Physicochemical Properties of Liposomes Affecting Apoptosis Induced by Cationic Liposomes in Macrophages

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Purpose. Cationic liposomes are expected to be useful as nonviral vectors for gene delivery. Cationic liposomes showed cytotoxicity, and we proposed that the cytotoxicity is through apoptosis. In this study, we examined the effects of liposomal properties, such as liposomal charge, size, membrane fluidity, and PEG coating, on the induction of apoptosis in the macrophage-like cell line RAW264.7.

Methods. RAW264.7 cells were treated with liposomes, and the induction of apoptosis was evaluated by monitoring the changes in DNA content by flow cytometry. The association of liposomes with cells and the generation of reactive oxygen species (ROS) were also measured by flow cytometry.

Results. The induction of apoptosis of RAW264.7 cells was dependent on the concentrations of stearylamine or cholesterol, a component of cationic liposomes. A significant correlation was observed between the degree of apoptosis and association of cationic liposomes with the cells. Coating the liposomal surface with polyethylene glycol (PEG) decreased the association of cationic liposomes with RAW264.7 cells and reduced the induction of apoptosis. Liposomal size also affected the induction of apoptosis, and larger liposomes showed a higher degree of apoptosis induction. Furthermore, ROS, which were required for the induction of apoptosis by cationic liposomes, were generated in a cholesterol content-dependent manner, and ROS generation was also decreased by PEG coating as the association and the induction of apoptosis were reduced.

Conclusions. The degree of apoptosis is related to the extent of association of cationic liposomes with cells and is related to the generation of ROS.

KEY WORDS: apoptosis; cationic liposome; macrophage; PEG-coating; reactive oxygen species.

INTRODUCTION

Liposomes are of considerable interest as carriers for controlled delivery of drugs because many substances can be encapsulated in aqueous and lipid phases (1). Cationic liposomes are currently expected to be good candidates as nonviral vectors because DNA readily forms a complex with cationic liposomes via electrostatic interactions (2,3). It is well known that cationic liposomes show cytotoxic effects (4,5). However, there have been few detailed studies of the cytotoxicity of cationic liposomes in the cells with which they interact, and the cause of the cytotoxicity remains unclear. To clarify the mechanism of cytotoxicity of cationic liposomes and regulate the expression of cytotoxicity would be advantageous for the development of safe nonviral vectors.

Apoptosis has been shown to play an indispensable role in multiple physiologic and pathologic processes, such as embryonic development, homeostatic maintenance of tissues and organs, maturation of the immune system, autoimmune disease, oncogenesis, and tumor progression (6,7). Recently, we investigated whether the cytotoxicity of cationic liposomes is a result of apoptosis, and we showed that cationic liposomes induced apoptosis in mouse splenic macrophages, the mouse macrophage-like cell line RAW264.7, and the mouse immature B-cell line WEHI 231 but not in rat hepatocytes or human hepatoblastoma HepG2 cells (8,9). The process of apoptosis induced by cationic liposomes was suggested to involve reactive oxygen species (ROS) production by lipoxygenase (8–10).

When liposomes are injected intravenously, they are rapidly removed from the blood circulation and taken up by macrophages in the reticuloendotherial system (RES) such as the liver and spleen (11–13). The interaction of liposomes with macrophages has been studied, and liposomal properties such as charge, size, and lipid composition have been shown to affect liposomal uptake by macrophages (14–16).

In this study, we examined the effects of liposomal lipid composition and size on the induction of apoptosis by cationic liposomes in RAW264.7 cells. The effects of polyethyleneglycol (PEG) coating on the induction of apoptosis were also examined. Furthermore, the effects of liposomal properties on the generation of ROS, which were required for the induction of apoptosis by cationic liposomes, were investigated. The findings obtained here indicated that the degree of apoptosis is related to the extent of association of cationic liposomes with cells and is related to the generation of ROS.

MATERIALS AND METHODS

Materials

The mouse macrophage-like cell line RAW264.7 was obtained from Riken Cell Bank (Ibaraki, Japan). Phosphatidylcholine (PC) from egg yolk was obtained from Nippon Oil and Fat Co. Ltd. (Tokyo, Japan). Phosphatidylserine (PS) from calf brain, stearylamine (SA), and cholesteryl 3β-*N*-(dimethylaminoethyl) carbamate (DC-Chol) were obtained from Sigma (St. Louis, MO). Cholesterol (Chol) was obtained from Wako Pure Chemical (Tokyo, Japan). Dipalmitoylphosphatidylethanolamine-PEG (DPPE-PEG; average MW of PEG is 2,000) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Birmingham, AL). *N*-[1-(2, 3-Dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium (DOTMA) was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). *N*-(α-Trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG) was from Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes, Inc. (Eugene, OR).

