Morphometry of Platelet Internal Contraction

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Blood platelets have a characteristic discoid shape supported by a circumferential band of microtubules. Following stimulation by aggregating agents or foreign surfaces, platelets lose their discoid form, extend pseudopods, and undergo a process of internal reorganization. Randomly dispersed cytoplasmic organelles become concentrated in cell centers within rings of microtubules and masses of microfilaments. Questions have been raised about this process and its contractile nature by studies demonstrating that platelet microtubules dissolve within seconds after activation and reassemble several minutes later in new locations. Earlier investigations showed that Taxol, a microtubule-stabilizing agent, did not inhibit platelet shape change, internal transformation, secretion, aggregation, or clot retraction. In the present study the diameters of microtubule coils in discoid platelets treated or not treated with Taxol and in platelets activated by thrombin, ADP, and a foreign surface were measured. The results of the study reveal no significant differences in diameters of microtuble rings in control or Taxol-treated cells. However, after activation by ADP, thrombin, or the grid surface, the diameter of coiled microtubules decreased by 30% or more. The results support the concept that internal transformation is a contractile event. (Am J Pathol 1984, 115:412–417)

PLATELETS in circulating blood have a characteristic discoid form.[1] The lentiform appearance is supported by a coiled microtubule lying just under the cell membrane along its greatest circumference.[2–5] Aggregating agents cause platelets to lose their discoid shape, become irregular with multiple pseudopods, and develop internal reorganization.[6] Randomly dispersed organelles become compressed together in cell centers and encircled by close-fitting rings of microtubules and masses of microfilaments.[7,8]

Constriction of peripheral microtubule bands into tight coils in platelet centers has been considered a manifestation of internal contraction.[9–11] Some workers, however, have suggested that the physical changes observed in the position of microtubules are not related to contraction.[12,13] Their studies have shown that microtubules dissolve almost completely within seconds after platelets are stimulated and reassemble 1–5 minutes later in new positions. Such a process would not require internal contraction for relocation of microtubules.

Recently we used the microtubule stabilizing agent Taxol[14–17] to reexamine this question. Taxol prevented disassembly of platelet microtubules by cold[18] and antimitotic agents, such as vincristine.[19] However, Taxol did not inhibit platelet shape change, pseudopod formation, internal reorganization, secretion, aggregation, or clot retraction.[20] The present study has measured the diameters of microtubule rings in control and activated Taxol-treated and untreated platelets. Under conditions in which microtubules are protected from disassembly, activation by ADP, thrombin, and foreign surfaces regularly caused reductions in the diameters of the microtubule coils.

Materials and Methods

General

Blood for this study was obtained from normal donors after informed consent was given. Samples extracted by venipuncture were mixed immediately with citrate–citric acid–dextrose, pH 6.5 (93 mM sodium citrate, 70 mM citric acid, and 140 mM dextrose) in a ratio of 9 parts blood to 1 part anticoagulant.[7,21] Platelet-rich plasma (C-PRP) was separated from whole blood by centrifugation at 100*g*.

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Reagents

Taxol was obtained from the National Products Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. The agent was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM. A volume of 10 μl added to 1 ml of C-PRP yielded a final concentration of 10^{-4} M Taxol and 1.0% DMSO. Adenosine diphosphate (ADP) and thrombin were made up as stock solutions for regular use in platelet aggregation studies and kept frozen between experiments.[7,21,22]

Experimental Studies

Samples of C-PRP were combined with a final concentration of 10^{-5} M ADP or thrombin in the amount of 0.2 U/ml, inverted once, and allowed to stand without stirring. The procedure allows platelets to undergo shape change without aggregating.[22] Surface activation was accomplished by allowing drops of C-PRP to settle on carbon-stabilized, Formvar-coated grids for 30 or 60 minutes. Previous studies have shown that all stages of platelet physical alteration are present after exposure to grids for these periods of time.[23]

