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Invited Review

Three-dimensional ultrastructure of in situ membrane skeletons in human erythrocytes by quick-freezing and deep-etching method

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Summary. The erythrocyte membrane skeleton is a network of some structural proteins, principally composed of spectrin, actin and protein 4.1, which is also attached to the cytoplasmic surface of the lipid membrane. The purpose of the present review is to describe the three-dimensional ultrastructure of in situ membrane skeletons in human erythrocytes. The in situ membrane skeletons are densely organized, and the spectrin itself has a property of extension or contraction with reacting the external mechanical forces. The lengths between the intersections are changed according to the mechanical strength, and also vary even in one erythrocyte. To clarify the in situ organization of membrane skeletons, the quick-freezing and deep-etching method should be used for fresh unfixed erythrocytes because of their fragility during preparation steps.

Key words: Membrane skeleton, Quick-freezing, Spectrin, Actin, Protein 4.1, Ankyrin

Introduction

In mature erythrocytes, the supramolecular structure under the erythrocyte membrane maintains its biconcave shape as a structural support for lipid bilayer, and keeps essential properties of elasticity and flexibility during its circulatory travels. To withstand the mechanical forces of blood flow over a several-month life span, the erythrocyte membrane components must be assembled into a highly durable structure. The erythrocytes, which flow through the microcirculation, are usually subjected to the shear stress, and have either a parachute-like shape or an elliptical one (Mohandas et al., 1983). The spectrin, actin and protein 4.1 are principal structural proteins of membrane skeletons, and the molecular balance of spectrin and actin is probably important for keeping their structures. The major roles of the membrane skeletons are to prevent the erythrocyte membrane from fragmentation against the external forces and to regulate the receptor-cytoskeleton or channel-cytoskeleton interaction (Branton et al., 1981; Bennett, 1990; Marchesi, 1985; Lazarides, 1987; Coleman et al., 1989). One functional model has been reported, which represents a complex of the lipid layer, the membrane skeleton and integral membrane proteins as a «trilayer couple», while the erythrocyte undergoes its shape transformation.

Therefore, it is necessary to visualize the ultrastructure of in situ erythrocyte membrane skeletons, especially in the condition of flowing through blood vessels. For this objective, the quick-freezing method is the best way to get morphological information about the native erythrocytes. The purpose of this review is to clarify the ultrastructures of in situ membrane skeletons in erythrocytes, which were obtained by the quick-freezing and deep-etching (QF-DE) method in combination with erythrocyte-splitting method (Ohno, 1992). In the first part, we will briefly summarize the molecular structures of major erythrocyte proteins. The second part deals with some morphological approaches to visualize three-dimensional ultrastructure of in situ membrane skeletons by electron microscopy.

I. Molecular components of the membrane skeleton

The literature concerning the proteins of erythrocyte membrane skeletons has been a main subject of repeated reviews (Palek and Sahr, 1992; Bennet and Gilligan, 1993). The basic membrane architecture includes the lipid bilayer, the integral proteins spanning the lipid bilayer, and a meshwork of proteins which form a membrane skeleton along the cytoplasmic surface (Fig. 1). The protein-protein binding sites of major skeletal proteins, such as spectrin, actin, protein 4.1 and ankyrin, have been often discussed in the previous reviews (Winkelmann and Forget, 1993; Conboy, 1993; Peters and Lux, 1993).

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(A) Spectrin

The spectrin is a flexible molecule, which is composed of a series of contiguous and homologous motifs with their molecular sequence of approximately 106-amino acid residues (Fig. 2). The complete sequences of the human erythrocyte 280kDa alpha subunit (Sahr et al., 1990) and the 246 kDa beta subunit (Winkelmann et al., 1990), deduced from their cDNA sequences, have indicated that most of the molecules are composed of these repetitive segment motifs or repeats. One segment consists of a three-helix bundle (Speicher and Marchesi, 1984), and a molecular model of the interface between two tandem peptide segments suggests that their hydrophobic interactions constrain the intersegmental flexibility (Yan et al., 1993). The human erythroid alpha subunit has been reported to contain 22 domains in total. The domains, 1-9 and 11-19, are repeated structures, and the domain 10 in SH3 (Src-homology domain 3) motif (Musacchio et al., 1992). Recently, it was reported that the rat epithelial Na^+-channel was bound to alpha spectrin in vivo, whose binding is mediated by SH3 domain of the alpha spectrin (Rotin et al., 1994). The two domains, 20 and 21, are related to 106-residue repeats with an 8-residue insertion, and the C-terminal domain, 22 includes EF-hands motifs. Recently, it was reported that EF-hand calcium-binding proteins played a central role in calcium-mediated signal transduction (Chazin, 1995) with the calcium-induced conformational change (Trave et al., 1995).

The beta subunit of spectrins is composed mainly of three domains, I-III. Domain I consists of the first 273 amino acids and functions for binding with actin and protein 4.1. The actin-binding domain of the human erythrocyte spectrin is localized on a stretch of 140 amino acids at the amino-terminus of the beta spectrin from the first 47th alanine through the first 186th lysine (Karinch et al., 1990). Domain II contains 17 repeats, most of which are composed of 106-amino acids. Domain III also consists of 52 amino acids at the carboxyl-terminus and is related to both spectrin self-association and phosphorylation. Therefore, it is highly suggested that the alpha and beta spectrin molecules are organized to have signal-regulated spring conformations.

The alpha and beta subunits in antiparallel orientation are associated to each other in a side-to-side fashion to produce the heterodimer, which is firm for the subunit interaction. This antiparallel association has been reported to require a two-step process (Speicher et al., 1992). The spectrin heterodimer assembly involves the initial contact of complementary nucleation sites on each molecular subunit. The nucleation sites have a specific lock structure, which defines the unique side-to-side pairing of homologous segments in both subunits (Fig. 2, arrows). After the initial binding of nucleation sites, the remainders of both subunits are quickly associated to each other along their full length to reconstitute a flexible heterodimer with supercoiling of each molecule. These spring-like alpha and beta spectrins probably have stronger mechanical stability than only one molecule.

The binding sites between the N-terminal region of alpha subunit and C-terminal region of beta subunit, which form the alpha-beta complexes, have been already identified (Fig. 2, and Fig. 1, C and C' boxes). The head-to-head association region is homologous to the 106-residue repetitive motif, which comprises most of both amino acid sequences (Speicher et al., 1993; Kennedy et al., 1994). Moreover, this interaction enables the heterodimers to associate to each other and form larger oligomeric structures (Liu et al., 1984). Some biochemical studies have demonstrated that a variety of assemblies in the erythrocyte proteins are responsible for the abnormality in erythrocyte shapes, such as hereditary spherocytosis and hereditary elliptocytosis (Becker and Lux, 1995).

The spectrin has also been known to interact with phosphatidylserine and phosphatidylethanolamine, which are preferentially localized in the inner leaflet of the lipid membrane layer, at multiple sites including the

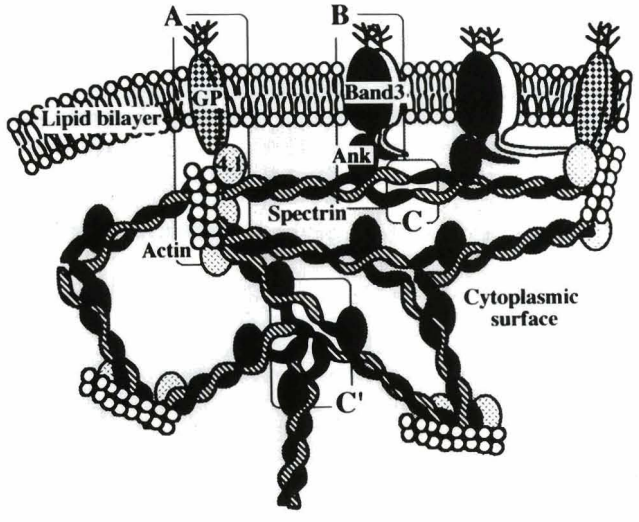

Fig. 1. A schematic representation of the skeletal protein constituents and their assembly. Ank: ankyrin; GP: glycophorin. **A box.** Glycophorin-protein 4.1-spectrin/actin. **B box.** Spectrin-ankyrin-band 3. **C and C' boxes.** Spectrin-self associations, tetramer (C) and oligomer (C').