Preparation of Liposomes

Multilamellar liposomes composed of various phospholipids, cationic amphiphiles, and Chol were prepared by our

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previous methods (17). Briefly, 10 μmol of lipids in chloroform at various ratios were dried under reduced pressure into a thin film on the inner face of a glass tube. Following hydration with 1.0 ml of physiologic saline, multilamellar vesicles were prepared by vigorous vortexing. Liposomes were passed through a membrane filter (0.45 μm; Iwaki Co. Ltd., Tokyo) before use. To prepare DiI-labeled liposomes, 40 nmol DiI was added to 10 μmol liposomal lipids. When necessary, liposomes were passed through a polycarbonate membrane (pore sizes 0.8, 0.6, 0.2, and 0.1 μm, Nucleopore, Pleasanton, CA) using LiposoFast (Avestin, Inc., Ottawa, ON, Canada). Liposomal size was measured with a dynamic light-scattering spectrophotometer (DLS-7000, Otsuka Electronics, Tokyo, Japan). Liposomal ζ-potential was determined by rotating prism microelectrophoresis with a Pen Kem Laser Zee model 501 by measuring the electrophoretic mobility of liposomes in saline.

DNA Content

RAW264.7 cells (5×10^5) suspended in RPMI1640 medium supplemented with 10% FCS were treated with liposomes for 24 h at 37°C and then fixed with 70% ethanol at 4°C overnight. The cells were centrifuged at $500 \times g$ for 5 min, and ethanol was thoroughly removed. The cell pellets were resuspended in phosphate–citrate buffer and allowed to stand at room temperature for at least 30 min. After centrifugation at $500 \times g$ for 5 min, the cell pellets were suspended in RNase A solution (100 μg/ml, Amresco Inc., Dallas, TX) and incubated at 37°C for 30 min to deplete RNA. RNase A-treated cells were suspended in 1.0 ml of propidium iodide (PI, 50 μg/ml, Molecular Probes, Inc., Eugene, OR), and DNA contents in cells were evaluated by flow cytometry (FACSCalibur, Becton Dickinson).

Liposome-Cell Association

RAW264.7 cells (1×10^6) were incubated with DiI-labeled liposomes (0.5 μmol/ml) at 37°C for 1 h. The cells were washed with PBS, and then the association of liposomes was measured by flow cytometry.

Measurement of Intracellular Peroxide Levels

To investigate ROS generation, peroxide-sensitive 2′,7′-dichlorodihydrofluorescein diacetate (5 μM, DCFH/DA, Molecular Probes, Inc., Eugene, OR) was preloaded into RAW264.7 cells for 15 min. The cells were incubated with liposomes (0.5 μmol/ml) at 37°C for 30 min, and then the changes in fluorescence intensity were investigated by flow cytometry.

Membrane Fluidity

Membrane fluidity of liposomes with various cholesterol contents was estimated by fluorescence polarization using 1,6-diphenyl-1,3,5-hexatriene (DPH, Molecular Probes, Inc., Eugene, OR) as a fluorescence probe with excitation and emission wavelengths of 360 nm and 430 nm, respectively (18). Briefly, a tetrahydrofuran solution of DPH (1 mM) was diluted 1,000-fold with vigorously stirred liposome solution (0.1 μmol/ml). After incubation at 37°C for 3 min, fluorescence intensity was measured through a polarizer situated parallel