Preparation of Thin Sections

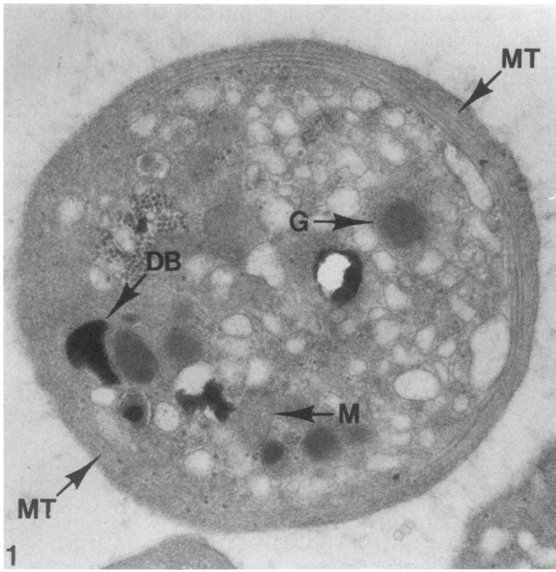
Control and experimental samples were prepared for study in the electron microscope according to methods reported in detail in previous publications.[7,18-22] Samples combined with ADP or thrombin, inverted once, and allowed to stand without stirring were fixed at 1 and 3 minutes after exposure to thrombin and 2 and 5 minutes after addition of ADP. Briefly, samples were combined with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3 (a 10% solution of a 1:1 mixture of 1) 2.4 M NaCl, 0.1 M KCl, 46 mM $MgSO_4$, 64 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 2) 0.13 M $NaHCO_3$, 8.4 mM $NaH_2PO_4 \cdot 7H_2O$, 3.8 mM anhydrous KH_2PO_4, and 0.1 g/l phenol red). After 15 minutes at 37 C, the samples were sedimented to pellets, and the supernatant was discarded and replaced with 3% glutaraldehyde in the same buffer. Fixation was continued at 4 C for 60 minutes. The cells were then washed in buffer and combined with 1% osmic acid in veronal acetate (0.02 N HCl, a 20% stock solution containing 0.14 M sodium barbital and 0.145 M sodium acetate, and a 6.8% solution of a stock solution containing 1.7 M NaCl and 18 mM $CaCl_2$). After exposure to the second fixative for 1 hour the cells were dehydrated in a graded series of alcohol and embedded in Epon 812. Contrast of thin sections cut from plastic blocks on an ultramicrotome was enhanced with uranyl acetate and lead citrate. Observations were made in a Philips 301 electron microscope.

Preparation of Whole Mounts

Samples of C-PRP were prepared at 30 or 60 minutes for ultrastructural study after negative staining according to the method of Small.[24] The Triton X-100 glutaraldehyde mixture consisted of 0.5% triton X-100 and 0.25% glutaraldehyde. Detergent extraction and fixation were carried out at room temperature. Grids carrying spread platelets were washed briefly in Tris-buffered saline followed by a cytoskeleton buffer (NaCl, 127 mM; KCl, 5 mM; Na_2HPO_4, 1.1 mM; KH_2PO_4, 0.4 mM; Na HCO_3, 4 mM; glucose, 5.5 mM; $MgCl_2$, 2 mM; EGTA, 2 mM; PIPES, 5 mM; ph 6.0–6.1). After washing in the cytoskeletal buffer the cells were transferred to the Triton X-100 glutaraldehyde mixture for 1 minute. After a brief wash in cytoskeleton buffer, the grids were stored on coverslips on the same buffer containing 2.5% glutaraldehyde for 2 hours before negative staining for electron microscopy. Staining in sodium silicotungstate was carried out at room temperature. Grids were rinsed two times in distilled water and transferred sequentially through 4 drops of bacitracin (40 mg/ml in water; Sigma Chemical Co., St. Louis, Mo) in a plastic Petri dish and drained briefly on the edge with filter paper. They were then passed through four drops of 3% sodium silicotungstate, finally drained of excess stain, and allowed to air-dry.