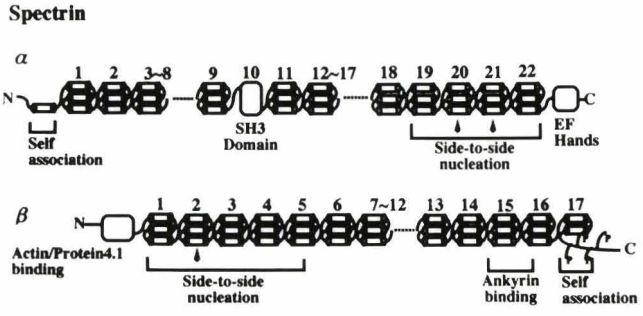

Fig. 2. A molecular model of spectrin structures. The alpha and beta molecules are depicted as a series of helical repeating segments. Repeats with an 8-residue insert are indicated by arrows.

tail-end region (Cohen et al., 1986; Sikorski et al., 1987). In certain hemolytic disorders such as hereditary spherocytosis, the weak spectrin-lipid interaction is representative of the well-known lipid membrane instability, resulting in a loss of lipid components (Chasis et al., 1988).

(B) Protein 4.1

Protein 4.1 plays a crucial role in stabilizing the spectrin cross-links between actin filaments (Ungewickell et al., 1979; Cohen and Korsgren, 1980; Fowler and Taylor, 1980). A spectrin-actin-binding domain (Fig. 3, 10kDa domain) of protein 4.1 is encoded by two exons, which are translated into the alternative N-terminal 21 amino acids, and a constitutive exon encoding the C-terminal 59 amino acids (Discher et al., 1995). Precisely, the protein 4.1-spectrin interaction requires the 21-amino acid alternative cassette plus the 43 N-terminal residues of the constitutive domain. Moreover, the protein 4.1-actin interaction requires a putative actin-binding domain with the 26 N-terminal residues of the constitutive domain (Schischmanoff et al., 1995). Protein 4.1 deficiency, including structural and functional defects of the protein 4.1, transforms the biconcave erythrocytes into the elliptical ones (Palek and Sahr, 1992; Becker and Lux, 1995).

Protein 4.1 also binds to the erythrocyte membrane through at least two sites; a high affinity site for the glycophorin C and A (Fig. 1, A box) (Anderson and Lovrien, 1984; Chasis and Mohandas, 1992; Hemming et al., 1995), and a low affinity site associated with band 3 (Lombardo et al., 1992; Jöns and Drenckhahn, 1992) (Fig. 3, 30kDa domain). This glycophorin-protein 4.1 association is regulated by a polyphosphoinositide cofactor (PIP/PIP_2) (Anderson and Marchesi, 1985). The Ca^{++} treatment leads to a reduction of PIP and PIP_2 in erythrocytes, which were concomitantly transformed into echinocytes. It has also been reported that the Ca^{++} and calmodulin treatment competitively inhibits protein 4.1 association with inside-out erythrocyte vesicles (Lombargo and Low, 1994). None of the splicing isoform lacks the calmodulin-binding domain, which is located at the 30kDa N-terminal chymotrypsin-resistant domain, suggesting that protein 4.1 may be subjected to calmodulin regulation (Marfatia et al., 1994).

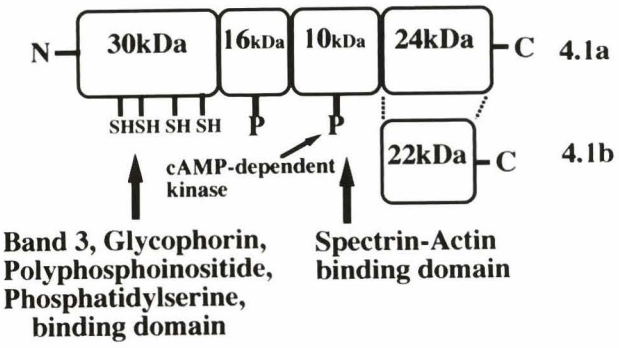

Fig. 3. A molecular model of human erythrocyte protein 4.1. It is composed of four functional domains, designated by using chymotrypsin cleavage technique. The difference in 4.1a and 4.1b is attributed to the 24kDa or 22kDa carboxyl-terminal domain.

Several protein kinases, including protein kinase C and cAMP-dependent kinase, are reported to phosphorylate protein 4.1. Moreover the phosphorylation causes dramatic reduction of spectrin capacity to bind to F-actin (Ling et al., 1988) (Fig. 3, 10kDa and 16kDa domains). However, in protein 4.1-deficient erythrocytes, only the spectrin-actin binding domain is required to restore their normal stability. Therefore, the conformational changes of protein 4.1 induced by the second messengers, such as Ca^{++} and polyphosphoinositide, may lead to morphological changes of erythrocytes due to the alteration of the membrane skeletons. The 30kDa domain is also reported to have a binding site with phosphatidylserine vesicles (Cohen et al., 1988) (Fig. 3, 30kDa domain). So the protein 4.1-deficient erythrocytes were found to show an altered pattern of membrane-lipid interaction.

(C) Actin

The erythrocyte actin is composed of only a beta subtype, which is usually found in a variety of non-muscle cells. Unlike the actin in other cells, the erythrocyte actin appears to be organized as short and double helical F-actin filaments with about 12 to 16 monomers' length (Pinder and Gratzer, 1983). The state of actin polymerization is functionally important for erythrocyte morphology. Some chemical compounds, which inhibit actin polymerization, usually increase the membrane flexibility, though other compounds, which promote its polymerization, often increase the rigidity of the erythrocyte membrane (Nakashima and Beutler, 1979). Irreversibly sickled erythrocytes (ISE) remain sickled even under the conditions in which they are well oxygenated and the hemoglobin is depolymerized. The molecular difference of the beta subtype actin between ISE and normal erythrocytes has been demonstrated, which shows the formation of a disulfide bridge between cystein 284 and cystein 373 in the ISE beta actin (Shartava et al., 1995). Therefore, it is probable that the flexibility of the erythrocyte membrane is effected by the construction of the beta subtype actin.

The beta subtype actin of erythrocytes is localized at the lipid membrane-cytoskeletal interface, resembling that of non-erythroid cells (Hoock et al., 1991). The pericyte beta actin is also localized beneath the plasma membrane, especially in moving regions (Herman, 1987; Hoock et al., 1991). It is well-known that mature erythrocytes do not move voluntarily, but they must always restore their discoid shape against external mechanical forces. So, the beta subtype actin is probably concerned with the rapid sol-gel transformation in the flexible erythrocyte membranes.

Other proteins bound with the short actin include

protein 4.9, which has been reported to bundle F-actin in vitro (Siegel and Branton, 1985). Moreover, the tropomyosin lining grooves of actin protofilaments (Fowler and Bennett, 1984) and the adducin, a Ca^{++}-calmodulin binding protein, stimulate the addition of spectrin to F-actin in a protein 4.1-independent manner (Gardner and Bennett, 1987; Mische et al., 1987). So these proteins with the spectrin may also regulate the length of F-actin in erythrocytes.

