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Direct measurement of the extravasation of polyethyleneglycol-coated liposomes into solid tumor tissue by in vivo fluorescence microscopy

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Abstract

The extravasation of liposomes of different sizes into solid tumors after i.v. injection was visualized by in vivo fluorescence microscopy in mouse neuroblastoma C-1300-bearing mice. Liposomes composed of distearoylphosphatidylcholine/cholesterol (1/1 molar ratio) and 6 mol% distearoylphosphatidylethanolamine derivative of polyethyleneglycol (PEG) were prepared. The PEG-coated liposomes were fluorescently labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) as a liposome marker or with doxorubicin (DXR) as an aqueous-phase marker. Liposomes with an average diameter of 100–200 nm showed the greatest tumor accumulation. With time after injection of DiI-labeled liposomes, the tumor interstitial fluorescence intensity increased. Most fluorescent spots were located outside and around the vessel wall, indicating extravasation of intact liposomes. The perivascular distribution was heterogeneous. We also obtained the same fluorescence localization pattern with DXR released from extravasated liposomes after injection of DXR-encapsulated liposomes. No fluorescence from extravasated liposomes was detected in normal s.c. tissue; the fluorescent spots were observed only in the vessel wall. Our results indicate that small-size long-circulating liposomes are able to traverse the endothelium of blood vessels in tumors and extravasate into interstitial spaces. Moreover, encapsulated drug was released from extravasated liposomes in the tumor.

Keywords: Liposomes; Polyethyleneglycol; Extravasation

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1. Introduction

The recently developed amphipathic polyethyleneglycol (PEG)-coated long-circulating liposomes (PEG-liposomes) have great potential as a drug delivery system. The authors and other groups have demonstrated that the inclusion of PEG significantly reduces the reticuloendothelial system (RES) uptake of liposomes and results in the prolonged circulation of liposomes (Klibanov et al., 1990; Blume et al., 1990; Allen et al., 1991; Maruyama et al., 1992). Most importantly, small unilamellar PEG-liposomes, around 100 nm in average diameter, showed high accumulation into solid tumors (Gabizon and Papahadjopoulos, 1988). Recent studies have shown that PEG-liposomes encapsulating anthracyclines such as doxorubicin (DXR) exhibit improved therapeutic efficacy and reduced toxicity after i.v. injection in solid tumor-bearing mice (Papahadjopoulos et al., 1991; Gabizon, 1992; Unezaki et al., 1995). Generally, the capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal organs (Jain and Gerlowski, 1986). Due to the increased circulation time of PEG-liposomes and the leakiness of the microcirculation in the solid tumors, PEG-liposomes containing anticancer drugs have been shown to accumulate preferentially in the tumors. However, the mechanism of the extravasation of PEG-liposomes in solid tumor tissue is not understood, though possible pathways of transendothelial transport have been suggested (Yuan et al., 1994).

To examine the extravasation of intact liposomes encapsulating drugs in solid tumor tissue, we have designed an experiment to visualize directly the transvascular transport of liposomes. PEG-liposomes were fluorescently labeled with DiI (lipid marker) or DXR (aqueous-phase marker) and in vivo fluorescence microscopy was used. This combination allows us to observe directly the normal and tumor microcirculation in anesthetized mouse and to obtain quantitative information on the extravasation of PEG-liposomes.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) (COATSOME MC-8080) and distearoylphosphatidylethanolamine (DSPE) (COATSOME ME-8080) were kindly donated by Nippon Oil and Fats, Tokyo, who also provided monomethoxy polyethyleneglycol succinimidyl succinate (PEG-OSu), with an average M_r of 1000. DSPE-PEG derivatives were synthesized from DSPE and PEG-OSu as described previously (Maruyama et al., 1992). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Molecular Probes, Eugene, USA. Doxorubicin (DXR) was kindly provided by Kyowa Hakko Kogyo, Tokyo. Cholesterol (CH), [^{3}H]cholesteryl hexadecyl ether and other chemicals used were of reagent grade.

