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Lipoxygenase May Be Involved in Cationic Liposome-Induced Macrophage Apoptosis

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The purpose of this study was to determine the source of reactive oxygen species (ROS) generation and the contribution of ROS to the apoptosis of RAW264.7 cells induced by cationic liposomes. Cationic liposome-induced apoptosis was inhibited by lipoxygenase inhibitors, but not inhibitors of NADPH-oxidase, xanthine oxidase or cyclooxygenase. ROS generation induced by cationic liposomes was also inhibited by the lipoxygenase inhibitor NDGA. Furthermore, lipid peroxidation was observed following liposome treatment, but the apoptosis was not inhibited by the antioxidant α-tocopherol. These findings suggested that lipoxygenase is responsible for ROS generation, and ROS but not lipid peroxidation acts as a key mediator in the progress of apoptosis induced by cationic liposomes.

Key Words: **apoptosis; macrophages; cationic liposome; reactive oxygen species; lipoxygenase.**

Cationic liposomes are currently expected to be good candidates as nonviral vectors, because DNA readily forms a complex with cationic liposomes via electrostatic interactions (1). Cationic liposomes show cytotoxic effects (2), however, there have been few detailed studies of the cytotoxicity of cationic liposomes in the cells with which they interact.

Apoptosis is essential for development and maintenance of homeostasis during embryogenesis, cell growth and elimination of damaged cells in eukaryotic organisms (3). Recently, we investigated whether the cytotoxicity of cationic liposomes is due to apoptosis, and we showed that cationic liposomes induced apoptosis in mouse splenic macrophages (4), the mouse macrophage-like cell line RAW264.7 (4), and the immature B cell line WEHI 231 (5), but not in rat hepatocytes or human hepatoblastoma HepG2 cells (4). The process of apoptosis induced by cationic liposomes was suggested to involve reactive oxygen species (ROS) (4, 5), but the mechanism of ROS generation is still unclear.

ROS has been implicated in the apoptotic cell death pathway following treatment with a variety of agents including TNF-α (6), Fas (7), ceramide (8) and nerve growth factor withdrawal (9). Mitochondria play a key role in the regulation of apoptosis, and the predominant source of ROS generated in most apoptotic cells is considered to be this organelle (10). However, the most important system for ROS generation in phagocytes is NADPH-oxidase. Recently, several reports have suggested that NADPH-oxidase contributes to the induction of apoptosis in leukocytes (11, 12). Furthermore, the catabolism of purine nucleotides, the metabolism of fatty acids, and many other biological reactions can produce ROS (13).

In this study, to determine the source of ROS generation, the effects of several inhibitors of NADPH oxidase, xanthine oxidase, cyclooxygenase, and lipoxygenase on cationic liposome-mediated apoptosis of RAW264.7 cells were investigated by evaluating DNA content using flow cytometry. Lipoxygenase inhibitors prevented the decreases in DNA content, DNA fragmentation, and the ROS generation induced by treatment with cationic liposomes, suggesting that lipoxygenase acts as a key enzyme to generate ROS, implicated in the apoptosis of RAW264.7 cells induced by cationic liposomes.

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, dipalmitoylphosphatidylcholine (DPPC) was obtained from Nippon Oil and Fat Co. Ltd. (Tokyo, Japan). Phosphatidylserine (PS) from calf brain and stearylamine (SA) were obtained from Sigma (St. Louis, MO). Diphenyl iodonium was obtained from Dojindo (Kumamoto, Japan); apocynin was from Aldrich Chemical Company Inc. (Milwaukee, WI); allopurinol and indomethacin were from Wako Pure Chemicals; nordihydroguaiaretic acid (NDGA), curcumin, and 5,8,11,14-eicosatetraynoic acid (ETYA) were from Sigma.

Preparation of liposomes. Liposomes of multilamellar vesicles (MLV) were prepared by vortexing and were passed through a membrane filter (0.45 μm; Iwaki Co. Ltd., Tokyo) before use. Lipid com-

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positions of liposomes were PC:cholesterol = 1:1 (PC-liposomes; neutral liposomes), PC:PS:cholesterol = 1.5:0.5:2 (PS-liposomes; anionic liposomes), and PC:SA:cholesterol = 1.5:0.5:2 (SA-liposomes; cationic liposomes).

DNA content and DNA fragmentation. RAW264.7 cells (1×10^6) suspended in RPMI1640 medium supplemented with 10% FCS were treated with liposomes in the presence of enzyme inhibitors for the indicated times, and then fixed with 70% ethanol at 4°C overnight. The cells were centrifuged at 500*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*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).

