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Membrane skeleton in fresh unfixed erythrocytes as revealed by a rapid-freezing and deep-etching method

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ABSTRACT

A rapid-freezing and deep-etching method for examining en face the cytoplasmic aspects of unfixed erythrocyte membranes is described, which provides improved resolution. Normal human erythrocytes were centrifuged, washed in a phosphate buffer solution and pelleted. Glass coverslips were coated with 3-aminopropyl triethoxy silane and glutaraldehyde to make erythrocytes stick to them. A drop containing the erythrocyte pellet was sandwiched between 2 coverslips. The attached erythrocytes were slowly split open in the cytosol buffer solution. The specimens on coverslips were rapidly frozen in an isopentane–propane mixture (−193 °C), deeply etched and rotary shadowed with platinum and carbon. Filamentous structures were observed to form fine networks on the cytoplasmic side of erythrocyte membranes. The length of the filaments was shorter than that previously reported for glutaraldehyde-fixed filaments. The number of intersections between filaments was increased as compared with the previous data. It is concluded that dense in situ networks of short filaments beneath erythrocyte membranes can be viewed in a relatively intact state by splitting fresh unfixed specimens followed by the rapid-freezing and deep-etching method.

Key words: Cytoskeleton; spectrin; actin.

INTRODUCTION

Ultrastructural studies have demonstrated a fine skeleton in the membrane of fixed erythrocytes (Tsukita et al. 1980; Nermut, 1981; Fujikawa, 1983; Shen et al. 1984, 1986; Byers & Branton, 1985; Liu et al. 1987; Lupu & Constantinescu 1989; Ursitti et al. 1991; Ohno, 1992; Ohno et al. 1993; Ursitti & Wade, 1993). It has been accepted that the network consists mainly of proteins such as spectrin, actin, band 4.1, band 4.9, ankyrin and tropomyosin (Marchesi, 1983; Bennett, 1985, 1989). The spectrin molecules are believed to be in the form of tetramers and form 2-dimensional networks, which are also attached to band 3 or glycophorin in the lipid bilayer. Their mutual binding serves the purposes of regulating the structure of membrane skeletons and thereby maintaining the shape of erythrocytes. Recently, isolated membrane skeletons were observed by electron microscopy with the negative staining technique, clarifying the ultrastructure of continuous networks in vitro (Timme, 1981; Shen et al. 1984, 1986; Liu et al. 1987; Derick et al. 1992). It was also demonstrated that spectrin filaments formed anastomosing networks in combination with actin filaments. In some reports, it has been stressed that actin proteins exist as short filaments with which spectrin filaments are connected (Byers & Branton, 1985; Shen et al. 1986; Liu et al. 1987). Although the spectrin and actin filaments interact with each other to form 2-dimensional networks, the in situ arrangement of these filaments is still unclear at present.

The ultrastructure of the membrane skeleton in erythrocytes has been examined by various techniques, which have included scanning electron microscopy of erythrocyte ghost preparations, negative staining and freeze-etching methods (Yu et al. 1973; Hainsfeld & Steck, 1977; Sheetz & Sawyer, 1978; Nermut, 1981; Mohandas et al. 1982; Ursitti & Wade, 1993). Conventional electron micrographs of fixed

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erythrocytes have also demonstrated fibrillar networks beneath the erythrocyte membrane (Tsukita et al. 1980). Routine electron microscopic techniques, however, that rely on chemical fixation, dehydration, heavy metal staining and other preparative steps, appear to cause artifacts that are too complex to permit the in situ ultrastructure to be portrayed. An erythrocyte splitting technique has recently been developed for the ultrastructural study of erythrocyte membranes, followed by a quick-freezing and deep-etching method (Ohno, 1992; Ohno et al. 1993). The purpose of this paper is to clarify the in situ appearances of the cytoplasmic aspects of unfixed erythrocyte membranes.

