Importance of Cholesterol Side Chain in the Membrane Stability of Human Erythrocytes

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Note

INTRODUCTION

Cholesterol is a major sterol in mammalian plasma membranes and has first been investigated as a modulator of the membrane fluidity in liposomes. For instance, cholesterol increases or decreases the bilayer fluidity below or above the phase transition temperature of phospholipids, respectively. Parts of cholesterol in biological membranes are concentrated in the sphingomyelin-rich domain of phospholipid bilayer. The micro domain that consists of the cholesterol–sphingomyelin complex is called lipid rafts. Signal-transducing proteins such as insulin receptor are embedded in such lipid rafts. Moreover, the activity of intramembrane proteases such as precepinil is modulated by cholesterol. Thus, cholesterol in biological membranes interacts with intramembrane proteins as well as phospholipids and plays an important role in the stability and functions of the membrane.

Recently, we have reported cholesterol effects on the membrane stability of human erythrocytes. In that report, we have demonstrated that cholesterol strengthens the bilayer–cytoskeleton interaction and suppresses the hemolysis under high hydrostatic pressure (simply, called pressure) or hypotonic conditions. However, the role of a short side chain of cholesterol is unknown. So, we have selected the plant sterols such as β-sitosterol and stigmasterol. Incorporation of sterols into the membrane using a sterol/methyl-β-cyclodextrin complex was confirmed by the mass spectrometry. Hemolysis of human erythrocytes under high hydrostatic pressure (200 MPa) or hypotonic conditions was suppressed by cholesterol, but not by β-sitosterol and stigmasterol. Moreover, the bilayer–cytoskeleton interaction was also strengthened by cholesterol, but not by β-sitosterol and stigmasterol. Taken together, we suggest that the short side chain of cholesterol plays an important role in the membrane stability of human erythrocytes.

Key words bilayer–cytoskeleton interaction; hemolysis; human erythrocyte; sterol; mass spectrometry; methyl-β-cyclodextrin

MATERIALS AND METHODS

Materials Compounds were obtained from the following sources: acetyl chloride and cholesterol, Nacalai Tesque (Kyoto, Japan); methyl-β-cyclodextrin (CD), Sigma-Aldrich (St. Louis, MO, U.S.A.); β-sitosterol and stigmasterol, Tama Biochemicals (Tokyo, Japan). All other chemicals were of reagent grade.

Hemolysis Human erythrocytes from the Fukuoka Red Cross Blood Center were washed with phosphate-buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). For reduction of membrane cholesterol levels, the erythrocytes (20% hematocrit) in 0.5% (w/v) CD-containing PBS, which was pre-incubated for 30 min at 37°C, were incubated for 1 h at 37°C and washed with PBS. For loading of sterols such as cholesterol, β-sitosterol, and stigmasterol, the erythrocytes (20% hematocrit) in a 0.5% CD/1.3 mM sterol complex-containing PBS were incubated for 1 h at 37°C and washed with PBS. Here, the CD/sterol complex-containing PBS was prepared as follows. Briefly, each sterol (approx. 0.2 mg) was dissolved in chloroform and the solvent was evaporated in a test tube. CD (0.5% (w/v)) was dissolved in PBS and incubated for 30 min at 37°C. To the test tube containing the thin-layered sterol, 0.4 mL of such CD solution was added. The tube was incubated for 1–2 h at 37°C to form the CD/sterol complex. To compress the erythrocytes, the cells in PBS were exposed to 200 MPa for 30 min at 37°C, and decompressed up to atmospheric pressure. Such decompressed samples were centrifuged for 1 min at 1000 × g and room temperature. Hemolysis was determined by measuring the supernatant optical density at 542 nm. For the hypotonic stress, the erythrocyte

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 suspensions in 10 mM sodium phosphate (pH 7.4) containing 54 mM NaCl were incubated for 10 min at 37 °C. Hemolysis was determined, as described above.9,13)

**Positive-Ion Electrospray Ionization Mass Spectrometry (ESI+−MS) of Sterols** For an ESI+−MS, the lipids were acetylated. A mixture of cholesterol, β-sitosterol, and stigmasterol was dissolved in chloroform at each same concentration (10 µM), and acetyl chloride (100 µM) was added into the sterol solution.14,15) The reaction solution was incubated for 1 h at room temperature and the solvent was evaporated by rotary evaporator. The membrane lipids were extracted from the erythrocytes by using chloroform and methanol (2 : 1 (v/v))16) and were acetylated. Briefly, acetyl chloride (0.6 M) was added into the chloroform solution containing the membrane lipids and stood for 4–12 h at room temperature. After the reaction, the solvent was evaporated to obtain lipids. Acetylated lipids were dissolved in 0.1 mL of chloroform–methanol (1 : 9 (v/v)) in the presence of NaOH (0.1 mM). ESI+ mass spectra (MS) were recorded on a JEOL JMS-T100LC instrument. Acetylated cholesterol, stigmasterol, and β-sitosterol were detected as a monomer of Na+ adduct at m/z 451, 477, and 479, respectively.14,15)

