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Cationic liposomes evoke proinflammatory mediator release and neutrophil extracellular traps (NETs) toward human neutrophils

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ABSTRACT

Cationic liposomes are widely used as nanocarriers for therapeutic and diagnostic purposes. The cationic components of liposomes can induce inflammatory responses. This study examined the effect of cationic liposomes on human neutrophil activation. Cetyltrimethylammonium bromide (CTAB) or soyaethyl morpholinium ethosulfate (SME) was incorporated into liposomes as the cationic additive. The liposomes' cytotoxicity and their induction of proinflammatory mediators, intracellular calcium, and neutrophil extracellular traps (NETs) were investigated. The interaction of the liposomes with the plasma membrane triggered the stimulation of neutrophils. CTAB liposomes induced complete leakage of lactate dehydrogenase (LDH) at all concentrations tested, whereas SME liposomes released LDH in a concentration-dependent manner. CTAB liposomes proved to more effectively activate neutrophils compared with SME liposomes, as indicated by increased superoxide anion and elastase levels. Calcium influx increased 9-fold after treatment with CTAB liposomes. This influx was not changed by SME liposomes compared with the untreated control. Scanning electron microscopy (SEM) and immunofluorescence images indicated the presence of NETs after treatment with cationic liposomes. NETs could be quickly formed, within minutes, after CTAB liposomal treatment. In contrast to this result, NET formation was slowly and gradually increased by SME liposomes, within 4 h. Based on the data presented here, it is important to consider the toxicity of cationic liposomes during administration in the body. This is the first report providing evidence of NET production induced by cationic liposomes.

1. Introduction

Nanomedicine for therapeutic and diagnostic use is presently in the spotlight. Liposomes serving as nanosystems are currently more available than the other nanoparticles used clinically [1]. Liposomes with positive charge have been proven to be good nanocarriers of DNA, siRNA, and vaccines for the formation of complexes [2,3]. Cationic liposomes can target the tumor vasculature for anticancer drug delivery and bioimaging [4,5]. Certain clinical trials have verified this possibility [6,7]. Cationic charge on a nanoparticulate surface not only promotes cellular delivery but also stimulates immune and inflammatory responses [8]. Cationic liposomes may be similar to double-edged swords, functioning as an effective drug/gene carrier but also leading to nanotoxicity.

Most of the nanocarriers used for medical purposes are administered intravenously, entering the circulation. In humans, 50–70% of circulating leukocytes are neutrophils [9]. Neutrophils are a type of polymorphonuclear leukocyte that act as the first immune cells that defend against pathogens [10]. These cells also participate in the inflammatory process, inducing tissue damage. The interaction of neutrophils with xenogeneic substances, such as microbes and nanoparticles, can cause cell activation. Although certain

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studies have reported the activation of neutrophils by metallic and polymeric nanoparticles [11,12], there is a lack of investigation pertaining to the effect of cationic liposomes on neutrophil-mediated inflammation. We aimed to explore the mechanisms by which cationic liposomes elicit neutrophil activation and cytotoxicity. The possible interaction between cationic liposomes and human neutrophils was also elucidated.

The immune and inflammatory responses are dependent upon the nature and concentration of cationic additives in nanosystems. We employed two cationic surfactants in liposomes for comparison: cetyltrimethylammonium bromide (CTAB) and soyaethyl morpholinium ethosulfate (SME). CTAB is a commonly used surfactant that is incorporated into liposomes and nanovesicles to assist delivery into cells [13,14]. SME can be utilized as a deodorant and sterilizer and is also loaded into nanoparticles to target the brain as well as hair follicles [15,16]. The viability, oxidative stress, degranulation, and intracellular Ca^{2+} influx of neutrophils in response to cationic liposomes and the liposomes' cytotoxicity were assessed in this study. Neutrophil extracellular traps (NETs) are a newly described mechanism of neutrophil activation. The cells' bactericidal capability and inflammatory response can be increased by the formation of NETs [17]. In the present work, we examined the role of NETs in neutrophil activation by cationic liposomes. The results demonstrated how cationic surfactants on liposomal bilayers influence cytotoxicity to and activation of neutrophils via oxygen-dependent and oxygen-independent mechanisms. CTAB and SME displayed different effects on neutrophils' morphological change and NET development.

