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Characterization and cytotoxicity of mixed polyethyleneglycol modified liposomes containing doxorubicin

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Abstract

Liposomes are recognized as one of the useful drug carriers, but have many problems to overcome before their clinical application. Liposomes, bonding peculiarly with serum protein (opsonization), are taken up by reticuloendothelial system (RES) cells in the liver and spleen. It is known that polyethyleneglycol (PEG) modification of the liposome surface induces the formation of a fixed aqueous layer around the liposomes due to the interaction between the PEG-polymer and water molecule, and thus prevents the attraction of opsonins. Namely, PEG-modified liposomes are able to escape trapping by the RES cells, and have a prolonged circulation time. In this study, the effects of different anchors with the same PEG molecular weight on the cell uptake and cytotoxicity of mixed PEG-modified liposomal doxorubicin (DOX) were examined.

The fixed aqueous layer thickness (FALT) of liposomes covered with mixtures of PEG-molecules which differ in their chain length were increased, compared to that of the single PEG2000-modified liposome. Mixed PEG-modification of liposomes with different anchors (PEG2000-(1-monomethoxypolyethyleneglycol-2,3-distearoylglycerol (DSG): cholesterol (CHO) = 1:1)-modified liposome) led to an increase in the FALT, compared to that of each single PEG-modification. The uptake of DOX into Ehrlich ascites carcinoma cells by the liposomes covered with PEG-CHO was higher than the other liposomes. Thus, liposomes covered with PEG-DSG and PEG-CHO have an enhanced cytotoxicity.

In conclusion, it was confirmed that mix-modified liposomes using PEG-lipid with different anchors were superior.

Keywords: Liposome; Polyethyleneglycol; Doxorubicin; Fixed aqueous layer thickness (FALT)

1. Introduction

Polyethyleneglycol (PEG) coated liposomes are able to avoid from entrapment by the reticuloendothelial system (RES), one of many barriers to the in vivo application of liposomes as drug carriers. The prolongation of liposome circulation in the blood and passive targeting to the tumor has been achieved by modification of the liposome surface with PEG (Yuda et al., 1999; Sadzuka et al., 1995). Many studies have examined the interaction between the prolonged circulation time of liposomes and the conditions of the PEG-molecule on the liposome surface in terms of their physicochemical properties by PEG-modification (Kuhi et al., 1994; Torchilin et al., 1994; Janzen et al., 1996). The fixed aqueous layer thickness (FALT) around the liposomes was measured using interfacial electrochemistry and it was reported that the FALT of PEG-modified liposomes increased in comparison with PEG-unmodified liposomes (Shimada et al., 1995; Zeisig et al., 1996). We have reported the efficacy of mixed PEG-modification with different PEG-molecular weights on the FALT, cellular uptake and biodistribution of liposomes (Sadzuka et al., 2002, 2003). The FALT of mixed PEG modified liposomes increased, compared to that of each single PEG modified liposome. In this mixed modification, the maximum FALT was shown for the liposome most suitable to the mixed ratio. On the other hand, mixed PEG modification of the liposome containing doxorubicin (DOX) had a tendency to improve the biodistribution and increase in the antitumor activity compared to that of the other modifications. As for the character of the liposome, FALT was proven to be one of the physical factors that decide biochemical property of the PEG-modified liposome (Sadzuka et al., 2002). Furthermore, mixed PEG modification of liposomes containing DOX had the tendency to have increased cytotoxicity. We indicated that if the PEG-modified liposome accumulated in tumors in vivo, it transferred into the tumor cell and was found to be cytotoxic. Namely, we expected that mixed PEG-modified liposomes containing DOX are better (Sadzuka et al., 2005).

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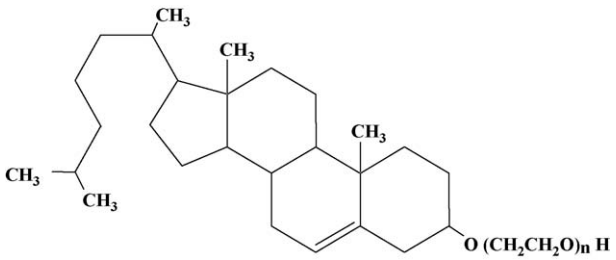

Fig. 1. Chemical structure of cholesterol-PEG (PEG-CHO).

