Blood platelet formation *in vitro*. The role of the cytoskeleton in megakaryocyte fragmentation

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Summary

We have developed a unique *in vitro* model that promotes differentiation of megakaryocytes into platelets. When megakaryocytes isolated from guinea pig bone marrow were cultured on hydrated rat tail collagen gels, cells spontaneously formed elongated, beaded processes that fragmented to yield cytoplasmic pieces with the same size and internal composition as individual platelets. Addition of nocodazole at the initiation of cultures blocked process formation, while addition of nocodazole to cells with previously established processes resulted in their retraction. The addition of taxol to cultures resulted in abnormally thick processes that were tightly adherent to the underlying substratum, and did not bead or fragment. Cytochalasin D accelerated process formation and fragmentation of megakaryocytes cultured on collagen gels by twofold. On the basis of these results, we propose a model for platelet formation in culture that involves the following steps: adherence of megakaryocytes to the underlying extracellular matrix; dilation of the demarcation membrane system and breakdown of the actin-rich peripheral zone; microtubule-based extension of pseudopodia, which are no longer adherent to the substratum; and fragmentation into platelets by the coalescence and fusion of demarcation membrane vesicles with the plasma membrane. We feel that this distinctive culture system closely approximates thrombocytopoiesis *in vivo*, thus allowing detailed elucidation of this important process.

Key words: megakaryocyte differentiation, microtubules, cytochalasin D, nocodazole, actin, taxol.

Introduction

Platelet formation from megakaryocytes has been actively studied, but this process is still incompletely understood (for review, see Stenberg and Levin, 1988). Megakaryocytes *in vivo* have long, often-beaded processes extending from the hematopoietic space into the vascular sinuses. These beads have been proposed to separate from the processes, resulting in the formation of either individual platelets or proplatelets (Lichtman *et al.* 1978; Mohandas and Prenant, 1978; Weiss, 1970). Prior studies have shown that cultured megakaryocytes can form long processes that appear to be the first step in platelet formation (Albrecht, 1957; Leven and Yee, 1987; Levine *et al.* 1985; Radley and Scurfield, 1980; Thiery and Besis, 1956). Modulation of the megakaryocyte cytoskeleton *in vitro* by cytochalasin and vincristine has shown that the proper organization of microfilaments and microtubules is essential for platelet formation. Disruption of the actin cytoskeleton by cytochalasin results in augmentation of cytoplasmic process formation (Leven and Yee, 1987), while vincristine inhibits process formation and reverses process elongation (Handagama *et al.* 1987; Radley and Haller, 1982). Although these studies suggest that one can observe varying degrees of megakaryocyte differentiation *in vitro*, it has not previously been shown that cytoplasmic fragments of the same size and internal organization of platelets can form in culture. To understand the role of the megakaryocyte cytoskeleton in platelet formation, we have established an *in vitro* system that does result in fragmentation and platelet formation. Using this culture system, we have examined the role of the cytoskeleton in generation of platelets and now report that the disruption of either microtubules or actin filaments can alter the process of fragmentation and platelet formation.

Materials and methods

Materials

The following chemicals were obtained from Sigma Chemical Corporation (St Louis, MO): rhodamine goat anti-rabbit IgG, nocodazole, cytochalasin D (CD). Matrigel was obtained from Collaborative Research, Inc. (Bedford, MA). Rabbit anti-tubulin antibody was from Miles Scientific (Elkhart, IN). All culture reagents were purchased from GIBCO (Grand Island, NY). Other reagents were at least of analytical grade. Guinea pigs were obtained from Camms Labs, (Wayne, NJ).

Taxol was a kind gift from Dr Matthew Suffness, Chief, Natural Products Branch, National Cancer Institute.

Megakaryocyte isolation

Guinea pig bone marrow megakaryocytes were isolated on bovine serum albumin density gradients by modifications of the method of Levine and Fedorko (1976), as described in the procedures of Hill *et al.* (1989). Megakaryocytes were identified morphologically using Wright–Geimsa-stained cytospin preparations. Purity of megakaryocyte preparations was 86 %.

Megakaryocyte cultures

All cells were cultured in Dulbecco's modified Eagle's (DME) medium with 10 % fetal bovine serum and 0.1 mg ml^{-1} gentamycin at 37°C in a 5 % CO_2 – 95 % humidified air atmosphere. Megakaryocytes (100 000 cells per 35 mm dish) were cultured either directly in 35 mm plastic tissue culture dishes (Falcon) or in 35 mm dishes coated with either 1.0 ml of type 1 collagen gel isolated from rat tail tendon (Lee *et al.* 1984) or 100 μl of reconstituted basement membrane matrix (Matrigel). Nocodazole and CD (1 μg ml^{-1}) and taxol (10 μM) were added to the media at the time that the cultures were initiated. The cultures were observed and photographed with a Nikon inverted microscope using a 20×, 0.4 NA phase objective.

To be sure that CD did not affect membrane transport, control and CD-treated cells were cultured on collagen gels for 24 h, and glucose uptake was measured by the addition of 10 μCi ml^{-1} tritiated glucose. After 24 h, the cells were collected on Millipore filters, which were washed in cold DME. The dried filters were placed in scintillation vials with Ecolite (West Chemical, San Diego, CA), and counted in a Beckman model LSC9000 scintillation counter (Beckman Instruments, Irvine, CA). Glucose uptake was the same in CD-treated and control cells.

Immunofluorescence

Megakaryocytes were prepared for indirect immunofluorescence and examined as described by Leven and Nachmias (1982), except that rhodamine-labelled secondary antibody was used. Cells were observed and photographed with 100×, 1.24 NA objective, on a Nikon inverted microscope equipped with epifluorescent illumination.

Electron microscopy

Megakaryocyte cultures were fixed and embedded according to the method of Tablin and Taube (1987). Cultures were sectioned *en face* and at right angles to the culture dishes, and sections were stained with uranyl acetate and lead citrate, and examined on a Hitachi HS 600 transmission electron microscope.

Results

When we cultured guinea pig megakaryocytes on collagen or Matrigel to approximate their *in vivo* extracellular environment, we observed differing responses to the different types of extracellular matrix (ECM). Many cells cultured on both substrata began to adhere to the underlying matrix within 24 h of plating. Megakaryocytes cultured on rat tail collagen gel showed no alteration in morphology until 36–45 h after the cultures were established. At that time, many cells spread and formed elongated processes and produced cell fragments (Fig. 1A). Small pseudopodia first appeared at the edges of the spread cytoplasm and elongated at the same time as the spread cytoplasm was reorganized, apparently by incorporation into the processes. Pro-platelet fragments, equal to the size of several individual presumptive platelets, as well as individual platelet-sized fragments, were released into the medium of these cultures. Approximately 25–30 % of the cells underwent morphogenesis to form processes and presumptive platelets. The rest of the cells remained rounded but were still adherent to the substratum. Megakaryocytes cultured on Matrigel spread very irregularly and formed fewer wide processes, which did not fragment (Fig. 1B). These thick processes appeared to be strongly adherent to the matrix.

