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Membrane perturbations induced by the interactions of zinc ions with band 3 in human erythrocytes

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ABSTRACT

Of group 12 metals, zinc is an essential element to maintain our life, but other metals such as cadmium and mercury are toxic in cellular activities. Interactions of these metals with biomembranes are important to understand their effects on our living cells. Here, we describe the membrane perturbations induced by these metals in human erythrocytes. Of these metals, Zn^{2+} ions only induced the erythrocyte agglutination. Histidine residues in extracellular domains of band 3 participated in Zn^{2+}-induced agglutination. Interestingly, it was found that band 3-cytoskeleton interactions play an important role in Zn^{2+}-induced agglutination. In contrast with Hg^{2+} and Cd^{2+} ions, Zn^{2+} ions greatly suppressed pressure-induced hemolysis by cell agglutination. Such a suppression was removed upon dissociation of agglutinated erythrocytes by washing, indicating the reversible interactions of Zn^{2+} ions with erythrocyte membranes. Excimer fluorescence of pyrene indicated that spectrin is denatured by a pressure of 200 MPa irrespective of hemolysis suppression. Taken together, these results suggest that the agglutination of erythrocytes due to the interactions of Zn^{2+} ions with band 3 is stable under pressure, but spectrin, cytoskeletal protein, is denatured by pressure

1. Introduction

Membrane stability and functions are ingeniously regulated by the interactions among components such as proteins, lipids, and sugars [1]. Of biomembranes, erythrocyte membranes are characterized by their deformability and stability [2]. Interactions between membranes components in human erythrocytes have been intensively investigated using simplified systems [3]. However, it seems to be preferable to use whole cells in the analysis of such interactions. Hemolysis reflects sensitively the perturbation in the membrane structure of whole cells [4]. In particular, we expect that pressure-induced hemolysis provides useful information about membrane perturbations in whole cells [5].

When human erythrocytes are exposed to a pressure of 200 MPa, hemolysis, vesiculation, and fragmentation are observed [6]. Here, vesiculation or fragmentation is defined by the formation of particles with diameters below 0.65 μm or between 0.65 and 3 μm, respectively. Pressure-induced hemolysis is suppressed by facilitation of vesiculation and fragmentation [5–7]. For instance, vesicles are formed on the membrane surface of 49 °C-treated erythrocytes, where spectrin is denatured [5]. Upon exposure of such erythrocytes to high pressure, vesiculation is facilitated so that the hemolysis is suppressed [5]. On the other hand, the hemolysis at 200 MPa of trypsin-treated erythrocytes is enhanced due to the suppression of fragmentation [7]. Thus, pressure-induced hemolysis is largely affected by vesiculation and fragmentation.

Recently, we have reported that pressure-induced hemolysis is enhanced by mercury ions [6]. Mercury ions bind to Cys-189 of aquaporin 1 and inhibit water transport [8]. Under such conditions, the fragmentation under pressure is suppressed so that the hemolysis is remarkably enhanced [6]. Thus, mercury ions show the dramatic effects on pressure-induced hemolysis. Therefore, it is of interest to examine the interactions of the erythrocyte membrane with other metal ions such as Zn^{2+} and Cd^{2+} of group 12. Particularly, zinc is an interesting element because it plays an essential role in catalytic and structural functions of many proteins [9]. For instance, Zn^{2+} ions participate in catalytic reactions in metalloenzymes such as carboxypeptidase A [10], thermolysin [11], and carbonic anhydrase [12]. As seen in transcription factors, zinc atoms stabilize the DNA binding motifs [13]. Interestingly, Zn^{2+} ions modulate the aggregation of amyloid-β in Alzheimer's disease [14]. In Zn^{2+}-treated erythrocytes, moreover, it is well known that Zn^{2+} ions induce the cluster of band 3, anion transporter [15]. On the other hand, Cd^{2+} ions induce the destruction of cytoskeletal structure in

Abbreviations: DEPC, diethylpyrocarbonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DMPC, dimyristoylphosphatidylcholine; DNDS, 4, 4′-dinitrostilbene- 2,2′-disulfonate; NPIA, N-(1-pyrenyl) iodoacetamide; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; PBS, phosphate-buffered saline, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4; 5P8, 5 mM sodium phosphate, pH 8; SEM, scanning electron microscope; TBS, tris-buffered saline, 17 mM Tris, 123 mM NaCl, pH 7.4; TM, transmembrane

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erythrocyte ghosts [16]. Therefore, anemia is observed in cadmium-administered mammals [17]. In the present work, we demonstrate the responses of human erythrocytes to the group 12 metal ions. Of these metal ions, Zn^{2+} ions only induce the agglutination of erythrocytes. So, properties of this erythrocyte agglutination are described on the basis of Zn^{2+}- band 3 interactions and pressure-induced hemolysis.