2.2. Animals and tumor models

Tumor-bearing mice were prepared by inoculating s.c. a suspension ($\sim 1 \times 10^7$ cells) of mouse neuroblastoma C-1300 cells directly into the hind leg of female A/J mice (8 weeks old, and weighing 20–25 g). Single tumor cells were isolated with 0.1% collagenase (in 0.6% glucose-PBS solution) from s.c. C-1300 neuroblastoma. The viability of these cells was > 90% as judged from trypan blue exclusion. The prepared mice were tested when the estimated tumor mass volume (calculated as $1/2 \times \text{length} \times \text{width}^2$) reached about 1000–1500 mm^3 (Coebett et al., 1978).

2.3. Liposome preparation

Liposomes were prepared using the extrusion technique, following the REV method (Szoka and Papahadjopoulos, 1978). DSPC/CH in a molar ratio of 1:1 were used for the conventional liposomes and DSPC/CH/DSPE-PEG in a molar ratio of 1:1:0.13 for PEG-liposomes. Liposomes labeled with a trace of [^{3}H]cholesteryl hexadecyl ether were prepared for the biodistribution study. DiI-labeled liposomes were prepared by the addition of DiI (chloroform solution) at 4 μg/mg lipid

in the REV method. DXR-encapsulating liposomes were prepared by the pH gradient method as described previously (Unezaki et al., 1995; Mayer et al., 1986). In this case, 300 mM citric acid (pH 4.0) was used instead of saline in the REV method. The pH of the liposome suspension was raised to pH 7.8 with 1 N NaOH. This liposome suspension was mixed with DXR solution dissolved in distilled water at a drug-to-lipid weight ratio of 0.2, and the mixture was incubated with periodic mixing for 10 min at 60°C. The free fluorescent dye or DXR in the resulting preparation was removed by gel filtration on a Sephadex G-50 column. The average diameter of the liposomes was measured by dynamic laser light scattering using a Nicomp 370 HPL submicron particle analyzer (Pacific Scientific, CA, USA.). The amount of entrapped DXR was determined with a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm, Hitachi F-3000) after dilution with 0.3 N HCl-50% ethanol.

2.4. Experimental procedure

The microvascularization of normal and tumor tissue was examined directly by in vivo fluorescence microscopy after injection of DiI-labeled liposomes (1 mg lipids/mouse) into the tail vein of C-1300-bearing mice. At the selected time, a mouse was anesthetized with sodium pentobarbital and placed on a microscope stage. A part of the surface skin over the tumor or normal s.c. tissue on the leg was peeled and the exposed target region was fixed on a slide glass with adhesive tape, then examined directly in a fluorescence microscope (TMD 300 with excitation filter B-2A 450–490, Nikon) at 400 × magnification. This method allows us to observe the normal and tumor microcirculation of anesthetized mouse via the fluorescence microscope, as well as the extravasation of DXR-encapsulated liposomes (DXR-liposomes) in tumor tissue. Free DXR or liposomal DXR was injected via the tail vein into mice at 5 mg DXR/kg. The fluorescence from tissue was measured directly as described above. DXR concentration in tissue was determined by HPLC assay as described previously (Unezaki et al., 1995).

3. Results

3.1. Characterization of PEG-liposomes

To test the effect of size on the liposomal biodistribution and localization into the tumor tissue, PEG-liposomes of 63, 133, 198, and 388 nm in average diameter were prepared, and injected into C-1300-bearing mice via the tail vein. The biodistribution results at 6 h post injection are presented in Fig. 1. As previously reported (Maruyama et al., 1992), DSPE-PEG effectively prolonged the circulation of DSPC/CH liposomes by decreasing the liver uptake, for liposomes with an average diameter of 100–200 nm. However, when the average diameter of the PEG-liposomes was > 300 nm, the circulation time was decreased relative to that of the small liposomes. These large PEG-liposomes were accumulated efficiently in the spleen, as previously reported (Klibanov et al., 1991). The PEG-liposomes of the size which showed the most prolonged circulation were accumulated most efficiently in the C-1300 solid tumor.