Not all of the cells underwent morphogenesis at the same time and rate, allowing us to observe megakaryocyte morphogenesis through a stratified culture system. Electron microscopic examination of guinea pig megakaryocytes cultured on collagen gels showed variable mor-

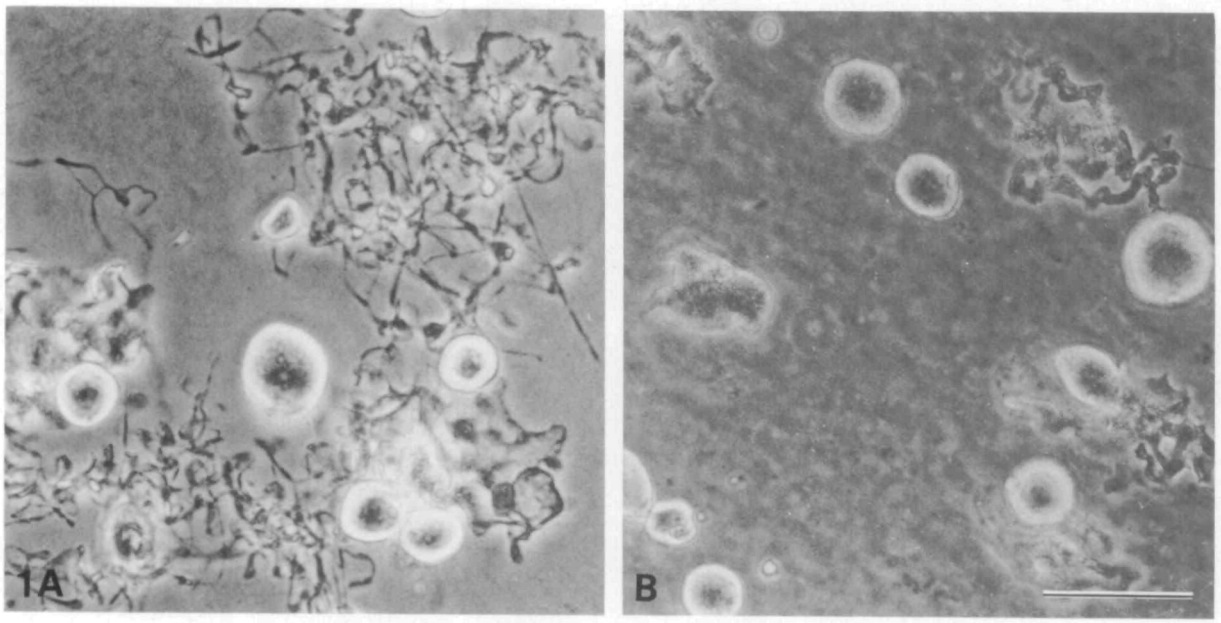

Fig. 1. Megakaryocytes cultured on extracellular matrix (ECM). (A) Guinea pig megakaryocytes were cultured for 48 h on rat tail collagen gels. Many cells have formed a complex array of beaded cytoplasmic processes, although some cells remain unchanged. (B) Megakaryocytes cultured for 48 h on Matrigel. These cells form thick irregular processes that are tightly adherent to the underlying matrix, but do not form thin processes or fragment. Bar, 60 μm.

phology that depended on proximity of the cells to the matrix. Cells in the early stages of morphogenesis were found uppermost in the culture, extending pseudopodia down toward the collagen gel (Fig. 2). These pseudopodia contained only thin filaments and polyribosomes (Fig. 2, inset). The overall organization of these cells was similar to that of megakaryocytes described by other investigators, in which alpha and dense granules and the demarcation membrane system (DMS), a series of membranous vesicles, were excluded from the cell cortex by an actin-rich peripheral zone. As cells became adherent to the matrix, there was distention of the DMS (Fig. 3), as well as reorganization of the cytoplasm at the periphery of the cell. Dilated DMS separated the cytoplasm into thin strips, the most peripheral of which contained thin filaments, microtubules and polyribosomes (Fig. 4). In some areas

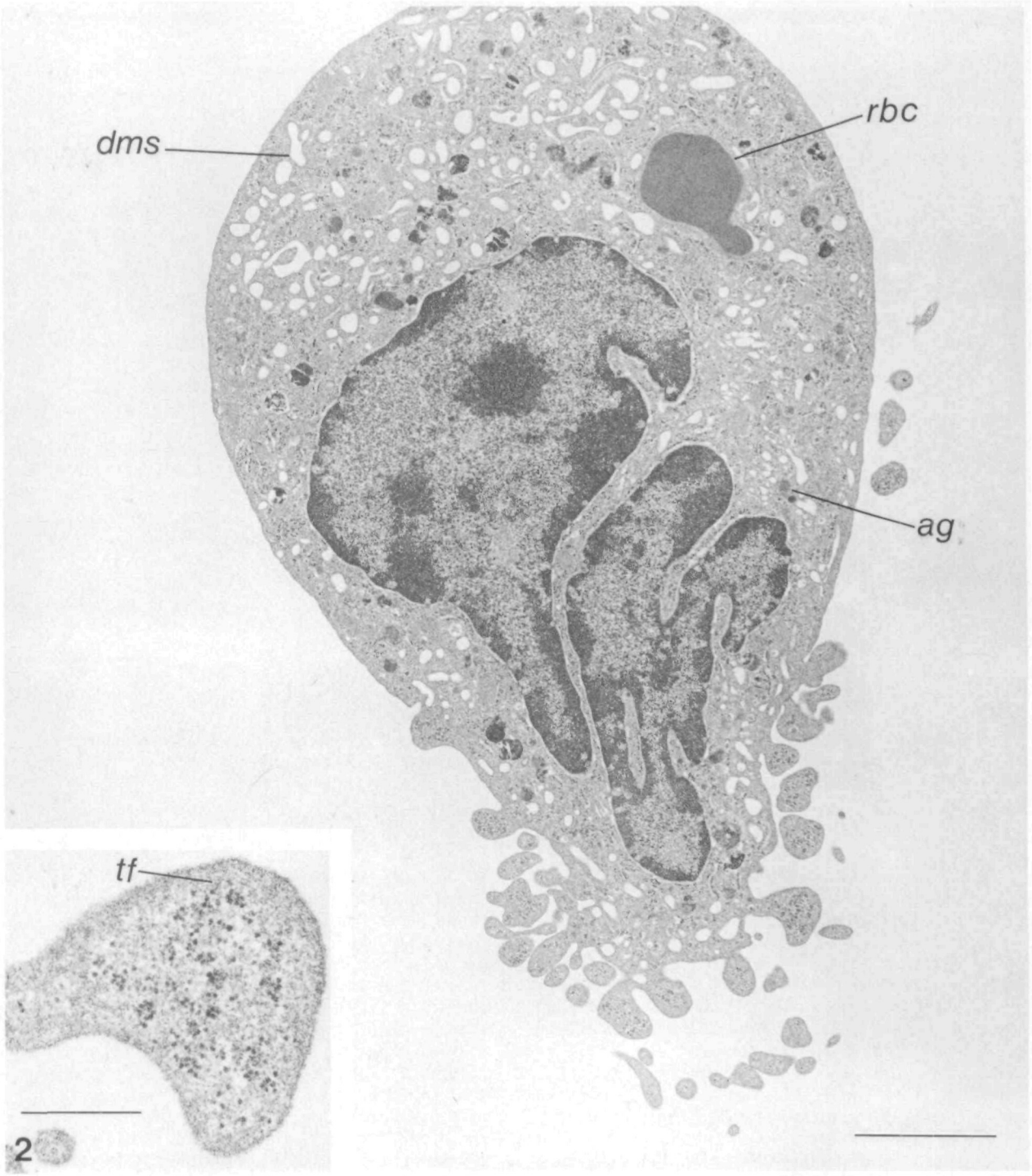

Fig. 2. Megakaryocyte with pseudopodia extending toward the collagen gel. A portion of a culture cut at right angles to the collagen gel shows a highly polar cell extending many pseupodia in the direction of the ECM located below the bottom of this micrograph. The 'trailing' edge of the cell contains the majority of alpha granules (ag) and demarcation membrane vesicles (dms), as well as a portion of an erythrocyte (rbc). Bar, 3 μm. Inset: at higher magnification a pseudopodium demonstrates cortical thin filaments (tf) and polyribosomes. Bar, 0.5 μm.

