Effects of cholesterol on the membrane stability of 49˚C-heated human erythrocytes

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Abstract

Cholesterol is a major sterol in human erythrocyte membrane and plays an important role in the membrane stability. However, there is little information about the contribution of cholesterol to the membrane stability of heated erythrocytes. So, we examined the effect of cholesterol on the hemolysis and vesiculation of 49˚C-heated human erythrocytes. The hemolysis at 49˚C of cholesterol-depleted erythrocytes increased due to the suppression of vesiculation, compared to that of intact or cholesterol-loaded ones. Heat-induced vesicles revealed that upon loading of cholesterol into the erythrocyte membrane, cholesterol levels in the vesicle membrane increase, whereas spectrin levels decrease. Moreover, the tight linkage of the bilayer to cytoskeleton was induced by cholesterol. These results suggest that cholesterol protects the membrane structure against the heat stress through the strong bilayer-cytoskeleton interaction.

Keywords: cholesterol, human erythrocyte, heat stress, hemolysis, vesicle

1. Introduction

Vesiculation *in vivo* is involved in many biological processes [1]. Vesicles released from many cells play an important role in intercellular communication [1]. For instance, the transformation of normal cells into cancer ones is induced by cancer cell-derived vesicles [2]. Moreover, vesicles derived from senescent erythrocytes are observed in the blood and contribute to the removal of breakdown products of membrane proteins such as band 3, anion exchanger [3]. On the other hand, the vesiculation *in vitro* occurs in erythrocytes exposed to various stresses such as heat [4], pressure [5], and ATP depletion [6]. Of these vesicles, heat vesicles are produced by simply incubating erythrocytes at 49˚C [7]. In 49˚C-treated erythrocytes, spectrin is denatured [8]. Cytoskeletal proteins such as spectrin and actin are essential in the membrane stability of erythrocytes [9]. Spectrin is linked to band 3 *via* ankyrin, anchor protein [9]. Band 3 is a major transmembrane protein in erythrocytes [10]. Thus, the cytoskeleton is associated with the lipid bilayer and can mediate the stability and deformability of the erythrocyte membrane [9]. Therefore, the abnormal interactions of lipid bilayer with cytoskeleton induce the membrane instability so that the hemolysis and vesiculation occur, as seen in spherocytosis [11]. Heat-denatured spectrin also induces the abnormal interaction of lipid bilayer with cytoskeleton [7]. Thus, vesicles are formed on the membrane surface and parts of them are detached from the surface and released into the medium [7]. Here, vesicles produced by heating are referred to as heat vesicles.

Cholesterol is a major lipid in human erythrocyte membrane and largely contributes to the membrane stability [12]. It is well known that the membrane fluidity is mediated by the cholesterol-phospholipid interaction [13]. However, recent research demonstrates that cholesterol also interacts with membrane proteins and is able to regulate the activity of intramembrane enzymes such as precenilin [14, 15]. Here, precenilin is an enzyme that cleaves amyloid precursor protein [15]. Recently, we have reported that the bilayer-cytoskeleton interaction in human erythrocyte membrane is mediated by cholesterol [16]. So, it is of interest to examine how the stability of the erythrocyte membrane against heat is affected by cholesterol. In the present work, we describe the cholesterol effect on properties of the hemolysis and vesiculation in 49˚C-treated erythrocytes.

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2. Materials and Methods

2. 1 Materials

Compounds were obtained from the following sources: cholesterol, Nacalai Tesque; 2, 5-dihydrobenzoic acid (2, 5-DHB), Tokyo Kasei; methyl-β-cyclodextrin (CD), Sigma-Aldrich. All other chemicals were of reagent grade.

