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Prolonged Retention of Doxorubicin in Tumor Cells by Encapsulation of γ-Cyclodextrin Complex in Pegylated Liposomes

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Abstract

For further increase of retention of doxorubicin (DOX) in tumor cells, we prepared the pegylated liposomes entrapping the complex of DOX with γ-cyclodextrin (γ-CyD) (complex-in-liposome), and then examined the physicochemical properties and the *in vitro* cellular uptake/release, compared with those of pegylated liposomes entrapping DOX alone (DOX-in-liposome). The particle sizes of these liposomes were almost comparable, and the entrapment ratios of both DOX and γ-CyD in liposomes were more than 90%. The release of DOX from liposomes in the fetal calf serum (FCS) was significantly inhibited by entrapment of γ-CyD in the liposomes. The cellular uptake of DOX into Colon-26 cells, a mouse rectal carcinoma cell line, after incubation with these liposomes was almost equivalent. However, the cellular release of DOX from cells in the complex-in-liposome system was markedly slower than that in the DOX-in-liposome system. These results suggest the potential use of liposomes containing the DOX/γ-CyD complex for high retention of DOX in tumor cells.

Abbreviations: complex-in-liposome – pegylated liposomes entrapping the complex of doxorubicin with γ-cyclodextrin; CH – cholesterol; CyD – cyclodextrin; DSPC – distearoylphosphatidylcholine; DSPE-PEG2000 – distearoylphosphatidylethanolamine-polyethylene glycol 2000; DOX-in-liposome – pegylated liposomes entrapping doxorubicin alone; DOX – doxorubicin; EPR – enhanced permeability and retention; FCS – fetal calf serum; PEG – polyethylene glycol

Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of 6–8 glucose units through α-1,4-glycosidic bonds and have been utilized as carriers for improvement of pharmaceutical properties such as solubility, stability and bioavailability [1]. Sterically stabilized liposomes can increase the accumulation of the encapsulated drugs into tumor tissues by the process of ''passive targeting'' due to the effect of the enhanced permeability and retention (EPR) [2–4]. Recently, pegylated liposomal doxorubicin preparations for the treatment of metastatic cancers are approved in USA, Europe, Canada and Israel [5]. The preparations certainly provide a favorable advantage over conventional doxorubicin in terms of cardiac safety [6], but further improvement of a pharmacological efficacy of these preparations has been desired. One of the methods is to increase a cellular uptake of drug-encapsulating liposomes in tumor cells [7].

So far, many attempts have been made to increase the DOX concentration in tumor cells, for example, the modification of a liposomal surface with various targeting ligands such as transferrin, folate and antibody to tumor cells [8]. Meanwhile, the potential use of liposomes containing a drug/CyD inclusion complex has been reported for improvement of the pharmacokinetic behavior and therapeutic efficacy of drugs [9–12], especially the incorporation of the complex of dexamethasone with CyD in liposomes which allowed to increase the retention of the drug and CyD to liver and spleen after intravenous administration of the liposomes in rats, compared to the complex of dexamethasone with CyD [13]. To improve the doxorubicin (DOX) retention in tumor cells, we prepared pegylated liposomes entrapping the DOX complex with γ-CyD (complex-in-liposome). And their physicochemical properties such as a particle size and an entrapment ratio, the *in vitro* release of the drug from liposomes in fetal calf serum

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(FCS) and the *in vitro* cellular uptake/release, were compared with those of pegylated liposomes entrapping DOX alone (DOX-in-liposome).

Experimental

Materials

γ-CyD and DOX hydrochloride were donated from Nihon Shokuhin Kako (Tokyo, Japan) and Mercian (Tokyo, Japan), respectively. Distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) were donated from Daiichi Pharmaceutical (Tokyo, Japan). Cholesterol (CH) was obtained from Nacalai Tesque (Kyoto, Japan). RPMI-1640 culture medium and FCS were obtained from Nissui Pharmaceutical (Tokyo, Japan) and JRH Biosciences (Renexa, KS), respectively. Other chemicals and solvents were of analytical reagent grade.