The encapsulation of DXR into PEG-liposomes with an average diameter of 137 ± 38 nm was done by the pH gradient method. This size range of liposome was chosen because they exhibited an elevated tumor accumulation (Table 1). DXR was entrapped in the liposomes with approximately 98% efficiency. The presence of DSPE-PEG did not interfere with this procedure. DXR-PEG-liposomes were injected into C-1300-bearing mice via the tail vein, and at 6 h post injection the tumors were excised and the DXR concentration was assayed. Table 1 shows the DXR levels in tumor 6 h after the administering of free DXR and liposomal DXR. As previously reported (Unezaki et al., 1995), free DXR was cleared quickly from the blood circulation and DXR concentration in the tumor was < 1 μg/g tissue. Administering of DXR-PEG-liposomes produced increased the accumulation of DXR in the tumor compared with administration of the free drug, and resulted in a tumor concentration of 12.46 μg/g tissue at 12 h after injection. It is clear from these results that the high tumor accumulation of DXR directly parallels the high blood concentration of long-circulating liposomes.

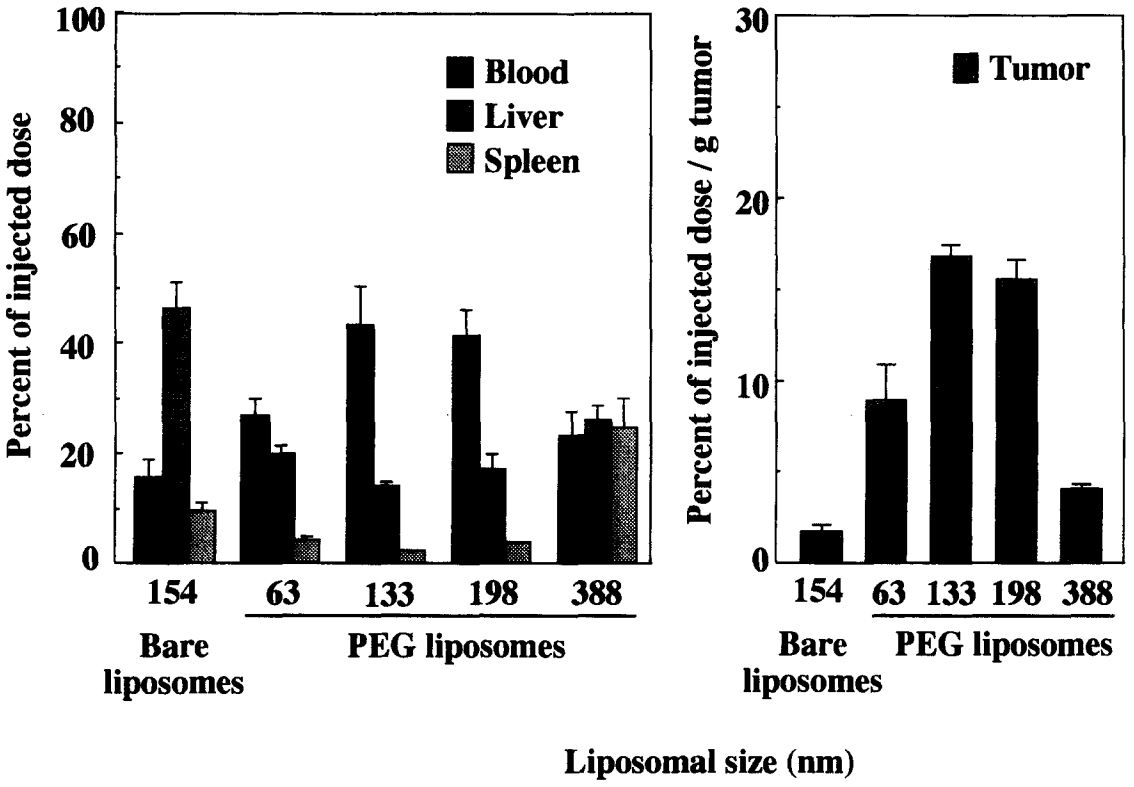

Fig. 1. Size-dependent biodistribution of PEG-liposomes at 6 h after injection. [^{3}H]Cholesteryl hexadecyl ether-labeled liposomes were injected into C-1300-bearing mice. Results are given as means ($n = 3$) ± S.D.

3.2. Localization of DiI-PEG-liposomes in normal and tumor tissue