2. 2 Hemolysis and vesiculation of human erythrocytes at 49 ˚C

Human erythrocytes were obtained from the Fukuoka Red Cross Blood Center. The erythrocytes were washed three times with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). To reduce the membrane cholesterol, the erythrocytes (20 % hematocrit) in 0.5% 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 [17]. For loading of cholesterol, the erythrocytes (20 % hematocrit) in 0.5% CD-1.3 mM sterol complex-containing PBS, which was pre-incubated for 1~2 h at 37˚C, were incubated for 1 h at 37˚C. After the incubation, the erythrocytes were washed with PBS. For the heat treatment, the erythrocytes in PBS were incubated for 1 h at 49 ˚C. The distribution of particles contained in such heat-treated erythrocyte suspensions was analyzed using an EPICS XL-MCL flow cytometer (Coulter, USA) [18]. Heat-treated erythrocyte suspensions were centrifuged for 10 min at 1,000 × g and room temperature (~25˚C), and the supernatants were used for the measurement of hemolysis and the vesicle preparation. For the hemolysis, the absorbance of the supernatant was measured at 542 nm. For the vesicle preparation, the supernatants were filtered using a Milipore filter (pore size, 3 μm). The filtrates were centrifuged for 20 min at 20,000 × g and 4 ˚C. The pellets containing vesicles were used for the lipid extraction and SDS-PAGE.

2. 3 SDS-PAGE of membrane proteins

Ghosts were prepared from erythrocytes using 5 mM sodium phosphate, pH 8 (5P8) at 0˚C. Membrane proteins in ghosts and vesicles were separated by SDS-PAGE using 8% acrylamide gels, according to the method of Laemmli [19]. Protein bands were detected by the Coomassie brilliant blue staining.

2. 4 Cholesterol contents in the membrane

Membrane lipids were extracted from erythrocytes using chloroform and isopropyl alcohol (2:3 by volume) [20]. In erythrocyte lipids, the cholesterol concentration was determined by the method of Zlatkis *et al*. [21] and the amount of total phospholipids was estimated by the method of Ames [22]. In heat vesicles, lipids were extracted using chloroform and methanol (2:1 by volume) [23] and detected by a matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in linear positive-ion mode. As a matrix, 2, 5-DHB (10 mg/mL) was dissolved in a mixed solvent of chloroform and methanol (2:1 by volume) [24]. The lipids were dissolved in a mixed solvent of chloroform and methanol (1:9 by volume). As samples for MS, equal volumes of lipids and 2, 5-DHB were mixed [24]. The measurement was carried out using an Autoflex speed MALDI-TOF mass spectrometer (Bruker). Cholesterol was observed as H^+ adduct ($M + H^+ - H_2O$) at m/z 368.9 [25].

2. 5 Bilayer-Cytoskeleton Interaction

Open ghosts were suspended in 7 volume of 5P8 and incubated for 30 min at 37˚C. After the incubation, ghost suspensions were centrifuged for 20 min at 20,000 × *g* and 4˚C [26]. The protein concentration in the supernatant was determined by the method of Lowry *et al*. [27].

3. Results

3. 1 Hemolysis of heated erythrocytes is suppressed by cholesterol

Depletion or loading of membrane cholesterol was performed by incubating intact erythrocytes with a CD or a CD-cholesterol complex, respectively. The resulting cholesterol levels were decreased by about 30% of the original level upon depletion and increased by about 60% upon loading (Fig. 1A).

When intact erythrocytes were heated at 49˚C, the hemolysis was induced. So, we examined the cholesterol effect on such event. Hemolysis at 49˚C was enhanced upon cholesterol depletion, but not upon cholesterol loading (Fig. 1B). The proportion of hemolysis, fragmentation, and vesiculation in erythrocytes exposed to stresses such as high pressure is able to be estimated by a flow cytometry [16, 18]. Figure 2 shows the flow cytometry of 49˚C-treated erythrocyte suspensions, where cholesterol levels in the erythrocyte membrane are modulated. The regions **a**, **b**, **c**, and **d** in Fig. 2 contain mother cells, fragmented particles, vesicles, and hemolyzed cells, respectively [16, 18, 28]. Compared with heating of intact erythrocytes (Fig. 2B), cholesterol-depleted cells at 49 ˚C hemolyzed readily due to the suppression of vesiculation (Fig. 2A). On the other

