Cytoskeletal Reorganization of Human Platelets after Stimulation Revealed by the Quick-Freeze Deep-Etch Technique

Takao Nakata and Nobutaka Hirokawa
Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, Tokyo 113, Japan

Abstract. We studied the cytoskeletal reorganization of saponized human platelets after stimulation by using the quick-freeze deep-etch technique, and examined the localization of myosin in thrombin-treated platelets by immunocytochemistry at the electron microscopic level.

In unstimulated saponized platelets we observed cross-bridges between: adjoining microtubules, adjoining actin filaments, microtubules and actin filaments, and actin filaments and plasma membranes.

After activation with 1 U/ml thrombin for 3 min, massive arrays of actin filaments with mixed polarity were found in the cytoplasm. Two types of cross-bridges between actin filaments were observed: short cross-bridges (11 ± 2 nm), just like those observed in the resting platelets, and longer ones (22 ± 3 nm). Actin filaments were linked with the plasma membrane via fine short filaments and sometimes ended on the membrane. Actin filaments and microtubules frequently ran close to the membrane organelles. We also found that actin filaments were associated by end-on attachments with some organelles. Decoration with subfragment 1 of myosin revealed that all the actin filaments associated end-on with the membrane pointed away in their polarity.

Immunocytochemical study revealed that myosin was present in the saponin-extracted cytoskeleton after activation and that myosin was localized on the filamentous network. The results suggest that myosin forms a gel with actin filaments in activated platelets. Close associations between actin filaments and organelles in activated platelets suggests that contraction of this actomyosin gel could bring about the observed centralization of organelles.

PLATELETS, which play an important role in the maintenance of hemostasis have been a favorite subject for the study of cytoskeletal reorganization of actin filaments and their related proteins in nonmuscle cells because platelets undergo dynamic morphological changes from disks to irregular forms with several pseudopods and centralize their organelles after mechanical or chemical activation (23, 26). Platelets contain not only actin, myosin, and tubulin, but also actin-binding protein (ABP), α-actinin, vinculin, talin, and other actin-related proteins (8, 31, 32, 36, 39, 40). Recent studies also demonstrated the existence of microtubule-associated proteins (24, 38). Biochemical studies have demonstrated changes in some cytoskeletal components with increase of actin, myosin, and other proteins in the Triton-insoluble cytoskeletons after activation (2, 4–6, 23, 30), and immunofluorescence studies of redistribution of these contractile proteins have supported these ideas (8, 9, 36, 39, 40). Redistribution of several membrane proteins was studied at the electron microscopic level (27, 33, 41, 42).

A detailed observation of three-dimensional architecture in situ in both resting and activated platelets at the molecular level is necessary for a more complete understanding of the reorganization of the platelet cytoskeleton.

There have been a number of studies on the morphology of platelet cytoskeleton, using both the negative stain technique (13, 28, 29) or thin sections (10, 13, 44) with transmission electron microscopy and in whole mounts with high voltage electron microscopy (25, 26, 29) and scanning electron microscopy (43). Boyles et al. (3) described the cytoskeletal organization of resting platelets in detail in thin sections after a modified extraction and fixation with lysine. However, structural information on the relationship between filaments, and between filaments and membranes in the activated platelets, is lacking. In the conventional thin-section method it is quite difficult to reconstruct three-dimensional images, and in whole-mount cells the extensive overlap of the cytoplasmic constituents interferes with detailed observation. Triton models have been used for the study of platelet cytoskeletons in thin sections and in whole mounts. However, because the surface membrane and the membrane skeleton could not be well preserved, the relationship between membrane and cytoskeleton could not be analyzed.