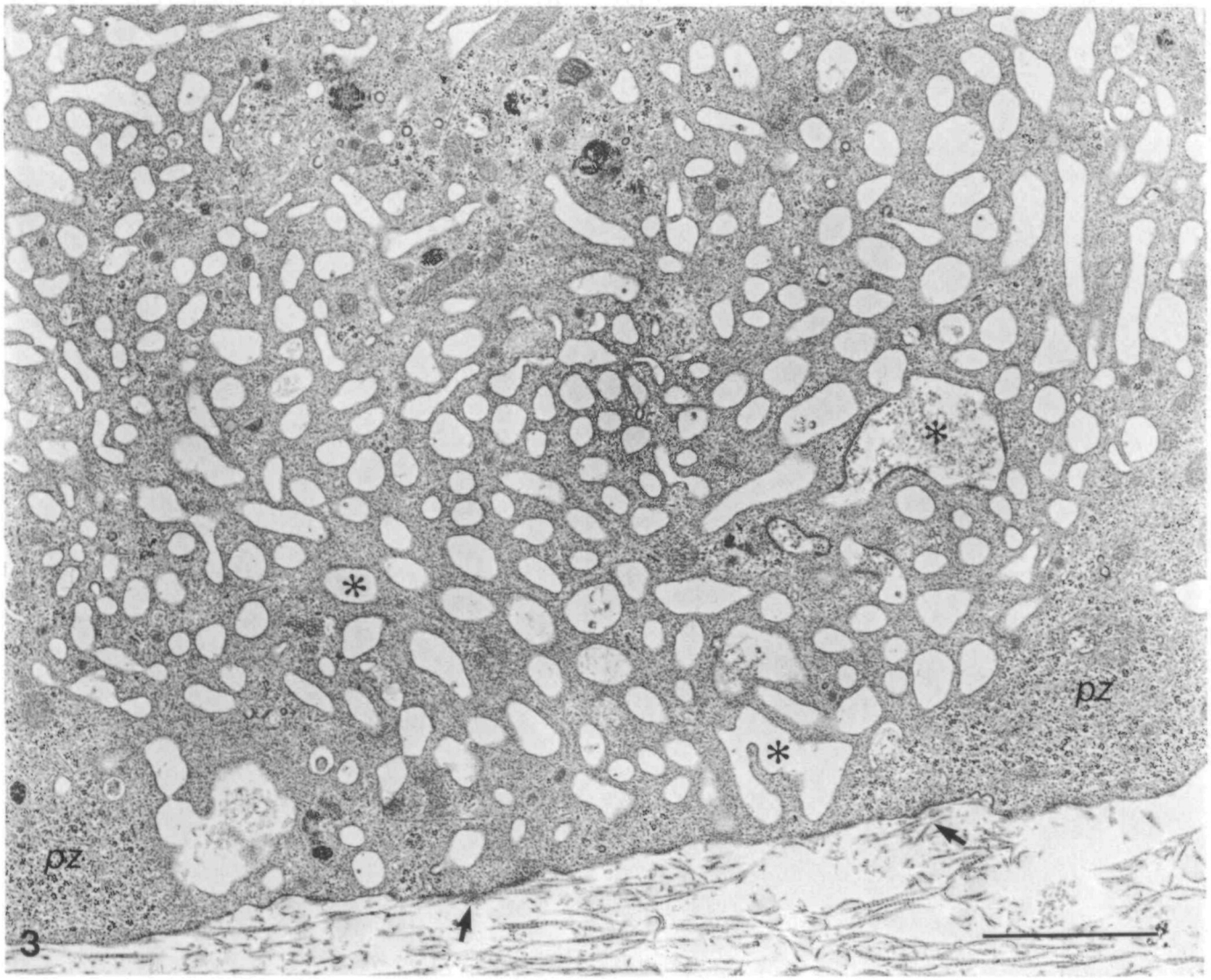

Fig. 3. Megakaryocyte adherent to the collagen gel. Several areas of close association between the plasma membrane and the matrix (arrows) are visible. The peripheral zone (pz) is no longer continuous, and the DMS (*) has begun to dilate. The majority of the cellular organelles are still more centrally located. Bar, 2 μm.

dilated DMS appeared to fuse with the plasma membrane (Fig. 4), apparently allowing these strips to peel away from the interior of the cell and resulting in long thin cytoplasmic processes that were no longer adherent to the underlying matrix. These fine processes contained longitudinal microtubules, alpha granules, mitochondria and membranous (DMS) vesicles (Fig. 5). Granules and DMS were not seen in processes in which microtubules did not extend to the end of the pseudopodia. An intriguing observation was the association of DMS vesicles at points of constriction along the processes, and their apparent fusion with the plasma membrane (Fig. 6). Interestingly, microtubules within beaded processes and at the end of the processes appeared to change orientation towards a more circumferential arrangement, similar to that of mature platelets (Figs 6 and 7). Individual free-floating fragments were morphologically similar to normal circulating platelets, and contained alpha granules, circumferential microtubules and cortical microfilaments (Fig. 8).

Effect of cytoskeleton-disrupting drugs on fragmentation

The actin-disrupting agent CD did not prevent process formation and fragmentation by guinea pig megakaryocytes cultured on collagen, and in fact appeared to accelerate the process. Guinea pig megakaryocytes cultured on collagen gels with the addition of 1 μg ml^{-1} CD displayed fragmentation that appeared similar to that of control cultures, with the exception that fragmentation occurred within 24 h, rather than the 48 h observed in the control cultures. The processes of CD-treated cells appeared somewhat thinner than those of control cultures, lacked long arrays of thin filaments, and only contained small numbers of short, thin filaments, which were associated with the plasma membrane. Microtubules, DMS vesicles, mitochondria and alpha granules were present in the processes in an arrangement similar to that of cells grown in the absence of CD (Fig. 9). Interestingly, although guinea pig megakaryocytes did not spontaneously form thin processes or fragment on Matrigel, process formation and fragmen-

tation were observed in cells on Matrigel with CD (Fig. 10).

