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Stability and antitumor effects of all-*trans* retinoic acid-loaded liposomes contained sterylglucoside mixture

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Abstract

The aim of this study was to develop an intravenous delivery of all-*trans* retinoic acid (ATRA) for the treatment of cancer. Two kinds of liposomes composed of dipalmitoylphosphatidylcholine and cholesterol with sterylglucoside (SG) mixture (SG liposomes) or without SG (non-SG liposomes) were prepared. A limited amount of ATRA was incorporated into liposomes approximately 3 mol%. ATRA-loaded SG liposomes were more stable at 4 °C with light protection for 24 days than non-SG liposomes; maintaining 83.1% ATRA and the average diameter 198.5 nm (36 days), and in 80% rat serum for 120 min. SG seems to increase the ATRA-loaded efficiency in liposomes and stability of liposomes compared with cholesterol. The mean survival time of mice given SG liposomes (18.2 days) indicated a greater antitumor activity than saline (14.1 days) ($P < 0.001$) without change of mean body weight. It is concluded that the current ATRA-loaded SG liposomes are potentially applicable for hepatic metastasis of M5076 tumor.
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Keywords: All-*trans* retinoic acid; Liposomes; M5076; Sterylglucoside; Stability; Antitumor effect

1. Introduction

Retinoids are natural and synthetic analogs of vitamin A that normally play a critical role in growth, vision, reproduction, differentiation and immune functions. All-*trans* retinoic acid (ATRA) is a retinoid that has been proven effective against acute promyelocytic leukemia (APL) (Lazzarino et al., 1996; Mehta, 1989), primary murine plasma cell tumors (Swaminathan et al., 1999), squamous cell carcinoma of the skin (Giannini et al., 1997), isolated tissue culture systems (Montenegro et al., 1996) and in human clinical trials (Estey et al., 1996; Sun et al., 1999). ATRA is also an inducer of apoptosis for hepatoma cells (Parthasarathy and Mehta, 1998; Cortesi et al., 1999; Holmes et al., 2000). However, it was reported that oral administration of ATRA was not effective for hepatic metastasis in mice (Murakami et al., 1998; Sakukawa et al., 1998). Therefore, a new dosage form of ATRA is expected for the treatment of hepatic metastasis.

Liposomes are non-toxic, biodegradable lipid vesicles that can alter the distribution and bioavailability of drugs (Lasic et al., 1991; Sharma and Sharma,

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1997). The use of liposomes as drug carriers has been exploited to improve the therapeutic index of several antimicrobials (Price et al., 1992, 1994; Trafny et al., 1999; Yu et al., 2002) and anticancer agents (Sharma and Straubinger, 1994; Zelphati and Szoka, 1996; Ishida et al., 1998). The incorporation of ATRA into liposomes has allowed the preparation of a new intravenous (i.v.) ATRA formulation that significantly improved the potency and duration of ATRA activity only in cases of squamous carcinoma (Parthasarathy et al., 1994), lymphoma (Sundaresan et al., 1997) and leukemia (Estey et al., 1999).

In our previous work, doxorubicin-loaded liposomes modified with sterylglucoside (SG, SG liposomes) have been shown to accumulate in the liver, resulting in an increased life-span of mice bearing hepatic metastasis of M5076 tumor (Shimizu et al., 1998). There is no report to apply ATRA-loaded liposomes on hepatic metastasis effectively. Therefore, ATRA-loaded SG liposomes were designed to develop a new application of ATRA in cancer.

The aim of this study was to characterize ATRA-loaded SG liposomes in stability and evaluate their antitumor effects for mice bearing hepatic metastasis of M5076 tumor.

2. Materials and methods

2.1. Materials

DPPC was purchased from NOF Co. Ltd. (Tokyo, Japan). Cholesterol (Ch) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). SG was provided by Ryukakusan Co. (Tokyo, Japan). SG is a mixture of steryl β-D-glucosides consisting of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%) and brassicasterol (7.2%) (Fig. 1). ATRA was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Rat serum was purchased from Rockland Inc. (PA, USA). All other chemicals used were of reagent grade.

