The Substructure of Human Platelet Microtubules

By JAMES G. WHITE

BEHNKE AND ZELANDER have recently described the filamentous substructure of platelet marginal band microtubules observed in negatively stained whole mount preparations. Each microtubule was composed of up to 12 evenly spaced 35 Å subfilaments in parallel association. Beading of the subfilaments was observed, but a relationship of beads on adjacent subfilaments was not commented upon. At points where microtubules were fractured the subfilaments appeared to disintegrate into 35 Å globular subunits. Intact subfilaments spraying out from the broken microtubules were not delineated.

A similar study of platelet microtubules in negatively stained preparations was underway in this laboratory when the work of Behnke and Zelander was published. General findings are in close agreement with the results of their excellent investigation, and require no additional elaboration. Significant differences, however, were noted in the number of subfilaments comprising single microtubules, in the relationship of beads on adjacent subfilaments to one another, and in the intact appearance of long segments of subfilaments derived from broken tubules. The aspects of microtubule substructure observed in this study and not previously reported are herein described.

MATERIALS AND METHODS

Citrate platelet rich plasma was prepared from the blood of normal human donors by methods previously described. Separation was accomplished at room temperature and the samples of citrate platelet rich plasma were maintained at 37 C. during experimental procedures. Small portions of the platelet-rich plasma were drawn into plastic syringes, and #26 gauge needles were joined to the hubs. Tiny drops were pushed to the tips of the needles, and touched to the surface of drops of triple distilled water. Platelets spread on the surface of the water were picked up on formvar coated carbon stabilized copper grids. After one minute the fluid was removed from the grid by touching the edge with filter paper. A drop of 2 per cent phosphotungstic acid (PTA) buffered with potassium hydroxide to pH4 was placed on the grid, and immediately removed. Another drop of stain was left on the grid surface for one minute before removal. The grids were air-dried and examined in the Phillips 200 electron microscope. Observations on this type of preparation were essentially identical to those reported by Behnke and Zelander, and will not be further detailed.
The preparations which yielded significant differences were obtained in a slightly different manner. Five ml. samples of citrate platelet rich plasma were concentrated by sedimentation at 1200 rpm for 15 minutes in an International U.V. centrifuge at room temperature. Four ml. of supernate were discarded and replaced with 5 ml of triple distilled water. The sample was centrifuged again and all but 0.5 ml of the supernate was removed. Residual platelets were resuspended in the half ml. volume. Small drops of this material were spread on distilled water, transferred to grids, dried and stained with PTA in the manner described above.

RESULTS

The appearance of negatively stained platelets and intact bundles of microtubules in spread preparations has been described in the previous reports.\textsuperscript{1,2} Marginal band microtubules in platelets swollen initially by exposure to distilled water resembled those in platelets prepared by the spreading technic alone. The initial ballooning of platelets, however, caused greater disruption of the marginal bundle of microtubules, and promoted spreading of the individual cells over a larger area of the grid surface (Fig. 1). Though few intact bundles of tubules remained, individual microtubules could be observed with greater clarity (Fig. 2a and 2b). A single microtubule was usually evident following the inner curvature of the spread surface. Portions of these long tubules were composed of 6 or 7 subfilaments in parallel association. Areas of greater width occurred frequently along the course of the tubules in which larger numbers of parallel subfilaments were identified.

In some platelets fully spread, broken microtubules were evident in the hyaloplasm. The substructure of these tubules was of interest. As many as 12 to 15 subfilaments could be identified in fully spread tubules (Fig. 3a and 3b). Individual subfilaments revealed a sawtooth pattern suggestive of beading. The subunits appeared stacked together like the coils of a rope rather than links of a chain. This apparent helical wind of subfilaments was accentuated by a linear pattern cutting diagonally across the long axis of the spread microtubules at an angle of 50–60°. The period of the diagonal pattern was 40 Å. Since the diameter of each subfilament was 35–40 Å the dimensions of each unit of the subfilament were approximately 35–40 Å by 40 Å.