MATERIALS AND METHODS

Splitting of erythrocytes

From each of 2 normal volunteers 10 ml venous blood was withdrawn, with consent, into syringes containing 0.1 % heparin. The blood was washed and centrifuged in a test tube (3000 ***g*** for 10 min) 3 times in phosphate buffered saline with Mg/EGTA (150 mM-NaCl/5 mM-NaH_2PO_4/2 mM-NaN_3/2 mM-$MgCl_2$/1 mM EGTA, pH 7.5) to obtain erythrocyte pellets. In order to prepare replicas of the exposed cytoplasmic sides of their surface membranes, erythrocytes were split open using the method devised by one of the present authors (Ohno, 1992; Ohno et al. 1993). Briefly, a coverslip was coated with 3-aminopropyl triethoxy silane and 1 % glutaraldehyde so that erythrocyte membranes readily adhered to the glass surface. A drop of unfixed erythrocyte pellets was put on the coverslip and another coverslip placed over it to sandwich the erythrocytes. The overlying coverslip was gently pressed with forceps to make the erythrocytes adhere to both coverslips. Another phosphate buffer containing Mg/EGTA (5 mM-NaH_2PO_4/2 mM-$MgCl_2$/1 mM EGTA, pH 7.5) was allowed to run between them (Byers & Branton, 1985). The coverslips were then detached from each other and the erythrocytes were split open mechanically. Soluble proteins were removed by thorough washing with the phosphate buffer and the erythrocyte membranes were rapidly frozen as described in the following section.

Preparation of rapidly frozen and deep-etched replicas

The split erythrocytes on coverslips were drained with filter paper to remove excess fluid and then plunged into a liquid isopentane–propane mixture cooled in liquid nitrogen (~ -193 °C) (Ohno, 1992). They were then transferred into liquid nitrogen, shaken sharply to remove the adherent isopentane–propane mixture and stored in liquid nitrogen. The frozen specimens were put into an Eiko FD-3S freeze-fracture apparatus equipped with a turbo molecular pump and deeply etched at a vacuum of 10^{-7}–10^{-8} Torr and a temperature of -95 °C for 15–30 min. After deep-etching, erythrocyte membranes were first shadowed for several seconds with platinum at an angle of 25° without stage rotation to make fine structures clear and then rotary shadowed up to a total metal thickness of ~ 2 nm. They were additionally shadowed with carbon at an angle of 90°.

A drop of 2% collodion in amyl acetate solution was put onto the replicas as soon as the specimens were taken out of the machine to prevent the replicas from breaking into pieces during the following digestion procedure. The replicas, coated with dried collodion, were treated as described in previous papers (Ohno, 1992; Ohno et al. 1993). They were picked up onto Formvar-coated copper grids and immersed in amyl acetate solution to dissolve the dried collodion. All replicas were observed in a Hitachi HS-9 electron microscope at an accelerating voltage of 75 kV.

Electron micrographs were printed from the inverted negative films to make platinum deposits appear white. As the filaments of networks were relatively straight, morphometric analyses to measure their length between intersection points and to count the number of intersections were performed with the Digigramer-G analyser system (Mutoh Co., Tokyo, Japan), as previously reported (Ohno, 1992; Ohno et al. 1993). All values were expressed as means $\pm$ S.D.

RESULTS

Figure 1*a* illustrates an overall view of the exposed cytoplasmic or internal aspect of the erythrocyte membrane. Networks attached to the membrane could be identified and the edge of the membrane was occasionally curled up (Fig. 1*a*, inset). At higher magnification, the networks were made up of filamentous components forming angular lattice units with intersections (Fig. 1*a*, *b*). Some fibrillar networks were frequently broken owing to their fragility, and large holes or nude areas were sometimes apparent in the networks (Fig. 1*b*). The number of filaments at the intersections was counted, where 3 or 4 filaments were common; 5 or 6 filaments were relatively rare.

Some exposed cytoplasmic networks were partly detached from the erythrocyte membranes because of the washing procedures (Fig. 2) so that the network