2.2. Preparation of liposomes

Two kinds of liposomes loading ATRA are as follows: 60 μmol DPPC, 10 μmol SG, 1–10 μmol (1.0–9.1 mol%) ATRA and 30 μmol Ch for SG liposomes; and 60 μmol DPPC, 1–10 μmol (1.0–9.1 mol%) ATRA and 40 μmol Ch for non-SG liposomes were prepared according to the reverse-phase evaporation method. Briefly, DPPC, Ch and ATRA were dissolved in chloroform and SG was in chloroform–methanol mixture (1:2). A mixture of lipids and ATRA were dried under reduced pressure. Chloroform (3 ml), isopropylether (3 ml) and 1/10 diluted phosphate-buffered saline (1/10 PBS, pH 7.31, 4 ml) with or without 50 mM calcein was added to the dried film and sonicated at 45 °C for 3 min to make w/o emulsion. The organic solvent in the w/o emulsion was removed under reduced pressure (∼100 mmHg) and flushed with a gentle stream of nitrogen (500 ml/min) at 45–50 °C. Liposomes were extruded through a polycarbonate membrane (Nuclepore, MA, USA) with a pore size of 200 nm at ∼50 °C. The recovery of DPPC was 72.5±19.4% (S.D.). Aliquots of liposomes were analyzed for measurement of ATRA and calcein concentration in the percentage of 10% Triton-X 100 (Shimizu et al., 1996a). The particle size distribution of liposomes was determined using a dynamic laser light scattering instrument (Model ELS-800, Ostuka Electronics Co., Ltd., Japan) and lipid concentration of liposomes was determined using a Wako phospholipid B test (Wako Pure Chemical Ind., Ltd., Osaka, Japan).

2.3. HPLC assay

Determination of ATRA was extracted from liposome suspension by adding chloroform; the HPLC system consisted of a Shimadzu LC-10AD high-pressure pump, a Shimadzu SIL-10A autoinjector, a Shimadzu SPD-10A UV spectrophotometric detector (340 nm) and a YMC-Pack ODS-A column (150 mm × 4.6 mm i.d.). The mobile phase consisted of acetonitrile/1% ammonium acetate (85:15%, v/v) and a flow rate of 1.0 ml/min, using all-*trans* retinol acetate for internal standard. The storage stability of liposomes was examined at 25 and 4 °C and open (did not cover with aluminum foil) or close (cover with aluminum foil) condition to protect from light influence, because ATRA was very weak by light (Regazzi et al., 1997). SG liposomes or non-SG liposomes entrapped calcein were prepared by the above method, and then entrapped calcein and free calcein

