Microtubule Coils in Spread Blood Platelets

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The fate of the circumferential bundle of microtubules in activated platelets has been a subject of disagreement. Thin sections of stimulated platelets fixed at multiple intervals following exposure to aggregating agents have revealed that the circumferential band is constricted into a tight ring around centrally concentrated organelles. However, studies of detergent-resistant platelet cytoskeletons fixed and either negatively stained or critical point dried after activation on polylysine-coated grids have revealed that microtubule rings disappear, leaving only fragments in the peripheral cytoplasm of spread cells. The present study has employed immunofluorescence on glass slides and the whole mount technique with detergent extraction and either negative staining or critical point drying to evaluate the fate of microtubules in surface-activated platelets treated with or without the microtubule stabilizing agent, taxol. Significant numbers of microtubule coils were visible in control and taxol-treated platelets stained indirectly with a fluorescein-coupled antibody to tubulin 30 to 60 minutes after surface activation on glass. Coils of microtubules were also visible in dendritic forms and in significant numbers of spread platelets on negatively stained or critical point dried whole mounts in the electron microscope. The findings support the concept that microtubule disassembly is not an integral step in early phases of platelet activation.

A COILED MICROTUBULE forming a circumferential band lies just under the cell membrane, along its greatest circumference, in resting platelets.1,2 The important role of the microtubule bundle in supporting the discoid shape of platelets is well established.3 Following exposure to aggregating agents, platelets lose their lentiform appearance and become irregularly spherical with multiple pseudopods.4 The fate of the peripheral band of tubules in stimulated cells has been a subject of debate.

Examination of thin sections of plastic-embedded platelets fixed at intervals after activation revealed that circumferential bands of microtubules were constricted into tight rings around centrally concentrated organelles.5 Recent investigations employing taxol, a microtubule-stabilizing agent, have supported this earlier concept.6 Immunofluorescence investigations have also demonstrated that microtubule coils do not disassemble during early phases of platelet activation.7 However, biochemical studies have suggested that the microtubules disassemble almost completely in platelets after stimulation and reassemble in new locations one to four minutes later.8,9 Morphological support for this proposal has come mainly from examination of detergent-resistant cytoskeletons in whole mount preparations following negative staining or critical point drying.10-14 Only fragments of microtubules were found in fully spread platelets, and coils of microtubules were rarely noted, except in very early stages of shape change.

The purpose of the present study was to examine the fate of microtubules in spread platelets after surface activation. Samples of washed platelets were allowed to settle on glass slides for one to 60 minutes and were then stained indirectly with fluorescein-conjugated antibody to tubulin. Drops of platelet-rich plasma were fixed and simultaneously extracted with detergent before negative staining or critical point drying, after contact with grid surfaces for intervals of one to 60 minutes. Both taxol-treated and untreated control platelets were examined. Remnants of the circumferential bundle of microtubules were present in most platelets, whether or not they had been treated with taxol, and microtubule coils were commonly found in fully spread cells.

MATERIALS AND METHODS

General

After informed consent, blood was obtained from healthy donors whose platelets had been evaluated in previous studies from our laboratory.3 Samples obtained by venipuncture were mixed immediately with citrate–citric acid, pH 6.5, in a ratio of nine parts blood to one part anticoagulant. Platelet-rich plasma (C-PRP) was separated from whole blood by centrifugation at 100 g for 20 minutes at 23 °C and maintained at room temperature until used in specific experiments.

Immunofluorescence7

Samples of C-PRP were mixed with an equal volume of the citrate anticoagulant and centrifuged to pellets. The platelets were resuspended in phosphate-buffered saline (PBS; NaCl, 8 g; KCl 0.2 g; KH₂PO₄, 0.2 g; Na₂HPO₄, 1.15 g/L adjusted to pH 7.2 with 1.0 mol/L NaOH). Twenty microliters of washed platelets was placed on each of a number of polylysine-coated glass slides, which were inserted in a moist chamber to settle at room temperature. At intervals of 1, 5, 15, 30, and 60 minutes, slides were fixed in metha-