(D) Ankyrin

Ankyrin is a large, pyramid-shaped protein (Tyler et al., 1980), which is composed of three domains: the 62kDa spectrin-binding domain; the 89kDa membrane binding domain; and the 55 kDa regulatory one (Fig. 4). The amino acid sequence of the 89kDa domain is dominated by a tandem array of 24 ANK repeats, which form four folded subdomains (Fig. 4, membrane-binding domain; Fig. 1, B box). It contains two separate binding sites for a Cl^-/HCO_3^- anion exchanger, band 3; the repeat subdomain D2, and both D3 and D4 subdomains (Michaely and Bennett, 1995). Moreover, the 89kDa domain can bind to tubulin, Na^+ channel and Na^+/K^+-ATPase in vitro (Davis and Bennett, 1984; Devarajan et al., 1994) and the 62kDa domain can bind to intermediate filaments, vimentin and desmin (Georgatos et al., 1987).

II. Ultrastructures of the membrane skeleton

The membrane skeleton of erythrocytes was first demonstrated by scanning electron microscopy (Hainfeld and Steck, 1977) and by the negative staining technique in Triton X-treated erythrocytes (Sheetz and Sawyer, 1978). In addition, by the use of tannic acid in the fixation for conventional electron microscopy, a spider-like configuration was morphologically obtained as the membrane skeleton (Tsukita et al., 1980). In the following first section, we will discuss the ultrastructure of membrane skeletons, which have been examined by the negative staining technique in combination with the spread erythrocyte preparations. Then we will mention the QF-DE method to obtain the three-dimensional ultrastructure of the in situ membrane skeletons, which

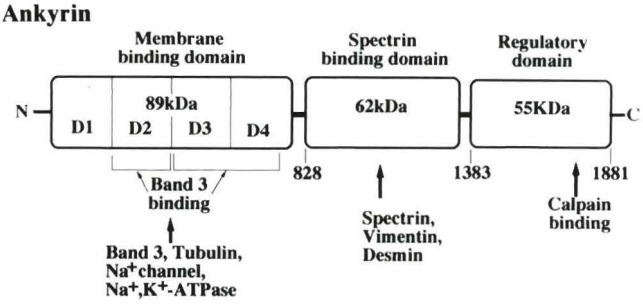

Fig. 4. A molecular model of human erythrocyte ankyrin, which is composed of three functional domains as defined by the chymotrypsin cleavage treatment.

are organized under the different conditions.

(A) Membrane skeletons visualized by low angle shadowing and negative staining techniques

Ultrastructural studies have been focused on the organization of spectrin and actin along the cytoplasmic side of erythrocyte membranes. Some clear images of actin crosslinked by spectrin were obtained by a low angle shadowing technique, as revealed by electron microscopy (Cohen et al., 1980). When the G-actin is polymerized in the presence of spectrin with their molecular ratios close to those of the erythrocyte, the formed networks are composed of clustered spectrin with granular structures of about 25 nm in diameter. By the negative staining technique with spread erythrocyte membrane skeletons, the meshworks consisted of junctional complexes crosslinked by long flexible spectrin molecules, which have been identified as F-actin (Gilligan and Bennett, 1993). In the spread membrane skeleton, the rod spectrin proteins act as nucleating sites for the elongation of actin filaments, when they are incubated with G-actin under sufficient conditions for the filament elongation (Byers and Branton, 1985). On the other hand, considering 13 actin monomers per oligomer, the surface area of an erythrocyte, and biochemically measured actin contents, there would be about 250 oligomers/μm^2 in the intact erythrocyte membrane. In fully spread membrane skeletons, the length of spectrin tetramers is about 200 nm, which would give a density of 25-29 oligomers/μm^2 depending on whether or not they form a square or hexagonal lattice. Thus, the protein density in the spread meshwork must be 9- to 10-fold less than that in the intact membrane. The extended membrane skeleton appears to be primarily a hexagonal lattice with actin protofilaments at the center of hexagons, which are interconnected by spectrin tetramers (~85%) and three-armed hexamers (~10%) (Liu et al., 1987). The Fourier analysis of the spectrin molecule visualized by the negative staining technique indicates that the alpha and beta subunits twist around each other to form a helical structure (McGough and Josephs, 1990). The pitch and diameter of the helix vary with the extension degree of the spectrin molecule, suggesting that it functions as an elastic spring. The spread membrane skeletons, as revealed by the negative staining technique, match the biochemical data. However, another question is how these spread membrane skeletons are compacted under the condition of in situ erythrocytes.

(B) In situ membrane skeletons visualized by QF-DE method

(i) Fresh intact membrane skeletons

It has been described that fresh membrane skeletons are compactly distributed on the cytoplasmic side of lipid membranes, organizing the filamentous network. By our erythrocyte-splitting method, in combination

with the QF-DE method (Ohno, 1992), the membrane skeletons, which are underlying the lipid membrane, have been visualized in three dimensions (Figs. 5, 6, 8, 9, 11, 12). One is visualized from the cytoplasmic side of erythrocytes, which are split open to remove the upper erythrocyte membrane (Figs. 5, 9). In the proper condition of erythrocytes attached to the coverslip, the membrane skeleton is not extended even by the external mechanical forces during the erythrocyte-splitting procedure, and fine compact networks can usually be observed (Fig. 5). Some exposed networks are partially detached from the erythrocyte lipid membrane, indicating the contracting property (Fig. 5b,c). These skeletal structures are designated as «fresh unfixed membrane skeletons». Morphometric data show that the length of each filament between intersections is significantly shorter than that in the glutaraldehyde-fixed networks, as reported before (Ohno et al., 1994) (Fig. 5d). It has also been described that the long-time glutaraldehyde fixation alters the organization of the membrane skeleton itself, because of its strong covalent chemical reaction (Ohno et al., 1994). The other is visualized from the outside of erythrocytes, which were prepared by stripping off the lipid bilayer (Fig. 6a-c). The membrane skeletons are quite fragile, and so it is difficult to get large intact areas of membrane skeletons. By tugging force at the intact mesh structures, many filaments are broken to produce holes (Fig. 6a,c). The characteristic finding of the quick-frozen networks is that their lengths are various depending on parts even within one erythrocyte. So it is suggested that the membrane skeleton is morphologically changed in the circulating blood flow.

In specimen preparation, the concentration of magnesium ion had an effect of stability on unfixed membrane skeletons just before the quick-freezing step and influenced the organization of F-actin in erythrocytes (Shen et al., 1986). When the F-actin was prepared in 10mM magnesium acetate, some network arrays of filaments were usually produced. On the contrary, they displayed paracrystals at higher magnesium concentrations (20mM) (Pinder et al., 1995). The caldesmon was also purified from erythrocytes (Terrossian et al., 1994), and bound to the F-actin localized as protofilaments in the presence of free magnesium. Moreover, this magnesium-dependent attachment is closely related to the results reported for myosin (Fowler et al., 1985) and tropomyosin (Fowler and Bennet, 1984). Recently, a tropomyosin binding protein, tropomodulin, was discovered in the human

