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Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice

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Abstract

We have examined the size dependence of extravasation and interstitial localization of polyethyleneglycol-coated liposomes (PEG-liposomes) in the solid tumor tissue by means of electron microscopic observation. Liposomes composed of distearoyl phosphatidylcholine, cholesterol and distearoylphosphatidylethanolamine derivative of polyethyleneglycol (PEG) were prepared in various size ranges. PEG-liposomes with an average diameter of 100–200 nm showed the most prolonged circulation time and the greatest tumor accumulation in all the solid tumors employed in this experiment. Although large PEG-liposomes with a diameter of 400 nm showed a short circulation time in normal mice, the results in splenectomized mice indicated that they do have an intrinsic prolonged circulation character in vivo. However, large PEG-liposomes could not extravasate into solid tumor tissue. These results indicate that the size of liposomes is critical for extravasation. The electron microscopic observations revealed the almost exclusive engulfment of extravasated liposomes by tumor-associated macrophages; very few were taken up by tumor cells.

Keywords: Liposomes; Polyethyleneglycol; Extravasation

1. Introduction

Recently developed amphipathic polyethyleneglycol (PEG)-coated liposomes (PEG-liposomes) have great potential as drug delivery systems. It has been reported that the inclusion of PEG significantly reduces the reticuloendothelial system (RES) uptake of liposomes and results in the prolonged circulation of liposomes (Blume and Cevec, 1990; Klibanov et al., 1990; Allen et al., 1991; Maruyama et al., 1992). More importantly, these so called long-circulating liposomes (100–200 nm in mean diameter) are able to traverse the endothelium of blood vessels in solid tumors and are extravasated into the interstitial spaces, resulting in significantly greater accumulation compared with conventional liposomes (Huang et al.,

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1991; Yuan et al., 1994; Unezaki et al., 1996). Generally, the capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of healthy tissue (Jain and Gerlowski, 1986; Dvorak et al., 1988). Under such conditions, liposomes of small size and longer half-life in the circulation will have more opportunity to traverse the tumor capillaries. Thus, in terms of drug delivery to solid tumors, these long-circulating liposomes offer several advantages over customary liposomal formulations. Recent studies have shown that PEG liposomes encapsulating anthracyclines such as doxorubicin (DXR) exhibit improved therapeutic efficacy and reduced toxicity after intravenously (i.v.) injection into solid tumor-bearing mice (Papahadjopoulos et al., 1991; Gabizon, 1992; Unezaki et al., 1995). The mechanisms of the extravasation and interstitial movement of PEG-liposomes in solid tumor tissue, however, are less well understood, although, a knowledge of them is essential to develop strategies for PEG-liposome-mediated tumor therapy. Here, we further characterize the size dependence of extravasation and interstitial movement of PEG-liposomes. The pathway of extravasation and the final localization of liposomes were visualized by electron microscopy in solid tumor-bearing mice.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) (COATSOME MC-8080) and distearoylphosphatidyl ethanolamine (DSPE) (COATSOME ME-8080) were kindly donated by Nippon Oil and Fats (Tokyo), who also provided monomethoxy polyethyleneglycol succinimidyl succinate (PEG-OSu) (SUNBRIGHT VFM4101) with an average molecular weight of 2000. An amphipathic PEG (DSPE-PEG) was synthesized from DSPE and PEG-OSu, as described previously (Maruyama et al., 1992). Gold chloride was purchased from Sigma (St. Louis, MO). Glutaraldehyde solution and osmium tetroxide (EM grade) were purchased from Electron Microscopy Sciences (Washington, PA). Araldite M, cacodilic acid and propylene oxide were from Nisshin EM (Tokyo, Japan). Dodecenylsuccinic anhydride (DDSA), POLY/BED 812 and DMP-30 were from Polysciences Inc. (Warrington, PA). Na[^{125}I] was from New England Nuclear Japan. ^{125}I-labeled tyrarninylinulin (^{125}I–TI) was used as an aqueous tracer to measure liposomal biodistribution in vivo. TI was synthesized according to Sommerman et al. (1984), and radiolabeled with ^{125}I by using the IODEGEN procedure (Pierce Chemical, Rockford, IL). Cholesterol (CH) 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 ($\approx 1 \times 10^7$ cells) of S-180 or Ehrlich cells directly into the hind leg of male ddY mice (7 weeks old, weighing 25–30 g). Mouse colon carcinoma cells (Colon 26) ($\approx 1 \times 10^7$ cells) or B16 melanoma cells ($\approx 2 \times 10^6$ cells) were inoculated into the hind leg of male BALB/c mice (7 weeks old, weighing 2–25 g) or female C57BL/6 mice (7 weeks old, weighing 17–22 g), respectively. Liposome localization experiments were performed when the tumor size was in the range from 8 to 10 mm in diameter.

