Platelet Activation and Cytoskeletal Reorganization: High Voltage Electron Microscopic Examination of Intact and Triton-extracted Whole Mounts

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ABSTRACT The sequential changes in the three-dimensional organization of the filamentous components of human platelets following surface activation were investigated in whole-mount preparations. Examination of intact and Triton-extracted platelets by high voltage electron microscopy provides morphological evidence of increased polymerization of actin into the filamentous form and an increased organization of the cytoskeletal elements after activation. The structure of resting platelets consists of the circumferential band of microtubules and a small number of microfilaments randomly arranged throughout a dense cytoplasmic matrix. Increased spreading is accompanied by cytoskeletal reorganization resulting in the development of distinct ultrastructural zones including the peripheral web, the outer filamentous zone, the "trabecular-like" inner filamentous zone, and the granulomere. These zones are present only in well-spread platelets during the late stages of surface activation and are retained following Triton extraction. Extraction of the less stable cytoplasmic components provides additional information about the underlying structure and filament interactions within each zone.

The morphological transformation that accompanies platelet activation has been attributed to alterations involving both the distribution and the degree of polymerization of the various contractile and structural proteins within platelets. Contractile proteins have been implicated in many of the events of activation including centralization and secretion of granules, aggregation, and clot retraction (1, 7, 8, 35, 48). Recent biochemical studies of thrombin-induced platelet activation have demonstrated a time-dependent incorporation of actin into the filamentous form and an increase in the amount of myosin associated with the cytoskeleton (5, 20, 37). The organization of these platelet contractile proteins, particularly actin and myosin, has been studied at the light microscopic level through immunocytochemical techniques (9, 11, 12, 33, 35). These studies have indicated that there are temporal changes in the whole-cell distribution of actin and myosin within platelets. While they are initially uniformly distributed, actin and myosin are segregated to selective areas as spreading progresses. Other studies employing transmission electron microscopy (TEM)[1] have yielded considerable information regarding the types of filaments within platelets and overall platelet structure (22, 23, 42, 44–48). Only a very small part of the platelet cytoskeleton is visible in conventional TEM thin sections hindering an accurate determination of the overall three-dimensional organization and in situ relationships of the filamentous cytoskeletal elements. Whole-mount preparations have therefore been increasingly utilized to study platelet ultrastructure (2, 22, 23, 25, 26, 29, 42). However, masking of the cytoskeletal elements by the extensive overlap of other cytoplasmic constituents in intact whole-mount preparations can interfere with the analysis of the interaction of the filamentous elements with each other or with other intracellular structures. Studies with cultured cells

[1] *Abbreviations used in this paper:* HVEM, high voltage electron microscopy; PHEM buffer, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9; TEM, transmission electron microscopy.

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(4, 16, 32, 39, 43) have established that extraction of living cells with nonionic detergents such as Triton X-100 will remove the less stable cytoplasmic components while leaving behind a cytoskeleton consisting of three major filament types: microtubules, microfilaments, and intermediate filaments. Application of extraction procedures has proved beneficial for TEM visualization of the platelet cytoskeleton in whole-mount preparations (20, 26, 30, 31, 34). The purpose of the present study was to utilize extraction techniques on platelet whole-mount preparations in conjunction with high voltage electron microscopy (HVEM) to document the sequential changes in the three-dimensional organization of the filamentous components of the cytoskeleton following activation. A comparison of intact and Triton-extracted whole mounts has permitted the examination of the structural relationships between the different filamentous elements and has provided a more definitive ultrastructural model of platelets.

MATERIALS AND METHODS

Platelet Preparation: Platelets were obtained from normal healthy adult volunteers by venous puncture. Blood samples (10 ml) were collected in polypropylene tubes containing 10 mM EGTA and mixed by gentle inversion. Platelet-rich plasma was prepared by centrifugation of whole blood at 180 *g* for 10 min. Platelets were separated from plasma proteins by passage through a Sepharose CL-4B column (41) having a 40-ml bed volume. The column was equilibrated at room temperature with a calcium-free Tyrodes buffer, pH 7.3 (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgCl_2 \cdot 6H_2O$, 1 g/l dextrose, and 2 g/l albumin). Platelets were collected in the void volume, deposited on Formvar-coated Ni maxtiform grids, and allowed to settle and adhere at 37°C in a moist chamber.

