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Changes in the morphology of cell-size liposomes in the presence of cholesterol: Formation of neuron-like tubes and liposome networks

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Abstract

Spontaneous changes in the morphology of cell-size liposomes (dioleoylphosphatidylcholine, DOPC and egg PC) as model cells were investigated in the presence of cholesterol. Tube structures and liposome networks connected by the tubes were observed in the presence of 5–30% cholesterol by dark-field and laser-scanning microscopy. Furthermore, in the presence of more than 40 mol% of cholesterol, the tubes disappeared and changed to small liposomes. Thus, cholesterol induced a morphological change in giant liposomes from tubes to small liposomes. These phenomena may be related to the role of cholesterol in the morphological changes in living cells such as neurons.

Keywords: Giant liposome; Cholesterol; Lipid bilayer; Tubular network; Neuron

1. Introduction

Cholesterol is distributed ubiquitously in biomembrane. Extensive studies have examined the many functions of cholesterol. Lipid model systems can provide important insights into the roles of biomembrane components. For example, studies of lipid–cholesterol interactions in a model system have contributed to our understanding of the formation of lipid rafts and related membrane microdomains, such as caveolae [1,2]. In contrast, there have been few detailed studies on the morphological changes in lipid–cholesterol membrane, in comparison to the many studies on phase separation and lateral diffusion. Recently, giant liposomes of several micrometers in diameter have attracted growing interest because they can be directly observed by optical microscopy [3–6]. We report here the morphological changes in lipid membrane structure, cell-size giant liposomes, liposome-tube networks and small vesicles in the presence of cholesterol. Neurons have a unique membrane morphology, which includes long tube structures (myelin axons or dendrites) and small synaptic vesicles. Approximately 25% of the total amount of cholesterol in humans is present in the brain. However, the role of cholesterol in the nervous system is still poorly understood [7]. Recently Pfrieger reported that cultured neurons from the mammalian nervous system require cholesterol to form efficient synapses and cholesterol influences synapse development and stability [8]. Studies on the effects of cholesterol on morphological changes in cell-size liposomes may provide important information regarding the physicochemical role of cholesterol in the neuronal membrane.

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2. Materials and methods

2.1. Lipids

Dioleoylphosphatidylcholine (DOPC) was obtained from Fluka. Cholesterol and $CoCl_2 \cdot 6H_2O$ were obtained from Sigma Chemical Co. 3-*sn*-Phosphatidylcholine, from Egg Yolk (Egg PC), and $MgCl_2 \cdot 6H_2O$ were obtained from Wako Chemical Co. Methyl-β-cyclodextrin (M-β-CD) was obtained from Tokyo Kasei Kogyo Co. 2-[4-(2-Hydroxy-ethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2′,7′-{[*bis*(carboxymethyl)amino]methyl}-fluorescein (Calcein) were obtained from Dojin Chemical Co. *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) was obtained from Molecular Probes (Eugene, OR).

2.2. Preparation of tubular network

DOPC and Egg PC were dissolved and stored at 10 mM in a chloroform:methanol solution (2:1, v/v). Cholesterol was dissolved and stored at 1 mM in chloroform:methanol (2:1, v/v). Suspensions (chloroform:-methanol=2:1) of phospholipids and cholesterol (various molar fractions of cholesterol) were prepared in test tubes. The organic solvents were evaporated slowly under argon gas flow, and dried in vacuo for 30 min. A thin film of lipids then formed at the bottom of the test tubes. The films were swelled by adding 10 mM HEPES buffer (10 mM $MgCl_2$, pH 7.0), where the final concentration of lipid was 1 mM, and incubated for 1 h at 40 °C. We found that such incubation eliminated a tubular-network structure that was spontaneously formed from DOPC or Egg PC film without cholesterol. After treatment, only spherical liposomes were observed.