Liposome preparation

Liposomes were prepared from DSPC/CH (1:1, molar ratio) and 6 mol% of DSPE-PEG according to a dehydration–rehydration method [14]. Briefly, lipid mixtures were dissolved in chloroform, and the solvent was removed under reduced pressure by a rotary evaporator. Three ml of 300 mM citric acid (pH 4.0) were added to the resulting lipid film, and the suspensions were agitated for 5 min and freeze-thawed three times, followed by an extrusion 10 times through two stacked polycarbonate membranes (pore sizes 1.0, 0.2 and 0.1 μm) to adjust the particle sizes according to the extrusion method [15]. Liposome sizes were measured by a submicron particle analyzer N4 Plus (Beckman Coulter, Fullerton, CA) at room temperature. DOX and DOX/γ-CyD encapsulations were performed using the pH gradient method [16]. Briefly, the pH of the liposome suspensions was raised from pH 4.0 to pH 7.8 with 1 N NaOH. The liposome preparations were mixed with the aqueous DOX and DOX/γ-CyD solutions preheated at 60 °C at the drug-to-lipid weight ratio of 0.2. This mixture was incubated with periodic mixing for 10 min at 60 °C. After preparing liposome-entrapped DOX (DOX-in-liposome), non-entrapped DOX and γ-CyD were removed by the ultracentrifugation (109,000×*g*, 1 h, 4 °C) using a Optima TLX ultracentrifuge (Beckman Coulter, Fullerton, CA).

Physicochemical properties of liposomes

The entrapment ratio of DOX was determined using a F-4500 fluorescence spectrometer (Hitachi, Tokyo, Japan) by diluting liposomes with 0.3 M HCl-50% ethanol. The wavelengths for excitation (Ex) and emission (Em) used were 470 nm and 590 nm, respectively. The entrapment ratio of γ-CyD was measured by anthrone-sulfuric acid method [17]. Briefly, 3 ml of anthrone reagent were added to 0.5 ml of the suspension containing complex-in-liposome. The tube was covered with a glass ball and was heated for 10 min in boiling water. After quenching with cold water, absorbance of the suspension was measured using a U-2000A spectrophotometer (Hitachi, Tokyo, Japan) at 620 nm.

In vitro release of DOX from liposomes

The *in vitro* release of DOX from liposomes in FCS was performed at 37 °C. Briefly, liposome suspensions (0.2 ml) were added to FCS (2 ml), and then incubated with stirring at 37 °C. At appropriate intervals, the reaction solution (2.2 ml) was ultracentrifuged (266,000×*g*, 10 min, 4 °C) using a Optima TLX ultracentrifuge (Beckman Coulter, Fullerton, CA), and the amounts of DOX released from liposomes to FCS were determined with a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm).

Cellular uptake and release of DOX

Colon-26 cells, a mouse rectal carcinoma cell line, were used. For cellular uptake experiments, the cells (3×10^6 cells/dish) were incubated with 1 ml of RPMI-1640 culture medium containing liposomes (DOX-in-liposome or complex-in-liposome) at the concentration of 8.6 μM DOX in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. At appropriate intervals, after washing three times with phosphate buffered-saline (PBS, pH 2.5) to remove the membrane-bound liposome, the cells were lysed with 1 N NaOH, and then the amounts of DOX in the cells were determined with a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm). For the release experiments from the cells, the cells (3×10^6 cells/dish) were incubated for 2 h with 1 ml of RPMI-1640 culture medium containing liposomes (DOX-in-liposome or complex-in-liposome) at the concentration of 8.6 μM DOX in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After washing three times with PBS (pH 2.5), the cells were incubated with 1.1 ml of fresh medium supplemented with 10% FCS at 37 °C. At appropriate intervals, the amounts of DOX released from the cells to the medium were determined using a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm).

Data analysis

Data are given as means ± SEM. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

Results and discussion

Characterization of liposomes entrapping DOX or DOX-γ-CyD complex

It is well known that CyDs interact with various drugs and lipids. Among α-, β- and γ-CyDs, γ-CyD has the highest solubility in water, the highest interaction with DOX, the lowest interaction with phospholipids and CH and the lowest hemolytic activity [18, 19], i.e. the stability constant of DOX/γ-CyD complex in a buffer solution (pH 4) at 25 °C was determined to be 665 M^{-1}, which is almost the same value (670 M^{-1}) in a buffer solution (pH 7) as reported by Bekers [20], and the extent of phospholipids released from Caco-2 cells into culture medium after treatment with γ-CyD were significantly lower than those of α-CyD [21]. Therefore, we sought to use γ-CyD because of the lowest interaction with liposomal membrane and the highest interaction with DOX in liposomes.

