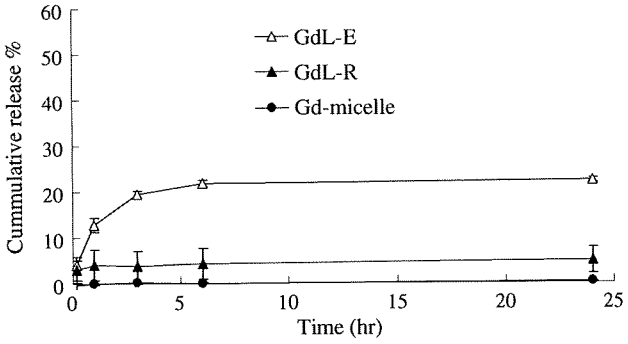

Fig. 2. Release profiles of Gd from Gd-micelle or Gd-DTPA from Gd-liposomes prepared by ethanol injection method (GdL-E) and reverse phase evaporation method (GdL-R) in PBS (pH 7.4) at 37°C. Data represent mean±S.D. (n=3).

Pharmacokinetics of the Gd-micelle and Gd-liposome

As shown in Fig. 3, at 10 min after the intravenous injection, 33.3% of the injected dose was found in blood for the Gd-micelle, and 40.0% and 50.3% for the Gd-liposome of GdL-E and GdL-R, respectively. At a dose of 33 μmol Gd/kg, the $T_{1/2}$ of the Gd-micelle was 10.2±3.9 h. Besides, the $T_{1/2}$ of GdL-E at a dose of 6.75 μmol Gd/kg and GdL-R at a dose of 2.65 μmol Gd/kg were 5.9±0.5 h and 6.0±1.0 h, respectively. In a previous study, we showed that Gd-DTPA was very rapidly cleared from the bloodstream with a minute's order half-life (19). Therefore, the detected Gd in blood is considered to be Gd-DTPA encapsulated in the liposome in a quantitative manner for measurements 6 h post intravenous injection. On the other hand, the main purpose of this study is the ABC phenomenon of a polymeric micelle MRI contrast agent, and PEGylated liposome is used as a positive control for the ABC phenomenon. Therefore, detection of liposome with Gd measurements is appropriate for the present purpose.

Effect of the First Dose on the Distribution of the Gd-micelle

The effects of the first dose on the distribution of the Gd-micelle injected a second time were evaluated. When the second dose of Gd-micelle was fixed at 33 μmol/kg, there was no significant difference of percent injected doses in plasma, kidney, and spleen between various first doses of the Gd-micelle from 0 to 100 μmol/kg (Fig. 4A). A dose of 100 μmol Gd/kg is the clinical dose of Gd-DTPA (17). Interestingly, the distribution of Gd-micelles in plasma, kidney, spleen and liver with the first injection of the empty liposome was similar to that with the first injection of saline. For the liver, the percent injected dose after a first dose of 33 μmol/kg and 100 μmol/kg was significantly higher than in the control saline group, possibly due to the incomplete elimination of the first dose of the Gd-micelle in liver at day 7 because of high doses of polymeric micelles (67.3 mg ~ 203.9 mg polymer/kg). The dose of 2 μmol Gd/kg of the Gd-micelle was the minimum at which Gd was detectable by means of ICP 6 h after injection. As shown in Fig. 4B, when the second dose of the Gd-micelle was decreased to 5 μmol/kg and 2 μmol/kg, the distribution was similar to that of 33 μmol/kg (Fig. 4A). Hence, the results showed that the tissue distribution of the Gd-micelle at the second dose of 33, 5, or 2 μmol/kg was not affected significantly except in liver by pre-administration of the Gd-micelle or the empty liposome. Although Gd in the first dose may interfere with the Gd accumulation in liver following the second dose injection, Gd-micelle as the first dose for micelle-forming properties are

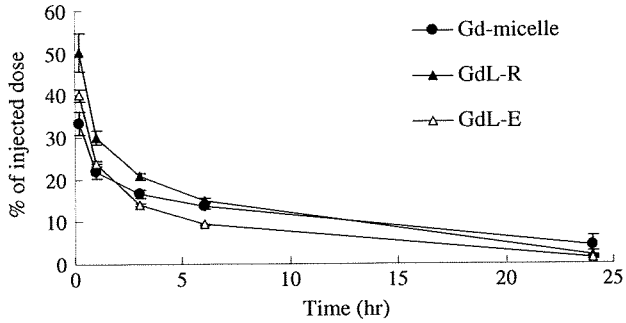

Fig. 3. Plasma elimination profiles of Gd following a single intravenous injection of Gd-micelle at a dose of 33 μmol Gd/kg and Gd-liposome including Gd-liposome prepared by ethanol injection method (GdL-E) at a dose of 6.75 μmol Gd/kg and Gd-liposome prepared by reverse phase evaporation method (GdL-R) at a dose of 2.65 μmol Gd/kg. Data represent mean±S.D. (n=3–4).

