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Involvement of protein kinase Cδ in induction of apoptosis by cationic liposomes in macrophage-like RAW264.7 cells

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ABSTRACT

We have recently demonstrated that reactive oxygen species (ROS) play an important role in RAW264.7 cell apoptosis induced by cationic liposomes composed of stearylamine (SA-liposomes). In this study, we investigated whether protein kinase Cδ PKCδ) is involved in apoptosis induced by cationic liposomes. Tyrosine phosphorylation, nuclear localization, and cleavage of PKCδ were observed following the treatment of cells with SA-liposomes, suggesting that SA-liposomes activate PKCδ. Rottlerin, a specific inhibitor of PKCδ, inhibited ROS generation and also suppressed apoptosis. Cell surface proteoglycans may contribute to PKCδ activation by SA-liposomes. These findings suggest that PKCδ is strongly associated with apoptosis induced by SA-liposomes.

1. Introduction

Successful gene therapy depends on the use of efficient gene transfer vectors [1,2]. Viral vectors and non-viral vectors have been investigated extensively, and cationic liposomes are considered to be a superior candidate among non-viral vectors because of their low immunogenicity and the absence of a risk of viral infection [3]. However, toxicity is a barrier limiting the clinical applications of cationic liposomes [4]. Accordingly, clarifying the mechanism of cytotoxicity of cationic liposomes and regulating this cytotoxicity should facilitate the development of safe cationic liposomes for use as non-viral vectors.

Apoptosis plays a major role in development, homeostasis, and many disease processes [5,6]. In our previous study, we demonstrated that the cytotoxicity of cationic liposomes is a result of apoptosis [7–11], and that cationic liposome-induced apoptosis exhibited the following features: the generation of reactive oxygen species (ROS) [7,9–11]; the activation of p38 mitogen-activated protein kinase (MAPK) [11]; the activation of caspase-8 [11]; the cleavage of Bid and its translocation to the mitochondria and the release of cytochrome *c* [11]; and the activation of caspase-3 [10]. However, it remains unclear how cationic liposomes lead to ROS generation.

Protein kinase C (PKC) isozymes comprise a family of at least 10 related serine–threonine kinases that play important roles in the regulation of various cellular processes, including cell proliferation, differentiation, malignant transformation, and apoptosis [12,13,18]. On the basis of their structures and co-factor requirements, the PKC isomers are divided into classic PKCs (α, β, and γ), novel (δ, ε, η, and θ) and atypical (ζ and λ) groups. For instance, PKCα, β, ε and ζ have been shown to suppress apoptosis, while PKCδ is a critical pro-apoptotic signal in many cell types [12,15–19]. Various apoptotic agents including etoposide, γ-irradiation, and Fas-ligand have been shown to induce PKCδ phosphorylation [13,14], nucleus translocation [15], and caspase-dependent cleavage [16]. The apoptotic function of PKCδ has been associated with the activation of multiple signaling proteins, such as c-Jun NH_2-terminal kinase [17] and p38 [18]. However, the involvement of PKCδ in apoptosis induced by cationic liposomes has not yet been clarified.

We demonstrate that PKCδ activation and subsequent ROS generation could be involved in macrophage-like RAW264.7 cell apoptosis induced by cationic liposomes composed of stearylamine (SA-liposomes). These findings are the first observations of their kind and may elucidate a novel mechanism by which cationic liposomes regulate cell apoptosis, and lead to the development of safe non-viral vectors.

Abbreviations: SA-liposomes, cationic liposomes composed of stearylamine; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; MAPK, mitogen-activated protein kinase; PC, phosphatidylcholine; PI, propidium iodide; PKC, protein kinase C; PG, proteoglycan; ROS, reactive oxygen species; SA, stearylamine

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2. Materials and methods

2.1. Materials

Stearylamine (SA) was obtained from Sigma Chemical Co. Ltd. (St. Louis, MO). Phosphatidylcholine (PC) from egg yolk was obtained from Nippon Oil and Fat Co. (Tokyo, Japan). Cholesterol, propidium iodide (PI) were obtained from Wako Pure Chemicals (Osaka, Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), ROS detection reagents were obtained from Molecular Probes (Eugene, OR). Rottlerin was obtained from Calbiochem (La Jolla, CA). ChondroitinaseABC was obtained from Sigma Co. Ltd. (St. Louis, MO).