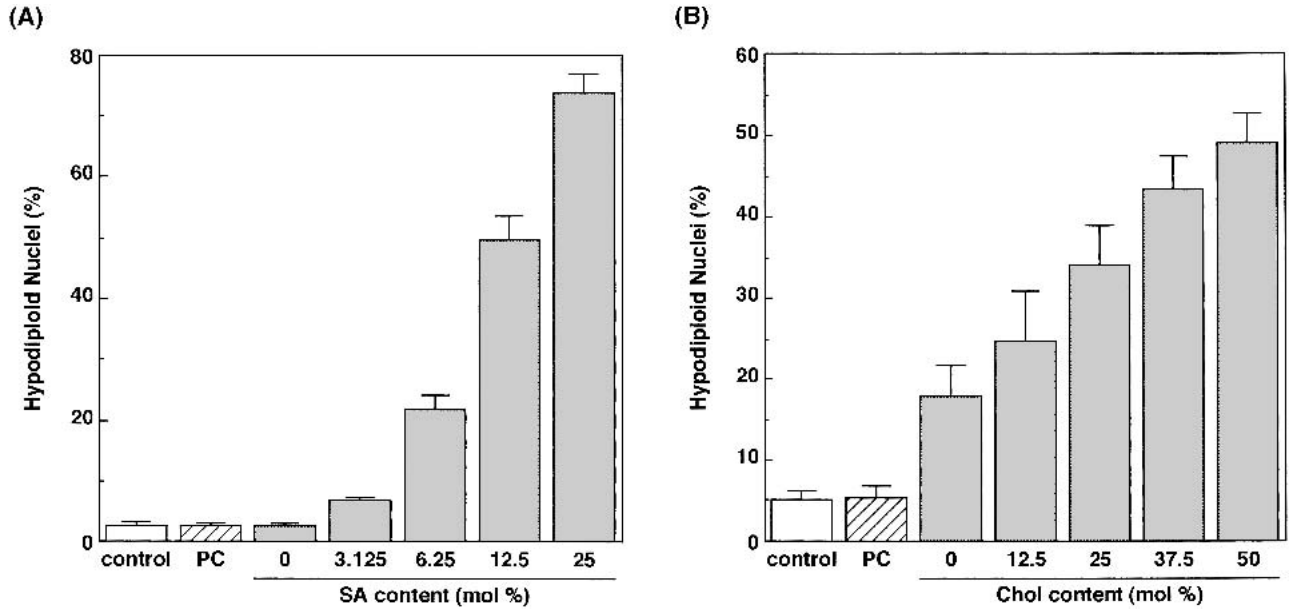

Fig. 1. Comparison of apoptosis induced by cationic liposomes with various stearylamine or cholesterol contents. RAW264.7 cells (5×10^5) were treated with liposomes (0.5 μmol/ml) for 24 h, and DNA contents were measured by flow cytometry following PI-staining. (A) Compositions of liposomes were SA:PC:Chol = 0:1:1 (SA content 0%, ζ– potential –0.50 ± 1.10 mV), SA:PC:Chol = 0.25:3.75:4 (SA content 3.125%, ζ–potential +0.77 ± 0.75 mV), SA:PC:Chol = 0.5:3.5:4 (SA content 6.25%, ζ–potential +22.03 ± 3.50 mV), SA:PC:Chol = 1:3:4 (SA content 12.5%, ζ–potential 32.86 ± 4.10 mV), SA:PC:Chol = 2:2:4 (SA content 25%, ζ–potential +42.60 ± 0.61 mV), and PC only (SA content 0%, Chol content 0%, ζ–potential –2.20 ± 1.84 mV). (B) Compositions of liposomes were SA:PC:Chol = 1:7:0 (Chol content 0%), SA:PC:Chol = 1:6:1 (Chol content 12.5%), SA:PC:Chol = 1:5:2 (Chol content 25%), SA:PC:Chol = 1:4:3 (Chol content 37.5%), SA:PC:Chol = 1:3:4 (Chol content 50%), and PC only (SA content 0%, Chol content 0%). Mean diameter of liposomes (SA:PC:Chol = 1:3:4) was 380 nm. Results represent the means ± SD of three experiments.

