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Effects of mixed polyethyleneglycol modification on fixed aqueous layer thickness and antitumor activity of doxorubicin containing liposome

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Abstract

Polyethyleneglycol (PEG) has often been used for the modification of liposomes, but it is difficult to insert PEG on the surface of liposomes, and the effects of modification are not marked enough. In this study, we examined the fixed aqueous layer thickness (FALT) of single or mixed PEG (molecular weight, 340, 500, 900, 2000)-modified doxorubicin (DOX) liposomes, and physical character and biological properties of these liposomes. On single PEG-modification, as the PEG-molecular weight increased, FALT also increased, but the ratio of the increase was reduced. While on modification by a mixture of PEG2000 and PEG with a short polyoxyethylene chain (PEG340 or PEG500), FALT increased compared with the single PEG2000-modified value. Moreover, when liposomes were modified by mixture of PEG2000 and PEG500, we recognized the most suitable mixed rate (PEG2000, 500 = 2:1), showed the maximum FALT. On the other hand, in vivo, as increase of FALT, DOX concentrations increased in the plasma and in the tumor, decreased in the liver. Furthermore, liposomes with remarkable increase of FALT showed enhancement of antitumor activity. As a result, the connection among increase of FALT and improvement of circulation in blood, the involvement of antitumor activity of DOX of these liposomes was suggested. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Polyethyleneglycol; Stealth liposome; Doxorubicin; The fixed aqueous layer thickness (FALT); Passive targeting

1. Introduction

Many drugs are under development for the purpose of treating diseases, but many of them have problems such as side effects. Chemotherapy plays an important part in treatment of cancer, but side effects appear when effective activities are expected. Liposomes are the principal means of effectively using medicines and are broadly recognized as a drug delivery system (DDS; Gabizon et al., 1988; Goren et al., 1990). However, when liposomes are administered by intravenous injection, they are rapidly taken up by reticuloen-

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dothelial system (RES) cells in the liver and spleen (Berestein et al., 1984; Sadzuka et al., 1995). This phenomenon reduces the effectiveness of liposomes as a drug carrier if the aim is long-tern circulation in the blood or targeting of tissues other than the RES. Therefore, techniques for avoiding catch by RES cells and increasing the effectivity of liposomes based on modification on the surface of liposomes have been developed.

Polyethyleneglycol (PEG) has often been used to prolong the circulation time of liposomes (Sadzuka et al., 1995). With PEG modification of the liposome surface, a fixed aqueous layer is formed around the liposomes by interaction between the PEG-polymer and water molecule (Shimada et al., 1995), and prevents the attraction of opsonins, because, serum proteins cannot bind to the water gathered on the surface of the liposomes. As a result, PEG-modified liposomes escape trapping by the cells of the RES, have a prolonged circulation time, and accumulate in tumors by passive targeting (Wu et al., 1993; Schmitz et al., 1992; Papafadjopoulos et al., 1991). However, it is difficult to insert PEG, which can be use to enlarge the fixed aqueous layer thickness (FALT), on the surface of liposomes, so effects of modification by PEG are not exhibited enough. Furthermore, effects of modification by PEG cannot be shown by size or charge, as a common character of liposomes. In other words, no really effective method for PEG-modification of liposomes is available. Until now, the relation between the effect of hybrid liposome without antitumor agents on the inhibition of growth of glioma cells and FALT was reported (Matsumoto et al., 2000). However, for liposome containing antitumor agent, there are few reports, which show the relation between tissue distribution resulted in avoiding trop by RES and FALT, moreover there is no report on mixed PEG-modified liposomes.

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most widely used anticancer agents on the clinical scene, because of its broad spectrum of anticancer activity (Blum and Carter, 1974). After injection, DOX immediately disappears in the blood (Benjamin et al., 1977; Shinozawa and Oda, 1981; Bots et al., 1983; Eksborg et al., 1986; Yesair et al., 1972), and causes severe bone marrow suppression and cardiotoxicity (Minow et al., 1975; Von Hoff et al., 1979). On the other hand, it is shown that liposomal DOX causes a reduction of cardiotoxicity, and increase of antitumor activity (Forssen and Tokes, 1981; Herman et al., 1983; Van Hoesel et al., 1984; Balazsovits et al., 1989). Liposomal DOX has been used clinically and shown to be effective against Kaposi's sarcomas in patients with AIDS in the USA and Europe (Coukell and Spencer, 1997).