When cultures were treated with nocodazole throughout the entire culture period, megakaryocyte process formation and fragmentation was inhibited entirely. Cells were still able to adhere to the underlying matrix, but there was no dilation of the DMS, and no pseudopodia were formed (data not shown). If nocodazole was added to megakaryocyte cultures that were already undergoing morphogenesis, processes retracted and fragmentation ceased. These

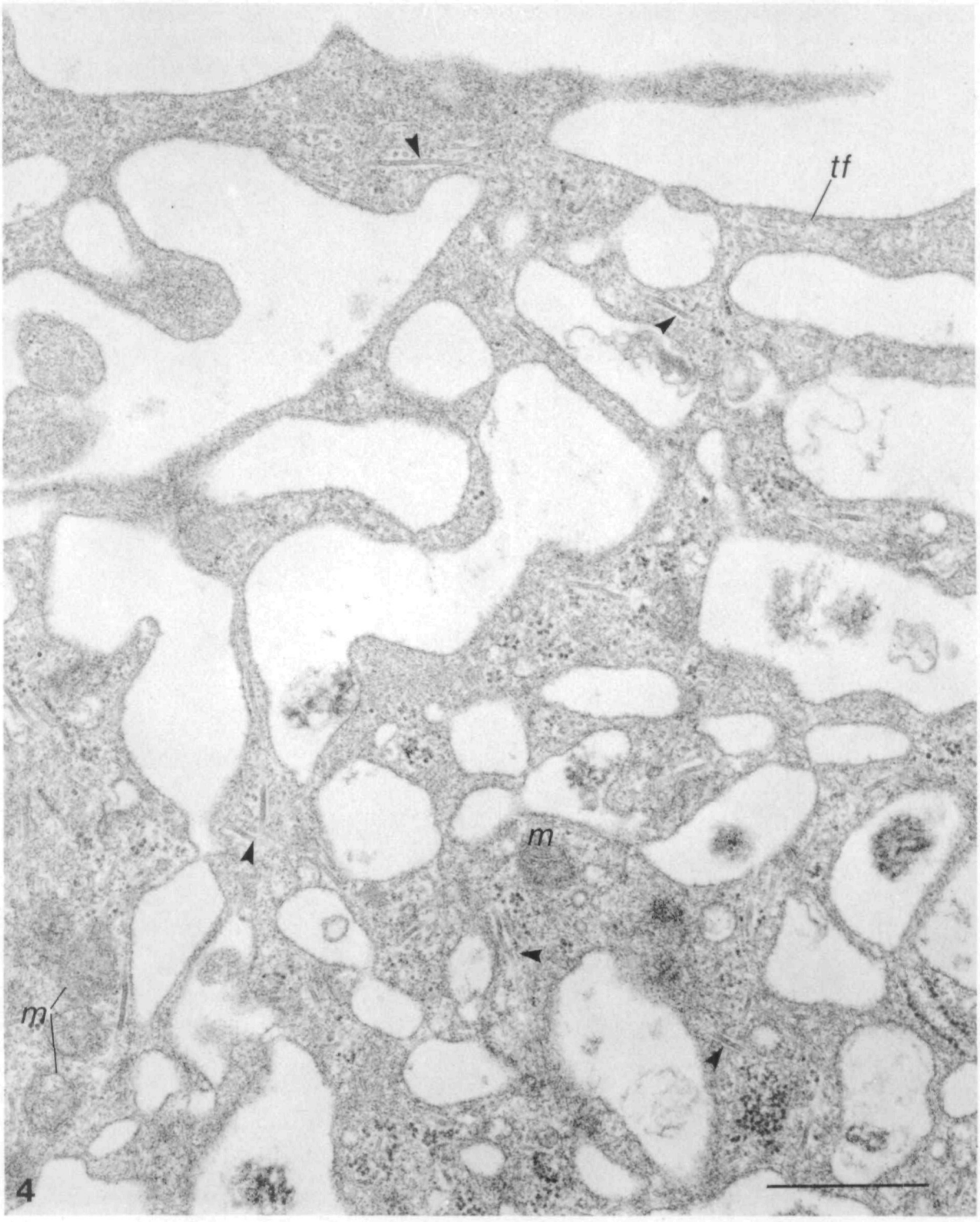

Fig. 4. A higher magnification of a portion of a cell similar to that seen in Fig. 3 demonstrates the organization of cytoskeletal elements. The most peripheral aspects of the cytoplasm, seen at the top of the micrograph, contain numerous microtubules (arrowheads) arrayed in many different planes. Thin filaments (tf) and polyribosomes are also present in these areas. An adjacent area closer to the center of the cell, at the bottom of the micrograph, also contains mitochondria (m). Bar, 0.5 μm.

cells were morphologically indistinguishable from large round megakaryocytes that had not undergone morphogenesis (data not shown).

Cultures that contained 10 μM taxol exhibited abnormal process formation. These cells produced long, thick processes that were tightly adherent to the underlying substrata (Fig. 11), and which contained large arrays of microtubules as well as cortical thin filaments (Fig. 12). These cells rarely beaded, and then beading only occurred at the ends of cytoplasmic processes (Fig. 11). They did not undergo fragmentation.

Discussion

The role of the cytoskeleton during differentiation has been studied by many investigators. Under most circumstances extension of pseudopodia by cells involves changes in the actin cytoskeleton. However, our results provide two