Extraction and Cytoskeleton Preparation: The extraction protocol developed by Schliwa and van Blerkom (39) has provided the best structural preservation in our platelet system. The extraction buffer PHEM has the following composition: 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9.

Well-spread platelets were washed briefly with PHEM then lysed with 0.15% Triton X-100 in PHEM buffer for 15 s to 1 min. Optimal results were obtained at 45 s with grids being gently agitated during the extraction process. After extraction, grids were washed well with buffer and fixed in PHEM-buffered 1% glutaraldehyde, 0.2% tannic acid for 30 min at room temperature.

To examine platelet cytoskeletons in the early stages of activation, gel filtered platelets were deposited on polylysine-coated Formvar grids. Platelets were allowed to adhere for 1–5 min then lysed with Triton in PHEM buffer. Specimens were washed well with PHEM and fixed as above. The use of polylysine was necessary to ensure adherence during short incubations. The coated grids were prepared by placing a drop of freshly prepared 0.1% poly-L-lysine (90,000 mol wt, Sigma Chemical Co., St. Louis, MO) on the grid for 5 min. The grids were washed well with distilled water and used immediately.

Nonextracted Whole Mounts: Whole mounts of unstimulated platelets were prepared by collecting an equal volume of gel-filtered platelets directly off the column into 0.2% glutaraldehyde. Briefly fixed platelets were deposited on polylysine-coated grids, washed with protein-free buffer, and fixed with 0.1 M HEPES-buffered 1% glutaraldehyde, 0.2% tannic acid, 0.05% saponin (27), pH 7.2 for 30 min at room temperature. Well-spread forms were obtained after 30 min adherence on Formvar grids and fixed as above.

Electron Microscopy: Subsequent to glutaraldehyde fixation, all preparations were postfixed in 0.1 M HEPES-buffered 0.05% OsO_4 for 20 min, stained in 1% uranyl acetate (aqueous), and dehydrated through a graded series of alcohol to absolute ethanol, itself dried by storage over a molecular sieve. Samples were dried by the critical-point method in a critical-point dryer equipped with an in-line molecular sieve filter and a hydrophobic water-excluding microporous filter to remove trace H_2O from liquid CO_2. Samples were evaporatively coated with a thin layer of carbon and stored over a molecular sieve until examined with the AEI·EM.7 1 MeV electron microscope of the Madison HVEM Facility (University of Wisconsin). Stereo pair micrographs were taken at tilt angles appropriate for specimen thickness and magnification (18).

RESULTS

The general structural characteristics of unstimulated platelets are illustrated in Fig. 1. Platelets at this stage possess an overall discoid shape 2–3 μm diam and lack microspikes or pseudopodia. Fine hair-like projections are present on the glycocalyx which composes the platelet external surface. The circumferential microtubular band is present in the peripheral margin. The interior contains numerous organelles including various types of granules, mitochondria, and the internal membraneous systems. A few strand-like structures can occasionally be seen but identification is difficult against the cytoplasmic matrix. Long, ordered microfilaments are not present.

