**Effects of Cholesterol on Membrane Stability of Human Erythrocytes**

Takeo Yamaguchi* and Toshihiro Ishimatu

*Department of Chemistry, Faculty of Science, Fukuoka University; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan.

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**INTRODUCTION**

Human erythrocyte membrane is characterized by its stability and deformability. Cholesterol contributes to the stability and function of the membrane. Membrane stability of the erythrocyte has been mainly examined under hypotonic conditions, but not under high hydrostatic pressure. So, the effect of cholesterol on the membrane stability of human erythrocyte was examined under a pressure of 200 MPa. As with hypotonic hemolysis, the pressure-induced hemolysis was enhanced by depletion of cholesterol from the intact erythrocyte membrane, whereas suppressed by cholesterol loading to the intact one. Enhancement of such hemolysis was associated with the suppression of fragmentation, whereas the hemolysis was suppressed by the facilitation of vesiculation. Cholesterol induced the tight linkage of the lipid bilayer with cytoskeleton. Taken together, these results suggest that the erythrocyte membrane stability is affected by such tight linkage by cholesterol.

**Key words** bilayer–cytoskeleton interaction; cholesterol; human erythrocyte; hemolysis; flow cytometry; vesicle

**MATERIALS AND METHODS**

**Materials** Cholesterol and methyl-β-cyclodextrin (CD) were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich (U.S.A.), respectively. All other chemicals were of reagent grade.

**Pressure Treatment of Human Erythrocytes** Human erythrocytes were obtained from the Fukuoka Red Cross Blood Center. The erythrocytes were washed three times with phosphate-buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). To decrease the membrane cholesterol contents, the erythrocytes were incubated for 1 h at 37°C at a 20% hematocrit in 0.5% CD-containing PBS, which was preincubated for 30 min at 37°C, and washed with PBS. For the loading of cholesterol, the erythrocytes were incubated for 1 h at 37°C at a 20% hematocrit in PBS containing 0.5% CD-1.3 mM cholesterol, which was preincubated for 1–2 h at 37°C. After the incubation, the erythrocytes were washed with PBS. For the pressure treatment, the erythrocytes in PBS were incubated for 30 min at 200 MPa and 37°C, and decompressed up to atmospheric pressure. Such pressure-treated erythrocyte suspensions were used for measurements of hemolysis and flow cytometry.

**NOTE**

*To whom correspondence should be addressed. e-mail: takeo@fukuoka-u.ac.jp

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for 30 min. The samples in l-butyalcohol were frozen at −5°C and dried. The dried samples were coated with Pt. The cell morphology was observed using an SEM (model JSM-LV, JEOL).

For the hypotonic hemolysis, the erythrocytes were suspended in 10 mM sodium phosphate (pH 7.4) containing 54 mM NaCl, incubated for 10 min at 37°C, and centrifuged for 1 min at 1000 × g. The degree of hemolysis was estimated, as mentioned above.

**Analysis of Erythrocyte Membrane Lipids** Membrane lipids were extracted from erythrocytes by using a mixed solvent of chloroform and methanol (v/v = 2:1).18) The cholesterol concentration was determined by the method of Zlatkis et al.19) The total phospholipids were estimated by the method of Ames.20)

**Bilayer–Cytoskeleton Interaction** To extract the cytoskeletal proteins from the membrane, open ghosts, which were prepared from the erythrocytes using 5 mM sodium phosphate, pH 8 (5P8) at 0°C, were suspended in 7 volume of 5P8 and incubated for 1 h at 37°C.21) After the incubation, ghost suspensions were centrifuged for 20 min at 20000 × g and 4°C. The protein concentration in the supernatant was determined by the method of Lowry et al.22)