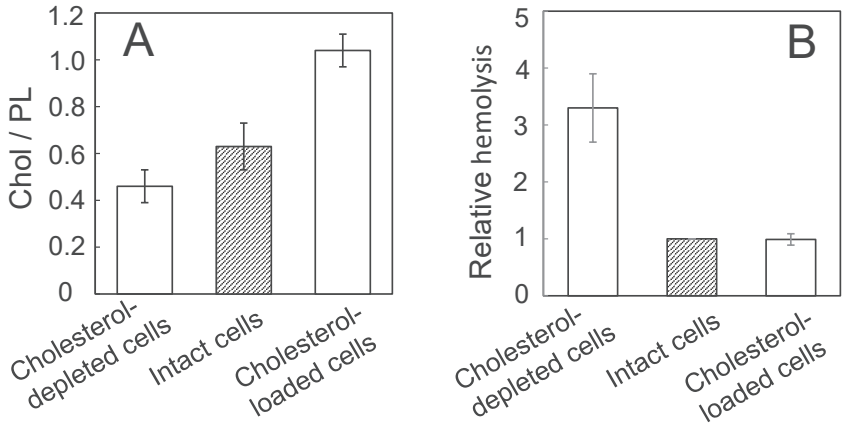
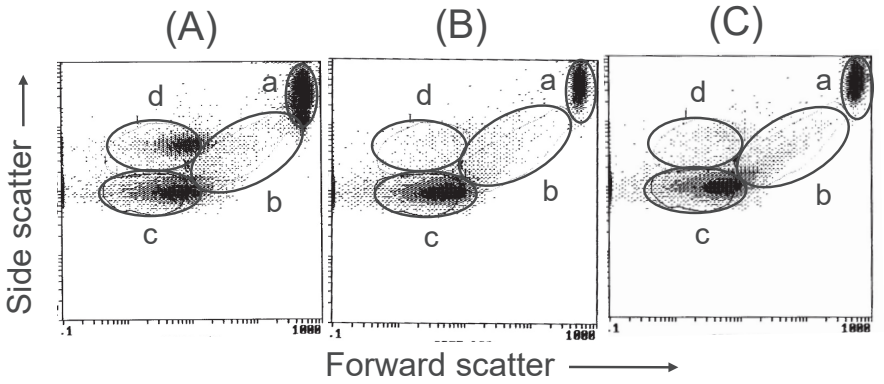


Fig. 1 Effects of cholesterol on the hemolysis of 49 ˚C-heated human erythrocytes. (A) Cholesterol levels of intact erythrocyte membrane were modulated using a CD or CD-cholesterol complex. Cholesterol (Chol) levels are normalized by total phospholipids (PL) in the membrane. (B) Hemolysis at 49 ˚C of cholesterol level-modulated erythrocytes. Values are means ± SD for three independent experiments.



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51.7	7.6	15.0	10.1	33.2	8.4	40.6	3.3	38.7	11.4	34.6	3.5

Fig 2 Flow cytometry of 49˚C-treated erythrocyte suspensions. Cholesterol-depleted (A), intact (B), and cholesterol-loaded (C) erythrocytes were incubated for 1 h at 49˚C. After the incubation, the erythrocyte suspensions were used for flow cytometric measurements.

hand, the response of cholesterol-loaded erythrocytes to heating was similar to that of intact cells (Fig. 2C).

3. 2 Properties of heat vesicles

Membrane proteins in heat vesicles were examined using a SDS-PAGE. Band 3 and spectrin were observed as major membrane proteins (Fig. 3A). In various vesicles such as ATP-depleted [28] and pressure-induced ones [5], band 3 is the most constituent protein, whereas spectrin levels are modulated [5-7, 28]. So, spectrin levels in heat vesicles were expressed as the ratio of band intensities of spectrin and band 3. As cholesterol levels in the erythrocyte membrane increased, spectrin levels in heat vesicles decreased (Fig. 3B).

Furthermore, MALDI-TOF mass spectra of lipids were measured to examine cholesterol levels in heat vesicles. Figure 4A shows the part of mass spectrum of heat vesicle lipids from intact erythrocytes. Cholesterol was identified as a mass peak at m/z 369 [24, 25]. The peak intensity of cholesterol was normalized by that of sphingomyelin at m/z 703 on the same mass spectrum. Thus, the cholesterol levels in heat vesicles produced from intact erythrocytes were about 0.6-fold of those in the intact erythrocyte membrane. Moreover, cholesterol levels in vesicle membranes reflected those in erythrocyte membranes, *i.e.*, cholesterol-poor heat vesicles were produced from cholesterol-depleted erythrocytes, whereas cholesterol-rich vesicles from cholesterol-loaded ones (Fig. 4B).