Evaluation of Microtubule Constriction

Thin sections of control and activated platelets were photographed at the same magnification. Whole mounts were also recorded on film at the same microscope settings. Diameters of microtubule rings were measured with calipers and expressed in centimeters. A minimum of 30 control, ADP and thrombin-activated platelets with and without Taxol were measured and recorded. No differences in shape change induced by ADP at 2 and 5 minutes or thrombin at 1 and 3 minutes were observed. Therefore, the data were pooled. A similar number of control and Taxol-treated discoid, dendritic, and spread platelets activated by contact with grid surfaces were photographed and analyzed. Catalase crystals with a lattice spacing of 81.2 Å were photographed at the same on-scope magnification on each strip of 35-mm film containing 40 pictures of platelets. During the course of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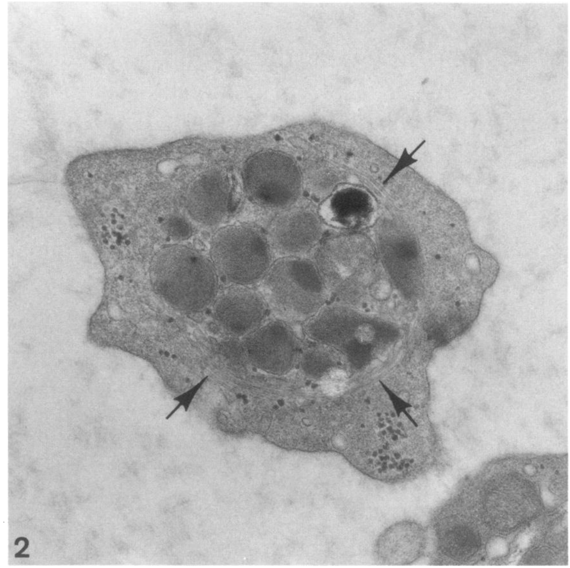
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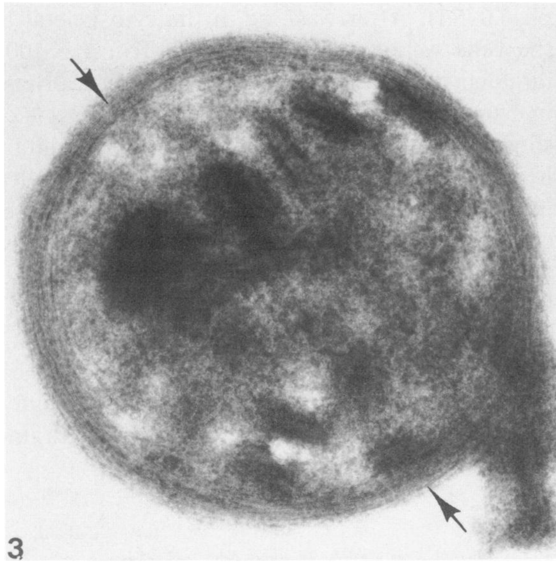
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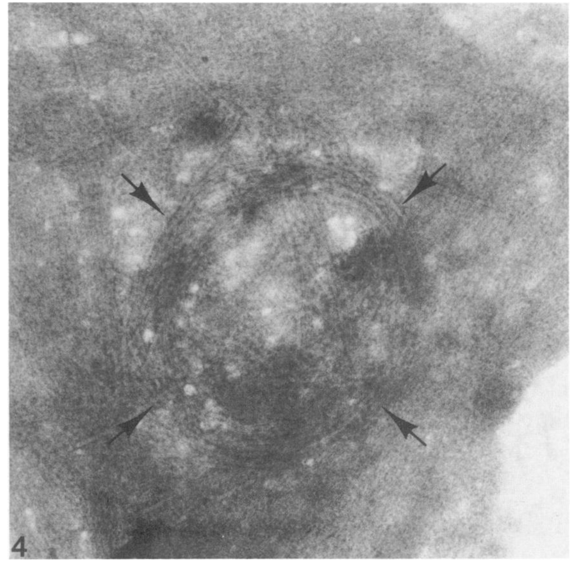
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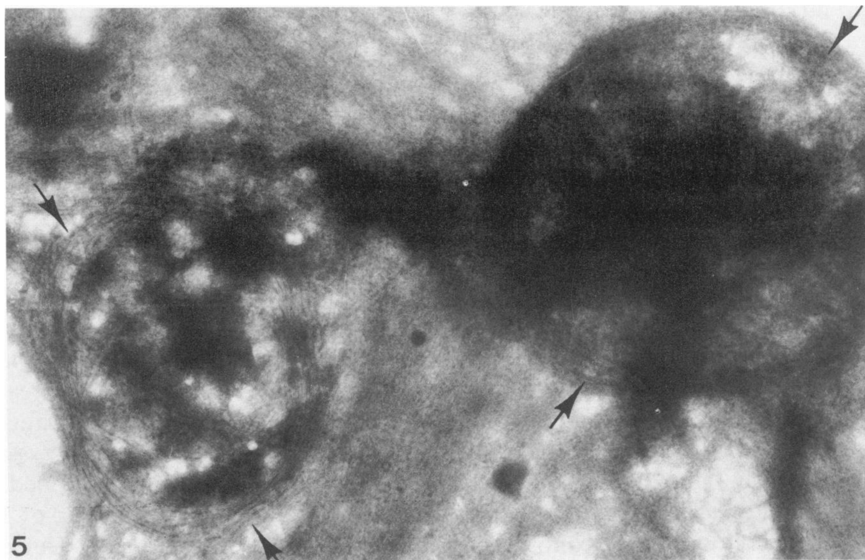
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these investigations no significant change at 4200 or 5500 on-scope magnifications was detected in the lattice spacing of catalase. Means and standard deviations derived from measurements of microtubule coil diameters and the statistical significance were determined with the use of the Student *t* test.

