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Enhanced delivery of doxorubicin to tumor by long-circulating thermosensitive liposomes and local hyperthermia

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Doxorubicin (DXR) was encapsulated in long-circulating, thermosensitive liposomes (180–200 nm), prepared from dipalmitoylphosphatidylcholine (DPPC)/distearoylphosphatidylcholine (DSPC) (9:1 (m/m)) and 6 mol% of ganglioside G_{M1} (G_{M1}), with 95–98% entrapping efficiency by the pH-gradient method. 45% of the entrapped DXR was released from these G_{M1}/DPPC/DSPC liposomes by incubation at 42°C for 5 min in 20% serum or saline (this degree of release was lower than that of hydrophilic drugs such as cisplatin, due to the basic and amphiphilic nature of DXR). Inclusion of G_{M1} (6 mol%) endowed DPPC/DSPC liposomes with prolonged circulation ability, resulting in increased blood levels of liposomes and decreased reticuloendothelial system uptake over 6 h after injection. Concomitantly, DXR levels in blood remained high for long time. Accumulation of DXR into tumor tissue of tumor-bearing mice (mouse colon carcinoma 26) by local hyperthermia after injection of DXR loaded, long-circulating, thermosensitive (DXR-G_{M1}/DPPC/DSPC) liposomes was 2.5-times or 6-times higher than that after treatment with DXR-DPPC/DSPC liposomes or free DXR in combination with hyperthermia, respectively. Furthermore, the treatment with DXR-G_{M1}/DPPC/DSPC liposomes and hyperthermia resulted in effective tumor-growth retardation and increased survival time. Our results indicate that the combination of drug-loaded, long-circulating, thermosensitive liposomes with local hyperthermia at the tumor site could be clinically useful for delivering a wide range of chemotherapeutic agents in the treatment of solid tumors.

Introduction

Liposomes have demonstrated considerable promise as a carrier for the delivery of drugs in vivo. Enhancement of therapeutic efficacy and reduction of toxicity of a variety of drugs have been demonstrated with liposome-encapsulated dosage forms [1,2]. However, one of the drawbacks is that most liposomes intravenously injected into animals are rapidly removed from the blood circulation by uptake primarily in the liver, but also in the spleen, and very slightly in the lung and bone marrow [3]. Recently, it has been demonstrated that liposomes containing ganglioside G_{M1} exhibit a prolonged circulation time in the blood, because their uptake affinity by the reticuloendothelial system (RES) cells in liver and spleen is significantly lower than that of the ordinary liposomes [4,5]. More importantly, this type of liposome (approx. 100 nm in mean size) showed a higher level of uptake by solid tumor than did ordinary liposomes [5]. Furthermore, our recent study demonstrated that administration of this type of liposome (approx. 200 nm in mean size) encapsulating doxorubicin at a dose of 5 mg/kg to L1210 tumor-bearing mice significantly increased the mean survival times as compared with free drug and the drug encapsulated in ordinary liposomes [6,7]. Thus, liposomes with longer circulation half-lives stand a better chance of penetrating the leaky vasculature of the solid tumor, and they release their contents slowly over a long period in the blood circulation.

It is known that the lamellar structure of liposome membrane at the gel-to-liquid-crystalline transition phase is somewhat loose and porous. Therefore, it is possible to design liposomes which can release entrapped drugs preferentially at their liquid-crystalline phase-transition temperature [8,9]. For example, large unilamellar vesicles (LUV) composed of DPPC/DSPC (9:1 molar ratio) showed a very sharp increase of

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release rate between 40°C and 41°C [10]. Unlike LUV, however, small unilamellar vesicles (SUV) showed only a small release rate increase. In model experiments with mouse leukemia L1210 or sarcoma 180, it was shown that a drug encapsulated in such thermosensitive liposomes drug was accumulated 4-fold more efficiently in the tumor heated at 42°C than in the unheated contralateral control tumor in the same animals [8,9,11,12].

It is well-known, however, that the residence time of such liposomes in the blood circulation is very short, due to their rapid removal by liver and spleen, and this is obviously disadvantageous for local drug release by hyperthermia. If the thermosensitive liposomes can be endowed with a prolonged circulation life time, it may be possible to achieve much higher drug levels in tumors subjected to hyperthermia than could be obtained with ordinary thermosensitive liposome. However, little information is available on liposomes which have both thermosensitivity and prolonged circulating ability.

The purpose of the present study was to establish the optimum conditions for preparing long-circulating, thermosensitive liposomes from the viewpoint of effective drug release in response to hyperthermia, and to estimate the drug biodistribution and chemotherapeutic activity in colon 26 tumor-bearing mice treated with an anticancer drug encapsulated in such long-circulating, thermosensitive liposomes in combination with local hyperthermia. For the present study, doxorubicin (DXR) was selected to be encapsulated for the following reasons. (1) DXR, an anthracycline aminoglycoside, is a potent anticancer chemotherapy agent with a broad spectrum of antitumor activity. (2) Encapsulation of DXR into liposomes markedly reduces the cardiotoxicity of the drug [13–15]. (3) DXR exhibits strikingly enhanced cytotoxicity against tumor cells when combined with 42–43°C hyperthermia [16]. (4) DXR can be efficiently entrapped in liposomes by the pH gradient method [17]. Thus, DXR is a good candidate for encapsulation in long-circulating, thermosensitive liposomes, and such a formulation should be experimentally and clinically useful.

