

Induction of apoptosis in macrophages by cationic liposomes

Yukihiko Aramaki*, Shuhei Takano, Seishi Tsuchiya

Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0355, Japan

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Abstract The effects of liposomes on apoptosis in macrophages were evaluated from DNA content and DNA fragmentation. Cationic liposomes composed of different kinds of cationic lipids induced apoptosis in mouse splenic macrophages and the macrophage-like cell line, RAW264.7 cells. Generation of reactive oxygen radicals from macrophages treated with cationic liposomes was detected using flow cytometry, and further apoptosis was inhibited by the addition of oxidant scavenger, *N*-acetylcysteine. From these findings, the production of reactive oxygen species may be important in the regulation of apoptosis induced by cationic liposomes.

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Key words: Apoptosis; Macrophage; Cationic liposome; Reactive oxygen species

1. Introduction

Liposomes are of considerable interest as carriers for the controlled delivery of drugs because many substances can be encapsulated in aqueous and lipid phases. One of the most well-known observations in the field of liposome research is that intravenously injected liposomes are rapidly taken up by macrophages in the reticuloendothelial system (RES) such as the liver and spleen [1]. Since this characteristic is advantageous for directing antigens or immunomodulating agents to macrophages, many investigators have used liposomes as adjuvants, depots for the slow release of antigens, and targeting agents for delivery of antigens to macrophages, which are one of antigen-presenting cells [2,3]. However, the changes liposomes exert in these cells with which liposomes interact remain unresolved.

Recently, the effects of liposomes on macrophage functions were investigated; Fcγ receptor-mediated phagocytosis was up-regulated [4,5] and nitric oxide production from macrophages stimulated with lipopolysaccharide was inhibited by anionic liposomes [6,7]. In this study, we examined whether liposomes cause apoptosis in cells with which liposomes interact.

Apoptosis plays a major role during development, homeostasis, and in many diseases including cancer and acquired immunodeficiency syndrome [8]. Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and eventual disintegration into membrane-enclosed apoptotic bodies [9,10].

Since liposomes are preferentially taken up by the liver and spleen, the effects of liposomes on the induction of apoptosis

of macrophages and hepatocytes were investigated by evaluating DNA content and DNA fragmentation. Cationic liposomes induced apoptosis in mouse splenic macrophages and the mouse macrophage-like cell line RAW264.7, but not in hepatocyte and human hepatoblastoma, HepG2 cells. Apoptosis induced by cationic liposomes depended on liposomal charge, but not on lipids composing liposomes, and the interaction of cationic liposomes with macrophages may have directly caused apoptosis. The oxidant scavenger *N*-acetylcysteine (NAC) inhibited macrophage apoptosis induced by cationic liposomes, thus, the involvement of reactive oxygen species (ROS) in this phenomenon was suggested.

2. Materials and methods

2.1. Materials

BALB/c mice and Wistar rats were purchased from SLC Co. Ltd. (Shizuoka, Japan). RAW264.7 and HepG2 cells were provided from Riken Cell Bank (Ibaraki, Japan). Phosphatidylcholine (PC) from egg yolk was donated by Nippon Oil and Fat Co. Ltd. (Tokyo, Japan). Phosphatidylserine (PS) from calf brain and propidium iodide (PI) were obtained from Sigma Co. Ltd. (St. Louis, MO, USA). Stearyl amine (SA) was obtained from Wako Pure Chemicals (Tokyo, Japan). 1,1'-Dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes (Eugene, OR, USA). *N*-(α -Trimethylammonio-acetyl)-didodecyl-D-glutamate chloride (TMAG) was from Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2. Preparation of liposomes

Liposomes of multilamellar vesicles (MLV) were prepared by vortexing and were passed through a membrane filter (0.45 μ m; Corning Glass Works, Corning, NY, USA) before use. Lipid compositions of liposomes were PC:cholesterol = 1:1 (PC-liposomes, neutral liposomes), PC:PS:cholesterol = 1.5:0.5:1.5 (PS-liposomes, anionic liposomes), and PC:SA:cholesterol = 1.5:0.5:1.5 (SA-liposomes, cationic liposomes). Small unilamellar vesicles (SUV) of SA-liposomes were prepared by sonication.