2.3. Preparation of splenectomized tumor-bearing mice

At five days after s.c. inoculation of Colon 26 cells, the mice were splenectomized under anesthesia induced by an intraperitoneal injection of sodium pentobarbital, and used after 1 day.

2.4. Liposome preparation

Liposomes were prepared by extrusion, using the hydration method. Briefly, a lipid mixture composed of DSPC/CH/DSPE-PEG (10:10:1, m/m) in chloroform was evaporated to form a dried thin film in a round-bottom flask. The lipid film was hydrated with saline containing ^{125}I-TI as a liposomal marker for the distribution study, and vortexed at 60°C. The resulting PEG-liposomes were extruded several times through Nuclepore

polycarbonate filters (0.4, 0.2, 0.1, or 0.05 μm in series, Nomura Science, Tokyo) to control the size. The extrusion was performed at 60°C in a thermostatic extrusion device (Extruder, Lipex Biomembrane, Canada). The extruded liposomes were then chromatographed on a Bio-gel A1.5 m column to remove free ^{125}I-TI. The average diameter of the liposomes were measured by dynamic laser light scattering using a Nicomp 370 submicron particle analyzer (Pactfic Science, CA).

2.5. Preparation of PEG-liposomes entrapping colloida1 gold

Preparation of PEG-liposomes entrapping colloidal gold was done by a modified procedure according to Huang et al. (1991). A thin lipid film (10 μmol phospholipid) composed of DSPC/CH/DSPE-PEG (9:10:1 m/m) was hydrated with freshly prepared 6.36 mM gold chloride 60 mM citric acid 15 mM K_2CO_3 solution. The PEG-liposomes were then extruded ten times through a membrane filter with a pore size of 0.1 μm at 60°C. Immediately after final extrusion, the pH of the liposome suspension was raised to 6.0 by adding 1 M KOH and the suspension was then incubated at 60°C for 30 min. After gold particles had formed, the unencapsulated free gold and excess citrate were removed by passing the liposome suspension through a column of Bio-gel A1.5 m. Finally, homogeneous colloidal gold-PEG-liposomes were obtained from the supernatant separated by centrifugation at $7000 \times g$. The average size of the liposomes was 130 ± 21 nm in diameter, as determined by submicron particle analyzer.

2.6. Electron microscopy

Colloidal gold-PEG-liposomes were injected into Colon 26 tumor-bearing mice via the tail vein. At 24 h after injection, the mice were retrogradely perfused with 0.08 M cacodylate buffer solution containing heparin (0.25 g l^{-1}) and procaine hydrochloride (5 g l^{-1}) through the aorta abdominalis, and fixative (2% glutaraldehyde) was introduced for 1 min. Then, the tumor tissue was excised, cut into small blocks in the same fixing solution and further fixed by immersion in 2% glutaraldehyde for 2 h. The blocks were washed in buffer, postfixed in 1% OsO_4 solution in buffer for 1 h, dehydrated in an ethanol series and embedded in an Epon–Araldite mixture (Sasaki, 1990). Ultrathin sections were cut with an ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate and were examined under a HITACHI 7000TEM electron microscope (Tokyo).

3. Results

3.1. Effects of liposome size on biodistribution and tumor accumulation

To test the effect of size on the liposomal biodistribution, PEG-liposomes composed of DSPC/CH/DSPE-PEG (10:10:1 m/m) and of different sizes were prepared and injected i.v. in to Colon 26 tumor-bearing mice. The levels of PEG-liposomes remaining in blood and the uptake by liver, spleen and tumor at different times are shown in Fig. 1, compared with the results for bare liposomes (DSPC/CH (1:1 m/m), 120 nm average diameter). 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 120 nm average diameter. Such liposomes were accumulated more efficiently into the Colon 26 solid tumor tissue than bare liposomes. However, when the average diameter of the PEG-liposomes was 400 nm, the circulation time was decreased and the uptakes by liver and spleen were increased relative to the 120 nm liposomes. In particular, the spleen uptake of such large PEG liposomes was significantly higher. This accumulation in spleen reached 17% of the injected dose at 6 h post injection. These large PEG-liposomes did not accumulate in the solid tumor tissue.