2. Materials and methods

2.1. Materials

Compounds were obtained from the following sources: diethylpyrocarbonate (DEPC), Nacalai Tesque; dimyristoylphosphatidylcholine (DMPC), Sigma; 4, 4′-dinitrostilbene-2,2′-disulfonate (DNDS), Tokyo Kasei; 4, 4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), eosin-5-maleimide, and N-(1-pyrenyl) iodoacetamide (NPIA), Molecular Probes; octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$), Nikko Chemicals. All other chemicals were of reagent grade.

2.2. Erythrocyte agglutination

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) by centrifugation for 10 min at 1000*g* and 4 °C. The washed erythrocytes were incubated for 10 min at 37 °C in TBS (17 mM Tris, 128 mM NaCl, pH 7.4) containing 4 mM $ZnCl_2$, 4 mM $CdCl_2$, or 10 μM $HgCl_2$. The cell morphology was observed using a light microscope (Olympus, model IX-71). To examine changes in sedimentation speeds due to cell agglutination, erythrocyte suspensions at a 0.5% hematocrit in TBS containing 0.5 mM Zn^{2+} ions were preincubated for 10 min at room temperature (~25 °C) and entered into an optical microcell (sample volume 0.1 mL). The turbidity at room temperature was monitored at 700 nm. For dissociation of agglutinated erythrocytes, the transparent well plate of U-bottom type was used. Erythrocyte suspensions (0.5 mL) at a 1% hematocrit in TBS containing 0.5 mM Zn^{2+} ions were incubated for 10 min at 37 °C and centrifuged at 900*g* for 1 min. The pellets were incubated in 0.5 mL of TBS containing 10 mM monosaccharides (N-acetylglucosamine, galactose, mannose, fucose, and N-acetylneuraminic acid) or 1 mM amino acids (all amino acids on extracellular loops of band 3) for 1 h at 37 °C. After the incubation, the erythrocyte suspensions were entered into wells and the dissociation of agglutinated erythrocytes was examined after 1 h at room temperature.

2.3. DMPC vesicles

Liposome suspensions were prepared by sonicating DMPC (0.74 mM) in PBS. Erythrocyte suspensions at a 10% hematocrit were prepared using these liposomes and incubated for 5 h at 37 °C. After the incubation, the erythrocyte suspensions were centrifuged for 20 min at 3000 g at 4 °C. The supernatants were filtered through a Millipore filter of pore size 3.0 μm. The filtrates were centrifuged for 20 min at 20,000 g and 4 °C. The pellets as DMPC vesicles were suspended in TBS containing 0.5 mM Zn^{2+} ions and used to measure particle size by dynamic light scattering using a Zetasizer Nano S (Malvern Instruments). Ghosts from intact erythrocytes and DMPC-treated ones were prepared using 5P8 (5 mM sodium phosphate, pH 8). Membrane proteins in DMPC vesicles and ghosts were separated by SDS-PAGE using 8% acrylamide, according to the method of Laemmli [18]. The gels were stained with Coomassie blue.

2.4. Zinc-induced erythrocyte agglutination under various conditions

For chemical modification of erythrocytes, cell suspensions at 20% hematocrit in PBS were incubated with 7 mM DEPC for 30 min at 0 °C or 50 μM DIDS for 30 min at 37 °C. These erythrocytes were washed three times with PBS. The chemically modified erythrocytes were suspended in TBS containing 0.5 mM Zn^{2+} ions and the agglutination was estimated from the measurement of turbidity, as described above. To examine effects of pH on agglutination, erythrocytes were preincubated in TBS (pH 5.8, 6.6, 7.4, 8.0, or 8.6) containing 0.5 mM Zn^{2+} ions for 10 min at room temperature. Similarly, the agglutination of erythrocytes suspended in these buffers was estimated from the turbidity at 700 nm.