PEG-alkyl lipid derivatives have different physicochemical properties depending on the molecular weight of the PEG-moiety, the fatty acid length as the anchor, and the conformation of the spacer unit as the connection between the lipids and PEG. We generally used a PEG-molecular weight in PEG-lipid of 1000–5000 to modify the liposomes. With the increase of PEG-molecular weight, the PEG-lipid becomes more difficult to incorporate into the liposomal membrane. The PEG-lipid is easy to withdraw from liposomes with short chain fatty acids as anchors in the PEG-lipid. Furthermore, it is known that the dissociation of PEG lipid from liposomes differs according to the conformation of the anchor unit (Sadzuka et al., 2003; Hirota, 1998). In order to increase the antitumor effect by the liposomalization of DOX, it is necessary to have the opposite character with sufficient balance in vivo. The liposomes have a prolonged circulation time and they most retain their stability to be delivered to the target tumor cells. On the other hand, when the liposomes are taken into the target tumor cells, they must be burst in the tumor cells or around the tumor cells, and release the drug. Thus, in the case of the preparation of PEG-modified liposomes, it is important to choose PEG-lipid derivatives according to each purpose.

In this study, we used PEG-cholesterol (PEG-CHO) (Fig. 1), which has different characteristics with the alkyl anchor as the surface material of the liposome modification, and we investigated single or mixed PEG-modified liposomes. We modified liposome with mix PEGs that had different PEG-molecular weights (with same anchor) or different anchor units (with same molecular weight) of the PEG-lipid and we examined the characteristics of PEG-CHO lipids, single and mixed PEG-CHO modified liposomes containing DOX in vitro, and we evaluated the their usefulness.

2. Materials and methods

2.1. Materials

The DOX used to prepare liposomes was a gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan), and 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 NOF Co. Ltd. (Tokyo, Japan). 1-Monomethoxypolyethyleneglycol-2, 3-distearoylglycerol (PEG-DSG), with PEGs of average molecular weight of 2000 (PEG2000-DSG) and cholesterol-polyethyleneglycol (PEG-CHO), with a PEG of an average molecular weight of 500 (PEG500-CHO), 700 (PEG700-CHO), 900 (PEG900-CHO), 1000 (PEG1000-CHO) and 2000 (PEG2000-CHO), were a gift from NOF Co. Ltd. (Tokyo, Japan). All other chemicals were commercial products of reagent grade.

2.2. Determination of the octanol/water value and the critical micelle concentration (c.m.c.) value of the PEG-CHO lipids

Determination of the octanol/water value was determined according to our previous paper (Sadzuka et al., 2003). To calculate the octanol/water value, the PEG-lipid concentration was determined in the water layer using the picrate method, in the previous study (Shimada et al., 2000). The c.m.c. value was determined using the fluorescence probe method (Brito and Vaz, 1986) employing 8-anilino-naphthalene-1-sulfonate (ANS, Tokyo, Kasei).

2.3. Preparation of the PEG-CHO incorporating liposomes

All liposomes were prepared according to a modification of the method of Bangham (Bangham et al., 1965). DSPC/Cholesterol/DSPG (100:100:60 μmol) (the PEG-modified liposomes were prepared by adding 15 μmol PEG-lipids) were dissolved in a chloroform/methanol mixture (4:1, v/v) and the mixture was perfectly dispersed by sonication. The chloroform and methanol were then evaporated to dryness under a stream of nitrogen gas. The thin lipid film was evacuated in a desiccator and then hydrated with 10 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 at 60 °C after 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 to obtain liposomes homogeneous in size, and the liposome suspension was extruded. This liposome was expressed as the plain liposome (PL). In this regard, single PEG-modified empty liposomes were coated with a single molecular weight PEG2000-DSG and PEG500-CHO, PEG1000-CHO and PEG2000-CHO, where the anchor part of the PEG-lipid was cholesterol. Meanwhile the mixed PEG-modified empty liposomes were coated with PEG2000-DSG and PEG2000-CHO. The PEG modified liposomes were expressed as detailed below:

- PEG500-CHO modified liposome (PEG-CHO(500))
- PEG1000-CHO modified liposome (PEG-CHO(1000))
- PEG2000-CHO modified liposome (PEG-CHO(2000))
- PEG-CHO 2000:500 = 1:1 modified liposome (PEG-CHO(2000:500 = 1:1))
- PEG-CHO 2000:500 = 2:1 modified liposome (PEG-CHO(2000:500 = 2:1))
- PEG-CHO 2000:1000 = 1:1 modified liposome (PEG-CHO(2000:1000 = 1:1))

- PEG2000-DSG: PEG2000-CHO = 1:1 modified liposome (PEG-(DSG(2000):CHO(2000) = 1:1)).