2.2. Preparation of liposomes

Cationic liposomes were prepared as described previously [11]. Lipid compositions of liposomes were PC:SA:cholesterol = 1.5:0.5:2.0 (by mole). The mean diameter of liposomes and Zeta potential were about 400 nm and +35 mV, respectively. SA-liposomes composed of DiI (0.1%/total lipids) as fluorescence marker were used for the liposome binding experiment.

2.3. Apoptosis detection assays

Cells from mouse macrophage-like cell line RAW264.7 were purchased from the Riken Cell Bank (Ibaraki, Japan) and cultured as described previously [11]. Cell culture plates were washed to remove non-adherent cells, adherent cells were treated with liposomes, and cell apoptosis was evaluated by our previous methods [11], namely, determining DNA content. DNA content was measured by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA) using propidium iodide (PI).

2.4. Western blotting

Phosphorylated PKCδ (Tyr301, Thr505) and the full length (80 kDa) of PKCδ were analyzed by Western blotting as described previously [11]. For the determination of phosphorylation of PKCδ Tyr301, Thr505), antibodies recognizing phosphorylated Tyr301 and Thr505 of PKCδ (both antibodies were obtained from Cell Signaling Technology (Beverly, MA)) were used. The change in the full length of PKCδ was evaluated using antibody recognizing regulatory domain of PKCδ. Cell Signaling Technology (Beverly, MA)). Band intensity was analyzed with NIH Image in each case.

2.5. Confocal microscopy

For the determination of PKCδ, RAW264.7 cells (4×10^5/ml) were treated with liposomes (0.5 μmol lipid/ml) for given periods. The cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. Following cell washing twice with PBS, cells were permeabilized with permeabilization buffer (0.2% saponin, bovine serum albumin in PBS) at room temperature for 5 min, and then blocked with blocking buffer (10% goat serum albumin in PBS) at room temperature for 1 h. Following washes with PBS, cells were incubated with an antibody recognizing catalytic domain of PKCδ Santa Cruz Biotechnology, Inc. (California, USA)) overnight and mounted in Vectashield Hard Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Localization of PKCδ was observed by confocal microscopy (FV1000D; Olympus, Japan).

2.6. Statistical analysis

Data are given as the mean ± S.D. The statistical significance of differences was determined by ANOVA with Duncan's test for multiple comparisons. The *P* value for significance was set at 0.005.

3. Results

It is well known that numerous apoptotic stimuli including etoposide, γ-irradiation, and Fas-ligand induce phosphorylation and nucleus translocation of PKCδ [12–15]. Then, the full length of PKCδ