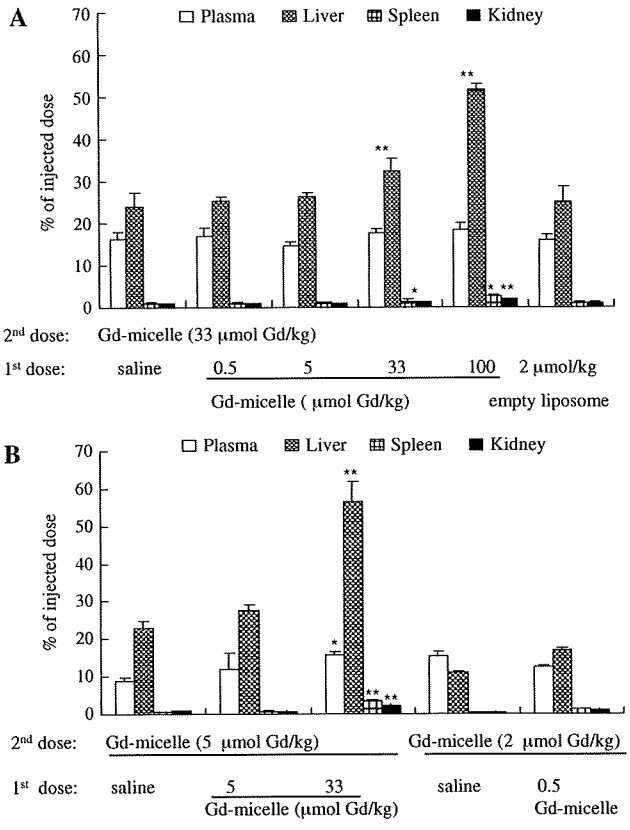

Fig. 4. Effect of the first dose on the tissue distribution of Gd-micelle. The second dose of Gd-micelle with 33 μmol/kg (**A**) or 5 μmol/kg or 2 μmol/kg (**B**) was intravenously injected at day 7 after the first injection of 0.5, 5, 33, 100 μmol/kg of Gd-micelle or the empty liposome at a dose of 2 μmol lipid/kg. Tissues including blood, liver, spleen, and kidney were taken out 6 h after the second injection of Gd-micelle. Data represent mean±S.D. (n=3, 6). P values apply to differences between the saline group and Gd-micelle or liposome treated group. *p<0.05, **p<0.01.

Gd-content-dependent, and Gd-free polymeric micelle is different from the Gd-containing micelle in size and micelle forming characteristics.

Effect of Time Interval Between the Two Injections on the Distribution of the Gd-micelle

Since it was reported that the ABC effect was maximized when the interval between the two injections of liposome was 10 days in mice (7,12), we changed the time interval for the injection of Gd-micelle at a dose of 33 μmol/kg from 3 days to 10 days. No significant difference in plasma Gd levels (15 ~ 18% dose) was observed between the control group and the groups with different time intervals 6 h after the second injection (Fig. 5). The control group was given the Gd-micelle at 33 μmol/kg after a first injection of saline. The Gd% of injected dose in the liver was much higher at day 3 after the second injection than that on other days, probably due to the incomplete elimination of the first dose of the Gd-micelle.

Effect of the First Dose on the Distribution of the Gd-liposome

Since a lower dose of lipid in the first injection results in a more significant ABC, the effects of dose were investigated. The first dose of the Gd-micelle (5 μmol Gd/kg), empty GdL-E (2 μmol lipids/kg), or GdL-E (2 μmol lipids/kg corresponding to 0.45 μmol Gd/kg) was given with a second dose of GdL-E at 10 μmol lipids/kg corresponding to 2.26 μmol Gd/kg. As shown in Fig. 6, the first injection of the Gd-micelle resulted in a similar percentage of the injected dose of the Gd-liposome in plasma, liver, spleen, and kidney in comparison with the saline group. On the other hand, after the second injection of GdL-E, the Gd concentrations in plasma and kidney were too low to be detected, with the first injection of the empty GdL-E and the GdL-E. At that time, the %dose in the liver significantly increased, but that in spleen significantly decreased as compared to saline ($p<0.05$).