Results

Thin Sections

Many previous investigations have shown that the methods used here to collect blood, separate C-PRP, and fix cells for electron microscopy maintain the discoid form of most platelets.[7,8,18-22] It was not difficult, therefore to find platelets sectioned sagittally or in the equatorial plane (Figure 1). Groups of hollow circular profiles were identified at the polar ends of platelets in sagittal section. Microtubules were evident as a circumferential band under the cell membrane of platelets cut in the equatorial plane. Measurement of coil diameters was made from the outermost microtubule in thin sections and in whole mounts. Incubation in Taxol appeared to improve the discoid appearance of platelets, although it did not increase the frequency of coils present in the band.[18-20] The mean diameter of microtubule coils in platelets incubated with Taxol was slightly greater than that of untreated platelets. However, the difference was not significant (Table 1).

Platelets incubated with 0.2 U of thrombin or 10^{-5} M ADP revealed a variety of shape changes. Those chosen for measurement were platelets in which the organelles were closely apposed in the central zone of the cell (Figure 2). Platelets demonstrating this degree of internal transformation were evident at all time intervals with both agents. Occasional small 3–4-platelet aggregates and fibrin strands were evident in thrombin-stimulated samples. For the most part, however, the cells remained single. Taxol treatment had no apparent influence on internal transformation. Just as many cells with organelles concentrated in cell centers and enveloped by tight rings of microtubules and microfilaments were evident in Taxol-treated cells stimulated by agonists as in control platelets after exposure to thrombin and ADP.[20] Diameters of microtubule rings were considerably reduced in activated platelets whether or not they were treated first with Taxol (Table 1). The degree of reduction compared to mean diameters in unstimulated platelets varied considerably but was always significant.