In this study we carefully examined the cytoskeletal organization of unstimulated and thrombin-activated platelets, focusing on the interaction of filaments with each other and the relationship between membrane and cytoskeletons. We

1. Abbreviations used in this paper: ABP, actin-binding protein; SI, myosin subfragment 1.
used the quick-freeze deep-etch method, which allows us to observe three-dimensional cytoskeletal architecture with high resolution. Furthermore, by using the milder detergent saponin, we could examine membrane–cytoskeleton interactions. We also used taxol in an extraction buffer to preserve microtubules in order to study their related structures and their redistribution on activation.

We have identified actin and myosin, possible candidates for the main components of filamentous networks of saponin-extracted cells, by myosin subfragment 1 (S1) decoration and by immunocytochemistry (36). We studied the reorganization of these cytoskeletal components at the electron microscopic level, their distribution, and the polarity of actin filaments in the cytosol. Our results provide a detailed morphological basis for an understanding of the changes in the platelet cytoskeleton after thrombin activation.

Materials and Methods

Preparation of Platelets

Platelet-rich plasma (gift of Dr. Y. Shibata, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, Tokyo, Japan) was used within 2 d after blood was drawn from donors. The plasma was centrifuged at 2000 g for 5 min at 37°C and washed by resuspension in buffers that were preequilibrated to 37°C. The first two washes included 120 mM sodium chloride, 13 mM trisodium citrate, and 30 mM dextrose, pH 7.0. The third wash was with 154 mM sodium chloride, 10 mM Tris-HCl and 1 mM EDTA. After each wash platelets were isolated by centrifugation at 2000 g for 5 min at 37°C. The washed platelets were resuspended in a stabilizing buffer containing 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, and 1 mM EDTA, pH 7.4, and were incubated for 30–60 min at 37°C (11).

For the preparation of unstimulated discoid platelets we used stabilizing buffer containing 10 ng/ml prostacyclin (gift of Dr. Y. Shibata), again based on Fox et al. (11). For activation, platelets were incubated for 3 min with 1 U/ml human plasma thrombin (Sigma Chemical Co., St. Louis, MO) in stabilizing buffer at room temperature (23). Shapes of the platelets in these samples were examined by Nomarski differential interference contrast microscopy before further processing. Many of the platelets in the resting preparation were discoid with few filopodia. Activation by thrombin in the stabilizing buffer caused platelets to change shape and form spurs, but cell clumping was not apparent. These fresh samples of unstimulated and activated platelets were centrifuged at 2000 g for 3 min at room temperature and quick-frozen.

Chemical Permeabilization of Platelets

Small aliquots of suspensions of unstimulated and activated platelets were gently added to ~40 times volume of an extraction solution containing 0.02% saponin, 10 μM taxol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin in 0.1 M KCl, 30 mM Heps at pH 7.2, 5 mM MgCl₂, 2 mM EGTA (KHMe buffer), and incubated for 5 min at room temperature (18). The suspensions were centrifuged at 2000 g for 3 min at room temperature and the pellets were quick-frozen.

Decoration of Actin Filaments by S1

Myosin subfragment S1 was prepared from chicken skeletal muscle (35). Saponin-extracted platelets were incubated in 2 mg/ml of S1 in the KHMe buffer with 10 μM taxol, 1 mM PMSF, 10 μM leupeptin for 30–40 min at room temperature. They were washed in the same buffer and quick-frozen (17, 20).

Quick-Freeze, Freeze-Fracture, and Deep-Etch Methods

Samples were quick-frozen using a liquid helium–cooled machine as described previously (18, 18). The surfaces (<10 μm) of the frozen samples were fractured in a Balzers 301 (Balzers, Fürstentum, Liechtenstein) at −196°C at a vacuum of c<1–2 × 10⁻⁸ torr and deep-etched for 6–30 min at −95°C. Replicas were made by rotary shadowing with platinum and carbon. Tissues were dissolved in chromic sulfuric acid overnight and cleaned with distilled water. Replicas were examined at 100 kV with a JEOL 1200EX electron microscope.