**RESULTS**

**Effects of Cholesterol on Membrane Stability of Human Erythrocytes** To examine effects of cholesterol on the membrane stability of human erythrocytes, the amount of membrane cholesterol was modulated by using a CD or a CD-cholesterol complex. The cholesterol content was evaluated for total membrane phospholipid one. Pressure (200 MPa)-induced hemolysis and hypotonic one15) were enhanced upon depletion of cholesterol from intact erythrocyte membranes (cholesterol/phospholipid approx. 0.6 at molar ratio), whereas suppressed upon cholesterol loading (Fig. 1). Moreover, when the cholesterol-depleted erythrocyte membranes were reloaded with cholesterol to cholesterol/phospholipid approx. 1.6, the hemolysis at 200 MPa and hypotonic lysis were suppressed, as seen in cholesterol loading of intact erythrocyte membranes (Fig. 1). These results suggest that the membrane stability under pressure or hypotonic stress is dependent on cholesterol content.
The shape of 200MPa-treated erythrocytes was examined using a SEM (Fig. 2). The shape of intact erythrocytes exposed to a pressure of 200MPa changed from biconcave disk to spherical form, and many vesicles were observed on the surface of mother cells (Fig. 2A). Here, mother cells only were observed since other particles such as open ghosts and released vesicles were removed by centrifugation. In cholesterol-depleted erythrocytes, pressure-treated cells became spherical and vesicles were formed on the cell surface (Fig. 2B). In cholesterol-loaded erythrocytes, on the other hand, few spherical and vesicles were formed on the cell surface (Fig. 3A). Here, the size of closed particles is as follows: mother cells > fragmented particles > vesicles. Upon facilitation of fragmentation and vesiculation, the hemolysis at 200 MPa is suppressed (Fig. 3C). Moreover, the effect of fragmentation on the hemolysis at 200MPa is more dominant than that of vesiculation. When cholesterol-depleted erythrocytes were exposed to a pressure of 200MPa, particles in regions a and b deceased and particles in regions c and d increased (Fig. 3B). This indicates that the hemolysis enhances upon suppression of fragmented particles. In cholesterol-loaded erythrocytes, on the other hand, the vesiculation was facilitated so that pressure-induced hemolysis was significantly suppressed (Fig. 3C).

**Effects of Cholesterol on the Bilayer–Cytoskeleton Interaction**

Membrane stability of human erythrocytes is affected by the bilayer–cytoskeleton interaction. Parts of cytoskeletal proteins such as spectrin and actin are detached from the bilayer when ghosts are exposed to hypotonic buffer. The amount of detached proteins reduces under tight linkage between bilayer and cytoskeleton. So, the effect of cholesterol on such detachment was tested. The amount of detached proteins from the bilayer under hypotonic buffer significantly enhanced upon depletion of cholesterol from intact membrane, and reduced upon sterol loading (Fig. 4A). Thus, cytoskeletal proteins became refractory to such detachment under cholesterol loading (Fig. 4B). These results suggest that cholesterol strengthens the bilayer–cytoskeleton interaction.

**DISCUSSION**

Under hypotonic conditions, the erythrocytes are swollen and hemolyzed. The swollen cells are characterized by reduction of the surface area-to-volume ratio. Upon compression at 200MPa of erythrocytes under isotonic conditions, the cell volume decreases and the hemolysis occurs upon release of large vesicle. For instance, hereditary spherocytic (HS) erythrocytes are more fragile under hypotonic buffer, but not under compression at 200MPa. Moreover, the erythrocytes treated with 4,4’-disothiocyanostilbene-2,2’-disulfonate (DIDS), an inhibitor of anion transporter band 3, exhibit a 10% increased cell volume due to water uptake. In DIDS-modified erythrocytes, the hypotonic hemolysis is enhanced, whereas the hemolysis at 200MPa is suppressed. Interestingly, in cholesterol-depleted erythrocytes that exhibit a decreased surface area-to-volume ratio, hypotonic hemolysis is independent. Furthermore, denatured spectrin in 49°C-treated erythrocytes is tightly attached to the bilayer.

In these erythrocytes, 200MPa-induced hemolysis and hypotonic one are suppressed. Similar results are obtained in cholesterol-loaded erythrocytes (Fig. 1). These results suggest the modulation of the bilayer–cytoskeleton interaction by cholesterol.

Pressure-induced hemolysis is greatly suppressed by facilitation of vesiculation, as with 49°C-heated erythrocytes or ATP-depleted ones and also of fragmentation, as seen under hypertonic buffer of normal cells. The present work shows that the hemolysis at 200MPa is suppressed by the facilitated vesiculation in cholesterol-loaded erythrocytes, whereas is enhanced by the suppressed fragmentation in cholesterol-depleted cells, as with ATP-rich ones. Why does cholesterol...
loading into the membrane facilitate the vesiculation under pressure? In 49°C-treated erythrocytes and ATP-depleted ones, the bilayer interacts tightly with cytoskeleton so that the vesiculation under pressure is facilitated.6,23) This work also shows the tight linkage of the bilayer with cytoskeleton by cholesterol. This tight linkage induces the facilitated vesiculation under pressure.23) Cholesterol is distributed in both leaflets of lipid bilayer. β-Hydroxyl group at C-3 of cholesterol is located in the interface of the bilayer, whereas its isoctyl chain is oriented in the hydrophobic region of the bilayer.20) Thus, it seems unlikely that cholesterol interacts directly with cytoskeletal proteins such as spectrin. Therefore, cholesterol may mediate indirectly the bilayer–cytoskeleton interaction.