2. Materials and methods

2.1. Preparation of liposomes

A thin-film hydration method was utilized to generate liposomes. Soybean phosphatidylcholine (3.2% of the final concentration; American Lecithin, Oxford, CT, USA) and cholesterol (0.8%) were dissolved in a mixture of chloroform and ethanol (2:1). CTAB (Sigma–Aldrich, St. Louis, MO, USA) or SME (Forestall®, Croda, East Yorkshire, UK) at a concentration of 2% was incorporated into the mixture in certain cases. The organic solvent was evaporated in a rotary evaporator at 50 °C. The residual solvent was removed under vacuum overnight. Water was added to hydrate the film by probe-type sonication (VCX600, Sonics and Materials, Danbury, CT, USA) at 35 W for 30 min.

2.2. Average diameter and zeta potential

The mean diameter (*z*-average) and zeta potential of the liposomes were measured using a laser-scattering method (Nano ZS90, Malvern, Worcestershire, UK). The liposomal dispersions were diluted 100-fold with water before testing. The determination was repeated three times per sample for three batches.

2.3. Human neutrophils

Human neutrophils from healthy volunteers (20–30 years old) were purified using a protocol approved by the Institutional Review Board at Chang Gung Memorial Hospital. All subjects were required to provide written informed consent. The neutrophils were isolated by sedimentation prior to centrifugation in a Ficoll-Hypaque gradient and hypotonic lysis of the erythrocytes [18].

2.4. Cell viability determination based on WST-1 assay

The survival rate of the neutrophils was measured using the Cell Proliferation Reagent WST-1 (Roche, Indianapolis, IN, USA). The neutrophils (1×10^6 cells/ml) were treated with liposomes for 15 min at 37 °C. The treated medium was centrifuged at $200 \times g$ for 8 min. The supernatant was discarded. The WST-1 reagent was added, and after a 1-h incubation at 37 °C, the absorbance was determined at 450 nm.

2.5. Membrane damage determination based on LDH release

The LDH value after liposomal treatment was detected using a commercially available method (CytoTox 96®, Promega, Madison, WI, USA). Neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C for 2 min and subsequently treated with liposomes for 15 min. The membrane damage was determined based on LDH release into cell-free medium as a percentage of the total LDH. The total LDH was measured by treating neutrophils with 0.1% Triton X-100.

2.6. Intracellular superoxide anion production

Hydroethidine (HE) is a probe for detecting intracellular superoxide anion. Neutrophils (2.5×10^6 cells/ml) were loaded with HE (10 μM) at 37 °C for 5 min. Liposomes were added to the cell medium for 15 min at 37 °C. Subsequently, Hank's balanced salt solution (HBSS) at 4 °C was used to stop the reaction, and the fluorescence was monitored by flow cytometry.

2.7. Degranulation determination based on elastase release

The elastase substrate used was MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide. After supplementation with the substrate (100 μM), neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C for 2 min and then treated with liposomes for 15 min. The absorbance at 405 nm was determined after 15 min to measure the elastase release.

2.8. Intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Neutrophils (3×10^6 cells/ml) were loaded with Fura-2/AM reagent (2 μM) at 37 °C for 40 min. After being washed, the neutrophils were resuspended in HBSS. Liposomes were added to the cell medium for 15 min at 37 °C. The fluorescence spectrometry at λ_{ex} of 340/380 and λ_{em} of 505 nm was examined during continuous stirring. The detailed processes have been described by Hwang et al. [19]. 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM) at a concentration of 20 μM was added to the medium to suppress Ca^{2+} homeostasis if necessary. The BAPTA-AM was pipetted into the neutrophil suspension in Ca^{2+}-free solution before incubation with liposomes.

2.9. Scanning electron microscopy (SEM)

Neutrophils (1.8×10^7 cells/ml) were incubated with liposomes for 5 min. Phorbol myristate acetate (PMA) at a concentration of 10 nM was used as the positive control. The suspension was centrifuged at $200 \times g$ at 4 °C for 8 min. Glutaraldehyde (0.5%) was added to the neutrophils after supernatant removal. The neutrophils were fixed and dehydrated before SEM visualization. Gold palladium alloy was employed as sputter coating.

2.10. Quantification of NETs

Measurement of extracellular DNA reflects the level of NET formation. A total of 1×10^6 neutrophils (100 μl) were allowed to incorporate the membrane-impermeable DNA dye SYTOX Green (50 μl) at a concentration of 10 μM for 5 min at 37 °C. Liposomes