Immunocytochemistry of Myosin in Saponin-extracted Cells

Saponin-extracted platelets were prefixed with 2% paraformaldehyde and 0.1% glutaraldehyde in the KHMe buffer for 1 h at room temperature. The samples were extracted further with 0.1% Triton in PBS for 30 min and then washed three times with 50 mM glycine in PBS. After blocking with 10% normal goat serum for 1 h at 4°C, they were incubated overnight at 4°C with 100-fold–diluted rabbit anti-human platelet myosin rod serum (kindly provided by Dr. K. Fujitawa, National Cardiovascular Center, Osaka, Japan) or preimmune serum. They were washed twice in PBS and once in Tris-buffered saline (TBS) at pH 8.2 with 0.1% normal goat serum, and further incubated with 20-fold– diluted 5 nm colloidal gold–labeled goat anti–rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium) in TBS with 1% normal goat serum overnight at 4°C. Then they were washed four times with TBS and postfixed with 3% glutaraldehyde, 0.1% tannic acid in TBS for 30 min. After osmification with 1% OsO₄ in 0.1 M phosphate buffer for 15 min on ice, the samples were stained with 0.5% uranyl acetate, dehydrated, and embedded in Epon 812 by the conventional method.

Results

Unstimulated Platelets

Submembranous Actin Filaments Have a Mixed Polarity in Unstimulated Platelets. Discoid-shaped cells 2–3 μm in diameter with many organelles randomly dispersed in the cytoplasm were observed in both fresh and saponin-extracted samples. The cytoplasm of fresh platelets was filled...
with abundant granular materials, probably soluble proteins, making it difficult to observe filamentous structures clearly. However, marginal microtubule bundles were recognizable (Fig. 1). A network of 9-nm filaments, on whose surface a 5-nm regular banding pattern was observed, was the main component of the cytoplasm of the saponin-treated platelets. Just inside the membrane were arrays of 9-nm filaments that had a tendency to run parallel to the membrane and to the circumferential microtubule bundles (Figs. 2 and 3f). These filaments were connected by fine short cross-links with an average length of 11 ± 2 (SD) nm and a diameter of 4–6 nm (Fig. 3a). These filaments also had lateral connections with the surface membrane (Fig. 3b and c). 9-nm filament bundles could be decorated with S1 and each filament showed a characteristic double helical structure, although the cross-links between the filaments were removed (Fig. 3d). A careful examination of the surface of the helix shows that gyri are somewhat angular; the asymmetry of each gyre, which appears flatter on one side than on the other, allows us to determine the polarity of the filaments (17, 21). As shown in Fig. 3d, the actin filaments within the bundles had a mixed polarity.

In unstimulated platelets the network of 9-nm filaments in the center of the cytoplasm was looser than in the submembranous area (Fig. 2). Relatively long filaments extended in all directions in the cytoplasm, which could also be decorated with S1 and were found to have a mixed polarity.

**Microtubules in Marginal Bands Are Cross-linked with Each Other and with Submembranous Actin Filament.**
The coilings of the microtubules in ~10 loops along the greatest circumference was observed in the discoid platelets. Some globular structures (11 nm in diameter) were attached to the surfaces of the microtubules, and cross-bridges 4–6-nm in diameter and 10.5 ± 2.5(SD) nm long linked microtubules (Figs. 1 and 3e). We also carefully examined the relationship between marginal microtubule bundles and submembranous actin filaments. Sometimes we found lateral connections between microtubules and actin filaments (Fig. 3f); however, the connections were irregularly spaced and infrequent compared with those between neurofilaments and microtubules in axons (19).

