Evidence for An Involvement of Actin in the Positioning and Motility of Centrosomes

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ABSTRACT Cultured human polymorphonuclear leukocytes exposed to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) spread on the substratum and undergo centrosome splitting. The two centrioles may separate by a distance of several micrometers, each being surrounded by an aster of microtubules. Here we show that the centriole/aster complexes are in constant, rapid motion through the cytoplasm, carrying with them some of the cytoplasmic granules while pushing aside others, or deforming and displacing the nucleus. An analysis of this unique motility phenomenon was undertaken. We show that intact microtubules are required for TPA-induced centrosome splitting and aster motility, but not for cell spreading. More importantly, disruption of the actin network inhibits both centrosome splitting and cell spreading, and even reverses splitting (induces convergence and fusion of asters) in polymorphonuclear leukocytes pretreated with TPA alone. These observations indicate the existence of a dynamic relationship between microtubules and actin networks and provide evidence for a role of actin in determining the position of the centrosome by way of interaction with the microtubules radiating from it.

As early as 1883, van Beneden realized that a line through the centrosome and the center of the nucleus establishes an axis that gives a cell a polar organization (34). Recently, the idea of the centrosome as a determinant of cell polarity has received renewed interest when it was discovered that some cell types apparently relocate the centrosome in response to certain stimuli, thereby reorienting the cell axis. Thus, migrating 3T3 cells, polymorphonuclear leukocytes (PMNs), and newt eosinophils are reported to position the centrosome between the nucleus and the leading lamella (1, 6, 15, 18). In cultured neuroblastoma cells, neurite outgrowth and, therefore, initiation of cell polarity, is associated with an aggregation of its many nucleation sites (centrioles) from a loose distribution around the nucleus to a cluster in the region of the axon hillock behind the neurite (31). Finally, cultured endothelial cells migrating into an in vitro wound shift the centrosome towards the cell side facing the wound and concomitantly reorganize the microtubule system (9, 10, 16). Despite some reports to the contrary (3, 21, 30), these and other observations were taken as evidence that cell polarity and the direction of cell migration are determined by the position of the centrosome and the arrangement of the microtubules associated with it.

However, precisely how the centrosome receives the information to set up cell polarity and communicates it to the microtubules, and how the microtubules transmit this information to the rest of the cell, is not understood. Evidence from both in vivo and in vitro studies indicates that microtubules can interact with both actin filaments and intermediate filaments (e.g., references 8, 11, 24, and 26). These interactions are likely to be of importance for many cellular activities, conceivably including those leading to cell polarity determination and directional migration. It needs to be considered, however, that if such interactions occur, they can work both ways: not only can microtubules communicate information about cell polarity and asymmetry to the rest of the cytoskeleton, but the latter, particularly the dynamic actin-based network, is likely to influence the microtubule system. The question of cause and effect is central to the issue of cell polarity determination, but at present it cannot be answered clearly on the basis of the available evidence.

In this study, we took advantage of a unique experimental model that allowed us to investigate the dynamic relationship between microtubule organization and actin-based contractile networks. Use is made of human PMNs in which centrosome splitting (27) was induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). We show here that...
centrosome/microtubule complexes in TPA-treated cells are in constant, rapid motion, and that their movement depends upon, and apparently is directed by, the contractile machinery of the cell. Our observations indicate a role for actin-based contractile networks in determining the position of the centrosome and the spatial organization of microtubules, and we discuss the possible implications of these findings for the current understanding of the role played by microtubules in directional cell migration.

MATERIALS AND METHODS

Source of Cells: The PMNs used in this study were derived from freshly drawn venous blood of six healthy adults between the ages of 25 and 37. No statistically significant differences in the frequency of TPA-induced centrosome splitting was detected between samples derived from different individuals.

