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Effect of hybridized liposome by novel modification with some polyethyleneglycol-lipids

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ABSTRACT

This study is about hybridized liposome contained doxorubicin (Hy-LDOX) that has dual properties of stability in blood and incorporation in tumor cells. We used two kinds of polyethyleneglycol-lipids which are 1-monomethoxypolyethyleneglycol–2,3-distearoylglycerol (PEG–DSG) with an alkyl anchor and cholesterol–PEG (PEG–CHO) with a cholesterol anchor. Hy-LDOX was evaluated on antitumor activity (in vivo), DOX uptake into tumor cells, and DOX cytotoxicity (in vitro). Both tumor size and tumor weight in the Hy-LDOX group were decreased, compared with those in the control group. Hy-LDOX had increased DOX uptake into P388 leukemia cells, compared with the single PEG–DSG modified liposomes. Moreover, the IC_{50} value, used as the index of the effect of cytotoxicity, significantly decreased in Hy-LDOX. We suggested that these results of DOX uptake and cytotoxicity contributed to PEG–CHO on liposomal membrane. The PEG modified liposome with only PEG–CHO cannot have a prolonged circulation time, but the Hy-LDOX which was modified with mixing PEG-lipids (PEG–DSG and PEG–CHO) showed stability in blood and incorporation in tumor cells.

As the result of these experiments, Hy-LDOX were observed to be useful in terms of cell transition at target site, as shown by high DOX uptake into cell, and high cytotoxicity because PEG–CHO has good incorporated into tumor cell. Hence, it is expected that Hy-LDOX has novel functions.

1. Introduction

Liposomes are one of the most useful drug carriers in drug delivery system (DDS). However, they are taken up by reticuloendothelial system (RES) cells in the liver and spleen (Senior, 1987; Jones and Nicholas, 1991; Allen, 1998). It is known that polyethyleneglycol (PEG) modification on the surface of the liposomal membrane leads to a prolonged circulation time in the blood (Allen et al., 1991; Klibanov et al., 1990; Lasic et al., 1991; Blume and Cevc, 1993) and this modification has clinical applications (Ranade, 1989; Gregoriadis, 1988; Juliano, 1989; Huang et al., 1987; Fidler, 1989; Hong and Tseng, 2001). The effects of PEG modification on the pharmacokinetics in vivo cannot currently be judged from the physicochemical properties of liposomes. In contrast, a measurement method for the fixed aqueous layer thickness (FALT) around liposomes was developed (Shimada et al., 1995). FALT is formed by interaction of polyoxyethylene chain and water molecule. By this measurement, the FALT of PEG modified liposomes was clarified to increase, in comparison with that of non-PEG modified liposomes (Shimada et al., 1995; Zeisig et al., 1996).

In our previous paper, we reported that mixed PEG modified liposomes contained a mixture of PEG of short and long polyoxyethylene chains were useful for drug delivery (Sadzuka et al., 2002, 2003). The mixed PEG modification of liposomes increased the FALT, compared to that of a single PEG modification. Furthermore, we confirmed that mixed PEG modification of doxorubicin (DOX)-containing liposomes improved their circulation time in the blood, their ability to escape from RES and their biodistribution. It also increased antitumor activity as the FALT increased (Sadzuka et al., 2002, 2003, 2005). Therefore, it is believed that measurement of the FALT is useful for monitoring the development of PEG modified liposomes. Moreover, in our previous paper, we have clarified the physicochemical characteristics of mixed PEG modified liposomes with different anchor units (with same molecular weight of PEG moiety) (Sadzuka et al., 2006; Sugiyama and Sadzuka, 2007). We used PEG–2,3-distearoylglycerol (PEG–DSG) which has an alkyl anchor, and cholesterol–PEG (PEG–CHO) which has a cholesterol anchor and different surface characteristics. The FALT of mixed PEG–CHO modified liposomes with different length PEGs also increased more than that of the single PEG modified liposomes, as that of mixed modified liposomes with the DSG anchor. However, the increases of FALT of PEG–CHO modified liposomes were small. In modification by a mixture of PEG2000–DSG and PEG2000–CHO, the FALT around the liposomes increased with the

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PEG2000–DSG ratio. The FALT of (DSG:CHO = 3:1) modified liposome showed the maximum value.

In this study, we used PEG–DSG:PEG–CHO modified liposome (hybridized liposome contained DOX, Hy-LDOX, the most suitable mix modified liposome (DSG:CHO = 3:1) (Sugiyama and Sadzuka, 2007)) and examined the effect of Hy-LDOX on the tissue distribution of DOX and antitumor activity (in vivo), the DOX uptake into tumor cells, and DOX cytotoxicity (in vitro). Hence, we clarified the usefulness of Hy-LDOX.