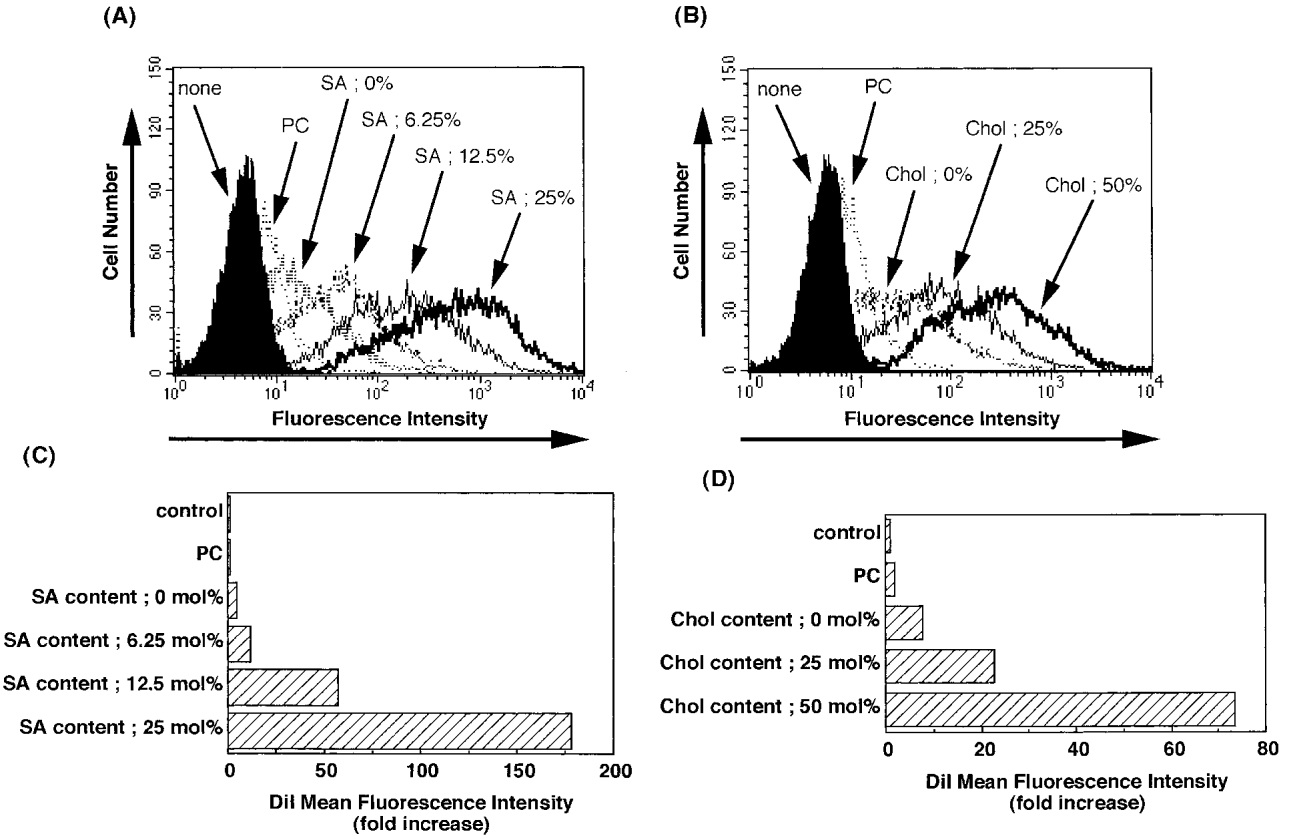

Fig. 2. Effects of stearylamine content (A,C) or cholesterol content (B,D) on the association of liposomes with RAW264.7 cells. RAW264.7 cells (1×10^6) were treated with DiI-labeled liposomes (0.5 μmol/ml) for 1 h at 37°C, and association of DiI-labeled liposomes was examined by flow cytometry. Flow cytometric histograms are shown in A and B, and the mean fluorescence intensities are shown in C and D. Compositions of liposomes were the same as described in Fig. 1.

(I_0) and perpendicular against the vertically polarized exciting beam (I_{90}). The fluorescence polarization (P) value was then calculated according to the formula:

$$P = (I_0 - I_{90})/(I_0 + I_{90})$$

RESULTS AND DISCUSSION

Effects of Stearylamine Content and Cholesterol Content

We recently investigated whether the cytotoxicity of cationic liposomes occurs through apoptosis and showed that cationic liposomes composed of various cationic lipids induced apoptosis in the mouse macrophage-like cell line RAW264.7 cells and mouse splenic macrophages as determined by monitoring DNA content and DNA fragmentation (8). Following intravenous injection, liposomes are rapidly removed from the blood circulation and taken up by macrophages in the reticuloendothelial system such as the liver and spleen (11–13). Liposomal properties such as charge and lipid composition influenced the recognition of liposomes by macrophages. Therefore, we investigated the effects of SA content or Chol content on the induction of apoptosis by cationic liposomes in RAW264.7 cells. The changes in DNA content of RAW264.7 cells following liposome treatment were measured by flow cytometry. As shown in Fig. 1A, the percentage of cells with hypodiploid DNA content increased with increasing SA concentrations in liposomes, and 70% of cells underwent apoptosis when cells were treated with cationic liposomes composed of 25 mol% SA.

Furthermore, the effects of Chol content of cationic liposomes containing 12.5 mol% SA on apoptosis were investigated, and the percentage of cells with hypodiploid DNA increased in a manner dependent on Chol content of the liposomes (Fig. 1B).