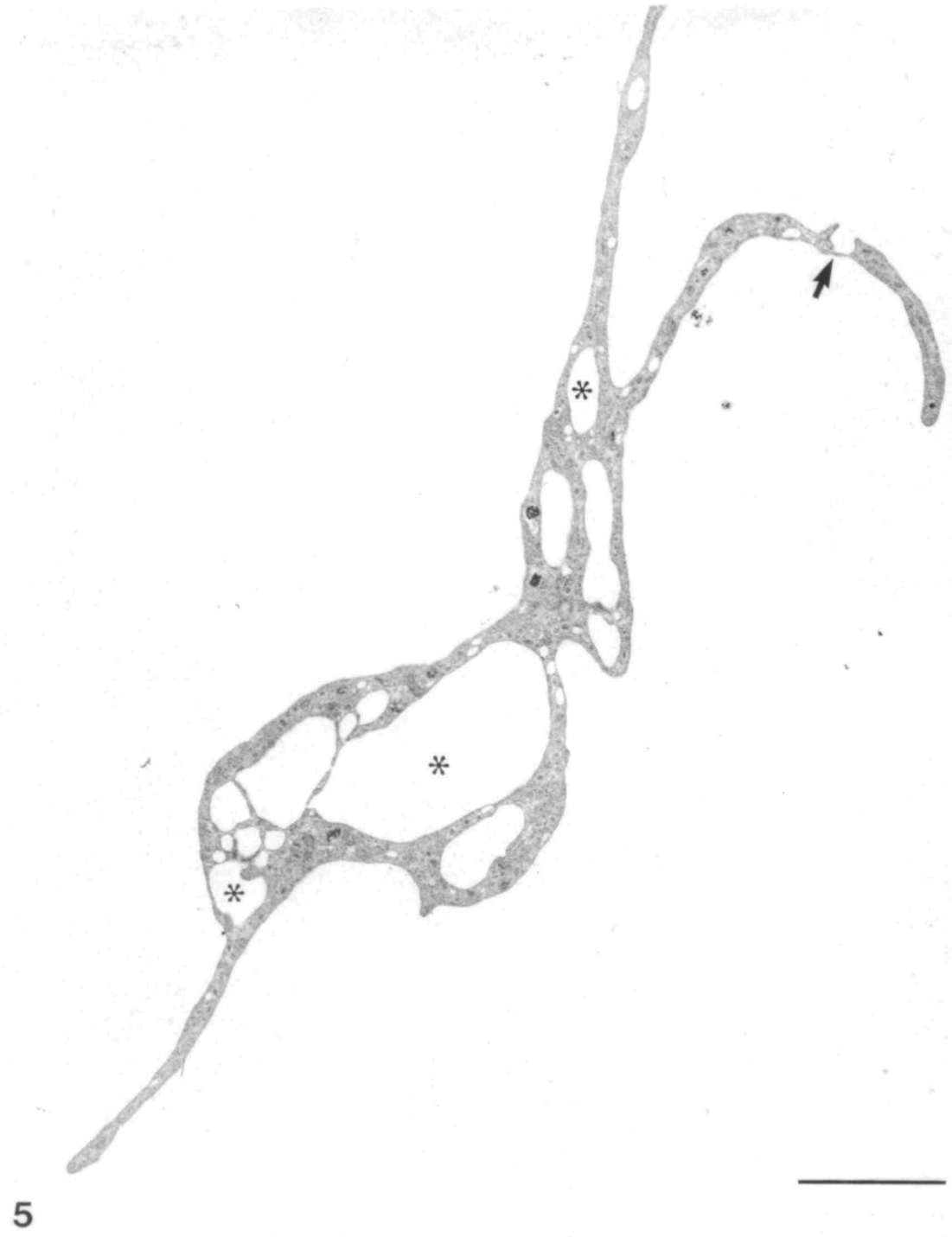

Fig. 5. An *en face* view of an elongated cytoplasmic process that is no longer adherent to the collagen gel. Several areas of extremely dilated DMS (*) are seen. A putative site of DMS–plasma membrane fusion is indicated by an arrow. Bar, 3 μm.

lines of evidence that megakaryocyte process formation may be controlled instead by the reorganization and/or elongation of microtubules. Megakaryocyte processes contain highly regulated microtubule arrays in the form of coils, which may impose specialized controls on process formation and fragmentation. Nocodazole, a drug that depolymerizes microtubules (DeBrabander *et al.* 1981), has profound effects on the megakaryocyte's ability to produce platelets in culture. Depolymerization of megakaryocyte microtubules results in inability of the cells to form processes or to undergo fragmentation. In addition, already formed pseudopodia retract when nocodazole is added to the cultures, strongly suggesting that it is the depolymerization of microtubules that is responsible for process retraction. Our results are similar to those previously observed when megakaryocytes in liquid culture were exposed to vincristine (Radley and Haller, 1982). Megakaryocyte process formation may be similar to process formation in *Actinosphaerium nucleofilum* (Tilney and Porter, 1967; Tilney, 1968). The large rounded cells of this pond organism extend axopodia containing highly ordered arrays of microtubules that retract when treated

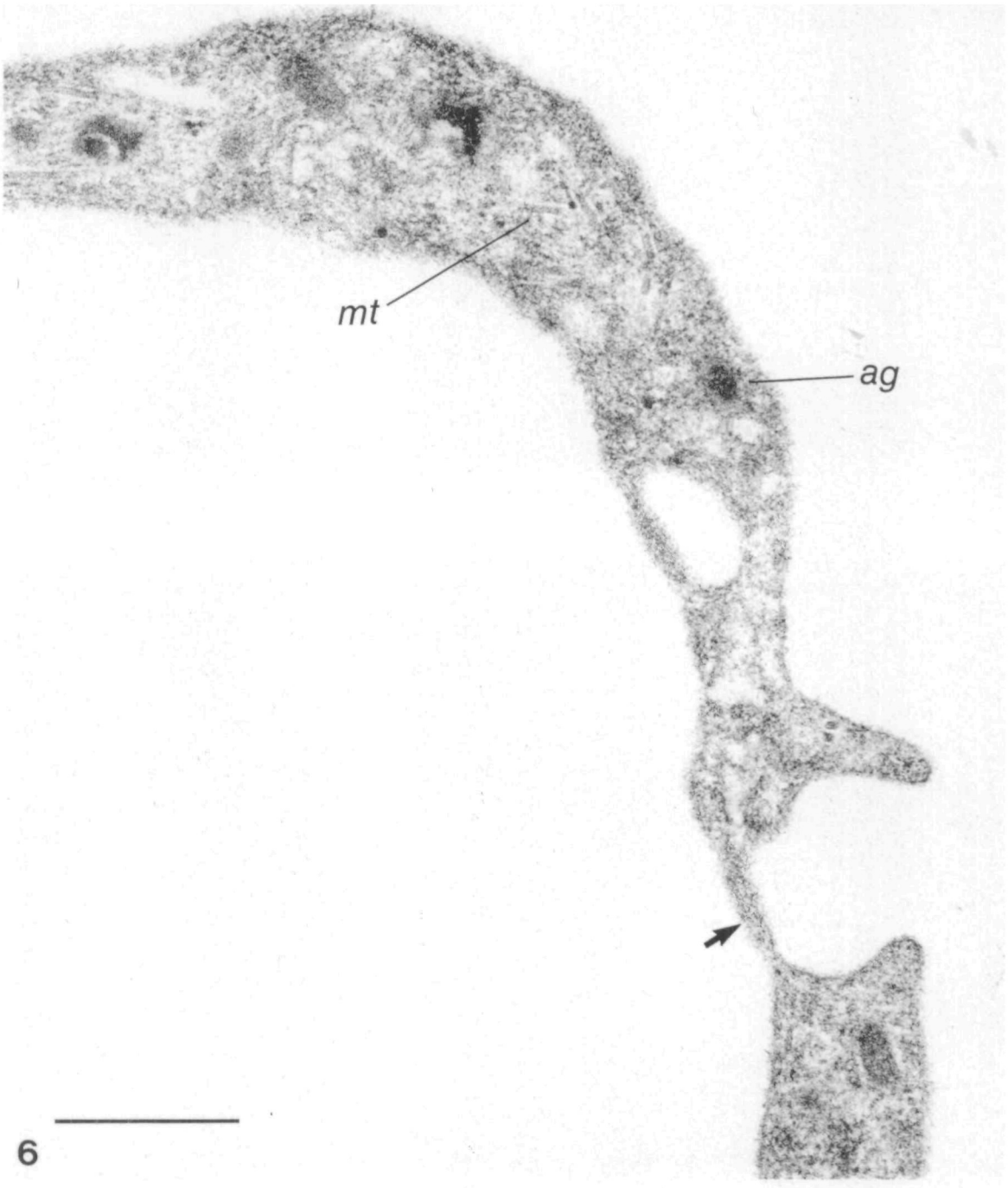

Fig. 6. A high-magnification view of Fig. 5 demonstrates the putative site of vesicle coalescence and fusion with the plasma membrane (arrow). This tangential section demonstrates the reorganization of microtubules (mt) from a linear to a non-linear array. Bar, 0.5 μm.

with colchicine. Neurite outgrowths of PC12 and other neuronal cells (Bray *et al.* 1978; Jacobs and Stevens, 1986) also contain highly ordered microtubule arrays that respond to microtubule disrupting drugs in a similar manner (Bray and White, 1988; Jacobs and Stevens, 1986; Letourneau *et al.* 1987).

