Microtubule-organizing Centers and Cell Migration: Effect of Inhibition of Migration and Microtubule Disruption in Endothelial Cells

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ABSTRACT We have previously shown that microtubule-organizing centers (MTOC's) become preferentially oriented towards the leading edge of migrating endothelial cells (EC's) at the margin of an experimentally induced wound made in a confluent EC monolayer. To learn more about the mechanism responsible for the reorientation of MTOC's and to determine whether a similar reorientation takes place when cell migration is inhibited, we incubated the wounded cultures with colcemid (C) and cytochalasin B (CB), which disrupt microtubules (MT's) and microfilaments (MF's), respectively. The results obtained showed that the MTOC reorientation can occur independent of cell migration since MTOC's reoriented preferentially toward the wound edge in the CB-treated cultures, even though forward migration of the EC was inhibited. In addition, the MTOC reorientation is inhibited by C, indicating that it requires an intact system of MT's and/or other intracellular structures whose distribution is dependent on that of MT's.

In animal cells, many cytoplasmic microtubules (MT's) emanate from the centrosome, which consists of a pair of centrioles and associated material forming the microtubule-organizing center (MTOC). The MT's assembled in this region are thought to be responsible for the maintenance of an asymmetric cell shape, and to control the distribution of other cell organelles in the cytoplasm (1–5). We have previously shown that when stationary endothelial cells (EC's) start to translocate, the MTOC becomes positioned in front of the nucleus towards the direction of migration of the endothelial cell (EC) sheet (6). Others have shown that the MTOC's in single migrating 3T3 cells are preferentially located toward the front end of the cell (7). A reorientation of the MTOC similar to that in EC's (6) has been reported in polymorphonuclear leukocytes migrating towards a chemotactic stimulus (8) and more recently in sheets of migrating fibroblasts (9).

The purpose of the present studies was to determine (*a*) whether the MTOC reorientation occurs independently or as a result of cell movement, and (*b*) whether it requires intact MT's and/or microfilaments (MF's). Using cytochalasin B (CB) to disorganize the MF's and inhibit cell migration, we have shown that after induction of the wound the MTOC reorientation occurs even when cell migration is inhibited. We also show that this reorientation is inhibited by colcemid (C), an agent that causes MT's to depolymerize, a finding that suggests that the reorientation requires the presence of MT's and/or other intracellular structures whose distribution is dependent on that of MT's.

MATERIALS AND METHODS

Cell Culture: The harvesting and culturing of EC's from the porcine thoracic aorta has been previously described in detail (10). EC's subcultured from one to three times were used for the experiments.

In Vitro Wounds: Confluent monolayers of EC's grown on glass coverslips were mechanically wounded with a flat-edged Teflon spatula so as to remove the cells from one-half of each 22 × 44-mm glass coverslip as previously described (9). The cultures were examined at various times after wounding.

Immunofluorescence: Fixation of EC's with methanol and acetone and indirect staining with fluorescein goat anti-rabbit IgG (Hyland Diagnostics, Costa Mesa, CA) after incubation with antisera to tubulin (11) and to myosin (12) and tropomyosin (13) have been previously described (14).

Colcemid and Cytochalasin Treatment: Colcemid (C) (No. 890-3014 Gibco Laboratories, Grand Island NY) at a final concentration of 0.75 μg/ml in PBS or Cytochalasin B (CB) in DMSO (dimethyl sulfoxide, Fisher Scientific Co., Pittsburgh, PA) (Sigma No. C-6762, Sigma Chemical Co., St. Louis, MO) at a final concentration of 2.5 μg/ml was added to the EC culture 1 h before wounding. Time-lapse cinemicrography showed that these concentrations were the lowest that inhibited the forward migration of cells. After the monolayer was wounded, the cells were further incubated with C or CB and observed with time-lapse cinemicrophotography over a 22-h period. At various