Preparation of PMNs: Monolayers consisting mostly of PMNs were prepared by the clot procedure (22). A drop of blood was added to clot in a cova- slip for 40-45 min at 37°C in a moist chamber. The clot was rinsed off with Gey's tissue culture medium supplemented with 10% human AB serum and placed in a sterile 35-mm plastic tissue culture dish containing Gey's medium with 10% serum. In these preparations, PMNs constituted 85-90% of the cells. They were identified on the basis of cell size, nuclear shape, and granule content. Only cell types in these preparations include monocytes, eosinophils, lymphocytes, platelets, erythrocytes, and macrophages; platelet aggregates were excluded.

Experimental Treatment: PMNs on coverslips were exposed to TPA (20-50 ng/ml), nocodazole (2-10 µg/ml), colcemid (5 µg/ml), or cytochalasin D (0.5-2 µg/ml), either alone or in one of the following combinations: TPA for 25 min, followed by nocodazole or cytochalasin D for an additional 30 min in the presence of TPA; TPA for 25 min, followed by cytochalasin D for 2-60 min in the presence of TPA; nocodazole or cytochalasin D for 25 min, followed by nocodazole or cytochalasin D for 25 min, followed by TPA for 25 min. The compounds were added from stock solutions made up in dimethyl sulfoxide at the following concentrations: TPA, 1 mg/ml; nocodazole, 2 mg/ml; colcemid, 5 mg/ml; and cytochalasin D, 2 mg/ml. The final concentration of dimethyl sulfoxide did not exceed 0.11%, which, when used alone, had no detectable effect on the morphology and behavior of PMNs.

Recording of Cell Behavior: Coverslips with attached PMNs were mounted on glass slides using spacers derived from No. 1 coverslips, sealed with VALAP (vaseline + lanoline + paraffin at 1:1:1), and placed on the heated stage of a Leitz Aristoplan microscope equipped with a 65 video camera (Dage-MTI Inc., Michigan City, IN) with a newvicon tube, which was used for real-time recording of the movement of the PMNs on the coverslips. The movement of the PMNs was recorded at a magnification of the cell of 250 x. Micrographs were taken at 0.5-5 sec intervals, and the images were stored on a video tape. The video tape was then analyzed by computer (MicroVide, Barco Photo, Belgium) to determine the number of movements and the duration of each movement.

RESULTS

 Movements of the Centrosome/Microtubule Complex

Treatment of randomly locomoting PMNs with TPA induces a "fried egg" morphology with the lobed nucleus usually occupying a central position (28). A study of the behavior of TPA-treated PMNs by time-lapse video microscopy at high magnification shows a wealth of cytoplasmic movements that fall into three categories: (a) ruffling activity in the cell periphery and upper cell surface; (b) saltatory movement of cytoplasmic particles or vesicles; and (c) an unprecedented form of curvilinear movement of cytoplasmic domains or entities. The latter form of movement was observed in several hundred cases. On the basis of the following four lines of evidence, the movement of these entities is identified as a representation of the movement of the centrosome/microtubule complexes: (a) two or three such motile cytoplasmic domains are found per cell, at a frequency that roughly corresponds to that of centrosome splitting after TPA treatment; (b) their movement is abolished by treatment with microtubule inhibitors; (c) the pathways of saltatory movements are centered upon such motile cytoplasmic domains and displace along with them; and (d) correlative light microscopic and fine structural analysis. The latter provides the most direct evidence for the congruence of motile domains with aster centers. For these studies, cells attached to gold grids were video-taped for time periods of 3-8 min to follow the movement of presumptive centrosome/microtubule complexes, quickly lysed in microtubule stabilizing buffer while still under microscopic observation, and processed for whole-mount electron microscopy. In all ten cases examined so far, the exact position of the microtubule asters in the fixed cell could always be predicted with remarkable accuracy from the recording of the live cell before fixation (Fig. 1). Thus, even...
though the asters themselves are not visible in the light microscope, their movements can easily be followed in live cells after TPA treatment. Unfortunately, these movements are difficult to document in a series of still photographs, but they are clearly seen in time-lapse recordings (cf. video presentation at the 24th ASCB Meeting, reference 7a). The moving asters are seen to push aside, drag along, or displace nuclear lobes or other cytoplasmic organelles, and to approach the cell periphery within a few micrometers. The average rate of aster movement, whose characteristics are similar to those described by Bessis (5), is ~0.2 μm/s, but it may reach up to 0.6 μm/s. Frequently, asters may also remain on one location for time periods of several tens of seconds. Thus, the motile "entities" seen in live cells are identical with centrosome/aster complexes, and these asters are in rapid motion in many cells.