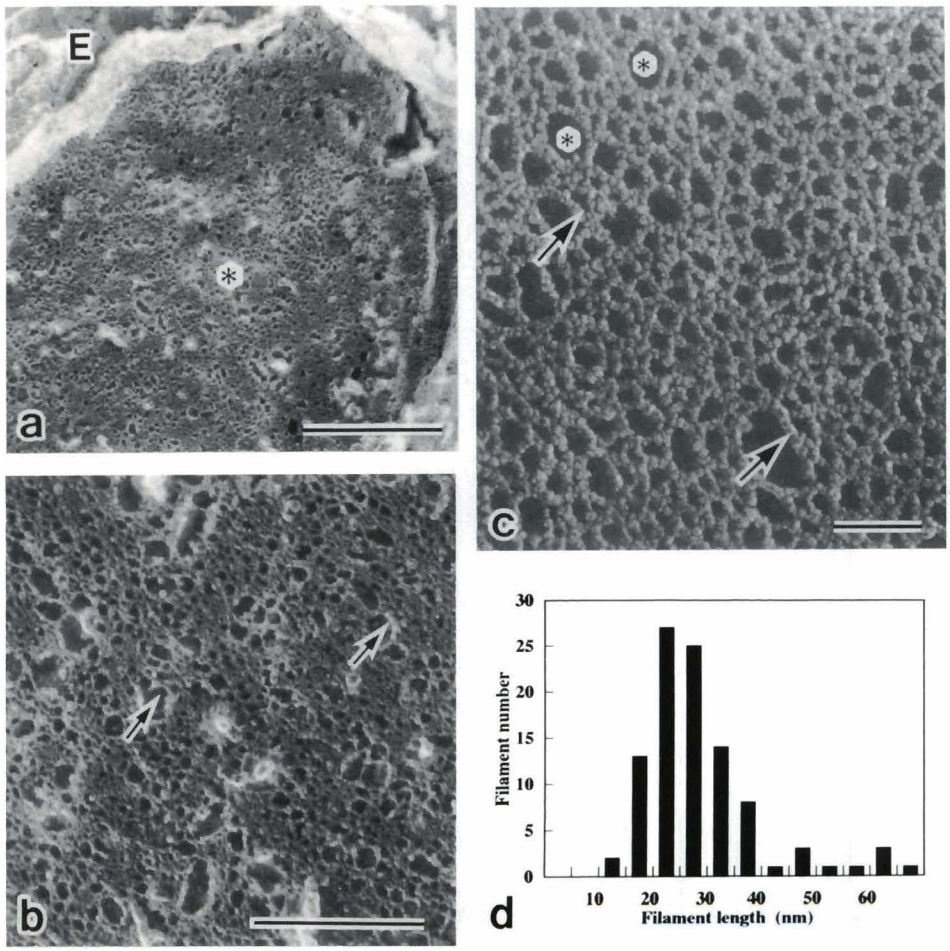

Fig. 5. Replica electron micrographs of the membrane skeleton of fresh unfixed erythrocytes. **a.** Lower magnification of an overview of the split erythrocyte (asterisk). E; edge of the split erythrocyte. **b.** Higher magnified micrograph of the cytoplasmic side of an erythrocyte. Note that the lengths between intersections vary from place to place. Networks are partly destroyed (arrows). **c.** Some filamentous networks (arrows) are detached from the erythrocyte membrane, because of the buffer washing procedures. Damaged networks are contractible and form nude areas (asterisks). **d.** A histogram showing the length distribution of 100 filaments. Classes are composed of 0≤x<5, 5≤x<10, ..., 65≤x<70 nm. Mean±SD, 29.2±11.1 nm. a. x 18,200; bar: 100 μm. b. x 45,000; bar 500 nm. c. x 111,500

erythrocyte membrane skeleton (Fowler et al., 1993). So the actomyosin system might be involved in the molecular mechanism for erythrocyte shape changes, including membrane deformability and resistance. If the concentration of magnesium ion is reduced to 0.05mM in the hour-long incubation, the total membrane skeletons extend from 6 μm to 11 μm in their diameter. Moreover, the addition of dithiothreitol causes further expansion of the membrane skeleton disks to 15 μm (Shen et al., 1986). It is reported that the reducing agent may have this effect through its ability to inhibit the self-association of alpha and beta spectrins (Beaven and Gratzer, 1980). So the different ion concentrations before the quick-freezing step are critical to keep the

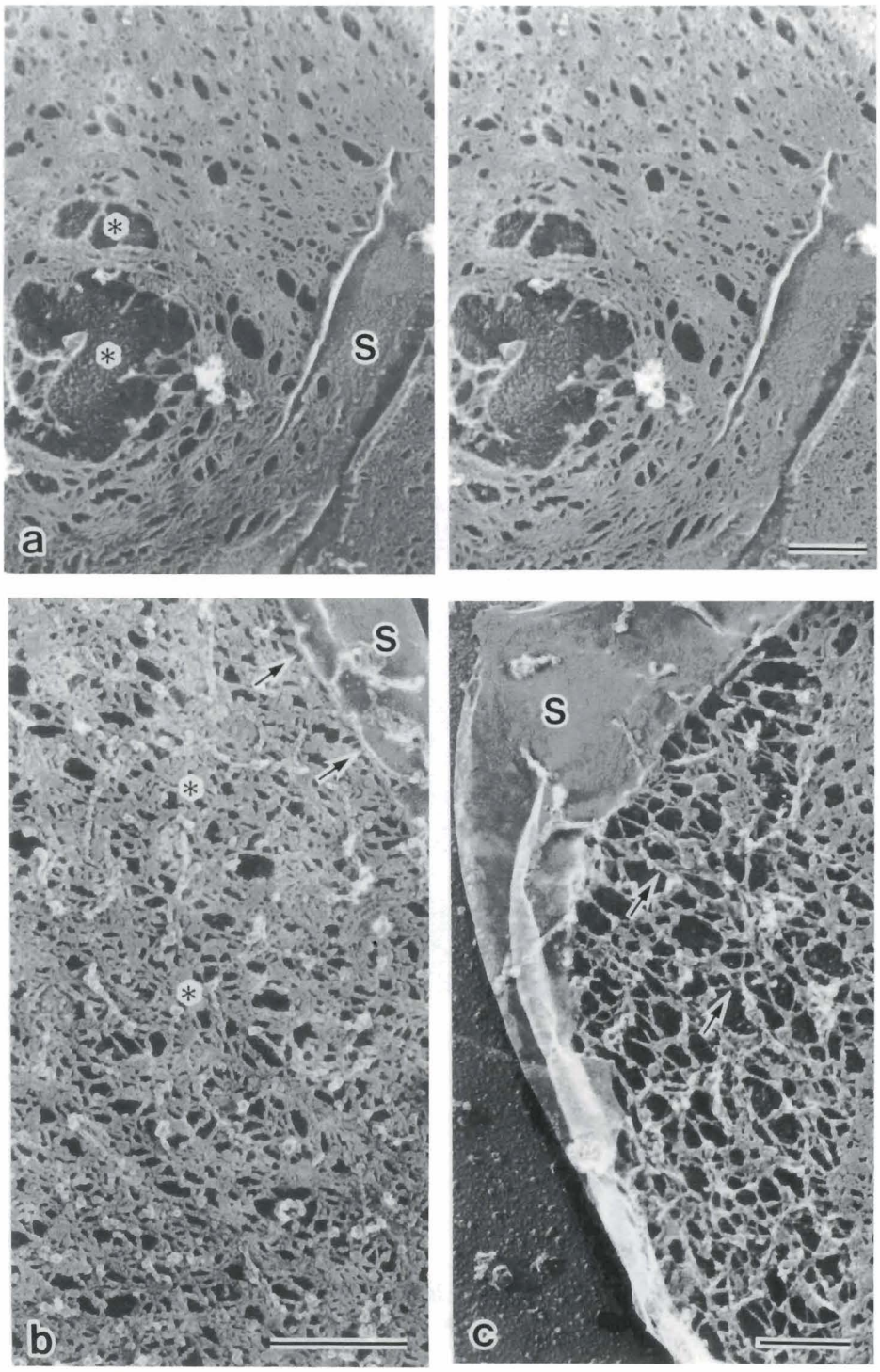

Fig. 6. Replica electron micrographs of membrane skeletons, as viewed from the upper side. The upper lipid membrane is stripped off and the underlying networks are observed. Note the fragility of the membrane skeletons. S: erythrocyte membrane surface. **a.** In the splitting procedure, mechanical stripping forces induce the membrane skeleton to be modified. Three-dimensional membrane skeletons are viewed as stereo-pictures. The membrane skeletons are condensed, and some are transformed into thick filamentous structures. Large holes (asterisks) are observed. x 21,900; bar: 500 nm. **b.** Some lipid membranes are curling at their edge (arrows) and fine meshworks are observed underneath (asterisks). x 37,700; bar: 500 nm. **c.** Some parts of the membrane skeleton are subjected to mechanical force (arrows), which induces the irreversible deformability during specimen preparation. x 25,600; bar: 500 nm.

organization of F-actin with accessory proteins.