2.3. Preparation of mouse splenic macrophages and rat hepatocytes

Mouse splenic macrophages were prepared as described by Suzuki et al. [11]. Briefly, the spleens were teased through sterile stainless steel screens (mesh 150) into ice-cold RPMI 1640 medium containing 10% fetal calf serum (FCS). The single cells were treated with Tris-(hydroxymethyl) aminomethane-buffered ammonium chloride to lyse the contaminated erythrocytes and then washed three times with RPMI 1640 medium containing FCS. The splenic macrophages were removed by adherence to plastic Petri dishes, and non-adherent cells were prepared. Splenic macrophages were treated with liposomes at the indicated concentrations for 24 h.

Rat hepatocytes were isolated from male Wistar rats by the *in situ* collagenase perfusion method [12]. Isolated cells were suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1 mixture) medium (Gibco BRL) supplemented with 10% FCS and were routinely 60–70% single and 90–95% viable.

2.4. Cell culture

Mouse splenic macrophages and RAW264.7 cells suspended in RPMI 1640 medium supplemented with 10% FCS and rat hepatocytes and HepG2 cells suspended in DMEM and DMEM/F12 medium supplemented with 10% FCS were incubated with PC-, PS- or

*Corresponding author. Fax: (81) (426) 76-3182.
E-mail: aramaki@ps.toyaku.ac.jp

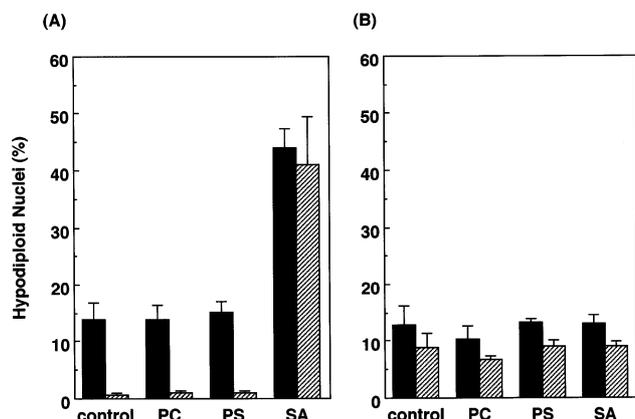


Fig. 1. Effects of liposomes on apoptosis of macrophages and hepatocytes. Cells were treated with three kinds of liposomes (0.5 μmol lipid/ml) for 24 h, and the percentage of cells showing DNA degradation was examined using flow cytometry. Results represent the mean \pm S.D. of three experiments. A: Spleen macrophages (shaded columns) and RAW264.7 cells (striped columns). B: Rat primary hepatocytes (shaded columns) and HepG2 cells (striped columns).

SA-liposomes for the indicated time. DNA content and DNA fragmentation were evaluated using a flow cytometer and agarose gel electrophoresis, respectively.

2.5. DNA content

Liposome-treated macrophages or hepatocytes (1×10^6 cells) were fixed with 70% ethanol at 4°C overnight. The cells were then 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 $1000 \times g$ for 5 min, the cell pellets were suspended in RNase A solution (100 $\mu\text{g}/\text{ml}$, Amresco Inc., Dallas, TX, USA) and incubated at 37°C for 20 min to deplete RNA. RNase A-treated cells were suspended in 1.0 ml of propidium iodide (PI, 50 $\mu\text{g}/\text{ml}$) and the cells were subjected to flow cytometry (Becton Dickinson, FACSCalibur).

2.6. DNA fragmentation

Liposome-treated RAW264.7 cells (1×10^6 cells) were lysed using a lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100) at 4°C for 45 min. The cell lysate was incubated with RNase A (400 $\mu\text{g}/\text{ml}$) at 37°C for 60 min to digest RNA, and then further incubated with proteinase K (400 $\mu\text{g}/\text{ml}$, Merck,) for 60 min to digest protein. DNA was extracted using phenol:chloroform:isoamyl alcohol = 25:24:1 according to the method of Fukuda et al. [13]. The extracted DNA was dissolved in TE buffer (10 mM Tris buffer (pH 7.4) containing 1 mM EDTA), and subjected to 2% agarose gel electrophoresis as described previously [14].