The DOX-containing liposomes were prepared as referred to above and DOX 10 mg (18 μmol) was added when the lipid film was prepared. DOX liposomes without PEG coatings were referred to as plain liposomal DOX (PLDOX), while those coated with PEG-CHO (molecule weight: 500, 1000, 2000, 2000:500 (1:1, 2:1, mol/mol), 2000:1000 (1:1, mol/mol)) were expressed as PEG-CHO(500)-LDOX, PEG-CHO(1000)-LDOX, PEG-CHO(2000)-LDOX, PEG-CHO(2000:500 = 1:1) -LDOX, PEG-CHO(2000:500 = 2:1)-LDOX, PEG-CHO(2000:1000 = 1:1)-LDOX, respectively. PEG2000-DSG:PEG2000-CHO = 1:1 modified liposome expressed PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX.

Each liposome suspension was dialyzed against 9.0% sucrose in 10 mM lactate buffer (pH 4.0) for 16 h to remove the untrapped DOX.

2.4. Physicochemical characteristics of liposomes

The particle sizes and zeta potentials of the liposomes were measured with an electrophoretic light scattering apparatus (ELS 8000; Otsuka Electronics, Co. Ltd. Osaka, Japan). Zeta potentials were measured with various concentrations of NaCl and plotted against κ, that is, $3.3\sqrt{c + 0.0056}$ (c, concentration of NaCl), the slope giving the position of the slipping plane or FALT in nm units (Sadzuka et al., 1995; Sadzuka and Hirota, 1997). Based on this theory, the thickness of the fixed aqueous layer of each liposome was estimated.

2.5. Determination of the incorporated ratio of PEG-CHOs using the picrate method

We determined the amount of PEG-CHOs in the sample by direct determination using the picrate method (Shimada et al., 2000). On the mixed PEG-modified liposomes, the amount of each PEG-lipid was determined with utilizing the difference of the value of octanol/water partitioning (Sadzuka et al., 2003).

2.6. Residual PEG-CHOs on liposomal membranes

The inside of the PEG-modified liposomes were composed of 9.0% sucrose in 10 mM lactate buffer (pH 4.0). The outside of the PEG-modified liposomes were 10 mM Tris–HCl–150 mM NaCl buffer (pH 7.4). Each PEG-modified liposome suspension was centrifuged at 30,000 × g for 2 h to remove the PEG-CHOs that had not been incorporated into the liposomal membranes. 10 mM Tris–HCl–150 mM NaCl (pH 7.4) was added to the pellet, followed by sonication. This suspension was mixed with the same volume of FBS, and then incubated at 37 °C for a definite time. After incubation, the sample suspension was centrifuged at 30,000 × g for 2 h, and the amount of PEG-lipid was determined with the picrate method. On the other hand, as a control, 10 mM Tris–HCl–150 mM NaCl buffer was added to the pellet instead of FBS, and then the same procedure was performed. This value in the control group was expressed as the 100% residual PEG-lipid ratio.

2.7. Determination of the encapsulation efficiency of DOX

Liposomes encapsulating DOX before and after dialysis were dissolved in chloroform–isopropanol (1:1, v/v), and then the amount of trapped DOX was calculated with a fluorescence spectrophotometer, Hitachi F2000 (Hitachi Ltd., Tokyo), at an excitation wavelength of 500 nm and an emission wavelength of 550 nm.

2.8. Effects of PEG-CHO modification on the DOX uptake into tumor cells

We examined the effect of liposomalization on the DOX uptake and PEG modification. Ehrlich ascites carcinoma cells (5.0×10^6 cells/mL) were added DOX-containing liposomes (DOX concentration, 5.0 μg/mL), and then the Ehrlich cells were incubated at 37 °C for 30 min. For determination of the time course of the intracellular drug concentration, aliquots of the cell suspension were removed at intervals. The aliquot was cooled on ice and then centrifuged at 150 × g for 3 min. The cells were washed and resuspended in 1.0 mL of ice-cold 10 mM phosphate buffer (pH 7.8). The concentration of the drug in the organic phase was determined as described in Section 2.7.