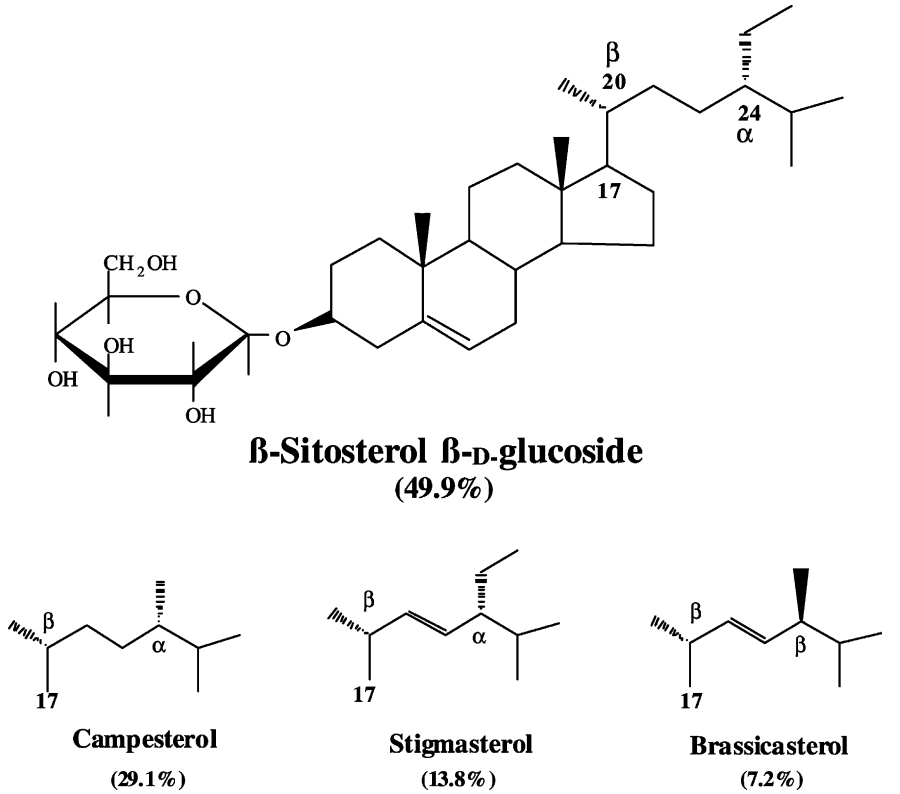

Fig. 1. Chemical structure of the soybean-derived sterylglucoside mixture (SG). The numbers in parentheses represent the mixture ratio in SG.

was separated by passing through a Sephadex G-50® column (Pharmacia, Sweden) with the 1/10 PBS. ATRA concentration was calculated as per the equation: $(C_p/A_p) \times G_f$, where C_p is the ATRA amount for liposome preparation after gel filtration, A_p is 1/10 PBS amount for liposomes preparation (=4.0 ml) and G_f is the dilution constant for gel filtration (=0.111).

2.4. Stability analysis

The storage stability of liposomes with 2.9 mol% ATRA was monitored at 4 and 25 °C at open (no-cover with aluminum foil) or close (cover with aluminum foil) condition by measuring particle size and ATRA concentration. Entrapped ATRA was separated from free ATRA through a Sephadex G-50® column (Pharmacia, Sweden) with the 1/10 PBS. The amount of ATRA in free fraction and liposomal fraction was determined by HPLC ($n = 3$). The ATRA-loading efficiency of liposomes was calculated according to the following equation:

$$\text{ATRA-loading efficiency (\%)} = \left(1 - \frac{A_f}{A_t}\right) \times 100$$

where A_f is the amount of free ATRA and A_t is the total amount of ATRA. The stability of liposomes in 80% rat serum was monitored by the release of calcein from the liposomes entrapped calcein after gel filtration. After liposomes were incubated for 120 min at 37 ± 0.5 °C, calcein release from each liposome was measured by fluorescence spectrophotometer (F4010, Hitachi Co., Ltd., Japan) at the excitation of 520 nm and the emission of 490 nm. The total calcein concentration was expressed as the sum of the liposomal concentration and released calcein from liposomes (Shimizu et al., 1996a).

Table 1
Effects of ATRA/lipid (mol%) on entrapment efficiency and particle size of SG and non-SG liposomes after gel filtration[a]

Liposomes	ATRA/lipid (mol%)	ATRA (μM)	Entrapment efficiency (%)	Mean particle size
SG liposomes	0	–	–	162.1 ± 1.7
	1	23.7 ± 4.2	84.5 ± 15.2	171.6 ± 6.3
	2.9	47.3 ± 19.4	56.7 ± 23.3	174.3 ± 4.7
	4.8	52.8 ± 26.7	38.0 ± 19.2	157.8 ± 2.0
	9.1	125.0 ± 19.4	45.0 ± 0.7	167.4 ± 5.3
Non-SG liposomes	0	–	–	172.6 ± 1.9
	1	28.8 ± 19.2	103.6 ± 69.0	155.4 ± 3.7
	2.9	53.5 ± 25.1	64.2 ± 30.1	175.6 ± 4.7
	4.8	110.6 ± 49.7	79.6 ± 35.8	182.8 ± 11.0
	9.1	140.0 ± 66.9	50.4 ± 24.1	191.3 ± 6.7

[a] Each value represents the mean ± S.D. ($n = 3$).

2.5. Animal and antitumor activity

Specific pathogen-free female C57BL/6 mice (19–20 g, 7-week old) were purchased from Tokyo Animal Experiment Center (Tokyo, Japan). Mouse murine histiocytoma M5076 tumor cells were supplied by Dr. T. Yamori of the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan) and kept in vivo as a solid tumor in C57BL/6 mice.