2. Materials and methods

2.1. Materials

The DOX used to prepare the liposomes was a gift from Mercian Co., Ltd. (Tokyo, Japan). Adriacin® injection 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 the liposomes, were purchased from NOF Co., Ltd. (Tokyo, Japan). 1-Monomethoxypolyethyleneglycol–2,3-distearoylglycerol (PEG–DSG), with PEGs of an average molecular weight of 2000 (PEG2000–DSG) and cholesterol–polyethyleneglycol (PEG–CHO), with a PEG of an average molecular weight of 2000 (PEG2000–CHO), were gifts from NOF Co., Ltd. (Tokyo, Japan). RPMI 1640 Medium "Nissui" (2) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were commercial products of reagent grade.

2.2. Liposomal preparation and characterization

All liposomes were prepared according to our previous paper (Sugiyama and Sadzuka, 2007). DSPC/cholesterol/DSPG/DOX (100:100:60:18 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 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 60 °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 at above the Tc. DOX-containing liposomes without PEG coatings were referred to as plain liposomal DOX (PLDOX). These PEG modified liposomes were expressed as detailed below.

PEG2000–DSG modified liposome: PEG–DSG(2000)-LDOX
PEG2000–CHO modified liposome: PEG–CHO(2000)-LDOX
PEG2000–DSG:PEG2000–CHO = 1:1 modified liposome: Hy-LDOX(1)
PEG2000–DSG:PEG2000–CHO = 3:1 modified liposome: Hy-LDOX(3).

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. The amount of loaded DOX into liposomes were measured using the fluorescence spectrophotometer (F-2000; HITACHI High-Tech Co., Ltd. Tokyo, Japan) and were calculated by standard curve.

The particle sizes and zeta potentials of the liposomes were measured with an electrophoretic light scattering apparatus (ELS 8000; Otsuka Electrophoretics, Co., Ltd. Osaka, Japan). Zeta potentials were measured with various concentrations of NaCl and plotted against κ, that is, $3.3 \times \sqrt{(c + 0.0056)}$ (c: concentration of NaCl), the slope giving the position of the slipping plane or fixed aqueous layer thickness (FALT) in nm units (Zeisig et al., 1996; Sadzuka et al., 2002). Based on this theory, the thickness of the fixed aqueous layer of each liposome was estimated.

2.3. Tissue distribution

Animal experiments were approved by the institutional animal care and use committee at University of Shizuoka. Male DBA/2 mice (body weight, 20–25 g, 5 weeks old) were obtained from Japan SLC Ltd. (Shizuoka, Japan). P388 leukemia cells (5.0×10^5 cells/animal) provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan) were transplanted onto the backs of mice. On day 9 after transplantation, tumor-bearing mice were injected intravenously with DOX-containing liposomes at a dose of 2.5 mg/kg. At 6 or 24 h after injection, the mice were sacrificed by cervical dislocation, blood was collected from the heart, and then the tumor, heart and liver were removed and weighted. The DOX concentrations in the plasma and tissues were determined by fluorophotometry (Sadzuka et al., 1995).

2.4. Antitumor activity

P388 leukemia cells (5.0×10^5 cells/animal) were transplanted onto the backs of mice, and the DOX-containing liposomes were injected intravenously at a dose of 2.5 mg/kg at 5, 8 and 11 days after tumor inoculation. The mice were sacrificed by cervical dislocation on the 12th day after inoculation, and the tumor was removed and weighted. The DOX concentration in the tumor was determined as described in Section 2.3.

2.5. Incorporated ratio of PEG-lipids on liposomal membranes

The inside of the PEG modified liposomes was composed of 9.0% sucrose in 10 mM lactate buffer (pH 4.0). The outside of the PEG modified liposomes was composed of 10 mM Tris–HCl-150 mM NaCl buffer (pH 7.4). Each PEG modified liposomes suspension was centrifuged at 30,000 × g for 2 h to remove the PEG-lipids that had not been incorporated into the liposomal membranes. 10 mM Tris–HCl-150 mM NaCl buffer (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 to remove PEG-lipids that had withdrawn from the liposomal membranes. The amount of PEG-lipids was determined with the picrate method (Favretto and Tunis, 1976). 10 mM Tris–HCl-150 mM NaCl buffer (pH 7.4) was added to the pellet, followed by sonication and each aliquot were samples. Each sample were added 10 mL of sodium nitrate–picrate reagent (3.3 M $NaNO_3$ and 20 mM picric acid/0.1 M NaOH) and leaved 10 min. After 10 min, each sample was added 5.0 mL of 1,2-dichloroethane and mixed heavily. Therefore, the samples were centrifuged at 150 × g for 10 min. The amount of PEG-lipids in the organic phase was determined at 378 nm with an absorption spectrophotometer (U-1000/1100; HITACHI High-Tech Co., Ltd. Tokyo, Japan). As a control, 10 mM Tris–HCl-150 mM NaCl buffer was added to the pellet instead of FBS, and then the sample procedure was performed. This value of the control group was set as the value for the 100% mark of the residual PEG-lipid ratio.