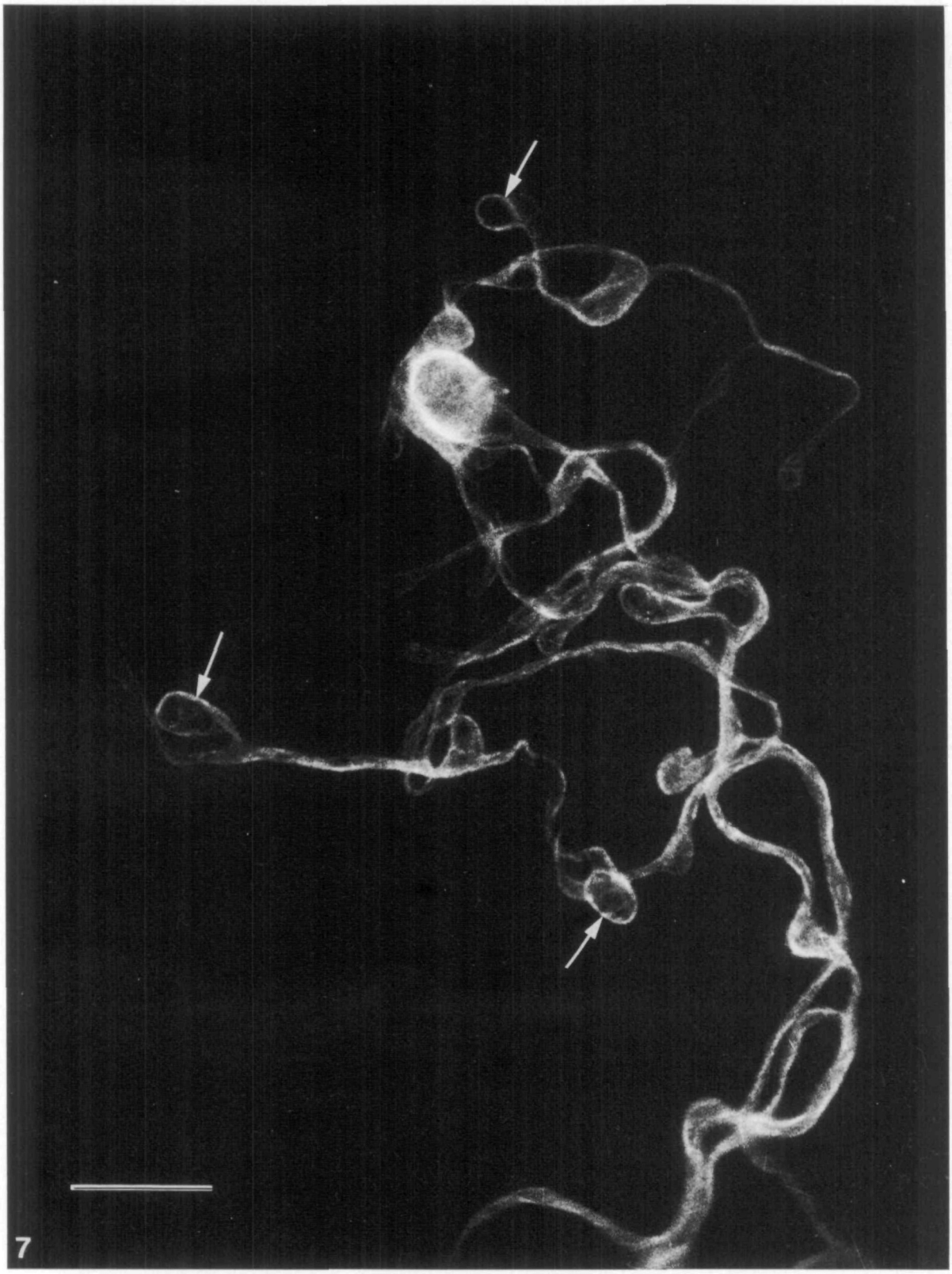

Fig. 7. Guinea pig megakaryocyte stained with anti-tubulin antibody. This cell demonstrates bundles of longitudinally oriented microtubules throughout the ctyoplasmic processes, except where 'beading' has occurred. In these regions the microtubules assume a circumferential orientation (arrows) similar to that seen in platelets. The cell body is not present in this micrograph. Bar, 15 μm.

The treatment of megakaryocyte cultures with taxol, a drug that stabilizes microtubules and incorporates free monomer onto polymerized microtubules (Schiff *et al.* 1979), also supports the concept of a microtubule basis for pseudopodial extension. However, it must be noted that taxol-treated megakaryocytes with stabilized microtubules produced abnormally thick processes that were tightly adherent to the extracellular matrix and that did not bead or fragment, suggesting that a dynamic rearrangement of microtubules from linear to circumferential arrays is necessary for complete platelet formation. This rearrangement occurs at the same time as the beading of cytoplasmic processes, but it is unclear how these two events are spatially related.

A second line of evidence implicating microtubules in platelet formation is the insensitivity of this process to CD. CD acts to shorten actin filaments and may bind actin dimers (Cooper, 1987). Much to our surprise, process fragmentation and platelet formation not only proceeded but were accelerated in CD-treated cells, occurring in half of the normally anticipated time (24 instead of 48 h). Furthermore, the addition of CD to cultures of guinea pig megakaryocytes on Matrigel resulted in beaded process formation and fragmentation. These observations suggest