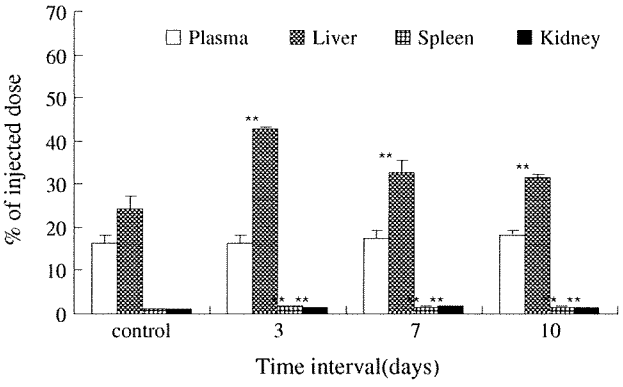

Fig. 5. Effect of the time intervals on the tissue distribution of Gd-micelle. The second dose of Gd-micelle at 33 μmol/kg was intravenously injected at day 3, day 7, or day 10 after the first injection of the same micelle at 33 μmol/kg. The control group was referred to the second dose of Gd-micelle at a dose of 33 μmol/kg with the first injection of saline. Tissues including blood, liver, spleen, and kidney were taken out at 6 h after the second injection of Gd-micelle. Data represent mean±S.D. ($n=3$). *P* values apply to differences between the control group and treated group. $*p<0.05$, $**p<0.01$.

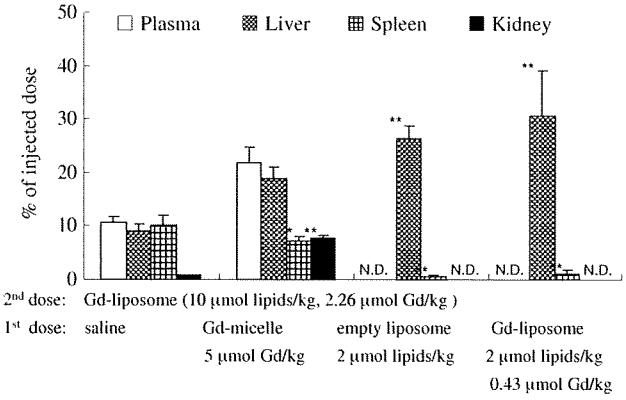

Fig. 6. Effect of the first dose on the tissue distribution of Gd-liposome (GdL-E). The second dose of GdL-E with 10 μmol lipids/kg and 2.26 μmol Gd/kg was intravenously injected at day 7 after the first injection of Gd-micelle (5 μmol Gd/kg), empty GdL-E (2 μmol lipids/kg), and GdL-E (2 μmol lipids/kg and 0.43 μmol Gd/kg). Tissues of blood, liver, spleen, and kidney were removed 6 h after the second injection of GdL-E liposome. Data represent mean±S.D. ($n=3$). *P* values apply to differences between the saline group and Gd-micelle or liposome treated group. $*p<0.05$, $**p<0.01$. N.D. The Gd concentration was too low to be detected by ICP.

Therefore, the data herein show that the accelerated clearance of Gd-liposome at 10 μmol lipids/kg corresponding to 2.26 μmol Gd/kg was induced by both the Gd-liposome and empty liposome, but not by the Gd-micelle. This finding indicates that Gd ions at the first dose of 0.45 μmol/kg did not affect the induction of ABC caused by liposomes.