To examine the extent of encapsulation of DOX and γ-CyD into liposomes, their entrapment ratios in liposomes were determined by a fluorescence spectroscopic method and the anthrone-sulfuric acid method [17], respectively. As shown in Table 1, the entrapment ratios of DOX in DOX-in-liposome and complex-in-liposome were 96.4% and 91.1%, respectively. Likewise, the entrapment ratio of γ-CyD in the complex-in-liposome was 90.7%. Hence, these results indicate that DOX as well as γ-CyD are efficiently encapsulated in liposomes, and γ-CyD changes the entrapment ratio of DOX in liposomes only very slightly. On the other hand, mean diameters of DOX-in-liposome and complex-in-liposome in the suspension were 137.2 nm and 135.3 nm, respectively. Therefore, there seems to be no significant difference between the diameters of these liposomes under the present experimental conditions.

In vitro release of DOX from liposomes

Stability of liposomes strikingly affects cellular uptake and *in vivo* pharmacokinetic behaviors. Next, we examined the effects of γ-CyD on the *in vitro* release of DOX from liposomes in FCS at 37 °C. Figure 1a and b show the initial burst release of DOX (a) from liposomes to FCS immediately after addition of liposomal suspensions to FCS and the time course of the following release of DOX (b), respectively. It is evident that the release of DOX from complex-in-liposome was slower than that from DOX-in-liposome in these experimental conditions. The inhibitory effects of γ-CyD on the release of DOX from liposomes to FCS may be due to the complexation of DOX with γ-CyD in liposomes and/or the stabilization of liposomal membrane by γ-CyD.

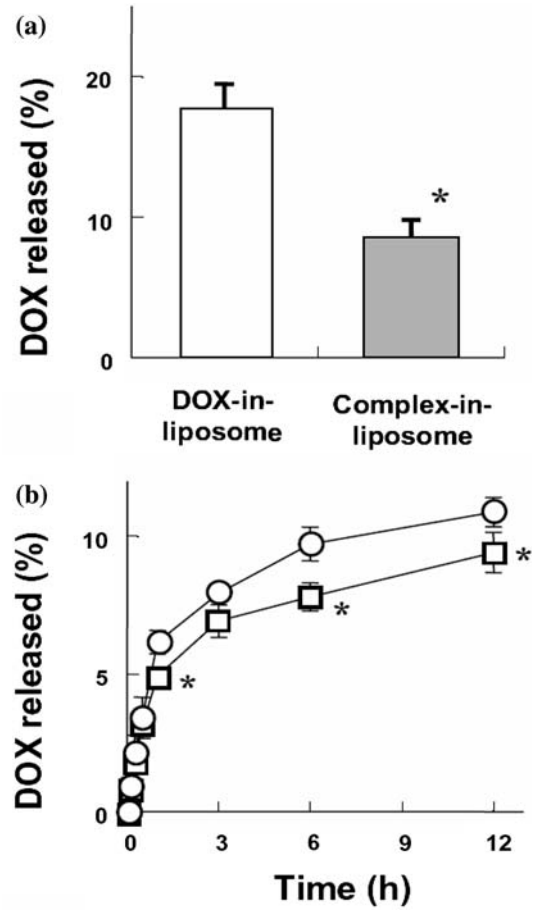

Figure 1. Initial release of DOX (a) and time course of the following release (b) of DOX from pegylated liposomes in FCS. The *in vitro* release of DOX from liposomes in 91% FCS was performed at 37 °C. At appropriate intervals, the reaction solution was ultracentrifuged, and then the amounts of DOX released from liposomes to FCS were determined using a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm). (a) Open column, DOX-in-liposome; hatched column, complex-in-liposome. Each value represents the mean ± SEM of 7–8 experiments. $*p < 0.05$ *versus* DOX-in-liposome. (b) Open circle, DOX-in-liposome; open square, complex-in-liposome. Each point represents the mean ± SEM of 7–8 experiments. $*p < 0.05$ *versus* DOX-in-liposome.

Table 1. Mean diameters of liposomes and entrapment ratios of DOX and g-CyD

	DOX-in-liposome	Complex-in-liposome
Mean diameter[a] (nm)	137.2 ± 1.2	135.3 ± 1.5
Entrapment ratio of DOX (%)	96.4 ± 0.5	91.1 ± 1.1
Entrapment ratio of γ-CyD (%)	–[b]	90.7 ± 1.0

[a]Liposome size was determined by dynamic light scattering at 25 °C.
[b]Not determined.
Each value represents the mean ± SEM of 3–4 experiments.

