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Design of vesicles of 1,2-di-*O*-acyl-3-*O*-(β-D-sulfoquinovosyl)-glyceride bearing two stearic acids (β-SQDG-C_{18}), a novel immunosuppressive drug

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Abstract

The immunosuppressive effects of synthetic sulfo-glycolipids in the class of sulfoquinovosyl-diacylglycerols (SQDG), including stereoisomers, were interesting in development of a promising clinical drug. Especially, 1,2-di-*O*-stearoyl-3-*O*-(6-deoxy-6-sulfo-β-D-glucopyranosyl)-*sn*-glycerol (β-SQDG-C_{18}) was thought to be a valuable candidate because of the preliminary observations of its high inhibitory activities in spite of low toxicities. The problem of using this material is to find an applicable way avoiding its low solubility in water. The vesicle formation of β-SQDG-C_{18} is advantageous to i.v. administration in its chemico-structural character. With preparation in water, β-SQDG-C_{18} was hard to form vesicles, because its hydrophilicity was strong. We examined the suitable parameter of the vesicle forming condition. It was possible to take a balance between the hydrophilicity and the hydrophobicity of the β-SQDG-C_{18} molecule to be optimized to form vesicles in 150 mM PBS. In addition, we demonstrated the strong immunosuppressive activity of β-SQDG-C_{18} vesicles. This is the first report of the preparation method of β-SQDG-C_{18} vesicles, which should facilitate in vitro and in vivo application.

Keywords: Sulfo-glycolipids; SQDG; β-SQDG-C_{18}; Vesicle; MLR; Immunosuppressive drug

1. Introduction

Sulfoquinovosyl-diacylglycerols (SQDG) were reported to exert extensive biological effects, such as DNA polymerase inhibition [1–6], antitumor activity [7,8], P-selectin receptor inhibition [9] and reverse transcriptase inhibition in HIV [10], as well as AIDS antiviral influence [11]. We reported previously biological activities of sulfo-glycolipids from a fern [1], an alga [3,4], spinach [6] and a marine invertebrate [7]. There is great variety in the long chain fatty acids in natural SQDG [12–14], making then very difficult to isolate one kind. To study biological activities of SQDG precisely, we have developed systematic methods to synthesize α- and β-configurated SQDG chemically, including their stereoisomers [15–17]. We have reported that the synthesized SQDG exhibit strong immunosuppressive activities [18,19]. α- and β-SQDG were found to inhibit human mixed lymphocyte reaction (MLR) in vitro

Abbreviations: β-SQDG-C_{18}, 1,2-di-*O*-stearoyl-3-*O*-(6-deoxy-6-sulfo-β-D-glucopyranosyl)-*sn*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; MLR, mixed lymphocyte reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; SQDG, sulfoquinovosyl-diacylglycerols

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[19]. In particular, 1,2-di-*O*-stearoyl-3-*O*-(6-deoxy-6-sulfo-β-D-glucopyranosyl)-*sn*-glycerol (β-SQDG-C_{18}) exhibited the strongest inhibitory effect, also prolonging rat skin allograft rejection in vivo [18,19]. Moreover, in our preliminary studies none of these SQDG demonstrated any cytotoxic effects in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in vitro or pathologically evident toxicity at the concentrations used in vivo. It is well known that immunosuppressive drug FK506 was widely used for human organ transplantation; however it often induces neuropathy [20]. It is, therefore, important to continue to develop new agents with low toxicity for a long time use. Thus, β-SQDG-C_{18} has been developed for this purpose as a promising clinical agent (Fig. 1).

To develop the immunosuppressive drug for medical use, we first-time achieved mass-production of β-SQDG-C_{18} on a factory scale. A problem occurred for in vivo uses, β-SQDG-C_{18} was hard to dissolve in water. If not perfectly dissolved, i.v. injection of β-SQDG-C_{18} would be dangerous since peripheral blood vessels might become clogged. To solve this problem, we have paid attention to molecular assemblies, especially vesicle formation [21–26]. The vesicles are usually known to be formed in the system of amphiphiles, e.g., double chained amphiphiles [22]. β-SQDG-C_{18} has two hydrophobic tails consisting of stearyl esters that are connected to the hydrophilic sulfo-glucose moiety, so that it might be expected to form vesicles.

In the present report, we describe suitable conditions for the formation of β-SQDG-C_{18} vesicles, based on turbidity, microscopic observation, trapping efficiency and differential scanning calorimetry (DSC). In vitro MLR assay and particle size analysis were carried out for the obtained vesicles. The preparation of β-SQDG-C_{18} vesicles should facilitate further investigation of the potential of these non-toxic agents in in vitro experiments and in vivo uses, with the final goal of clinical application.