We investigated whether the size of liposomes affects the level of tumor uptake in other solid tumor models. The results are summarized in Fig. 2. PEG-liposomes with 120 nm average diameter, which exhibited the most prolonged circulation, showed the highest accumulation into all the solid

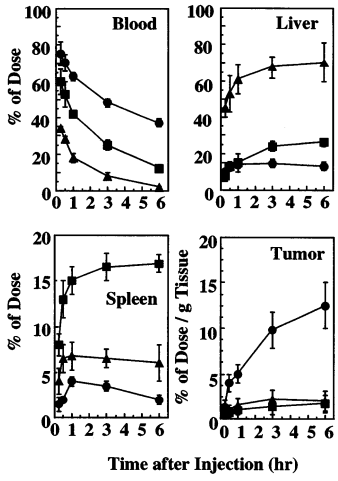

Fig. 1. Size-dependent biodistribution of PEG-liposomes composed of DSPC/CH/DSPE-PEG (10:10:1 m/m), compared with that of bare liposomes (DSPC/CH (1:1 m/m) in lipid composition and 120 nm in an average diameter). Liposomes containing ^{125}I-TI with a defined diameter of 120 or 400 nm were injected i.v. at a dose of 500 μg lipid per mouse. (●) PEG-liposomes with 120 nm in average diameter. (■) PEG-liposomes with 400 nm in average diameter. (▲) Bare-liposomes with 120 nm in average diameter.

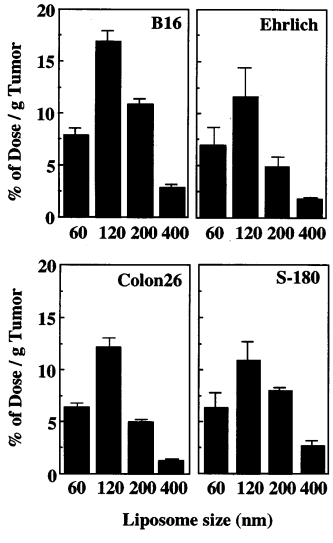

Fig. 2. Effect of liposomal size on the accumulation of PEG-liposomes in various solid tumors. Liposomes were injected into tumor-bearing mice via the tail vein, and the biodistribution was estimated at 6 h after administration.

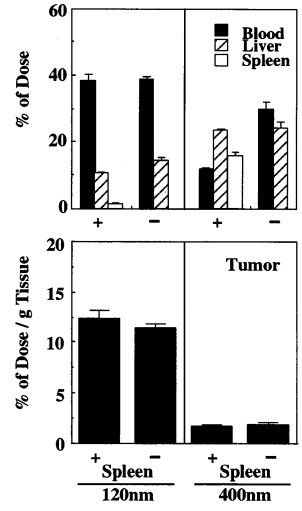

Fig. 3. Biodistribution and tumor uptake of PEG-liposomes of small (120 nm) or large (400 nm) size in splenectomized Colon 26 tumor-bearing mice. Biodistribution and tumor uptake were estimated at 6 and 24 h after administration, respectively.

tumors employed in this experiment. Increasing the diameter resulted in lower tumor accumulation in all solid tumor models. However, the accumulation levels of PEG-liposomes of the same size varied widely among the different solid tumors. Conversely, the smallest liposomes (60 nm) showed rather low tumor accumulation. These results confirm earlier observations with other tumors such as mouse neuroblastoma C-1300 (Unezaki et al., 1996) that small (100–200 nm) PEG-liposomes not only have a prolonged circulation time, but also exhibit favorable tumor accumulation, and the accumulation of smallest one (63 nm) is rather low.