2.5. Band 3-cytoskeleton interactions

To label band 3 with eosin-5-maleimide, erythrocytes (20% hematocrit) in PBS were pretreated with 0.5 mM DNDS at 37 °C for 10 min and treated with eosin-5-maleimide (0.1 mg/ml) for 1 h at room temperature. After incubation, the erythrocytes were washed three times with PBS, twice with PBS containing 0.5% (w/v) bovine serum albumin, and then three times with PBS. Ghosts were prepared from eosin-5-maleimide-labeled erythrocytes using 5P8 and incubated in 140 mM KCl, 10 mM NaCl, 5 mM sodium phosphate, 1 mM $MgCl_2$, pH 8 at 37 °C for 1 h. These resealed ghosts (at 5% hematocrit) were incubated for 10 min at 37 °C in TBS (pH 7.4 and 8.6) and then solubilized by 0.5% (w/v) $C_{12}E_8$ in TBS (pH 7.4 and 8.6) for 10 min at 15 °C. Thus, solubilized samples were centrifuged at 20,000*g* for 20 min at 4 °C. The fluorescence of eosin was measured with excitation at 490 nm and emission at 546 nm using a model FP-750 spectrometer (JASCO). The proportion of band 3 interacted with cytoskeleton was estimated from fluorescence intensities of eosin-5-maleimide in supernatants and pellets.

2.6. Pressure-induced hemolysis of human erythrocytes

To examine the effects of metal ions on pressure-induced hemolysis, the erythrocytes suspended at a 0.3% hematocrit in TBS containing 4 mM $ZnCl_2$, 4 mM $CdCl_2$, or 10 μM $HgCl_2$ were preincubated for 10 min at 37 °C and then exposed to a pressure of 200 MPa for 30 min at 37 °C in the presence of these metal ions, as previously described [4,6]. For reversibility of metal ion binding to the membrane, these preincubated erythrocytes were washed three times with TBS. Washed erythrocytes were suspended in metal-free TBS and compressed up to 200 MPa. After the decompression, the erythrocyte suspensions were centrifuged for 1 min at 3000*g* and room temperature. The absorbance of the supernatant was measured at 542 nm.

2.7. Membrane damages under pressure

Erythrocyte suspensions at a 1% hematocrit were preincubated in TBS containing 1 mM Zn^{2+} ions for 10 min at 37 °C and exposed to a pressure of 200 MPa for 10 min at 37 °C. After the decompression, the agglutinated erythrocytes were dissociated with 1 mM EDTA, washed three times with buffer, and fixed for 2 h at room temperature using 1% glutaraldehyde. The fixed cells were washed in PBS and dehydrated by ethylalcohol. The dehydrated samples were immersed in *t*-butylalcohol-ethylalcohol mixed solution (v/v=1:1) for 30 min and then 100% *t*-butylalcohol for 30 min. The samples in *t*-butylalcohol were frozen at −5 °C and dried with a JEOL freeze dryer (model JFD-310). The dried samples were coated with Pt using a JEOL sputtering outfit (model JFC-1600). The cell morphology was observed using a scanning electron microscope (SEM) (model JSM-LV, JEOL).

For fluorescence measurements, pressure-treated erythrocytes were dissociated with EDTA and used to prepare ghosts, as mentioned

above. Resealed ghosts in PBS were treated with NPIA (10 μg/ml) for about 20 h at 0 °C. The NPIA-labeled ghosts were washed several times with PBS, and then solubilized by 1% (v/v) Triton X-100 for 1 h at 0 °C. The solubilized samples were centrifuged at 20,000g for 20 min at 4 °C. The pellets (Triton shells) were suspended in PBS. Fluorescence spectra of NPIA were measured using a model FP-750 spectrometer with excitation at 345 nm (slit width, 5 nm).

3. Results

3.1. Agglutination of erythrocytes by Zn^{2+} ions

Human erythrocytes were incubated with Zn^{2+}, Cd^{2+}, or Hg^{2+} ions. Zn^{2+} ions induced the agglutination of erythrocytes (Fig. 1A and B). However, the agglutinated erythrocytes were dissociated by washing with buffer. On the other hand, no such an agglutination was observed in the erythrocytes treated with Cd^{2+} or Hg^{2+} ions under our conditions (Fig. 1C and D). In the case of Hg^{2+} ions, a low concentration of the metal ions (10 μM) was used to avoid the hemolysis.

3.2. Involvement of band 3 in Zn^{2}-induced erythrocyte agglutination

To examine the contribution of band 3 in Zn^{2+}-induced erythrocyte agglutination, band 3-rich vesicles were prepared using DMPC liposomes (Fig. 2A, lane 3) [19]. When Zn^{2+} ions were added to the suspensions of band 3-rich vesicles, the size of the vesicles increased (Fig. 2B).