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nol at -10°C for ten minutes followed by brief immersions (three times for three minutes each) in acetone at -10°C. A stock monoclonal antibody to β-tubulin (Amersham, Arlington Heights, Ill) was diluted to a concentration of 0.01 mg/mL in PBS with 1% bovine serum albumin (BSA). Slides were washed once with PBS, and the platelet spots covered with 20 to 40 µL of the antitubulin antibody. After 30 minutes, the slides were washed with PBS and the spots covered with a fluorescein-conjugated antimouse globulin antibody (Cappel, Cochranville, Pa). Following incubation for another 30 minutes, the slides were washed well with PBS and mounted under coverslips in a solution of p-phenylene diamine–PBS–glycerin, after the method of Johnson et al. The slides were studied under phase-contrast and phase-fluorescence in a Zeiss photomicroscope equipped with an ultraviolet power source and appropriate excitation and barrier filters. Platelets were observed and photographed through epifluorescence optics and 60x and 100x planapo objectives. In some experiments, samples of platelet-rich plasma were incubated for 30 minutes with 10⁻⁴ mol/L taxol (see below) before washing and resuspension in PBS containing 10⁻⁴ mol/L taxol.

Detergent-Extracted Cytoskeletons

**Critical point drying.** The method employed by Mattson and Zuickes was followed closely. Platelets were allowed to settle and spread on formvar-coated carbon-stabilized copper grids that had been pretreated with 0.1% polylysin. A drop of C-PRP was placed on a grid, and the platelets allowed to settle out for 1, 5, 15, 30, or 60 minutes in a moist chamber. The grids were then washed by immersion three times in PBS. Extraction and fixation were accomplished simultaneously. Grids were placed on drops of 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.2, containing 0.5% Nonidet for 15 minutes. They were then transferred to 2.5% glutaraldehyde without detergent for an additional 15 minutes. The grids were then rinsed in distilled water and dehydrated in a graded series of alcohols. They were then transferred to freon and critical point dried by the method of Anderson.

**Negative staining.** Additional samples of detergent-extracted platelets were prepared at 1, 5, 15, 30, or 60 minutes for ultrastructural study after negative staining, according to the method of Small as modified from Hoglund et al. 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 mmol/L; KCl, 5 mmol/L; Na₂HPO₄, 1.1 mmol/L; KH₂PO₄, 0.4 mmol/L; NaHCO₃, 4 mmol/L; glucose, 5.5 mmol/L; MgCl₂, 2 mmol/L; EGTA, 2 mmol/L; PIPES, 5 mmol/L; pH 6.0-6.1). After washing in the cytoskeleton buffer, the cells were transferred to the Triton X-100–glutaraldehyde mixture for one minute. Following a brief wash in cytoskeleton buffer, the grids were stored on coverslips on the same buffer containing 2.5% glutaraldehyde for two hours before negative staining for electron microscopy. Staining in sodium silicotungstate was carried out at room temperature. Grids were rinsed twice in distilled water and transferred sequentially through four drops of bacitracin (40 mg/mL in water; Sigma Chemical Co, St Louis) 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 and then finally drained of excess stain and allowed to air dry.

**Taxol**

Several published reports have suggested that microtubules are disassembled during the spreading of platelets on grids. Therefore, taxol, used in the present study to stabilize microtubules, was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md. The agent was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mmol/L. A volume of 10 µL added to 1 mL of platelet-rich plasma yields a final concentration of 10⁻⁴ mol/L taxol and 1.0% DMSO. Samples of C-PRP were incubated with taxol at a concentration of 10⁻⁴ mmol/L for 30 minutes. Subsequently, drops of treated platelets were mounted on grids for the same time intervals as untreated cells and then prepared for ultrastructural study after either critical point drying or negative staining.

**Frequency of Various Shapes and Microtubule Coils in Spread Platelets**

Whole grid squares were photographed at low magnification in the transmission electron microscope. Usually two to three grid squares provided enough platelets to determine the percentages of different forms in at least 100 cells. The frequency of microtubule coils was determined in higher magnification photographs of spread platelets in the same grid squares and as many additional squares as necessary to reach at least 100 cells. Seventeen different blood samples from ten donors were used in the present investigation.