Spectrin is mainly attached to the bilayer via linking proteins such as ankyrin and protein 4.1R.15) Ankyrin links spectrin to the transmembrane protein band 3, whereas protein 4.1R links spectrin to the transmembrane protein glycophorin C.21) Interestingly, cholesterol binds transmembrane proteins.31) The cholesterol-binding motif is the following amino acid consensus sequences: L/V-X(1–5)-Y/F-X(1–5)-K/R (CRAC) and K/R-X(1–5)-Y/F-X(1–5)-L/V (CARC).31) The both motifs are located on the same transmembrane segment in membrane receptors.31) Similar motifs are observed in band 3 that comprises 14 transmembrane segments.32) Indeed, the interaction of cholesterol with band 3 is reported.30,33) As shown in Fig. 4C, cholesterol binding motifs are located on the fifth transmembrane segment (TM 5) of band 3.32) Here, the CRAC (LIIFYTEFS539K) motif is located in the outer leaflet of lipid bilayer and CARC in its inner leaflet. Moreover, the TM 6 or 7 of band 3 has CRAC or CARC motif, respectively.32) Importantly, the interaction of spectrin with the bilayer is modulated by the binding of DIDS to Lys-539, which is located at exofacial site on the TM 5.26) Thus, the TM 5 might be predominant than TMs 6 and 7 as a modulator of the bilayer–cytoskeleton interaction. Another transmembrane protein such as glycoporphin A interacts with cytoskeletal proteins upon ligand binding.34) Binding motif for cholesterol is observed as a CRAC on the transmembrane segment of glycoporphins A and C (Fig. 4C). Thus, it seems likely that the interaction of the bilayer with cytoskeleton is modulated by the binding of cholesterol to the transmembrane proteins such as band 3, glycoporphins A, and C. So, we propose that the membrane stability of erythrocytes under pressure is mediated by the cholesterol–transmembrane protein interaction, which is followed by the bilayer–cytoskeleton interaction.

Roles of cholesterol in the membrane stability have been mainly explained by cholesterol–phospholipid interactions.12,35) Cholesterol in lipid bilayer induces the ordering of hydrocarbon chains in phospholipids so that the liquid ordered phase is formed.15) Such ordered phase is also induced by high pressure.56) Interestingly, giant liposomes composed of egg phosphatidylcholine (PC) only show a spherical form at a pressure of 285 MPa, but a budded form after decompression.55) However, cholesterol-containing egg PC liposomes remain spherical during such pressure treatment,53) suggesting the stabilization of the liposome structure under pressure by cholesterol. On the other hand, the compression at 200 MPa of cholesterol-loaded erythrocytes induces the vesiculation. This vesiculation is ascribed to the tight linkage of bilayer with cytoskeleton,23) via cholesterol–transmembrane protein interactions. Thus, we have demonstrated that the modulation of bilayer–cytoskeleton interaction by cholesterol plays an important role in the mechanical stability of human erythrocyte membrane. Further studies on cholesterol–phospholipid interactions under pressure using liposomes composed of erythrocyte membrane phospholipids promise to supplement our idea.

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

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**Fig. 4. Effects of Cholesterol on Protein Detachment from the Membrane and Cholesterol Binding Amino Acid Sequences**

(A) Ghosts were prepared from intact (none) erythrocytes, cholesterol-depleted (−) or cholesterol-loaded (+) ones. For detachment of cytoskeletal proteins, these ghosts were incubated in 5P8 for 30 min at 37°C. (B) Relation between detached proteins and cholesterol contents. (C) Transmembrane segments (TM) of band 3 (glycoporphin A (Human #P02724), and glycoporphin C (Human #P04921). Under lines of straight and wave present CARC and CRAC motifs, respectively. Values are means ± S.D. for three independent experiments. * **p < 0.01 vs. None.
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