Localization of DiI-liposomes in normal or tumor tissue was observed directly by fluorescence microscopy. The mean diameter ± S.D. of the DiI-labeled PEG-liposomes used was 126 ± 35 nm. Each photograph shown in Fig. 2 is one of many similar photographs taken from various surface areas of the tumor and normal s.c. tissue. At 30 min after liposome injection, fluorescence was detected in tumor blood vessels (Fig. 2A). This indicates that the tumor vascular network was hypervascular as compared with normal s.c. tissue, but the distribution and blood flow were both heterogeneous. With time after injection, fluorescence intensity in the tumor increased and tumor interstitial fluorescent spots (patches) were observed at about 6 h after injection. As shown in Fig. 2B, at 2 days after injection, most fluorescent spots were located outside and around the vessel wall. These results indicate that the fluorescence exhibited by DiI probably represents extravasated intact liposomes. In our previous experiments

Table 1
DXR levels in tumor after i.v. injection of free DXR or liposomal DXR into C-1300-bearing mice at a dose of 5 mg DXR/kg

DXR administration	DXR concentration (μg/g tumor)		
	1 h	12 h	24 h
Free DXR	0.33 ± 0.15	0.57 ± 0.12	0.46 ± 0.10
DXR-PEG-liposome	6.25 ± 0.48	12.46 ± 3.72	7.81 ± 1.46

The mean diameter ± S.D. of DXR-PEG-liposomes used was 137 ± 38 nm. Results are given as means ($n = 3$) ± S.D.