Materials and Methods

Materials. Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) (COATSOME MC-6060 and MC-8080, respectively) were kindly donated by Nippon Oil & Fats. Ganglioside G_{M1} (G_{M1}) and inulin were purchased from Funakoshi and Wako, respectively. [^{3}H]Cholesteryl hexadecyl ether, ^{125}I and ^{51}Cr were from New England Nuclear. ^{125}I-labeled tyraminyl inulin ([^{125}I]TI) was used as a tracer for liposomal biodistribution in vivo. TI was synthesized according to Sommerman et al. [18], and radiolabeled with ^{125}I by using the Iodo-Gen procedure (Pierce). Doxorubicin (DXR) was a kind gift from Kyowa Hakko Kogyo.

Liposome preparation. Liposomes were prepared with DPPC/DSPC (9:1 molar ratio) and an appropriate amount of G_{M1} by the reverse-phase evaporation method [19]. The lipid mixture was dissolved in isopropyl ether/chloroform (1:1 (v/v)). This lipid solution was mixed with 300 mM citric acid (pH 4.0). When liposomes were prepared for biodistribution experiments or estimation of integrity in vivo, an appropriate amount of [^{125}I]TI or [^{3}H]cholesteryl hexadecyl ether was added at this step, respectively. The mixture was sonicated to give a w/o emulsion with a bath-type sonicator (Iuchi VS-150), and the organic solvent in the emulsion was evaporated gradually at 60°C to form an LUV suspension. Liposomes were extruded through Nuclepore filters (0.4 and 0.2 μm). Liposome size was measured by a Nicomp 370 submicron particle analyzer (HIAC Pacific Scientific).

Doxorubicin encapsulation. The encapsulation of DXR into G_{M1}/DPPC/DSPC liposomes was done by employing the pH gradient method developed by Mayer et al. [17,20]. Briefly, the pH of the liposome suspension, initially at pH 4.0, was raised to pH 7.8 with 1 M NaOH. The liposome preparation was subsequently heated to 60°C for 5 min. These liposomes were then mixed with a preheated (60°C) DXR solution dissolved in distilled water at a drug-to-lipid weight ratio of 0.2. This mixture was incubated with periodic mixing for 10–15 min at 60°C. The resulting preparation was finally passed through a Sephadex G-50 column.

The amount of liposomally entrapped DXR was determined with a fluorescence spectrometer (Hitachi F-3000) as follows: The liposomes were diluted with 0.3 M HCl-50% ethanol and then the fluorescence intensity was measured (excitation at 470 nm and emission at 590 nm).

In-vitro temperature-dependent drug release. Temperature-dependent release profiles of DXR from thermosensitive liposomes were estimated by incubation of the liposomes at various temperatures as follows. The liposomes were diluted with saline, 50% (v/v) fetal bovine serum or 1/15 M phosphate-buffered saline (PBS (pH 4.0)). 1 ml of each sample was placed in a plastic tube and incubated for 5 min in a water bath (Haake F2) maintained at constant temperature. The released DXR was separated from the liposomal suspension by ultrafiltration and assayed by fluorescence spectrometry as described under doxorubicin encapsulation. A Centrisart (molecular weight cut-off 20000, Sartorius) was used for this procedure.

Biodistribution and stability of DXR-liposomes in vivo. DXR-Liposomes labeled with [^{125}I]TI or [^{3}H] cholesteryl hexadecyl ether were injected via the tail vein into male Balb/c mice weighing 22–25 g at a dose

of 5 mg DXR/kg. At different times after injection, mice were anesthetized, bled via the retro-orbital sinus and killed by cervical dislocation. Major organs were collected and counted for ^{125}I using a gamma counter. For scintillation counting, a 0.1-g portion of tissue or whole tissue when it weighed less than 0.1 g was added to 2 ml of Soluen-350 (Packard) and stood at 55°C for 24 h followed by 100 μl of hydrogen peroxide. Subsequently, 10 ml Hionic-Fluor (Packard) scintillation cocktail was added. Samples were left for at least 24 h prior to counting in a Aloca LSC-3000 scintillation counter. Data are expressed as % injected dose in each organ or blood.

Correction factors for the blood content in various organs were determined by examining the distribution of ^{51}Cr-labeled erythrocytes 30 min after i.v. injection [21]. Briefly, red blood cells were isolated from male Balb/c mice of the same age and weight, washed with sterile isotonic saline solution, and incubated for 1 h at 37°C with $Na_2^{51}CrO_4$ (2×10^7 cells in 1 ml, 100 μCi ^{51}Cr). Free ^{51}Cr was removed from the cells by centrifugation. The labeled red blood cells were then injected (i.v.) into tumor-bearing mice for determination of the blood content of various organs. The correction factors for various organs and tumor were relatively small (less than 1%), with liver being the one with greatest correction, which was only approx. 6%. The weight of total blood was assumed to be 7.3% of the body weight [22].

For measurement of DXR concentration, plasma (100 μl) was homogenized and extracted with butanol/toluene (1:1 (v/v)), then the extracts were subjected to HPLC assay according to the method of Masuike et al. [23]. The HPLC system consisted of a Hitachi 655A-12 high pressure pump, a Hitachi F1000 fluorescence detector (excitation 470 nm, emission 585 nm) and a Nucleosil$_{10}$ C_{18} column (GL Science). The mobile phase was 1 M formic acid/methanol (1:1 (v/v)) and a flow rate of 1.5 ml/min was used.