2.6. Effects of PEG modified liposome on the DOX uptake into tumor cells

We examined the effects of liposomalization and PEG modification on the DOX uptake. P388 leukemia cells (5.0×10^6 cells/mL) were suspended with DOX-containing liposomes (DOX concentration, 10 μg/mL), and then the P388 leukemia cells were incubated at

37 °C for 60 min. For determination of the time course of the intracellular DOX concentration, aliquots of the cell suspension were removed in definite time, 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 PBS(−). The concentration of DOX in the cells was determined as described in Section 2.3.

2.7. Effects of PEG-lipids modification on DOX cytotoxicity

The P388 leukemia cell suspension was seeded in a 96 well plate (FALCON), and then incubated at 37 °C for 24 h. After incubation, the DOX-containing liposomes (DOX concentration, 0.01–10 μg/mL) were added to the cell suspension, 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 2 h. The absorbance at 450 nm was determined. The probability of cell survival without drug exposure was expressed as 100 %. We determined the probability of cell survival in each sample.

2.8. PEG-lipids uptake into tumor cells

PEG2000–DSG solution or PEG2000–CHO solution was added to the P388 leukemia cells suspension (1.0×10^6 cells/mL) that final concentrations were 0.35, 0.70 and 1.0 mM, respectively, and incubated at 37 °C. The cell suspension was removed in the definite time, cooled on ice and then centrifuged at 150 × *g* for 3 min. The cells were washed two times and resuspended in PBS(−). The amount of PEG-lipids in cells was determined with the picrate method in Section 2.5.

2.9. Statistical analysis

Statistical analysis was performed using one–factor ANOVA.

3. Results

3.1. Physical characteristics of PEG modified liposomes

The amount of DOX entrapped in all liposomes was about 90%. The average particle sizes of all liposomes were 120–170 nm. The FALT of PLDOX, PEG–DSG(2000)-LDOX, PEG–CHO(2000)-LDOX, Hy-LDOX(1) and Hy-LDOX(3) was 0.39 ± 0.05 nm, 2.61 ± 0.31 nm, 2.28 ± 0.46 nm, 3.11 ± 0.23 nm and 3.93 ± 0.43 nm, respectively. Hence, the FALT of Hy-LDOX(3) showed the maximum value.

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

DOX concentration in the plasma at 6 h after Hy-LDOX(3) administration was 29.47 μg/mL plasma (Fig. 1(A)). It reached 4.5 times ($p < 0.001$) of that of the PLDOX group (6.62 μg/mL plasma). On the other hand, the DOX concentration in the Hy-LDOX(1) group was equal to that of the PLDOX group. At 6 h after administration, the order of DOX concentration in the plasma was Hy-LDOX(3) ≫ Hy-LDOX(1) = PLDOX. The DOX concentration in the Hy-LDOX(3) group was also higher than that of other groups at 24 h after administration.

In the tumor (Fig. 1(B)), the DOX concentration in the Hy-LDOX(3) group was 55.04 μg/g protein, which was 11.4 times ($p < 0.01$) of that in the PLDOX group (4.84 μg/g protein). Along with the DOX concentration in the plasma, the DOX concentration in the Hy-LDOX(1) group was equal to that of PLDOX group. Hence, at 6 h after administration, the DOX concentrations in tumors reflected those of the blood, and the order was Hy-LDOX(3) ≫ Hy-LDOX(1) = PLDOX.

The DOX concentrations in the PLDOX group and the Hy-LDOX(1) group transferred from blood to tumor time dependently.

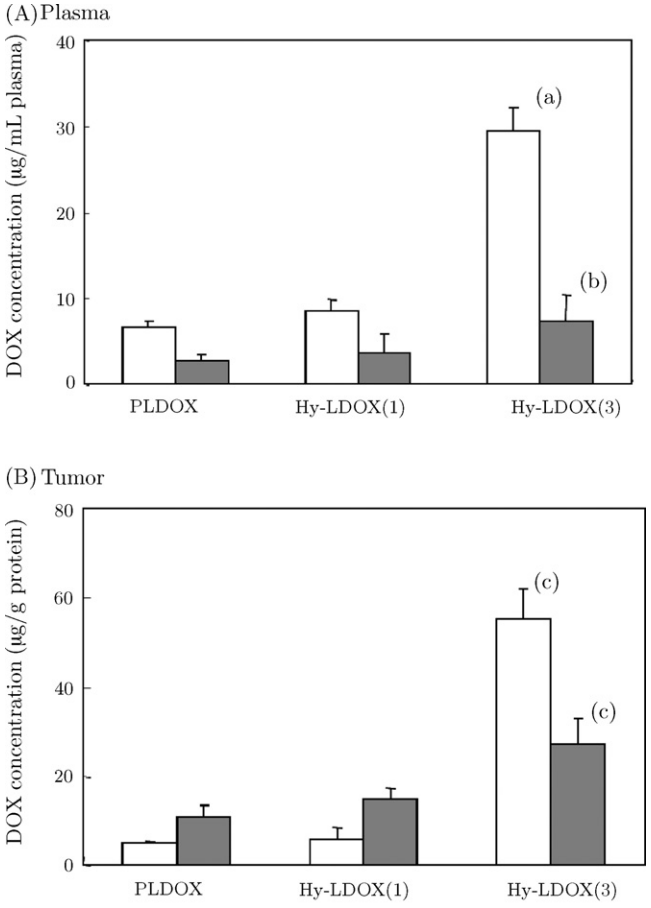

Fig. 1. Effects of PEG modification on the DOX concentrations in plasma and tumor. Each column represents the mean ± S.D. of 4–6 mice. (A) Significant differences from the level of the PLDOX group are indicated by (a) $p < 0.01$ and (b) $p < 0.001$. (B) Significant difference from the level of the PLDOX group is indicated by (c) $p < 0.05$. □ 6 h; ■ 24 h.