**Bilayer–Cytoskeleton Linkage** Open ghosts were prepared from the sterol-loaded erythrocytes using 5 mM sodium phosphate, pH 8 (5P8) at 0°C, suspended in 7 volume of 5P8, and incubated for 30 min at 37 °C.17) After the incubation, ghost suspensions were centrifuged for 20 min at 20000 × g and 4 °C. For the protein concentration in the supernatant, the method of Lowry et al. was used.18)

**RESULTS**

**Membrane Stability of Sterol-Incorporated Human Erythrocytes** ESI+ MS of a mixture of cholesterol, β-sitosterol, and stigmasterol, which were pre-acetylated and dissolved into the organic solvent, was measured at the same concentration. No significant difference was observed in the peak intensities of cholesterol, β-sitosterol and stigmasterol (Fig. 2). Therefore, we can estimate the relative concentration of sterols from their peak intensities on MS.

We examined whether each sterol was incorporated into the intact erythrocyte membrane using a CD/sterol complex. As shown in Fig. 3A, each sterol was additionally incorporated into the erythrocyte membrane without affecting original cholesterol content that was estimated from the peak intensity of cholesterol at m/z 451. Here, the peak intensity of cholesterol was normalized using that of phosphatidylcholine at m/z 780 (Fig. S1). The additionally loaded sterol contents were 20–40% of original cholesterol content in intact erythrocyte membrane (Fig. 3B).
Membrane stability of the erythrocytes that incorporated each sterol into their intact membranes was examined by measuring the hemolysis under a pressure of 200 MPa and hypotonic conditions. Hemolysis at 200 MPa of sterol-loaded erythrocytes was suppressed by cholesterol, but enhanced by β-sitosterol or stigmasterol (Fig. 4A). Similar results were also obtained in hypotonic hemolysis (Fig. 4B). Moreover, in order to confirm the sterol effect on the membrane stability, cholesterol was depleted up to 40% of original level by a CD and then sterols were incorporated into cholesterol-depleted membranes using a CD/sterol complex. With regard to sterol contents, 2-fold sterol of the residual cholesterol was loaded in each case (Fig. 5A). As shown in Fig. 5B, the hemolysis under pressure (200 MPa) and hypotonic conditions increased upon depletion of cholesterol from intact erythrocyte membrane and showed results similar with those in Fig. 4 for each sterol loading. Taken together, these results suggest that by contrast with cholesterol, plant sterols such as β-sitosterol and stigmasterol make the erythrocyte membrane unstable under pressure or hypotonic conditions.

**Effects of Sterols on Detachment of Cytoskeletal Proteins from the Erythrocyte Membrane under Hypotonic Buffer**

When erythrocyte ghosts are incubated in a hypotonic buffer, cytoskeletal proteins such as spectrin are detached from the membrane. Such detached proteins are released into the buffer. So, we examined the sterol effect on such protein detachment using a sterol-loaded erythrocyte membrane. Such protein detachment was suppressed by cholesterol, but not by
plant sterols such as \( \beta \)-sitosterol and stigmasterol (Fig. 6).

**DISCUSSION**

ESI\(^+\) MS have shown that the depletion of cholesterol from erythrocyte membrane and the loading of sterols into the cholesterol-depleted membrane are performed using a CD and a CD/sterol complex, respectively.