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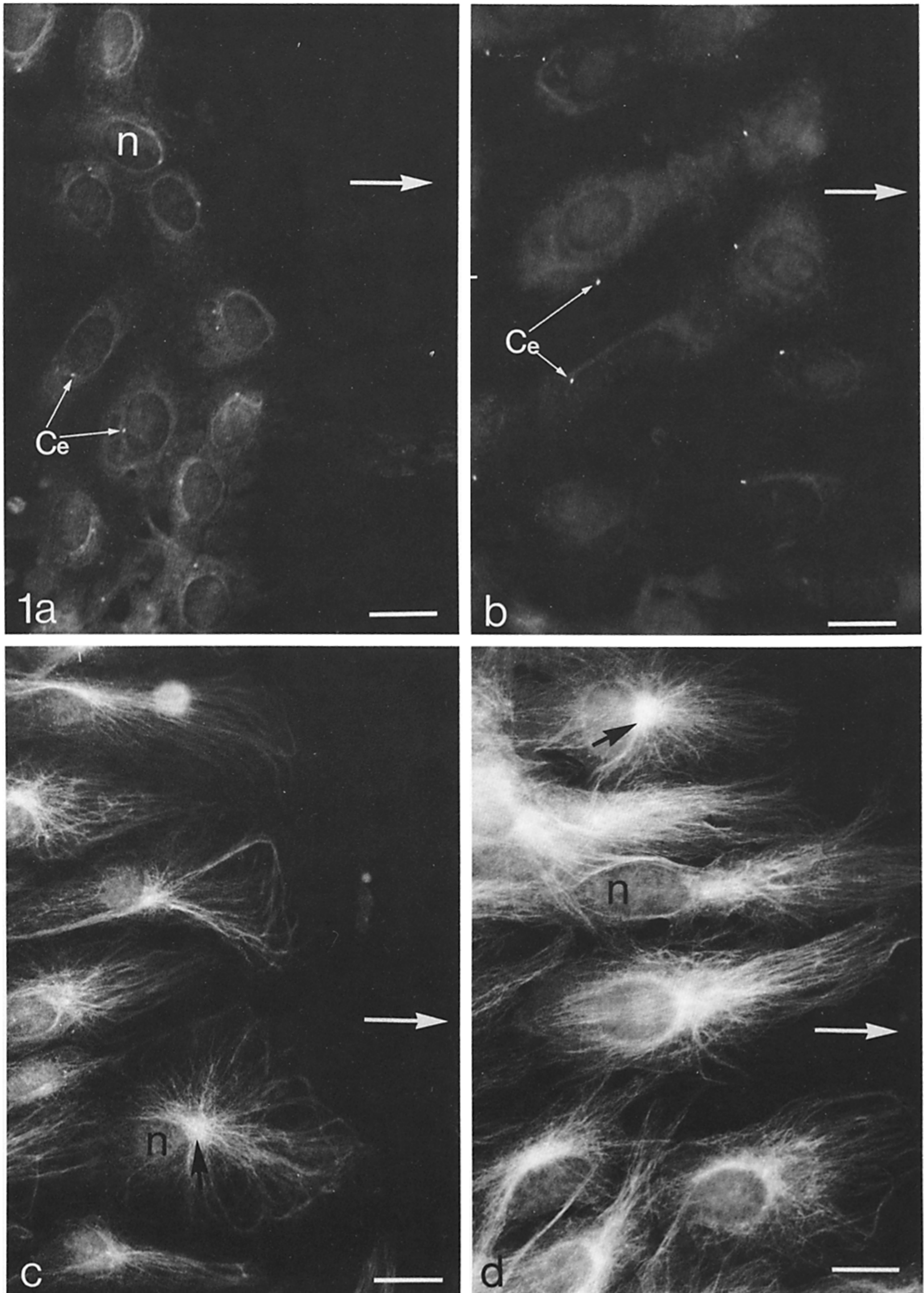

FIGURE 1 Immunofluorescent staining of EC's in wounded cultures treated with C and then fixed and stained with antitubulin serum (*a*) 4 h and (*b*) 22 h after wounding. Note loss of MT's and presence of stained centrioles (*Ce*) that are randomly distributed relative to the wound edge. Similarly stained EC's fixed at (*c*) 5 h and (*d*) 9 h after C has been washed out in a wounded culture previously treated for 4 h with C. Note that the MT's have now reassembled and that the MTOC's (*arrow*) are preferentially oriented toward the wound edge. The large arrow is placed perpendicular to the wound edge and indicates the direction of movement of the EC sheet after removal of C. *n*, nucleus. Bar, 20 μm. $\times$ 6,500.

times the cells were fixed and processed for immunofluorescence. The distribution of the MTOC's visualized by staining with antisera to tubulin was determined as previously described (6). MTOC's in cells in the first and second rows along the wound edge were counted separately. Wounds incubated with CB were also stained with antisera to myosin and tropomyosin to visualize the distribution of microfilaments. In some cultures, the C and CB were removed at various time intervals by washing three times with PBS and adding new growth medium to the culture dishes. The cells were then incubated in normal growth medium and fixed at different time intervals after removal of the drug. 200 cells were counted in both the first and second row of cells on each wounded-monolayer coverslip. Three sets of wounds were counted at each time point, and three separate experiments were carried out for each experimental condition.

Cinemicrophotography: Time-lapse cinemicrography was carried out to observe the behavior of cells after wounding in the presence and absence of drugs as previously described (6). Cultures were photographed with a 20X phase-constrast objective and a ¼ relay lens on a Nikon Inverted Microscope Model M. The extent of cell movement, ruffling activity, and the change in cell shape were observed.