Cellular uptake and release of DOX

To examine the effects of γ-CyD on DOX retention in cells, the cellular uptake and the following cellular release of DOX were evaluated in Colon-26 cells. As shown in Figure 2a, there was insignificant difference in the cellular uptake behaviors of DOX between DOX-in-liposome and complex-in-liposome. However, the following cellular release of DOX in the

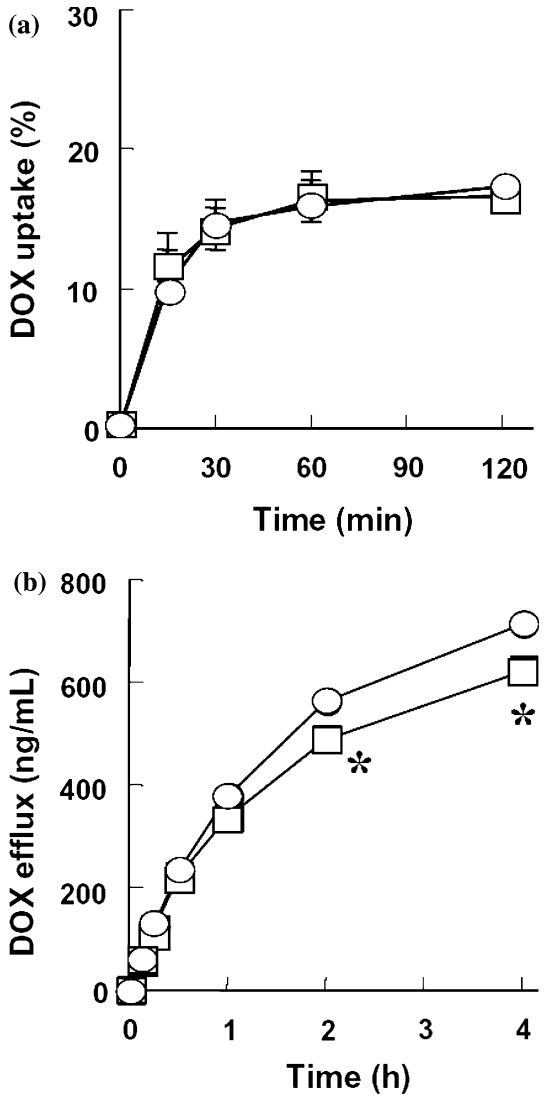

Figure 2. Cellular uptake (a) and release (b) of DOX. (a) Colon-26 cells (3×10^6 cells) were incubated with DOX-in-liposome or complex-in-liposome (8.6 μM DOX) at 37 °C. After washing three times with PBS (pH 2.5), the cells were lysed with 1 N NaOH. The amounts of DOX retained in the cells were determined using a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm). Open circle, DOX-in-liposome; open square, complex-in-liposome. Each point represents the mean ± SEM of 4 experiments. $*p < 0.05$ *versus* DOX-in-liposome. (b) After uptake of two liposomes into Colon-26 cells for 2 h, the cells were washed three times with PBS (pH 2.5), and then fresh medium supplemented with 10% FCS was added to the dish and incubated at 37 °C. At appropriate intervals, the release amounts of DOX from the cells in the medium were determined using a fluorescence spectrometer (Ex: 470 nm, Em: 590 nm). Open circle, DOX-in-liposome; open square, complex-in-liposome. Each point represents the mean ± SEM of 4 experiments. $*p < 0.05$ *versus* DOX-in-liposome.

complex-in-liposome system was significantly slower than that of DOX in the DOX-in-liposome system (Figure 2b). These results suggest that complex-in-liposome results in the prolonged retention of DOX in tumor cells. Recently, Xiong et al. reported that the *in vitro* intracellular uptake and *in vivo* therapeutic efficacy for the encapsulated DOX, namely, increased intracellular uptake of liposomal DOX results in improvement in antitumor activity [7]. Hence, the prolonged retention of DOX in the complex-in-liposome system might lead to high accumulation of DOX in tumor cells, followed by potent antitumor efficacy. However, the mechanism for the slower cellular release of DOX in the complex-in-liposome remains unknown. Some possibilities may be envisaged, e.g. the formation of a membrane-impermeable complex of DOX with γ-CyD in cells and the inhibitory effects of γ-CyD on drug efflux pumps in cells. Elaborate study is further required to clarify this mechanism.

In conclusion, we revealed here the potential use of pegylated liposomes entrapping DOX/γ-CyD complex for the high retention in cells, compared with the DOX-in-liposome. Hereafter, further studies regarding the effects of γ-CyD on anti-tumor activity of DOX in various tumor cells including multidrug-resistant cells *in vitro* should be necessary. Currently, we are investigating biopharmaceutical properties of DOX/γ-CyD complex-in-liposome using tumor-bearing mice.

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