In this study, we used 1-monomethoxy-polyethyleneglycol-2, 3-distearoyl-glycerol (PEG–DSG; molecular weight, 340, 500, 900, 2000) to coat the surface of liposomes, and examined FALTs of single or mixed PEG-modified DOX liposomes. Moreover, we examined the effects of PEG-modification of the liposome on the tissue distribution and antitumor activity of DOX in vivo, and elucidated the connection between physical character and biological properties of these liposomes. Consequently, we performed a physical character to obtain an index of passive targeting and achieve a more effective PEG-modification of the liposome.

2. Materials and methods

2.1. Materials

The DOX, used to prepare liposomes, was a gift from Meiji-seika Co., Ltd. (Tokyo, Japan). That used to prepare the DOX solution (DOXsol) was purchased from Kyowa Fermentation Co., Ltd. (Tokyo, Japan). L-α-distearoylphosphatidylcholine (DSPC) and L-α-distearoylphosphatidyl-DL-glycerol (DSPG), used to prepare liposomes, were purchased from Nippon Oil & Fat Co., Ltd. (Tokyo, Japan). 1-Monomethoxypolyethyleneglycol-2, 3-distearoylglycerol (PEG–DSG), with PEG of an average molecular weight of 340 Da (PEG340), 500 Da (PEG500), 900 Da (PEG900), 2000 Da (PEG2000), was a gift from Nippon Oil & Fat Co., Ltd.

2.2. Liposome preparation

All liposomes were prepared according to a modification (Sadzuka et al., 1995) of the method of Bangham et al. (1965).

DSPC/cholesterol/DSPG/DOX (100:100:60:18 μmol) were dissolved in a chloroform/methanol mixture (4:1, v/v). The chloroform and methanol were evaporated under a stream of nitrogen gas. The thin lipid film was placed in a desiccator, which was evacuated, and then the lipid film was hydrated with 10.0 ml of 9.0% sucrose in 10 mM lactate buffer (pH 4.0) in a water bath at 75 °C for 10 min. The suspension was sonicated for 20 min above the phase transition temperature (Tc) with nitrogen gas bubbling. The liposome suspension was extruded through two stacked polycarbonate membrane filters with 0.2 μm pores, and then passed five times through polycarbonate membrane filters with 0.1 μm pores at above the Tc, to obtain a homogeneously-sized liposome suspension. The PEG-modified liposomes were prepared by adding 15 μmol PEG–DSG, of single molecular weight or a mixture of two kinds, when lipid films were made. Each liposome suspension was dialyzed against 9.0% sucrose in 10 mM lactate buffer (pH 4.0) for 16 h to remove untrapped DOX. Empty liposomes, i.e. without DOX, with the same lipid composition, were also prepared.

Empty liposome without a PEG–DSG coating was referred to as plain liposome (PL). On the other hand, single PEG-modified empty liposomes which were coated with a single molecular weight PEG-DSG (molecular weight, 340, 500, 900, 2000) were, respectively, called PEG (340), PEG (500), PEG (900), and PEG (2000), while mixed PEG-modified empty liposomes, which were coated with two kinds of PEG–DSG, were called PEG900: 500(1:1, mol/mol)-liposome (PEG (900:500 = 1:1)), PEG2000, 340(1:1, mol/mol)-liposome (PEG (200:340 = 1:1)), PEG2000: 500(1:2, mol/mol)-liposome (PEG (2000:500 = 1:2)), PEG2000: 500(1:1, mol/mol)-liposome (PEG (2000:500 = 1:1)), PEG2000: 500(2:1, mol/mol)-liposome (PEG (2000:500 = 2:1)), PEG2000: 500(4:1, mol/mol)-liposome (PEG (2000:500 = 4:1)).