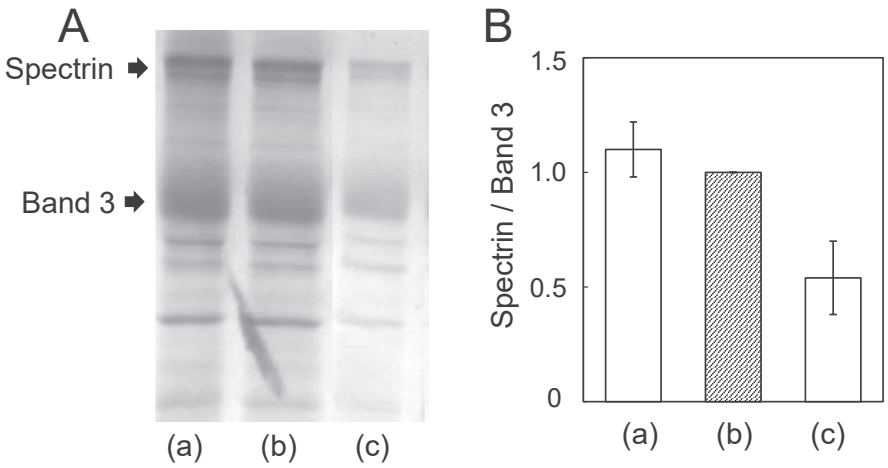

Fig. 3 Spectrin levels in the heat-vesicle membrane. Heat vesicles were prepared by heating cholesterol-depleted (a), intact (b), and cholesterol-loaded (c) erythrocytes at 49˚C. (A) SDS-PAGE of heat vesicles. (B) Spectrin levels in heat-vesicle membrane. Spectrin levels were normalized by band 3. Values are means ± SD for three independent experiments.

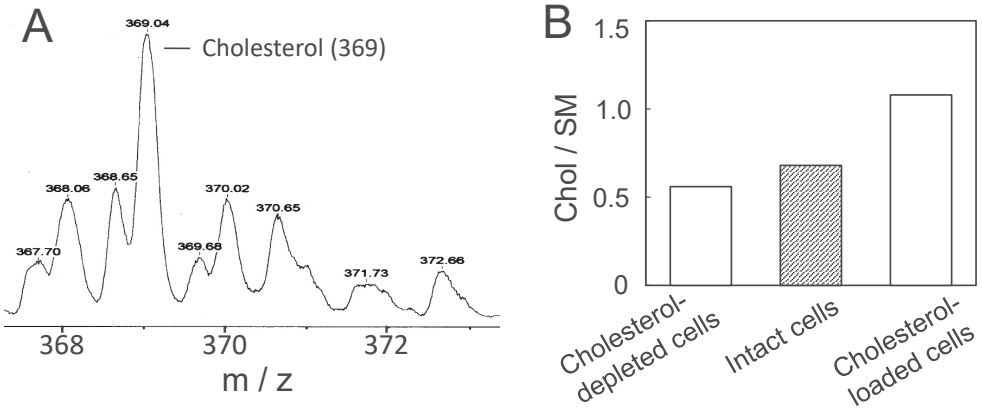

Fig. 4 MALDI-TOF mass spectrum of heat vesicles and cholesterol levels in heat vesicles. (A) MALDI-TOF mass spectrum of heat vesicles from intact erythrocytes. (B) Cholesterol levels in heat vesicles produced from cholesterol-modulated erythrocytes. Peak intensity of cholesterol (Chol) at m/z 369 was normalized by that of sphingomyelin (SM) at m/z 703.