In the initial stages of spreading after adhesion, platelets

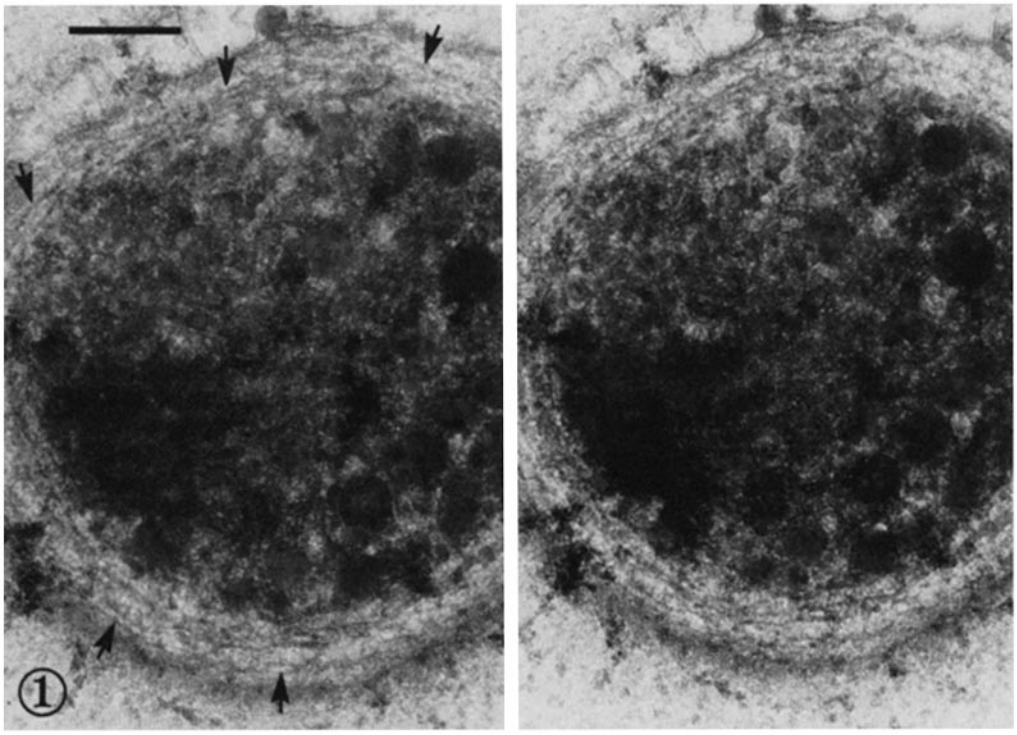

FIGURE 1 HVEM stereo pair whole mount. Representative unstimulated, nonextracted platelet. Platelet possesses characteristic discoid shape and lacks pseudopodia. The circumferential microtubular band (arrows) is present at the cell margin. Numerous organelles are seen against the dense cytoplasmic matrix. Bar, 0.5 μm. × 29,787.

undergo a transformation from the discoid shape to a predominately spherical shape with short pseudopodia (Fig. 2). The predominant structural feature is the circumferential microtubular band which is visible in the peripheral margins of platelets. The cytoplasm in the interior of platelets contains oval to spherical granules amidst a granular-appearing background material. Microfilaments, 5–7.5 nm diam, are present as a randomly arranged, loosely intertwined network throughout the interior and as closely packed longitudinal arrays composing the core of the developing pseudopodia.

As spreading proceeds, further changes in the organization of the filamentous elements become apparent (Fig. 3). Extension of the cytoplasm beyond the circumferential band after 2–5-min adherence produces more stellate-shaped platelets. Increased spreading is accompanied by the appearance of a more extensive array of microfilaments throughout the cytoplasm of the entire platelet. The filaments are present within prominent microfilament bundles which radiate from the center out into the pseudopodia as well as discrete filaments within a net-like arrangement throughout the cell body. The