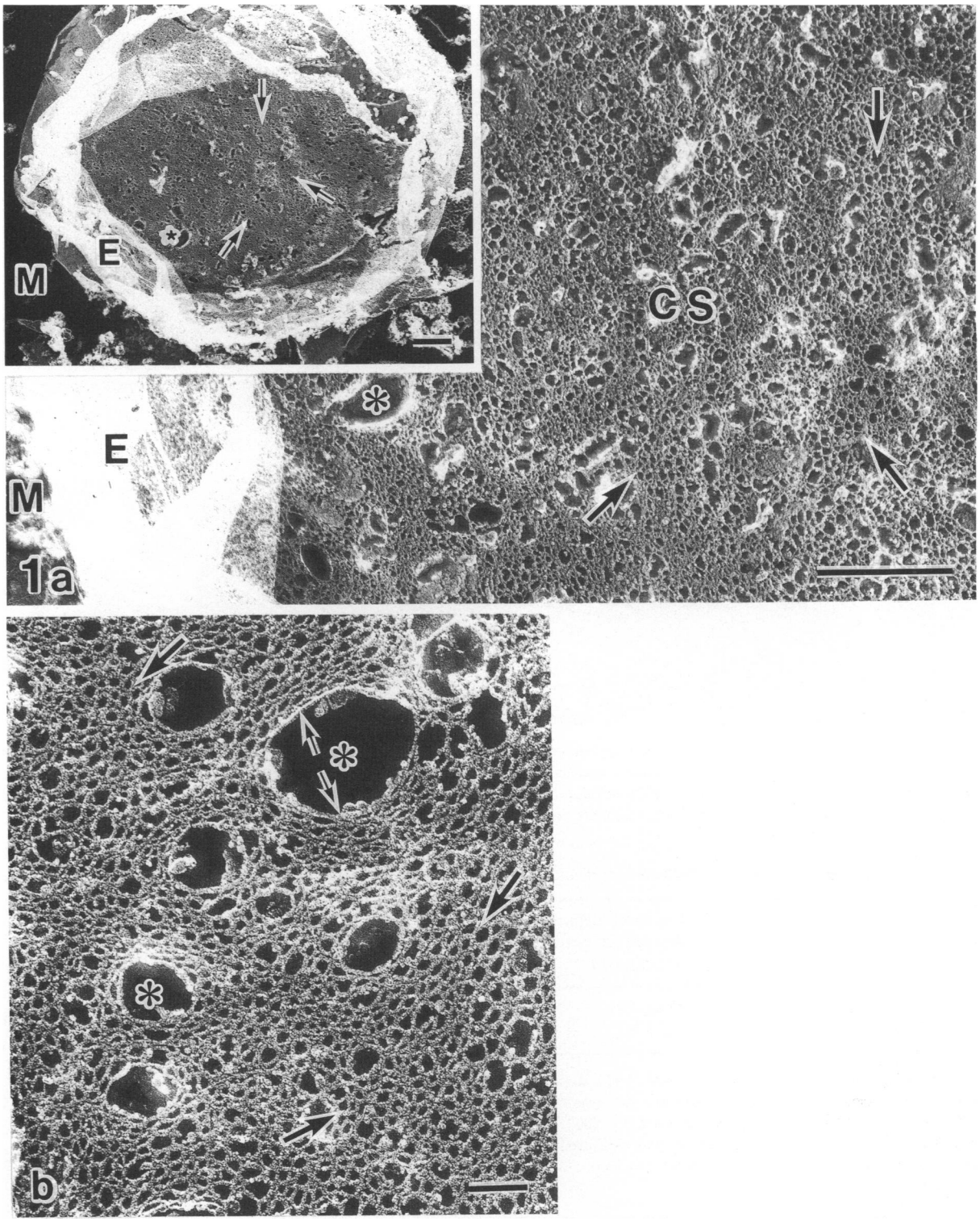

Fig. 1. Electron micrographs of the replicated cytoplasmic sides of fresh unfixed erythrocyte membranes. (*a*) An erythrocyte has been split open to expose the cytoplasmic side (CS) of the membrane. Filamentous networks (arrows) are seen to be attached to the membrane. Asterisks, damaged networks. M, extracellular matrix. E, curved edge of erythrocyte. ×42700; bar, 0.5 μm. Inset. Lower magnification of an overview of the erythrocyte. ×11700; bar, 0.5 μm. (*b*) Network structures are well preserved in some parts (large arrows), but damaged networks are shrunken (small arrows) to form nude areas (asterisks). ×92100; bar, 0.1 μm.

organisation was slightly distorted. The networks had elastic properties, being stretched when attached to the lipid membrane and contracted when detached. Morphometric data (see Table) showed that the lengths of filaments between intersections were significantly shorter than those in the glutaraldehyde-fixed filament networks as previously reported (Ohno, 1992) and that the number of filament intersections dis-