For determination of DNA fragmentation, DNA was extracted from liposome-treated cells using phenol: chloroform: isoamylalchol = 25:24:1 according to the method of Fukuda *et al.* (14). The extracted DNA was dissolved in 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA, and subjected to 2% agarose gel electrophoresis as described previously (4).

ROS generation. ROS generation was measured by our previously described method (4). 2′,5′-Dichlorofluorescein diacetate (5 μM, DCFH-DA, Molecular Probes, Inc.) was preloaded into RAW264.7 cells for 15 min. The cells were incubated with liposomes (0.5 μmol lipid/ml) at 37°C for 30 min, and then the change in fluorescence intensity was investigated by flow cytometry (FACSCalibur, Becton–Dickinson).

Lipid peroxidation. RAW264.7 cells (5×10^6) suspended in RPMI1640 medium supplemented with 10% FCS were treated with liposomes for the indicated times. Then the cells were analyzed for 2-thiobarbituric acid reactive substance (TBARS) according to the method of Beuge and Aust (15), and the extent of lipid peroxidation was expressed as absorbance at 532 nm/μg protein.

TABLE 1

Effects of Various Antioxidants on Apoptosis Induced by Liposomes

Compounds	Hypodiploid nuclei (%)
None	2.05 ± 0.52
PC-liposome	2.18 ± 0.25
PS-liposome	2.35 ± 0.17
SA-liposome	49.41 ± 0.97
SA-liposome + NAC (10 mM)	45.64 ± 4.71
SA-liposome + NAC (25 mM)	38.61 ± 1.59
SA-liposome + NAC (50 mM)	21.13 ± 0.36
SA-liposome + DPI (1 μM)	53.67 ± 0.56
SA-liposome + DPI (5 μM)	52.83 ± 0.29
SA-liposome + DPI (10 μM)	55.02 ± 0.62
SA-liposome + Apocynin (50 μM)	53.55 ± 0.75
SA-liposome + Apocynin (100 μM)	54.62 ± 0.16
SA-liposome + Apocynin (250 μM)	55.88 ± 0.28
SA-liposome + Allopurinol (0.5 mM)	52.01 ± 0.49
SA-liposome + Allopurinol (1 mM)	53.32 ± 0.85
SA-liposome + Allopurinol (5 mM)	55.28 ± 0.19
SA-liposome + Indomethacin (0.1 mM)	49.27 ± 0.81
SA-liposome + Indomethacin (0.5 mM)	49.52 ± 0.84
SA-liposome + Indomethacin (1 mM)	50.43 ± 0.92
SA-liposome + NDGA (10 μM)	46.41 ± 4.65
SA-liposome + NDGA (25 μM)	39.79 ± 1.76
SA-liposome + NDGA (50 μM)	31.11 ± 2.63
SA-liposome + curcumin (5 μM)	43.31 ± 0.81
SA-liposome + curcumin (10 μM)	36.06 ± 2.26
SA-liposome + curcumin (20 μM)	25.31 ± 4.21
SA-liposome + ETYA (25 μM)	45.99 ± 9.71
SA-liposome + ETYA (50 μM)	40.57 ± 6.08
SA-liposome + ETYA (100 μM)	35.67 ± 6.22

RESULTS AND DISCUSSION

Effects of Lipoxygenase Inhibitors on Apoptosis

It has been suggested that ROS may play a critical role in regulating apoptosis (6–9), and the predominant source of ROS generated in most apoptotic cells is the mitochondria (10, 16, 17). We previously reported that ROS generation was involved in the apoptosis of RAW264.7 cells induced by cationic liposomes, and that ROS generation led to changes in the mitochondrial membrane potential and release of cytochrome *c* (18). However, the mechanism of ROS generation by cationic liposomes is still unclear. The most important system for ROS generation in phagocytic cells is NADPH oxidase. Recently, several reports have suggested that NADPH oxidase contributes to the induction of apoptosis in leukocytes (11, 12). Furthermore, the catabolism of purine nucleotides, the metabolism of fatty acids, and many other biological reactions can produce ROS (13). To determine the source of ROS generation, the effects of specific inhibitors of NADPH oxidase, xanthine oxidase, cyclooxygenase and lipoxygenase on cationic liposome-mediated apoptosis of RAW264.7 cells were investigated by evaluating DNA contents by flow cytometry. As shown in Table I, 49% of RAW264.7 cells treated with SA-liposomes for 24 h had hypodiploid DNA content, indicating that half of RAW264.7 cells underwent apoptosis following treatment with SA-liposomes. Hypodiploid DNA contents of cells treated with PC- (neutral liposomes) and PS-liposomes (anionic liposomes) were low and similar to the control levels. Addition of *N*-acetylcysteine (NAC) decreased hypodiploid DNA content in a dose-dependent manner. NAC has been shown to be an oxidant scavenger (19); thus, ROS generation is important in the regulation of apoptosis by cationic liposomes. On the other hand, diphenylene iodium (DPI, an inhibitor of NADPH oxidase), apocynin (an inhibitor of NADPH oxidase), allopurinol (an inhibitor of xanthine oxidase), and indomethacin (an inhibitor of cyclooxygenase) showed no effects on hypodiploid DNA content, suggesting that these inhibitors have no protective effect against apoptosis of RAW264.7 cells, and NADPH oxidase, xanthine oxidase and cyclooxygenase do not contribute to the apoptosis induced by SA-liposomes that is required for ROS generation. However, hypodiploid DNA content decreased significantly when the cells were treated with the lipoxygenase in-