To observe the membrane skeleton from erythrocyte exterior, Triton X-100 has often been used to solubilize the membrane lipids and remove a majority of integral membrane proteins (Sheetz and Swayer, 1978) (Fig. 7a,b). Morphometric data show that the length of filaments is slightly longer than that revealed by the erythrocyte-splitting method (Figs. 5d, 7c). The air-dryness step of erythrocyte specimens before the quick-freezing step was reported to induce the spreading of network filaments (Ursitti and Wade, 1993). They pointed out that the surface tension of isolated membrane skeletons imposed during negative staining caused a mechanical loss of their interactions, which had been normally present in intact membrane skeletons. It has also been reported that purified human erythrocyte spectrins form large oligomeric structures without collaboration of other proteins (Morrow and Marchesi, 1981). So it is speculated that high concentrations of spectrins are usually maintained in vivo as necessary for the oligomerization. In such a situation, the spectrin easily forms the submembranous networks. Actually, 25-35% of medium-sized spectrin oligomers, in addition to 45-55% of tetramers, coexist in the erythrocyte membrane skeleton (Ursitti et al., 1991). Functionally, these fine mesh structures are effective to withstand external mechanical forces from all directions in the circulating blood stream.

In the spread membrane skeletons, the localization of their composed proteins, such as protein 4.1, dematin (protein 4.9), adducin and ankyrin, was demonstrated by employing immunogold labeling in combination with a negative staining technique (Derick et al., 1992). We have also examined the localization of spectrin proteins on intact membrane skeletons with our prepared monoclonal antibodies. Some areas in immunostained networks are decorated with globular structures, and other areas are heavily decorated to form sheet-like structures (Fig. 8), in comparison with the network in non-immunostained erythrocyte (Fig. 8, inset). So most of network structures are assumed to consist mainly of spectrin proteins, which is probably compatible with the skeletal model in oligomeric forms of spectrin molecules. Moreover, the organization of actin filaments is clarified on the network structures of erythrocytes by the immunostaining technique.

(ii) Membrane skeletons changed by external mechanical forces

During the procedure of our erythrocyte-splitting method, the membrane skeletons of erythrocytes are often stretched under the external mechanical force and their uniaxial extension can be detected on the lipid

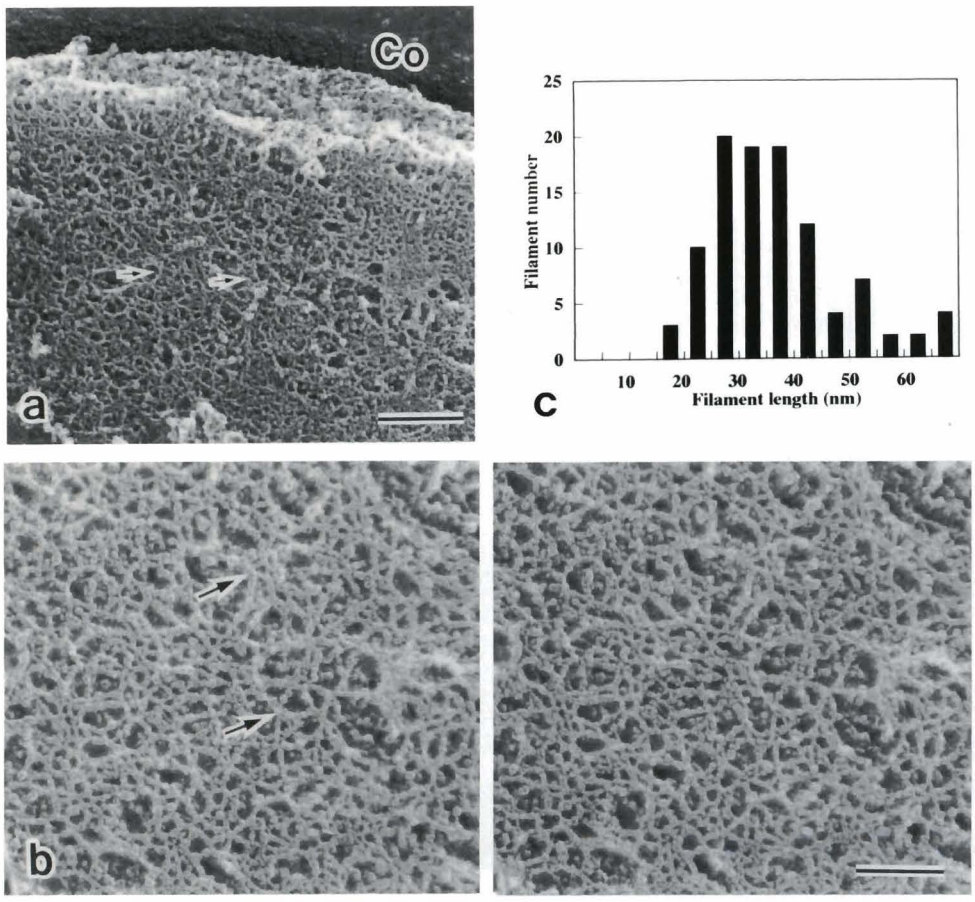

Fig. 7. Replica electron micrographs of membrane skeletons, prepared by prefixation with 2% paraformaldehyde and permeation with Triton X-100. **a.** Lower magnification of an erythrocyte. Co: the face of coverslip. x 21,000; Bar: 500 nm. **b.** Stereo views of higher magnified micrographs, showing their detailed substructure (arrows). x 57,600; Bar: 200 nm. **c.** Histogram of the length distribution of 100 filaments measured in Triton-permeabilized membrane skeletons shown in Fig. 7b. Classes are composed of $0 \leq x < 5$, $5 \leq x < 10$, ..., $65 \leq x < 70$ nm. Mean±SD, 36.7±11.6 nm.

membrane (Fig. 9a-c). In the circulatory blood stream, erythrocytes probably suffer from the external mechanical forces. For the rapid response to the mechanical stresses, the spectrin network has the intramolecular unfolding mechanism. When a strong mechanical force works beyond the erythrocyte limit of reversible deformability, then some junctional protein complexes are temporarily dissociated in the membrane skeletons (Weinstein et al., 1986). Among these junctions, the weakest points are probably head-to-head associations of the spectrin tetramer (Mohandas, 1991). However, the membrane skeletons undergo an active rearrangement, involving disconnection of existing protein-protein contacts and formation of new associations which permanently stabilize the erythrocytes in their deformed shape during the prolonged period (Liu et al., 1993). So, considering the membrane skeletal changes, the rapid or long-term mechanical effect must be examined for the erythrocyte shapes.