3. 3 Effects of cholesterol on detachment of cytoskeletal proteins from the erythrocyte membrane

The stability of the erythrocyte membrane is controlled by the interaction of the lipid bilayer with cytoskeleton [9]. Interestingly, the cytoskeletal proteins are detached from the lipid bilayer by incubating the erythrocyte membrane in hypotonic buffer [26]. The amount of detached proteins is affected by the strength of the bilayer-cytoskeleton linkage. For instance, in the tight linkage of bilayer to cytoskeleton the amount of detached proteins is decreased [7, 28]. To examine the cholesterol effect on the bilayer-cytoskeleton interaction, the erythrocyte membrane, in which cholesterol levels are modulated, was incubated in hypotonic buffer. The amount of detached cytoskeletal proteins was reduced upon increasing cholesterol levels (Fig. 5). This result suggests that cholesterol induces the tight linkage of bilayer to cytoskeleton.

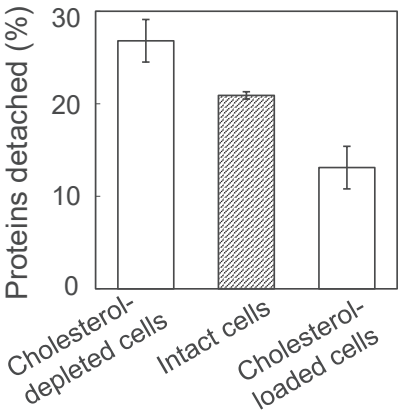

Fig. 5 Cytoskeletal proteins detached from the erythrocyte membrane. Open ghosts prepared from cholesterol-modulated erythrocytes were incubated in 5P8 for 30 min at 37˚C. Values are means ± SD for three independent experiments.

4. Discussion

In the present work, we have examined the cholesterol effect on the membrane stability of 49°C-treated erythrocytes. The hemolysis and vesiculation of erythrocytes are induced by heat stress at 49°C. Upon depletion of cholesterol from the intact erythrocyte membrane, such a hemolysis was enhanced due to the suppression of vesiculation. Similar results are also obtained in pressure (200 MPa)-treated erythrocytes [16]. Moreover, the effect of cholesterol-loading is clearly observed as the suppression of hemolysis due to the facilitation of vesiculation in 200 MPa-treated erythrocytes, whereas not apparent due to the less damage of intact erythrocyte membrane in the case of 49°C-treated cells [16]. Perhaps, the effect of cholesterol loading on heat stress may appear under severe conditions such as the long incubation at 49°C.

In 49°C-treated erythrocytes, denatured spectrin is tightly attached to the bilayer [7, 8]. Under such conditions, the hemolysis and vesiculation are induced [7]. Moreover, we have demonstrated the strong interaction of bilayer with cytoskeleton by cholesterol [16]. Upon exposure to 49°C of cholesterol-depleted erythrocytes, the hemolysis is facilitated and the vesiculation is suppressed, compared to the case of intact erythrocytes or cholesterol-loaded ones. These results suggest that the cholesterol-depleted erythrocyte membrane becomes more fragile and the membrane is hemolyzed before the vesiculation.

Upon exposure of erythrocytes to heat stress at 49°C, parts of vesicles formed on the membrane surface are released into the medium [7, 18]. The level of membrane cholesterol, which is expressed as the ratio with phospholipids, in heat vesicles released from intact erythrocytes is smaller than that of mother cells. Similar results are obtained in vesicles released from 140 MPa-treated erythrocytes [29]. Moreover, cholesterol levels in heat vesicles reflect those in the mother cell membrane, *i.e*., cholesterol-poor vesicles are released from cholesterol-depleted erythrocytes, as shown in Fig. 5. Membrane protein levels in vesicles are modulated by the bilayer-cytoskeleton interaction in mother cells. For example, when the erythrocytes are incubated for a long time in glucose-free medium, intracellular ATP is consumed [28]. In ATP-depleted erythrocytes, the cytoskeleton links tightly to the bilayer so that spectrin-poor vesicles are produced [28]. The present work also shows that cholesterol induces the strong interaction of the bilayer with cytoskeleton. As expected, the spectrin levels in heat vesicles increase in cholesterol-depleted erythrocytes, but decrease in cholesterol-loaded ones. Cholesterol is able to interact with transmembrane proteins such as band 3 as well as phospholipids [14, 30]. Therefore, the tight linkage of the bilayer to cytoskeleton by cholesterol may be induced through its binding to transmembrane proteins [16].

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