In-vivo studies with hyperthermia. Tumor-bearing mice were prepared by inoculating mouse colon carcinoma 26 cells ($1 \cdot 10^5$ cells) into the hind foot of Balb/c mice (male, 8-weeks-old, and weighing 22–25 g), and the tumor was allowed to grow for approx. 8 days, when the mean of its length and width was 8 mm. The mice were anesthetized with Nembutal (Dainabot). The free DXR or liposomal DXR was injected intravenously via the tail vein at a dose of 5 mg DXR/kg. Local hyperthermia using a radiofrequency oscillator (RF-hyperthermia HEH-100, Omron, Kyoto) with 42°C heating temperature was started at 5 min after drug administration and was continued for 20 min. The hyperthermia apparatus used in this study was modified to accommodate small animals. At 5 min after completion of the hyperthermia treatment, blood was collected from the retro orbital sinus and major organs were excised. Organs were stored at −20°C until assay. A 0.1 g portion of tissue or whole tissue when it weighed less than 0.1 g was used for measurement of DXR concentration. Samples were homogenized and extracted with butanol/toluene (1:1 (v/v)), then the extracts were subjected to HPLC assay as described above.

Evaluation of antitumor activity. Tumor-bearing mice were assigned at random into groups of 10. Treatment was started when the tumor had reached a diameter of 8 mm (= day 0), at approx. 8 days after tumor cell inoculation. The dose per injection was 5 mg DXR/kg body weight. Injections were performed i.v. via the tail vein. Local hyperthermia with 42°C for 20 min was done at 5 min after administration of free DXR or liposomal DXR. Tumor volumes were determined as

$$\text{Volume} = L \cdot W^2/2 \quad (1)$$

where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface [24]. Survival time was recorded in days after treatment of local hyperthermia.

Results

Encapsulation of DXR into G_{M1}/DPPC/DSPC liposomes by the pH-gradient method

The encapsulation of DXR into G_{M1}/DPPC/DSPC liposomes was done under conditions based on the information of Mayer et al. [17,20], i.e., ΔpH 3.8, a DXR/lipid weight ratio of 0.2, and a standing time of 10 min at 60°C at the loading step. DPPC and DSPC (9:1 molar ratio) were used to prepare thermosensitive liposomes and the mean diameters of all liposome preparations were controlled to within the range of 180–200 nm. Under these fixed conditions, DXR was entrapped into liposomes with 95–98% efficiency (data not shown), even in the presence of 3–9 mol% of G_{M1} in the lipid components. Furthermore, this process was completed within 5 min. The mean drug content of liposomes obtained by the above procedure was 3.92 mg DXR/20 mg lipid for G_{M1}/DPPC/DSPC liposomes. Since the ordinary method described in our previous report [25] give only 20% trapping efficiency (data not shown), the pH-gradient method is much more effective for the encapsulation of DXR into G_{M1}/DPPC/DSPC liposomes.

Effect of G_{M1} on temperature-dependent drug release from DPPC/DSPC liposomes

Temperature-dependent release of DXR from G_{M1}/DPPC/DSPC liposomes containing various amounts of G_{M1} was examined in saline, 50% (v/v) fetal bovine serum or 1/15 M PBS (pH 4.0). As shown in Fig. 1, 40–45% of entrapped DXR was released from liposomes containing 0–6 mol% of G_{M1} by incu-

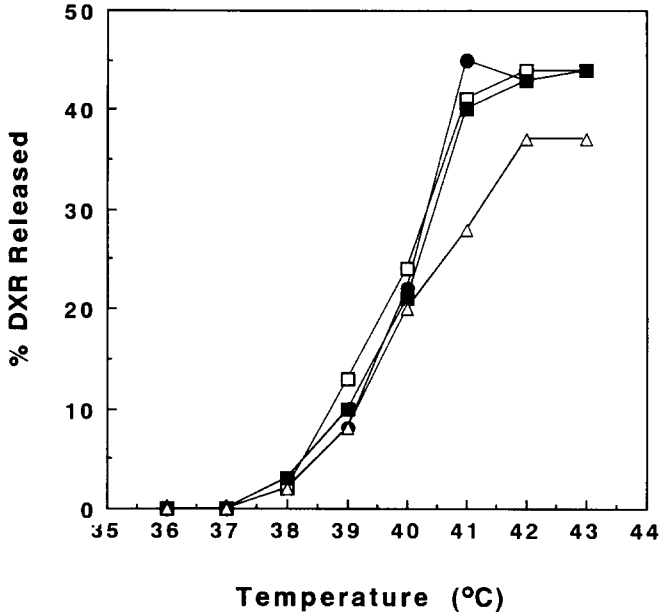

Fig. 1. Effect of G_{M1} inclusion on temperature-dependent release of DXR from liposomes. DXR-loaded liposomes were prepared from DPPC/DSPC (9:1, molar ratio) and G_{M1} in various molar ratios, and release of DXR from the liposomes incubated with saline was measured upon exposure to heating at various temperatures between 36°C and 43°C for 5 min, as described in Materials and Methods. The symbols ●, □, ■ and △ indicate 0, 3, 6 and 9 mol% of G_{M1} in DPPC/DSPC liposomes, respectively. Mean values of three to five independent experiments are plotted.