The elliptocytosis and spherocytosis are representative of abnormally shaped erythrocytes. Some morphological approaches, including the negative staining technique, the QF-DE method and the surface replica method, have been used to compare the intact membrane skeleton with that in the abnormal erythrocytes (Liu et al., 1990; Ohno et al., 1993; Yawata et al., 1994). The hemolytic anemias caused by deficiency or disfunction of erythrocyte membrane proteins are divided into two categories (Palek and Lambert, 1990). (1) Vertical protein interactions in erythrocytes involve the spectrin-ankyrin-band 3 connection and also a weak binding between spectrin and negatively-charged lipids of the inner leaflet. (2) Horizontal protein interactions are parallel to the plane of erythrocyte lipid membranes, which involve the spectrin heterodimers assembled into tetramers and their connections with oligomeric actin due to protein 4.1 and adducin. The hereditary spherocytosis (HS) is reported to be a disorder of the vertical protein interaction, and the hereditary elliptocytosis (HE) is due to the abnormal horizontal interaction (Palek and Lambert, 1990). It has been known that erythrocyte precursors of the HE are morphologically in discoid shape, and their shapes becomes progressively more elliptical as they age in vivo (Chien, 1987). We have already reported the less filamentous and more granular structures of membrane skeletons in the HE and HS erythrocytes than those of normal erythrocytes (Ohno et al., 1993). The abnormal shape changes are probably due to the balance between the regaining force of membrane skeletons and the effected external forces in the blood stream. The abnormal erythrocytes in an individual patient have a variety of erythrocyte shapes, and thus age-related structural changes should be considered to explain the finding.

Recently, we obtained a morphological finding that the external mechanical force was transmitted from the peripheral area of erythrocytes to the central one during the erythrocyte-splitting procedure (Fig. 9a). So, to clarify rapid morphological changes against the external force, the QF-DE method would be needed to get their native image. To some extent, the paraformaldehyde fixation can keep the in situ shape of stretched erythrocytes, but it is not sufficient. A novel method, which is referred to as «in vivo cryotechnique», will be used for visualizing the in vivo morphology of erythrocytes in the circulating blood flow (Ohno et al., 1995).

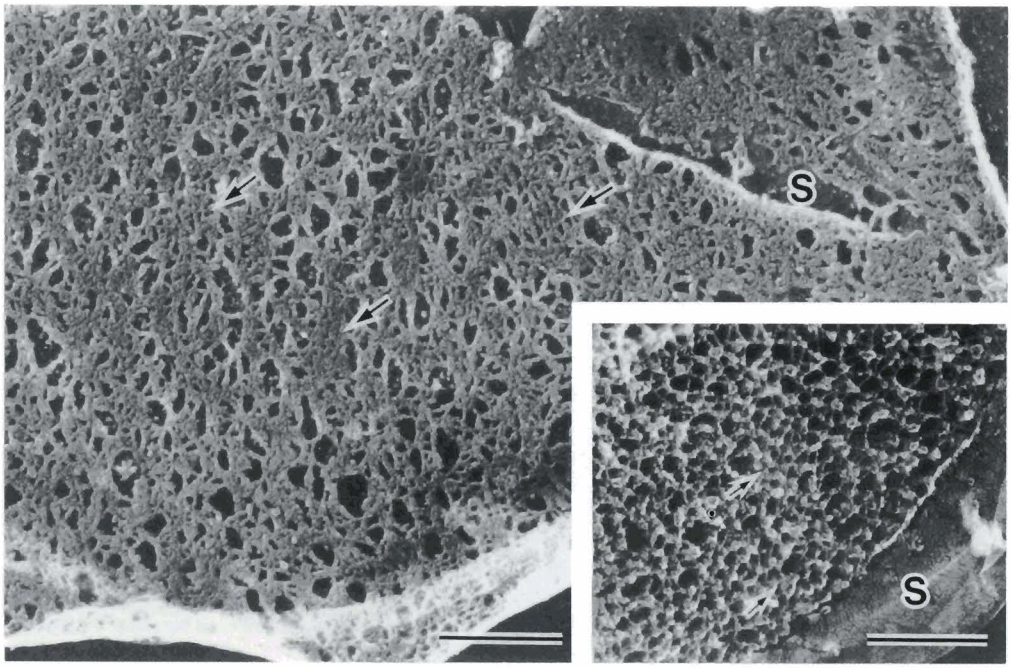

Fig. 8. Replica electron micrographs of the membrane skeletons, prepared by the erythrocyte-splitting method, which are additionally immunostained with monoclonal anti-spectrin antibody (arrows). Fine networks under the peeled lipid membrane are viewed from the extracellular space. x 31,900; bar: 500 nm. **Inset:** non-immunostaining of erythrocytes (small arrows). x 31,300; bar: 500 nm.

(C) Different organization of membrane skeletons in a single erythrocyte

It was demonstrated that the conversion of protein 4.1b into protein 4.1a was due to the deamidation of asparagine 502 (Inaba et al., 1992). Moreover, this conversion usually occurred in a time-dependent manner under physiological conditions (Molchanova et al., 1991). It is likely that the deamidation at this site causes the conformational change of protein 4.1 and alters its function during erythrocyte aging. The ratio of protein 4.1a/4.1b has a great variety among animal species, and is related to the mean erythrocyte life span in the circulating blood flow (Inaba and Maeda, 1988). Moreover, the irreducible complexion of hemoglobin with spectrin is a natural phenomenon of erythrocyte aging (Snyder et al., 1983). The inhibition of self-association in spectrin dimers was suggested to occur after producing their complexion with globin in vitro (Kiefer et al., 1995). So, it is necessary to clarify if different organizations of membrane skeletons can be observed during erythrocyte aging.

There are different organizations of membrane skeletons even within a single erythrocyte. Some types of filaments are very short and compactly distributed, though other types are long and loosely distributed (Fig. 10a,b). The decreased deformability with erythrocyte aging is probably related to changes of erythrocyte shapes, cytoplasmic viscosity, and membrane skeletons. If compact meshworks of the membrane skeletons are loosened in erythrocytes, they become more fragile and are easily trapped in the reticulo-endothelial system.

(D) Altered membrane skeletons in erythroid differentiation

From the molecular structure of spectrin, its membrane-association domain (MAD), not related to protein 4.1 and ankyrin, has been reported to be present in the beta-spectrin (Lombardo et al., 1994). The MAD1