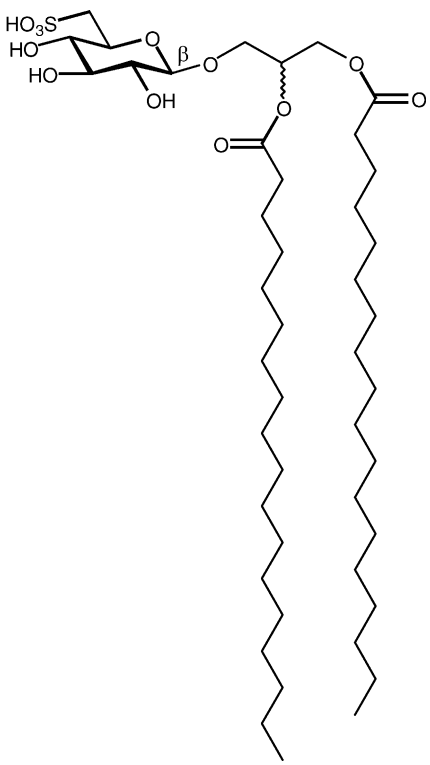

Fig. 1. Chemical structure of synthesized β-SQDG-C_{18}.

2. Materials and methods

2.1. Materials

β-SQDG-C_{18} was synthesized according to the procedures described previously [15–17]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from Nippon Oil and Fats and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Sigma Chemical, and calcein from Dojindo Laboratories. FK506 was obtained from Fujisawa Pharmaceutical. All other reagents were of analytical grade and purchased from Wako Chemical.

2.2. Preparation of vesicles

Vesicles were prepared using a conventional technique similar to the method described by Bangham et al. in 1965 [27]. Briefly, the DPPC and DSPC were dissolved in chloroform and β-SQDG-C_{18} in a chloroform:methanol:-water = 100:10:1 in a 10 ml round-bottom flask. The organic solvent mixture was removed using a rotary flash evaporator under reduced pressure, and the residual organic solvent was further removed by drying overnight under vacuum. The dried lipid films were hydrated with pure water, phosphate-buffered saline (PBS) and 0.1 mM calcein solution, respectively, followed by incubation at 54 °C (DPPC) or 64 °C (β-SQDG-C_{18} and DSPC) for 30 min. PBS concentration was changed from 0 to 600 mM. The flask was then shaken vigorously on a vortex mixer.

For in vitro assays, we attempted leveling by extrusion treatment [28,29], using various membranes. An Avanti Mini-extruder (Avanti Polar Lipids) was employed and the pore size of polycarbonate membranes (Nuclepore Filtration Products) was 600 or 100 nm, applied in size order. Vesicle suspensions were passed through the membranes under syringe pressure to regulate the size of the vesicles. The preceding procedures were carried out at a temperature above the transformation temperature of the lipids employed. The mean diameter of vesicles was measured at room temperature using a light scattering method (NICOMP 380ZLS, Particle Sizing System).

2.3. Optical microscopy of β-SQDG-C_{18} vesicles

β-SQDG-C_{18} vesicle suspension prepared at 150 mM PBS (pH 7.4) was examined under an inverted microscope

with a transmitted light differential interference contrast attachment (IMT-2, Olympus Optical).

2.4. Freeze fracture electron microscopy of β-SQDG-C_{18} vesicles

β-SQDG-C_{18} vesicle suspension prepared at 150 mM PBS (pH 7.4) was quickly frozen in liquid propane using a cryo-preparation apparatus (Leica EM CPC, Leica). The frozen sample was fractured in a freeze-replica with a making apparatus (FR-7000A, Hitachi Science) at −150 °C. The fractured surface was replicated by evaporating platinum at an angle of 45°, followed by evaporating carbon at normal incidence to strength the replica. It was then placed on a 150 mesh copper grid and observed under a transmission electron microscope (JEM-1200EX, JEOL).

2.5. Measurement of the amount of calcein trapped in vesicles

The measurement of trapping efficiency for calcein was performed according to the method reported by Oku et al. [30]. Forty microliters of vesicle suspension were diluted to 2 ml with PBS and the fluorescence of the suspension was measured before (F_t) and after (F_{in}) addition of 20 μl of $CoCl_2$ (10 mM). F_t represents the fluorescence of all the calcein present while F_{in} is the fluorescence from the initial compartment plus the unquenched fraction of the external compartment. Subsequently, 20 μl of 20% Triton-X was added and the fluorescence measured again. The latter addition destroys the integrity of the vesicles and the resultant fluorescence intensity (F_q) represents the equilibrium concentration of free calcein. The trapping efficiency is calculated from:

$$\text{Trapping efficiency}\ (\%) = \frac{F_{in} - F_q r}{F_t - F_q r} \times 100$$

where r is the dilution factor, 1.04 in the present case. The fluorescence intensities of F_t, F_{in} and F_q were measured under an appropriate temperature with a spectrofluorometer (RF-5000, Shimadzu) operated using excitation and emission wavelengths at 490 and 520 nm, respectively.