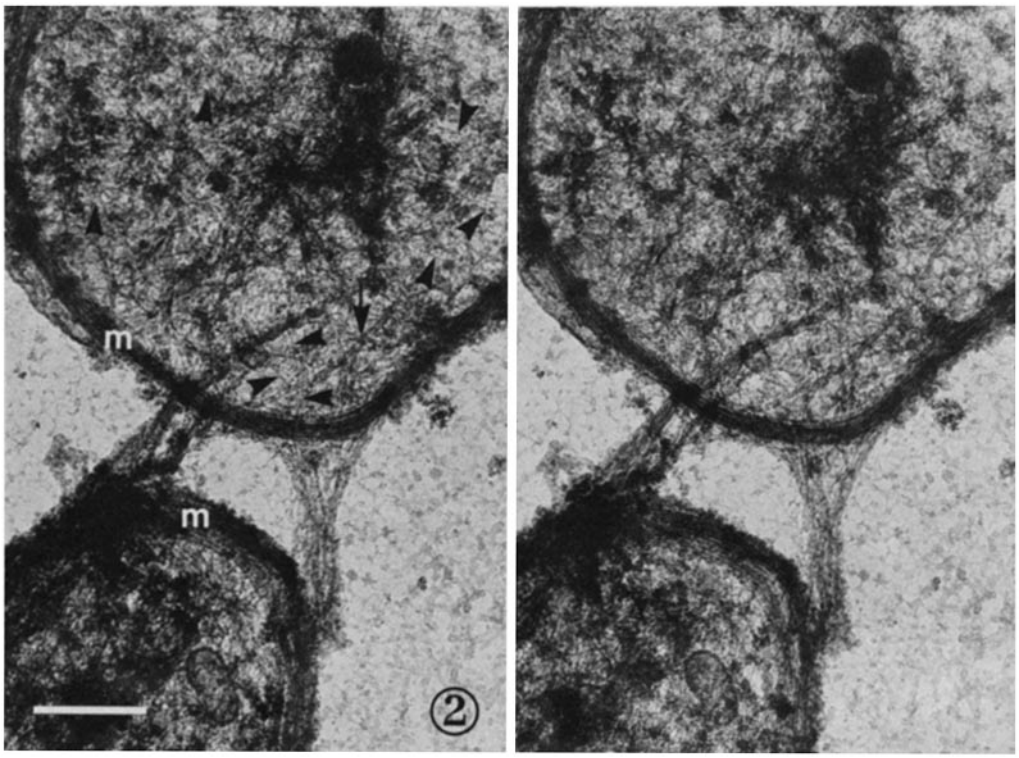

FIGURE 2 HVEM stereo pair whole mount. These platelets, after mild detergent extraction, show the overall spherical shape and short pseudopodia characteristic of the initial stages of spreading. The microtubular coil (*m*) remains at the periphery. A free end (arrow) winds into the cytoplasm. Microfilaments (arrowheads) form a loose network in the interior and extend out into the pseudopodia. Bar, 0.5 μm. × 30,600.

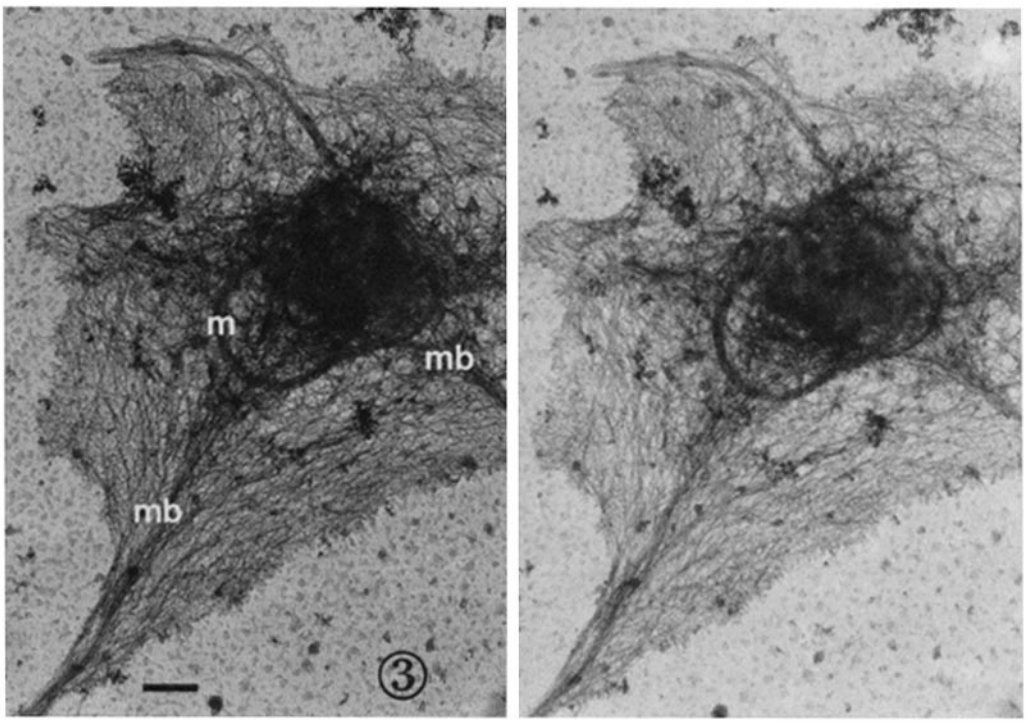

FIGURE 3 HVEM stereo pair whole mount. Extracted platelet in an intermediate stage of spreading. The microtubular coil (*m*) has been retained but is no longer circular. Increased numbers of microfilaments form a network throughout the interior. Prominent microfilament bundles (*mb*) radiate out into the pseudopodia. Bar, 0.5 μm. × 14,956.

central regions are occupied by tightly clustered electron-opaque granules which may be encompassed by the microtubular coil. If present, the coil is often no longer circular. The examination of many platelets during this stage reveals that the coil is retained in some and totally absent in others, indicative of a rapid depolymerization of microtubules. Retention of the band at this stage may be due in part to the interaction of microtubules themselves, or the interaction of a negatively charged material that surrounds them, (48) with the polylysine substrate, thus preventing microtubule dissociation and reorientation which Lewis et al. (24) have shown to normally follow activation. The intracellular redistribution of Ca^{++} associated with platelet shape change may play a role in this process (19, 21).