DOX liposome without a PEG–DSG coating was referred to as plain liposomal DOX (PL-DOX), while that coated with PEG (molecule weight: 500, 2000, or 2000:500 (2:1, mol/mol)) was, respectively, called PEG500, 2000, or 2000:500 = 2:1 liposomal DOX (PEG (500)-LDOX, PEG (2000)-LDOX, PEG (2:1)-LDOX).

2.3. Calculation of FALT

Zeta potentials of the liposomes were measured using an electrophoretic light scattering apparatus (ELS 800, Otsuka Electronics, Co., Ltd. Osaka, Japan). The zeta potential (V) was calculated from electrophoretic mobility (m^2/V S) applying the Smoluchowski equation (Ichino et al., 1990).

In this study, we calculated FALT using the Gouy–Chapmann theory (Verwey and Overbeek, 1948; Israelachivili, 1985; Kondo et al., 1992). According to this theory, zeta potential $\psi(L)$ as the electrostatic potentials at the position of the slipping plane L (nm) is expressed as:

$$\ln \psi(L) = \ln A - \kappa L$$

where A is regarded as a constant and κ is the Debye–Hückel parameter, $= \sqrt{C}/0.3$ for univalent salts, where C is the molality of electrolytes. If zeta potentials are measured from the changing concentration of NaCl and plotted against κ, the slope L gives the position of the slipping plane or thickness of the fixed aqueous layer in nm units (Shimada et al., 1995; Sadzuka and Hirota, 1997).

Based on this theory, the thickness of the fixed aqueous layer of each liposomes was estimated.

Although NaCl and sucrose were added to the weak buffer of 10 mM lactate buffer, zeta potentials were thought to be greatly affected by the salt even at concentrations as low as 10 mM lactate. Therefore, the ionization in 10 mM lactate buffer should be taken into consideration. Lactic acid has a pK_a of 1.27×10^{-4} at 25 °C. From the Henderson–Hasselbalch equation, about 56% of lactic acid was found to be dissociated at pH 4.0. Therefore, the electrolyte concentration C was regarded as the NaCl concentration plus 0.0056 M.

2.4. Tissue distribution

Male CDF_1 mice (body weight, 20–25 g, 5 weeks old) were obtained from Japan SLC, Ltd., (Hamamatsu, Shizuoka). Ehrlich ascites carcinoma cells (5×10^5 cells per animal) were transplanted onto the backs of mice. On day 14 after transplantation, tumor-bearing mice were injected intraperitoneally with DOXsol or DOX-containing liposomes at a dose of 2.5 mg/kg. At 2, 6, 24, 48, or 72 h after injection, the mice were sacrificed by cervical dislocation, blood was collected from the heart, then tumor, liver, and heart were removed and weighed. The DOX concentrations in the plasma and tissues were determined by fluorophotometry (Sadzuka et al., 1995). Tissue samples were homogenized in ten volumes (w/v) of 10 mM phosphate buffer (pH 7.8). Plasma samples were diluted similarly. Each suspension was mixed for 60 s with five volumes (v/v) of chloroform–isopropanol (1:1, v/v), and then centrifuged ($1200 \times g$, 15 min). The concentration of DOX in the organic phase was determined with a fluorescence spectrophotometer (excitation: 500 nm, emission: 550 nm).

2.5. Antitumor activity

Ehrlich ascites carcinoma cells (5×10^5 cells per animal) were transplanted into the backs of mice, and then DOXsol or DOX-containing liposomes were injected intraperitoneally at a dose of 2.5 mg/kg at 14, 17, and 20 days after tumor inoculation. The mice were sacrificed by cervical dislocation on the 23rd day after inoculation, and the tumor was removed and weighed. The DOX concentration in the tumor was determined in the same way.

2.6. Statistical analysis

Statistical analysis was carried out by Student's t-test.

3. Results

3.1. FALT around liposomes

FALTs around liposomes were estimated by the method based on the zeta potential (Table 1).

The negativity of zeta potentials of PEG-modified liposomes in 10 mM lactate buffer showed a decrease with an increases of FALT. With increasing NaCl concentration, the larger the FALT of liposome was, the more steeply the absolute value of zeta potential of liposomes decreased (Tables 1 and 2).