2.6. Differential scanning calorimetry

The phase transition temperature of β-SQDG-C_{18} vesicles was measured with a differential scanning calorimeter (8240B, Rigaku Denki). Twenty millimolars of β-SQDG-C_{18} vesicle suspension prepared in 150 mM PBS (pH 7.4) were placed in a sampling vessel, made of stainless steel (resistance to pressure at 50 atm, 3 mm × 5 mm (∅) for the vesicle size), which was then sealed. The measurement conditions were 1 K/min for the scanning rate, 40–70 °C for the scanning range and 0.1 mcal/s for the sensitivity.

2.7. Turbidity of β-SQDG-C_{18} suspensions

The turbidity of 1 mM β-SQDG-C_{18} suspension was measured at a room temperature and changes were determined with a spectrophotometer (MPS-2000, Shimazu) at 450 nm. Time-dependent variation was expressed as ($T_t/T_0 \times 100$), where T_0 and T_t denote the turbidity of the vesicle suspensions initially and at time t, respectively. The dynamic sedimentation of the vesicle suspension could be measured by comparison with the turbidity of the initial vesicle suspension.

2.8. Human MLR assay

Peripheral blood lymphocytes (PBL) from healthy human volunteers were obtained by density centrifugation. PBL obtained from one donor as stimulator cells, namely antigen presenting cells, were prepared by incubating cells at 106 cells/ml under the presence of mitomycin C for 20 min. After treatment, these cells were washed with PBS four times and suspended in RPMI 1640 (Nissui) supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum at 106 cells/ml. PBL obtained from another donor as responder cells were incubated on a nylon wool column (Biotest AG) for 1 h at 37 °C in 5% CO_2. T cells that did not bind to the nylon wool column were suspended in medium at 106 cells/ml and incubated with synthetic sulfolipids at 37 °C in 5% CO_2 for 1 h. Responder T cells and antigen presenting cells were mixed at a 1:1 ratio and aliquoted into triplicate wells of 96-well round-bottom plates. After 4 days of cultivation, [^{3}H]thymidine was added to the wells followed by culture for 24 h, and the cells were then harvested with a SKATRON harvester. The radioactivity of responder T cells was measured in an LS 6000 scintillation counter.

MTT assay was performed using Jurkat cell line according to the method described previously by Matsumoto et al. [19].

2.9. Statistical analysis

The data are expressed as the mean ± S.E. Statistical analyses were performed with Student's t-test for comparison of two groups. A P value < 0.05 was considered as statistically significant.

3. Results

3.1. Vesicle formation of β-SQDG-C_{18}

We investigated the effect of ionic strength on the turbidity of β-SQDG-C_{18} solution prepared at different PBS concentration (Fig. 2). Turbidity of the β-SQDG-C_{18} solution increased with the PBS concentration. However,

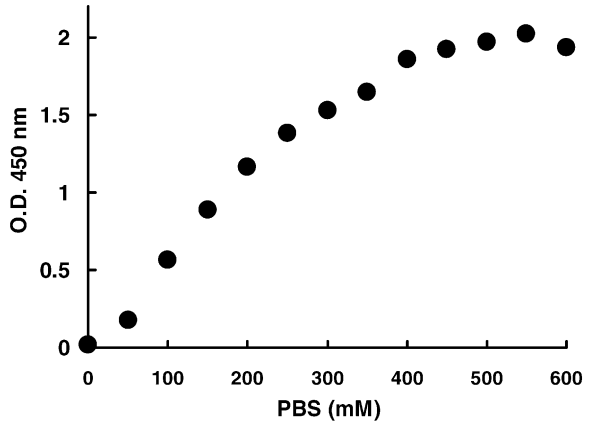

Fig. 2. Ionic strength effects on turbidity of β-SQDG-C_{18} solutions prepared at 0–600 mM PBS (pH 7.4).

any significant turbidity was not observed in the sample prepared in water. Microscopic studies were employed to follow the turbidity increase of β-SQDG-C_{18} suspensions, in addition to characterize the β-SQDG-C_{18} molecular assembly prepared at 150 mM PBS. Fig. 3 illustrates an optical micrograph (A) and a freeze-fracture electron micrograph (B) of β-SQDG-C_{18} vesicles prepared at 150 mM PBS. Optical and electron micrographs of the β-SQDG-C_{18} suspensions indicated intravesicular particles and particle size variation (approximately 200 nm–10 μm).