In contrast, the time course of DOX concentration in the plasma, in Hy-LDOX(3) group, was similar to that of the tumor.

In the heart, the DOX concentration in the Hy-LDOX(1) group was 6.0 times ($p < 0.01$) than that in the PLDOX group at 6 h after administration. On the other hand, it was shown that the Hy-LDOX(3) group was not significantly different from the PLDOX group.

In the liver, the DOX concentration of the Hy-LDOX(1) group and the Hy-LDOX(3) group was significantly decreased, i.e. 46.2% ($p < 0.001$) and 63.7% ($p < 0.05$), respectively, of that of the PLDOX group at 6 h after the administration.

3.3. Antitumor activity

The tumor sizes of each group gradually increased. After the administration of each liposome, these increases were suppressed, compared to that of control group. After the 12th day, the tumor sizes of the PLDOX group and the Hy-LDOX(3) group reduced to 74.9% ($p < 0.05$) and 67.3% ($p < 0.001$) of the control level, respectively.

Tumor weight is shown in Fig. 2(A). After PLDOX and Hy-LDOX(1) treatment, the tumor weights showed 79.7% and 95.4% of the control level (0.87 ± 0.28 g), respectively. Namely, these liposomes did not have an antitumor effect. In the Hy-LDOX(3) group, the tumor weight (0.52 ± 0.14 g) was reduced to 59.9% ($p < 0.05$) of the control level and 75.3% ($p < 0.05$) of that in the PLDOX group (0.69 ± 0.18 g). The order of DOX concentration in the tumor was

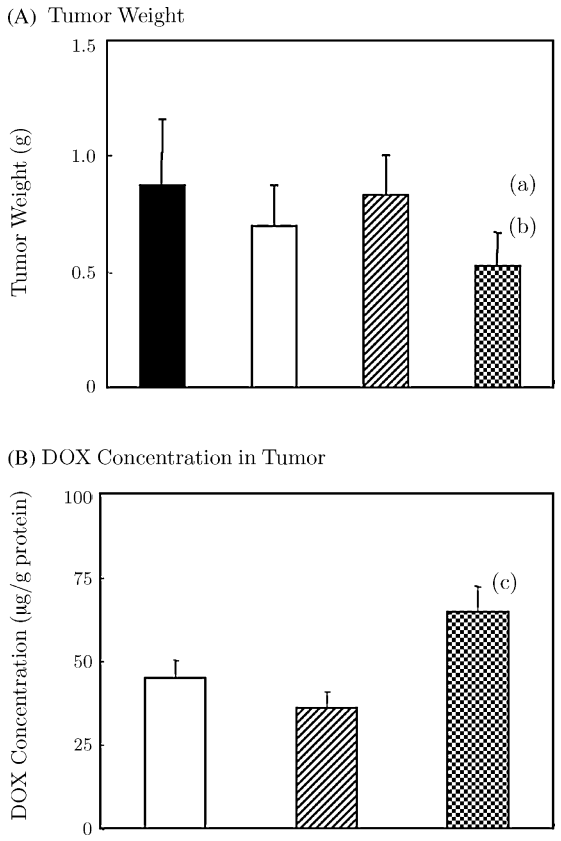

Fig. 2. Effects of PEG modification on tumor weight and DOX concentration in tumor. Each column represents the mean ± S.D. of 5–6 mice. (A) Significant difference from the level of the control group is indicated by (a) $p < 0.05$. Significant difference from the level of the PLDOX group is indicated by (b) $p < 0.05$. (B) Significant difference from the level of the PLDOX group is indicated by (c) $p < 0.01$. ■ Control; □ PLDOX; ▨ Hy-LDOX(1); ▩ Hy-LDOX(3).

Hy-LDOX(3) > PLDOX ≧ Hy-LDOX(1). The DOX concentration in the Hy-LDOX(3) group was 1.5 times higher ($p < 0.01$) than that in the PLDOX group (Fig. 2(B)).

3.4. Incorporation ratios of PEG-lipids into liposomal membranes

The incorporation ratio of PEG-lipids into the liposomal membranes was Hy-LDOX(1) (1.01 ± 0.02 mM) = PEG–CHO(2000)-LDOX (0.94 ± 0.02 mM) > Hy-LDOX(3) (0.83 ± 0.02 mM) > > PEG–DSG(2000)-LDOX (0.50 ± 0.01 mM). PEG–CHO containing liposomes tended to increase the incorporation ratios.