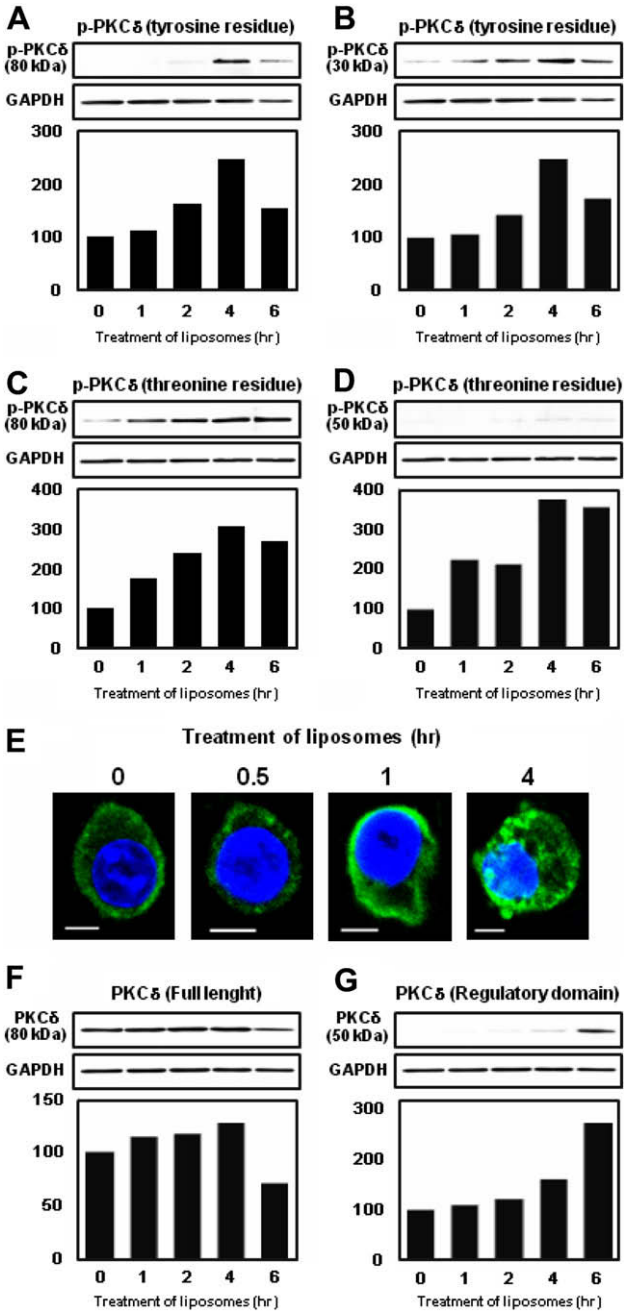

Fig. 1. Effects of SA-liposomes on PKCδ activation. RAW264.7 cells were treated with SA-liposomes (0.5 μmol lipid/ml) for the indicated period. Phosphorylation of PKCδ 80 kDa, tyrosine residue of full-length protein) is shown in (A), phosphorylation of PKCδ 30 kDa, tyrosine residue of catalytic domain) is shown in (B), phosphorylation of PKCδ 80 kDa, threonine residue of full-length protein) is shown in (C), phosphorylation of PKCδ (50 kDa, threonine residue of regulatory domain) is shown in (D), and fragmentation of full-length PKCδ (80 kDa; F) to the regulatory domain (50 kDa; G) was analyzed by Western blotting. Data expressed in (A–D), (F), and (G) were expressed as percentage values of band intensity compared with the control. Cells were treated with SA-liposomes for the indicated period, and translocation of the PKCδ (green) to the nucleus (blue) was evaluated by confocal microscopy (E). The figure is representative of three experiments showing similar results.

(80 kDa) is proteolytically cleaved into subunits composed of the catalytic domain (30 kDa) and the regulatory domain (50 kDa) after its activation [16]. Therefore, we examined PKCδ phosphorylation, nucleus translocation, and cleavage of PKCδ in order to evaluate the contribution of PKCδ to the apoptosis induced by cationic liposomes. In general, PKCδ is activated by phosphorylation of a tyrosine residue in the catalytic domain and a threonine residue in the regulatory domain [13,14]. Thus, we initially evaluated phosphorylation of both sites by Western blotting following treatment of cells with SA-liposomes. As shown in Fig. 1A and B, tyrosine phosphorylations were observed in the full length (80 kDa) and the catalytic domain (30 kDa) of PKCδ, and the highest band intensity was observed at 4 h. Fig. 1C and D indicates threonine phosphorylation of both full length (80 kDa) and regulatory domain (50 kDa), and the band intensity gradually increased up to 6 h. Next, we examined whether cationic liposomes induce nuclear translocation of PKCδ in macrophages by confocal microscopy. As shown in Fig. 1E, translocation of the PKCδ from the cellular membrane to the nucleus was observed in a time-dependent manner, and nuclear translocation was clearly observed by 4 h following the treatment. Furthermore, we examined whether cationic liposomes induce the fragmentation of PKCδ by Western blotting. As shown in Fig. 1F and G, the band intensity of the full length (80 kDa) of PKCδ decreased clearly at 6 h following the treatment of the cells with SA-liposomes. In response to this decrease, the band intensity of the regulatory domain (50 kDa) of PCKδ increased gradually and a clear increase was observed at 6 h after the treatment of SA-liposomes, indicating that fragmentation of PKCδ clearly occurred at 6 h after treatment.