3.2. Trapping efficiency of β-SQDG-C_{18} vesicles

The vesicles have aqueous compartments as observed in the lipid bilayers of biomembranes. Determining the volume of the aqueous compartment of the vesicles can be used to confirm vesicle formation. The ionic strength was the important factor for vesicle formation of β-SQDG-C_{18} (Figs. 2 and 3). To assess the relationship between the vesicle formation and the ionic strength, therefore, vesicles were prepared in a solution of the fluorescent dye, calcein. Trapping efficiency of the β-SQDG-C_{18} vesicles obtained at the different concentrations up to 600 mM PBS was investigated to confirm the extent of vesicle formation (Fig. 4A). The trapping efficiency gradually increased from 0 to 100 mM PBS and then reached to plateau (10.9% at 150 mM PBS). Then, the efficiency gradually decreased over 250 mM PBS.

The dependence of lipid concentration on trapping efficiency was investigated at 150 mM PBS (Fig. 4B). From 0 to 30 mM lipid concentrations, the efficiency increased with it. To know the effects of pH conditions in preparing β-SQDG-C_{18} vesicles, the relationship between trapping efficiency and pH of the solution was investigated (Fig. 4C). From pH 5.6 to 8.0, trapping efficiency of β-SQDG-C_{18} vesicles did not change under the PBS concentration fixed at 150 mM. Therefore, pH 7.4 was used in the following preparation.

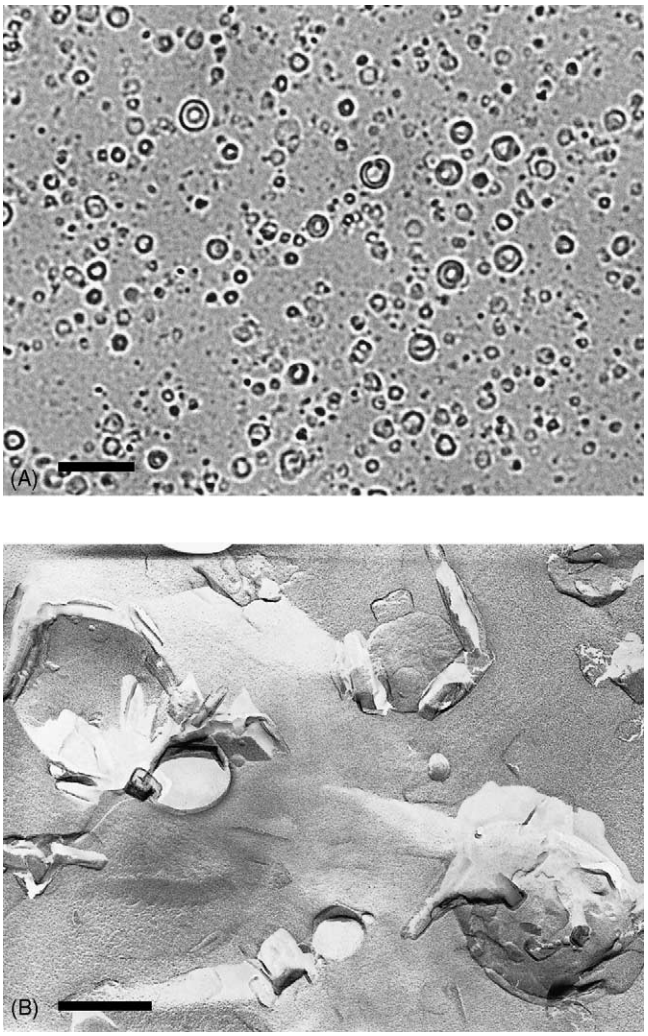

Fig. 3. (A) Optical micrograph (×600) and (B) freeze-fracture electron micrograph (×50,000) of β-SQDG-C_{18} vesicles prepared at 150 mM PBS (pH 7.4). Scale bar represents (A) 20 μm and (B) 500 nm.

3.3. Phase transition behavior of β-SQDG-C_{18} vesicles

The generally accepted concept for the lipid phase transition of gel to liquid crystal is that it is due to the conformational change in the acyl chain from solid-like to liquid-like state. Phase transition measurements were here employed with temperature-dependent curves for the trapping efficiency (Fig. 5A) and DSC analysis (Fig. 5B) at 150 mM PBS. The trapping efficiency of β-SQDG-C_{18} vesicles was maintained until 52 °C and suddenly decreased during 54–60 °C. As shown by DSC analysis, β-SQDG-C_{18} vesicles had sharp transition peak at around 57 °C.