2.3. Microscopic observation

Fifty microliters of the DOPC/cholesterol suspension was placed in a small cavity between glass slides (0.2 mm×22 mm×12 mm (thickness×width×length)). A dark-field and a fluorescence microscope (E600, Nikon) with a 100 W high-pressure Hg lamp as a light source were used to observe the tubular networks. The images were captured with a digital camera (C4743-95, Hamamatsu Photonics) and recorded and analyzed with an image processor (Aquacosmos, Hamamatsu Photonics).

2.4. Laser scanning microscopic observation

Twenty microliters of DOPC/cholesterol containing 0.1 nM Texas Red DHPE suspension of the network structure was placed described above. The images were obtained and analyzed by LSM 510 META (Zeiss).

2.5. Transmission electron microscopic observation

Electron microscopy was carried out using an H-600 (HITACHI) operated at an acceleration voltage of 100 kV. A lipid network was prepared by adding an aliquot of phosphotungstate (1.0 wt.%, pH 7.0) to swell the lipid film described above. Five microliters of the sample was placed on a hydrophilized 200-mesh copper grid with a carbon-coated collodion. The grid was then set between glass slides with a 0.2-mm-thick spacer and the whole assembly was aerated with OsO_4 vapor over 3 days, with care taken to avoid drying it out. The grid was allowed to dry at room temperature for at least 1 h before observation.

2.6. Semi-quantitative analysis of tubule formation

The rate of tubule formation was analyzed using the dark-field microscopic images. We assumed that all of the bright components in the images are from lipid structures. The obtained two-dimensional images were binarized, the total areas (pixels) containing tubules and other structures were calculated, and the average ratios of the tubule area were calculated. Image processing was performed using NIH image software (http://rsb.info.nih.gov/nih-image/Default.html).

2.7. Fluorescence quenching

In the fluorescence-quenching experiment, a DOPC/cholesterol film was swollen with 10 mM HEPES buffer (10 mM $MgCl_2$, pH 7.0) containing the hydrophilic fluorescence dye calcein (0.1 mol%). After 1 h of incubation, 0.1 mM of $CoCl_2$ solution was added to the DOPC/cholesterol suspension.

2.8. Network dismantling experiment

One hundred microliters of the DOPC/cholesterol (DOPC/Chol=1:0.1, in final) suspension of network structures was placed in a test tube and 100 μL of 10 mM methyl-β-cyclodextrin (M-β-CD) in 10 mM HEPES buffer (pH 7.1) was added at 1:1 (v/v).

3. Results

We investigated the changes in the morphology of giant liposomes obtained by natural swelling in the presence of cholesterol. Dried thin films of lipids with various contents of cholesterol formed at the bottom of test tubes. After the films were allowed to swell for 1 h at 40 °C, the structure that formed in the small-volume chamber on the glass slide was observed by dark-field or laser fluorescence microscopy. In the presence of 10 mol% of cholesterol, for example, DOPC liposome