On the other hand, the residual amounts of PEG-lipids in the liposomal membranes of single and mixed PEG modified liposomes decreased time dependently (Fig. 3). The residual amount of PEG–CHO(2000)-LDOX drastically decreased to 78.7% at 2 h after incubation. Hy-LDOX(1) was shown to decrease moderately in the early stages, whereas the residual amount of PEG-lipids at 2 h after incubation decreased markedly and decreased the level further to 79.8% and 62.7% after 3 and 6 h, respectively. In contrast, the residual amount of PEG-lipids in the Hy-LDOX(3) group slowly decreased until 4 h after incubation.

3.5. Effect of PEG modification on DOX uptake

Fig. 4 shows the effect of PEG modification on DOX uptake in P388 leukemia cells. After 20 min incubation, the intracellular levels of DOX were not different by the PEG modification. However, the DOX level in each liposome group

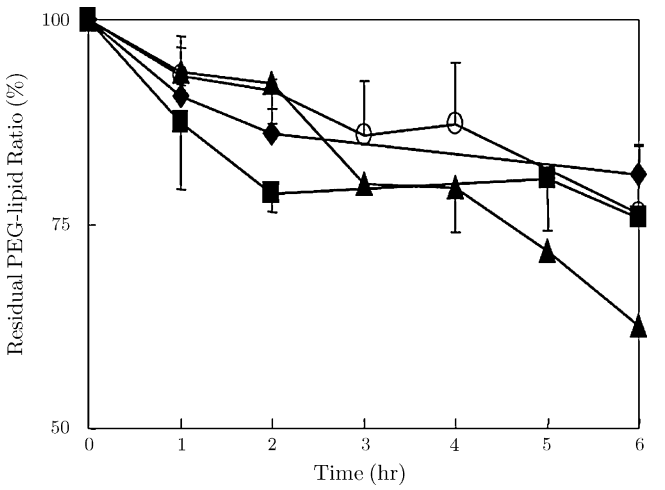

Fig. 3. Residual PEG-lipid in the liposomal membrane after incubation in 50% FBS. Each point represents the mean ± S.D. of 4 samples. ◆ PEG-DSG(2000)-LDOX; ▲ PEG-CHO(2000)-LDOX; ■ Hy-LDOX(1); ○ Hy-LDOX(3).

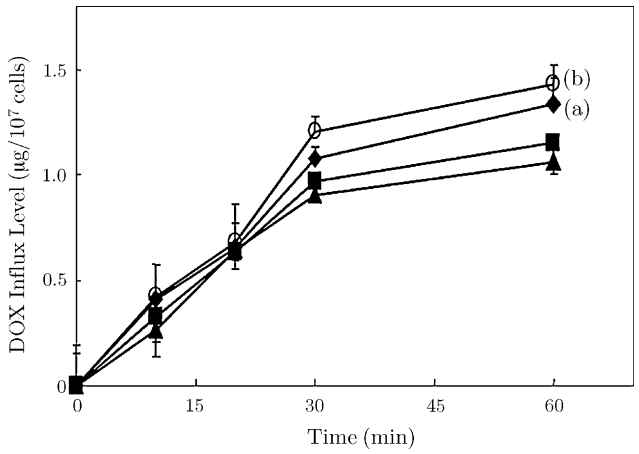

Fig. 4. Effects of PEG modification on DOX uptake in P388 leukemia cells. Each point represents the mean ± S.D. of 4 samples. Significant differences from the level of the PEG–DSG (2000)-LDOX group are indicated by (a) $p < 0.05$ and (b) $p < 0.001$. ▲ PEG-DSG(2000)-LDOX; ◆ PEG-CHO(2000)-LDOX; ○ Hy-LDOX(1); ■ Hy-LDOX(3).

after 30 min incubation was different and the intracellular level of DOX was PEG–CHO(2000)-LDOX ≧ Hy-LDOX(1) > Hy-LDOX(3) ≧ PEG–DSG(2000)-LDOX at 60 min. In particular, the DOX levels in the PEG–CHO(2000)-LDOX and the Hy-LDOX(1) groups increased 1.34 times ($p < 0.001$, 1.43 ± 0.23 μg/10^7 cells) and 1.26 times ($p < 0.05$, 1.34 ± 1.8 μg/10^7 cells), respectively, of that in the PEG–DSG(2000)-LDOX group. In addition, the difference of the modified PEG-lipids induced differences in the initial absorbed levels of DOX. The absorbed level in the PEG–CHO(2000)-LDOX group was the maximum value (1.42 ± 0.19 μg/10^7 cells) and that in the PEG–DSG(2000)-LDOX group was the minimum value (0.80 ± 0.11 μg/10^7 cells). The order of initial levels was PEG–CHO(2000)-LDOX > Hy-LDOX(1) > Hy-LDOX(3) > PEG–DSG(2000)-LDOX.