3.4. Dispersion stability of β-SQDG-C_{18} vesicle suspensions

We investigated the relative turbidity of DPPC, DSPC and β-SQDG-C_{18} vesicle suspensions in 150 mM PBS (Fig. 6). DPPC and DSPC were used as comparison

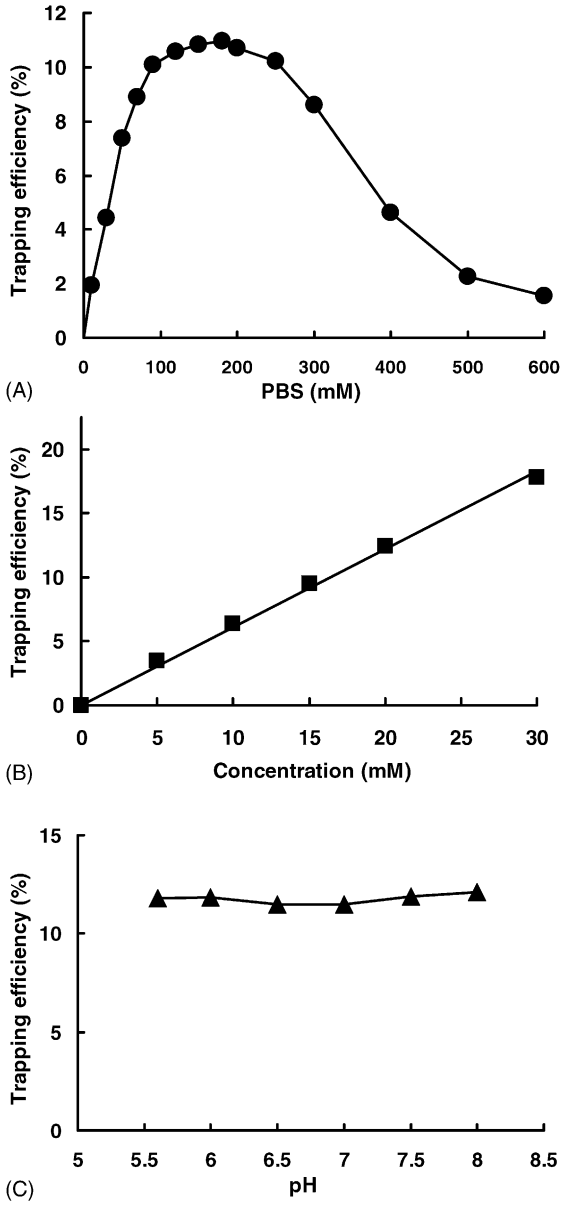

Fig. 4. (A) Ionic strength effects (●) and (B) the concentration-dependence on the trapping efficiency of (■) β-SQDG-C_{18} vesicles in 150 mM PBS (pH 7.4). (C) pH effects on the trapping efficiency of (▲) β-SQDG-C_{18} vesicles in 150 mM PBS (pH 5.6–8.0).

standards. DPPC and DSPC vesicle suspensions rapidly lost turbidity, and after 4 h only 15% of the original turbidity remained. On the other hand, decrease in the turbidity of β-SQDG-C_{18} vesicle suspension was not observed even after 6 h.

3.5. Effects of β-SQDG-C_{18} vesicles on the human MLR

We investigated the immunosuppressive effect and the cytotoxicity of β-SQDG-C_{18} vesicles with human MLR and MTT assay. Fig. 7A exhibits the MLR activity with β-SQDG-C_{18} assemblies prepared in water or 150 mM PBS (pH 7.4) by Bangham method [27]. FK506 (10 μg/ml) was used as a positive control. The assemblies of β-SQDG-C_{18}