2.7. Liposome association and ROS generation

RAW264.7 cells (5×10^5 cells/ml) were incubated with DiI-labeled liposomes (0.5 $\mu\text{mol}/\text{ml}$) at 37°C for 1 h. The cells were washed with

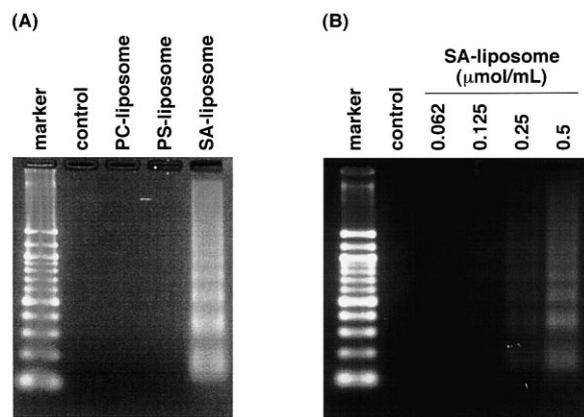


Fig. 2. Agarose gel electrophoresis of DNA extracted from RAW264.7 cells treated with liposomes. A: RAW264.7 cells were treated with three kinds of liposomes (0.5 μmol lipid/ml) for 24 h, and then DNA fragmentation was analyzed using gel electrophoresis. B: Effects of liposomal dose of SA-liposomes (0.062–0.5 μmol lipid/ml).

PBS and then the association of liposomes to the cells was measured by flow cytometry.

To investigate ROS generation, 2',5'-dichlorofluorescein diacetate (DCFH/DA, 5 μM) was pre-loaded to RAW264.7 cells for 15 min. The cells were incubated with liposomes (0.5 $\mu\text{mol}/\text{ml}$) at 37°C for 30 min, and then the change in fluorescence intensity was investigated by flow cytometry.

3. Results and discussion

3.1. DNA content and DNA fragmentation

Apoptosis is characterized by chromatin condensation, DNA fragmentation, and eventual disintegration into membrane-enclosed apoptotic bodies [9,10]. The induction of apoptosis in macrophages and hepatocytes by liposomes was evaluated from DNA content and DNA fragmentation. The changes in DNA content of macrophages and hepatocytes following treatment with liposomes (0.5 μmol lipid/ml) were examined using flow cytometry. As shown in Fig. 1A, 45% of splenic macrophages and RAW264.7 cells treated with cationic liposomes (SA-liposomes) for 24 h had hypodiploid DNA content. DNA contents of controls in splenic macrophages were higher than those of RAW264.7 cells, but those of PC- and PS-liposome-treated samples were similar to those of controls. In contrast, about 10% of rat hepatocytes and HepG2 cells had hypodiploid DNA content after treatment with liposomes irrespective of liposomal charge, and this value was similar to that of controls (Fig. 1B).

Table 1
Effect of various liposomes on apoptosis of RAW264.7 cells

| Lipid composition | Dose ($\mu\text{mol}/\text{ml}$) | Cationic lipid content (nmol/ml) | Hypodiploid nuclei (%) | ζ -potential (mV) |
|------------------------------|------------------------------------|----------------------------------|------------------------|-------------------------|
| Control (no stimulus) | – | – | 2.57 ± 0.15 | – |
| PC:Chol = 1:1 | 0.5 | – | 2.28 ± 0.17 | -0.51 ± 1.10 |
| PC:PS:Chol = 1.5:0.5:2 | 0.5 | – | 2.33 ± 0.10 | -23.84 ± 1.67 |
| PC:SA:Chol = 1.5:0.5:2 (MLV) | 0.5 | 62.5 | 38.72 ± 0.46 | 34.06 ± 1.30 |
| PC:SA:Chol = 1.5:0.5:2 (SUV) | 0.5 | 62.5 | 17.08 ± 0.46 | 34.62 ± 1.41 |
| PC:TMAG:Chol = 1.5:0.5:2 | 0.5 | 62.5 | 17.92 ± 3.99 | 17.78 ± 1.57 |
| DC-Chol:DOPE = 3:2 | 0.125 | 75 | 17.46 ± 1.14 | 58.51 ± 2.17 |
| Lipofectin | 0.01 | 54 | 59.03 ± 2.34 | 39.15 ± 1.94 |