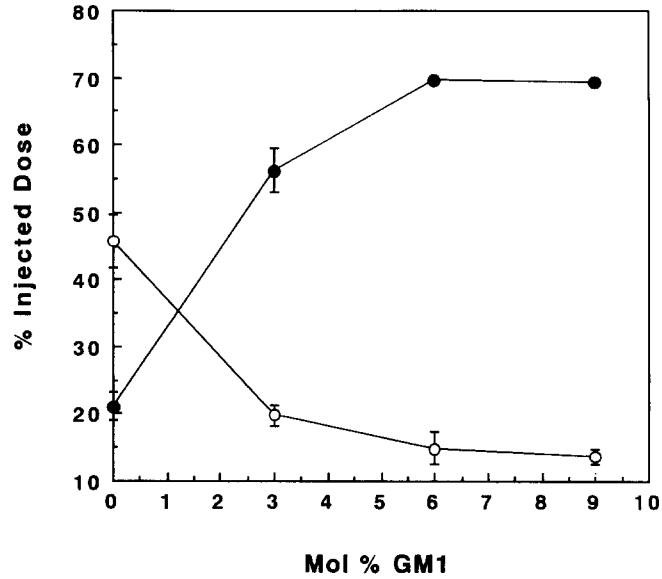

Fig. 2. Effect of increasing concentration of G_{M1} on blood residence and RES (liver and spleen) uptake of liposomes composed of DPPC/DSPC (9:1, molar ratio) at 30 min after injection into mice via the tail vein. Liposomes were labeled with [^{125}I]TI. The symbols ● and ○ indicate blood and RES, respectively. The average diameter of liposomes was in the range of 180–200 nm. Bars represent S.D. (n = 3–5).

bation at 42°C for 5 min with saline, but only 30% was released from those containing 9 mol% of G_{M1}. It was observed that the DXR release in serum was almost the same as that in saline. These results indicate that DXR was released from G_{M1}/DPPC/DSPC liposomes in a temperature-dependent manner, and inclusion of less than 6 mol% of G_{M1} did not interfere with this release. However, the degree of DXR release was relatively low, compared with that of cisplatin [10]. This relatively low efflux of DXR may be due to the basic and amphiphilic nature of the drug. We also measured the drug release under an acidic condition. 80% of entrapped DXR was released from G_{M1}/DPPC/DSPC

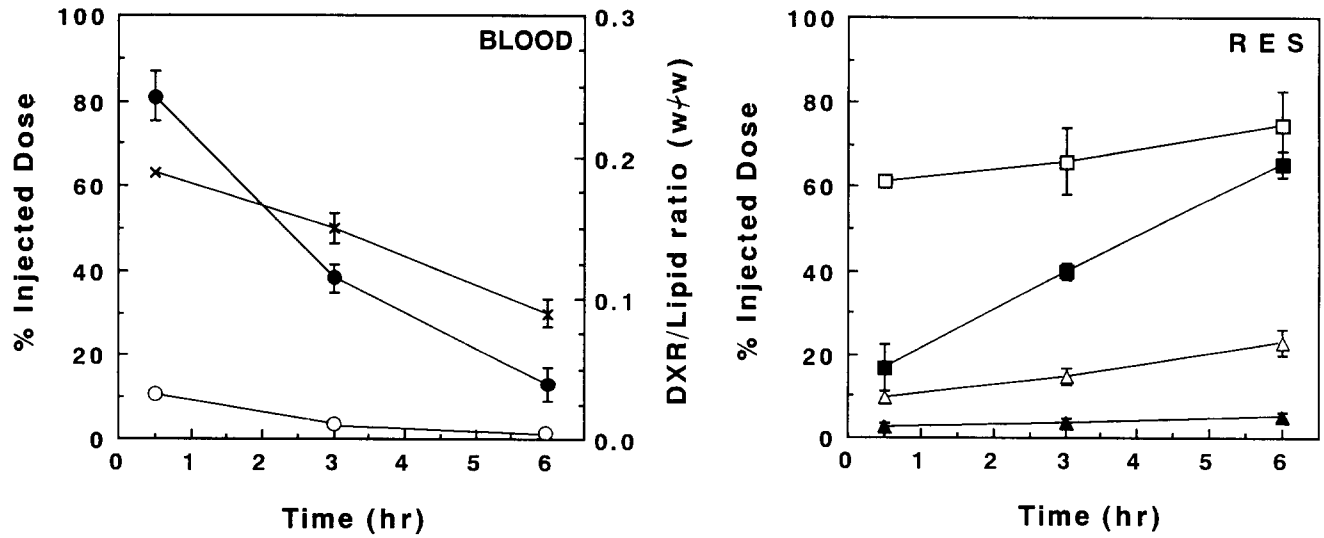

Fig. 3. Time-course of blood residence and RES (liver and spleen) uptake of DXR-DPPC/DSPC (9:1, molar ratio) liposomes or DXR-G_{M1}/DPPC/DSPC (0.13:9:1, molar ratio) liposomes. Circles, squares and triangles indicate blood, liver and spleen, respectively. Open symbols are DXR-DPPC/DSPC liposomes, and closed symbols are DXR-G_{M1}/DPPC/DSPC liposomes. The symbol × indicates the DXR/lipid ratio of DXR-G_{M1}/DPPC/DSPC liposomes. Liposomes were labeled with [^{3}H]cholesteryl hexadecyl ether. The average diameter of liposomes was in the range of 190–200 nm. Bars represent S.D. (n = 3–5).

containing 6 mol% of G_{M1} by incubation with 1/15 M PBS (pH 4.0) at 41°C for 5 min (data not shown). Since DXR is in ionic form at pH 4.0, the hydrophobic interaction between DXR and acyl residues of DPPC and DSPC would be less effective. On the other hand, since DXR is in non-ionic form in neutral or basic conditions, DXR can then interact with the acyl residues when it penetrates through the lipid layers. These liposomes were less leaky at temperatures below 37°C. These results suggest that the gel-to-liquid-crystalline phase transition of DXR-loaded G_{M1}/DPPC/DSPC liposomes occurred at approx. 40°C.