**Activated Platelets**

*Actin Forms a Gel of Mixed Polarity after Activation.* On activation with 1 U/ml thrombin for 3 min at room temperature, platelets changed their shape from disks to irregular forms (Fig. 4). In saponin-extracted cells, a network of 9-nm filaments was prominent throughout the cytoplasm surrounding membrane organelles (Fig. 5). Two types of arrange-
Figure 3. Higher magnification of the saponin-treated unstimulated platelets. (a) The arrows indicate some of the cross-bridges that interconnect actin filaments in the submembranous area. (b and c) Relationship between the plasma membrane and the actin filaments. Cross-bridges (arrows) link the actin filaments to the membrane. (d) SI-decorated submembranous actin filaments in unstimulated platelets. Actin filaments look like twisted rope just inside the plasma membrane. The polarity of the filaments is mixed (arrows). (e) Microtubule domain of saponin-treated discoid platelets. There are cross-bridges between microtubules (arrows), and granular materials (arrowheads) on the surface of the microtubules. (f) Sometimes we found cross-bridges between microtubules and actin filaments that had a tendency to run parallel to the marginal microtubule bundles in the submembranous area (arrows). Bars, 0.1 \( \mu \)m.

Figure 4. Untreated, quick-frozen, fresh, human platelet activated with 1 U/ml thrombin for 3 min. Single or small bundles of microtubules (arrows) dispersely enclose the membrane organelles. The cytoplasm is also filled with granular materials. Bar, 0.1 \( \mu \)m.

Some Actin Filaments Were Associated with the Membrane at Right Angles. In activated platelets, 9-nm filaments, which were associated with the surface membrane at one end and extended into the cytoplasm obliquely, were remarkably increased in number compared with unstimulated forms (Fig. 7, a and b). Some actin filaments were associated with...
the plasma membrane at right angles (Fig. 7 b). Cross-links between actin filaments and the membrane were also observed (Fig. 7, c and d).

SI decoration revealed that these filaments associated with plasma membrane at one end pointed away in their polarity (Fig. 7 f).

Two Types of Cross-Bridges Exist between Actin Filaments in Activated Platelets. Fine cross-links (11 \pm 2 nm) between actin filaments just like those observed in resting platelets (Fig. 3 a) were also found in the submembranous actin filament bundles after activation (Fig. 7 a). We also found other types of cross-bridges between actin filaments in the gel of actin filaments in the inner cytoplasm. These cross-bridges (22 \pm 3(SD) nm) were longer than those in the bundles of actin filaments beneath the plasma membrane (Fig. 7 e).

Actin Filaments Are Closely Associated with Membrane Organelles. On activation, membrane organelles accumulated, in many cases, at one side of the cell (12), facing the surface membrane across almost one layer of 9-nm filaments or microtubules. Many 9-nm filaments were closely associated with membrane organelles at one end, and some had a 5-nm banding pattern, suggesting that these were actin filaments (Fig. 8).

Myosin Localization at the Electron Microscopic Level
To examine the localization of myosin in activated platelets, we performed immunocytochemistry of platelet myosin in saponin-extracted cells. In activated platelets, myosin was present both in the filamentous network in the submembranous area and in the inner cytoplasm in saponin-extracted cytoskeletons, as indicated by clusters of gold particles (Fig. 9, a and b). Several gold particles were observed in the vicinity of the membrane organelles with which numerous 9-nm filaments associated (Fig. 9 a). In controls incubated with preimmune serum instead of the first antibody, very few gold particles were observed (Fig. 9 c).

Discussion
Reorganization of Microtubules and Their Related Structures after Activation
It is known that microtubules change their distribution on activation (7, 9, 28, 29). However, the precise details of their redistribution are still unclear. To answer these questions, we have carefully examined the relationship between microtubules and other cytoskeletal elements.
Our observations of both intact and saponin-extracted discoid platelets confirmed the existence of cross-bridges between microtubules (3), and we determined them to be 4–6 nm in diameter and 10.5 ± 2.5 nm in length. Lateral associations between microtubules may help to stabilize the marginal bands mechanically, which in turn may support the discoid shape of the unstimulated platelets. However, these cross-links were not the type of regularly repeating surface projections demonstrated in brain microtubules (19, 22). We also found granules on the surface of microtubules, with an average diameter of 11 nm, which might correspond to the observations of Kenney et al. (24) on isolated platelet marginal bands. The granular and rod-shaped structures shown in this study are possible candidates for the microtubule-associated proteins recently found biochemically (24, 38).