RAW264.7 cells were treated with liposomes (0.5 μmol lipid/ml) for 24 h and the percentage of cells showing DNA degradation was examined by flow cytometry. Liposome charges measured as the ζ -potential were determined in saline at 25°C. Results represent the mean \pm S.D. of three experiments. Mean diameters of SUV and MLV of SA-liposomes were 78 nm and 380 nm, respectively.

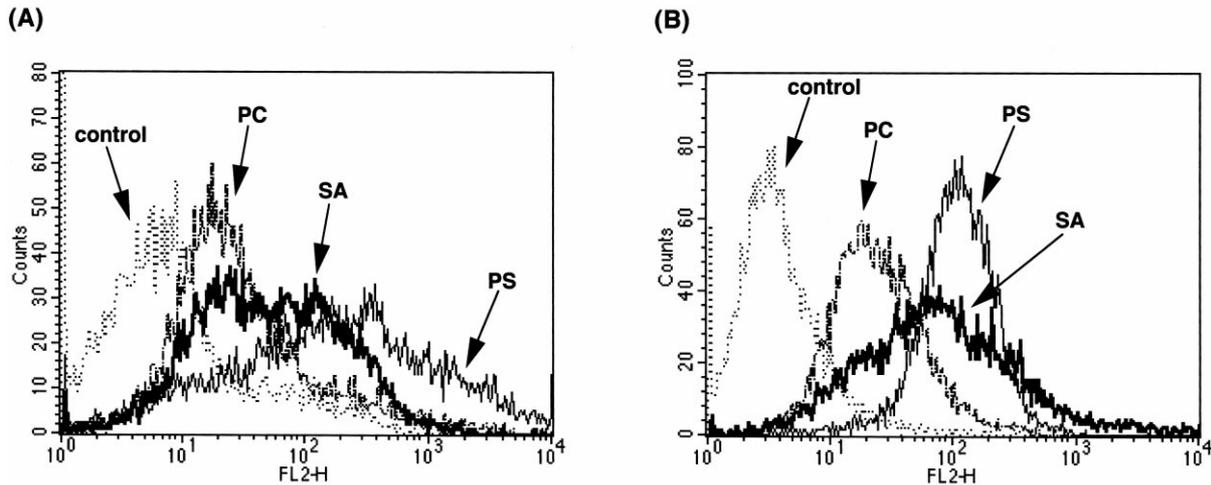


Fig. 3. Flow cytometric analysis of liposome uptakes in macrophages. Spleen macrophages (A) and RAW264.7 cells (B) were treated with DiI-labeled liposomes (0.5 $\mu\text{mol lipid/ml}$) for 1 h at 37°C, and the uptakes of DiI-labeled liposomes was examined using flow cytometry.

In cells undergoing apoptosis, nuclear DNA is fragmented into the size equivalent of DNA into mono- or oligonucleosomes [15]. DNA was extracted from RAW264.7 cells treated with liposomes, and DNA fragmentation was examined using 2% agarose gel electrophoresis. As shown in Fig. 2A, DNA ladders were observed in DNA extracted from SA-liposome-treated cells, but no DNA fragmentation was found in PC- or PS-liposome-treated cells. The DNA ladder was clearly observed when RAW264.7 cells were treated with SA-liposomes at concentrations of more than 0.25 $\mu\text{mol lipid/ml}$ (Fig. 2B). From these findings, SA-liposomes induced apoptosis in mouse splenic macrophages and RAW264.7 cells.