Effect of G_{M1} concentration on the blood residence time of DXR-G_{M1}/DPPC/DSPC liposomes

The effects of increasing concentration of G_{M1} on the blood residence time of DXR-loaded thermosensitive liposomes composed of DPPC/DSPC (9:1 molar ratio, labeled with [^{125}I]TI) and with a mean size of 180–200 nm were examined in mice via tail vein injection. The percent of the injected dose in blood and RES (liver + spleen) as a function of G_{M1} concentration at 30 min post-injection is shown in Fig. 2. It is clear that the blood levels and RES uptake of liposomes are dependent on the included amount of G_{M1}. The blood level was increased by the addition of G_{M1} up to 6 mol%, but reached a plateau thereafter. The RES uptake was correspondingly decreased by increasing G_{M1} concentration. These results suggested that thermosensitive liposomes required at least 6 mol% of G_{M1} to prolong the circulation time and avoid capture in the RES. The results shown in Fig. 1 and Fig. 2 demonstrate that DPPC/DSPC (9:1 molar ratio) liposomes containing 6 mol% of G_{M1} possess both thermosensitivity and a long circulation time.

Time-course of tissue distribution of DXR-G_{M1}/DPPC/DSPC liposomes

G_{M1}-thermosensitive liposomes (G_{M1}/DPPC/DSPC 0.13:9:1, average diameter of approx. 200 nm) were labeled with [^{3}H]cholesteryl hexadecyl ether and injected i.v. into mice via the tail vein. The biodistribution was measured up to 6 h after injection. As can be seen in Fig. 3, the amount of ordinary thermosensitive liposomes without G_{M1} rapidly decreased in blood and concomitantly accumulated in the RES, suggesting that liposomes of this lipid composition and this size range are readily taken up by the RES. On the other hand, the incorporation of 6 mol% of G_{M1} significantly increased the blood level of thermosensitive liposomes and decreased RES uptake within 6 h after injection. These effects, however, were relatively weaker than in the case of egg PC/CH or DSPC/CH liposomes incorporating 6 mol% of G_{M1}, as described by us [26]. It appears that the combination of G_{M1} and rigid lipids is important for prolonged circulation ability of liposomes. However, fluid lipids are necessary for the thermosensitivity.

The stability of DXR-G_{M1}/DPPC/DSPC liposomes in blood was estimated as a DXR-to-lipid ratio (Fig. 3).