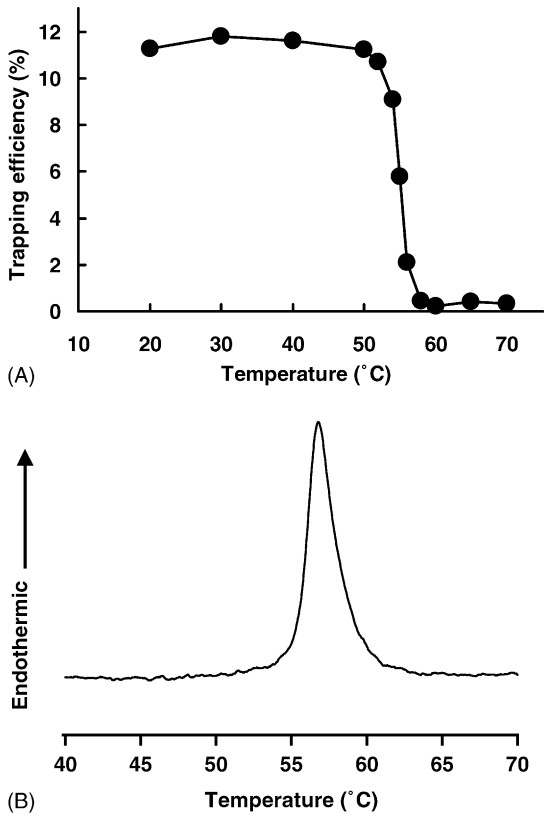

Fig. 5. (A) Temperature-dependent curves for the trapping efficiency and (B) DSC analysis of β-SQDG-C_{18} vesicles in 150 mM PBS (pH 7.4).

added to MLR at concentrations of 1–50 μg/ml. β-SQDG-C_{18} vesicles, prepared in PBS was clearly and dose-dependently inhibited the MLR activity ($P < 0.05$). On the other hand, β-SQDG-C_{18} prepared in water did not show any immunosuppressive effects. As shown in Fig. 7B, the addition of β-SQDG-C_{18} vesicles did not inhibit the cell growth in comparison with some toxic effect observed in FK506.

We attempted to investigate in vitro activities with leveling by extrusion treatment using various membranes.

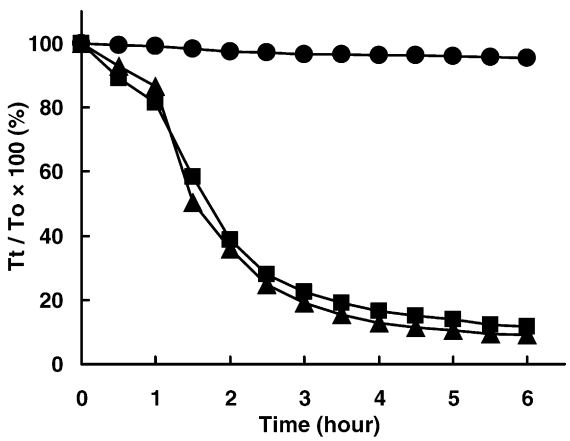

Fig. 6. Time-dependent variation of the turbidity of β-SQDG-C_{18} vesicle suspension. (●) β-SQDG-C_{18}, (■) DPPC and (▲) DSPC in 150 mM PBS (pH 7.4).

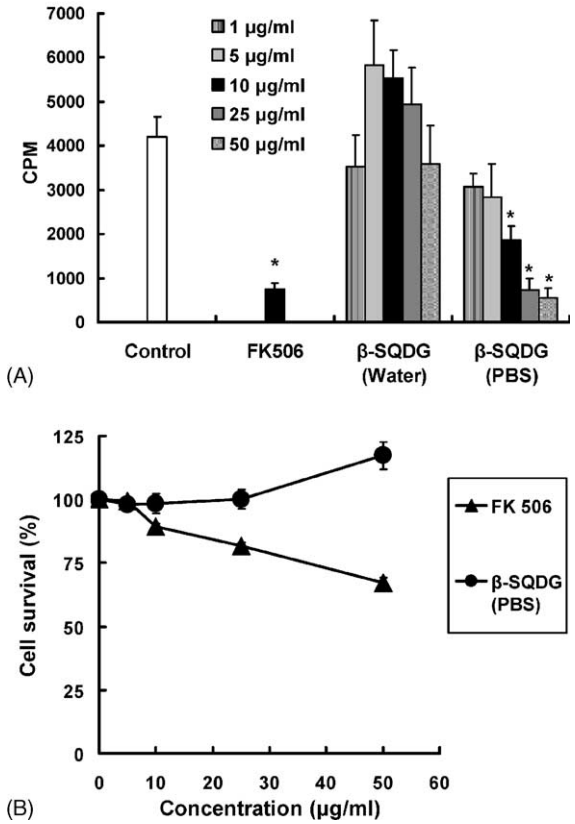

Fig. 7. (A) Inhibitory effects of vesicles of β-SQDG-C_{18} on human MLR. β-SQDG-C_{18} solutions are prepared in water or 150 mM PBS. Data are shown as the mean ± S.E. of three independent experiments. $^{*}P < 0.05$ vs. control values. (B) Cell growth inhibition by β-SQDG-C_{18} vesicles and FK506. Cell viability was determined by MTT assay. Data are shown as the mean ± S.E. of four independent experiments.