Effect of PEG on the Distribution of Gd-liposomes

Next, the effect of injecting a PEG homopolymer and PEG_{2000}-DSPE on the distribution of Gd-liposomes was examined. Since the encapsulation efficiency of Gd was low with the ethanol injection method, we prepared another Gd-liposome by the reverse phase evaporation method (GdL-R). The tissue distribution of GdL-R at 6 h after injection at a dose of 5 μmol lipids/kg was not significantly influenced by the pre-administration of 50 mg/kg PEG $_{500,000}$, 0.2 mg/kg PEG $_{500,000}$, or 0.3 mg/kg PEG_{2000}-DSPE 7 days before (Fig. 7). The dose of 0.2 mg/kg PEG $_{500,000}$ and 0.3 mg/kg PEG_{2000}-DSPE with the concentration of 0.04 mg/ml is similar to that of the 5 mol% PEGylated liposome (0.3 mg/kg PEG_{2000}-DSPE), which could produce the ABC phenomenon (Fig. 6). Hence, the first injection of $PEG_{500,000}$ saline or PEG_{2000}-DSPE saline failed to cause the ABC phenomenon after the second administration of Gd-liposome. Hence, only injections of PEG macromolecules did not induce the ABC effect.

DISCUSSION

In the present study, the influence of dose on the tissue distribution of Gd-micelles after repeated administrations was investigated. Many studies have found that a lower dose of lipid in liposomes or nanoparticles results in a greater ABC effect (6–8,12), and the magnitude of the ABC phenomenon reached a maximum when the time interval between two

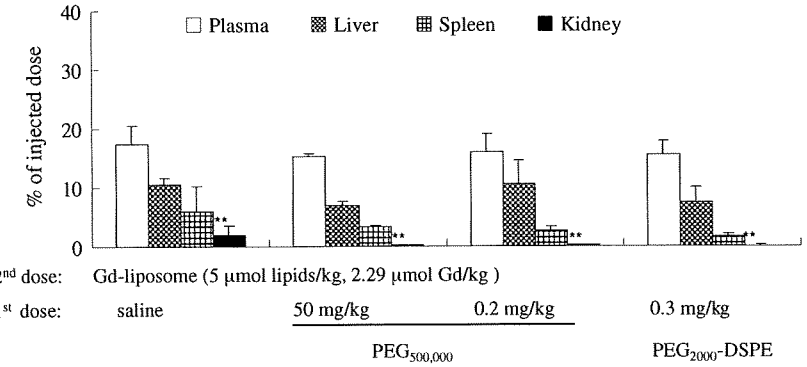

Fig. 7. Effect of $PEG_{500,000}$ and PEG_{2000}-DSPE on the tissue distribution of Gd-liposome (GdL-R). The second dose of Gd-liposome with 5 μmol lipids/kg and 2.29 μmol Gd/kg was intravenously injected at day 7 after the first injection of $PEG_{500,000}$ saline at a dose of 50 mg/kg or 0.2 mg/kg, and PEG_{2000}-DSPE saline at 0.3 mg/kg. The control group of GdL-R was injected at a dose of 10 μmol lipids/kg with the first injection of saline. Tissues of blood, liver, spleen, and kidney were removed at 6 h after the second injection of GdL-R. Data represent mean±S.D. (n=3–5). P values apply to differences between the saline group and $PEG_{500,000}$ or PEG_{2000}-DSPE treated group. $*p<0.05$, $**p<0.01$.

injections was 5–7 days in rats (5) and 10 days in mice (7,12). Hence, we investigated the distribution of the Gd-micelle at various doses and an interval of 3, 7, or 10 days between injections. We found that repeated injections of the Gd-micelle, even with the second dose reduced to 2 μmol Gd/kg (corresponding to 4 mg polymer/kg) and at different time intervals at a dose of 33 μmol Gd/kg (corresponding to 67.3 mg polymer/kg), did not result in an accelerated clearance. ABC of the second injection of the Gd-liposome was induced by the first injection of both the Gd-liposome and the empty PEGylated liposome, but not by the first injection of the Gd-micelle (Fig. 6), Gd-DTPA encapsulated in liposomes would not affect the ABC phenomenon. Therefore, our observation that the ABC phenomenon did not occur with the Gd-micelle is important as it means that injections of the Gd-micelle will not change the biodistribution of a second administration of diagnostic or therapeutic agents.