In single PEG-modified liposomes, the FALT around liposomes increased as the PEG-molecular weight increased. While when we mixed PEG2000 and PEG with a short polyoxyethylene chain, the FALT around the liposomes increased in comparison with the FALT of single PEG2000-

Table 1
Zeta Potentials of PEG-modified-liposomes and plane (not PEG-modified)-liposome in ionic solutions containing various concentration of NaCl

	ζ Potential (mV)			
	NaCl			
	0 mM	10 mM	50 mM	100 mM
PL	-42.90 ± 2.20	-39.89 ± 0.70	-36.43 ± 0.12	-31.99 ± 1.02
PEG (340)	-43.28 ± 2.35	-33.76 ± 4.02	-25.77 ± 0.50	-19.22 ± 1.01
PEG (500)	-40.20 ± 0.09	-32.13 ± 0.47	-22.04 ± 0.29	-17.54 ± 0.25
PEG (900)	-35.71 ± 3.12	-25.23 ± 4.21	-11.70 ± 1.62	-6.30 ± 1.18
PEG (2000)	-23.32 ± 0.44	-13.42 ± 1.12	-5.04 ± 0.93	-2.92 ± 0.25

Each liposome sample was prepared according to a modification of the method of Bangham et al. and Zeta potentials were measured in 10 mM lactete buffer containing various concentrations of NaCl. Results are expressed as mean ± S.D. ($n \geqq 3$).

Table 2
Particle size and FALT of PEG-modified liposomes and plane (not PEG-modified) liposome

	Particle size (nm)	FLAT (nm)
PL	128.8 ± 2.7	0.34 ± 0.05
PEG (340)	142.6 ± 0.8	0.94 ± 0.03
PEG (500)	121.3 ± 2.0	1.00 ± 0.01
PEG (900)	138.0 ± 1.9	2.10 ± 0.02
PEG (2000)	145.6 ± 2.7	2.52 ± 0.03
PEG (900:500 = 1:1)	145.8 ± 3.6	1.84 ± 0.13
PEG (2000:340 = 1:1)	151.7 ± 0.9	3.05 ± 0.04
PEG (2000:500 = 1:1)	139.1 ± 2.1	2.95 ± 0.03

Zeta Potentials were measured at various concentrations of NaCl (0, 10, 50, 100 mM) and plotted against κ. Results are expressed as mean $\pm$ S.D. ($n \geqq 3$).

modified liposomes. On the other hand, no remarkable difference was observed between sizes of each liposome.

The FALT around liposomes, which were modified by a mixture of PEG500 and PEG2000, was plotted against the PEG2000 mixing ratio (Fig. 1). FALT around the liposomes increased with the PEG2000 ratio, when PEG2000:500 = 2:1 showed the maximum value, and thereafter decreased to the same level as single PEG2000-modified liposomes.

DOX liposomes showed similar behavior.

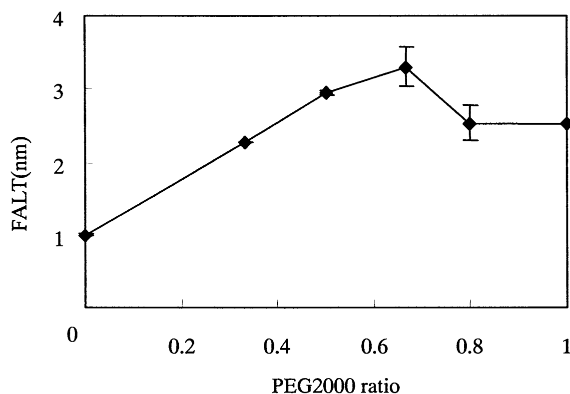

Fig. 1. Effect of PEG2000 ratio on the FALT of PEG(2000:500)-modified liposomes. When PEG2000 ratio is 0, PEG500 ratio is 1. Each point represents the mean $\pm$ S.D. of four samples.