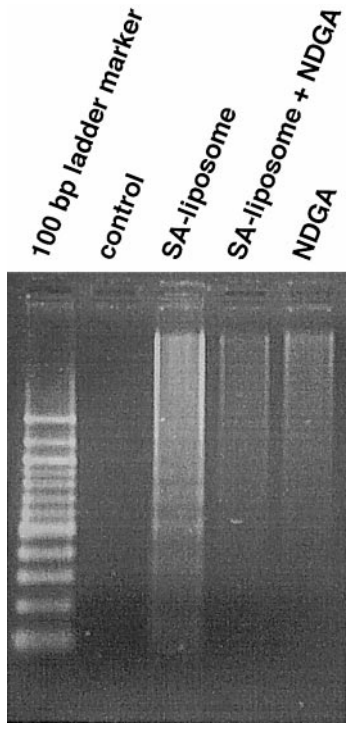

FIG. 1. Agarose gel electrophoresis of DNA extracted from RAW264.7 cells treated with liposomes. RAW264.7 cells (1×10^6) were treated with SA-liposomes (0.5 μmol lipid/mL) for 24 h with or without the lipoxygenase inhibitor NDGA (50 μM), and then DNA fragmentation was analyzed by gel electrophoresis.

hibitors, NDGA, curcumin and ETYA. In cells undergoing apoptosis, nuclear DNA is cleaved into fragments equivalent in size to DNA in mono- or oligonucleosomes (20). DNA was extracted from RAW264.7 cells treated with liposomes, and DNA fragmentation was examined using 2% agarose gel electrophoresis. As shown in Fig. 1, DNA ladders were observed in DNA extracted from SA-liposome-treated cells, but no DNA fragmentation was found when cells were treated with SA-liposomes in the presence of the lipoxygenase inhibitor NDGA. Conflicting results concerning the effects of lipoxygenase inhibitors on the induction of apoptosis were reported recently: lipoxygenase inhibitors were shown to be capable of inducing apoptosis in some cell systems (21), while some lipoxygenase inhibitors blocked apoptosis in some systems with induction of lipoxygenase activity by CD95 (22), hydrogen peroxide (23) or γ-irradiation (24). Lipoxygenase inhibitors suppressed the SA-liposome-induced apoptosis of RAW264.7 cells, suggesting that lipoxygenase is involved in the induction of apoptosis in this system.

ROS Generation

ROS generation after treatment of RAW264.7 cells with SA-liposomes was estimated fluorometrically using DCFH-DA. DCFH-DA, which is deacetylated to the nonfluorescent compound 2′,7′-dichlorofluorescein in the cells, can be oxidized to the fluorescent compound 2′,7′-dichlorofluorescein by a variety of peroxides (25). As shown in Fig. 2, the fluorescence intensity increased by treatment with SA-liposomes, indicating that cells demonstrated oxidation of DCFH-DA to 2′,7′-dichlorofluorescein. The pretreatment of cells with the lipoxygenase inhibitor NDGA restored the fluorescence intensity to the control level. These findings suggested