Broken or partially fractured microtubules were encountered near platelet surfaces (Fig. 4). At points of tubule discontinuity, individual subfilaments appeared to lose their intimate parallel association and spray out into adjacent hyaloplasm or to the cell exterior. The subfilaments from microtubules were difficult to distinguish from hyaloplasmic microfilaments unless their point of origin from a tubule could be defined (Fig. 5).

DISCUSSION

Considerable speculation concerning the origin and functional significance of the circumferential bundle of microtubules in platelets still exists. Most workers agree that the band of tubules plays a role in supporting platelet discoid shape.\textsuperscript{3,4,5,6} Changes which develop in marginal tubules during dynamic alterations in platelet shape, however, have raised questions concerning a more complex involvement of microtubules in platelet physiology. The
Fig. 1.—Whole mount of human platelet negatively stained with phosphotungstic acid (PTA). The cell is from a sample of platelet rich plasma (PRP) exposed to distilled water and spread by surface tension before mounting on the carbon stabilized formvar coated grid. Cells prepared in this manner appear more swollen than cells spread on the surface of distilled water alone, and are often ruptured. Granules (G) are not concentrated in the centers of the water swollen cells as they are in platelets of PRP placed directly on the surface of grids. A PTA positive coat (C) is evident covering the exterior surface of the plasma membrane. Fragments (F) of the surface coat are evident on the platelets and beyond their cell walls. Elements of the marginal bundle of microtubules (T) are located peripheral to the granules and inside the cell membrane. Even at this magnification the filamentous substructure of the microtubules is apparent. Mag × 28,800.

circumferential bundle of microtubules appears to be involved, even if passively, in a centripetally directed wave of contraction which develops inside platelets after exposure to aggregating agents.\textsuperscript{7,8,9} It is essential, therefore, to
Fig. 2.—Two examples of marginal band microtubules in negatively stained, swollen platelets. The circumferential bundle (left) is composed of a single tubule. Parallel subfilaments making up the tubule are evident (1). Variation in the number of subfilaments along its course appears to depend on whether the tubule collapsed on itself or opened up completely during the spreading process. As few as six and as many as fourteen subfilaments can be identified in this example. The microtubule (right) is spread more completely than the tubule shown in the previous illustration. Superimposed fragments of the cell coat cause difficulty in determining the precise number of subfilaments in parallel association, but up to fifteen can be identified at intervals along the course of this tubule. Mag. × (left) 64,400; (right) 69,000.

define precisely the structure and relationships of microtubules in order to elucidate their role in the platelet reaction.

The findings of the present study complement and extend the results of
Fig. 3.—Two examples of spread microtubules in which the structure of individual subfilaments can be identified. Each subfilament appears to be composed of alternating light and dark areas. The pattern resembles a twisted rope, suggesting that globular subunits are stacked in a helical fashion. The helical wind in each subfilament is accentuated by periodic diagonal lines (1) cutting across the spread tubules at an angle of 50°–60° with their long axes. This pattern indicates that the beads on adjacent subfilaments are not in exact parallel register. The tubule above contains 15 subfilaments and the example below has at least 14 elements in parallel arrangement. Mag. × (above) 145,000; (below) × 136,900.

previous investigations. Behnke and Zelander in their reports failed to find more than 11 or 12 subfilaments in the substructure of single microtubules.¹ ²

The presence of up to 15 subfilaments in tubules noted in this study appears to disagree with their observations. However, variation in the number of subfilaments is not unexpected since considerable differences in the diameter of platelet microtubules are evident in thin section.³ ⁴ ⁵ Furthermore, spindle microtubules which are reported to be narrower than platelet tubules have up to 13 subfilaments in parallel association.⁶

The beading of microtubule subfilaments observed in previous investigations was sharply accentuated in the platelets swollen by distilled water before spreading. Beads on individual subfilaments appeared to be stacked in an offset pattern suggestive of a helix. This apparent helical wind of stacked beads was reinforced by a diagonal periodic pattern which was not noted in earlier investigations of platelet microtubules. The regular diagonal lines at intervals of 40 Å suggest additional aspects of microtubule substructure.
Fig. 4.—Fractured marginal band microtubules. The spread microtubules in this illustration are broken near the cell surface. Inner elements of the tubule at (1) are intact and remain in parallel association along the inner curvature of the platelet wall. Outer subfilaments appear loosened from parallel association, and continue through the platelet surface. The free subfilaments are more clearly demonstrated emanating from the tubule at (2). This microtubule spreads over the surface of the first, and fans out into free subfilaments (*) radiating into the area exterior to the surface membrane. The free subfilaments spraying out of broken tubules appear slightly thicker than they do when bound in parallel association. Mag. × 125,400.