3.2. Effects of PEG-modification on the DOX distribution in vivo

To estimate the effects of increased FALT on the tissue distribution of DOX, we observed the tissue distribution of liposomes whose FALT was known.

3.2.1. Plasma (Fig. 2(A))

At 2 and 6 h after the administration, the order of DOX concentrations in plasma was PEG (2:1)-LDOX > PEG (2000)-LDOX > PEG (500)-LDOX > PLDOX = DOXsol. In the DOX sol group, the DOX concentrations in plasma was not detected at 2 h after administration. In the PLDOX or PEG (500)-LDOX groups, the DOX concentration in plasma was not detected at 24 h after administration, in contrast, the concentrations in plasma were detected at 24 h after administration in the PEG (2000)-LDOX or PEG (2:1)-LDOX groups, the level at 24 h after PEG (2:1)-LDOX administration being equal to that at 6 h after PEG (500)-LDOX administration.

3.2.2. Liver (Fig. 2(B))

In the liver, at 6 and 24 h after the administration of DOX, the order of DOX concentrations was PLDOX > PEG (500)-LDOX > PEG (2000)-LDOX > PEG (2:1)-DOX = DOXsol. In particular, when DOX levels showed the maximum value at 6 h after administration, the DOX levels of PEG (2000)-LDOX and PEG (2:1)-LDOX were 55 and 42% of that of PLDOX, respectively.

3.2.3. Tumor (Fig. 2(C))

In tumor, an increase in DOX levels caused by PEG-modification was observed at 24 h after administration. DOX levels of PEG (500)-LDOX, PEG (2000)-LDOX and PEG (2:1)-LDOX were 3.3, 5.7 and 9.1-fold higher than that of PLDOX, respectively. Notably, the DOX level of PEG(2:1)-LDOX was 2.23 μg/g protein at 72 h after administration, being equal to the maximum value of DOXsol.

3.2.4. Heart

In the heart, no significant difference was observed in DOX concentration, the DOX levels