**RESULTS**

**Immunofluorescence**

Many platelets were attached to polylysine-coated glass slides after one minute of incubation, and most were discoid in appearance. At five and 15 minutes, more platelets appeared to be attached than at one minute, and many had spread out on the grid surface. Examination under ultraviolet illumination revealed uniform fluorescent rings in all platelets at the 1-, 5-, and 15-minute intervals. At 30 minutes of exposure to polylysine-coated glass, few platelets remained discoid. Nearly all had spread into thin films with centrally concentrated masses of granules. Bright fluorescent rings were evident in nearly all platelets when examined under ultraviolet illumination (Fig 1A). Prior incubation with 10⁻⁴ mol/L taxol did not appear to influence spreading or persistence of fluorescent rings (Fig 1B). One hour after exposure to surface activation, fewer platelets remained adherent than at 30 minutes. All had spread into thin films with centrally concentrated granules. Fluorescent rings were still present in some platelets (Fig 1C). In many, the fluorescence resembled a spiral or had a radiating spoke-like appearance. Other platelets had a reticular fluorescence pattern or no definable organization. Control and taxol-treated platelets revealed similar changes (Fig 1D).

**Ultrastructure**

The fine structure of the detergent-resistant platelet cytoskeleton in whole mounts prepared for study by negative staining or critical point drying has been described previously. A small proportion of platelets adhere to the grid without losing their discoid
Fig 1. Indirect immunofluorescence of microtubules in surface-activated platelets. Examples B and D are from samples of C-PRP incubated with 10⁻⁴ mol/L taxol for 30 minutes before washing and resuspension in PBS containing 10⁻⁴ mol/L taxol. Platelets in A and B were allowed to settle on polylysine-coated glass slides for 30 minutes. In C and D, the cells were in contact with glass for one hour before fixation. Most of the platelets at both time intervals were spread. Brightly stained microtubule coils are present in nearly all control and taxol-treated platelets at 30 minutes (A and B). One hour after exposure to glass, some cells still contain fluorescent rings (C and D). In many, the ring has transformed into a spiral or stellate configuration, and on others, has become a reticular network. Very few platelets manifest a diffuse pattern with no cytoplasmic organization. (Mag x 260).

Fig 2. Ultrastructure of spread platelets fixed and simultaneously extracted in detergent one hour after surface activation and prepared by critical point drying. Most of the cells in this example are late dendritic or spread forms. Residual coils of microtubules are apparent in three cells (†), and substantial remains of dissociated coils or fragments of microtubule rings can be identified in most of the other cells. (Mag x 4,500).
Fig 3. Discoid platelet from a sample of C-PRP fixed with glutaraldehyde and simultaneously extracted with detergent one minute after contact activation on a polylysine-coated grid. The discoid cells are relatively resistant to penetration by the electron beam. In this example, however, a clear zone in the cytoplasm permits visualization of granules (G) and elements of the circumferential band of microtubules (1) at the cell border. (Mag x 14,300).

Figs 4 through 8. Dendritic forms prepared 15 to 30 minutes after contact with grid surfaces. Microtubules (1) encircle centrally concentrated organelles. Bands of microfilaments (MF), as in Fig 6, but not microtubules, extend into pseudopods. (Mag: Fig 6 x 6,500; Fig 7 x 8,450; Fig 8 x 8,450).

remnants of the circumferential band were evident in various arrangements. Coiled microtubules were found in 12% to 16% of spread platelets.

Negatively stained cytoskeletons. Platelets negatively stained after simultaneous fixation and detergent extraction appeared better maintained than critical point dried cells. Actin microfilaments were particularly well preserved. Microtubules were present in practically every platelet. Circumferential bands were evident in discoid cells (Fig 15). Microtubule coils and fractured fragments were present in dendritic forms (Figs 16 through 20). Coils of microtubules were

Figs 4 and 5. Early dendritic forms fixed and extracted with detergent five minutes after contact with the grid surface and subsequently dried by the critical point method. Platelets are still relatively opaque to the conventional electron beam at this stage, but circumferential bands of microtubules are evident in both cells peripheral to cytoplasmic organelles. (Mag: Fig 4 x 9,750; Fig 5 x 8,450).
Figs 9 through 14. Spread platelets from samples of C-PRP fixed and extracted at 15 to 60 minutes after contact with grid surfaces. Coils of microtubules (†) are evident in the central region of each cell. Bands of microfilaments are dispersed in a variety of configurations. (Mag × 6.175).