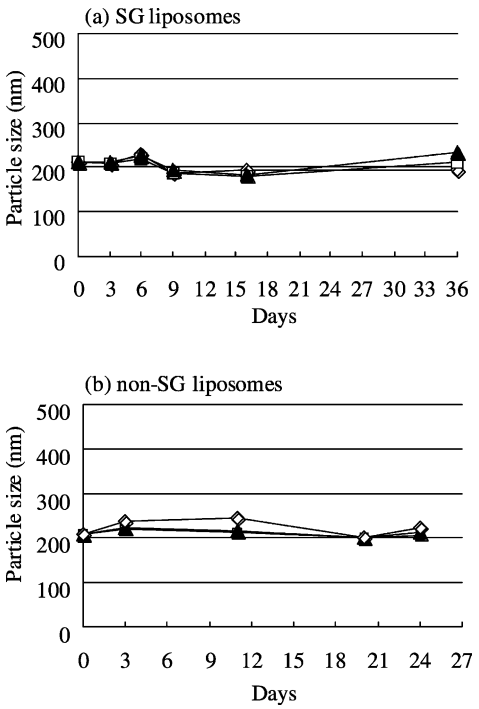

Fig. 2. Change of mean diameter of liposomes prepared with 2.9 mol% ATRA at 4 and 25 °C as a function of time. (◇) 25 °C (open), (□) 25 °C (close), (▲) 4 °C.

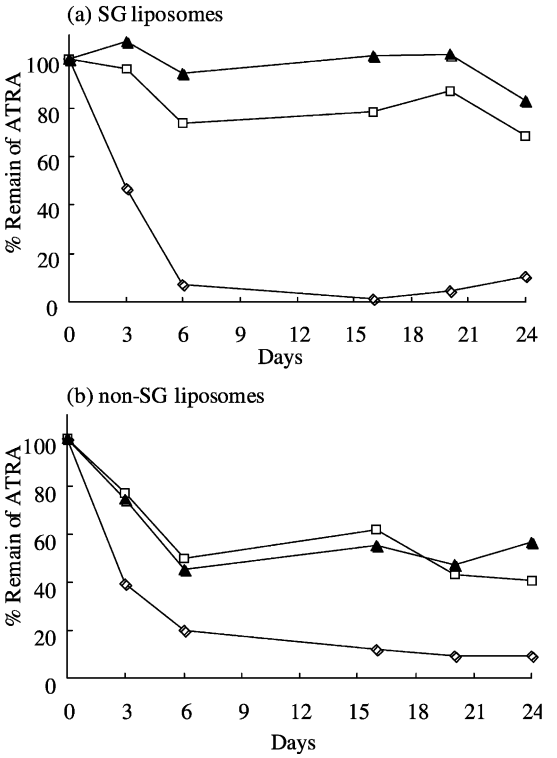

Fig. 3. Percentage of remaining ATRA in liposomes prepared with 2.9 mol% ATRA composed of SG liposomes (a) and non-SG liposomes (b). (◇) 25 °C (open), (□) 25 °C (close), (▲) 4 °C.

In the therapeutic experiments of liver metastasis cancer, groups of nine C57BL/6 mice were inoculated i.v. on Day 0 with 6×10^5 M5076 tumor cells. Treatment was initiated 8 days after inoculation. SG liposomes prepared with 2.9 mol% ATRA were used in this experiment. The animals were given ATRA-loading SG liposomes at 0.585 mg ATRA/kg weight by a single i.v. injection. The control group was given sterile saline. Mice were weighed every other day after saline or liposomal ATRA administration. The survival time was recorded in days following saline or liposomal ATRA administration. Antitumor activity was evaluated by comparing the mean survival time of the treated animals (T) with that of the controls (C), i.e. by calculation of the increase in life span (ILS), $(T/C - 1) \times 100$ (%). Body weight change (%) was calculated using the equation: $((W13 - W0)/W0) \times 100$, where W13 is the body