2.9. Effects of PEG-CHO modification on DOX cytotoxicity

Ehrlich ascites carcinoma cells or a P388 leukemia cells suspension was seeded in a 96 well plate (FALCON), and then incubated at 37 °C for 24 h. After incubation, the cell suspension was added the DOX-containing liposomes (DOX concentration, 0.01–10 μg/mL), and it was incubated at 37 °C for 48 h. Afterwards, this cell suspension was added WST-8 and it was then incubated at 37 °C for 3 h. The absorbance at 560 nm was calculated. The probability of cell survival without drug exposure was expressed as 100%. We determined the probability of cell survival in each sample.

2.10. Statistical analysis

Statistical analysis was performed using ANOVA.

3. Results

3.1. Octanol/water value and c.m.c. of PEG-CHO lipids

Table 1 shows that the order of the octanol/buffer partitioning value was PEG2000-CHO ≪ PEG1000-CHO ≒ PEG900-CHO ≒ PEG700-CHO ≒ PEG500-CHO in 10 mM Tris–HCl–150 mM NaCl buffer (pH 7.4). Namely, PEG2000-CHO exhibited slight hydrophilicity and the other PEG-CHOs exhibited remarkable lipid solubility. On the other hand, these values for all the PEG-CHO lipids in 10 mM lactate buffer (pH 4.0) were similar, and exhibited slight hydrophilicity.

Table 1
Octanol/buffer partitioning values of PEG-CHOs

	Tris–HCl buffer (pH 7.4)			Lactate buffer (pH 4.0)		
	Buffer[a]	Octanol[a]	Octanol/buffer partitioning values	Buffer[a]	Octanol[a]	Octanol/buffer partitioning values
PEG2000-CHO	0.564	0.436	0.773	0.650	0.350	0.538
PEG1000-CHO	0.184	0.816	4.438	0.624	0.376	0.602
PEG900-CHO	0.249	0.751	3.022	0.664	0.336	0.506
PEG700-CHO	0.245	0.755	3.089	0.635	0.365	0.575
PEG500-CHO	0.218	0.782	3.587	0.606	0.394	0.649

Octanol/buffer partitioning values of PEG-CHOs are measured by using the picrate method.
[a] Ratio of PEG when setting the additional amount (0.25 mM) to 1.

Table 2
c.m.c. values of PEG-CHOs

	c.m.c (μM)	
	Tris–HCl buffer (pH 7.4)	Lactate buffer (pH 4.0)
PEG2000-CHO	2.906	2.078
PEG1000-CHO	2.706	2.710
PEG900-CHO	2.409	2.122
PEG700-CHO	5.432	6.439
PEG500-CHO	7.932	5.517

c.m.c. values of PEG-CHO are estimated from the break point of ANS fluorescence. Each value represents the mean ($n \geq 3$).

Table 2 shows that the c.m.c. of PEG-CHO with different molecular weights was PEG2000-CHO ≒ PEG1000-CHO ≒ PEG900-CHO ≪ PEG700-CHO < PEG500-CHO in Tris–HCl buffer (pH 7.4). PEG2000-CHO exhibited 53.5% of PEG700-CHO and 36.6% of PEG500-CHO. On the other hand, this tendency was similar in lactate buffer (pH 4.0). As a result, it was suggested that the pH did not have an effect on the c.m.c. of each PEG-CHO lipid.

3.2. Particle size and FALT of PEG-CHO modified liposomes

As shown in Table 3, the FALT around the liposomes increased with the increase in PEG-molecular weight in single PEG-modified liposomes, but the FALT value of PEG-CHO(2000) was similar to PEG-CHO(1000). The FALT value of PEG-CHO(2000:500 = 2:1) or PEG-CHO(2000:1000 = 1:1), which is modified with different PEGs, increased more than that of the single PEG-modified liposomes. In particular, the FALT of the PEG-CHO(2000:500 = 1:1) modified liposome increased remarkably, and was 3.30 nm.

We examined mixed modified liposomes with the same PEG molecular weight and different PEG-lipids. We suggested that the FALT of PEG-(DSG(2000):CHO(2000) = 1:1) modified-liposomes were also increased more than those of the single modified-liposomes.

All liposomes were similar in size.