To confirm the contribution of PKCδ to the apoptosis induced by SA-liposomes, the effects of rottlerin, a specific inhibitor of PKCδ, on the apoptosis were examined by measuring DNA content using flow cytometry. As shown in Fig. 2, the proportion of cells with hypodiploid DNA content decreased to the control level when the cells were treated with rottlerin, suggesting that PKCδ contributes to cell apoptosis induced by SA-liposomes. In our previous study, we demonstrated that ROS generation is critical for the development of apoptosis induced by cationic liposomes in RAW264.7 cells [7,9–11]. However, it remains unclear how cationic liposomes lead to ROS generation. We thus investigated the PKCδ requirement for ROS generation by cationic liposomes. As shown in Fig. 3, ROS generation was suppressed when the cells were pre-

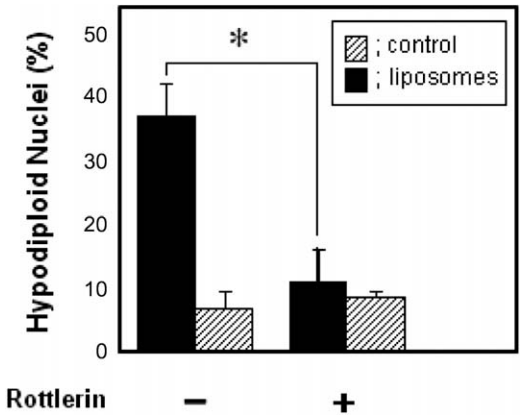

Fig. 2. Effects of rottlerin on hypodiploid DNA content in RAW264.7 cells treated with SA-liposomes. RAW264.7 cells were pretreated with rottlerin (4 μM) for 30 min and further incubated with or without SA-liposomes (0.5 μmol lipid/ml) for 24 h. Cells were then stained with PI, and hypodiploid DNA content was analyzed by flow cytometry. Data are presented as the mean ± S.D. from three independent experiments. * indicates P value <0.005 compared with rottlerin (−).

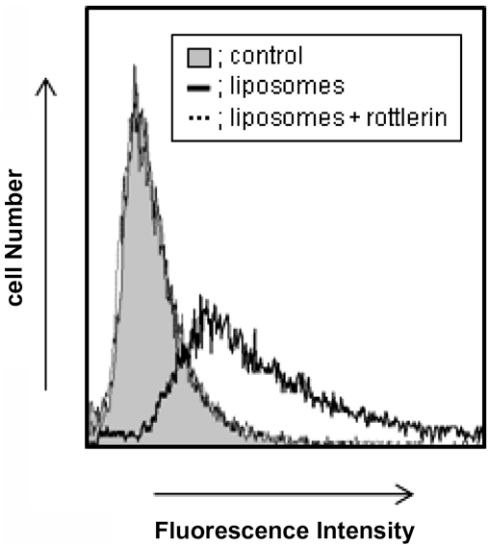

Fig. 3. Involvement of PKCδ activation in ROS generation in RAW264.7 cells treated with SA-liposomes. RAW264.7 cells (4×10^5 cells/ml) were pretreated with 4 μM rottlerin for 30 min, and then the cells were preloaded with H_2DCFDA (5 μM) for 15 min. Cells were treated with SA-liposomes (0.5 μmol lipid/ml) for 30 min, and subjected to flow cytometry. The figure is representative of three experiments showing similar results.

treated with rottlerin, suggesting the requirement of PKCδ for ROS generation.

Proteoglycan (PG) co-receptors exhibiting glycosaminoglycan chains modulate fundamental processes such as cell survival [21,22] and proliferation, and play an important role in cationic gene transfer [23]. Recently, the involvement of proteoglycans in the activation of PKCδ was suggested in dendritic cells [24]. Therefore, we examined whether the cationic liposome-induced PKCδ activation is associated with cell surface proteoglycans. As shown