**Mechanism of Centrosome Splitting and Motility**

We have studied the structural basis of centrosome/aster movements to elucidate how microtubular arrays might interact with the rest of the cytoskeleton. The chief assay used was immunofluorescence microscopy of centriole location. Although in this assay the parameter tested was reduced to the assessment of centriole position and "splitting" or "no splitting", it is a convenient and quantifiable test that allows us to survey large numbers of cells. Video microscopy of live cells and stereo high voltage electron microscopy were used in all experiments as essential supplementary techniques.

The effects of TPA alone or TPA and nocodazole or cytochalasin D are illustrated in Fig. 2 and are summarized in Table I. In agreement with reports by others (3), centrioles in randomly locomoting cells are located close to the nucleus in the cell center (Fig. 2, a and b), and are in close proximity to each other (Table I). Treatment with TPA at 50 ng/ml consistently induces centrosome splitting in 60–70% of the PMNs (Fig. 2, c and d; Table I). The effect of nocodazole on the frequency of centrosome splitting depends on the time at which this compound is administered. When cells are first treated with nocodazole for 20 min, followed by TPA for an additional 25 min in the presence of the drug, centrosome

![Figure 1](https://example.com/figure1.png)

**Figure 1** Correlation analysis of aster motility in a living cell and fine structure of the same cell as seen by whole-mount high voltage electron microscopy. (a) Tracing of the movements of the two asters in a TPA-treated cell over a time period of 125 s, taken from a time-lapse video recording. Numbers are times in seconds. (b) Last frame of that tracing (2:05 min), photographed from the video monitor using a Ronchi ruling. 2 s later, the cells were flushed with extraction medium (0.1% Triton X-100 in PHEM buffer). X 1,500. (c) Low power high voltage electron micrograph of the extracted cell after whole-mount preparation. X 1,500. (d) Stereo pair of the central cell area, showing the position of the two asters, each centered upon one centriole. The position of these asters corresponds precisely to that predicted from the video recording. X 4,200.

| Table 1 | Separated Centrioles in Human Neutrophils Treated with TPA and Nocodazole |
|---------|-------------------------------------------------|
| Split   | * Split | 1-3 μm | Not split | Not determined | Noc |
|         | %       | %      | %         | %             | %   |
| Random locomotion | 1.1 | 3.0 | 92.0 | 3.9 | 263 |
| TPA     | 71.0 | 5.0 | 17.5 | 6.5 | 175 |
| Noc → TPA | 0.5 | 5.5 | 85.0 | 9.0 | 154 |
| TPA → Noc | 76.0 | 0.5 | 23.0 | 0.5 | 171 |

Nocodazole (Noc): 1 μg/ml, 20 min. TPA: 50 ng/ml, 25 min. * Percentage of cells in which the state of centrioles could not be determined.
FIGURE 2  Corresponding phase contrast and immunofluorescence micrographs of centriole staining under various experimental conditions. (a and b) Random locomotion; (c and d) TPA treatment for 50 min at 40 ng/ml; in one of the two cells the centrioles are separated; (e and f) nocodazole (1 μg/ml) for 25 min, followed by TPA for 25 min in the presence of nocodazole; (g and h) TPA for 25 min, followed by 2 μg/ml cytochalasin D for 30 min in the presence of TPA; centriole pair is exactly in the center of the spread cells; (i and j) cytochalasin D for 30 min, followed by TPA for 25 min; cell spreading normally induced by TPA is completely inhibited, and centriole separation does not occur. Arrows indicate position of the centrioles. All micrographs x 1,000.

splitting does not occur, even though cells spread and develop a “fried egg” morphology (Fig. 2, e and f). In cells first treated with TPA and followed by nocodazole, the frequency of splitting is identical to that of cells treated with TPA alone (Table I). The complete absence of microtubules in these cells was confirmed by thin section and whole mount electron microscopy (Fig. 3). In both experimental conditions, no aster motility was observed in living cells. From this we conclude that intact microtubules are required to induce centrosome splitting upon TPA treatment, and that once splitting has occurred, microtubule disassembly does not affect the position of the split centrosomes.