DIDS specifically binds to band 3 and inhibits anion transport [3,20]. So, the effect of DIDS on Zn^{2+}-induced erythrocyte agglutination was examined. The agglutination of erythrocytes by Zn^{2+} ions was significantly suppressed by DIDS (Fig. 2C and D). These results suggest that the interactions of Zn^{2+} ions with band 3 play an important role in Zn^{2+}-induced erythrocyte agglutination.

3.3. Erythrocyte agglutination by the interactions of Zn^{2+} ions with histidine residues on band 3

Extracellular domains of band 3 may be considered as binding sites of Zn^{2+} ions. In band 3 glycoprotein, the sugar chain is linked to an asparagine-642 (Asn-642) on the loop between the transmembrane (TM) segments 7 and 8 [21]. To examine a possibility of the binding of Zn^{2+} ions to the sugar chain, monosaccharides which are contained in its oligosaccharide were added into the agglutinated erythrocyte suspensions [22]. However, Zn^{2+}-induced erythrocyte agglutinations were unaffected by all monosaccharides such as, N-acetylglucosamine, galactose, mannose, fucose, and N-acetylneuraminic acid. These results suggest that the sugar chain of band 3 is excluded from the binding sites of Zn^{2+} ions.

Next, amino acids contained in the loop regions of band 3 were examined as the binding sites of Zn^{2+} ions [21]. As with monosaccharides, amino acids were added into the agglutinated erythrocyte suspensions. Of amino acids tested, histidine was effective to dissociate the agglutinated cells (Fig. 3A). To confirm the possibility of histidine residues, the erythrocytes were treated with DEPC to chemically modify histidine residue [23]. The agglutination of DEPC-treated erythrocytes by Zn^{2+} ions was greatly suppressed (Fig. 3B). Furthermore, the buffer pH of erythrocyte suspensions was changed in the range of 5.8–8.6. At pH 5.8 below pKa (=6.0) of histidine, the agglutination of erythrocytes by Zn^{2+} ions was most inhibited. Then, the agglutination of erythrocytes increased with rising pH but inhibited at pH 8.6 (Fig. 3C). To examine why such a suppression at pH 8.6 occurred, the erythrocytes were treated with eosin-5-maleimide. In such erythrocytes, lysine-430 of band 3 is labeled with eosin-5-maleimide [24]. Resealed ghosts prepared from such erythrocytes were incubated in buffer of pH 8.6, solubilized with C12E8, and were centrifuged. The amount of band 3 interacted with cytoskeletal proteins was reduced (Fig. 3D).

3.4. Effects of group 12 metal ions on pressure-induced hemolysis

To examine membrane perturbations induced by group 12 metal ions, human erythrocytes were suspended in buffers containing Zn^{2+}, Cd^{2+}, or Hg^{2+} ions and were exposed to a pressure of 200 MPa. Pressure-induced hemolysis was greatly suppressed by Zn^{2+} ions, unaffected by Cd^{2+} ions, and remarkably enhanced by Hg^{2+} ions under our experimental conditions (Fig. 4A). Here, the erythrocyte agglutination by Zn^{2+} ions was also observed immediately after decompression. Moreover, to check the reversibility of the binding of

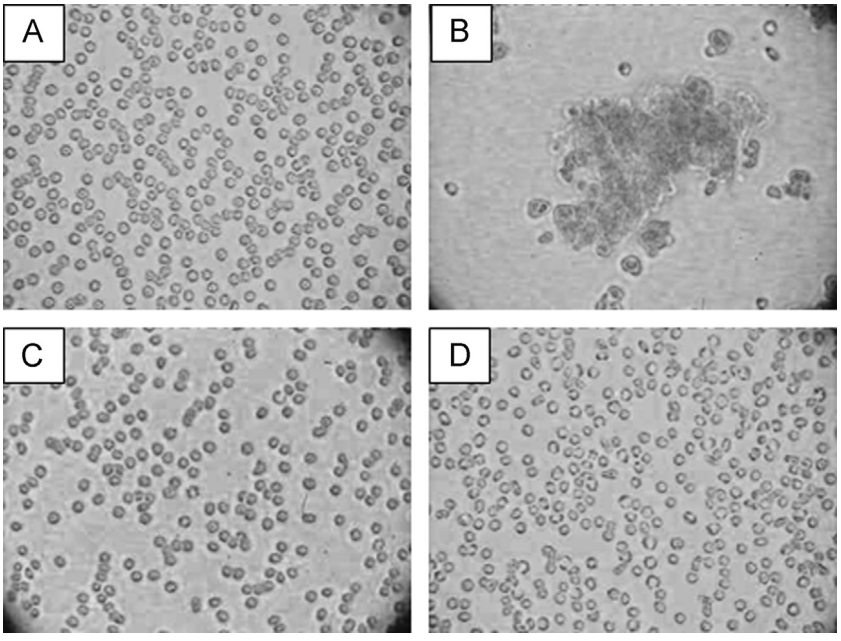

Fig. 1. Erythrocyte agglutination by Zn^{2+} ions. Erythrocytes in TBS (A) or 4 mM Zn^{2+} (B), 4 mM Cd^{2+} (C), 10 μM Hg^{2+} (D)-containing TBS were incubated for 10 min at 37 °C and observed under a light microscope.

