Archives of Biochemistry and Biophysics 518 (2012) 89–94

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Involvement of actin cytoskeleton in macrophage apoptosis induced by cationic liposomes

Katsuki Takano, Kaori Sato, Yoichi Negishi, Yukihiko Aramaki *

Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

ARTICLE INFO

Article history:
Received 5 November 2011
and in revised form 9 December 2011
Available online 19 December 2011

Keywords:
Apoptosis
Cationic liposomes
Actin
PKCδ
Reactive oxygen species

ABSTRACT

We clarified whether actin cytoskeleton is involved in the macrophage apoptosis induced by cationic liposomes composed of stearylamine (SA-liposomes). Externalization of phosphatidylserine induced by SA-liposomes was suppressed by cytochalasin D, a specific inhibitor of polymerization of F-actin. Furthermore, activation of PKCδ and reactive oxygen species (ROS) generation, which could be involved in the macrophage apoptosis, were inhibited by cytochalasin D. Microscopical observation revealed the co-localization of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled SA-liposomes and fluorescein-labeled phalloidin, which specifically binds to F-actin, and this co-localization was also inhibited by cytochalasin D. Co-localization of SA-liposomes and F-actin was also inhibited by the pre-treatment of cells with chondroitinase ABC. These findings could be the first observation concerning the contribution of the proteoglycan–actin cytoskeleton–ROS generation pathway to apoptosis induced by SA-liposomes in macrophages.

Introduction

Gene therapy has recently attracted significant attention as a source of hope for curing various genetic and intractable diseases [1]. The success of gene therapy depends on the development of vectors that can selectively deliver therapeutic genes to target cells efficiently and safely. Vectors proposed for gene delivery are classified into two categories, viral and non-viral vectors. Although viral vectors have provided a high level of gene transfection, concerns over their safety and immunological profile have steered research toward the development of efficient, non-viral vectors. Non-viral vectors, including cationic lipids, polymers, dendrimers, and peptides, have offered potential alternatives to solve these problems [2–4]. Compared with other non-viral carriers that have been widely used in gene delivery, cationic lipids have particularly excellent potential for gene delivery applications because of their low immunogenicity, robust manufacturability in terms of the ability to deliver large pieces of DNA, and ease of handling and preparation techniques [5].

Liposomes have been made into membranous vesicles composed essentially of naturally occurring phospholipids, and cationic liposomes are expected to be effective non-viral vectors as they readily form complexes with DNA drugs via electrostatic interactions [6]. However, many researchers have pointed out the cytotoxicity of cationic liposomes [7–9], and care must be taken when using cationic liposomes as a transfection agent. Understanding the mechanism of cytotoxicity due to cationic liposomes could facilitate the development of safe cationic liposomes as non-viral vectors.

In our previous study [10–14], we demonstrated that the cytotoxicity of cationic liposomes is a result of apoptosis, and the apoptosis was characterized as followings: cationic liposome-induced apoptosis involves the ROS-mediated activation of p38 MAP kinase and subsequent activation of caspase-8, as well as cleavage of Bid, a member of the bcl-2 family. Then, caspase-8-mediated cleavage of Bid and its translocation to the mitochondria are associated with the release of cytochrome *c* from mitochondria leading to the formation of an apoptosome. We have recently reported on the possible involvement of the lipid raft–PKCδ activation–ROS generation pathway in apoptosis induced by cationic liposomes [15,16]. However, detailed events induced at the membrane surface that generate apoptosis signals induced by cationic liposomes are unclear.

The actin cytoskeleton plays an essential role in a plethora of cellular functions, including endocytosis, motility, organelle and vesicle trafficking, cytokinesis and signal–response coupling [17]. Furthermore, recent reports have implicated changes to the dynamics of the actin cytoskeleton in the release of ROS from mitochondria and subsequent apoptosis [17,18].

In this study, we investigated whether actin cytoskeleton is involved in the macrophage apoptosis induced by cationic liposomes composed of stearylamine[1] (SA-liposomes). Co-localization of SA-liposomes and F-actin was found, and PKCδ activation and

* Corresponding author. Fax: +81 42 676 3182.
E-mail address: aramaki@ps.toyaku.ac.jp (Y. Aramaki).

[1] *Abbreviation used:* SA, stearylamine; ROS, reactive oxygen species; PC, phosphatidylcholine; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.

0003-9861/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2011.12.009

subsequent ROS generation, which could be involved in macrophage-like RAW264.7 cell apoptosis, were clearly inhibited by cytochalasin D, a specific inhibitor of actin. These findings suggested the involvement of actin in the development of apoptosis induced by cationic liposomes, SA-liposomes.