The percentage of cells with hypodiploid DNA content when cells were treated with liposomes composed of PC only (SA content 0 mol%, Chol content 0 mol%) was almost the same as untreated control (Fig. 1A,B).

To clarify whether different degrees of apoptosis of RAW264.7 cells induced by cationic liposomes composed of varying amounts of SA or Chol were related to the different extents of uptake, the association (sum of binding and uptake) of liposomes with RAW264.7 cells was investigated using DiI-labeled liposomes. The lipophilic carbocyanine dye

Table I. Effect of Cholesterol Content on the Fluidity of SA Liposomes

Liposome composition (molar ratio)	Chol content (%)	p value
SA:PC:Chol = 1:7:0	0	0.080
SA:PC:Chol = 1:6:1	12.5	0.091
SA:PC:Chol = 1:5:2	25	0.136
SA:PC:Chol = 1:4:3	37.5	0.182
SA:PC:Chol = 1:3:4	50	0.220

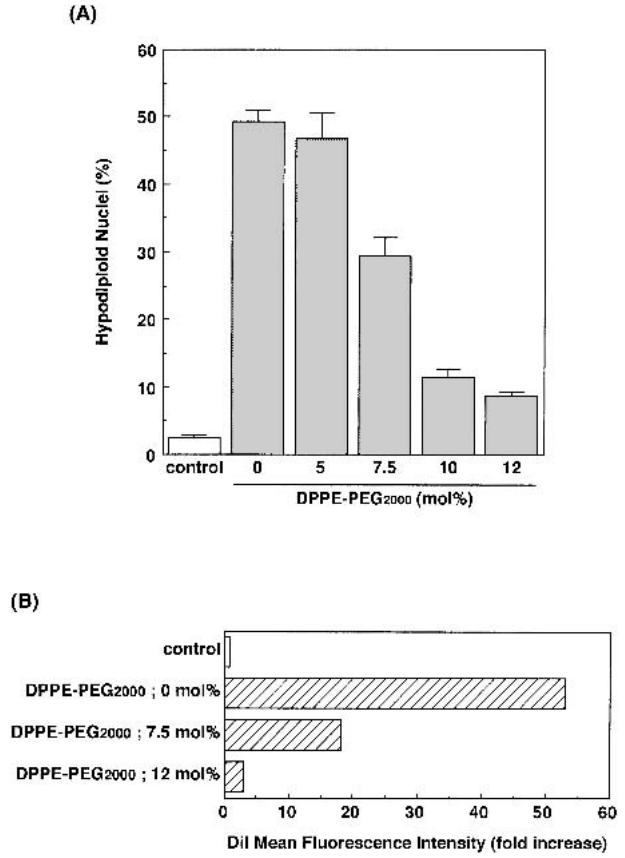

Fig. 3. Effects of PEG coating on apoptosis (A) and association (B) with RAW264.7 cells. Apoptosis and association were measured by flow cytometry as described in Materials and Methods. Compositions of liposomes were SA:PC:Chol = 1:3:4 (DPPE-PEG 0%); SA:PC:Chol:DPPE-PEG = 1:2.6:4:0.4 (DPPE-PEG 5%); SA:PC:Chol:DPPE-PEG = 1:2.4:4:0.6 (DPPE-PEG 7.5%); SA:PC:Chol:DPPE-PEG = 1:2.2:4:0.8 (DPPE-PEG 10%); SA:PC:Chol:DPPE-PEG = 1:2:4:1 (DPPE-PEG 12%).