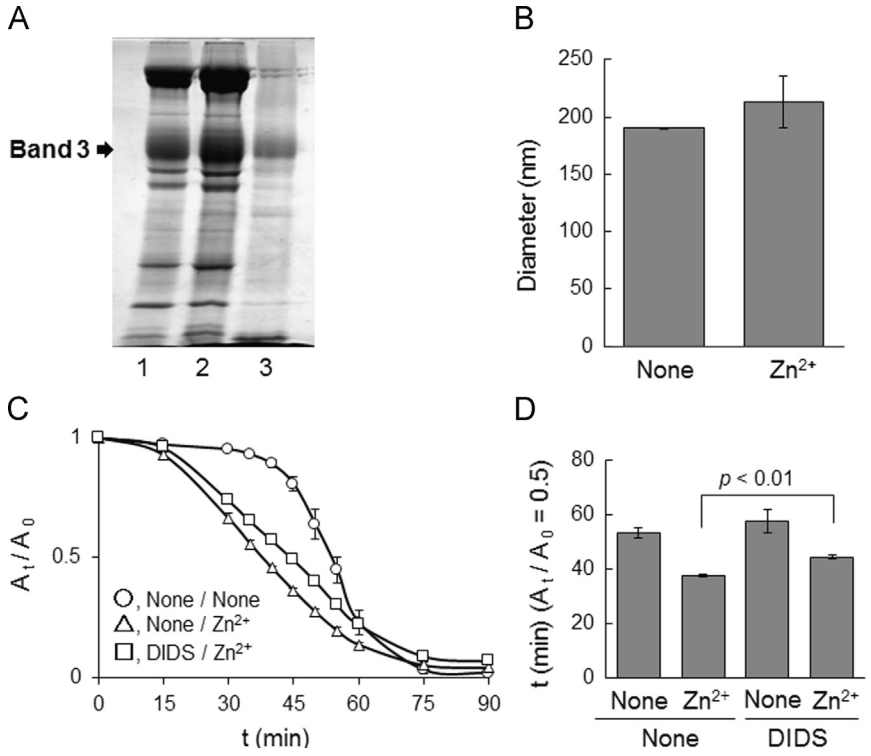

Fig. 2. Interactions of band 3 with Zn^{2+} ions. A, SDS-PAGE of band 3-rich vesicles. Erythrocytes in PBS containing DMPC liposomes were incubated for 5 h at 37 °C. Lane 1, ghosts from intact erythrocytes; lane 2, ghosts from DMPC-treated erythrocytes; lane 3, vesicles from DMPC-treated erythrocytes. B, Size of Zn^{2+}-treated vesicles. Vesicles from DMPC-treated erythrocytes were suspended in TBS containing 0.5 mM Zn^{2+} ions. C, DIDS effects on Zn^{2+}-induced agglutination of erythrocytes. Intact erythrocytes or 50 μm DIDS-treated ones were suspended in TBS containing 0.5 mM Zn^{2+} ions and turbidity of suspensions was monitored at 700 nm. A_0 and A_t are absorbance at 0 and t min of erythrocyte suspensions, respectively. Times (t min) corresponding to $A_t/A_0=0.5$ are shown in right panel. All values are means ± SD for three independent experiments.