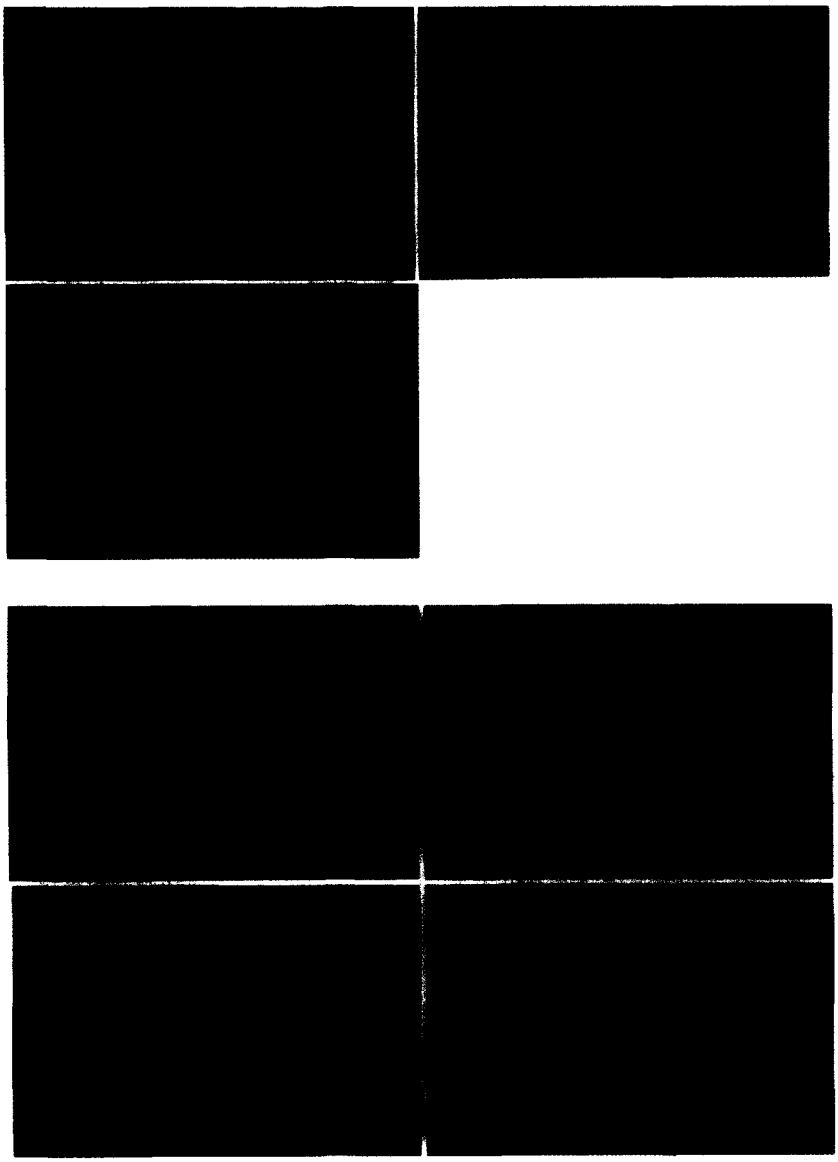

Fig. 2. Microscopic localization of DiI-PEG-liposomes or DXR-PEG-liposomes in normal and tumor tissue. The mean diameter ± S.D. of the DiI-PEG-liposomes used for A, B, D and E was 126 ± 35 nm, and that for C was 402 ± 48 nm. The mean diameter ± S.D. of the DXR-PEG-liposomes used for F was 133 ± 37 nm. (A) Fluorescence image of tumor microvasculature at 30 min after DiI-liposomes injection. (B) Liposome localization in the tumor was perivascular. The photograph was taken at 2 days after DiI-liposomes injection. (C) Fluorescence image of tumor microvasculature at 6 h after the large DiI-liposomes (402 ± 48 nm) injection. Extravasation of the large DiI-liposomes was not detected. Only fluorescent spots within the vessel wall were observed. (D) In normal tissue, extravasation of DiI-liposomes was not detected. Only fluorescent spots within the vessel wall were observed. The photograph was taken 1 day after DiI-liposomes injection. (E) In normal tissue, fluorescence image at 2 days after DiI-PEG-liposomes injection. Fluorescence intensity in the vessel wall gradually decreased. (F) Fluorescence image in tumor 1 day after DXR-PEG-liposomes injection. Focal extravasation is clearly visible. (G) Fluorescence image in tumor 1 day after free DXR injection.

(Unezaki et al., 1995), when DXR-PEG-liposomes were injected intravenously into mice implanted subcutaneously with colon 26 carcinoma, increased tumor accumulation was demonstrated and the highest drug level in the tumor was obtained at about 6 h after injection. Thus, the above observations correspond to those of previous studies. As shown in Fig. 1, size of liposome is an important factor for extravasation. Extravasation of DiI-PEG-liposomes with an average diameter of 402 ± 48 nm was not detected at 6 h after injection (Fig. 2C). Only fluorescent spots within the vessel wall were observed.

In normal s.c. tissue, extravasation of DiI-liposomes was not detected, and tumor interstitial localization of fluorescence could not be observed during 2 days (Fig. 2D and E). As shown in Fig. 2D, taken at 1 day after injection, only fluorescent spots in the vessel wall were observed, and the fluorescence intensity gradually decreased after injection (Fig. 2E).