The Triton-insoluble cytoskeleton of well-spread platelets is composed entirely of a branched filamentous network (Fig. 4). In contrast to platelets in the earlier stages, the structure of the cytoskeleton can be divided into four major domains which correlate well with the four structurally distinct zones within unextracted whole-mount preparations (Fig. 5). The terminal web in nonextracted platelets is composed of a densely packed meshwork of fine filaments. This arrangement is seen more clearly in the cytoskeleton preparations. Microtubules are absent from this zone, and microfilaments are the predominant filament type. Interior to the terminal web is the outer filamentous zone with its more loosely interwoven array of filaments. Two major classes of filaments can be differentiated in this zone: microfilaments and, occasionally, microtubules 19–21 nm in diameter. All filaments are discrete and uniform in diameter throughout their length. Numerous associations between filaments are visible throughout the region at higher magnification (Fig. 6). The associations are of two major types: side-to-side where filaments cross over each other or make parallel lateral contacts, and Y-shaped branch points due to end-to-side associations or the divergence of two apposed filaments. Free ends of filaments are occasionally seen. In contrast to the outer filamentous zone, the adjacent inner filamentous zone has a "trabecular-like" appearance in unextracted whole mounts. Filaments appear anastomotic and variable in diameter. The course and interactions of individual filaments is difficult to determine. Triton-extracted preparations demonstrate the underlying cytoskeletal structure of this inner filamentous zone. As opposed to the appearance of unextracted preparations, it is evident that the underlying structure of the inner filamentous zone is a densely packed, overlapping network of discrete filaments. Most filaments are orientated parallel to the long axis of the platelet but bend to completely encircle the granulomere region. There are numerous associations between filaments within the zone and with other filaments that extend into the inner zone from the outer filamentous zone. The granulomere region of unextracted platelets is composed of dense granules, electron lucent granules, and mitochondria suspended within a microfilament net (Fig. 5). Many of these organelles are extracted during Triton treatment. Consequently, while several granules may remain in the granulomere zone of extracted platelets, this zone often appears relatively empty when compared with the granulomere of unextracted whole mounts. Randomly arranged microfilaments originating in the inner filamentous zone crisscross the region; any unextracted granules remain within this network.

DISCUSSION

The formation of actin filaments from unpolymerized precursors after activation of platelets has been proposed as a result of recent investigations (13, 31). TEM examination of thin sections reveals very few actin filaments in unstimulated platelets; however, although individual filaments can be seen, this method does not permit optimal examination of the entire cytoskeleton. Nachmias and co-workers (29, 31), using a technique of rapid lysis and negative staining, examined