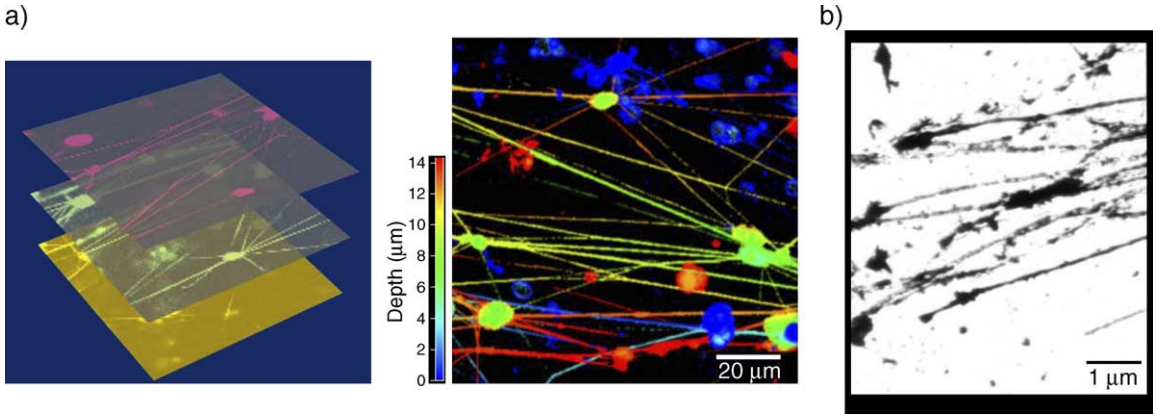

Fig. 1. (a) Laser-scanning microscopic images of the network. The lipid membrane was labeled by Texas Red DHPE. Left: Stacked image acquired over a depth range of 14 μm with Auto-Z. Right: Depth coding in rainbow color: blue in front and red at the back. (b) Transmission electron microscopic image.

networks connected by well-extended straight-chain tube-like structures were mainly formed. Laser scanning fluorescence microscopy showed that the structure was a three-dimensional liposome-tube network structure (Fig. 1a). From the TEM images, the average diameter of the tubes was estimated to be 150 nm (Fig. 1b). The changes in the morphology of the lipid structure were observed by dark-field microscopy in the presence of various concentrations of cholesterol (Fig. 2). The average probability of tube formation was plotted as a function of the cholesterol contents (Fig. 3). In the case of DOPC, a high probability was observed in the presence of 10–30 mol% of cholesterol, and relatively small liposomes were observed in the presence of more than 40 mol% of cholesterol. The tubes are under tension, and branch randomly. Tube formation was also observed in the case of egg PC, although at a lower probability.

To confirm that the tube structures had an interior space, a fluorescent-quenching experiment was performed. A network in the DOPC–cholesterol (10 mol%) system was

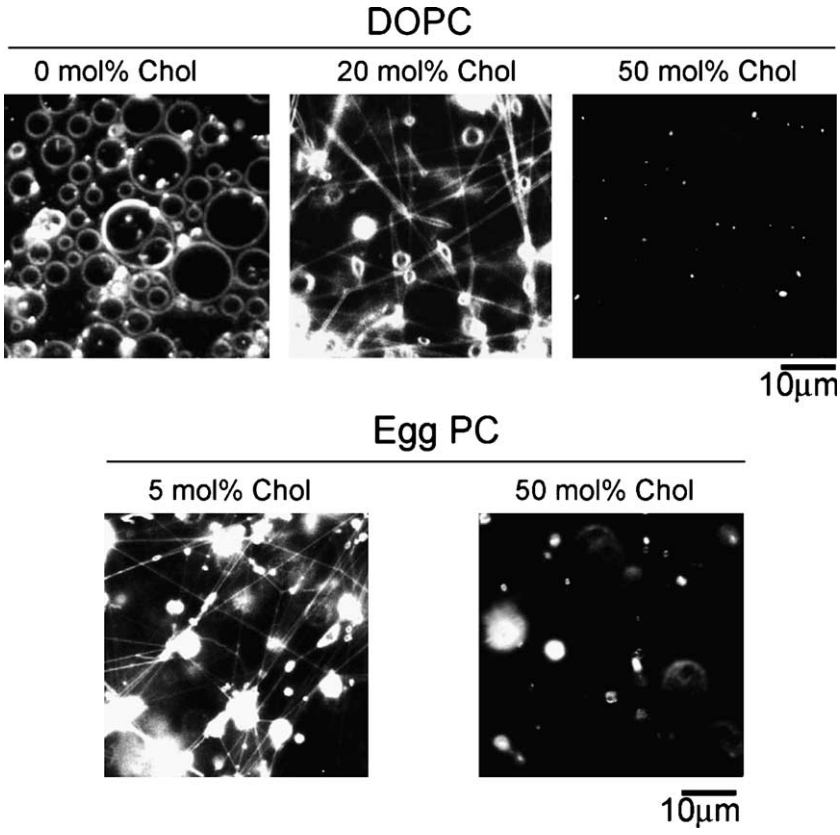

Fig. 2. Dark-field microscopic images of lipid structures with various concentrations of cholesterol.