A different pattern emerges when cells are treated with cytochalasin D. This compound prevents centrosome splitting when administered before TPA treatment; most interestingly, however, it reverses splitting in cells pretreated with TPA (Table II). Under both experimental conditions, the centriole pair occupies a position corresponding to the geometric center of the cell (Fig. 2, g-j). When live cells treated with TPA, followed by cytochalasin D, are observed by time-lapse video...
FIGURE 3 Thin sections of PMNs treated with 40 ng/ml TPA for 1 h (a), or TPA followed by 5 μg/ml nocodazole for 25 min in the continued presence of TPA (b). In a, the centrioles (arrows) are still relatively close together, and many microtubules radiate from the centrosomal area. No microtubules are found near the centrosome (b) or elsewhere in the cytoplasm after nocodazole treatment. × 22,000.

microscopy, they show just one aster precisely in the cell center. Cytoplasmic granules are radially aligned around it and undergo saltatory movement that follows radial pathways (7a). The aster center either does not move at all, or it performs a minimal “rocking” motion not exceeding 1–3 μm from its central position.

Cytochalasin D–induced reversal of centriole separation in TPA-treated cells occurs over a time period of 30–60 min (Table III). Initially, centrioles gradually come closer together until, at later time points, the vast majority of cells shows centrioles to be in very close proximity of each other in the cell center. Observation of live cells during cytochalasin treatment reveals a gradual, time-dependent decrease in the motile activity of asters that eventually become located in the cell center. These changes coincide with decreasing ruffling activity in the cell cortex.

High voltage electron microscopy of detergent-extracted whole mount preparations of cytochalasin D–treated cells shows marked changes in both the appearance of the actin-based filament network and the distribution of microtubules (Fig. 4). Cytochalasin treatment disorganizes the highly ordered cortical actin network. Ruffles are replaced by a shredded filament felt that is most prominent in the cell periphery. In cells treated with TPA alone, microtubules closely approach the cell margin and are embedded in, or become an integral component of, the peripheral three-dimensional filament network. Often they are curved, bent, or even seem to meander through the network (Fig. 4b; see also reference 28). In cytochalasin-treated cells (Fig. 4c), microtubules extend relatively straight into the cell periphery, pointing at the cell perimeter at more or less right angles. Their interaction with the disrupted filament network is much reduced.

As shown in Tables I and II, nocodazole treatment before administration of TPA and cytochalasin D treatment both before or after TPA treatment completely inhibit centrosome splitting. We have sought to determine whether removal of the microtubule or actin-active compounds will reverse this inhibitory action. Table IV demonstrates that in cells recovering from inhibitor treatment for 45 min in the presence of TPA, centrosome splitting is indeed initiated again. These experiments demonstrate that the inhibitor treatments were not damaging to the cells and that the TPA treatment per se did not affect the ability of the PMN’s cytoskeleton to respond to the action of perturbing agents. Similar observations were also made in experiments on cultured cells (29).