3.2. Biodistribution and tumor accumulation of large PEG-liposomes in splenectomized mice

To examine the size dependence of spleen uptake, the biodistribution of small (120 nm) or large (400 nm) PEG-liposomes was examined in splenectomized Colon 26 tumor-bearing mice. As shown in Fig. 3, large PEG-liposomes showed a high blood level in splenectomized mice at 6 h after injection, which was quite contrary to the

result in normal mice (Fig. 1), but was similar to that of small PEG-liposomes. These results showed clearly that PEG-liposomes with a diameter of 400 nm have an intrinsic prolonged circulation character in vivo. It was revealed that the short circulation time of large PEG-liposomes in normal mice was as a result of higher spleen uptake.

In the splenectomized tumor-bearing mice, the tumor accumulation of large PEG-liposomes at 24 h after injection was low and similar to that in normal tumor-bearing mice. It is interesting to note that PEG-liposomes with a diameter of 400 nm could not extravasate into solid tumor tissue in spite of their long circulation time in the splenectomized mice. These results indicate there are size-limited windows in the endothelial barrier for the extravasation of liposomes.

3.3. Electron microscopic observation of extravasation and localization of small PEG-liposomes in tumor tissue

Extravasation and localization of PEG-liposomes were examined by electron microscopy in Colon 26 tumor-bearing mice. PEG-liposomes of 126 ± 35 nm mean diameter, encapsulating colloidal gold particles, were used. As shown in

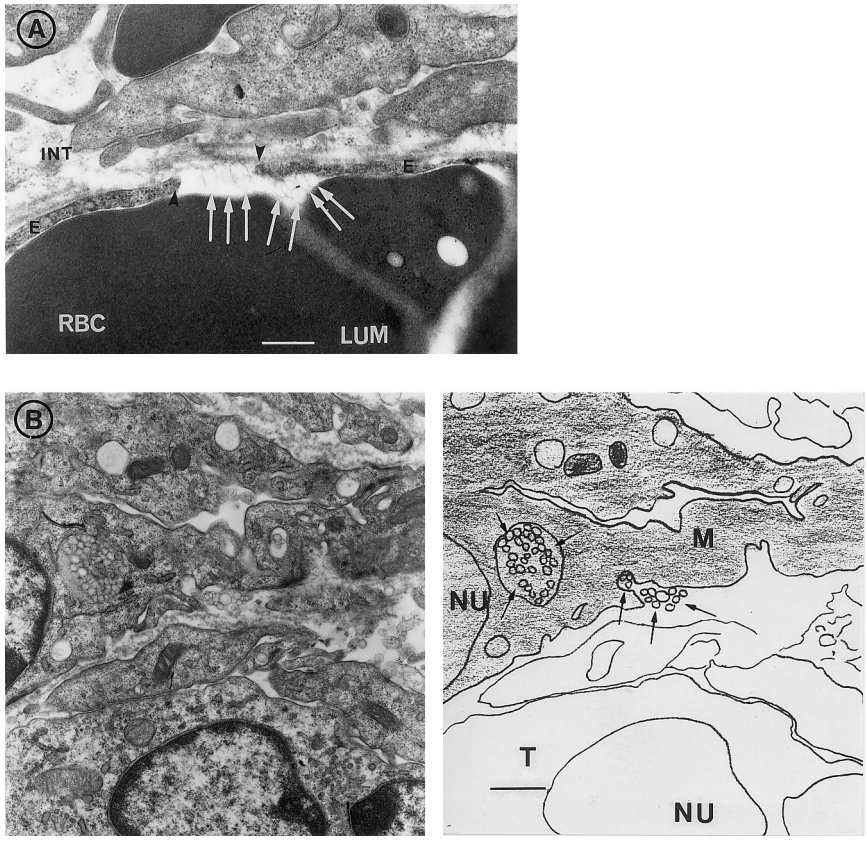

Fig. 4. Electron micrographs showing extravasation and localization of PEG-liposomes (126 ± 35 nm mean diameter) in Colon 26 solid tumor tissue. Liposomes were injected into mice via the tail vein, and the ultrathin sections of tumor tissue were prepared at 24 h after administration. (A) LUM, blood vessel lumen; RBC, red blood cell. E: endothelium; INT, interstitial space; Between arrowheads, the gap between adjacent endothelial cells; Arrows, liposomes across the gap; Bar, 500 nm; (B) M (Grey color), tumor-infiltrating macrophage in the interstitial space; Arrows, liposomes; T, tumor cell; Bar, 1 μm.