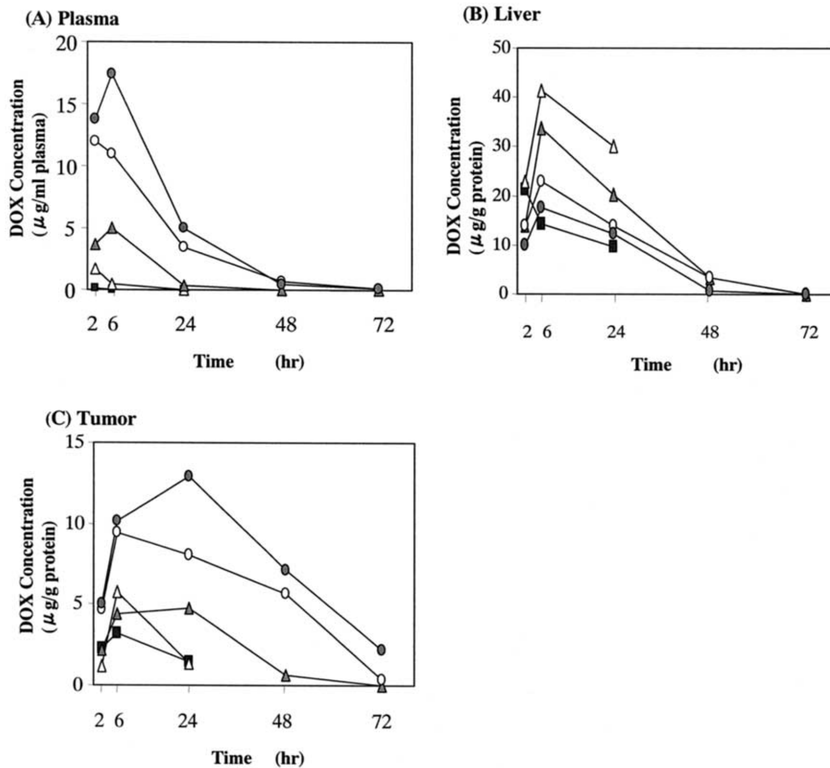

Fig. 2. Effects of PEG-modification on the DOX concentration in mice tissues after administration of DOXsol or DOX liposome (A) plasma, (B) liver, (C) tumor. Ehrlich ascites carcinoma cells (5×10^5 cells per animal) were transplanted onto the backs of mice. On day 14 after transplantation, tumor-bearing mice were injected intraperitoneally with DOXsol or DOX-containing liposomes at a dose of 2.5 mg/kg. At 2, 6, 24, 48, or 72 h after injection, the mice were sacrificed, blood was collected from the heart, and tissues were removed and weighed. Then DOX concentration in the tumor was determined. Each point represents the mean of three to six mice, each with no more than 10% variation between them. —■—, DOX sol; —△—, PLDOX; —▲—, PEG (500)-LDOX; —○—, PEG (2000)-LDOX; —●—, PEG (2:1)-LDOX.

showing a tendency to increase as for PLDOX and PEG (500)-LDOX, and to decrease as for PEG (2000)-LDOX and PEG (2:1)-LDOX in comparison with DOXsol (data not shown).

3.3. Antitumor activity

Fig. 3 shows the tumor weights and DOX concentrations in tumor after administration of DOX in each form. The tumor weight of control was 3.98 ± 1.48 g. After PLDOX treatment, the tumor weight was reduced to 79% of control. The decrease of tumor weight after PEG (500)-LDOX treatment was similar to that in the PLDOX group. In contrast, the tumor weight after PEG(2000)-LDOX treatment and PEG(2:1)-LDOX treatment was, respectively, 1.90 ± 0.99 and 1.10 ± 0.39 g, a 2.5 and 3.4-fold stronger effect than PLDOX (Fig. 3(A)).

On the other hand, after PEG (500)-LDOX treatment, the DOX concentration in the tumor hardly increased at all, compared with that after PLDOX treatment. After PEG(2000)-LDOX and PEG(2:1)-LDOX treatments, the DOX concentration in the tumor increased by 1.5-fold ($P < 0.001$, vs. PLDOX) and 2.4-fold ($P < 0.001$, vs. PLDOX) of that in PLDOX, respectively (Fig. 3(B)).

4. Discussion

The efficacy of antitumor drug-containing liposomes against tissue distribution and antitumor activity has been demonstrated, the passive targeting of tumor by PEG-modified DOX liposome being shown to enhance antitumor activity and reduce side effects (Sadzuka et al., 1995). In this study, we examined the effects of mixed PEG-modification of liposomes on the FALT around liposomes, and effects of increased FALT on the tissue distribution and antitumor activity of DOX in vivo.

To examine the effects of mixed PEG-modification of liposomes on the FALT, we compared FALTs of single PEG-modified liposomes and mixed PEG-modified liposomes. The FALT around liposomes was increased as the PEG-molecular weight increased, but the ratio of increase was reduced with PEG-molecular weight. Modeling of PEG-lipid, which modified the surface of liposomes, has shown that at least two regimes can be identified, 'mushrooms' (isolated grafts) and 'brushes' (extended chain conformations determined by the interaction between neighboring chains; Needham et al., 1997). This phenomenon could suggest that, because, PEG-lipid with a large molecular weight constructed more complete 'mushroom' structures than that with a small molecular weight, the increase of FALT was smaller than the increase of PEG-molecular weight. We tentatively calculated FALTs of PEG (500) and PEG (2000) on the basis of bond angle and bond length of polyoxyethylene chains. Consequently, when PEG (500) and PEG (2000) formed complete 'brushes', FALTs around liposomes were 4.18 and 16.72 nm. On the other hand, when they formed complete 'mushroom' structures, FALTs around liposomes were 0.73 and 2.52 nm, respectively. From this result, it was suggested that PEG-lipid with a large molecular weight constructed more complete 'mushroom' structures than that with a small molecular weight.