| TABLE II | Separated Centrioles in PMNs Treated with TPA and Cytochalasin D |
|----------|---------------------------------------------------------------|
|          | Split                                                        |
|          | >3 μm 1-3 μm Not split Not determined No.                   |
|          | %     | %     | %    |                  |
| TPA      | 65.9  | 2.6   | 24.1 | 7.3               | 151    |
| CD 1 μg/ml → TPA | 0.5  | 11.5  | 88.0 | 2.0               | 186    |
| CD 2 μg/ml → TPA | 0   | 0.5   | 97.0 | 2.5               | 147    |
| TPA → CD 1 μg/ml | 9.0 | 32.0  | 52.0 | 7.0               | 251    |
| TPA → CD 2 μg/ml | 4.0 | 20.0  | 73.0 | 3.0               | 191    |
| Cytochalasin D (CD): 1 and 2 μg/ml, 25 min, TPA: 50 ng/ml, 25 min. |

| TABLE III | Time Course of Cytochalasin D Effect on Centriole Separation |
|------------|-------------------------------------------------------------|
|            | Split                                                        |
|            | >3 μm 1-3 μm Not split Not determined No.                   |
|            | %     | %     | %    |                  |
| TPA        | 64.5  | 5.9   | 22.1 | 7.1               | 239    |
| TPA → 1 μg/ml CD | 60.6 | 9.1   | 24.8 | 5.5               | 165    |
| 5 min      | 60.6  | 9.1   | 24.8 | 5.5               | 165    |
| 10 min     | 49.7  | 18.8  | 21.8 | 9.7               | 166    |
| 20 min     | 29.7  | 24.1  | 40.5 | 5.7               | 158    |
| 30 min     | 15.1  | 25.8  | 50.3 | 8.8               | 159    |
| 60 min     | 6.0   | 4.9   | 77.0 | 11.8              | 144    |
| TPA: 50 ng/ml, 25 min. CD, cytochalasin D. |

The effects of the various experimental treatments on centrosome splitting and microtubule organization are diagrammatically summarized in Fig. 5.

DISCUSSION

Centrosome splitting and aster movement in TPA-treated PMNs was used as an experimental model to study the interrelationship of the centrosome/microtubule complex and actin networks. The value of the model lies in the fact that centrosome/aster activity is uncoupled from cell translocation, thus allowing a study of the cellular basis of aster motility per se. This study suggests that an actin-containing cellular component is involved in the positioning and activity of the centrosome, presumably by way of interaction with the mi-
TABLE IV

Recovery from Nocodazole and Cytochalasin Treatment

| Split | >3 μm | 1-3 μm | Not split | Not determined | No. |
|-------|-------|--------|-----------|---------------|-----|
| TPA   | 58.0  | 2.7    | 35.8      | 4.5           | 367 |
| Noc → TPA | 1.5 | 2.6    | 88.0      | 7.9           | 193 |
| Noc → TPA + rec | 52.2 | 1.5    | 38.7      | 7.6           | 257 |
| CD → TPA | 0    | 1.5    | 93.6      | 4.9           | 135 |
| CD → TPA + rec | 42.1 | 10.5   | 39.1      | 8.3           | 399 |
| TPA → CD | 2.0  | 6.4    | 85.8      | 5.8           | 312 |
| TPA → CD + rec | 40.6 | 14.4   | 37.1      | 7.9           | 355 |

TPA: 50 ng/ml, 25 min. Nocodazole (Noc): 1 μg/ml, 20 min. Cytochalasin D (CD): 2 μg/ml, 25 min. Recovery (rec): 45 min (in the presence of 50 ng/ml TPA).

FIGURE 4 (a) Cytoskeletal organization of PMNs treated with TPA followed by 2 μg/ml cytochalasin D for 30 min. One half of the cell is shown as a stereo pair. Both centrioles are located in the cell center (arrow; not discernible at this magnification). Microtubules (arrowheads) are more or less straight and radiate into the cell periphery marked by a completely disrupted actin felt. × 6,000. (b) Higher magnification of microtubules (arrowheads) in the cell cortex of a TPA-treated PMN. Microtubules are embedded in the three-dimensional filament network and appear to meander through it. (c) Cell treated with TPA, followed by cytochalasin D. Few structural connections of the microtubules to the shredded actin filament network are evident. × 33,000.