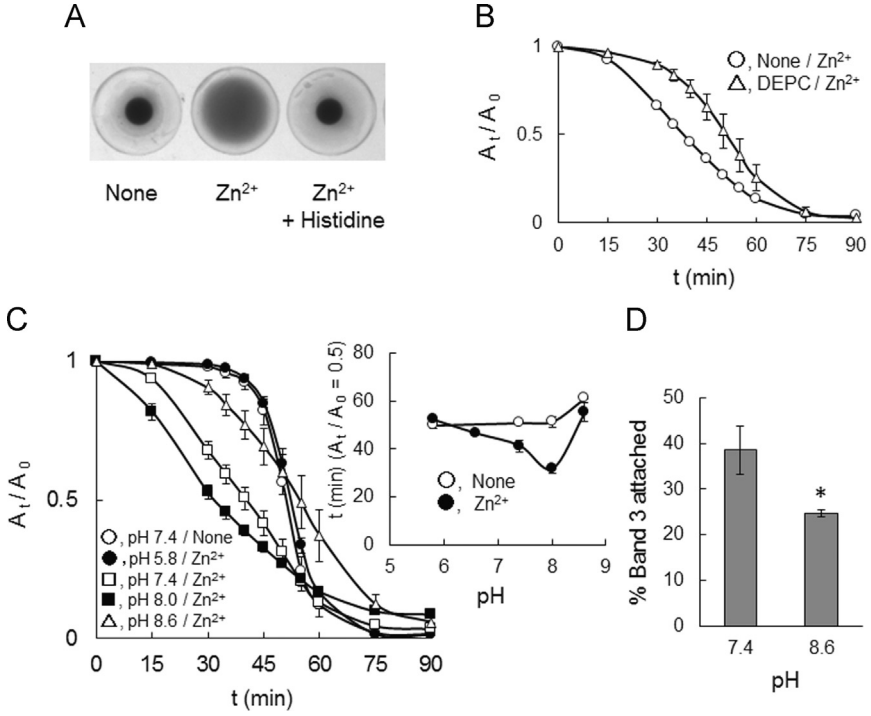

Fig. 3. Interactions of Zn^{2+} ions with histidines on band 3. A, Dispersed or agglutinated erythrocytes on agglutination plate. Left well, cells suspended in TBS; middle well, cells suspended in TBS containing 0.5 mM Zn^{2+} ions; right well, Zn^{2+}-treated cells suspended in TBS containing 0.5 mM histidine. B, DEPC effects on Zn^{2+}-induced agglutination. Intact erythrocytes or 7 mM DEPC- treated ones were suspended in TBS containing 0.5 mM Zn^{2+} ions. C, Effects of pH on Zn^{2+}-induced agglutination. Erythrocytes were suspended in TBS (pH 5.8–8.6) containing 0.5 mM Zn^{2+} ions. D, Band 3-cytoskeleton interactions. Eosin-5-maleimide-labeled ghosts were solubilized by 0.5% C12E8 in TBS (pH 7.4 and 8.6) and centrifuged. All values are means ± SD for three independent experiments. $^{*}p < 0.03$ vs. pH 7.4.

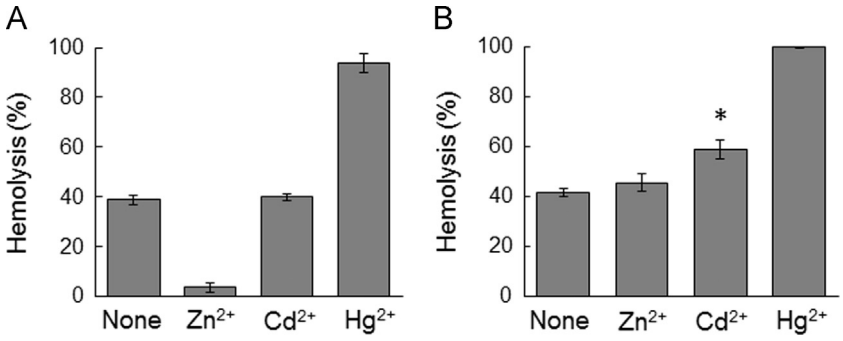

Fig. 4. Effects of group 12 metal ions on pressure-induced hemolysis. A, Erythrocytes were suspended in TBS containing 4 mM Zn^{2+}, 4 mM Cd^{2+}, or 10 μm Hg^{2+} ions and exposed to a pressure of 200 MPa in the presence of metal ions. B, Erythrocytes pretreated with metal ions in A were washed with TBS and compressed in the absence of metal ions. All values are means ± SD for three independent experiments. $^{*}p < 0.05$ vs. none.

Zn^{2+}, Cd^{2+}, or Hg^{2+} ions to the membrane, the erythrocytes preincubated with metal ions were washed with buffer. These erythrocytes were exposed to a pressure of 200 MPa in the absence of metal ions (Fig. 4B). In the case of Zn^{2+} ions, the values of hemolysis at 200 MPa were restored to original level. On the other hand, hemolysis at 200 MPa of Hg^{2+}-pretreated erythrocytes remained high levels, indicating the irreversible binding of Hg^{2+} ions. In the case of Cd^{2+} ions, the values of hemolysis at 200 MPa increased significantly. This suggests the slow process of membrane damages induced by Cd^{2+} ions.