We found a few distinct connections between microtubules and actin filaments, both in unstimulated and activated platelets. This finding suggested the possibility that actin filaments could have a direct influence on the movement of microtubules via the cross-links and vice versa.

In activated platelets, microtubule bundles became either smaller fascicles or appeared as single microtubules. They were relatively bare on their surface, which suggested a shape change of the platelets to irregular forms due to the loss of cross-links between microtubules on activation. Some microtubules ran close to the membrane organelles; however, we could not find a close association between them. From this structural information it seems unlikely that microtubules play a positive role in the centralization of the granules. Microtubules in some pseudopods extend from the microtubules enclosing the membrane organelles, as observed in previous studies. However, these microtubules do not seem to have special structures for motility such as are observed in cilia or flagella.

Reorganization of Actin Filaments

In activated platelets, actin filaments increased remarkably in number. We observed two groups of actin filaments: one group formed bundles running parallel to the surface membrane in the submembranous area, and the other extended into the inner cytoplasm, forming a network of actin filaments in all directions.

Regarding the organization of actin filaments in activated platelets, we have discovered a number of new structural features. (a) Actin filaments are connected to membranes not only laterally but also by end-on attachments. These associations increased greatly in number on activation, which suggests the reorganization of actin-related proteins that connect actin filaments and the membrane (2). (b) Cross-links between actin filaments also exist after activation. Fine cross-linkers (11±2 nm long) just like the ones observed in unstimulated platelets were found in actin filament bundles in the submembranous area and in the pseudopods. Long fine bridges (22±3 nm long) cross-link actin filaments at various angles. These longer and shorter cross-links are good candidates for actin-related proteins, such as ABP (35) and α-actinin. ABP is a long thin rod with dimensions of 160 ×
3 nm (14) and it appears much shorter when it cross-links actin filaments in vitro (20-40 nm long) (15). The longer cross-links we observed agree in size and shape with ABP wrapping around actin filaments at their branching points in rotary shadowed preparation in vitro (15). The localization of the shorter filaments mainly in the subplasmalemmal area agrees with that of α-actinin in the platelets (37). Furthermore, the ladderlike cross-linking of submembranous actin filaments by the shorter cross-links was just like the α-actinin copolymerized with actin in shape (34), although the length of the cross-links in vitro was somewhat longer (~30 nm) in average. Further study is necessary for the identification of these cross-links. (c) Membrane organelles were associated with actin filaments oriented at right angles (Fig. 8) or laterally (Fig. 5). These associations are greatly increased in number after activation. As apparent from the shape of the membrane organelles, the membranes with which actin filaments tended to associate at their tips are those of granules, and the membranes which actin filaments subtended are those of the surface-connected canicular system. Sometimes two membranes faced each other across one layer of parallel actin filament bundles. Our finding that the cytoplasmic surfaces of both the plasma and the canicular membranes are surrounded with actin filament bundles agrees with observation on osmotically shocked platelets by Zucker-Franklin (44).

(d) Actin filaments have mixed polarity in the submem-
Figure 8. This micrograph shows the close associations between membrane organelles and 9-nm filaments (arrows) in activated platelets after saponization. As seen from their banding pattern, these filaments are actin filaments. Bar, 0.1 μm.

branous area and in the gel of the filaments, except in the pseudopods, where actin filaments all point away. (e) S1 decoration of the activated platelets suggests that actin filaments associate with the membranes at their barbed ends.

Our results on the unstimulated platelets agree with those of Boyles et al. (3) in general. Furthermore, we found that submembranous actin filament arrays had a tendency to run parallel to the marginal microtubules. By decorating with S1 we identified them as actin and demonstrated their mixed polarity, which might be important for the initiation of actomyosin motility. We also confirmed the existence of cross-bridges between actin filaments and between actin filaments and the plasma membrane.