Whole Mounts

Contact with carbon stabilized Formvar-coated grids caused platelets to undergo marked physical changes.[25-27] The cells first become dendritic in appearance and then spread out into thin films. After 30 minutes the frequency of discoid platelets, early and late dendritic types, and spread forms becomes essentially stable. Microtubules were well preserved in the contact-activated platelets after fixation and simultaneous detergent extraction, negative staining, and air-drying. Circumferential bands were present at the periphery of all discoid platelets that could be penetrated by the 80–100-kv electron beam (Figure 3). Coils were also present in most dendritic platelets and in about 25% of spread platelets (Figure 4). The frequency of coils was higher in Taxol-treated spread platelets than in untreated control cells, but the difference varied from sample to sample. Mean diameters of microtubule circles in control and Taxol-treated spread platelets were significantly less than circumferential bands in discoid platelets (Figure 5), with an average reduction of greater than 30% (Table 1).

Discussion

The present investigation has examined the fate of the circumferential band of microtubules in discoid platelets following activation by thrombin, ADP, and foreign surfaces. Results of the study demonstrate that microtubule coils undergo significant reductions in diameter after stimulation. Thin sections of ADP-treated platelets revealed decreases of over 40%. After

←

Figure 1 – Discoid control platelet fixed after incubation with 10^{-4} M Taxol for 30 minutes. The cell has been sectioned in the equatorial plane. A circumferential bundle of microtubules (*MT*) lying under the surface membrane supports the lentiform appearance. Numerous granules (*G*), a few mitochondria (*M*), and occasional dense bodies (*DB*) are randomly dispersed in the cytoplasm. This platelet was photographed at the same magnification and on the same roll of film as the cell in Figure 2. (×24,000) **Figure 2** – Platelet from a sample of PRP incubated with Taxol (10^{-4} M) for 30 minutes, stimulated by exposure without stirring to thrombin (0.2 U/ml), and fixed for electron microscopy after 1 minute. The cell is irregular in form, and organelles are concentrated in the central area within a tight-fitting ring of microtubules (↑). Compared with the platelet photographed at the same magnification in Figure 1, the diameter of the microtubule ring in this cell is considerably reduced. (×24,000) **Figure 3** – Platelet from a sample of PRP incubated with 10^{-4} M Taxol for 30 minutes and exposed to a carbon-coated Formvar grid for a similar period. The cell was prepared by simultaneous fixation and detergent extraction followed by negative staining. A circumferential microtubule (↑) supports the discoid form of the cell, which was photographed at the same magnification on the same roll of film as the platelet in Figure 4. (×22,000) **Figure 4** – Fully spread control platelet prepared in the same manner as the cell in Figure 3. The diameter of the microtubule coil (↑) is considerably reduced compared to the discoid cell in the previous figure. (×22,000) **Figure 5** – Negative stain whole mount preparation. Discoid platelets are frequently deposited on or near fully spread cells. The differences in diameter of microtubule coils (↑) in the discoid platelet and fully spread cell is clearly apparent. (×21,000)

Table 1 – Morphometry of Platelet Internal Contraction

	n*	x̄†	s‡	p§	%‖
Thin Sections					
No Taxol					
Control	34	13.0	2.65	–	–
ADP	41	7.8	1.11	< 0.001	45.5
Thrombin	42	8.55	1.85	< 0.001	34.2
Taxol					
Control	32	13.92	2.61	–	–
ADP	41	7.60	1.28	< 0.001	45.4
Thrombin	43	9.55	2.03	< 0.001	28.5
Whole Mounts					
No Taxol					
Control	33	10.80	1.49	–	–
Activated	30	7.10	1.05	< 0.001	34.2
Taxol					
Control	32	10.75	1.37	–	–
Activated	42	7.35	1.73	< 0.001	31.6