3.6. Effects of PEG modification on DOX cytotoxicity

The order of the IC_{50} of each liposome on P388 leukemia cells was Hy-LDOX(1) = Hy-LDOX(3) ≧ PEG–CHO(2000)-LDOX > PEG–DSG(2000)-LDOX > DOXsol > PLDOX (Table 1). Cytotoxicity

Table 1
Cytotoxicity of liposomal DOX on P388 leukemia cells.

	IC_{50} (μM DOX)
DOXsol	4.60 ± 0.01
PLDOX	6.11 ± 0.05
PEG–DSG(2000)-LDOX	3.47 ± 0.18
PEG–CHO(2000)-LDOX	1.56 ± 0.10[a]
Hy-LDOX(1)	1.23 ± 0.04[b]
Hy-LDOX(3)	1.30 ± 0.03[b]

Cytotoxicities were determined by the WST-8 assay and expressed as IC_{50} (n = 5–8). Significant differences from the level of PEG-DSG(2000)-LDOX are indicated by (a) $p < 0.001$ and (b) $p < 0.01$.

was increased by PEG modification of the liposomes. The cytotoxicity of PEG–CHO(2000)–DOX, Hy-LDOX(1) and Hy-LDOX(3) which has PEG–CHO contained liposome were shown to be 2.2 times ($p < 0.001$), 2.8 times ($p < 0.01$) and 2.7 times ($p < 0.01$) of that of the PEG–DSG(2000)-LDOX group, respectively. Hence, PEG modified liposomes with PEG–CHO had increased cytotoxicity compared with single PEG–DSG modified liposomes.

3.7. Transition of PEG-lipids into tumor cell membrane

When PEG2000–DSG solution or PEG2000–CHO solution was added to the P388 leukemia cell suspension, the transition of PEG-lipids into the cell membrane increased with the increased concentration of PEG-lipids. Hence, the level of PEG-lipids into cell was dependent on the concentration of PEG-lipids.

At 0.35 mM of PEG-lipid (Fig. 5(A)), the transitional level into cell increased immediately after the addition of PEG-lipids. At 5 min after incubation, the concentrations of PEG2000–DSG and PEG2000–CHO were 0.55 ± 0.10% and 1.30 ± 0.03%, respectively. The level of PEG2000–CHO was 2.4 times ($p < 0.05$) that of the PEG2000–DSG group. Thereafter, the level of PEG-lipids decreased. At 0.70 mM of PEG-lipids (Fig. 5(B)), both PEG2000–DSG and PEG2000–CHO were taken up 1.20% at 5 min after incubation. The PEG2000–DSG group maintained this level. On the other hand, that of the PEG2000–CHO group decreased by 0.55 ± 0.07%. At 1.0 mM of PEG-lipid (Fig. 5(C)), the level of PEG2000–DSG into tumor cells was higher than that of PEG2000–CHO at 30 min after incubation, and this level was 2.3 times ($p < 0.001$) of that of PEG2000–CHO.

At 0.35 mM of PEG2000–DSG and PEG2000–CHO, the intracellular levels of PEG-lipids at 60 min after incubation were 4.5×10^{-4} mM (0.14 ± 0.02%) and 19.1×10^{-4} mM (0.55 ± 0.06%), respectively. The cell uptake level of PEG2000–CHO was 4.2 times ($p < 0.01$) higher than that of PEG2000–DSG (Fig. 5(A)). However, at 1.0 mM of PEG-lipids, the intracellular levels of PEG2000–DSG and PEG2000–CHO were 11.7×10^{-3} mM (1.17 ± 0.10%) and 4.8×10^{-3} mM (0.48 ± 0.02%), respectively. The uptake level of PEG2000–DSG was 2.4 times ($p < 0.001$) that of PEG2000–CHO (Fig. 5(C)).