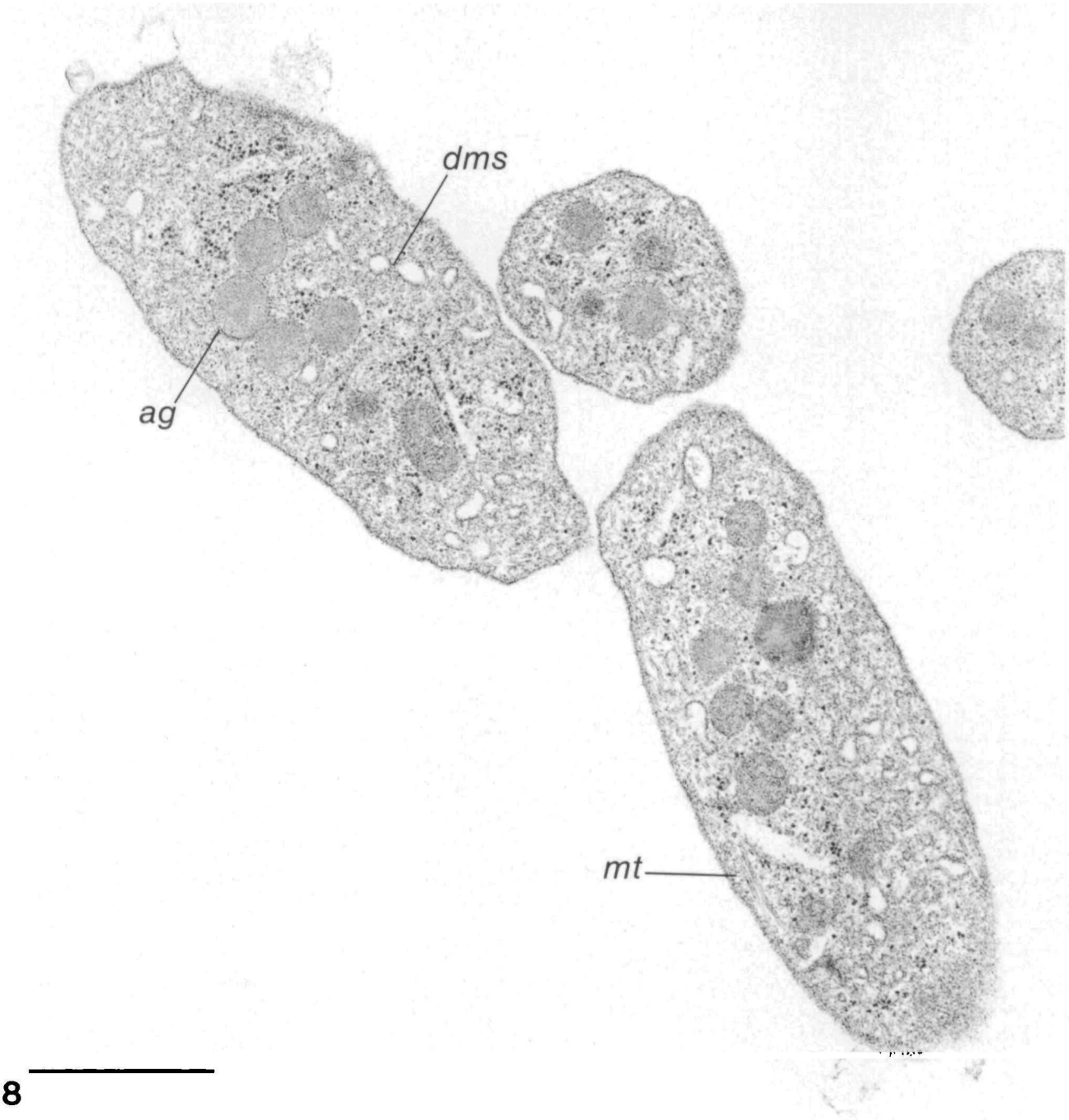

Fig. 8. Free-floating cytoplasmic fragments. Platelet-like fragments contain circumferential microtubules (mt) and probable cortical thin filaments as well as alpha granules (ag), mitochondria, dms vesicles and glycogen granules. Bar, 1 μm.

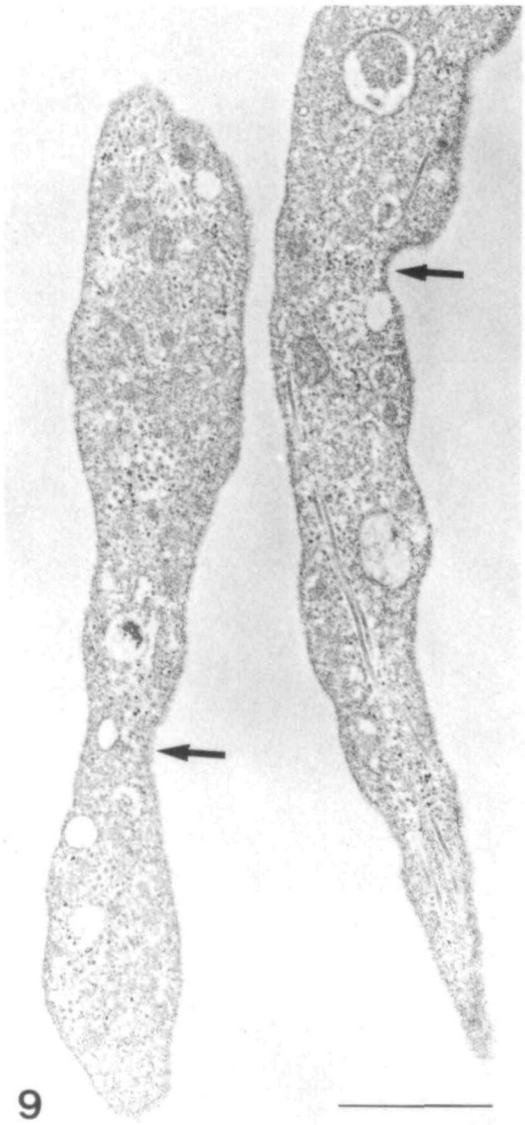

Fig. 9. Megakaryocytes cultured on collagen gels for 24 h in the continuous presence of CD (1 $\mu g\,ml^{-1}$). These cells are devoid of extensive arrays of cortical thin filaments; however, some membrane-associated thin filaments remain. Processes contain numerous elongate microtubules, mitochondria, DMS vesicles and alpha granules. In several areas along the processes, indentations (arrows) appear to be potential sites for DMS vesicle coalescence and fusion to form platelets. Bar, 1 μm.

that rearrangement of the actin-rich peripheral zone may be an important step in the process of platelet formation in this system, as first proposed by Leven and Yee (1987) for cells in liquid culture. In other cell types, treatment with cytochalasins has allowed microtubules to expand into previously actin-dense areas (Forscher and Smith, 1988; Letourneau *et al.* 1987). We propose that a similar mechanism occurs in megakaryocytes treated with CD. When the peripheral actin-rich zone is disrupted, microtubules, whose numerous arrays are adjacent to the peripheral zone, either elongate or reorient during the extension of pseudopodia. Thus, it appears that our culture system requires interaction between dynamic microfilament and microtubule systems for process elongation and fragmentation in megakaryocytes.

A distinctive feature of our culture system is the coalescence of vesicles within pseudopodia at putative sites of platelet formation. These vesicles are probably derived from the megakaryocyte DMS. Their movement down pseudopodia is likely to be mediated by the cytoskeleton, in light of the fact that they are not seen until microtubules extend to the tip of the pseudopod. DMS

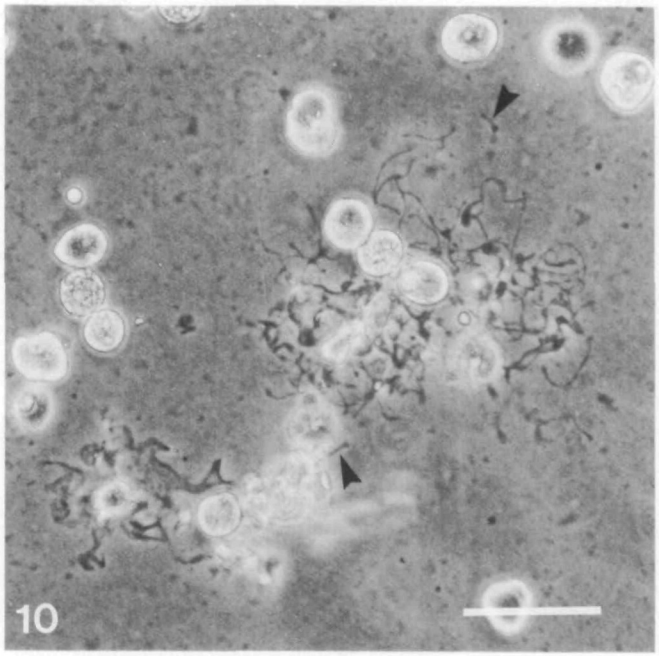

Fig. 10. Guinea pig megakaryocytes cultured on Matrigel in the presence of CD. Cells were cultured as described in Fig. 9. Several cells are undergoing fragmentation, and free-floating fragments (arrowheads) are present within the culture. Compare this to Fig. 1B where cells were cultured on Matrigel in the absence of CD, and in which cells were tightly adherent to the matrix with thick irregular processes that did not fragment. Bar, 60 μm.