Next, we compared FALTs of mixed PEG-modified liposomes. The FALT around liposomes modified by a mixture of PEG900 and PEG340 had a value between that of PEG (900) and that of PEG (340). When we mixed PEG2000 and PEG with a short polyoxyethylene chain (PEG340 or PEG500), the FALT around liposomes increased in comparison with the FALT of single PEG2000-modified liposomes. Moreover, when the surface of liposomes was modified by a mixture of PEG500 and PEG2000, the most suitable mix (PEG2000:500 = 2:1), showed a maximum

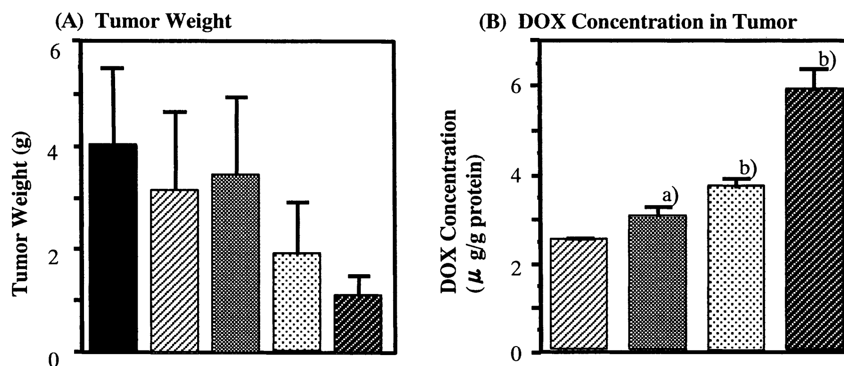

Fig. 3. Effects of PEG-modification on the change of tumor weight induced by DOX and DOX concentration in tumor of mice. Ehrlich ascites carcinoma cells (5×10^5 cells per animal) were transplanted into the backs of mice, and then DOX-containing liposomes were injected intraperitoneally at a dose of 2.5 mg/kg at 14, 17, and 20 days after tumor inoculation. The mice were sacrificed by cervical dislocation on the 23rd day after inoculation, and the tumor was removed and weighed, then DOX concentration in the tumor was determined. Each column represents the mean ± S.D. of four to six mice. Significant differences from the level of the PLDOX group are indicated by (a) $P < 0.01$ and (b) $P < 0.001$. ■, Control; ▨, PLDOX; ▩, PEG (500)-LDOX; ▦, PEG (2000); ▧, PEG (2:1-LDOX).

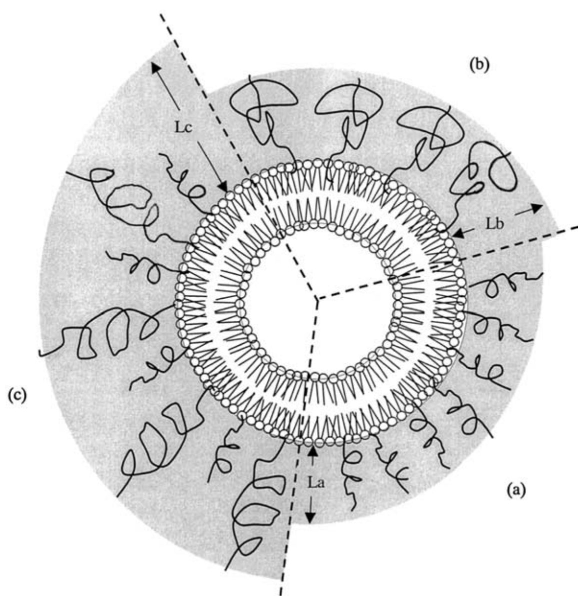

Fig. 4. Schematic representation of PEG-modification on the surface of liposomes. Section (a), single PEG modification by PEG with short polyoxyethylene chain, section (b), single PEG modification by PEG2000, and section (c), mixed PEG modification by mixture of PEG2000 and PEG with short polyoxyethylene chain. La, Lb and Lc represent the FALT of the liposome whose surface modified by (a), (b) or (c), respectively.