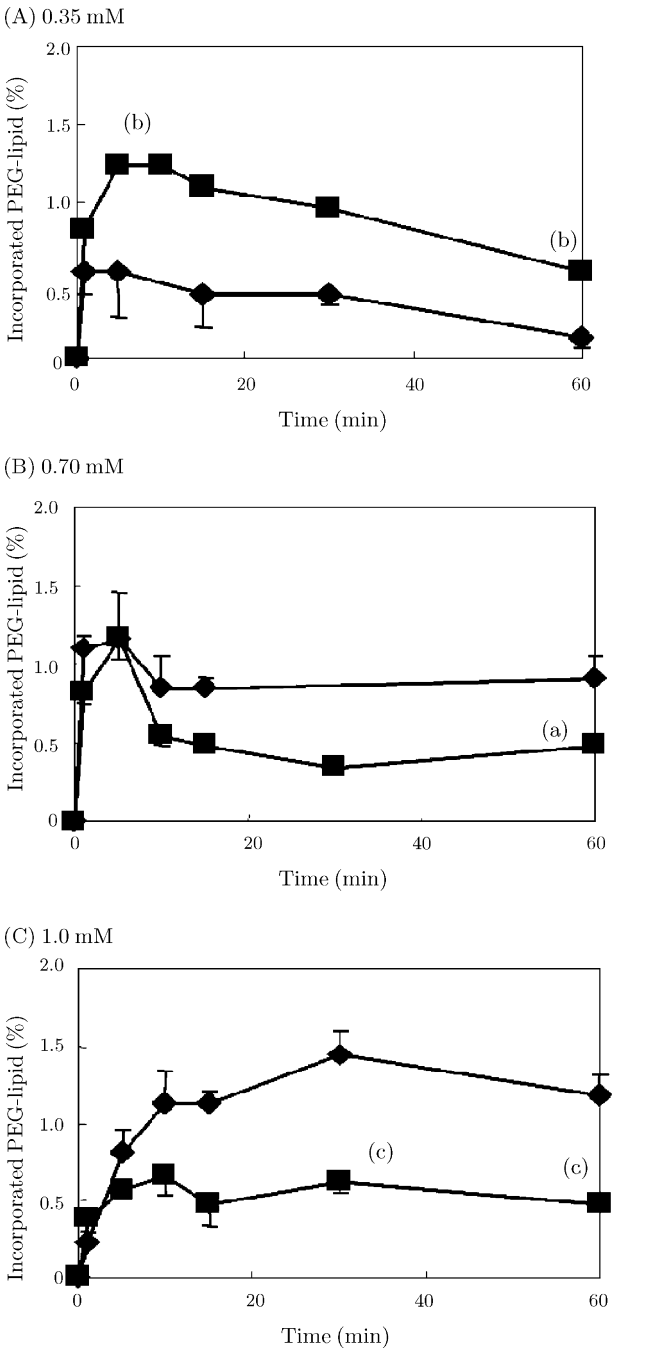

Fig. 5. Cell uptake of PEG-lipids in P388 leukemia cells. Each point represents the mean ± S.D. of 2–3 samples. Significant differences from the level of the PEG2000–DSG group are indicated by (a) $p < 0.05$, (b) $p < 0.01$ and (c) $p < 0.001$. ◆ PEG2000–DSG; ■ PEG2000–CHO.

4. Discussion

We examined the effects of Hy-LDOX in vivo and in vitro. Hence, we tried to clarify the connection between FALT and the characteristics of Hy-LDOX.

DOX concentration in the plasma at 6 h after administration in the Hy-LDOX(3) group was maximum and the Hy-LDOX(1) group was equal to that of the PLDOX group. In tumor, the DOX concentrations reflected those in the plasma concentration. The DOX concentration in the Hy-LDOX(3) group showed a greater level than the others. We focused attention on the DOX transition from the plasma to the tumor. The DOX concentrations in the PLDOX and the Hy-LDOX(1) groups transferred from the blood to the tumor time dependently. In contrast, the time course of the DOX concentration in the tumor for the Hy-LDOX(3) group was similar to that in the plasma (Fig. 1). It is generally known that liposomes accumulate in tumor by the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986). PLDOX and Hy-LDOX(1) groups were indicated to accumulate by EPR effect. On the other hand, Hy-LDOX(3) group was speculated to accumulate in the tumor by this mechanism and other pathways. In the Hy-LDOX(1) group, all tissues had lower DOX concentrations. We suspect that the DOX in the Hy-LDOX(1) group was eliminated from the body at 6 h after administration. Namely, after the release of PEG–CHO from the liposomal membrane, some

Hy-LDOX(1) was changed to PLDOX and this liposome was broken.

Both tumor size and tumor weight (Fig. 2(A)) in the Hy-LDOX(3) group were decreased, compared to those in the control group. Moreover, when Hy-LDOX(3) was injected three times according to the antitumor protocol, the DOX concentration in the tumor was especially high. We indicated that the antitumor activity depends on the increase of the DOX concentration in the tumor. Hence, Hy-LDOX(3) were suggested to be useful in vivo due to their the ability to escape from RES cells, their long-term circulation in the blood, the increase of DOX concentration in tumors after passive targeting, and the enhancement of antitumor activity.

We examined the effect of Hy-LDOX on DOX uptake into tumor cells and cytotoxicity. After 30 min of incubation, PEG–CHO contained liposomes had increased the DOX uptake into tumor cells compared to that of single PEG2000–DSG modified liposomes, and it was suggested that PEG–CHO has a high affinity for tumor cells. In particular, PEG–CHO(2000)-LDOX and Hy-LDOX(1) had significantly increased DOX uptakes compared with single PEG–DSG(2000)-LDOX. If DOX released from the liposome is taken up by passive diffusion, its behavior is similar to DOX solution. In our study, DOX uptake into tumor cell in liposome group differed from that in solution group (Sadzuka et al., 2003). It is indicated that the affinity for a particular cell varied by the modified PEG–lipids, because the amount of early absorption of DOX differed by any PEG modification. We had concerns about the effects of cell transition that the interaction of the cell and the liposome led to the disorder in three dimensions when PEG was incorporated into the liposomal membrane. In this study, it was shown that the increasing FALT due to the PEG modification of the liposome does not necessarily inhibit the cell uptake of the liposome, by the characteristics that PEG–CHO expresses.