Table 3
Particle size and FALT of PEG-CHO modified liposomes and plain (not PEG-modified) liposome

	Particle size (nm)	FALT (nm)
Plain liposome	128.34 ± 2.70	0.34 ± 0.05
PEG-CHO(500)	138.29 ± 7.67	1.41 ± 0.30
PEG-CHO(1000)	152.80 ± 1.56	2.24 ± 0.18
PEG-CHO(2000)	137.67 ± 5.51	2.28 ± 0.46
PEG-CHO(2000:500 = 1:1)	138.15 ± 1.88	3.30 ± 0.22
PEG-CHO(2000:500 = 2:1)	137.10 ± 0.70	2.53 ± 0.28
PEG-CHO(2000:1000 = 1:1)	138.02 ± 4.58	2.65 ± 0.31
PEG-(DSG(2000):CHO(2000) = 1:1)	139.30 ± 1.27	3.43 ± 0.70

Particle size and FALTs are expressed as the mean ± S.D. ($n \geq 3$).

3.3. Incorporated ratio and residual amount of PEG-CHOs in liposome

In single PEG-modified liposomes, the incorporated ratio of PEG2000-CHO in the liposomal membranes (73.72 ± 7.61%, 1.10 ± 0.01 mM) was lower than that of PEG500-CHO (99.31 ± 8.38%, 1.48 ± 0.01 mM). On the other hand, in mixed PEG-modified liposomes, the incorporated ratio was 75.19 ± 7.33%.

In both single and mixed PEG-modified liposomes, the residual amount of PEG-lipids in the liposomal membranes transiently decreased with 50% FBS (Fig. 2). In the single

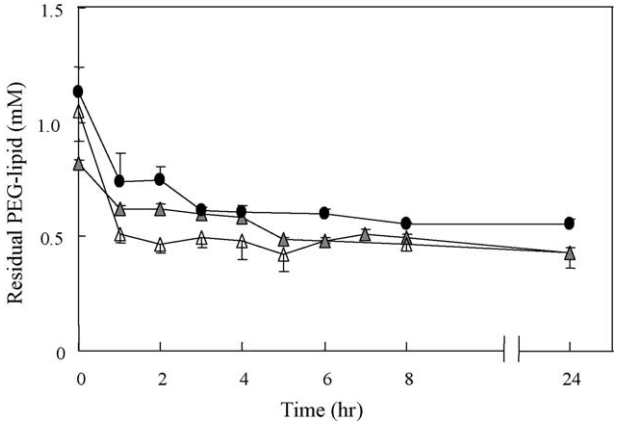

Fig. 2. Residual PEG-lipid in liposome membrane after incubation in FBS. Each PEG-modified liposome suspension was centrifuged (30,000 × g, 2 h). Tris–HCl–150 mM NaCl was added to the pellet. This suspension was mixed with 50% FBS, and incubated at 37 °C. The sample suspension was centrifuged (30,000 × g, 2 h), and the amount of PEG-lipids in this sample was determined using the picrate method. Each point represents the mean ± S.D. of four samples. ▲: PEG-DSG(2000); △: PEG-CHO(2000); ●: PEG-(DSG(2000):CHO(2000) = 1:1).

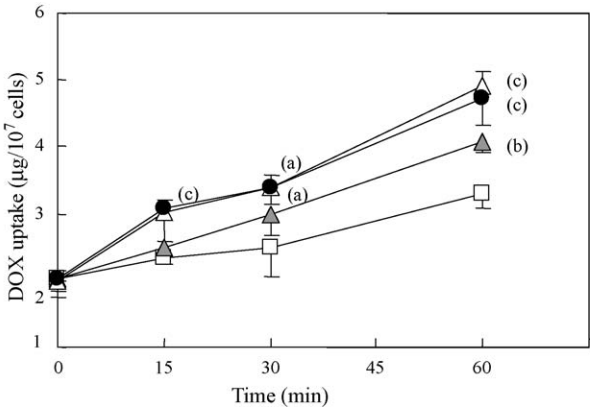

Fig. 3. Effects of PEG-modification on DOX uptake into Ehrlich ascites carcinoma cells. Ehrlich ascites carcinoma cells (5.0×10^6 cells/mL) were of DOX in the PLDOX (□), PEG-DSG(2000)-LDOX (▲), PEG-CHO(2000)-LDOX (△)or PEG-(DSG(2000): CHO(2000) = 1:1)-LDOX (●). Each point represents the mean ± S.D. of four samples. Significant differences from the level of PLDOX are inidicated by (a) $P < 0.05$, (b) $P < 0.01$ and (c) $P < 0.001$.

PEG-modified liposomes, the residual ratio of PEG2000-CHO decreased rapidly. In the mixed PEG-modified liposomes with the mixture PEG-(DSG(2000):CHO(2000) = 1:1), the residual ratio was higher than that of each single-modified liposome.

