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Enhanced antitumor activity of different double arms polyethyleneglycol-modified liposomal doxorubicin

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ABSTRACT

The effect of polyethyleneglycol (PEG)-modified liposome as a drug carrier has been demonstrated clinically. We designed and synthesized a novel PEG-lipid, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG (different double arms PEG, DDA-PEG) which had two PEG chains of 500 and 2000 in one molecule to develop more useful PEG-modified liposome. DDA-PEG-modified liposomal doxorubicin (DDA-LDOX(7.5)) had the biggest fixed aqueous layer thickness (FALT) compared with other PEG-lipid-modified liposomes even if the added amount was a few. It was thought that FALT was the indication of blood circulation time. In DOX uptake in tumor cells, DDA-LDOX(7.5) group increased the DOX concentration in tumor cells because it had contact ability with tumor cells. Hence, DDA-LDOX(7.5) which has long circulation time in the bloodstream and contact ability with tumor cells, also had a strong antitumor effect on mice bearing M5076 ovarian sarcoma cells which were DOX low sensitive cells according to the expression of multidrug resistance protein. Furthermore, this liposome maintained a high DOX concentration in a tumor for a long time. These results indicated that the useful antitumor effect of DDA-LDOX(7.5) against M5076 ovarian sarcoma cells is a promising DDS carrier for therapies against drug resistant tumors.

1. Introduction

The effect of liposome as a drug carrier is demonstrated by Doxil® and AmBisome®. Liposomes may play an important role in cancer and gene therapies. Most liposomes used in therapy or being studied is modified polyethyleneglycol (PEG) on the liposomal membrane surface (Allen et al., 1991; Klibanov et al., 1990; Lasic et al., 1991; Blume and Cevc, 1993) to exert enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986) this mechanism is avoidance from reticuloendothelial system (RES) cells (Senior, 1987; Jones and Nicholas, 1991; Allen and Gregoriadis, 1988). In our previous paper, the efficacy of a PEG-modified liposome was shown to be linked to the fixed aqueous layer thickness (FALT) around the liposome (Sadzuka et al., 2002), while other physical factors could not evaluate biological efficacy (Kuhi et al., 1994; Torchilin et al., 1994; Janzen et al., 1996). Doxorubicin (DOX), an antitumor agent, containing a PEG-modified liposome with thick FALT increased antitumor activity based on long time circulation in the bloodstream and accumulation in tumors via the EPR effect (Shimada et al., 1995; Zeisig et al., 1996; Matsuo et al., 1997). Moreover, the importance of the determination of the PEG layer on liposome is also suggested from *the Draft Guidance on Doxorubicin Hydrochloride* (liposome injection/intravenous; Food and Drug Administration, USA) to confirm its quality (www.fda.gov).

As for FALT, antitumor activity of the modified liposomal DOX with two different PEGs (MIX-LDOX) was stronger than a single PEG-modified liposome and it was confirmed from biological data (Sadzuka et al., 2003). The PEGs using mixed PEG-modification need different physical characteristics. Especially, the liposome had superior to antitumor activity when M.W. 500 and M.W. 2000 polyoxyethylene chains of PEG-lipid were 1:1 (Sadzuka et al., 2002, 2003, 2005). However, it was unclear how the two kinds of PEGs were modified in terms of uniformity and stability around the liposome because liposome was added to each PEG. Moreover, PEG2000 was easily separated compared with PEG500 from liposomal membrane in vivo, the modification ratio was not always the same during systemic circulation. To solve these problems is very important to show any antitumor effect of a PEG-modified liposome. Therefore, we designed and synthesized a novel PEG-lipid, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG (500

Abbreviations: DDA-PEG, different double arms PEG; DOX, doxorubicin; DSPC, L-α-distearoylphosphatidylcholine; DSPG-Na, L-α-distearoyl-phosphatidyl-DL-glycerol-Na; EPR, enhanced permeability and retention; FALT, fixed aqueous layer thickness; FBS, fetal bovine serum; PEG, polyethyleneglycol; PEG-DSG, 1-mono-methoxypolyethyleneglycol -2,3-distearoylglycerol; RES, reticuloendothelial system.

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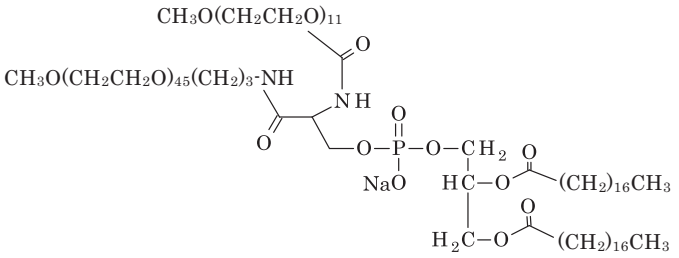

Fig. 1. Chemical structure of DSPE-PEG (500, 2000) (DDA-PEG).

and 2000). The novel PEG-lipid had two PEG chains of 500 and 2000 in one molecule and it was named different double arms PEG (DDA-PEG) (Fig. 1). PEG500 and PEG2000 were always modified at 1:1 in the DDA-PEG-modified liposome.