FALT. This phenomenon was thought to occur as follows. The mixture of PEG2000 and PEG which had a short polyoxyethylene chain increased the FALT around liposomes in comparison with that of single PEG2000-modified liposomes, because, 'mushroom' structures of PEG-lipid which has a large molecular weight became longer as a result of insertion of PEG-lipid with a short polyoxyethylene chain into the interval of a large one (Fig. 4). At this time, if the amount of PEG-lipid, which had a small molecular weight, was too high, the FALT around that liposome had a value between that of two single PEG-modified liposomes, because of the increase in area occupied by PEG-lipid, which had a small molecular weight. If the amount of PEG-lipid, which had a small molecular weight, was too low, the FALT around that liposome had a similar value to that of single PEG2000-modified liposome, because, the effects of PEG-lipid, which had a small molecular weight, were minimal.

Furthermore, it was suggested that single PEG-modification by the PEG-lipid, which had a large molecular weight, couldn't display sufficient ability, whereas the mixture of PEGs, which had a long and short polyoxyethylene chain, exhibited a stronger effect on PEG-modification.

We next investigated effects on the DOX concentration in tissues of mice after administration of mixed PEG-modified liposomal DOX. Tissue distribution after intraperitoneal injection of each liposome was studied, it clarified that intraperitoneal administration of plane liposome or PEG modified liposome is superior to intravenous administration (Sadzuka et al., 1997). In the plasma, the DOX concentration increased with increase of FALT, PEG-modified liposomes of large FALT being shown to maintain a high DOX level. In the livers, we observed an accumulation caused by liposomalization and avoidance from RES caused by PEG-modification. This avoidance was elevated by increase of FALT. This phenomenon showed that a fixed aqueous layer was formed on the surface of liposomes by PEG modification, and prevented the attraction of opsonins: as a result, the trapping by cells of the RES was avoided (Wu et al., 1993; Schmitz et al., 1992; Papafadjopoulos et al., 1991). This result was supposed that the ability to avoid trapping by RES was caused by increase of FALT. While, in the tumor, the time course of the DOX concentration showed a relation between increase of FALT and maintenance of a high DOX level for a long time, namely, the increase in DOX concentration in tumor caused by passive targeting was observed on mixed PEG-modification. In the heart, although a remarkable difference was not observed in the DOX concentrations among groups, DOX levels were slightly decreased in PEG (2000)-LDOX and PEG (2:1)-LDOX in comparison with DOXsol (data not shown). As a result, the side effects may be reduced for PEG (2000)-LDOX and PEG (2:1)-LDOX.

By the way, for PEG (2000), approximately half of the amount initially added PEG2000 was incorporated into liposomes, as opposed to all of the amount initially added PEG500 for PEG (500). While for PEG (2000:500 = 2:1), approximately half of PEG2000 and all of PEG500 were incor-

porated into liposomes, respectively, these rates were equal to single PEG modification. While the longer the PEG chain length, the larger the stripping on the liposome membrane (Sadzuka et al., 2001). These results suggested that effectiveness of mixed PEG modification did not depend on the increasing of the incorporation rate and stability of PEG on the liposome membrane, we suspect that the improvement of tissue distribution depended on the increase of FALT by modification on the surface of liposomes.

For the increase of FALT and improvement of tissue distribution caused by mixed PEG-modification, we investigated the effects of PEG-modification on the antitumor activity. The administration of PEG (2000)-LDOX and PEG (2:1)-LDOX caused a reduction of tumor weight as compared with PEG (500)-LDOX and PL-DOX; PEG (2:1)-LDOX showing a significant reduction of tumor weight. Moreover, the DOX concentration in the tumor showed PEG(2:1)-LDOX > PEG(2000)-LDOX > PEG(500)-LDOX > PLDOX, this order agreeing with that of FALT. Namely, it was suggested that although the effectiveness of PEG-modification was not significant the liposomes whose FALT increased slightly, the liposomes with a remarkable increase of FALT showed a reduction of tumor weight based on an increase of DOX concentration in tumor, and furthermore, an enhancement of antitumor activity.

As the result of these experiments, it was concluded that the mixed PEG-modification on the surface of liposomes gave the increase of FALT and maximum FALT induced by the most suitable mixture. Moreover, regarding the connection between increase of FALT and improvement of circulation in blood, the involvement of antitumor activity of DOX of these liposomes was suggested.

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