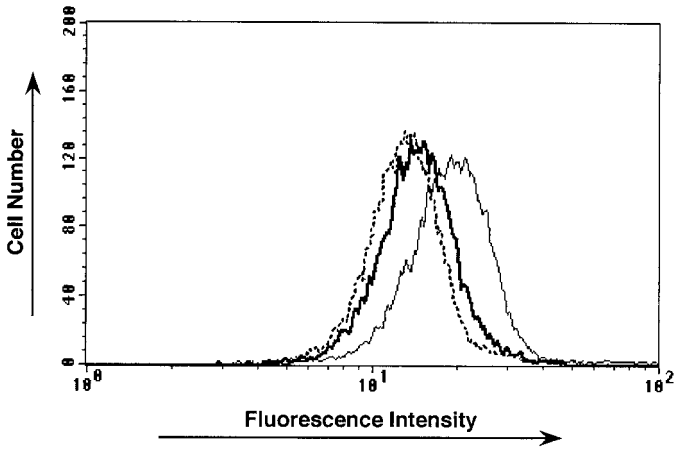

FIG. 2. Flow cytometric analysis of intracellular peroxide levels in RAW264.7 cells treated with liposomes. RAW264.7 cells (1×10^6) pre-loaded with 5 μM DCFH/DA for 15 min were treated with liposomes (0.5 μmol lipid/mL) for 30 min with or without the lipoxygenase inhibitor NDGA (50 μM), and then subjected to flow cytometry. Dotted line; untreated control, solid line (narrow), SA-liposomes; solid line (broad), SA-liposomes with NDGA

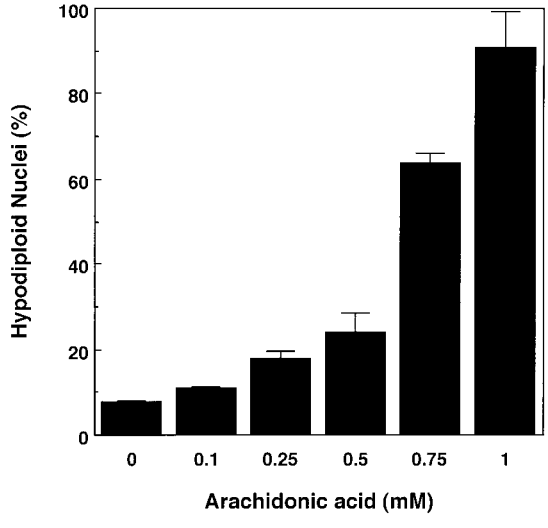

FIG. 3. Apoptosis induced by AA in RAW264.7 cells. (A) Flow cytometric analysis of DNA content in cells treated with AA. Cells (1×10^6) treated with the indicated concentrations of AA for 24 h were stained with propidium iodide and subjected to flow cytometry. Results represent the means ± SD of three experiments. (B) Agarose gel electrophoresis of DNA extracted from RAW264.7 cells treated with AA.

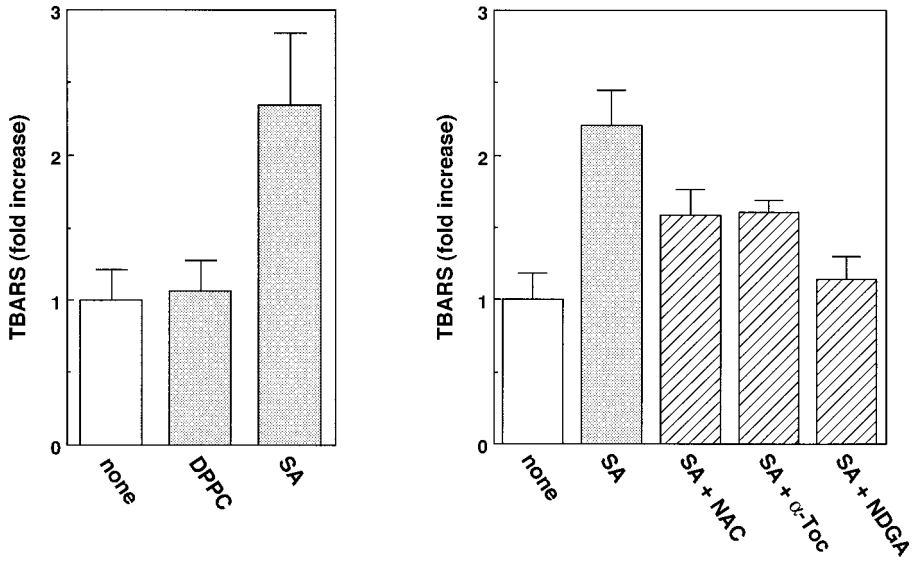

FIG. 4. The effects of NAC, lipoxygenase inhibitor, and α-tocopherol on lipid peroxidation induced by liposomes. Lipid compositions of DPPC- and SA-liposomes were DPPC:cholesterol = 1:1 and DPPC:SA:cholesterol = 1.5:0.5:2, respectively. RAW264.7 cells (5×10^6) were treated with liposomes (0.5 μmol lipid/mL) in the presence of NAC (50 mM), lipoxygenase inhibitor (NDGA, 50 μM), or α-tocopherol (40 μM) for 24 h, and the lipid peroxidation was evaluated by the measurement of TBARS. Results represent the means ± SD of three experiments.

that lipoxygenase plays an important role in ROS generation from RAW264.7 cells induced by SA-liposomes.