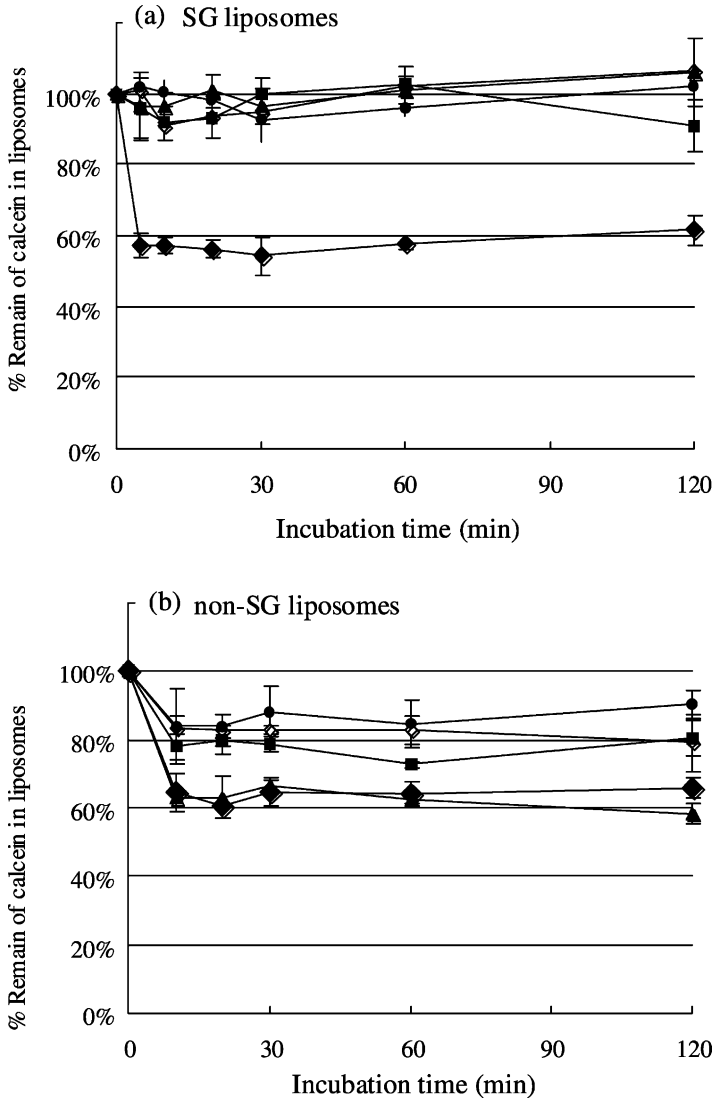

Fig. 4. Time-course of calcein release from liposomes in 80% rat serum. ATRA in the lipid mixture was 0% (◇), 1.0% (■), 2.9% (▲), 4.8% (●), 9.1% (◆). Each value represents mean ± S.D. ($n = 3$).

weight of mice on Day 13 and W0 is the body weight of mice on Day 0.

The statistical significance of the results was evaluated by the non-parametric test of Kruskal–Wallis for the survival experiment and the Student's *t*-test for all other experiments.

3. Results

3.1. Characterization of ATRA-loaded liposomes

Since ATRA is lipophilic compound, the entrapment of ATRA in the lipid bilayer may be limited. The effect of ATRA concentration added in the lipid on entrapment efficiency and the cumulative particles size of ATRA-loaded liposomes is shown in Table 1. Increasing ATRA/lipid mol% increased the particle size of non-SG liposomes slightly above 2.9 mol% ATRA. Meanwhile, the entrapment efficiency of ATRA gradually decreased as the quantity of lipid increased. ATRA concentration in SG and non-SG liposomes was increased with ATRA/lipid mol% from 1.0 to 9.1 mol%. ATRA amount in SG liposomes was almost the same level with that in non-SG liposomes prepared with ATRA of 2.9 mol% and showed the higher entrapment efficiency of ATRA, i.e. 56.7 and 64.2%, respectively. The entrapment efficiency in non-SG liposomes showed diverse value compared with that in SG liposomes. Non-SG liposomes seemed unstable when incorporating ATRA in liposomal membrane. It is known that incorporating cholesterol increases the rigidity of the liposomes at less than 50 mol%. Cholesterol might intercalate the phospholipid bilayer where ATRA molecules might be accommodated.