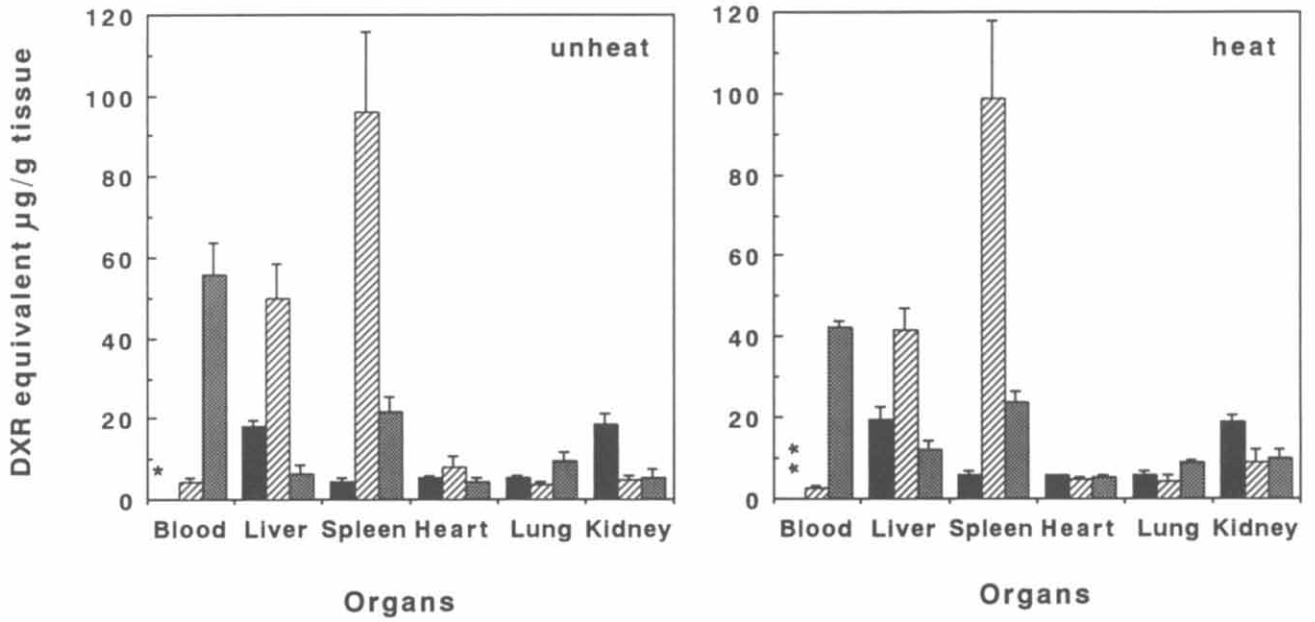

Fig. 4. Tissue DXR levels after the administration of free or thermosensitive liposome-encapsulated DXR to colon carcinoma 26-bearing mice with or without local hyperthermia. The symbols ■, ▨ and ▩ indicate free DXR, DXR-DPPC/DSPC and DXR-G_{M1}/DPPC/DSPC liposomes, respetively. Free DXR or liposomal DXR was injected intravenously via the tail vein at a dose of 5 mg/kg. Local hyperthermia with 42°C heating temperature was applied at the tumor site 5 min after drug administration and was continued for 20 min. DXR concentrations in major organs were assayed at 5 min after termination of hyperthermia, as described in Materials and Methods. * and ** are 0.069 and 0.071 μg/g, respectively.

The drug-to-lipid ratio (w/w) of DXR-G_{M1}/DPPC/DSPC liposomes prior to injection was 0.19. The plasma samples obtained 0.5 h after injection indicated a drug-to-lipid of 0.19 and this dropped to a ratio of 0.09 at 6 h. Thus, this formulation was leaky in blood.

DXR distribution in mice

The tissue-associated DXR level was measured after injection of DXR-loaded thermosensitive liposomes, with or without local hyperthermia. Free DXR and encapsulated DXR were administered to tumor-bearing mice via the tail vein at the dose of 5.0 mg/kg. Hyperthermia was applied at the tumor site as described in Materials and Methods. Untreated, mice were killed at 30 min after injection. DXR levels in tissue were determined by HPLC, and the data are summarized in Fig. 4. There were no marked differences in tissue levels of DXR between groups with and without hyperthermia, except in the kidney. For free DXR, the blood level was very low (0.07 μg/g) and the kidney level was 19.3 μg/g, suggesting that the free drug was cleared quickly from the kidney. For DXR-DPPC/DSPC liposome formulations, the blood level was also low (4.5 μg/g without hyperthermia) and high levels of DXR were observed in liver (52.5 μg/g) and spleen (98.5 μg/g), presumably as a result of accumulation of DPPC/DSPC liposomes therein (Fig. 3). DXR level in blood after administration of DXR-G_{M1}/DPPC/DSPC liposomes was significantly increased as compared with the above two formulations, while the distribution of DXR to liver and spleen was decreased. Thus, the incorporation of G_{M1} into liposomes resulted in increased blood levels of DXR and decreased drug levels in liver and spleen. These distribution characteristics of DXR directly paralleled those of the liposomes themselves. DXR levels in kidney were increased after hyperthermia for 20 min, suggesting that leaked DXR was accumulated in the kidney, as well as free DXR. A decreased blood level of DXR was observed after heating. These results indicate that DXR was released from G_{M1}/DPPC/DSPC liposomes in vivo in a thermosensitive manner.

Accumulation of DXR in the heart was almost the same after free drug and liposomal formulation treatments, with or without hyperthermia. In lung, DXR showed a tendency to accumulate after G_{M1}-liposome formulation treatment.

Accumulation of DXR in tumor tissue by local hyperthermia and antitumor activity

Tumor associated DXR levels after injection of DXR-thermosensitive liposomes with or without local heating were determined. As shown in Fig. 5, the combination of DXR-G_{M1}/DPPC/DSPC liposomes and hyperthermia, gave a value of 9.5 μg/g tumor, which was 2.5-times higher than that after DXR-DPPC/DSPC liposomes and hyperthermia and 6-times higher than that after free drug and hyperthermia. These levels with the liposomal systems were, of course, much higher than those obtained without hyperther-

TABLE I

Effects of thermosensitive liposome-encapsulated DXR with or without hyperthermia (42°C, 20 min) on tumor growth and survival of mice bearing colon carcinoma 26 tumor

Tumor-bearing Balb/c mice received 5 mg of DXR/kg i.v. in either free or liposome-entrapped form. Measurements were made of tumor volume (see Materials and Methods) and tumor growth ratio, which was obtained from the following equation:

$$\text{Tumor growth ratio} = \frac{\text{mean tumor volume at 7 or 14 days after treatment}}{\text{mean tumor volume at day of treatment (day 0)}}$$

Data are the mean (S.D.) of 10 mice per group.