3.5. *Membrane properties under pressure of erythrocytes agglutinated by Zn^{2+} ions*

The hemolysis at 200 MPa of erythrocytes agglutinated by Zn^{2+} ions was greatly suppressed, as mentioned above. So, the agglutinated erythrocytes after decompression were dissociated by washing with EDTA and the surface of the cell membrane was examined using a SEM. Upon exposure of intact erythrocytes to a pressure of 200 MPa, vesicles were observed on the membrane surface (Fig. 5A, left panel). Similar vesicles were observed in the case of compression of erythrocytes agglutinated by Zn^{2+} ions (Fig. 5A, right panel). Moreover, the denaturation of spectrin was examined using pyrene fluorescence (Fig. 5B). Excimer fluorescence was observed upon exposure of intact erythrocytes to a pressure of 200 MPa. Similar excimer fluorescence appeared upon compression of erythrocytes agglutinated by Zn^{2+} ions. These results suggest that the denaturation of spectrin occurs upon exposure of agglutinated erythrocytes to a pressure of 200 MPa.

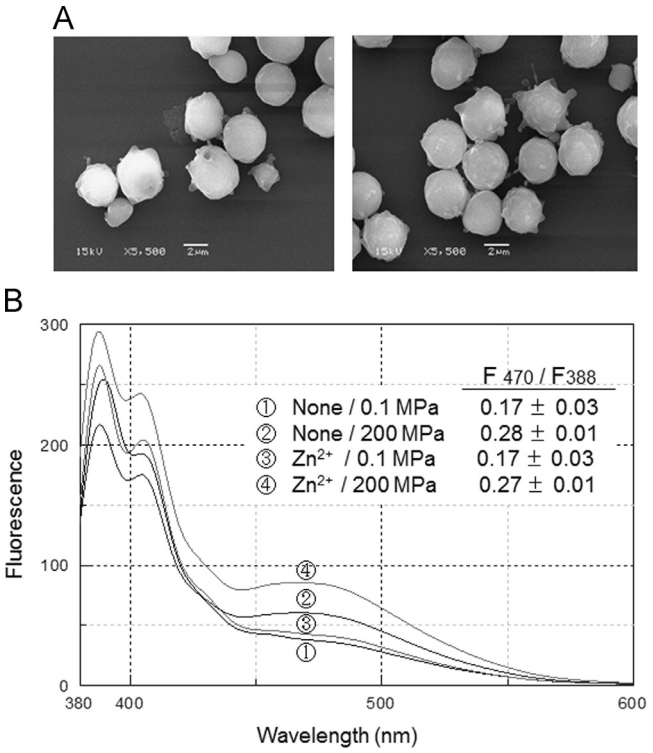

Fig. 5. Membrane properties at 200 MPa of erythrocytes agglutinated by 0.5 mM Zn^{2+} ions. A, SEM of pressure-treated erythrocytes. Left panel, intact cells; right panel, agglutinated cells were compressed and dissociated by 1 mM EDTA. B, Excimer fluorescence appears upon compression of agglutinated erythrocytes. Agglutinated cells were compressed, dissociated using EDTA, and used for ghost preparation. Resealed ghosts were labeled with NPIA. F_{388} and F_{470} are fluorescence intensities at 388 and 470 nm, respectively. All values are means ± SD for three independent experiments.

4. Discussion

Of the group 12 metal ions, Zn^{2+} ions induce the strong agglutination of human erythrocytes. It is well known that cytoskeleton-free band 3 dimers are clustered by Zn^{2+} ions [15]. This suggests one possibility that the interactions of Zn^{2+} ions with band 3 may induce the erythrocyte agglutination. This possibility has been confirmed by the results obtained from band 3-rich vesicles. Moreover, DIDS binds specifically to band 3 and perturbs the interactions of tetrameric band 3 with ankyrin which is linked to spectrin [3]. Upon DIDS treatment of erythrocytes, therefore, tetramers of band 3 shift to band 3 dimers that are free from the cytoskeleton [3]. In this paper, we demonstrated that the Zn^{2+}-induced agglutination of erythrocytes is suppressed by DIDS. This suggests the involvement of tetrameric band 3 linked to spectrin via ankyrin in Zn^{2+}-induced erythrocyte agglutination. Similar results are obtained from the effect of alkaline pH on agglutination. The erythrocyte agglutination by Zn^{2+} ions is also inhibited at pH 8.6, where the band 3-ankyrin interaction is loosened [25]. On the basis of these data, we propose that the interactions of Zn^{2+} ions with tetrameric band 3 rather than dimeric one are important in the erythrocyte agglutination. Concanavalin A-induced erythrocyte agglutination also demonstrates the importance of band 3-cytoskeleton interactions [26]. Interestingly, the interactions of transmembrane proteins such as cadherin with scaffold proteins are lacked in cancer cells so that the cell–cell adhesions are loosened [27,28]. Thus, the translocation seen in cancer cells is due to the weak cell–cell adhesions [28]. These data indicate that the interactions of transmembrane proteins with cytoskeleton play an important role in cell adhesions or agglutinations.