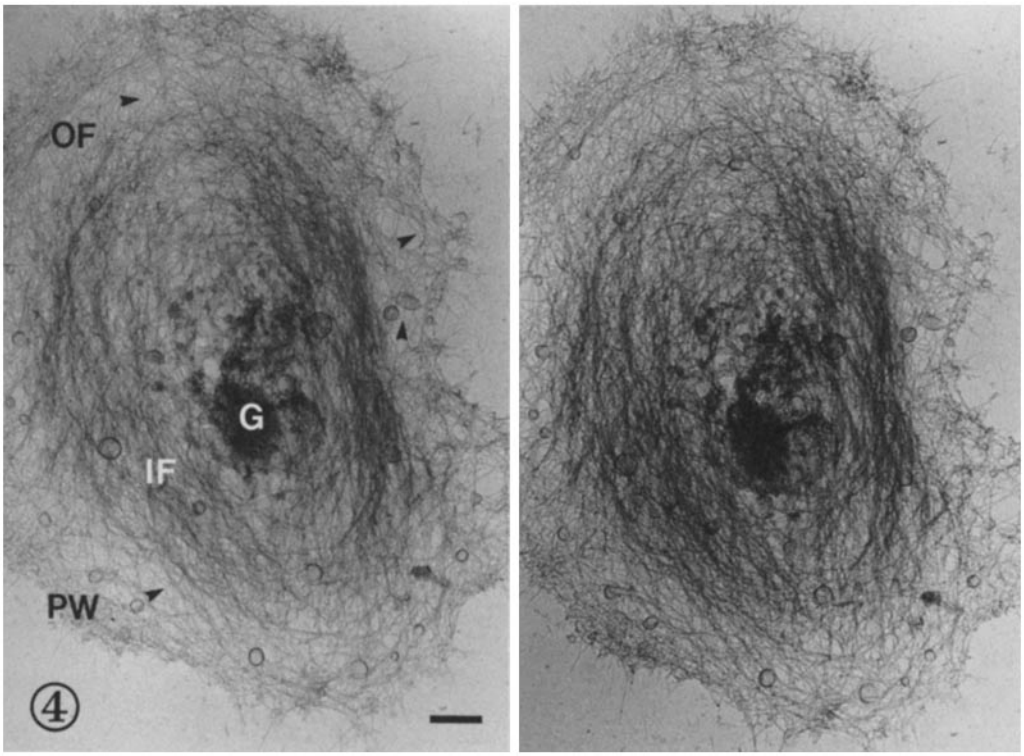

FIGURE 4 HVEM stereo pair whole mount. Triton-insoluble cytoskeleton of a well-spread platelet. The cytoskeletal components of the four structurally distinct zones that characterize this stage of activation are retained following extraction. These are the peripheral web (*PW*), the outer filamentous zone (*OF*), the inner filamentous zone (*IF*), and the granulomere (*G*). Numerous filament associations (arrowheads) are present throughout the outer filamentous zone. The inner filamentous zone consists of densely packed filaments which encircle the granulomere. Bar, 0.5 μm. × 15,108.

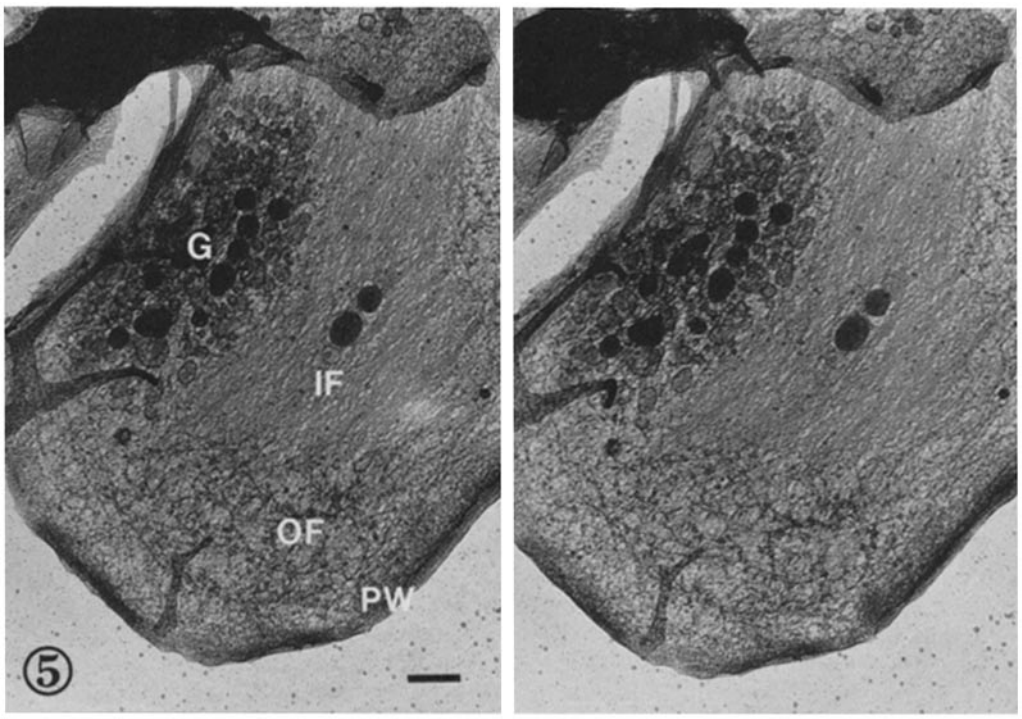

FIGURE 5 HVEM stereo pair whole mount. Nonextracted platelet at the same stage of spreading as the extracted platelet in Fig. 4. Note the dense filamentous meshwork of the peripheral web (*PW*), the outer filamentous zone (*OF*), the inner filamentous zone (*IF*), and the granulomere (*G*). Bar, 0.5 μm. × 14,000.