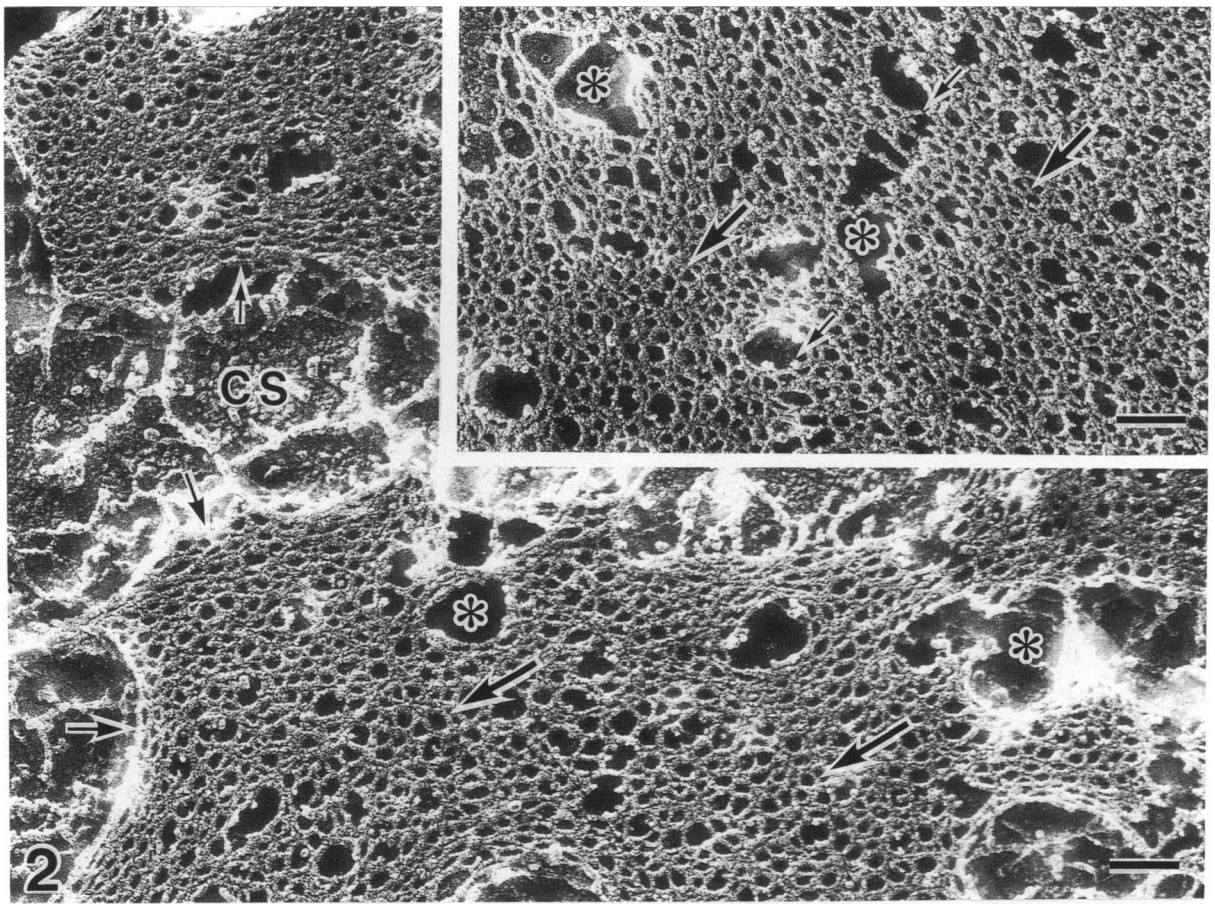

Fig. 2. Some filamentous networks (small arrows) have become detached from erythrocyte membranes and are more condensed than in other regions (large arrows). They are contracted to show the real cytoplasmic side (CS) of the erythrocyte membrane. In some parts, network structures are well preserved (large arrows), while in others networks are partly destroyed (asterisks). ×92100; bar, 0.1 μm. Inset. Large arrows indicate well preserved networks. Some filaments are interrupted (small arrows). Asterisks, nude areas. ×92100; bar 0.1 μm.

Table. *Morphometric data for fresh unfixed erythrocytes*

Case	Numbers of intersections per 0.25 μm² (mean ± S.D.)*	Length of filaments between intersections (mean ± S.D.)**
1	214.1 ± 24.8	23.6 ± 5.9
2	228.2 ± 36.2	22.7 ± 6.3

50 erythrocytes from each case were measured. * The number of filament intersections per 0.25 μm² cytoplasmic side was counted in 100 different areas for each erythrocyte; total n = 5000. ** The length of filaments between the intersections was measured in 100 different filaments for each erythrocyte; total n = 5000.

played a 3 or 4-fold increase as compared with our earlier findings (Ohno et al. 1993).