Fig 15. Discoid platelet from a drop of C-PRP fixed and detergent-extracted one minute after contact with a grid and then negatively stained. The circumferential band of microtubules (†) forms the margin of the cell. (Mag × 6.175).

Fig 16. An early stage of platelet activation. Cytoplasm extends beyond the bundle of microtubules (†). Microfilaments (MF) are prominent in the exterior and interior of the cell. (Mag × 10.725).
Figs 17 through 20. Early and late dendritic platelets fixed and extracted 5 to 60 minutes after contact with polylysine-coated grids and then negatively stained. Coils of microtubules (1) are apparent in central regions of the cytoplasm. The cell in Fig 18 is from C-PRP incubated with 10^{-4} mol/L taxol before exposure to the grid surface for 30 minutes. (Mag: Fig 17 ×13,975; Fig 18 × 10,400; Fig 19 × 7,800; Fig 20 × 10,725).

found in 38% of fully spread cells (Figs 21 through 26).

The reason for persistence of more microtubule coils in immunofluorescent preparations and negatively stained whole mounts than in critical point dried platelets is uncertain. However, examination at high magnification (Figs 27 and 28) suggests that the critical point drying procedure may cause more damage to microtubules and actin filaments than negative staining.18

Effects of Taxol

Previous studies have shown that taxol stabilizes microtubules20,21 and prevents their disassembly by conditions such as exposure to antimitotic agents22 or low temperature,23 which ordinarily remove all microtubules from platelets. It was employed in the present study to prevent the loss of microtubules during surface activation. Coils of microtubules were frequently observed in platelets incubated with taxol at 10^{-4} mol/L before detergent extraction and critical point drying or negative staining (Figs 20 and 26). However, just as many microtubule coils were observed in spread platelet preparations that had not been exposed to taxol.

DISCUSSION

The present study has shown that coils of microtubules are present in a significant proportion of fully spread platelets after exposure to grids for up to one hour. Microtubule coils were evident in 12% to 16% of spread platelets after whole mounting, fixation, and simultaneous detergent extraction, postfixation, dehydration, and critical point drying. This procedure is generally thought to protect delicate cell structures and to be ideal for preserving detergent-extracted cytoskeletons.24 However, Small18 has reported that the steps involved in fixation and dehydration for critical point drying cause significant damage to filamentous elements and may have contributed to the injury of microtubule coils in spread platelets.

Coils of microtubules were also present in 38% of negatively stained, detergent-resistant platelet cytoskeletons. The forces exerted at the air-fluid interface during air drying of grids can seriously damage delicate cellular elements.16 Fixation in glutaraldehyde first does not appear to prevent the injury. Therefore, it is surprising that the filamentous elements and microtubules of spread platelets prepared by the negative stain procedure survived better than after critical point drying.18

Other investigators have had difficulty in identifying coils of microtubules in spread platelets. In studies of platelet cytoskeletons at rest and after spreading, Nachmias9,10,25 often observed straight fragments of microtubules, but no coils. In a recent review of the subject,25,26 Nachmias noted that considerable extension of the cytoplasm could occur in cells exposed to grids for a few seconds, while several coils of the microtubule remained intact. When platelets were
Figs 21 through 26. Spread forms from drops of C-PRP fixed and extracted 15 to 60 minutes after contact with grids. Coils of microtubules are evident in all of the cells. In some examples, such as Fig 24, the coil may have fractured during air drying. The platelets in Fig 26 are from a sample of C-PRP incubated with 10⁻⁴ mol/L taxol before placing on the grid for 30 minutes. (Mag: Fig 21 x6,825; Fig 22 x14,300; Fig 23 x10,400; Fig 24 x13,000; Fig 25 x10,400; Fig 26 x10,400).