Materials and method

Materials

Alexa Fluor 488 phalloidin was obtained from Invitrogen (Tokyo, Japan). Cytochalasin D and stearylamine (SA) were 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 was obtained from Wako Pure Chemicals (Osaka, Japan). Annexin V apoptosis detection kits were purchased from Biovision (California, USA). Vectashield Hard Set Mounting Medium with DAPI was purchased from Vector Laboratories (Burlingame, CA). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Molecular Probes (Eugene, OR).

Preparation of liposomes

Cationic liposomes were prepared as described previously [15]. The lipid composition of liposomes was 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. Cationic liposomes composed of DiI (0.1% of total lipids) as a fluorescence marker were used for the liposome uptake experiment.

Apoptosis detection assays

Mouse macrophage-like cell line RAW264.7 cells were purchased from the Riken Cell Bank (Ibaraki, Japan) and cultured as described previously [14]. Cell apoptosis was evaluated by our previous methods, namely, determining externalization of PS [14]. PS externalization was measured by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA) using Annexin V-FITC apoptosis detection kits.

Confocal microscopy

For the determination of co-localization of cationic liposomes and actin, RAW264.7 cells (5×10^5/ml) were treated with DiI-labeled SA-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 Alexa Fluor 488 phalloidin recognizing F-actin. Co-localization of cationic liposomes and F-actin was evaluated by confocal microscopy (FV1000D; Olympus, Japan).

For the determination of PKCδ, cells (5×10^5/well) were treated with SA-liposomes (0.5 μmol lipid/ml) for given periods. The cells were washed with PBS, and fixation and permeabilization were carried out by the same procedure as mentioned above. The permeabilized cells were incubated with FITC-labeled antibody recognizing the 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).

ROS generation

ROS generation was estimated fluorometrically using 2′,7′-dichlorodihydrofluorescein deacetate (5 μM, DCFH/DA, Molecular Probes Inc., Eugene, OR). DCFH/DA, which is deacetylated to the non-fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCFH) in the cells, can be oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by a variety of peroxides. RAW264.7 cells (4×10^5/ml) pre-loaded with 5 μM DCFH/DA for 15 min were incubated with liposomes (0.5 μmol lipid/ml) at 37 °C for 30 min, and then subjected to flow cytometry.

Statistical analysis

Data are given as the mean ± SD. The statistical significance of differences was determined by ANOVA with Duncan's test for multiple comparisons.

Results

Involvement of actin in macrophage apoptosis

We recently demonstrated that PKCδ activation and subsequent ROS generation are critical for the development of apoptosis induced by SA-liposomes in RAW264.7 cells [15]. However, the detailed surface events that generate apoptosis signals induced by cationic liposomes are unclear. Actin cytoskeleton is an integral component of the plasma membrane-associated cytoskeleton and plays an important role in the determination of cell polarity, shape, and membrane mechanical properties including phagocytosis [17]. Recently, Gourlay and Ayscough reported the contribution of actin cytoskeleton in ROS generation in the development of cell death [18]. Actin can be disrupted by a number of pharmacological agents, and cytochalasin D can selectively inhibit polymerization of F-actin. Therefore, to clarify whether actin cytoskeleton could be involved in macrophage apoptosis induced by SA-liposomes, the effects of cytochalasin D on PKCδ activation and ROS generation were investigated by confocal microscopy and flow cytometry, respectively. As shown in Fig. 1A, the nuclear translocation of PKCδ, which is one of the features of PKCδ activation, was suppressed when the cells were pre-treated with cytochalasin D. Moreover, ROS generation was also suppressed by cytochalasin D pre-treatment (Fig. 1B). Having established that the activation of PKCδ and ROS generation by SA-liposomes occurs through actin cytoskeleton in RAW264.7 cells, the effects of cytochalasin D on the apoptosis induced by SA-liposomes were evaluated by measuring the externalization of PS using flow cytometry. As shown in Fig. 2, SA-liposome-induced PS externalization was suppressed, and the proportion of PS externalized cells decreased to the control level when the cells were pre-treated with 5 μg/ml cytochalasin D for 1 h. These results suggest that actin cytoskeleton contributes to cell apoptosis induced by SA-liposomes.

Co-localization of liposomes and actin

To confirm the involvement of actin cytoskeleton in macrophage apoptosis, we initially examined the interaction between SA-liposomes and actin in RAW264.7 cells using DiI-labeled SA-liposomes and Alexa Fluor 488 phalloidin, which specifically binds to F-actin. As shown in Fig. 3A, a cluster-like structure of phalloidin was observed at 5 min, and the dual images of red fluorescence generated from DiI-labeled SA-liposomes and green fluorescence from Alexa Fluor 488 phalloidin can be clearly seen at 15 min. The yellow color of a cluster-like structure denotes the co-localization of the SA-liposomes and F-actin, and the yellow fluorescence