The use of saponin as a detergent in this study made it possible to clearly preserve and visualize membrane–cytoskeleton associations. We also completely avoided the influence of chemical fixation by freezing the samples for replicas without fixatives. For these advantages we had to suffer the possibility of partial activation of the platelets in unstimulated preparation, although we used a calcium-chelated prostacyclin containing extraction buffer with a low concentration of saponin (0.02%). Even if it is the case, judging from the lens shape of the platelets with few filapodia, our unstimulated preparation may be at least in the very early stage of activation and is worthy of comparison with thrombin-activated preparation.

In S1 decoration of both unstimulated and activated platelets, cross-links between actin filaments were removed. It is reasonable to interpret this result to mean that the cross-links attached to actin filaments were competitively extracted with an excess of S1 of skeletal muscle (21).

Localization of Myosin

We performed immunocytochemistry of myosin in saponin-extracted activated platelets to answer two questions. Does myosin exist in the filamentous network we observed (Fig. 5)? If myosin exists, where is it present in the activated platelets?

We saponized the samples without fixation, just as in the preparation for quick-freezing, to see the influence of the permeabilization and found that many myosin molecules existed in saponin-extracted activated cytoskeletons, as shown biochemically in Triton-extracted cytoskeleton (23, 30).

In activated platelets, myosin was detected in the network of filaments. The distribution of myosin interspersed with ac-

Figure 9. Immunocytochemical localization of myosin in a saponin-extracted platelet that was exposed to polyclonal antibodies to human platelet myosin followed by immunogold-labeled second antibody. (a) Thrombin-stimulated platelets. Myosin molecules localize on the filamentous network in the cell body. Several gold particles and numerous filaments were observed in the vicinity of the membrane organelle in the lower right quarter. (b) A higher magnification of the filamentous network. 5-nm gold particles tend to form clusters. (c) Control-activated platelet that was incubated with preimmune serum. Only a few gold particles are identified. Bars, 0.1 μm.
tin filaments in mixed polarity in these areas strongly suggests contractile gel formation in activated platelets.

Although it is true that myosin exists in saponin-extracted platelets on activation (Fig. 9 a), we could not definitely identify myosin filaments in replicas (Fig. 5). A number of factors which make it difficult to identify myosin in quick-freeze deep-etch images can be considered. The size of myosin filaments in platelets are only 10–11 nm wide compared with a 9-nm wide actin filament (31). Furthermore the quantity of myosin is ~8–10% that of actin (31), which makes it difficult to identify myosin filaments in tremendous networks of fibrils (Fig. 5). In SI-decorated platelets almost all the filaments were decorated with SI (Fig. 6). However, this does not always mean that myosin exists in nonfilamentous form. Platelet myosin could be extracted with the excess of SI. Clustering of the gold labels suggested that myosin did not exist in one molecule but in oligomers, which in turn suggested that myosin formed filaments in the network (Fig. 9 a).

Some possible models have been proposed for the mechanism of actin–myosin interaction that could produce organelle centralization (7). In our results, actin filaments formed parallel bundles on the surface of the surface-connecting canicular system, which can play the role of a rail. On the other hand, a number of filaments are associated with granules by their tips. So there is the other possibility that membrane organelles can move on the rail of actin filaments with the help of myosin motors (l). Concerning this model, we can consider two ways in which myosin molecules may be involved in membrane organelle centralization. (a) Myosin filaments may slide between the rail of actin filaments and the other actin filaments associated with the membrane organelles, and thus membrane organelles could be transported to the center of the cell. (b) Myosin molecules are attached to the membrane organelles in some way, and these myosin–membrane organelles complexes move along the rail of actin filaments. Microtubules did not seem to have a close relation with actomyosin gels.

Further identification of each actin-related protein at the electron microscopic level based on these morphological data is expected to elucidate the mechanism of motility of the human platelets after activation.

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