Fig 27. Coil of microtubules in spread platelet fixed and simultaneously extracted with detergent before critical point drying 60 minutes after exposure to the grid. Although the coil itself is distinct, individual tubules appear to have fused. Actin filaments also appear to have melted into a sheet. Although the changes in this cell appear extreme, they are not unusual in platelets after detergent extraction and critical point drying. (Mag x 19,500).
allowed to spread more fully, the granular cytoplasm consisted entirely of a delicate network of interlacing fibrils. The breakdown of microtubule coils in platelets stimulated in suspension typically occurred at about the same time as formation of microfilaments.

Mattson and Zuiches studied the detergent-resistant cytoskeletons of platelets fixed and critical point dried two hours after contact with grid surfaces.\textsuperscript{12,13} They observed single microtubules coursing in a curved fashion through the periphery of fully spread platelets. Coils of microtubules were rarely, if ever, observed. Lewis and associates\textsuperscript{14} have recently used the whole mount technique, employing fixation and critical point drying without detergent extraction, to evaluate the fate of microtubules during adhesion and the release reaction. The coiled microtubule of the resting cell was disassembled upon cell activation and then reassembled in patterns that conformed to cell shape. As a result, microtubules were either radially arranged or in parallel bundles in the long axis of spread cells. However, diffuse circular arrangements were observed in some flattened platelets that had reached the terminal adhesion state.

Taxol was used in the present study to stabilize microtubules. Previous investigations have shown that taxol can prevent disassembly of platelet microtubules after exposure to antimitotic agents or low temperature, which uniformly remove all tubular elements from the cells.\textsuperscript{22,23} However, taxol does not prevent disassembly of microtubules in platelets lysed in suspension by exposure to cocaine or Triton X-100 (data not presented). Coils of microtubules were clearly visible in detergent-resistant cytoskeletons of taxol-treated dendritic and spread platelets. However, coils were no more frequent in taxol-treated spread platelets than in those that had not been treated, possibly because some spread platelets are in a prelytic state. The failure of Mattson and Zuiches\textsuperscript{12,13} to identify microtubule coils in spread platelets fixed at two hours may also be a reflection of overexposure to the grid, resulting in irreversible damage. A stage of microtubule disassembly, followed by reassembly, observed by Lewis et al\textsuperscript{14} was not apparent in taxol-treated or untreated platelets in the present investigation.

Immunofluorescence microscopic studies add considerable support to the observations obtained at the ultrastructural level in the present investigation. Debus et al\textsuperscript{7} used indirect immunofluorescence to define microtubule coils in platelets fixed immediately after centrifugation onto glass slides or after settling for two hours. Brightly stained rings were present at the periphery of all discoid platelets. In cells that had lost their discoid morphology, become spherical, and extended pseudopods, the microtubular band had become a spiral ring from which one or more arms extended in a radial fashion. Diameters of the microtubule rings decreased coincident with pseudopod formation. Loss of ring structure and unravelling of the marginal band of microtubules were observed, but only in spread platelets that had been in contact with glass for two hours.

Results of indirect immunofluorescence microscopy of platelets at several intervals during spreading on glass slides in the present investigation were similar to those reported by Debus et al.\textsuperscript{7} Coils of microtubules were present in discoid platelets and most dendritic and spread cells up to 30 minutes after exposure to glass. At 60 minutes, significant breakdown of microtubule coils was evident, though some coils persisted. No evidence of a phase in which microtubule coils disassembled and then reformed was observed in our study or the investigation reported by Debus et al.\textsuperscript{7}
Earlier studies from our laboratory demonstrated that platelet microtubules do not dissolve in activated platelets. Instead, the circumferential bands were constricted into tight rings around centrally concentrated organelles. Treatment with taxol to prevent microtubule disassembly supported the previous findings. Microtubule disassembly was not necessary for shape change, internal contraction, secretion, irreversible aggregation, or clot retraction. Results of indirect immunofluorescence and investigation of detergent-resistant platelet cytoskeletons prepared by the whole mount method support the earlier observations made on thin sections of platelets embedded in plastic. Coils of microtubules were regularly present in platelets through the dendritic stage and in many spread cells. Their persistence with or without taxol pretreatment into the terminal phase of platelet interaction with grids is inconsistent with the concept that microtubule disassembly is an essential stage in platelet activation, occurring before actin filament assembly.

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