Effects of Arachidonic Acid on Apoptosis

Activation of phospholipase A_2 (PLA_2) results in the release of arachidonic acid (AA) from phosphatidylcholine and phosphatidylethanol-amine. The released AA is consequently oxidized either by lipoxygenase or cyclooxygenase, finally resulting in the formation of leukotrienes and prostaglandins. As mentioned above, lipoxygenase could be involved in the progression of macrophage apoptosis induced by cationic liposomes. Thus, we examined the effects of AA on the apoptosis of RAW264.7 cells. As shown in Fig. 3, hypodiploid DNA contents increased by treatment of cells with AA in a dose-dependent manner. These findings suggested that AA is released by the action of PLA_2 following SA-liposome treatment, and that lipoxygenase contributes to oxidation of the released AA. Experiments to clarify how PLA_2 is activated following cationic liposome treatment are currently underway in our laboratory.

Lipid Peroxidation

ROS generation has been shown to induce lipid peroxidation and protein oxidation in mitochondrial membranes, and then lead to cytochrome *c* release (26, 27). Thus, the changes in lipid peroxidation following liposome treatment were investigated by measurement of the formation of thiobarbituric acid reactive substances (TBARS). In this experiment, PC composing PC- or SA-liposomes was exchanged to DPPC having saturated acyl chains to avoid the contamination of lipid peroxidants in PC. As shown in Fig. 4, TBARS values increased when the cells were treated with SA-liposomes but not DPPC-liposomes, indicating that the lipid peroxidation was induced by SA-liposomes. However, TBARS values were restored to the control levels by addition of the lipoxygenase inhibitor NDGA, ROS scavenger NAC, or antioxidant α-tocopherol. Furthermore, apoptosis was not inhibited by addition of α-tocopherol (Fig. 5), but was inhibited by NAC and NDGA as mentioned above. These findings suggested

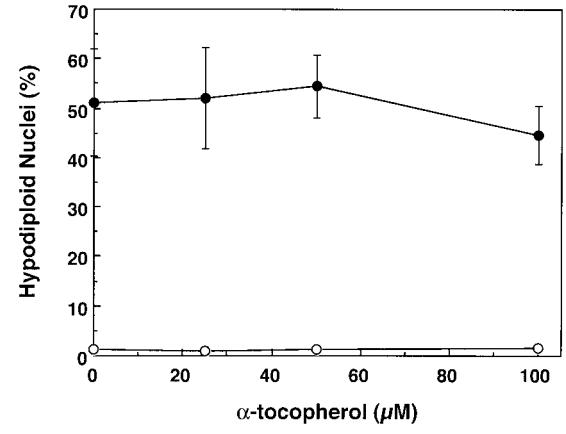

FIG. 5. The effects of α-tocopherol on macrophage apoptosis. RAW264.7 cells (5×10^6) were treated with SA-liposomes (0.5 μmol lipid/ml, closed circle) in the presence of α-tocopherol at the indicated concentrations for 24 h, and DNA contents were measured by FACS following PI-staining. Open circle represents DNA contents when the cells were treated with α-tocopherol at the indicated concentrations for 24 h. Lipid composition of SA-liposomes was DPPC:SA:cholesterol = 1.5:0.5:2. Results represent the means ± SD of three experiments.

that lipid peroxidation was not involved in the induction of apoptosis of RAW264.7 cells following SA-liposome treatment. Recently, we reported that SA-liposomes induce a decrease in mitochondrial membrane potential and the release of cytochrome *c* from mitochondria (18). Consequently, ROS generated by lipoxygenase following SA-liposome treatment may predominantly interact with mitochondria and induce apoptosis in RAW264.7 cells.

Cationic liposomes have been reported to cause cytotoxicity (2). We recently reported that cationic liposome composed of DC-cholesterol and lipofectin have the potential to induce apoptosis in macrophages as well as SA-liposomes, and the contribution of ROS to apoptosis by these cationic liposomes was indicated (4, 5). Recently, Dokka *et al.* (28) reported that LipofectAMINE cationic liposomes, frequently used as a plasmid transfection agent, showed lung cytotoxicity, and ROS was suggested to play a key role in cationic liposome-mediated cytotoxicity. The results observed here, for the first time, clearly demonstrated that lipoxygenase is responsible for ROS generation, and that ROS acts as a key mediator in the progression of apoptosis induced by SA-liposomes.

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