DISCUSSION

Erythrocyte membranes have been examined by means of transmission and scanning electron microscopy (Hainsfeld & Steck, 1977; Tsukita et al. 1980; Lupu & Constantinescu, 1989). Artificially spread networks have been examined by the negative staining technique (Shen et al. 1984, 1986; Byers & Branton, 1985; Liu et al. 1987; McGough & Josephs, 1990; Derick et al. 1992), but this procedure has not been applied to structures in situ. The present splitting technique of unfixed erythrocytes, in combination with the rapid-freezing and deep-etching method, has made it possible to remove soluble proteins from the erythrocyte membranes so that we obtained ultrastructural observations on their cytoplasmic aspects. It is not completely excluded, however, that the ultrastructure of networks was affected by washing in buffer and the centrifugation of unfixed erythrocytes.

The present study reveals ultrastructural features of erythrocyte membrane skeletons that were difficult to observe for isolated erythrocyte proteins. These anastomosing membrane skeletons have been reported for erythrocyte ghosts using the freeze-etching (Nermut, 1981; Mohandas et al. 1982) and negative staining (Timme, 1981) methods. The fibrillar networks have also been visualised beneath erythrocyte membranes by electron microscopy following tannic acid fixation (Tsukita et al. 1980; Fujikawa, 1983).

From our results, it is clear that the membrane skeletons in fresh unfixed erythrocytes differ from those in glutaraldehyde-fixed erythrocytes as reported before (Ohno, 1992; Ohno et al. 1993). We have reduced the fixation artifacts and obtained images of the membrane skeleton that originated directly from fresh unfixed erythrocytes. The direct visualisation of the membrane skeleton in situ may lead to further investigations to identify its molecular architecture.

The isolated membrane skeleton contained 2 major filamentous elements, such as long spectrin filaments and short actin filaments (Shen et al. 1986; Liu et al. 1987). It can be speculated that spectrin–actin networks could extend over the entire cytoplasmic aspect of the erythrocyte membrane. Purified spectrin dimers and tetramers have been directly visualised by low-angle shadowing and electron microscopy (Shotten et al. 1978; Cohen et al. 1980; Liu et al. 1984). The spectrin dimer appears as a flexible rod, about 100 nm long. Moreover, 2 spectrin dimers can bind each other to form a tetramer rod, nearly 200 nm long. Recently, it was reported that membrane skeletons in vitro are composed of hexagonal lattice structures (Liu et al. 1987; Derick et al. 1992). In our previous paper, we reported that 2 different sizes of filaments were localised on the cytoplasmic side; one is long (82.6 ± 23.3 nm) and the other short (30.6 ± 7.6 nm) (Ohno, 1992). However, it was not clear that they were spectrin filaments, because the latter were too short as compared with reported data on length of spectrin filaments (Nermut, 1981; Byers & Branton, 1985). In the present study, we were able to detect many short filaments (23.6 ± 5.9 nm in Case 1 and 22.7 ± 6.3 nm in Case 2), as shown in the Table, which are significantly different ($P < 0.01\%$) from the previous long filaments (82.6 ± 23.3 nm), perhaps because of intramolecular interactions of unfixed spectrin filaments. Although the filaments from our morphometric data proved to be short in length, another possibility is that spectrin tetramers may be contracted under the normal erythrocyte membranes. It is also clear that elasticity in the filaments could be detected in broken networks, as shown in Figures 1*b* and 2. We have not yet identified each protein in networks with antibodies. The present network structures are thought to consist mainly of spectrin proteins because they are the major components of the membrane skeleton (Ungewickell & Gratzer, 1978; Shotton et al. 1979; Goodman & Weidner, 1980; Tyler & Branton, 1980).

We have found a difference in the cytoplasmic aspects of erythrocyte membranes between glutaraldehyde-fixed erythrocytes and fresh unfixed ones. Fresh unfixed erythrocytes were demonstrated to have finer networks than those observed in fixed erythrocytes as reported before (Ohno, 1992; Ohno et al. 1993). The networks in unfixed erythrocytes were characterised by increased numbers of intersections and a relative predominance of short filaments. The fact that filament intersections were less abundant in glutaraldehyde-fixed erythrocytes (Ohno et al. 1993) suggests that their ultrastructural appearances could be accounted for by a relative predominance of broken filaments and a consequent defective organisation of the filamentous networks. In normal erythrocytes in situ, spectrin tetramer filaments might be in a contracted state and play an important role in imparting flexibility and deformability to the erythrocytes (McGough & Josephs, 1990). It is also suggested that, in situ, spectrin dimers may be associated with each other as hexamers and octamers, rather than tetramers (Ursitti & Wade, 1993). In the cytoplasmic aspects of the glutaraldehyde-fixed erythrocytes (Ohno et al. 1993), filamentous structures are reduced, suggesting that the alterations in the membrane skeletons are induced by preparative damage after fixation.

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