Treatments	Tumor volume		Tumor growth ratio		Survival time (days)		*T/C* (%)
	Day 7	Day 14	Day 7	Day 14	Range	Mean	
Control	3.0±0.7	6.9±1.5	12.3±2.8	27.7±6.0	21–35	29.4±4.2	100
Free DXR	1.6±3.9	3.9±0.4	6.7±1.6	15.6±1.6	21–35	29.4±5.2	100
DXR-DPPC/DSPC	1.4±0.3	4.0±1.0	5.6±1.2	16.3±4.0	21–28	25.2±3.4	88
DXR-G_{M1}/DPPC/DSPC	1.4±0.2	4.0±0.7	5.6±1.0	16.2±2.8	21–35	26.6±4.2	90
Heat	1.7±0.4	4.8±0.7	6.9±1.5	19.1±2.7	21–35	30.1±4.5	102
Free DXR + heat	1.6±0.4	3.0±1.0	6.7±1.6	12.3±4.0	21–42	31.5±5.6	107
DXR-DPPC/DSPC + heat	0.9±0.2	1.3±0.8	3.7±0.8	5.3±3.2	28–60	39.8±8.4	135
DXR-G_{M1}/DPPC/DSPC + heat	0.5±0.1	0.9±0.1	2.3±0.4	3.6±0.7	42– > 60	46.4 [a]	162

[a] One mouse survived over 60 days.

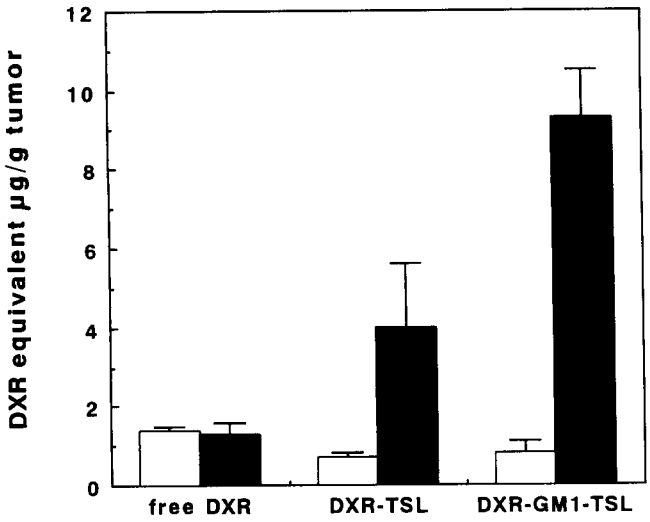

Fig. 5. Tumor DXR levels after the administration of free DXR or thermosensitive liposome-entrapped DXR to colon carcinoma 26-bearing mice with or without local hyperthermia at the tumor site. The symbols ■ and □ indicate with hyperthermia and without hyperthermia, respectively. For details, see Materials and Methods and the legend to Fig. 4.

mia. Since G_{M1} prolongs the circulation life time, and thus increases the amount of liposomes which pass the heating site, the amount of DXR released at the heating site is expected to be higher. Thus, it was suggested that the entrapped DXR was efficiently released from G_{M1}/DPPC/DSPC liposomes by hyperthermia at the tumor site and entered the tumor tissue by simple diffusion.

The antitumor activity of free DXR or thermosensitive liposomal DXR with or without hyperthermia is summarized in Table I. Hyperthermia alone (without administration of drugs) caused tumor growth retardation, compared with the control. On the other hand, the groups without hyperthermia (free DXR and the two DXR liposomal formulations) all showed no tumor growth retardation and no change in survival time. The system of free drug and hyperthermia was ineffective, giving the same result as heating alone. The combinations of systemic liposomal DXR and local hyperthermia effectively retarded tumor growth and increased survival time. As shown in Table I, DXR-G_{M1}/DPPC/DSPC and hyperthermia showed more effective tumor growth retardation and concomitantly increased survival time than did DXR-DPPC/DSPC and hyperthermia, and induced tumor growth regression in four mice at 14 days.

Discussion

Although thermosensitive liposomes could be useful for treatment of selected tumors in combination with local hyperthermia, they have had drawbacks such as low stability and short circulation half-life in the blood. However, in previous reports [4,26], we and others have presented evidence indicating that incorporation of G_{M1} into liposomes results in prolonged circulation times. The purpose of this study was to examine the usefulness of G_{M1}-containing thermosensitive liposomes in comparison with bare thermosensitive liposomes, which are cleared relatively quickly by the RES. Our results clearly indicate that the combination of DXR-containing, long-circulating, thermosensitive G_{M1}/DPPC/DSPC liposomes and local hyperthermia results in a higher drug distribution to the tumor (Fig. 5) and more effective retardation of tumor growth than the non-G_{M1}-containing thermosensitive DPPC/DSPC liposomal system (Table I).

Heat-induced drug release from thermosensitive liposomes is thought to be primarily determined by the lipid composition and the liposomal type. The drug release from such liposomes was previously examined by measuring the release of cisplatin at a temperature near the phase transition [10]. The results demonstrated that LUVs composed of DPPC/DSPC (9:1 molar ratio and 180–200 nm in mean diameter) showed a very sharp release of their content between 40°C and 41°C. In this experiment, we used similar conditions to prepare DXR-loaded, long-circulating, thermosensitive liposomes. But, as shown in Fig. 1, the release rates of DXR from DPPC/DSPC and G_{M1}/DPPC/DSPC liposomes were only about 50% at 42°C. Possibly DXR, which is an amphipathic and basic compound, interacts hydrophobically with acyl residues in the lipid bilayer. As described by Magin and Weinstein [12], it has been considered that drugs to be encapsulated in thermosensitive liposomes should generally be hydrophilic, with a low affinity for the lipid bilayer. However, it was reported that liposomes composed of DPPC/CH (8:2 molar ratio) exhibited the thermosensitive release of DXR with a release rate of 90% at 42°C [27].