The IC_{50} value, used as an index of the effect of cytotoxicity, significantly decreased in the PEG–CHO contained liposomes compared with the single PEG–DSG modified liposomes. In particular, the cytotoxicities of Hy-LDOX(1) and Hy-LDOX(3) as PEG–CHO contained liposomes were shown to be 2.8 times ($p < 0.01$) and 2.7 times ($p < 0.01$) of that of PEG–DSG(2000)-LDOX, respectively. These results were reflected in the DOX uptake (Fig. 4), and the high cell uptake of the PEG–CHO was considered to be related to the increase of cytotoxicity. It is possible that the PEG-lipids formed micelles after withdrawn from liposome. But micelle in both PEG–DSG and PEG–CHO were considered to be of similar level. In PEG–CHO modified liposome, unstable PEG–CHO on liposomal membrane withdrew and then returned to the liposomal membrane, or the cholesterol units of withdrawn PEG–CHO were inserted into the cell membrane and this PEG chain and other chains on the liposomal membrane interacted with each other. The mechanism of the cell fusion with PEG is informed by the fact that PEG is hydrogen bonding in solution, which causes free water to be absent, changes the water structure of outer aqueous phase near the membrane surface and changes the hydration state of polar radicals in the lipid bilayer membrane (Aldwinckle et al., 1982; Saez et al., 1982; Blow et al., 1978; Maggio and Lucy, 1978). The transition phase temperature is increased (Tilcock and Fisher, 1979) and structural deficits occur (Boni et al., 1981) by the presence of PEG. Hence, it is thought that PEG modified liposomes contained PEG–CHO increase cell transition because the cell fusion with PEG causes interactions between PEG and glycoprotein on the cell membrane surface and conformational changes of both PEG–CHO and the cell membrane surface.

Then, we considered the transition of PEG-lipids into tumor cells to explain the increased cell uptake of liposomes contained PEG2000–CHO. In this study, we used various concentrations (0.35, 0.70 and 1.0 mM) of PEG-lipids solution. When PEG modified liposome is prepared, 1.5 mM of PEG-lipids is usually added to the liposomal suspension. The incorporated PEG-lipid into the liposomal membrane usually totals about 45–60% of the added amount, producing a solution similar to 0.70 mM PEG solution. We used 0.35 mM PEG solution, because it was speculated that PEG-lipids which withdraw from the liposome membrane lead to an increase in cell accumulation of liposomes. Furthermore, a 1.0 mM PEG solution was used to find the accumulation levels into tumor cell in conditions of excess PEG-lipids. When PEG2000–DSG or PEG2000–CHO solutions was added to the P388 leukemia cell suspension, the accumulation of PEG-lipids into the cell membrane increased with increasing concentration of PEG-lipids. It is known that the PEG-lipids incorporated into the liposomal membrane are drafted by buffer solution or plasma protein. In this study, the reason for the increased DOX concentration in tumor cells was speculated to be that PEG-lipids which withdrew from liposome membrane transfer to the cell membrane were brought back into contact with the cell membrane via interactions induced by the PEG-lipids in the membrane. At 60 min after incubation, when the 0.70 and 1.0 mM solutions were added, the PEG–DSG concentration in the cell was higher than the PEG–CHO concentration. On the other hand, with the 0.35 mM solution, the cell uptake level of PEG–CHO was 4.2 times ($p < 0.01$) higher than that of PEG2000–DSG (Fig. 5: 60 min). When PEG solution is more diluted, like actual cell uptake or the cytotoxicity experiment, PEG2000–CHO is easily taken up by the cell. If PEG2000–CHO in aqueous solution is too concentrated, PEG2000–CHO cannot transfer into the cell to form clusters among the anchor units of PEG2000–CHO. At 6 h after incubation, the percentages of residual amounts of PEG from the liposomal membrane in the plasma for PEG2000–DSG, which is stable in the liposomal membrane, and PEG2000–CHO, which is not, were 6.3% and 15.2%, respectively. These values were equivalent to 0.095 and 0.23 mM, and the concentrations were less than that of the original concentration (0.35 mM). Free PEG-lipids concentration of DOX uptake and cytotoxicity in these experiments is surmised not over 0.35 mM. Namely, this result represents in vitro data of increased DOX uptake into the tumor cell and increased cytotoxicity based on the cell transition of PEG2000–CHO. These increases were higher than those of PEG2000–DSG.

In conclusion, we indicated that Hy-LDOX(3) created a multiplier effect on a lot of factors not only concerning the FALT but also the modification stability in the blood and cell transition of PEG-lipids as the indicator of improvement of circulation in the blood and antitumor activity. Moreover, we demonstrated that Hy-LDOX(3) is useful in terms of cell transition at a target site. This was shown by the high uptake of DOX into cells and the high cytotoxicity displayed due to the increased transition of PEG2000–CHO. Namely, we confirmed that Hy-LDOX(3) had novel abilities with blood circulation and high cytotoxicity.

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