3.2. Effects of liposomal lipid composition

The effects of liposomes on apoptosis of RAW264.7 cells were investigated using liposomes composed of different kinds of cationic lipids, and the induction of apoptosis was evaluated as DNA content. All cationic liposomes induced apoptosis in RAW264.7 cells (Table 1). The effect of liposomal size on the apoptosis was investigated using SA-liposomes of SUV and MLV, and MLV induced higher apoptosis comparing to SUV. This result may come from the difference in the amount of cationic lipid taken up by RAW264.7 cells. Lipofectin and DC-Chol are often used as DNA transfection agents. Lipofectin composed of dimethyldioctadecyl ammonium bromide and dioleoyl phosphatidylethanolamine (DOPE) showed the highest apoptosis in low concentrations. Although liposomes composed of DC-Chol and DOPE showed a high ζ -potential, the intensity of apoptosis was relatively low. These findings suggest that apoptosis induced by cationic liposomes corresponds not only to cationic charge but also to the structure of the cationic lipid composing liposomes.

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3.3. Association of liposomes

Cationic liposomes are taken up by a variety of cells through electrostatic interaction between the cationic charge of the liposomes and the anionic charge of the cells, whereas in anionic liposomes macrophages take them up through scavenger receptors [16,17]. To clarify whether apoptosis of RAW264.7 cells induced by liposomes came from the differences in the amount of liposomes taken up, the association (sum of binding and uptake) of liposomes by RAW264.7 cells was investigated using DiI-labeled liposomes. DiI is a fluorescence probe and the association of liposomes can easily be evaluated using flow cytometry. As shown in Fig. 3, the fluorescence intensity of cells incubated with anionic liposomes (PS-liposomes) at 37°C for 60 min was higher than that of cationic liposomes (SA-liposomes) in both splenic macro-

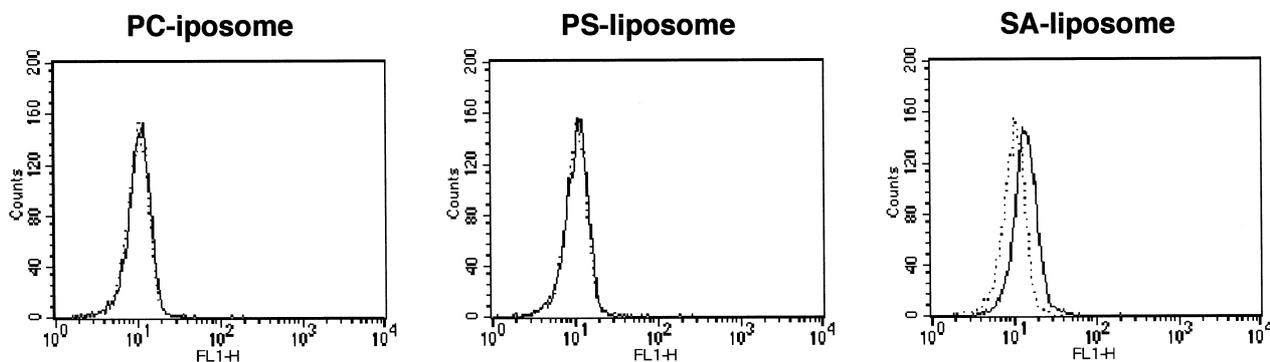


Fig. 4. Flow cytometric analysis of intracellular peroxide levels in RAW264.7 cells treated with liposomes. RAW264.7 cells pre-loaded with 5 μM DCFH/DA for 15 min were treated with liposomes (0.5 $\mu\text{mol lipid/ml}$) for 30 min, and subjected to flow cytometry. Dotted line, untreated control; solid line, SA-liposome-treated.