3.4. Effect of mixed PEG-CHO modification on DOX uptake by Ehrlich ascites carcinoma cells

The intracellular level of DOX was differed according to the PEG modification–unmodification and molecular weight of PEG in the PEG-DSG of the liposomes. The order of DOX uptake by Ehrlich ascites carcinoma cells after 30 min incubation was PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX ≒ PEG-CHO-(2000)-LDOX > PEG-DSG(2000)-LDOX ≫ PLDOX, namely the level of cell uptake was affected by the PEG-modification of liposomes or the kind of PEG-molecular weight. In particular, the order of DOX uptake by cells after 60 min incubation of PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX and PEG-CHO(2000)-LDOX group increased 1.4 and 1.5 times ($P < 0.001$) in the case of PLDOX, respectively. In all experiment, additional lipid level as liposome were equal in the medium, and thus did not affect DOX uptake (Fig. 3).

3.5. Cytotoxicity of mixed PEG-CHO modified liposomes containing DOX on Ehrlich ascites carcinoma cells and P388 leukemia cells

IC_{50} of each liposome on Ehrlich ascites carcinoma cells was PLDOX ≫ DOXsol > PEG-DSG(2000)-LDOX > PEG-CHO(2000)-LDOX > PEG-(DSG(2000):CHO (2000) = 1:1)-LDOX (Table 4). Namely, the cytotoxicity increased with the DOX uptake by cells. In particular, the cytotoxicity of PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX was 10 times of that of PLDOX. Empty liposome which prepared in the same way didn't have cytotoxicity. Namely, we have expected that the lipid in each liposome had no effect on the cytotoxicity of each formulation.

Table 4
Cytotoxity of DOXsol and liposomal doxorubicin on Ehrlich ascites carcinoma cells

	IC_{50}(μM DOX)
DOXsol	3.45
PLDOX	6.30
PEG-DSG(2000)-LDOX	2.85
PEG-CHO(2000)-LDOX	1.67
PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX	0.60

Ehrlich ascites carcinoma cells (1.0×10^6 cells/mL) were incubated with DOXsol, PLDOX, PEG-DSG(2000)-LDOX, PEG-CHO(2000)-LDOX or PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX. Cytotoxicities were determined by the WST-8 assay and expressed as IC_{50} ($n = 5$–7).

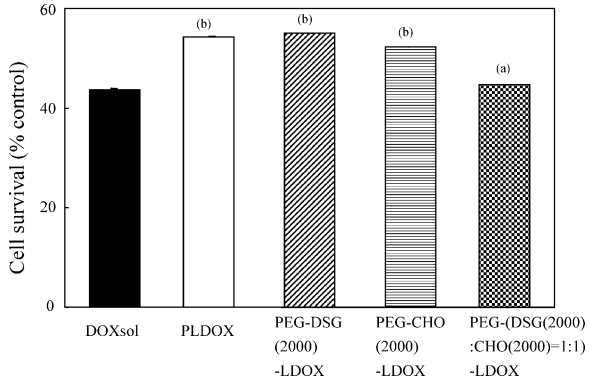

Fig. 4. Cytotoxicity of DOXsol and liposomal doxorubicin to P388 leukemia cells. P388 leukeima cells (1×10^6 cells/mL) were incubated with DOXsol, PLDOX, PEG-DSG(2000)-LDOX, PEG-CHO(2000)-LDOX or PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX as 0.01 μg/mL DOX concentration. Cytotoxicities were determined by the WST-8 assay and expressed as the mean ± S.D. ($n = 3$–8). Significant differences from the level of PLDOX are indicated by (a) $P < 0.05$ and the level of DOXsol are indicated by (b) $P < 0.05$.

On the other hand, the cytotoxicity of liposomal DOX (0.01 μg/mL as DOX concentration) on P388 leukemia cells was PLDOX ≒ PEG-DSG(2000)-LDOX < PEG-CHO(2000)-LDOX < PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX (Fig. 4). Namely, PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX had a stronger cytotoxic reaction than PLDOX.

4. Discussion

The correlation of FALT and the prolonged circulation in the blood or the antitumor effect of the liposome had been clarified for single and mixed PEG-DSG modified liposomes (Sadzuka et al., 2002). These results have suggested that FALT was one of the important factors that defined the pharmacokinetics of the PEG-modified liposomes. Namely, an increase of the FALT led to improvements of the escape ability from RES, the circulation in the blood, and an enhancement of the tumor accumulation of liposomes. Then, we recognized that the antitumor effect also increased (Sadzuka et al., 2002). Furthermore, the increase of FALT was induced by using two PEG-lipids which had different characteristics (Sadzuka et al., 2003).