Although there was no difference in the release rate of DXR at 42°C between DPPC/DSPC and G_{M1}/DPPC/DSPC (6 mol% of G_{M1}) liposomes (Fig. 1), the drug concentration in the tumor provided by G_{M1}/DPPC/DSPC liposomes was 2.5 times higher than that by DPPC/DSPC liposomes. This result suggests that a much larger amount of these long-circulating, thermosensitive liposomes passed the heating site during hyperthermia for 20 min compared with the thermosensitive liposomes without G_{M1}. Thus, long-circulating ability is effective for thermosensitive liposomal drug delivery in combination with hyperthermia. This system is expected to be very useful for antitumor therapy. Nevertheless, we still need to obtain the faster possible release of DXR from liposomes at the heating site in view of the rapid blood circulation time (about 12 s in mouse [28]). Development of long-circulating, thermosensitive liposomes which have a high DXR release rate is in progress.

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References

1 Gregoriadis, G. (1988) Liposomes as Drug Carriers: Recent Trends and Progress, John Wiley and Sons, New York.
2 Ostro, M.J. (1987) Liposomes from Biophysics to Therapeutics, Marcel Dekker, New York.
3 Poste, G. (1983) Biol. Cell 47, 19–37.
4 Allen, T.M. (1989) in Liposomes in the Therapy of Infections Diseases and Cancer (Lopez-Bernstein, G. and Fidler, I.J., eds.), pp. 405–415, Alan R. Liss, New York.
5 Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
6 Maruyama, K., Okamoto, A., Kojima, S., Suginaka, A. and Iwatsuru, M. (1992) Drug Deliv. Syst. 7, 345–350.
7 Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A. and Iwatsuru, M. (1992) Proceedings of the Conference 2nd Liposome Research Days, Leiden University, The Netherlands, June 17–19, 1992. (Abstract)
8 Yatvin, M.B., Weinstein, J.N., Dennis, W.H. and Blumenhal, R. (1978) Science 202, 1290–1293.
9 Weinstein, J.N., Magin, R.L. Cysyl, R.L. and Zaharko, D.S. (1980) Cancer Res. 40, 1388–1395.
10 Iga, K., Hamaguchi, H., Igari, Y., Ogawa, Y., Toguchi, H. and Shimamoto, T. (1989) Int. J. Pharm. 57, 241–251.
11 Yatvin, M.B., Muhlensiepen, H., Porschen, W., Weinstein, J.N. and Feinendegen, L.E. (1981) Cancer Res. 41, 1602–1607.
12 Magin, R.L. and Weinstein, J.N. (1983) in Liposome Technology, Vol. III (Gregoriadis, G., ed), pp. 137–155, CRC Press, Boca Raton.
13 Olson, F., Mayhew, E., Maslow, D., Rustum, Y. and Szoka, F. (1982) Eur. J. Cancer 18, 167–176.
14 Herman, E.H., Rahman, A., Ferrans, V.J., Vick, J.A. and Schein, P.S. (1983) Cancer Res. 43, 5427–5432.
15 Van Hoesel, Q.G.C.M., Steerenberg, P.A., Crommelin, J.A., Van Dijk, A., Van Oost, W., Klein, S., Douze, J.M.C., De Wildt, D.J. and Hillen, F.C. (1984) Cancer Res. 44, 3698–3705.
16 Hahn, G.M., Braun, J. and Har-Kedar, I. (1975) Proc. Natl. Acad. Sci. USA 72, 937–940.
17 Mayer, L.D., Bally, M.B., Hope, M.J. and Cullis. P.R. (1985) Biochim. Biophys. Acta 816, 294–302.
18 Sommerman, E.F., Pritchard, P.H. and Cullis, P.R. (1984) Biochem. Biophys. Res. Commun. 122, 319–324.
19 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
20 Mayer, L.D., Tai, L.C.L., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R. and Bally, M.B. (1989) Cancer Res. 49, 5922–5930.
21 Liu, D., Mori, A. and Huang, L. (1991) Biochim. Biophys. Acta 1066, 159–165.
22 Wu. M.S., Robbins, J.C., Bugianesi, R.L., Ponpipom, M.M. and Shen, T.Y. (1981) Biochim. Biophys. Acta 674, 255–258.
23 Masuike, J., Odake, J., Kohagura, M., Noda, T. and Takemoto, Y. (1984) Yakugaku Zasshi 104, 620–623. (Japanese)
24 Corbett, T.H., Griswold, D.P., Roberts, B.J., Peckham, J.C. and Schabel, F.M. (1978) Cancer Treat. Rep. 60, 1471–1488.
25 Unezaki, S., Matsumura, M., Ohtani, Y., Takahashi, N., Maruyama, K., Okamoto, A. and Iwatsuru, M. (1992) Drug Deliv. Syst. 7, 37–41.
26 Maruyama, K., Yuda, T., Okamoto, A., Ishikura, C., Kojima, S., and Iwatsuru, M. (1991) Chem. Pharm. Bull. 39, 1620–1622.
27 Tomita, T., Watanabe, M., Takahashi, T., Kumai, K., Tadakuma, T. and Yasuda, T. (1989) Biochim. Biophys. Acta 978, 185–190.
28 Kaplan, H.M., Brewer, N.R. and Blair, W.H. (1983) in The Mouse in Biomedical Research, Vol. 3 (Foster, H.L., Small, J.D. and Fox, J.G., eds.), pp. 248–292, Academic Press, New York.