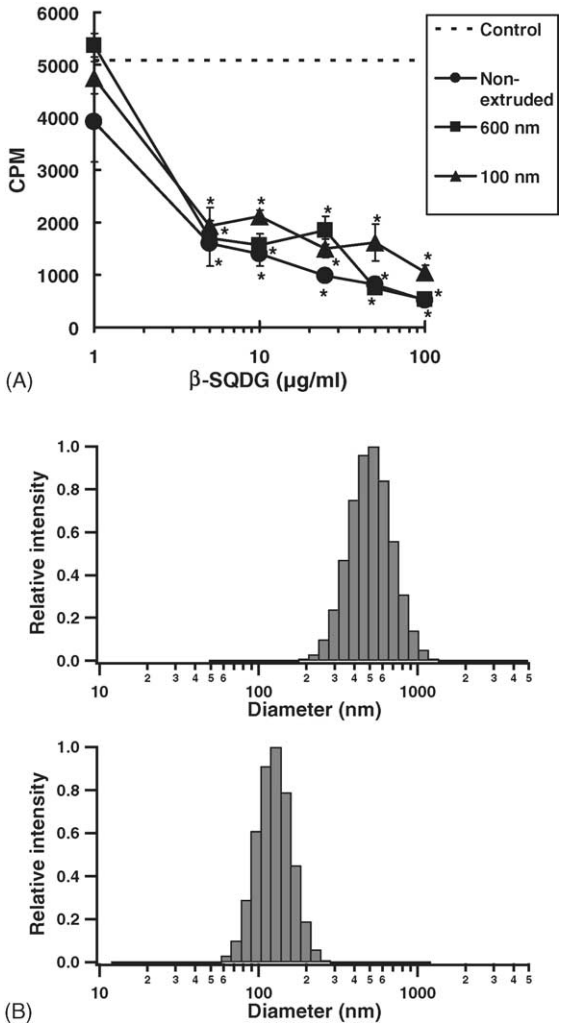

Fig. 8. (A) Inhibitory effects of β-SQDG-C_{18} vesicles of different size on human MLR. The numbers in figure represent the pore size of the membranes used for extrusion. Data are shown as the mean ± S.E. of three independent experiments. $^{*}P < 0.05$ vs. control values. (B) Particle size distribution of β-SQDG-C_{18} vesicles extruded with 600 nm (upper panel) or 100 nm (lower panel) pore size membranes.

MLR activities with vesicles in different sizes are shown in Fig. 8A. Vesicle samples were obtained by extrusion with 600 or 100 nm pore size membrane, in addition to the sample without extrusion. The particle size range of non-extruded vesicles was from 200 nm to 10 μm (Fig. 3). Extrusion through a pore size of 600 or 100 nm resulted in vesicles with average size of 550 ± 14 or 117 ± 4 nm, respectively (Fig. 8B). Each vesicle suspension inhibited dose-dependently the MLR activity in a concentration range from 1 to 100 μg/ml ($P < 0.05$).

4. Discussion

4.1. Preparation and characterization of β-SQDG-C_{18} vesicles

In the present paper, we have examined the assemblies of β-SQDG-C_{18} because of its chemico-structural suitability (negatively charged headgroup and two hydrophobic tails) for vesicle formation. First we investigated the condition of forming vesicles and its characterization. In general, molecular assemblies of amphiphile having negatively charged head groups are strongly affected by the ionic strength [31]. Therefore, we investigated the effect of ionic strength on the turbidity and the trapping efficiency of β-SQDG-C_{18} assemblies. The turbidity increased with ionic strength (Fig. 2). Turbidity depends essentially on the extent of molecular aggregation present in the liquid phase [32]. Turbidity change of β-SQDG-C_{18} solution would be resulted from some formational change of β-SQDG-C_{18} assemblies depending on the ionic strength. At 150 mM PBS, vesicle formation of β-SQDG-C_{18} was confirmed by micrographs (Fig. 3). The trapping efficiency gradually increased from 0 to 100 mM PBS and then reached to plateau. Then, the efficiency gradually decreased over 250 mM PBS (Fig. 4A). β-SQDG-C_{18} was hard to form vesicle structure in water, possibly because its hydrophilicity was relatively strong to its hydrophobicity in water. The ionic strength such as 150 mM produced a decrease in the effective area of the β-SQDG-C_{18} head group, which

resulted in an optimum balance between the hydrophilicity and the hydrophobicity in the β-SQDG-C_{18} molecule to closed vesicle formation. At higher ionic strength, β-SQDG-C_{18} molecule was hard to form closed vesicle structures.