For Gd-micelle, Gd was chelated to the micelle and thus existed in the form of micelle as shown in Fig. 1, which was consistent with the release results in Fig. 2 that Gd did not leak from the Gd-micelle in PBS (pH 7.4). Gd concentration in plasma, therefore, will reflect the pharmacokinetic behavior of the Gd-micelle. For Gd-liposomes, although the Gd concentration in plasma contained both the leaked Gd-DTPA from the Gd-liposome and the encapsulated Gd-DTPA in the Gd-liposome, the leaked-free Gd-DTPA is reported to be very rapidly cleared from the bloodstream with a minute's order half-life (19), and thus the detected Gd in blood is considered to be only the Gd-DTPA encapsulated in the liposome 6 h after intravenous injection in this study. Compared to GdL-R, the leakage of Gd-DTPA from GdL-E was faster, resulting in the lower Gd concentration (the encapsulated Gd) of GdL-E in blood in Fig. 3. Most importantly, the purpose of this study is to investigate if the distribution for the second dose of the Gd-liposomes or Gd-micelle was affected after pre-administered with the first dose or not. Therefore, the leakage of Gd-DTPA from the Gd-liposomes will not influence this study. In addition, many studies demonstrated that ABC phenomenon for empty liposome was observed determined by [H^3]-labelled or ^{99m}Tc-labelled method (5–9).

It is believed that macrophages in the RES play an important role in ABC, and liposomes were mainly located in Kupffer cells after a second injection (5,8).When hepatosplenic macrophages were depleted, no enhanced clearance of liposomes was observed (6). The induction of ABC with liposomes could be attributable to a 150 kDa serum factor (5), anti-PEG IgM (9,11,12,22,23), anti-PEG antibody (10), or anti-PEG IgG antibody (24).

Whereas the mechanism of the immune response on repeated injections of liposomes has not been fully elucidated yet, the enhanced clearance effect can still be divided into two phases: the induction phase following the first injection and the effectuation phase following the second injection (6). According to this theory, there are two very important factors: one is the biological material (e.g. antibody) produced in the induction phase, the other is the recognition of the antibody by the second dose. For the effectuation phase, it was reported that the ABC phenomenon was induced by the second dose of a PEGylated liposome, but not of a liposome lacking a PEG-coating (23). This indicates that PEG is essential for the nanocarrier to recognize the antibody in the effectuation phase. In this study, the ABC phenomenon was not observed after repeated injections of the Gd-micelle at different doses and time intervals. This ABC failure of Gd-micelle may be caused by the failure for the production of biological material in the induction phase (data not shown) or/and for the recognization by the antibody in the effectuation phase. Even if the first injection was of empty liposome, the second injection of the Gd-micelle did not produce the ABC phenomenon either. This suggests that the antibody produced by the empty liposome in the induction phase is not recognized by the PEG moiety of the Gd-micelle. Therefore, not only PEG, but also other factors such as structure and hydrophobic character affect recognition.

For the induction phase, the ABC phenomenon was not observed when the amount of PEGylated lipid of liposomes in the first injection was more than 10 mol% (7,8). We have previously reported the accelerated clearance of [^{3}H]-labeled PEGylated liposomes in mice pre-administered empty PEG-PBLA polymeric micelles (16). Furthermore, repeated

injections of PEG-PLA nanoparticles also produced the ABC phenomenon (12). Hence, the structure and component of nanocarriers has a considerable impact on the induction phase of ABC. From a structural perspective, the Gd-micelle formed through ionic interactions; therefore, it does not have any hydrophobic part (Fig. 1). In contrast, the PEG-PBLA micelle is composed of both a hydrophilic part, PEG, and a hydrophobic part, PBLA. Similarly, PEGylated liposomes possess a hydrophilic PEG chain and a hydrophobic bilayer membrane. The immunogenicity of an antigen can be affected by factors such as the physical and chemical properties of the antigen, its dose, and so on (25). The reasons why the Gd-micelle evaded the ABC phenomenon have not yet been elucidated at the present stage. The absence of a hydrophobic part may be a key for this elucidation because the other ABC-phenomenon-positive PEG-based carrier systems possess hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. We are currently investigating the ABC phenomenon induced by other kinds of polymeric micelles and nanoparticles. It is hoped that these experiments will provide more evidence for the mechanism of the ABC phenomenon.

CONCLUSIONS

The Gd-micelle did not induce ABC following its pre-administration at various doses and time intervals. In contrast, the Gd-liposome induced the phenomenon when it or an empty PEGylated liposome, but not the $PEG_{500,000}$ macromolecule or PEG_{2000}-DSPE, was pre-administered. ABC-phenomenon-positive PEG-based carrier systems possess a hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. The absence of a hydrophobic part of Gd-micelle may be a key factor for not producing the ABC phenomenon.

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