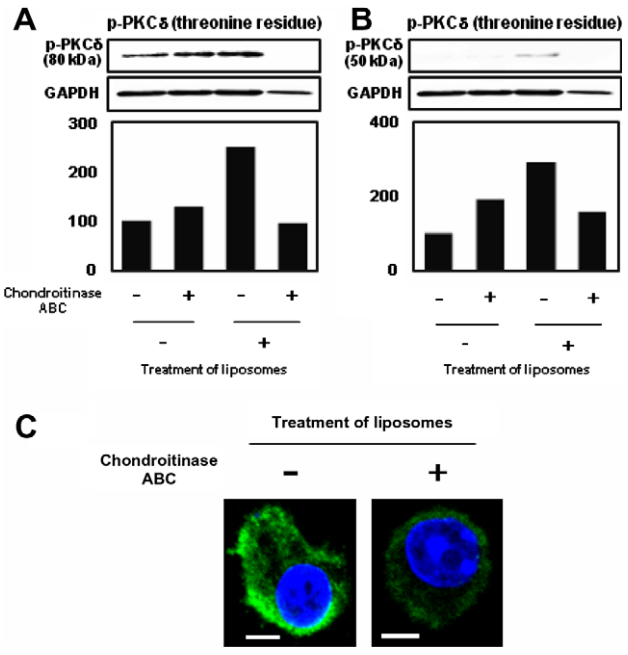

Fig. 4. Effects of chondroitinase ABC on PKCδ activation induced by SA-liposome treatment. RAW264.7 cells were pretreated with chondroitinase ABC (2 units/ml) for 30 min, and then treated with or without SA-liposomes (0.5 μmol lipid/ml) for 4 h. Following treatment, phosphorylation of PKCδ at a threonine residue was analyzed by Western blotting (A), and localization of PKCδ was examined by confocal microscopy (B). The figure is representative of three experiments showing similar results.

in Fig. 4A and B, the threonine phosphorylation of PKCδ induced by SA-liposome treatment was inhibited completely by the treatment of chondroitinase ABC. Consistent with this result, nuclear translocation of PKCδ was inhibited by the enzyme treatment (Fig. 4C). Furthermore, SA-liposome binding to RAW264.7 cells and ROS generation were inhibited when cells were treated by the enzyme (Fig. 5A and B). These results indicate that the activation of PKCδ is strongly related to the glycosaminoglycans, especially chondroitin sulfate on the plasma membrane.

4. Discussion

In our previous study, we demonstrated that cationic liposomes induced apoptosis in a macrophage-like cell line, RAW264.7, and speculated that the following mechanism was involved: treatment of cationic liposomes led to ROS generation, which activated p38 MAPK, and p38 MAPK signaling may also have regulated caspase-8 activity by phosphorylating caspase-8 itself. Then, caspase-8-mediated cleavage of Bid and its translocation to the mitochondria were associated to the release of cytochrome *c*, leading to the formation of an apoptosome [11]. However, the detailed molecular mechanism of cell apoptosis induced by cationic liposomes is still unclear. To clarify the mechanism by which cationic liposomes generate ROS, we focused on PKCδ in this study. The ubiquitously expressed isoform PKCδ has been shown to regulate cell apoptosis and survival in various cells [12–16]. We thus investigated the requirement for activated PKCδ in cationic liposome-induced apoptosis. By treatment of cationic liposomes, phosphorylation, nuclear translocation, and fragmentation of PKCδ were observed in RAW264.7 cells (Fig. 1). Furthermore, cationic liposome-induced apoptosis was inhibited in the presence of rottlerin, a specific inhibitor of PKCδ (Fig. 2). On the other hand, no inhibitory effects were observed when cells were treated with Go 6976, a specific inhibitor of PKCα and β (data not shown), indicating that PKCα and β could not be involved in apoptosis induced by cationic liposomes. Many studies concerning PKCδ function in apoptosis have been reported, and proteolytic cleavage and activation by caspase are shown to be an important step in apoptosis [15,16]. On the other hand, PKCδ may lie upstream of caspase [19]. Recently, Talior et al. [20] reported that ROS generation was associated with increased activity of PKCδ, and rottlerin inhibited ROS generation in adipocytes. In this study, ROS generation that is crucially important for the development of apoptosis induced by cationic liposomes was inhibited by rottlerin (Fig. 3), and which suggests that PKCδ could lie upstream of ROS generation induced by cationic liposomes.