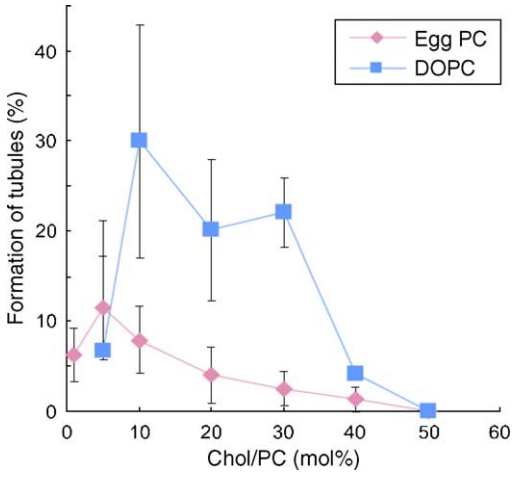

Fig. 3. Tubule formation with various concentrations of cholesterol with lipids.

formed by swelling with a solution containing calcein as a hydrophilic fluorescence probe, and a fluorescence quencher [Co(II) ion] was then added to the network solution. Since the cobalt ion cannot penetrate the lipid membrane, if the tube does not have interior spaces (such as cylindrical micelles), all of the fluorescence signals in the system will be quenched. After such treatment, liposomes and their connecting networks still show fluorescence signals (Fig. 4a). Thus, the tubular components have internal hydrophilic spaces.

To obtain further information about the stability of the tubular structures in the presence of cholesterol, the effect of cyclodextrin was investigated. Upon the addition of methyl-β-cyclodextrin to the DOPC/cholesterol (10/1 in mol%) tube network, the tube structures immediately disappeared and only spherical liposomes remained. This disappearance of the tubes is likely due to the removal of cholesterol from the tubes by the formation of an inclusion complex between cholesterol and cyclodextrin. A similar experiment was carried out after the fluorescence-quenching experiment described above. After the tube structures changed to liposomes, a fluorescent signal was observed inside the liposomes (Fig. 4b). The disappearance of the network proceeded without crucial disruption of the closed liposomal membrane. The results show that cholesterol plays an important role in stabilizing the tube structure.

4. Discussion

There have been few reported studies on the construction of a tube network-structure based on giant liposomes. Orwar and coworkers constructed a nanotube-connected (~100 nm in diameter and 20–30 μm in length) giant liposome system by a subtle mechanical procedure using a carbon microfiber as tweezers [9]. Evans et al. reported tube formation from giant liposomes by micro-pipette suction for microspheres attached to the liposomal membrane [10]. These liposome-tube networks were artificially formed on the surface of a two-dimensional substrate by fabrication methods. On the other hand, we previously reported that a three-dimensional giant liposome-tube network formed spontaneously upon the addition of a series of glycosphingolipids, gangliosides [11]. Although many studies have reported the behavior of phospholipid–cholesterol systems, to the best of our knowledge, there has been no previous report on the formation of a stable three-dimensional liposome-tube network by natural swelling in the presence of cholesterol.

Tube formation is caused by a local force acting on the lipid bilayer membrane of a liposome or erythrocyte ghost [12–14]. The phospholipid bilayer of DOPC or Egg PC is relatively a ‘soft’ membrane because of their unsaturated alkyl chains. It has a thermodynamic phase-transition point that is much lower than room temperature and shows a polymorph structure (i.e., budding, tubular, pearling) as a transient state structure. The dried film tends to have disordered parts (meshed form) on the layered lipids. Hydration and swelling from the meshed layer of soft lipids readily produce flabby network-like structures as transient state. However, such structures are unstable and the systems move toward stable states such as spherical liposomes through the relaxation of membrane tension. In general, the presence of cholesterol increases the order and packing density within the membrane in the liquid crystalline phase. The stability of the transient states of the higher-order structure of the lipid bilayer membrane such as a liposome-tube network is related to surface tension (membrane elastic bending modulus) at the equilibrium state [15]. Chen and Rand reported that the bending modulus of DOPC bilayer shows an increase of 30% with increasing of the concentration of cholesterol up to 50 mol% [16]. The increase of the bending modulus in the presence of an appropriate amount of cholesterol may stabilize the transient network structure.