We commonly used PEG-lipids with alkyl anchors. In this paper, we used PEG-CHO with a cholesterol anchor, which has other characteristic of PEG-modification, and studied the new

usefulness of this PEG modification agent and PEG modification method. We modified the liposomes with PEGs that had the same PEG-molecular weight and two different anchor units, and examined that the mixed PEG-modified liposomes affected the FALT, the incorporation of the PEG-lipid into the liposomal membrane, and the cytotoxicity toward tumor cells.

PEG-CHO lipids were classified into three classes, PEG2000-CHO, PEG1000-CHO and PEG900-CHO, and PEG700-CHO and PEG500-CHO according to the octanol/buffer partitioning value or the c.m.c. value. And so, we used PEG2000-CHO, PEG1000-CHO, or PEG500-CHO as the combinations of the molecular weight with different physical properties, and we performed the mixed modifications by changing the mix ratio.

In single PEG-modified liposomes, the FALT order was PEG-CHO (2000) > PEG-CHO (1000) > PEG-CHO (500). This result was the same with the alkyl anchor. Namely, the FALT around the liposomes was increased with the increase of the PEG-molecular weight. On the other hand, in mixed PEG-modified liposomes with long polyoxyethylene chains and short polyoxethylene chains, the FALT around the liposomes increased more than the single PEG-modified liposomes. Namely, in both the single and mixed PEG-modified liposomes, with the same molecular weight for the PEG-DSG and PEG-CHO, the FALT was the same, and it was reported that the increased density of the PEG on the liposomal membrane was induced to change from the mushroom structure to the brush structure (Ishiwata et al., 1995; Bradley et al., 1998). From this result, it is expected that the mushroom structure of long polyoxyethylene chains is transformed into a brush structure because the short polyoxyethylene chains full into the gap of the long polyoxyethylene chains, and the conformation change theory was approved.

On the other hand, the FALT of a mixed (PEG2000-DSG and PEG2000-CHO with the same PEG-molecular weight and different anchor unit) modified liposome was 3.43 nm, more than that of the single PEG2000-DSG modified liposomes (2.52 nm) or single PEG2000-CHO modified liposomes (2.28 nm). This phenomenon was expected to be based on the difference in the insertion state of the PEG anchor unit on the liposome surface. We expected that PEG-CHO is more difficult to insert in to the liposomal membrane than PEG-DSG, or that part of the anchor unit of PEG-CHO was inserted, because the anchor unit of PEG-DSG is a straight chain, and that of PEG-CHO is big in three dimensions. On the other hand, we indicated that the cholesterol anchor, which is a single chain was able to be easily introduced, compared with the DSG anchor, which has two chains, causing some kind of mixed PEG modification interaction. Furthermore, the PEG incorporation ratios of the mixed PEG-CHO modified liposomes were equal to or any more than those of the single PEG-DSG modified liposomes. It is thought that the increase of FALT in PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX is based on the following reasons. (1) Only a part of the anchor units of PEG-CHO come in the liposomal membrane, the PEG chain of PEG-DSG pushes up the PEG chain of PEG-CHO. (2) PEG-CHO, where one chain is inserted into the deep position, the PEG chain of PEG-CHO pushes up the PEG chain of PEG-DSG, and PEG-DSG changes from the mushroom structure to the brush structure.