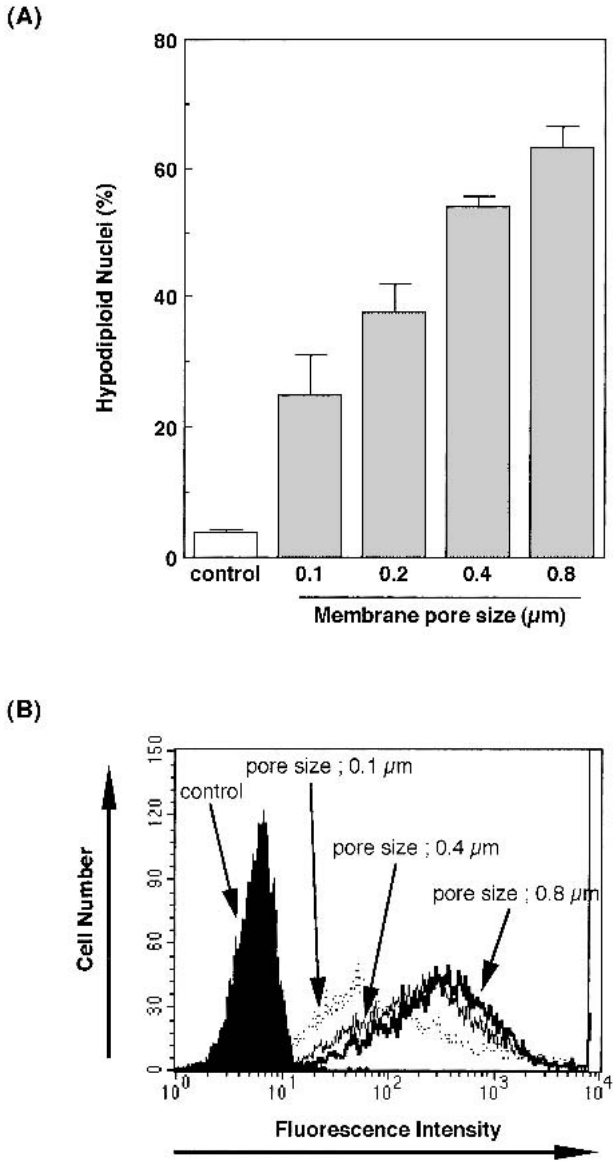

Fig. 4. Effects of liposomal size on apoptosis (A) and association (B) with RAW264.7 cells. Apoptosis and association were measured by flow cytometry as described in Materials and Methods. Compositions of liposomes were SA:PC:Chol = 1:3:4 (SA content 12.5%, Chol content 50%). Mean diameters of liposomes passed through the membranes with different pore sizes (0.8, 0.4, 0.2, or 0.1 μm) were 705, 336, 205, and 116 nm, respectively. Control: cells without treatment with liposomes.

DiI is a fluorescent liposomal lipid marker, and the associations of liposomes can easily be evaluated using flow cytometry. As shown in Fig. 2, DiI fluorescence intensity increased in proportion to SA or Chol content of liposomes, indicating that association of liposomes to the cells is dependent on SA or Chol content. Furthermore, DiI fluorescence intensity of liposomes composed of PC only was low and showed almost the same value as that of a control that was not treated with any liposomes.

Chol has membrane-stabilizing effects on phospholipid bilayers such as reducing membrane fluidity and decreasing membrane permeability above the transition temperature (19,20). Thus, the membrane fluidity of cationic liposomes with various Chol contents was measured. As shown in Table I, the p values of liposomes increased with Chol content, indicating that liposomal membrane fluidity decreased with increasing Chol content. There have been numerous reports concerning the uptake of liposomes by macrophages, and Chol-rich liposomes are taken up preferentially by macrophages rather than Chol-poor liposomes (13,21). In our previous study (8), cationic liposomes composed of SA and anionic liposomes composed of PS, both types of liposomes containing the same amount of Chol, showed the same association to RAW264.7 cells. However, apoptosis was observed when the cells were treated with the cationic liposomes. Thus, Chol may affect the association of liposomes to RAW264.7 cells, but the induction of apoptosis depends on the amount of SA that would be taken up into the cells.

We reported previously that cationic liposomes composed of SA did not induce apoptosis in rat hepatocytes or human hepatoblastoma HepG2 cells (8). Therefore, we conducted an experiment to clarify whether cationic liposomes [SA:PC:Chol = 1:3:4 (w/w)] were associated with rat hepatocytes or HepG2 cells using DiI-labeled liposomes. As expected, no marked increase in fluorescence intensity of cells was observed in rat hepatocytes or HepG2 cells (data not