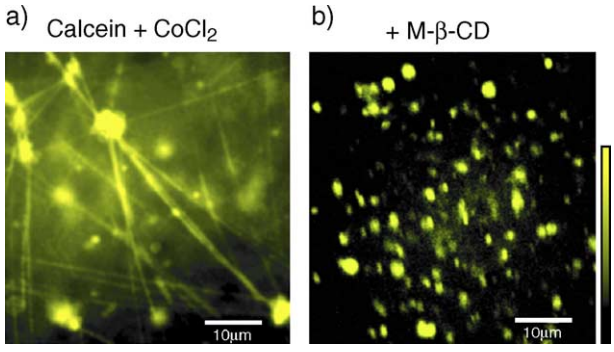

Fig. 4. Microscopic images from the fluorescence-quenching and network dismantling experiment: (a) The interiors of the network tubes are stained with the hydrophilic fluorescence dye calcein. Cobalt ion was added to the external media. (b) Methyl-β-CD was added to the network suspension after the quenching experiment.

Tubes are intermediate structures in the morphological change from giant liposomes to small liposomes or small aggregates. The transient anisotropic structures of tubes should be stabilized by the cooperative effect of cholesterol and phospholipids. We previously reported that gangliosides with small packing parameters (P=0.4–0.5) stabilize tubular structures of DOPC (P~1) and the difference in the lipid compositions between the inner and outer layers of bilayer membrane is essential for the transition from liposome to tube [11]. A higher ganglioside molar fraction in the outer layer stabilizes the tubes.

On the other hand, the packing parameter of cholesterol (P>1) is larger than DOPC due to the wedge shape [17]. In this case, a higher existence of cholesterol in the inner layers might stabilize the tubes. With an increase of molar fraction of cholesterol, the difference in the lipid compositions decreases and finally low curvature liposomes mainly form. In lipids/cholesterol systems, Mui et al. [18] and also Agirre et al. [19] pointed out that the generation of the differences in the compositions between internal and external monolayer areas (transbilayer area asymmetry) induces non-spherical structures such as tubes. Appropriate lateral and transversal redistribution of cholesterol would sterically stabilize regions of high curvature [18]. Further study to understand the effect of cholesterol in detail is under investigation.

The connected liposome-tube network is also interesting with regard to living systems. *E. coli* uses a well-known intercellular tube structure called a 'conjugation tube' for plasmid transfer [20]. Recently, intercellular nanotubular structures that create complex networks have also been found in mammalian cells [21]. These structures (50–200 nm in diameter and up to several cell diameters long) are thought to facilitate the selective transfer of membrane vesicles and organelles. From a morphological perspective, neurons show unique membrane structures, such as tubes and synaptic vesicles. Recently, the functions of cholesterol in the brain have attracted growing interest. Most of the cholesterol in the central nervous system is located in the myelin sheath (i.e., oligodendroglia) and plasma membrane of astrocytes and neurons. The cholesterol content in the biomembrane of the brain is relatively higher than that in other tissues. Furthermore, more than 70% of the brain cholesterol is associated with myelin and cholesterol contains 40 mol% of total lipid in myelin [7]. The high enrichment of cholesterol with myelin leads to a reduction in the permeability to ions, via high resistance and low capacity. Pfrieger has suggested that cholesterol is an essential component of synapses and predicted that their formation, function and stability are sensitive to disturbances in cholesterol metabolism [8]. In this study, we found that an appropriate amount of cholesterol stabilized the tube structure, while a higher concentration of cholesterol stabilized small liposomes. Cholesterol can control the morphology of giant liposomes. These findings may be related to the role of cholesterol in the brain. The shape of cellular biomembrane may be physicochemically controlled by the distribution of cholesterol.

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