Fig. 4(A), many wide inter-endothelial junctions were observed in tumor sections containing blood vessels. Liposomes escaped from the gaps between adjacent endothelial cells and extensively penetrated into the extravascular and interstitial space among tumor cells. This photograph is typical of many similar photographs of tumor tissue at 24 h after liposome injection. It is noteworthy that the extravasated liposomes were seen exclusively in tumor-infiltrating macrophages in the interstitial space, as shown in Fig. 4(B). Very few gold particles were observed in tumor cells. It appears that liposomes were taken up by macrophages and were not taken up by tumor cells in vivo.

4. Discussion

To better understand the mechanism of uptake of PEG-liposomes by solid tumor tissues, and to improve therapeutic efficacy of the encapsulated agents, it would be advantageous to know the pathway and the final localization of liposomes in tissues following i.v. injection.

Our results showed that a relatively high level of tumor accumulation of PEG-liposomes correlated very well with a relatively high concentration of liposomes in the blood. Large PEG-liposomes accumulated predominantly in the spleen, as shown in Fig. 1. However, the results in splenectomized mice revealed that large PEG-liposomes also have long-circulating activity (Fig. 3). Previous studies (Yuda et al., 1999) showed that PEG reduced the opsonization of liposomes in blood by providing a steric barrier. The spleen is a spongy organ that contains the white pulp and the red pulp. The red pulp of the spleen consists of a meshwork of reticular fibers with reticular cells accompanied by fixed macrophages. Aged or damaged red blood cells are filtered in this meshwork and are ingested by the splenic macrophages. Although the spleen function is complex, it was suggested that large PEG-liposomes accumulate by filtration and are taken up by splenic macrophages.

In morphological, physiological and pharmacological studies of microvascular permeability, leakage of particles or macromolecules was observed at postcapillary venules by electron microscopic observation. In general, tumor vessels are inherently leaky, because of the wide inter-endothelial junctions, large numbers of fenestrations and transendothelial channels, and discontinuous or absent basement membrane. Thus, the permeability of the endothelial barrier in newly vascularized tumors is increased compared to that in normal tissues. In this study, the localization of PEG-liposomes into the interstitial space between tumor cells by a process of extravasation from tumor vessels was revealed by the electron microscopic observations (Fig. 4(A)). Small PEG-liposomes can avoid rapid uptake by the RES and can remain in the blood circulation for a relative long period of time. Therefore, they have more opportunities to extravasate through discontinuous capillaries. The size limitation of the PEG-liposomes for tumor accumulation is of practical significance. The results in the splenectomized mice (Fig. 3) revealed that large PEG-liposomes could not extravasate in spite of their long circulation time in blood. Elevated tumor uptake of the PEG-liposomes has an optimal liposome size range which lies within the range for affording prolonged liposome circulation. Therefore, effective tumor uptake of liposomes depends on the prolonged circulation time and the size 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 generally have continuous and nonfenestrated vascular endothelia, and extravasation of macromolecules or liposomes is highly restricted.

It is very interesting that the extravasated liposomes were engulfed exclusively by the tumor associated macrophages, and were not taken up by tumor cells. Since DSPE-PEG used in this study has ester-linkage between PEG and liposome, PEG chains are cleavable in the physiological condition. In less flow condition in interstitial space among tumor cells, it is probable that PEG chains were removed and cleaved gradually from extravasated PEG-liposomes, re-

sulted in their increased uptake by tumor-associated macrophages. These results argue against the idea that increased therapeutic efficiency of DXR encapsulated in PEG-liposomes against mouse colon carcinoma is as a result of tumor uptake (Unezaki et al., 1995). Storm et al. (1988) have demonstrated the ability of macrophages to engulf and process liposomes entrapping DXR and then to release the free drug. Thus, internalization of the drug encapsulating liposomes by tumor cells is not necessarily required to explain the high antitumor response. The role of tumor-associated macrophages in the delivery of liposomal drugs to solid tumors seems to be critical. Further studies would be worthwhile.

The present study provides a possible mechanism for increased tumor accumulation of long circulating PEG-liposomes. However, microvascular permeability, a critical factor in liposome tumor localization, may vary considerably among different tumors. We have utilized these findings to develop a superior thermosensitive liposomal formulation (Ishida et al.). Furthermore, an application of this targeting system to tumor-associated macrophages will be reported elsewhere.

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