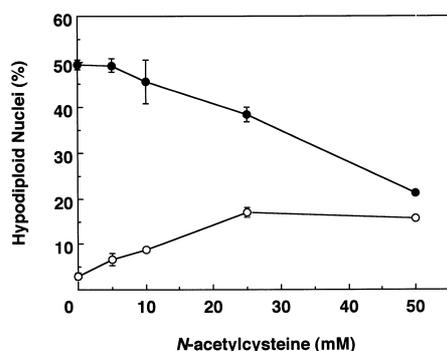


Fig. 5. Effect of NAC on apoptosis induced by liposomes in RAW264.7 cells. RAW264.7 cells were treated with SA-liposomes (0.5 $\mu\text{mol lipid/ml}$) for 24 h in the presence of NAC (0–50 mM), and the percentage of cells showing DNA degradation was examined using flow cytometry. Results represent the mean \pm S.D. of three experiments. Open circle, untreated control; closed circle, SA-liposome-treated.

phages and RAW264.7 cells. These findings indicate that the difference in amount of liposomes associated with macrophages was not involved in the induction of apoptosis.

3.4. Involvement of ROS in the apoptosis

Recently, Brown and Savill [18] reported that macrophages secrete Fas ligand (Fas-L) into the culture supernatant following the phagocytosis of opsonized zymosan. Therefore, we conducted an experiment to clarify whether RAW264.7 cells secrete a factor that induces apoptosis into the culture supernatant following treatment with cationic liposomes. Culture supernatant obtained from RAW264.7 cells treated with SA-liposomes was added to naive RAW264.7 cells, but no apoptosis was observed. Furthermore, the expression of Fas-L mRNA was investigated with RT-PCR following the treatment of cationic liposomes. However, no expression of Fas-L mRNA was observed (data not shown). These findings suggest that the secretion of apoptosis-inducing factor or the expression of Fas-L could be negligible.

It has been reported that ROS are major effectors in apoptotic cell death [9,19,20]. Thus, the production of ROS after the treatment of RAW264.7 cells with liposomes was estimated fluorometrically using DCFH-DA (Eastman Kodak) as the substrate according to the method of Kohno et al. [21]. As shown in Fig. 4, RAW264.7 cells were incubated with liposomes for 30 min, and the fluorescein intensity of cells increased by the treatment with SA-liposomes, but not PC- or PS-liposomes. Furthermore, the effects of NAC on the induction of apoptosis by SA-liposomes was investigated, and apoptosis of RAW264.7 cells was reduced by NAC in a dose-dependent manner, and a complete inhibition was observed at 50 mM of NAC (Fig. 5). NAC has been shown to be an oxidant scavenger and to induce intracellular GSH levels [22], thus, ROS production and intracellular thiol levels are important in the regulation of apoptosis by cationic liposomes.

Recently, Suzuki et al. [23] reported that ROS generation was very important in Fas-mediated apoptosis of human B lymphoma cells, and NADPH oxidase activation was responsible for Fas-mediated ROS generation because the generation was inhibited by the addition of diphenylene iodium (DPI), a specific inhibitor of NADPH oxidase. To determine ROS gen-

eration via NADPH oxidase activation in cells sensitive to liposome-mediated apoptosis, the effect of DPI on SA-liposome-mediated apoptosis of RAW264.7 cells was investigated. However, no inhibition of apoptosis induced by SA-liposomes was observed (data not shown). Hence, ROS generation through NADPH oxidase activation may not be involved in SA-liposome-mediated apoptosis of RAW264.7 cells.

DNA is polyanionic and easily forms a complex with cationic liposomes via electrostatic interactions. Cationic liposomes are therefore candidates as non-viral vectors [24,25]. Furthermore intravenously injected liposomes are rapidly taken up by macrophages in the liver and spleen [2,3], and this characteristic is advantageous for directing antigens or immunomodulating agents to macrophages, therefore many investigators have used liposomes as adjuvants. Nakanishi et al. [26] reported that cationic liposomes functioned as an adjuvant enhancing humoral and cellular immunity. In this study, cationic liposomes were shown to induce apoptosis, which may deleteriously affect application of cationic liposomes as non-viral vectors or immune adjuvants. It is well known that cationic liposomes show cytotoxicity [27], and apoptosis may be one of the causes of this cytotoxicity.

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