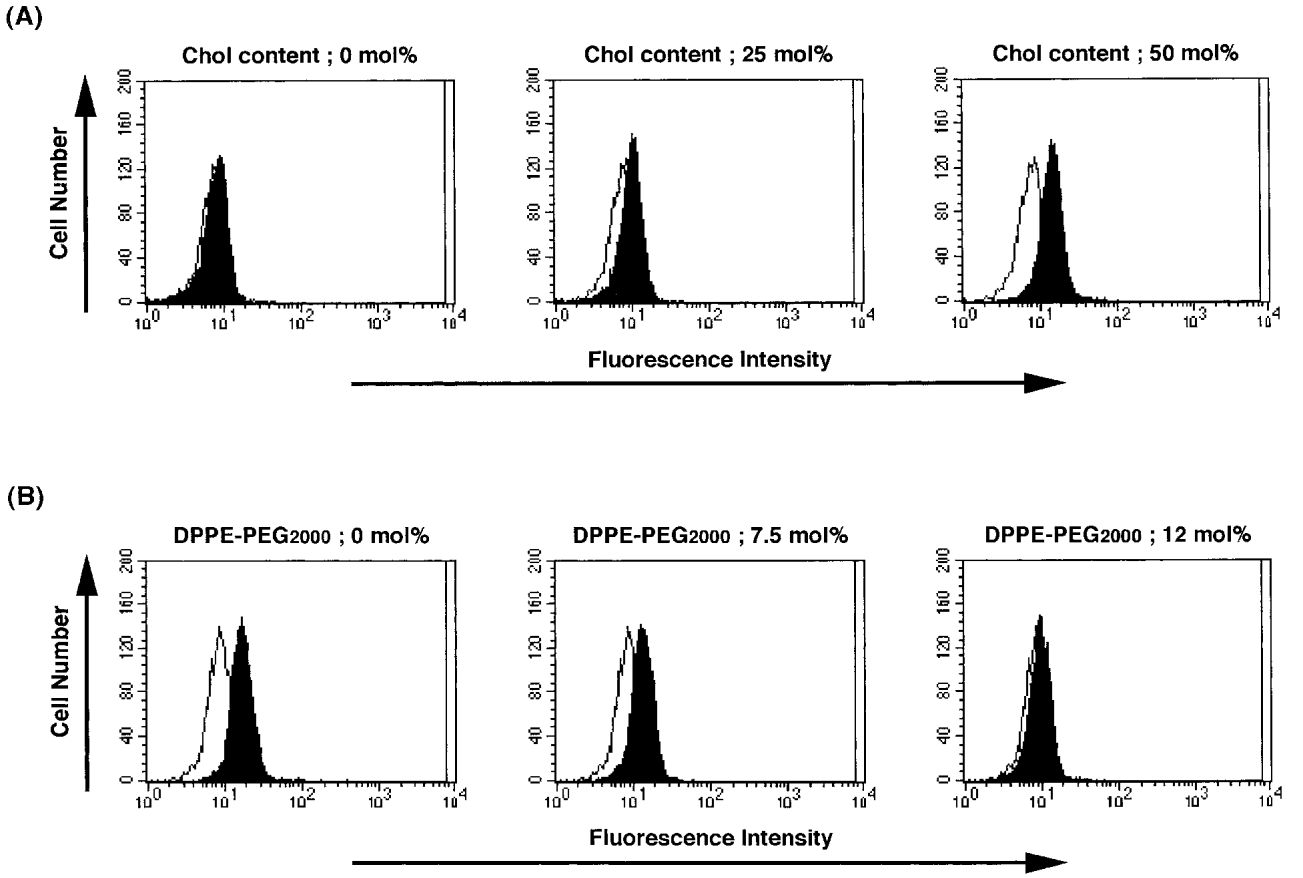

Fig. 5. Effects of cholesterol content (A) or PEG coating (B) on intracellular peroxide levels in RAW264.7 cells treated with cationic liposomes. RAW264.7 cells (1×10^6) preloaded with 5 μM DCFH/DA for 15 min were treated with liposomes (0.5 μmol/ml) for 30 min and then subjected to flow cytometry. Lipid compositions of liposomes used in A were SA:PC:Chol = 1:7:0 (Chol content 0%); SA:PC:Chol = 1:5:2 (Chol content 25%); SA:PC:Chol = 1:3:4 (Chol content 50%); and in B, SA:PC:Chol = 1:3:4 (DPPE-PEG 0%), SA:PC:Chol:DPPE-PEG = 1:2.4:4:0.6 (DPPE-PEG 7.5%); SA:PC:Chol:DPPE-PEG = 1:2:4:1 (DPPE-PEG 12%). Open histograms, untreated control; shaded histograms, liposome-treated cells.

shown). These findings also suggested that the association of liposomes with cells is a critical step in the induction of apoptosis by cationic liposomes.