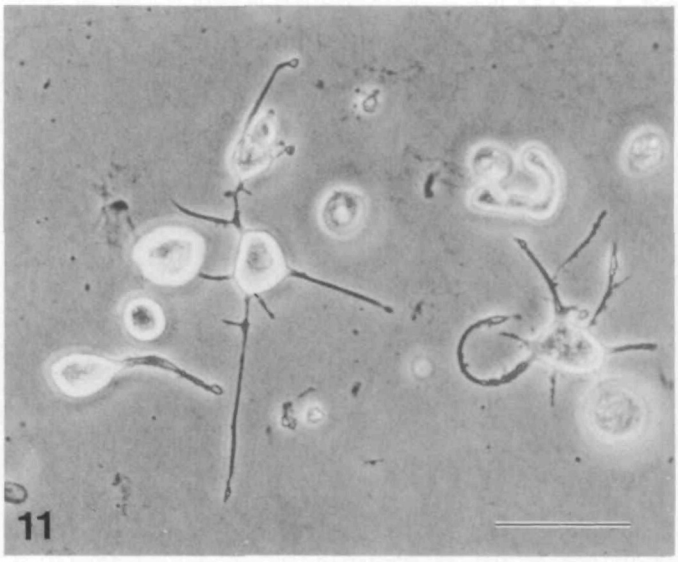

Fig. 11. Megakaryocytes cultured on collagen gels in the presence of taxol. These cells have produced long thick processes that are adherent to the matrix. No areas of beading are seen except at the very tips of the pseudopodia. Bar, 50 μm.

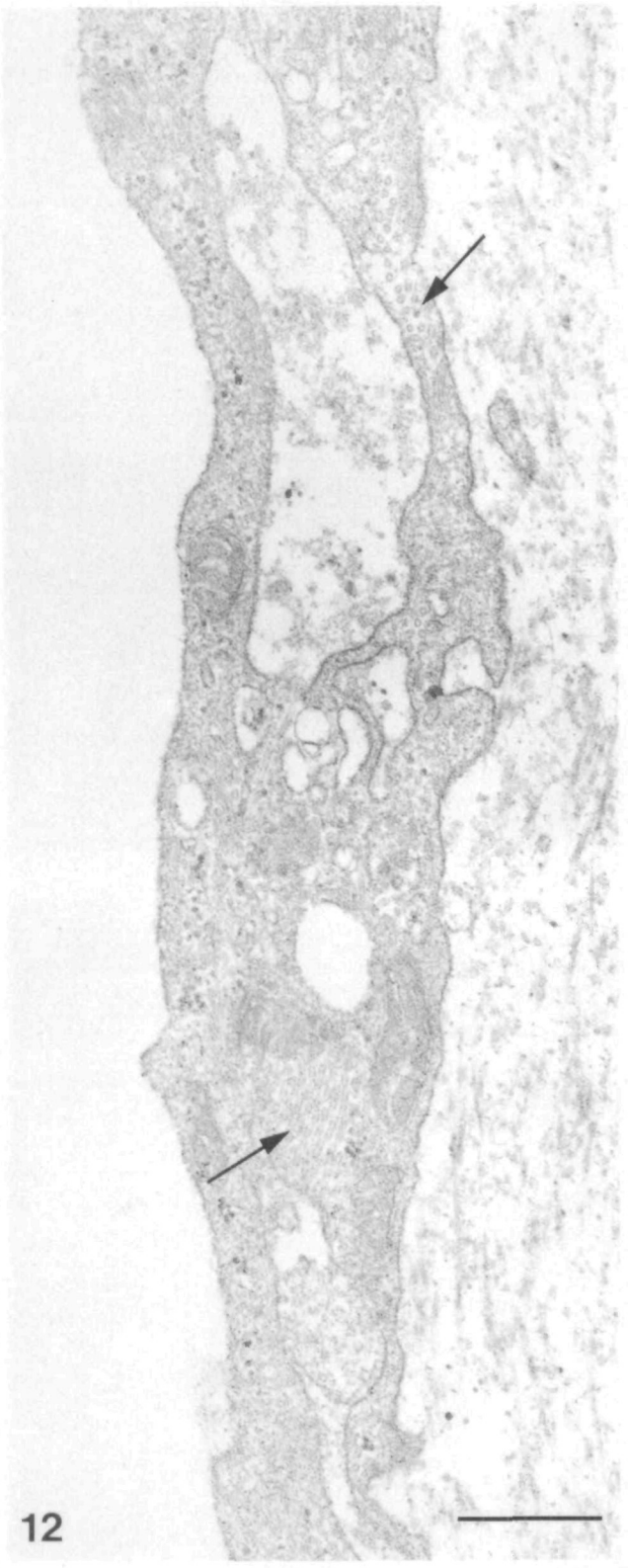

Fig. 12. A high-magnification view of cells cultured as in Fig. 11 shows the extensive arrays of microtubules (arrows), which are present in both longitudinal and cross-section. These processes appear to be closely adherent to the matrix. Bar, 0.5 μm.

vesicle transport in megakaryocyte processes may be comparable to the movement of vesicles along neurite microtubules (Cheng and Reese, 1987; Pfenninger and Bunge, 1974; Pfenninger and Maylie-Pfenninger, 1981*a*,*b*; Spooner *et al.* 1974). On the basis of preliminary studies with integrin antibodies, we speculate that during megakaryocyte differentiation, integrin receptors may be organized along the surface of the pseudopod at sites that designate fusion. Once anchored at these sites, coalescence of vesicles could occur by a mechanism analogous to the organization of the membrane–microfilament interaction of endothelial cells and fibroblasts by their vitronectin and fibronectin receptors (Dejana *et al.* 1988). According to this model, membrane fusion would also be possible in CD-treated megakaryocytes, which still contain some membrane-associated thin filaments.

Although a number of theories of platelet production have been proposed, this is the first report of megakaryocyte fragmentation and platelet production that defines the interactions of the cytoskeleton during this process. Our model for platelet formation consists of several steps, the first of which is the adherence of cells to the extracellular matrix, accompanied by the distension of the DMS. The second step is the formation of thin pseudopodia that are not tightly adherent to the extracellular matrix. This process involves the rearrangement of the actin-rich peripheral zone, which permits microtubule elongation and/or reorientation. The third step is microtubule-driven pseudopod formation and movement of the DMS and granules into the processes. The final step in platelet formation is the pinching off of pro-platelets and individual platelets by coalescence of the DMS-related vesicles and their fusion with the plasma membrane. This is a most intriguing and unusual observation, and although previous models have described the rearrangement of membranes during platelet formation, vesicle coalescence and fusion have not been previously reported.

Radley and Haller (1982) have proposed a 'flow' model of platelet formation in which the DMS is the source of the additional membrane necessary for pseudopodia formation. Our studies support this part of their model. However, they also propose that platelets are formed by a mechanism similar to cytokinesis, in which the actin-rich cleavage furrow separates daughter cells. This is clearly not the case in our culture system. Furthermore, treatment of megakaryocytes with CD does not block fragmentation, but actually shortens the time course of platelet formation. It has been suggested, on the basis of the freeze-fracture studies of megakaryocytes and platelets by Zucker-Franklin and Peterssen (1984), that the DMS alone forms the platelet plasma membrane and is distinct from the megakaryocyte plasma membrane. These proposals remain controversial, however, and we suggest that the DMS is actually acting in a dual fashion in platelet formation. First, part of the widened DMS fuses with the megakaryocyte plasma membrane to provide increased membrane for pseudopodia; and second, additional DMS vesicles move down the pseudopodia to align and to fuse with the plasma membrane, eventually forming platelets. This fusion of DMS vesicles may account for the similarity of DMS and platelet plasma membrane intramembranous particle distributions as seen by Zucker-Franklin and Peterssen (1984). We anticipate that much of what governs megakaryocyte morphogenesis is related to the interactions of the actin and microtubule systems with megakaryocyte integrins and with the extracellular matrix. We are currently pursuing these investigations in order to

understand more clearly the role of these adhesion proteins in the regulation of platelet formation.

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