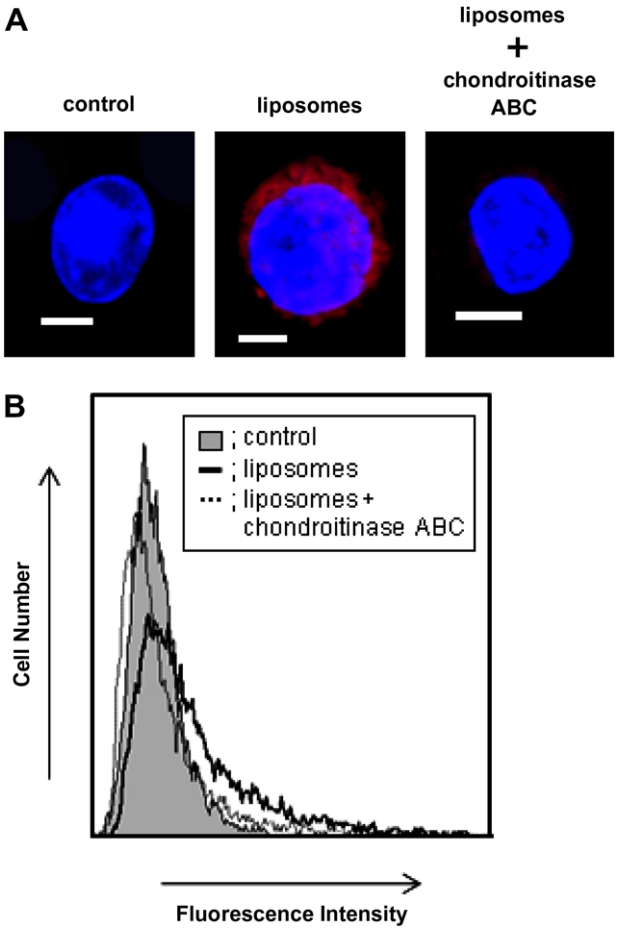

Fig. 5. Effects of chondroitinase ABC on the binding of SA-liposomes to cells and ROS generation. RAW264.7 cells (4×10^5 cells/ml) were pretreated with chondroitinase ABC (2 units/ml) for 30 min, and then treated with or without SA-liposomes (0.5 μmol lipid/ml) for 30 min. The binding of SA-liposomes composed of DiI (red) as fluorescence marker to cells was evaluated by confocal microscopy (A). In ROS generation experiment, enzyme treated cells were preloaded with H_2DCFDA (5 μM) for 15 min. Cells were treated with SA-liposomes (0.5 μmol lipid/ml) for 30 min, and subjected to flow cytometry (B).

Proteoglycans on the cell surface play an important role in cationic lipid-mediated gene delivery [23]. They consist of a core protein covalently linked to one or more sulfated glycosaminoglycans: heparin, heparan sulfate, hyaluronan, and chondroitin sulfate [21,22], and perform a wide variety of functions ranging from formation of extracellular matrix to cell–cell interaction and communication. Proteoglycans also function in the binding and entry of many viruses into cells [25]. Recently, You et al. reported that cross-linking proteoglycans on dendritic cells can activate PKCδ to induce the cell apoptosis [24]. Therefore, we examined the involvement of chondroitin sulfate in PKCδ activation and apoptosis of RAW264.7 induced by cationic liposomes. As shown in Fig. 4, phosphorylation and nuclear translocation of PKCδ were inhibited by chondroitinase ABC treatment, suggesting that the binding of cationic liposomes to chondroitin sulfate on the cell surface may lead to PKCδ activation. This point was supported by the findings that the binding of SA-liposomes to cells (Fig. 5A) and ROS generation (Fig. 5B) were completely inhibited by chondroitinase ABC treatment.

In conclusion, the mechanism of apoptosis induced by cationic liposomes is proposed as follows: cationic liposomes interact with macrophages through chondroitin sulfate on the plasma membrane. After interaction, PKCδ activation occurs, which leads to ROS generation. ROS generation may act to regulate a process downstream of apoptosis signals. Our finding is the first observation concerning the contribution of the chondroitin sulfate-PKCδ-ROS generation pathway to apoptosis in macrophages. We are now investigating how cationic liposomes induce PKCδ activation following the interaction with glycosaminoglycans. Clarification of the mechanism of apoptosis may lead to the safe application of cationic liposomes as non-viral vectors.

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