A sawtooth pattern perpendicular to the long axis of the tubules would have been compatible with a side by side association of beads on adjacent subfilaments. On the other hand the straight lines observed passing diagonally through the subfilaments suggest that the beads on adjacent subfilaments are not in exact parallel register. Thus, not only individual subfilaments, but the entire complex of parallel fibers making up a tubule appear to be associated in a helical array. A similar substructural arrangement has been delineated in microtubules of other cell types and in certain varieties of bacterial flagella.

Fractured microtubules observed in this study did not regularly break down
Fig. 5.—Hyaloplasmic microfilament and subfilaments of microtubules. The area of the swollen platelet shown in this illustration is filled with a complex meshwork of filamentous elements. The arrows indicate a site where subfilaments are in close parallel association. Similar short segments of microtubules are apparent elsewhere in the cell, but most of the filaments in the hyaloplasm appear unassociated. It is not possible to distinguish which of these elements are microfilaments and which are free subfilaments radiating from damaged microtubules. Mag. × 71,700.

into globular subunits as was previously suggested. Examples were noted in which subfilaments appeared to remain intact for long distances as they sprayed out of the broken tubules. Portions of subfilaments eminating from fracture sites appeared slightly thicker than the ends which were combined in parallel association. The apparent difference in diameter of parts of the same subfilament may be due to a staining artifact. Pooling of phosphotungstic acid in the trough between adjacent subfilaments of spread microtubules may cause the individual fibers to appear more narrow and farther apart than they actually are. Subfilaments which spray out of broken tubules are approximately 40–50 Å in diameter compared to 35–40 Å when bound in parallel association. The variations in diameter of the free and bound conditions of subfilaments are of considerable importance, since the free subfilaments do
not differ in appearance from hyaloplasmic microfilaments which are approximately 50 Å in diameter.16

The possibility that microfilaments and subfilaments of microtubules may be related has been intimated in previous studies. Bessis and Breton-Gorius6 noted fibriller elements in platelet hyaloplasm and suggested that some of the elements might be derived from microtubules. The fibrils they observed, however, were 60–80 Å in diameter. Bull17 also observed fibrils in platelet hyaloplasm, and found that they tended to align in a peripheral band in circular cells. The fibrils in his illustrations are identical in appearance to microfilaments, and their association in a peripheral band resembles a spread microtubule. Stephens18 working with the 22 S protein subunits derived from purified spindle microtubules demonstrated that the globular material will elaborate into filaments 40–60 Å in diameter under appropriate conditions. These several observations cannot be construed to indicate that subfilaments of microtubules, hyaloplasmic microfilaments and the thicker fibrils described by Bessis,6 Sixma,19 Bull17 and Zucker-Franklin20 are identical structures. However, the results of the present study do suggest that subfilaments of microtubules and hyaloplasmic microfilaments are practically indistinguishable on a morphological basis.

Behnke has suggested that the circumferential bundle of microtubules is in actuality a single long tubule coiled on itself.2 Furthermore, the coiled bundle appears to be under centrifugal tension, since individual elements were found to uncoil and straighten out in spread platelets. Observations on platelets swollen by distilled water before spreading appear to support Behnke’s thesis. A single microtubule was usually found just under the membrane of swollen, spread platelets. A reasonable explanation for disappearance of other tubules is that increase in platelet volume promoted expansion of the circumferential bundle. Uncoiling of the loops of a single long tubule as the circle enlarged would account for the finding of one tubule remaining in the marginal bundle of the swollen platelets.