3.2. Stability in storage

The stability of SG liposomes and non-SG liposomes prepared with 2.9 mol% ATRA at various storage conditions was monitored in terms of size of liposomes (Fig. 2) and remaining ATRA (Fig. 3). The mean particle size of SG liposomes and non-SG liposomes was not changed over 20 days at 4 and 25 °C. At 25 °C with opened condition, ATRA in SG liposomes and non-SG liposomes were very unstable and open condition since ∼7 and 20% ATRA remained after 6 days, respectively. At 25 °C with closed condition, SG liposomes were more stable than non-SG liposomes because ATRA was very weak by light (Regazzi et al., 1997). At 4 °C, SG liposomes were very stable showing 83.1% remaining ATRA until 24 days. However, ATRA incorporation in non-SG liposomes with closed condition declined much faster at 25 °C than that of SG liposomes. ATRA-loaded non-SG liposomes seemed unstable compared to SG liposomes.

3.3. Stability in 80% rat serum

After ATRA-loaded liposomes entrapped calcein were incubated in 80% rat serum, calcein released from liposomes was measured (Fig. 4). SG liposomes loaded with ATRA (except 9.1 mol%) were considerably stable, showing that ∼100% calcein remained until 120 min. SG liposomes with 9.1 mol% ATRA released ∼40% calcein after 5-min incubation, and then were almost constant for 5–120 min. While, non-SG liposomes were unstable, showing that ATRA remained ∼60–80% in liposomes, there was no clear dependence of liposomal stability in 80% rat serum on ATRA content in non-SG liposomes. Therefore,

Table 2
Effect of ATRA-loaded SG liposomes on hepatic metastasis of M5076 reticulosarcoma

Preparation	Dose (mg/kg)	Survival time (days)			ILS (%)[a]	Mean body weight change (%)[b] (S.D.)
		15 days	Mean (S.D.)	Median		
Saline		0/9	14.1 (0.9)	15		6.9 (5.8)
SG liposomes	0.585	7/9	18.2 (2.7)[c]	18	20.0	7.9 (2.1)

[a] Percentage increase in life span, $[(T/C - 1) \times 100\ (\%)]$, where T and C represent the median survival time (days) of the treated and control animals, respectively.

[b] Average and standard deviation of body weight change from 0 to 13.

[c] $P < 0.01$ compared to value for saline group.

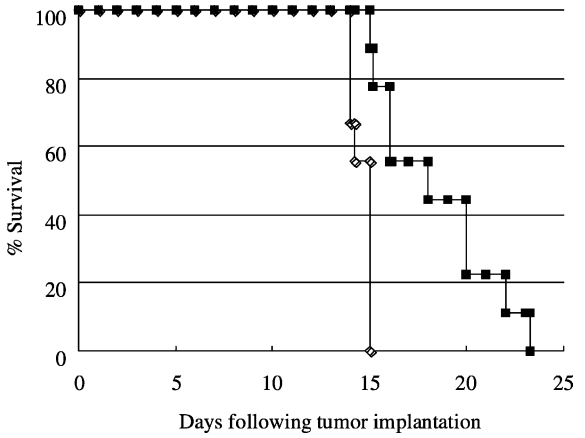

Fig. 5. Effect of ATRA-loaded SG liposomes on survival of mice injected at Day 9 via the tail vein following inoculation of hepatic metastasis M5076 tumor ($n = 9$). (◇) Control, (■) ATRA-loaded SG liposomes at a dose of 0.585 mg ATRA.

in in vivo experiment, ATRA-loaded SG liposomes were used.