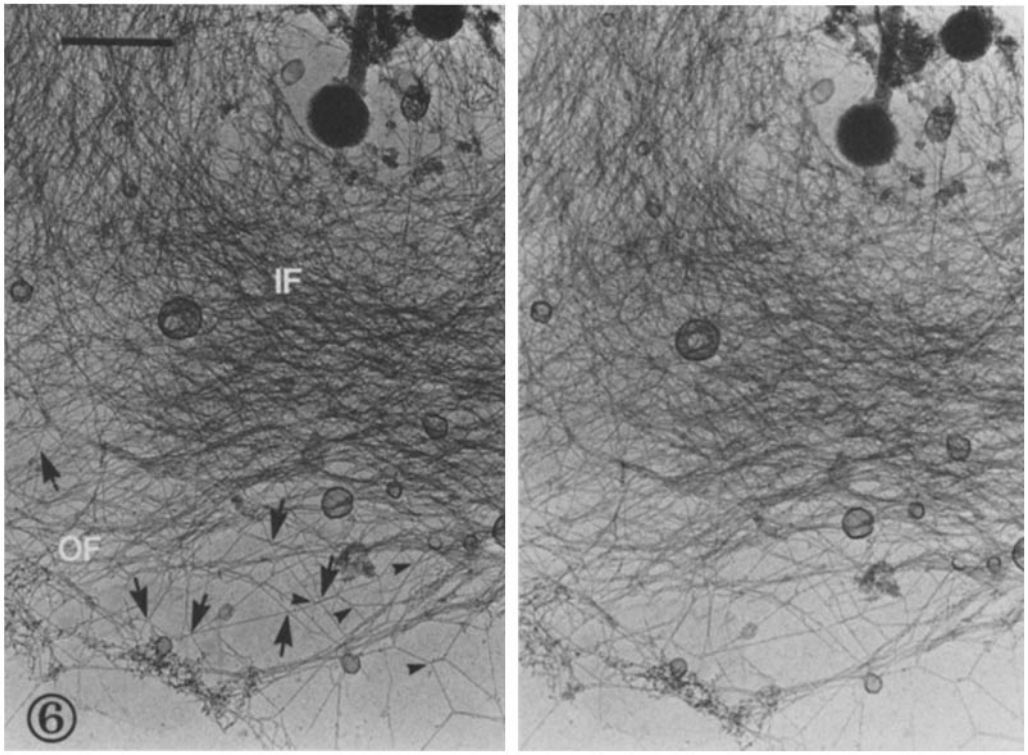

FIGURE 6 HVEM stereo pair whole mount of detergent-extracted, well-spread platelet. The closely packed discrete filaments of the inner filamentous zone (*IF*) are seen more clearly. Filament association resulting in Y-shaped branch points (arrowheads) are apparent in the outer filamentous zone (*OF*). Filament crossover points (arrows) can also be identified in the outer zone. Bar, 0.5 μm. × 30,000.

whole-mount preparations and found the cytoskeletal structure of resting platelets to consist of the circumferential microtubular coil which encompasses a granular, amorphous material thought to represent, in part, actin filament precursors. Few microfilaments were seen. HVEM examination of unstimulated platelets, characterized by a discoid shape and lack a pseudopodia, reveals the microtubular band in the cell margin and numerous organelles scattered diffusely throughout the cytoplasm as has been described by others (22, 26, 44, 46, 47). Microfilaments are not apparent in micrographs of platelets during this stage. However, very short actin filaments are difficult to visualize and could appear globular rather than filamentous. Biochemical assays have indicated that filamentous actin is present in populations of unstimulated platelets, but the estimates of the amount in the filamentous form vary from 31 to 50% (5, 6, 20). The assay provides no information on the length of actin filaments and is very sensitive to the buffers and method of platelet preparation. Thus, if the biochemical findings are correct with respect to the amount of actin in the filamentous form in unactivated platelets, the

microscopical data would suggest that this actin is in the form of extremely short filaments. These would be difficult to differentiate from other globular elements that compose the cytoplasmic matrix. However, it is also apparent that very early in the activation process a rapid conversion of G- to F-actin occurs. Microscopically, substantial numbers of well-developed actin filaments are observed in cytoskeletal preparations of extremely early stages in the spreading sequence. Therefore, care must be exercised to be certain a population of "unactivated" platelets is indeed comprised of totally unactivated forms.