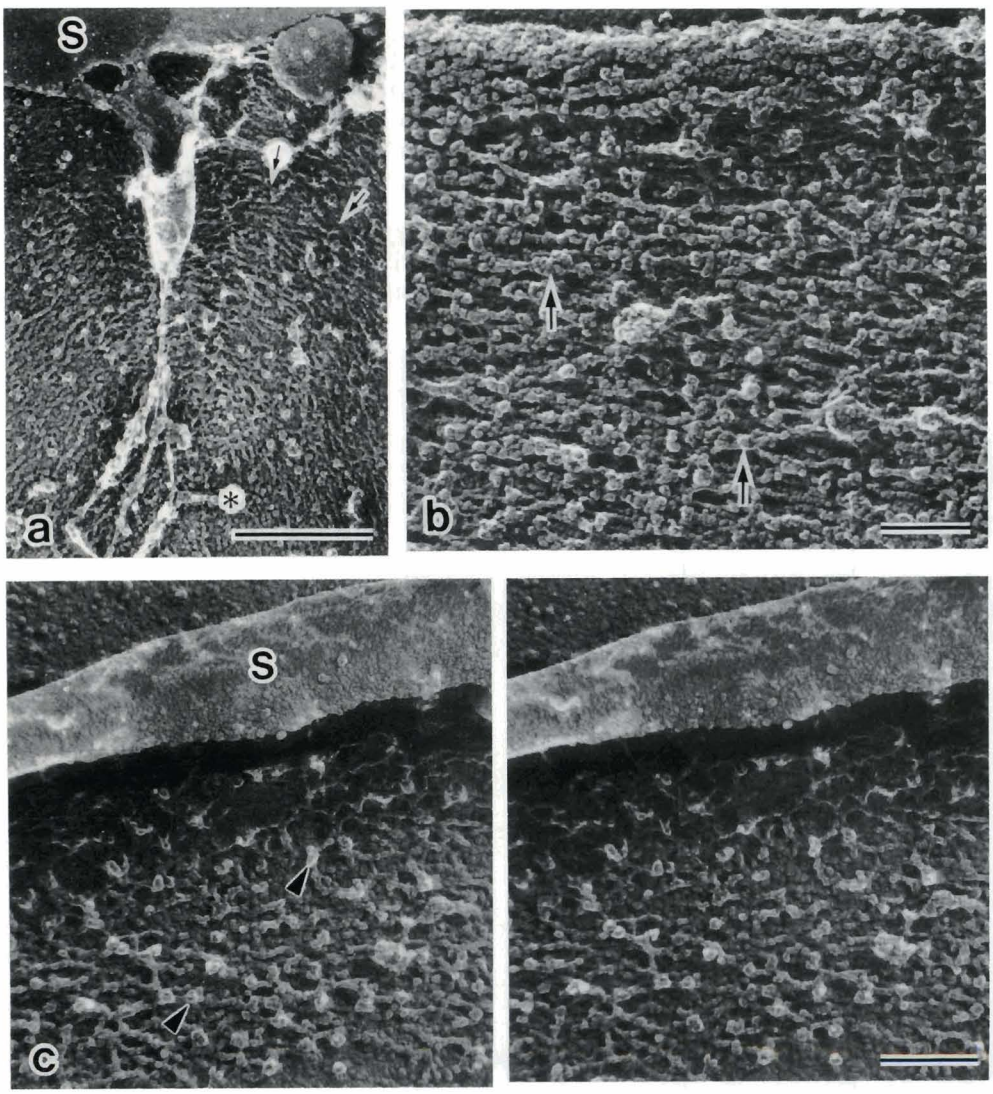

Fig. 9. Replica electron micrographs of the membrane skeletons attached to the cytoplasmic side of lipid membranes, which are formed under mechanical force in the process of the erythrocyte-splitting method. S: edge of the split erythrocyte surface. **a.** The meshwork in marginal areas (arrows) is affected by a stronger mechanical force than that in central areas (asterisk). x 35,100; bar: 500 nm. **b.** They are immunostained with monoclonal anti-alpha and beta-spectrin antibody and secondary peroxidase-conjugated antibody, and finally decorated with DAB reaction products (arrows). Spectrin proteins are extended in one direction and lie side by side.x 58,700; bar: 200 nm. **c.** Stereo pictures of extended membrane skeletons. They are immunostained with monoclonal anti-beta-spectrin antibody and secondary gold-conjugated antibody (arrowheads). x 65,300; Bar: 200 nm.

is represented as the site of all peptide fusions, including the spectrin repeat 1. The MAD2 is also identified in the C-terminal sequence (domain III) of both beta II (fodrin) and beta I$\sum$2 spectrin (non-erythroid muscle type). Moreover, it involves sequences in the pleckstrin homology (PH) domain (Lombardo et al., 1994; Ferguson et al., 1995). However, human erythroleukemia (HEL) cells have both erythroid and non-erythroid transcripts of the spectrin (Chu et al., 1994), indicating a possibility of the early switching in erythroid differentiation for preparing their escape from the bone marrow.

It has been reported that the erythroblast loses some microtubules and intermediate filaments just before its enucleation in the course of erythropoiesis. The microtubules usually exist as marginal bands (MB), resembling those of lower vertebrates, in erythroblasts and primitive erythrocytes (Koury et al., 1987). In avian erythrocytes, they are bundled at the peripheral edge of erythrocytes and are connected with their membrane skeletons (Fig. 11c,f). The strained MB in non-mammalian mature erythrocytes have been known to work as tension-supporting structures in response to the erythrocyte deformation (Joseph-Silverstein and Cohen, 1984; Waugh and Erwin, 1989). On the contrary, the intermediate filaments are assumed to play a role in anchoring the nucleus at the proper place in erythrocytes (Granger and Lazarides, 1982) (Fig. 11e). In human erythrocytes, the rapid disappearance of these two cytoskeletons is probably related to the maturation mechanism, which leads to the enucleation stage of erythroblasts.

The synthesis of alpha or beta spectrin proteins and their organization are often studied in erythroid progenitor cells (Koury et al., 1987; Hanspal et al., 1992). The maximal mRNA levels of spectrin, band 3, ankyrin and protein 4.1 are dependent on different developmental stages (Wickrema et al., 1994). The erythroid differentiation is usually accompanied by the dramatic alteration in its morphology, and the membrane structural properties are changed due to the reorganization of membrane skeletal proteins. The gene expression of a protein 4.1 isoform, which is associated with spectrin-actin binding domain, appears to be a very late event during erythropoiesis (Chasis et al., 1993). It is also concomitant with major changes in the erythrocyte membrane property. In addition, the deletion of the spectrin-actin binding domain of protein 4.1 induces dramatic membrane instability in mature erythrocytes and sometimes in hereditary elliptocytosis (Conboy et al., 1990; Lorenzo et al., 1994). The condensation of spectrin-actin interactions may be a prerequisite to erythrocyte release into the blood stream.

In the reticulocyte maturation, the membrane skeletal assembly and remodeling usually occur at later stages (Chasis et al., 1989). So membrane deformability in immature reticulocytes markedly decreased to approximately 10% of mature ones (Chasis et al., 1989). The assembly of membrane skeletons involves a number of different molecular changes, including an increased number of molecules of skeletal proteins, molecular changes in protein-protein interactions and also loss of the membrane lipid. The exosome formation is assumed to be a major route for removal of erythrocyte membrane

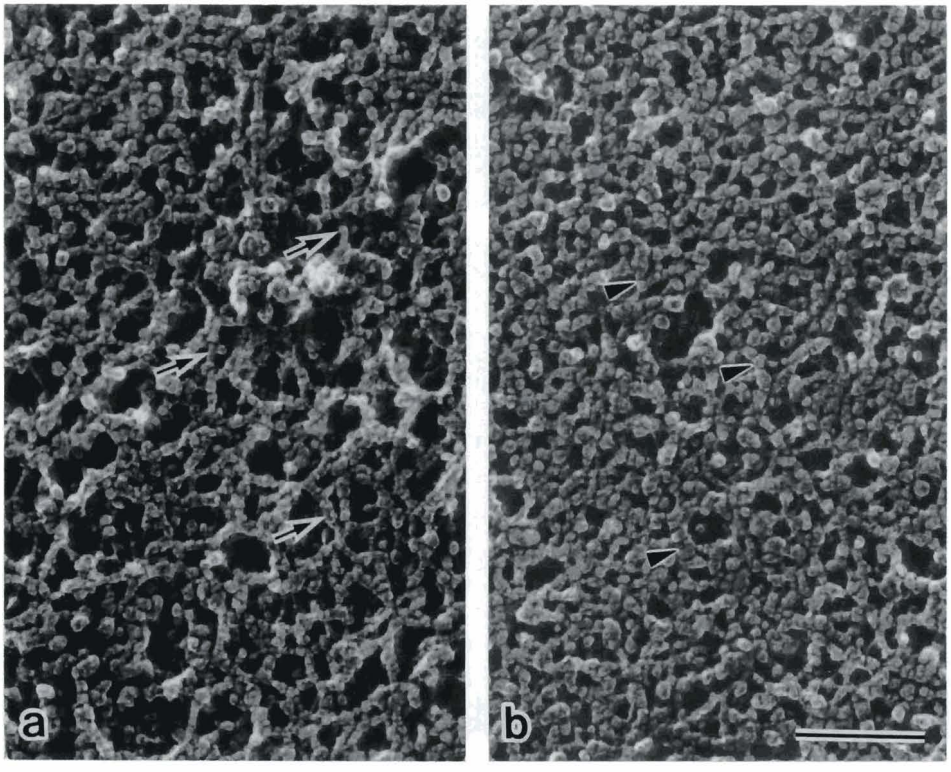

Fig. 10. Replica electron micrographs of membrane skeletons, prepared by prefixation with 2% paraformaldehyde and permeation with Triton X-100. Some erythrocytes have loose membrane skeletons (a; arrows) and others have compact ones (b; arrowheads). x 83,900; bar: 200 nm.