Effects of PEG Coating

Coating the liposomal surface with PEG has been shown to prevent phagocytosis by macrophages in the reticuloendothelial system and to prolong circulation (22,23). To determine whether the association of cationic liposomes with macrophages was indispensable for the induction of apoptosis, we prepared cationic liposomes with incorporation of DPPE-PEG at the indicated concentrations. Induction of apoptosis was significantly reduced when RAW264.7 cells were treated with liposomes containing 7.5 mol% or more of DPPE-PEG, and almost complete inhibition was observed with liposomes containing 12 mol% DPPE-PEG (Fig. 3A). Furthermore, the association of liposomes with the cells was also decreased by incorporation of DPPE-PEG at 7.5 mol% or more (Fig. 3B).

It has been reported that 5 mol% of PEG is enough to evade RES uptake of liposomes (22,23). However, the induction of apoptosis by cationic liposomes containing 5 mol% DPPE-PEG was still observed (Fig. 3). This discrepancy may come from the differences in the molecular weight of PEG or liposomal size, but the details are now unclear.

Effects of Liposomal Size

It has been reported that liposomal size is an important factor for uptake by macrophages and that larger liposomes are taken up more readily (14,21). Cationic liposomes [SA:PC:Chol = 1:3:4 (w/w)] of different diameters were prepared by the extrusion method, and the induction of apoptosis was evaluated as DNA content. As shown in Fig. 4A, cells treated with larger liposomes showed a higher percentage of hypodiploid DNA content, indicating that apoptosis was dependent on the liposomal size. Furthermore, the association of liposomes with the cells was increased in a liposomal size-dependent manner (Fig. 4B). These results suggested that liposomes with large sizes that are easily taken up by macrophages have a large amount of SA to be taken up to induce apoptosis.

ROS Generation

It has been suggested that ROS may play an important role in the regulation of apoptotic cell death (24,25). We reported previously that ROS production was important in the regulation of apoptosis induced by cationic liposomes (8–10,26). Furthermore, Dokka *et al.* (27) reported that cationic liposomes composed of LipofectAMINE, frequently used as a

plasmid transfection agent, showed lung cytotoxicity, and ROS were suggested to play a key role in this cytotoxicity. Therefore, we investigated the effects of Chol content and PEG coating on cationic liposome-induced ROS generation in RAW264.7 cells.

ROS generation was estimated fluorometrically using DCFH/DA. DCFH/DA, which is deacetylated to the nonfluorescent compound 2′,7′-dichlorodihydrofluorescein (DCFH) in the cells, can be oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by a variety of peroxides (28). As shown in Fig. 5A, no increase in fluorescence intensity was observed in cationic liposomes containing 0 mol% Chol, and the fluorescence intensity increased with increasing Chol content in cationic liposomes. This increased fluorescence intensity was reduced by the incorporation of DPPE-PEG and reached the control level at 12 mol% DPPE-PEG (Fig. 5B). These findings suggested that the generation of ROS by cationic liposomes was Chol content dependent and was inhibited by PEG coating similarly to the induction of apoptosis and the association with cells. Previously, we examined the effect of liposomal charge on ROS generation from RAW264.7 cells using three types of liposomes—anionic, neutral, and cationic—containing the same concentrations of Chol, and ROS generation was observed only in the cell treated with cationic liposomes composed of SA (8). Therefore, liposomal Chol may contribute to the association, and a cationic component such as SA in the liposomes may participate in ROS generation. Furthermore, we found that lipoxygenase is responsible for ROS generation and that ROS acts as a key mediator in the progression of apoptosis induced by cationic liposomes (10). Lipoxygenases catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides, and superoxide radicals are produced in this process (29). Therefore, DCFH may react with hydroperoxides or hydrogen peroxides that were produced by catalases with superoxide radicals and be converted into the fluorescent compound DCF. Because activation of phospholipase A_2 (PLA_2) results in the release of arachidonic acid (AA), which is then oxidized by either lipoxygenase or cyclooxygenase, finally resulting in the formation of leukotrienes and prostaglandins. ROS may be produced by the increase in AA that is released by the action of PLA_2 following cationic liposome treatment. Experiments to clarify how PLA_2 is activated following cationic liposome treatment are currently under way in our laboratory. Therefore, cationic liposomes could be taken up by cells, and then lipoxygenase is activated for production of ROS.

We have reported that ROS production is important in the apoptosis induced by cationic liposomes (8). Liposomal Chol may contribute to the association, and a cationic component such as SA in the liposomes may participate in ROS generation in macrophage apoptosis. Further studies are necessary to resolve ROS generation following the association of cationic liposomes to the cells.

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