FIGURE 5 Summary diagram of the observations on microtubule organization in PMNs under different experimental conditions. Numbers shown are the percentage of cells with separated centrioles as seen in immunofluorescence microscopy. Centrioles are represented by a bar and a dot, and microtubules are shown as solid lines. Intact and disrupted cortical actin filament networks are depicted as cross-hatched and stippled areas, respectively.
microtubules radiating from it. We show that microtubules are required for splitting to occur, and that an intact actin-based contractile network, presumably residing in the cell cortex, both initiates centrosome splitting and supports centrosome motility later on. It is very likely that the same mechanisms operate during transient centrosome splitting in the initial phase of a chemokinetic stimulus (27).

On the basis of the evidence presented in this paper, the following scenario can be envisioned to explain centrosome splitting and aster motility. In the initial phase of cell stimulation by either TPA or a chemoattractant, activation of the cell cortex leads to cell spreading and flattening, which in turn is likely to exert a force on the microtubules which extend into, and are embedded in, the peripheral filament network. That the force for splitting is exerted on the microtubules and not directly on the centrosome is indicated by the observation that microtubule disruption prevents TPA-induced splitting. The expanding cortex then literally pulls the microtubule aster apart, leading to the formation of mostly two, occasionally three, independent aster centers. The split condition is maintained as long as the cell cortex remains spread and active; it is reversed when chemokinetically stimulated, flattened cells return to a normal migratory morphology (27) or upon experimental disruption of the actin network (this paper). The simplest explanation of the cytochalasin experiments is that the microtubule asters, when released from forces normally exerted on their microtubules by the dynamic actin network, assume the most stable position at which all the microtubules are minimally bent and are of more or less equal length. This position would correspond to the geometric center of the cell. It is gradually reached by the asters in the course of cytochalasin D treatment as the functional integrity of the actin network is increasingly impaired. While these findings on the relationship between microtubule asters and actin networks were obtained on essentially nonpolar cells, we believe that they are of relevance for considerations of the role of microtubule asters in polar cells. In polar, migratory cells, a dynamic interaction between the microtubule system and the contractile machinery is likely to exist as well. Several pieces of experimental evidence support the notion that the cell cortex plays an important, if not dominant role in such cells. Leukocyte fragments derived from microtubule and centriole-free lamellipods are capable of directed locomotion and chemotaxis (14, 17). Colchicine-treated PMNs still can orient and migrate in a chemotactic gradient, although they may show wider angles of turn and appear to move more sluggishly (2, 4, 13, 35). Microtubule-free fragments of anterior lamellae of fish epidermal cells move with the same degree of persistency as intact cells (7). Finally, reorientation of the centrosome and the microtubules in wounded epithelial sheets is inhibited, at least initially, by cytochalasin B (10). These observations are consistent with the idea that the stimulus that causes the cell to assume a polar migratory morphology is received at the cell membrane and transmitted to the cell cortex, which, in turn, influences the position of the centrosome/microtubule complex. This chain of events depends upon the ability of actin filaments and microtubules to interact with each other, an assumption supported by both biochemical and structural evidence (11, 12, 20, 25).

The apparent importance of the cortex for polar organization does not mean that microtubules are dispensable and do not take part in directional locomotion. Rather, their role has to be re-defined: instead of setting up polarity, they serve to reinforce polar organization set up by the cell cortex. One possibility discussed in some detail by Trinkaus (32) is that the microtubules become passively oriented by the cell cortex, which will help stabilize the cortically determined asymmetry; thus, in a process of mutual reinforcement, cell polarity is stabilized. Once the centrosome/microtubule complex is positioned behind the leading lamella, it tends to exert a constraining influence on the cell’s ability to turn (23) and also serves to stabilize the bulk of the cytoplasm (17).

In summary, the evidence presented in this paper suggests that the contractile machinery affects microtubular organization and motility. This finding may lead to a re-interpretation of the role of the centrosome/microtubule complex in directional cell migration. Rather than being the determining factor in cell polarity, they may serve to reinforce the asymmetry set up by the cortical contractile machinery of the cell.

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