* Number evaluated.
† Mean.
‡ Standard deviation.
§ Significance of differences from control.
‖ Percent reduction from control.
The diameters of the microtubule rings were measured in thin sections of control and Taxol (10^{-4} M)-treated discoid platelets and in treated and untreated platelets after activation by thrombin (0.2 U/ml) or ADP (10^{-5} M). Differences between discoid cells incubated with Taxol and those not exposed to this agent were not significant. However, microtubule coils in ADP- or thrombin-activated platelets, whether or not they were preincubated with Taxol, were constricted in comparison with those in discoid cells. The reduction in the diameters of microtubule coils was greater than 30%. Similar reductions were observed in platelets after surface activation on carbon-stabilized Formvar grids. There were no differences in control cells as a result of treatment with Taxol. Surface stimulation, however, resulted in at least a 30% reduction in diameters of microtubule coils in spread platelets, compared with discoid control cells.

exposure to thrombin reductions in diameters of microtubule coils, compared with control cells, averaged about 30%. Platelets activated by contact with carbon coated grids revealed similar changes. Rings of microtubules were narrowed by over 30%.

The suggestion that the circumferential band of microtubules is constricted into tighter coils in activated platelets was made several years ago[7] and confirmed in many subsequent investigations.[8-11,18-22] Light-microscopic studies have supported the results obtained at the ultrastructural level.[28] Immunofluorescence microscopy employing an antibody specific for tubulin revealed the marginal band as a bright fluorescent ring in discoid platelets. At the time of pseudopod formation during activation on glass, the diameters of fluorescent rings were decreased. Evidence that microtubule rings dissolve following activation was not recorded in the light-microscopic investigation.

Biochemical studies employing colchicine binding assays, however, have indicated that microtubules become disassembled shortly after platelet stimulation by many different agents.[12,13] Within 1–4 minutes the microtubules repolymerize in new locations. Morphologic evidence supporting the concept of disassembly–reassembly was provided in one report.[13] Any need for a contractile process to force constriction of the circumferential band of microtubules would be obviated if this concept were correct.

The introduction of Taxol,[14-17] a potent microtubule-stabilizing agent, made it possible to respond to the questions raised to earlier work by the concept of disassembly–reassembly of microtubules in activated platelets.[12,13] Previous reports employing this agent demonstrated that Taxol prevents dissociation of microtubules in platelets exposed to cold or antimitotic agents.[18,19] Stabilization of platelet microtubules by Taxol did not prevent shape change, pseudopod formation, internal reorganization, or any other aspect of platelet physical or functional activity.[20] The studies demonstrated that disassembly of microtubules was not required for any phase of platelet activation.

The present investigation has confirmed and extended the earlier observations. Comparison of the diameters of microtubule coils in discoid and activated platelets has revealed significant reductions. Constriction of the rings was observed in platelets stimulated by ADP, thrombin, and foreign surfaces. The narrowing was observed whether or not the platelets were treated first with Taxol. However, the finding that diameters of microtubule coils decreased to the same extent in Taxol-treated platelets following activation as in untreated cells provides assurance that disassembly is not required and probably does not occur.

The mechanism involved in constriction of the microtubule coil in activated platelets is not yet clear. Most of the actin in unstimulated platelets is in an unassembled or globular form.[25,29] Activation results in rapid polymerization of actin filaments and their organization into bundles and a random gel.[30] Griffith and Pollard[31] have shown that mixtures of microtubules having microtubule-associated proteins (MAPs) with actin filaments have very high viscosities compared with the viscosities of the separate components. The authors suggest that actin filaments anchored to microtubules in the presence of MAPs may be responsible for some vital microtubule movements within cells. Close association of these constitutents in activated platelets may provide the tension necessary for internal transformation and for winding the peripheral microtubule into a tighter coil.

In conclusion, the present study has demonstrated that the circumferential microtubule in discoid platelets becomes wound more tightly in activated cells. Taxol was used in this study to prevent disassembly of

platelet microtubules. Since microtubules are not contractile elements, it is reasonable to suggest that they are acted upon by a contractile process that coils peripheral microtubules into tight rings around centrally concentrated organelles in activated platelets.

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