In this study, a novel PEG-lipid, DDA-PEG, modified liposomal doxorubicin (DDA-LDOX) was prepared. We demonstrated its usefulness by evaluating physical properties including FALT, drug uptake into tumor cells and cytotoxicity in vitro, as well as antitumor activity and blood circulation in vivo.

2. Materials and methods

2.1. Materials

The DOX used to prepare liposomes was a gift from Mercian 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-Na (DSPG-Na), used to prepare liposomes were purchased from NOF Co., Ltd. (Tokyo, Japan). 1-Mono-methoxypolyethyleneglycol-2,3-distearoylglycerol (PEG-DSG), with PEGs of average molecular weight of 2000 (PEG2000-DSG) and 500 (PEG500-DSG) were a gift from NOF Co., Ltd. (Tokyo, Japan). The novel PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG (DDA-PEG) were synthesized by NOF Co., Ltd. (Tokyo, Japan). RPMI1640 medium was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences. All other chemicals were commercial products of reagent grade.

2.2. Preparation of liposomal DOX

Liposomal DOX (LDOX) was prepared according to a modification of the method of Bangham (Bangham et al., 1965). Briefly, DSPC/cholesterol/DSPG-Na/DOX (100:100:60:18 μmol) were dissolved in a chloroform/methanol mixture (4:1, v/v) and the solution was dispersed by sonication. The chloroform and methanol were evaporated to dryness under a stream of nitrogen gas. A thin lipid film was hydrated with 10 mL of 9.0% sucrose in 10 mM lactate buffer (pH 4.0) in a water bath at 75 °C for 20 min. The suspension was sonicated for 20 min at 75 °C. The liposome suspension was extruded through polycarbonate membrane filters (pore size was 0.1 μm). PEG-modified-LDOX was also prepared using various PEG-lipid (15 μmol). When PEG-modified-LDOX was prepared, PEGs were added to lipids solution and done in a similar way to LDOX. PEG2000-DSG-modified-LDOX, PEG2000- and PEG500-DSG-modified-LDOX and DDA-PEG-modified-LDOX represent 2000-LDOX, MIX-LDOX and DDA-LDOX, respectively. DDA-LDOX was used two types in this study, namely, amount of PEG modification were 15 μmol and 7.5 μmol and represented as DDA-LDOX(15) and DDA-LDOX(7.5), respectively. 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.

2.3. Physicochemical characteristics of liposomes

The particle sizes and zeta potentials of the liposomes were measured with an electrophoretic light scattering apparatus (Zetasizer Nano ZS; Malvern Instruments Ltd., Worcestershire, UK).

Calculation of FALT involved measuring zeta potentials with various concentrations of NaCl 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 FALT in nm units (Shimada et al., 1995). Based on this theory, FALT of each liposome was estimated.

To measure the amount of DOX contained in each liposome, a choloroform:isopropanol mixture (1:1, v/v) was added to the liposomal suspension and mixed. Then, the amount of DOX contained in each liposome was determined using a fluorescence spectrophotometer (Hitachi F2000: Hitachi Ltd., Tokyo, Japan), at an excitation wavelength of 500 nm and an emission wavelength of 550 nm.

2.4. Loaded ratio of PEG-lipid around the liposomal membrane

The loaded ratio of PEG-lipids was defined as the ratio of PEG-lipids incorporated into the liposomal membrane. Micelles formed from PEG-lipid in each PEG-modified liposome suspension were removed. To separate PEG-lipid that were not incorporated into liposomal membranes, each PEG-lipid-modified liposome suspension was centrifuged at 30,000 × *g* for 2 h. Tris–HCl-150 mM NaCl buffer (10 mM) was added to the pellet, followed by sonication. We determined the amount of PEG-lipids in the samples by direct determination using the picrate method (Favretto and Tunis, 1976).

2.5. DOX uptake into tumor cells (in vitro)

Liposomal DOX (DOX concentration, 5.0 μg/mL) was added to M5076 ovarian sarcoma cell suspension (5.0×10^6 cells/mL), and then the cells were incubated at 37 °C for 30 min. To determine 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), and then mixed for 30 s with 5.0 mL of chloroform/methanol (4:1, v/v) and centrifuged at 450 × *g* for 15 min. We confirmed that the electrical binding liposomes to the outside of the tumor cell were removed from tumor cell fraction by this procedure. The concentration of the drug in the organic phase was determined with a fluorescence spectrophotometer, Hitachi F2000 (Hitachi Ltd., Tokyo, Japan), at an excitation wavelength of 500 nm and an emission wavelength of 550 nm.

2.6. Cytotoxicity of DDA-LDOX (in vitro)

An M5076 ovarian sarcoma cell suspension (1×10^6 cells/mL) was seeded in a 96 well plate (FALCON), and then incubated at 37 °C for 24 h. After incubation, to the cell suspension was added DOX solution, empty liposome and each LDOX (DOX concentration, 0.01–10 μg/mL), and the mixture was incubated at 37 °C for 48 h. Afterwards, WST-8 was added to this cell suspension and it was then incubated at 37 °C for 2 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.

2.7. Antitumor activity of DDA-LDOX (in vivo)

All animal experiments were approved by the Institutional Animal Care and Use Committee at Iwate Medical University. BDF_1 mice at 5-weeks old (male; 20–25 g body weight) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were given