Next, we examined the residual amount of PEG-lipid in both single and mixed PEG-modified liposomes. It was suggested that the liposomes, after administration into the blood, are diluted by the blood components, PEG-lipid, which is incorporated in the liposomal membranes is tinged with serum protein and breaks away. It was reported that PEG-lipids with short polyoxyethylene chains are easily incorporated in liposome membranes, and are hard to remove in the blood (Sadzuka et al., 2003); the release of PEG2000-DSG from the liposomal membrane is easier than that of PEG500-DSG (Sadzuka et al., 2003). We considered that the residual amount of PEG900-DSG was larger than that of PEG2000-DSG; therefore, it was difficult to withdraw. In this study, it was shown that PEG-CHO was more easily withdrawn than PEG2000-DSG, which is more easily broken away than the short chain PEG-DSG. We expected that PEG-DSG forms a cluster with cholesterol in the liposome constituent lipid and keep an even keel for the anchoring of its two chains. But, PEG-CHO is easily broken away because the cholesterol anchor of PEG-CHO is one chain. Namely, as for the increase of FALT in mixed modified liposomes, a possibility was suggested that a part of anchor unit of PEG-CHO was installed, and the PEG chain of PEG-DSG was pushing up the PEG chain of PEG-CHO. On the other hand, it was shown that the amount of residual as PEG-lipids increased by mixed modification with PEG-CHO and PEG-DSG, and thus it became stable in the serum. If PEG-lipids are too stably bound to the liposomal membranes, it is difficult to discard the liposome once the drug carried reaches the target region. It has no use as a drug carrier. Consequently, we suggested that the PEG modified stability for increasing the prolonged circulation time in the blood and the ease of slipping of moderate PEG-lipids, which bring about moderate drug release, are necessary. It is thought that a mixed modification with the characters of both the PEG-DSG lipids and PEG-CHO lipids would be useful.

It was reported that vesicles that consist of phospholipids containing a PEG-CHO derivative have high fluidity, instability of liposomal membrane and a high affinity for cell membranes (Carrion et al., 2001). Furthermore, it was also reported that the reduction of endocytosis by the charged influence of PEG-diacylphosphatidyl-ethanolamine on tumor cells is controlled by the neutral-charge of PEG-CHO (Carrion et al., 2001). On the other hand, it is said that the PEG-CHO modification of the liposome depresses the cell uptake because the hydrophilic layer on liposomal membranes inhibits the interaction with liposomes and serum proteins or a direct connection between liposomes and cells (Beugin-Deroo et al., 1998). And so, in order that a PEG-CHO lipid modified liposome might actually exert an influence on tumor cells, we evaluated the effect of PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX on the DOX uptake into tumor cells, and the cytotoxicity toward Ehrlich ascites carcinoma cells and P388 leukemia cells. After 30 min of incubation, PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX, PEG-CHO(2000)-LDOX and PEG-DSG(2000)-LDOX had significantly increased the DOX uptake into tumor cells in the case of PLDOX. In particular, as the cell uptake of PEG-CHO(2000)-

LDOX was dominantly high, it was indicated that PEG-CHO has a high affinity with tumor cells. In addition, it was suggested that liposomes modified with PEG-CHO and PEG-DSG also have these characteristics. The mechanism of the cell uptake of DOX in PEG-CHO modified liposomes is not yet clear, and a detailed examination is now needed. It has been suggested that there is a danger of the interaction of the cell and the liposome being blocked in three dimensions when PEG, which is a hydrophilic macromolecule exists on the liposomes surface (Hong et al., 1999). However, from these results, the increasing FALT due to the PEG modification of the liposome does not necessarily inhibit the cell uptake of the liposome, the characteristics the PEG-CHO lipid expresses, or the ease with which it is taken into tumor cells. Namely, it was reported that PEG-DSG may remain in its equilibrium state by releasing and adhering to the liposome membranes (Sadzuka et al., 2003). In addition, the IC_{50}, used as the index of the effect of cytotoxicity and the cell survival ratio on the Ehrlich ascites carcinoma cells and P388 leukemia cells, increased in the order of PLDOX, PEG-DSG(2000)-LDOX, PEG-CHO(2000)-LDOX and PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX. In particular, PEG-CHO(2000)-LDOX and PEG-(DSG(2000):CHO(2000) = 1:1)-LDOX showed an expected cytotoxicity compared with the case of PLDOX. Namely, we expected that modified liposomes with PEG-CHO lipid and high DOX uptake into tumor cells will cause not only the FALT but also the cell transfer and antitumor effect to be increased in vivo. If the hybrid type liposomes which have the characteristics of long-term circulation in the blood and the high cellular transfer of liposomes due to PEG modifications with alkyl and cholesterol anchors were formulated, their effectiveness in cancer chemotherapy is expected.

5. Conclusion

In mixed PEG-modified liposomes with long polyoxyethylene chains and short polyoxyethylene chains, the FALT around liposomes increased more than single PEG modified liposomes. Mixed PEG modification of liposome by different anchor led to increase in FALT, compared to that of each single PEG modification. The uptake of DOX in PEG-CHO modified liposome is higher than other liposomes. Mixed PEG modified liposomes with PEG-DSG and PEG-CHO (PEG2000-DSG:PEG2000-CHO = 1:1 modified liposome) were suggested to be effective to increase cytotoxicity.

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