Extracellular domains of band 3 seem to be the binding site(s) of Zn^{2+} ions. Particularly, the loop region between TM7 and TM8 in band 3 is attractive as Zn^{2+} binding site because concanavalin A binds to sugar chain attached to Asn-642 of this loop and induces the cell agglutination [21,29]. Zn^{2+}-induced agglutination is not affected

by the addition of monosaccharides contained in this sugar chain. Thus, sugar chain is not involved in Zn^{2+}-induced agglutination. On the other hand, the histidine of amino acids contained in loop regions dissociates the erythrocytes agglutinated by Zn^{2+} ions. His-547 or His 651 in human band 3 is located in the loop between TM5 and TM6 or between TM7 and TM8, respectively [21]. Here, Zn^{2+}-induced agglutination of erythrocytes is inhibited by electrostatic repulsion due to protonation of imidazole group in histidine, as seen at pH 5.8. Moreover, the chemical modification of histidines with DEPC inhibits Zn^{2+}-induced erythrocyte agglutination. In carboxypeptidase A, the catalytically active zinc atom is bound to His-69 and His-196 [10]. Binding of zinc atom with histidine is also seen in transcription factors [13]. These data suggest that the interactions of Zn^{2+} ions with histidine residues located in loop regions of band 3 play an important role in Zn^{2+}-induced erythrocyte agglutination.

For pressure-induced hemolysis, human erythrocytes show very different responses to group 12 metal ions. Hemolysis at 200 MPa of erythrocytes agglutinated by Zn^{2+} ions is greatly suppressed. Similar results are reported in erythrocytes agglutinated by anti-band 3 antibody [30] or concanavalin A [31]. Observation of 200 MPa-treated erythrocytes by SEM demonstrates that the membrane vesicles are formed upon compression of dispersed cells and agglutinated ones, as seen in Fig. 5. Pressure-induced hemolysis is associated with the release of large vesicles from the membrane surface [5,6,30]. However, such a vesicle release is suppressed in agglutinated erythrocytes. Thus, the decrease of apparent membrane surface area in erythrocytes agglutinated by Zn^{2+} ions may be ascribed to the suppression of pressure-induced hemolysis. Moreover, the excimer fluorescence of pyrene is observed upon compression of erythrocytes agglutinated by Zn^{2+} ions. Such excimer fluorescence appears when pyrene molecules approach within 2.4 Å each other [5]. For instance, the denaturation of spectrin occurs upon heating erythrocytes at 49 °C [5,32]. When denatured spectrin is labeled with NPIA, which is a SH reactive pyrene derivative, pyrene excimer appears [5]. This suggests that cysteine residues in denatured spectrin approach within 2.4 Å each other. Therefore, pyrene excimer represents conformational changes such as denaturation of spectrin induced under 49 °C [5] or 200 MPa [5,31]. Such denaturation of spectrin is also supported by the formation of membrane vesicles in pressure-treated erythrocytes [5]. In contrast with Zn^{2+} ions, Hg^{2+} ions facilitate the hemolysis at 200 MPa due to the suppression of the fragmentation [6]. On the other hand, the results obtained from the pressure-induced hemolysis of Cd^{2+}-treated erythrocytes indicate that the outer surface of their membranes is not perturbed by this metal ions. However, the hemolysis at 200 MPa after washing Cd^{2+}-pretreated erythrocytes shows a significant enhancement. It seems likely that parts of added Cd^{2+} ions are transported into erythrocytes, bind intracellular proteins such as spectrin with high affinity, and disrupt the cytoskeletal structure [16]. These results suggest that pressure-induced hemolysis sensitively reflects the interactions of the erythrocyte membrane with the group 12 metal ions and the compression at 200 MPa of erythrocytes agglutinated by Zn^{2+} ions induces the denaturation of spectrin.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.05.003.

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