Mechanism of the hemolysis in human erythrocytes provides the information about the membrane structure. For instance, pressure-induced hemolysis and hypotonic one are greatly affected by the bilayer–cytoskeleton interaction and the surface area-to-volume ratio, respectively.\(^9\),\(^19\),\(^21\) Particularly, much interesting information has been obtained from the analysis of pressure-induced hemolysis.\(^9\),\(^13\),\(^14\),\(^19\),\(^20\) So, our attention is focused on sterol effects on the membrane stability of erythrocytes under pressure. When erythrocytes are exposed to a pressure, the hemolysis, vesiculation, and fragmentation are observed.\(^9\),\(^14\),\(^20\) The proportion of these events is mainly affected by the bilayer–cytoskeleton interaction,\(^9\),\(^20\) where cytoskeletal proteins are linked to the bilayer via linker proteins such as ankyrin.\(^22\) Here, ankyrin is also associated with transmembrane proteins such as band 3, anion exchanger.\(^22\) The pressure-induced hemolysis is suppressed by the facilitated vesiculation or fragmentation.\(^9\),\(^14\),\(^20\) For instance, in ATP-depleted or 49°C-treated erythrocytes, cytoskeletal proteins are tightly linked to the bilayer.\(^20\),\(^23\) Upon compression of such erythrocytes, the vesiculation is facilitated so that the hemolysis is greatly suppressed.\(^20\),\(^23\) Similar results are reported in cholesterol-loaded erythrocytes.\(^9\) The present work reveals that cholesterol strengthens the bilayer–cytoskeleton linkage and suppresses the hemolysis, whereas \( \beta \)-sitosterol and stigmasterol induce no such tight linkage and enhance the hemolysis. It is interesting to examine how sterols modulate the bilayer–cytoskeleton interaction. Sterols are amphipathic molecules that comprise of a hydroxyl group at C-3 and a hydrophobic moiety such as steroid nucleus and hydrocarbon chain. Thus, the hydroxyl group of sterols is located in the interface region of lipid bilayer and the hydrophobic moiety is the center region of bilayer.\(^1\) Therefore, it seems unlikely that sterols directly interact with cytoskeletal proteins. Cholesterol binds transmembrane proteins such as band 3 in the erythrocyte membrane.\(^24\),\(^25\) The cholesterol recognition amino acid consensus motifs are L/V-X\(_{15}\)-Y/F-X\(_{15}\)K/R (CRAC) and K/R-X\(_{15}\)-Y/F-X\(_{15}\)L/V (CARC).\(^26\) For instance, in the case of CRAC on the fifth transmembrane segment of band 3, the hydroxyl group, rigid ring, and side chain of cholesterol interact with the polar group of lysine or arginine, the aromatic ring of tyrosine or phenylalanine, and the branched side chain of leucine or valine, respectively.\(^26\) Conformational changes of band 3 by ligands result in the tight linkage of band 3 to spectrin.\(^27\) Thus, it seems likely that cholesterol induces the...
tight association of bilayer with cytoskeleton, via its interaction with band 3. In cholesterol-loaded erythrocytes, therefore, the vesiculation under pressure is facilitated so that the hemolysis is suppressed. On the other hand, such tight linkage is the vesiculation under pressure is facilitated so that the hemolysis with band 3. In cholesterol-loaded erythrocytes, therefore, the bilayer–cytoskeleton interaction in plant sterol-treated erythrocytes remains normal, the pressure-induced hemolysis is enhanced. To explain this enhancement, we consider the sterol–phospholipid interactions. In cholesterol–phospholipid interactions, the hydroxyl group at C-3 of the sterol interacts with the hydrocarbon chains of phospholipids. Sterol effects on the phase transition temperature of DPPC and on the solubilization of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine by Triton X-100 demonstrate that cholesterol, compared with the plant sterols such as β-sitosterol and stigmasterol, interacts more favorably with phospholipids. Thus, cholesterol leads to the stronger phospholipid condensation than the plant sterols, suggesting that the bulky ethyl group at C-24 in plant sterols, compared with cholesterol, reduces the sterol effect on hypotonic hemolysis. The permeability of DPPC liposomes towards glucose is less reduced by the plant sterols, compared with cholesterol. Taking these facts into consideration, properties of hypotonic hemolysis in sterol-treated erythrocytes may be partially explained by the sterol–phospholipid interactions.

In this paper, we have demonstrated that, by comparison with the plant sterols, the isocytol side chain of cholesterol is important in the membrane stability of human erythrocytes. Particularly, the tight association of bilayer with cytoskeleton by cholesterol is an interesting phenomenon. Cholesterol can bind band 3, which is connected with spectrin via ankyrin. Therefore, it is necessary to examine whether such tight association is induced by the cholesterol–band 3 interaction. Moreover, further study on the interactions of sterols with erythrocyte phospholipids is also important to understand the enhancement of the hemolysis by plant sterols. Plant sterols such as β-sitosterol and stigmasterol are contained in soybeans, vegetable oil, etc. Absorption of plant sterols from intestine is suppressed in healthy individual. However, plasma concentrations of plant sterols are elevated in Sitosterolemia. Such patients are subjected to a risk of cardiovascular disease. The present work also suggests the increased risk of hemolysis under stresses if the erythrocyte membrane is loaded with plant sterols.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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