After activation, platelets undergo a characteristic shape change. The cytoskeleton preparations illustrate that shape change is accompanied by the appearance of increased numbers of filaments, which supports the conclusions of Carlsson et al. (5) and Jennings et al. (20). Our findings demonstrate that the filaments also become more highly organized during the course of activation. Filament arrays in the early stages of activation are randomly organized while those in latter stages are more structured. Associations between filaments are numerous in well-spread platelets but are rarely seen in platelets in the early stages of activation. Centralization of granules, which has been suggested to be associated with a contraction of the circumferential band (48), becomes more pronounced as activation proceeds.

Well-spread surface-activated platelets possess an ordered ultrastructure which can be divided into four major zones including the peripheral web, the outer filamentous zone, the trabecular-like inner filamentous zone, and the granulomere zone (2). These four structurally distinct zones are retained following Triton extraction. The peripheral web is composed of a tightly woven meshwork of microfilaments which is in agreement with the distribution of actin in thrombin-stimulated platelets reported by Painter and Ginsberg (33). This web is similar if not identical to the ruffle seen at the margin or leading edge of nucleated cells. Microtubules are not present in this zone. The adjacent outer filamentous zone is composed of microfilaments and microtubules arranged in a less dense array. In platelets in which tubules are present, filament-to-filament and filament-to-tubule associations are seen in this region. End-to-side actin filament associations resulting in Y- or T-shaped branches, which we observe to be prominent in the outer filamentous zone, have been described in preparations of purified actin (14, 15) as well as in whole nucleated cells (39). The significance of these filament interactions in platelet functions such as granule centralization and secretion or clot retraction remains to be evaluated; however, it has been shown that in platelets (3, 28) as well as in cultured cell systems (15, 39, 40), actin filaments all possess the same polarity wth respect to the cell body. Using heavy meromyosin subfragment-1 decoration, the arrowheads are directed towards the cell body and branch points while the polymerizing ends are located at the periphery or at the tips of pseudopodia. This orientation would enable coordinated force generation subsequent to the binding of bipolar myosin to adjacent actin filaments. While the bulk of platelet myosin has been localized to the platelet body (35), Painter and Ginsberg (33) have demonstrated that there is an actual redistribution of myosin during activation with myosin segregating centrally in a ring surrounding the granulomere as activation proceeds. Debus et al. (9) showed a similar distribution pattern with myosin located in pseudopodia during the dendritic stage but enriched in an area encircling the granulomere in the fully spread form. While we were unable to specifically identify individual myosin filaments in our preparation, the distribution of myosin described above would localize it within the inner filamentous zone.

The inner filamentous zone is the largest of the four zones present in well-spread platelets composing 60–70% of the cytoplasm. This zone is the only one that appeared significantly different in the extracted platelet preparations than it did in the unextracted, intact platelets. The appearance of this zone in nonextracted whole mounts is similar to the irregular three-dimensional microtrabecular lattice observed in whole mounts of cultured cells (49) and neurons (10). Schliwa et al. (38) have proposed that this appearance is possibly due to the fact that any cytoplasmic proteins are structure associated. This is based on their observation of a differential response of the microtubecular lattice to the type of detergent, Brij 58 vs. Triton X-100, employed in extraction. The very close packing of filaments in this zone, visible in the cytoskeleton preparations, may therefore contribute to the appearance of this zone in the nonextracted whole mounts. The exact nature of this structure remains controversial (17, 36).

Although the four major zones we have described are present in adherent platelets, distinct zones have also been observed in platelets that have been stimulated and fixed while in suspension. In such platelets, White (47), using conventional TEM thin sections, has described a sol–gel zone surrounding an organelle zone. This latter zone is analogous to the granulomere zone of the surface-activated platelets. The relationship of the sol–gel zone, described as the matrix of the platelet cytoplasm containing several fiber systems in various stages of polymerization, to the inner and outer filamentous zones is less precise.

We have provided morphological evidence that, together with previously reported biochemical evidence, suggests net polymerization of actin into the filamentous form and a reorganization of the platelet cytoskeleton during platelet activation. The Triton-insoluble cytoskeleton has provided additional insight into the underlying structure and the filament associations within the four structurally distinct zones seen in nonextracted surface-activated platelets. A number of areas require further investigation. These include a determination of the amount of actin in the nonfilamentous form in unstimulated platelets, the mechanism and significance of the disappearance of the microtubular band, and the localization of myosin with respect to microfilaments during the various stages of spreading. Elucidation of the nature of the changes in the structural organization of the filamentous cytoskeleton is required to understand the possible functional correlates of these changes such as granule centralization, granule release, and clot retraction.

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