Shipley et al. [12] reported the phase behavior of two SQDG from lipid extracts of mixed algal colony in water and suggested algal SQDG, with higher contents of unsaturated fatty acids, tend to form lamellar phase which transforms into vesicles than that with lower contents. We observed contrarily to the above, β-SQDG-C_{18} was hard to form vesicles in water. This difference between β-SQDG-C_{18} and natural lipid extracts of SQDG in water can be attributed to the fatty acids composition of SQDG, i.e. β-SQDG-C_{18} having saturated fatty acids (stearic acids), in contrast to that natural lipid extracts of SQDG are enriched in polyunsaturated fatty acids [12–14].

At 150 mM PBS, the vesicles of β-SQDG-C_{18} molecules were effectively formed. Under this condition, the trapping efficiency of β-SQDG-C_{18} vesicles increased linearly with its concentration from 0 to 30 mM (Fig. 4B), indicating that the vesicles were formed proportionally to the lipid concentration. Then, we examined the effect of pH value on the vesicle formation. The trapping efficiency was not modified by changing pH in the preparation procedure as shown in Fig. 4C. The pH value in this region did not affect the vesicle formation assuming the turbidity directly corresponded to the extent of the formation.

The gel to liquid crystal phase transition is a fundamental characteristic of vesicles. The trapping efficiency (Fig. 5A) and DSC analysis (Fig. 5B) were used to detect the phase transition temperature of β-SQDG-C_{18} vesicles. The phase transition was observed at around 57 °C in both measurements.

To avoid aggregation and the precipitation of drugs is essential for their in vitro and in vivo activities. β-SQDG-C_{18} vesicles were dispersed with stability under physiological condition of ionic strength and pH, comparing DPPC and DSPC (Fig. 6). The low pK_a value of the sulfonate group would suggest that the headgroup was ionized under the conditions. This high stability of dispersed β-SQDG-C_{18} vesicles were seemed to be resulted from the ionized headgroup of β-SQDG-C_{18}.

4.2. Immunosuppressive activity of β-SQDG-C_{18} vesicles

In vitro assays at the previous experiments, β-SQDG-C_{18} was first dissolved 2.3% dimethylsulfoxide and subsequently diluted with PBS since β-SQDG-C_{18} was hard to dissolve in water [18,19]. However, this dissolving method was not suitable when the β-SQDG-C_{18} concentration was required to increase in anticipated i.v. administration in vivo. Therefore, we must modify the method to be applicable to human body without containing any toxic organic solvent.

The present investigation of the suitable condition for vesicle formation, we elucidated β-SQDG-C_{18} formed vesicles in 150 mM PBS. In human MLR, β-SQDG-C_{18} vesicles could be demonstrated to possess immunosuppressive activity (Fig. 7A). The inhibition extent of β-SQDG-C_{18} vesicles were essentially the same as the data for original β-SQDG-C_{18} reported previously [18,19], indicating preservation of the function even by forming vesicle formation. Additionally, vesicles of β-SQDG-C_{18} exerted no overt cytotoxicity on the cells (Fig. 7B). As compared with vesicle formation, molecular assembly of β-SQDG-C_{18} prepared in water was not practical for in vitro application.

Considering i.v. administration, particle size regulation was needed because the size distribution of β-SQDG-C_{18} vesicles prepared by Bangham method [27] had a wide range and rat peripheral blood vessels are about 8 μm in diameter. Moreover, it is well accepted that the larger vesicles disappear more rapidly from the systemic circulation after i.v. administration than smaller vesicles [33,34]. Therefore, we assessed the immunosuppressive activity of β-SQDG-C_{18} vesicles made by extrusion technique. The potency of 600 or 100 nm extruded β-SQDG-C_{18} vesicles in MLR is similar to that of non-extruded β-SQDG-C_{18} vesicles (Fig. 8A). The immunosuppressive activity of β-SQDG-C_{18} vesicles was retained at a wide variety of vesicle sizes.

In conclusion, we confirmed that β-SQDG-C_{18} vesicles were formed under the physiological ionic strength and pH. β-SQDG-C_{18} vesicles could be demonstrated to possess the immunosuppressive effect in human MLR. To our knowledge, this is the first report of successful vesicle formation of β-SQDG-C_{18}, a potent immunosuppressive agent, and its applicability for in vitro experiments and in vivo uses. We are currently pursing experiments to test immunosuppressive effects of β-SQDG-C_{18} vesicles in vivo. Effect of i.v. administration with β-SQDG-C_{18} vesicles on rat skin allograft rejection will be described in a subsequent paper.

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