3.4. Antitumor activity

The antitumor efficacy of liposomal ATRA in the liver metastasis cancer model, is shown in Table 2 and Fig. 5. The mean survival time of mice given SG liposomes (18.2 days) indicated a greater antitumor activity than saline (14.1 days) ($P < 0.001$). The ILS value of mice given SG liposomes was 20.0%. Seven of the nine mice following administration of the SG liposomes survived for over 15 days, but mice given the saline did not survive (zero of the nine mice). The mean body weight change (%) of mice given SG liposomes and saline were $6.9 \pm 5.8\%$ and $7.9 \pm 2.1\%$, respectively, indicating no significance. The survival time of free ATRA (0.655 mg/kg) was the same as that of saline, and did not indicate an antitumor activity (data not shown). These findings indicated liposomal ATRA maintained active from antitumor effect.

4. Discussion

Wassall et al. (1988) reported that incorporation of ATRA into liposomal membrane increases upper position of acyl-chain fluidity because strongly hydrophilic carboxylic group of ATRA is assumed to project into the head group region of membrane. It was previously reported that SG was contained in liposomes until 26 mol% (Shimizu et al., 1996b) and sterol group of SG may be removed from the most stable position of the acyl chain of DPPC in SG liposomes because of the projection of glucose residue out of SG liposomes (Shimizu et al., 1996a). Therefore, membrane fluidity of SG liposomes at the hydrophobic part was increased at 30 °C (Muramatsu et al., 1994). Based on this information, both SG and ATRA seemed to increase the fluidity at the hydrophobic part in liposomal membrane. However, from the results of stability of ATRA-loaded liposomes in storage and rat serum, ATRA in SG liposomes were more stable than in non-SG liposomes, although particle size of SG liposomes and non-SG liposomes did not change at 4 and 25 °C over 30 days (Fig. 2). These results suggest that SG might lead to increased stability of liposomes loading ATRA compared with cholesterol.

Asai and Watanabe (2000) reported that the excess all-*trans* retinal (∼5 mol%) separated from the DPPC bilayer is stabilized as emulsion particles by the DPPC surface monolayer. The monolayer–bilayer equilibrium plays an important role in the structural formation in aqueous dispersions and excess all-*trans* retinal which separates from the liposomal bilayer and can be more stable dispersed as small particles. In these experiments, the size distribution of ATRA-loaded SG liposomes showed one peak (data not shown), which suggests that small particles of ATRA could not separate from liposomal bilayer. However, there is a possibility that the equilibrium of ATRA among liposomal membranes might play an important role to stabilize bilayer and SG enhances this equilibrium.

The activity of ATRA-loaded liposome was investigated as the antitumor effects because ATRA was sonicated and heated during the preparation of liposomes. SG liposomes with 2.9 mol% ATRA were selected because they were very stable in storage and rat serum and showed high entrapment efficiency of ATRA. Liposomal ATRA has useful anticancer effect against APL and the other type of leukemia (Drach et al., 1993; Hsu et al., 2001). However, it has not been reported that liposomal ATRA was effective against hepatic metastasis in vivo. ATRA bioavailability may be estimated at ∼50% on the basis of studies

conducted in volunteers (Regazzi et al., 1997). Murakami et al. (1998) reported that ATRA (2–8 mg/kg per day), administrated p.o. for 5 consecutive days per week for 3 weeks, did not affect the survival rate of mice bearing A549 experimental liver metastasis. In our study, dose of ATRA in SG liposomes was very low (0.585 mg/kg), which prolonged survival time in mice bearing hepatic metastasis (Fig. 5). It was suggested that liposomal ATRA was more effective for prolongation of survival time than free ATRA, indicating an increase of activity due to liver targeting, alteration of rates of metabolism, and decrease of toxicity due to altered pharmacokinetics.

Mehta (1989) reported that free ATRA, when administrated in CD-1 mice, exerted toxic effects at 25–30 mg/kg of body weight, while ATRA-loaded liposomes could tolerate much higher doses of ATRA (120 mg/kg), possibly due to an alteration of drug distribution in target tissue. In this study, dose of liposomal ATRA was very low (0.585 mg/kg) and, therefore, the difference of body weight change between SG liposomes and saline was not significant (Table 2). Alterations in biodistribution by SG liposomes may also be responsible for the increased effect and the decreased toxicity.

In conclusion, we could prepare very stable ATRA-loaded SG liposomes, showing high antitumor effect against hepatic metastasis of M5076 tumors.

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