proteins during the reticulocyte maturation (Johnstone et al., 1991). The membrane skeleton is often peeled off from the lipid membrane of mouse reticulocytes in phenylhydrazine-induced hemolytic anemia (Fig. 12). Bundled short filaments are visualized in the pattern of compact association. As the reticulocytes are more fragile than mature erythrocytes, their membrane skeletal structures are probably different from those of the mature erythrocytes. So, a further immunocytochemical study is needed to clarify the molecular organization of membrane skeletons in the reticulocytes.

Moreover, drastic changes of actin organization may occur in the maturation process of reticulocytes, because of the different polarization at the stage of their enucleation. It was reported that F-actin in immature erythroblasts showed a patchy distribution along the cell surface, but F-actin bundles could be detected as they matured (Koury et al., 1989). The F-actin in enucleating erythroblasts was compactly localized between an extruding nucleus and an incipient reticulocyte. Then,

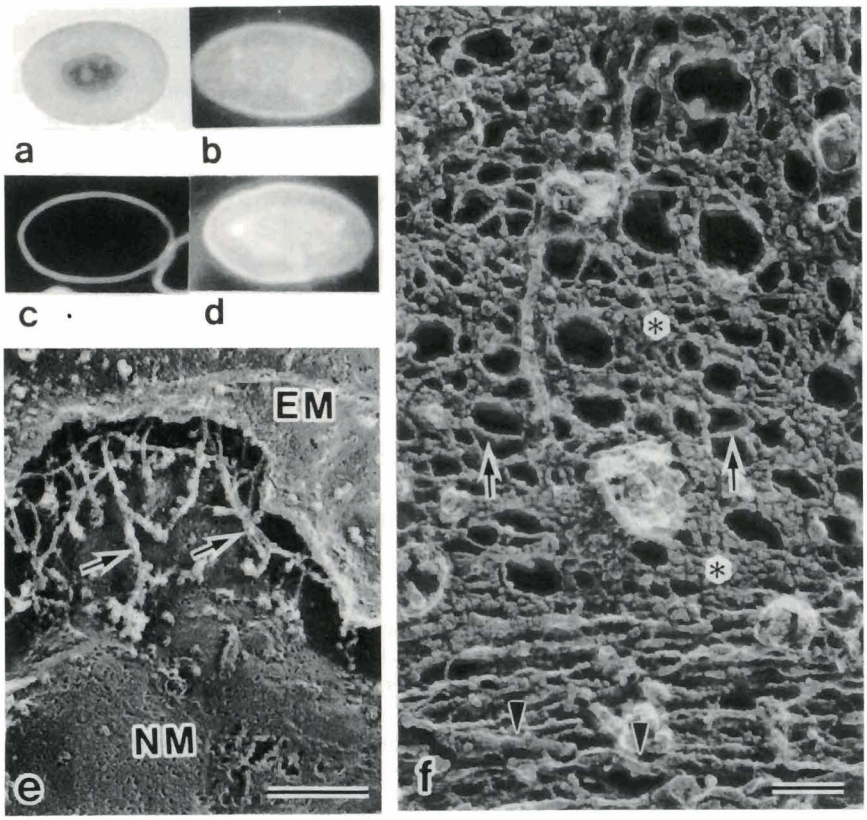

Fig. 11. (a-d) Light micrographs of chicken erythrocytes, which are stained for Wright-Giemsa (**a**), and immunostained for actin (**b**), for tubulin (**c**), and for spectrin (**d**). **e, f.** Replica electron micrographs of chicken erythrocyte membranes. e. Freeze-fractured erythrocytes, which are treated with saponin after paraformaldehyde fixation. Intermediate filaments (arrows) from the erythrocyte membrane (EM) to the nuclear membrane (NM). f. The membrane skeleton is composed of filamentous structures (arrows) and granular components (asterisks), in the chicken erythrocyte membrane, prepared by the cell-splitting method after paraformaldehyde fixation. Marginal bands are composed of several microtubules (arrowheads). a-d, x 1,850; e, x 26,000; Bar: 500 nm; f. x 45,000; bar: 200 nm.

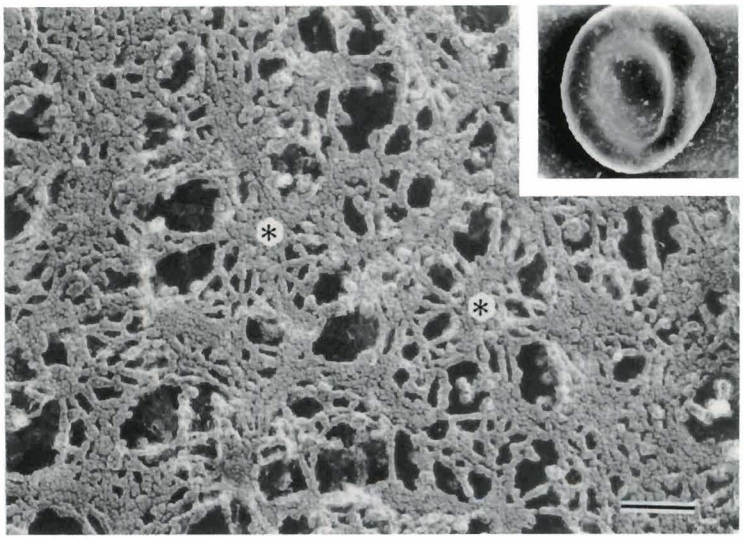

Fig. 12. Replica electron micrograph of membrane skeletons of mouse reticulocytes in phenylhydrazine-induced hemolytic anemia. The upper lipid membrane is easily stripped off during the erythrocyte-splitting method. Reticulocytes are more fragile than mature erythrocytes, and their membrane skeletons are compactly distributed (asterisks). x 46,900; Bar, 200 nm. **Inset:** an overview of the reticulocyte observed by scanning electron microscopy. x 4,800

the newly-formed reticulocyte exhibited the punctate pattern of F-actin. The mechanism of its redistribution is still obscure in reticulocyte development, which may play a key role in remodeling of F-actin in the erythrocyte membrane skeleton.

Concluding remarks

The erythrocyte is important for the transport of oxygen from lungs to peripheral organs in human bodies. Morphological elasticity is needed to circulate many times in vessels. It is well-known that the erythrocyte spectrin is a highly differentiated protein for this purpose. Recently, the switching mechanism in various genes of membrane proteins and also protein-protein interactions have clarified by many molecular genetic approaches. So our goal is to visualize the membrane skeletal organization in vivo under various physiological conditions and during erythroid differentiation.

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