ADP and other aggregating agents also cause platelets to increase in volume.21 However, the circumferential bundle of microtubules in platelets swollen by contact with ADP does not expand.8 Instead the bundle of tubules in ADP treated platelets is found in the interior of the cell closely encircling centrally clumped granules. As the influence of ADP diminishes the circumferential bundle of microtubules is restored to its original position under the cell wall. This sequence of events has suggested that the tendency of the marginal band of microtubules to expand in swollen platelets can be overcome by a centripetally directed wave of force triggered by ADP.9 The central shift and reduction in circumference of the marginal band in ADP altered platelets suggests that the loops have been wound into a tighter coil. Whatever the mechanism may be, the result indicates that the circumferential band is acted upon by, or involved in, the contractile response of blood platelets. If this were not the case the circumference of the band of tubules would be expected to enlarge as the platelet expands, or remain in
the same position as the contractile process moves other hyaloplasmic organelles to the platelet centers.

The nature of the relationship between marginal band microtubules and the platelet contractile mechanism is not known. There is no evidence to suggest that platelet microtubules can contract axially or that their subfilaments are composed of contractile proteins. However, if microtubules cannot contract, then some physical association between tubules and the contractile elements of the platelet appears necessary to explain the transformation produced in the cells by aggregating agents. Subfilaments radiating from the ends of a single coiled tubule making up the marginal bundles in unaltered platelets may provide the bridge between tubules and the contractile mechanism. A possible relationship between subfilaments of microtubules, hyaloplasmic microfilaments and contractile processes in platelets is by no means a novel consideration. Similar systems of tubules and microfilaments have been related to intracytoplasmic movements and contractile systems in a wide variety of cell types. The problem which remains is to determine the precise association between microtubule subfilaments and hyaloplasmic microfilaments, and the relationship of both structural elements to platelet contraction. Investigations to resolve these problems are in progress.

**Summary**

The substructure of the marginal bundle of microtubules supporting the discoid shape of platelets has been examined in negatively stained cells. Platelets were swollen first by exposure to distilled water, spread by surface tension, and mounted on carbon stabilized formvar coated grids. The findings differ from the work of previous investigators in the following ways:

1. Usually one microtubule remains in the circumferential bundle after pretreatment with distilled water.
2. The microtubules are composed of 12–15 subfilaments in parallel association. Each subfilament is composed of beads stacked in such a manner as to suggest a helical wind.
3. A diagonal pattern with a periodicity of 40 Å cuts across the long axis of the parallel subfilaments. This pattern suggests that globular subunits are 35–40 Å by 40 Å in size, and that beads on adjacent subfilaments are not in exact parallel register. Thus, the entire microtubule appears to be composed of subfilaments associated in a helical array.
4. At sites of partial fracture in microtubules, subfilaments lose their parallel association and spray out as free elements. The free subfilaments are essentially identical in appearance to hyaloplasmic microfilaments.

The relationship between subfilaments of microtubules, hyaloplasmic microfilaments, and the contractile mechanism of the platelet is discussed.

**SUMMARIO IN INTERLINGUA**

Le substructura del fasce marginal de microtubulos que supporta le forma discoide de plachettas esseva examineate in negativemente tincturate cellulas. Le plachettas esseva tumescite primo per lor exposition a aqua distillate. Alora illos esseva extendite per tension...
superficial e montate super retes a revestimento de formvar e stabilisation con carbon. Le constataiones differe ab illos de previe investigatores in le sequente punctos:

1. Usualmente un microtubulo remane in le fasce circumferential post pretractamento con aqua distillate.

2. Le microtubulos es componite de 12 a 15 subfilamentos in association parallel. Cata-un del subfilamentos es componite de perlas compilate in un maniera que suggestiona un disposition helical.

3. Un configuration diagonal con un periodicitate de 40 A transversa le longe axe del subfilamentos parallel. Iste configuration suggestiona que le perlas de adjacente subfilamentos non es disponite in precise parallelitate. Assi le integre microtubulo pare esser componite de subfilamentos que es associate in disposition helical.

4. Al sitos de fractura partial in le microtubulos, le subfilamentos perde br association parallel e se disperge como libere elementos. Le libere subfilamentos es essentialmente identic in lor apparentia con microfilamentos hyaloplasmic.

Es commentate le relation inter le subfilamentos de microtubulos, microfilamentos hyaloplasmic, e le mechanismo contractil del plachettas.

REFERENCES


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