It is difficult to see endothelial cells by microscopy, but the above results suggest that the accumulation of DiI-liposomes in normal s.c. vessel wall is probably related to specific properties of endothelial cells in normal vessels. The difference of vascular structure in tumor and normal tissue is probably closely related to differences in endothelial cell properties, and hence liposome-endothelial cell interaction is likely to be different in the two tissues.

3.3. Localization of DXR-PEG-liposomes in tumor tissue

We have also examined the localization of DXR-liposomes in tumor tissue. DXR was used as a water-soluble liposome-encapsulated marker. Fig. 2F and G shows fluorescence images of the tumor 1 day after injection of DXR-liposomes and free DXR, respectively. Most of the fluorescent spots were observed within the tumor mass (Fig. 2F). Their intensity was much stronger than that after free DXR administration (Fig. 2G). DXR is itself fluorescent, but this fluorescence is somewhat self-quenched when DXR is encapsulated in liposomes (Needham et al., 1992), so its presence in the tumor, as in Fig. 2F, indicates that DXR is released from the liposomes. This result has been confirmed by the tumor localization studies of DiI-liposomes described above. Perhaps DXR released from extravasated liposomes in the tumor would be able to penetrate into tumor cells.

4. Discussion

In this study, in order to visualize the extravasation of PEG-liposomes into the solid tumor, we have used DiI as a liposome lipid marker and DXR as an aqueous content marker. A lipophilic dye such as DiI is advantageous for a rapid and simple procedure to visualize liposomes in vivo. Furthermore, fluorescent DiI incorporated into the liposome lipid bilayer does not interfere with plasma proteins (Claassen, 1992). The size limitation of the PEG-liposomes is of practical significance, as shown by the data in Figs. 1 and 2A–C. Elevated tumor uptake of the PEG-liposomes exhibits an optimal liposome size range which coincided with the range shown in Fig. 1 for affording prolonged liposome circulation. Therefore, effective tumor uptake of liposomes may depend on the prolonged circulation of liposomes.

Generally, extravasation of circulating molecules from blood vessels is a function of both local blood flow and microvascular permeability. Normal tissues outside the RES are known generally to have continuous and nonfenestrated vascular endothelia, and extravasation of macromolecules or liposomes is greatly limited (Fig. 2D). On the other hand, the permeability of the endothelial barrier in newly vascularized tumors is increased compared to that in normal tissues. Under physiological tumor conditions, only liposomes with small size and prolonged circulation half-life will have a sufficient chance to encounter the leaky vessels of the tumor (Fig. 2B). The amount of blood circulating through a solid tumor is probably only a small fraction of the total and is likely to depend on the age, location and nature of the tumor.

We recently reported that PEG-coated thermosensitive liposomes exhibit a marked enhancement of the tumor accumulation and antitumor activity of encapsulated DXR in combination

with local hyperthermia (Unezaki et al., 1994) In that study, the entrapped DXR was efficiently released from thermosensitive liposomes in blood vessels by hyperthermia at the tumor site and entered the tumor tissue by simple diffusion. On the other hand, our present study shows that small-sized long-circulating liposomes can traverse the endothelium of blood vessels in the tumor, and extravasate into interstitial spaces. Internalization of the drug-encapsulating liposomes by tumor cells is not necessarily required to explain the antitumor response. We speculate that intact extravasated liposomes within the tumor area gradually break down and release the entrapped drug. Our observations (Fig. 2F and G) reveal that sustained local release and retention of DXR from extravasated liposomes are especially important for efficacy, because free DXR is quickly cleared from plasma and tissue. This is consistent with a recent study of the increased therapeutic efficacy of DXR encapsulated in PEG-liposomes against mouse colon carcinoma (Unezaki et al., 1995).

The present study provides a possible mechanism for increased tumor accumulation of long-circulating liposomes. PEG-liposomes may be available to increase the therapeutic index of anticancer drugs by transporting them through the endothelial barrier, via extravasation. We should note, however, that microvascular permeability, a critical factor in liposome tumor localization, may vary considerably among different tumors.

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