RESULTS

Effect of Colcemid

Examination of the C-treated cultures of EC's with time-lapse cinemicrography over a period of 22 h showed that there was complete inhibition of forward cell migration at the wound edge during this period. Although there was no translocation, considerable ruffling and extension and withdrawal of lamellipodia into the wounded region, however, did occur in the EC's lining the wound edge. Examination of cells at the wound edge by immunofluorescence with antiserum to tubulin showed a complete disruption of the microtubular network, and only an occasional single MT was seen in the cytoplasm in the C-treated cultures. The position of MTOC's in these cells could still be identified, however, by the staining of the centrioles (Fig. 1 *a* and *b*). Examination of the position of the centrioles in cells along the wound edge 4 h after wounding showed (Table I) that in 28% of the cells in the first row the centriole was located towards the wound edge, i.e., between the nucleus and the wound edge, in 34% away from the edge, i.e., between the nucleus and the monolayer behind the cell, and in 38% in the middle, i.e., along the side of the nucleus neither clearly toward nor away from the wound edge. The distribution of myosin and tropomyosin in C-treated cells as determined by immunofluorescent staining was similar to that in untreated cells.

REVERSAL OF COLCEMID TREATMENT: New cytoplasmic microtubules arising from the centriolar area were detected by immunofluorescence microscopy within the first hour after removal of C. After 5 h, all of the cells showed an extensive, apparently normal network of MT's (Fig. 1 *c*). Table II shows the position of MTOC's in the EC's at the wound edge at different time intervals in normal growth medium after 4 h in C treatment. Immediately after the change from C to normal growth medium, the MTOC's were still randomly distributed relative to the wound edge. The number of cells with MTOC's located towards the wound edge increased slowly over a 9-h period (Fig. 1 *d*) and reached a value of 64% (Table II). At the same time, the number of MTOC's located away from the wound edge decreased. EC migration began to occur about 5 h after C removal, indicating that the reorientation of MTOC's occurred only after the reappearance of most of the cytoplasmic microtubles and coincided with the onset of EC migration.

Effect of Cytochalasin B

Time-lapse cinemicrography during exposure of EC's to a concentration of 2.5 μgm/ml of CB for 4 h showed no forward movement of cells at the wound edge during this period, although some ruffling activity was detected. During longer periods of treatment with CB, mild arborization was evident in some of the cells and occasionally a cell at the wound edge showed a long process extending along (Fig. 2 *b*) or into the wounded area. Examination by time-lapse cinemicrography showed that cell migration into the wounded area was still inhibited in the cultures after 22 h of treatment with CB. Immunofluorescent staining for myosin and tropomyosin with the corresponding antiserum showed that, although the majority of cells retained microfilament cables after CB treatment, some of the fibers were smaller and stained less intensely. Use of DMSO, the solvent for CB, as a control did not affect cell shape, MTOC distribution, or cell migration.

Immunofluorescent labeling with antiserum to tubulin showed that MT's had remained intact after the CB treatment (Fig. 2 *a*, *b*, and *c*). MT bundles could be observed in all cells. As in the untreated cultures, MT's were more concentrated in the MTOC region.

Table III shows the distribution of the MTOC's in the cells at the wound edge at different time-periods after addition of CB. While only ~36% of the cells had their MTOC's facing the wound edge 4 h after addition of CB, this number did increase to 62% in 8 h and remained at about this level even after 22 h of CB treatment. Thus, although the reorientation of MTOC's is slower in CB-treated cultures and does not quite reach the levels seen in untreated control cultures, such reorientation does nevertheless take place in the first row of cells even when forward migration of the cells is inhibited.

In control cultures without drug treatment, significant reorientation of MTOC's takes place in the first as well as the second row along the wound edge (Fig. 2 *d*). For example, 74% of the cells in the first row and 68% of the cells in the second row from the wound edge had their MTOC's facing the wound edge 22 h after wounding (Table III). In CB-treated cultures, although significant reorientation could be detected in the first row along the wound edge, a similar reorientation failed to occur in the second row (Fig. 2 *c*) (Table III). While 62% of the cells in the first row of CB-treated wounds had their MTOC's

TABLE I

Effect of Colcemid on MTOC Reorientation in EC'S Along the Wound Edge

	Time after wounding			
	4 h		22 h	
	Colcemid X* SE	Control X SE	Colcemid X SE	Control X SE
Toward	28 ± 3.0	80 ± 1.5	38 ± 1.3	77 ± 2.0
Away	34 ± 3.5	12 ± 1.7	28 ± 0.1	10 ± 1.5
Middle	38 ± 1.0	8 ± 3.0	34 ± 1.3	13 ± 3.3

* Mean of three experiments.

TABLE II

Distribution of MTOC'S after Release from Colcemid Treatment in EC'S Along the Wound Edge

	Time after release from colcemid treatment		
	0 h	5 h	9 h
	X* SE	X SE	X SE
Toward	31 ± 1.0	57 ± 1.2	64 ± 3.8
Away	30 ± 1.0	16 ± 4.0	8 ± 2.3
Middle	39 ± 1.3	27 ± 5.0	28 ± 3.1

* Mean of three experiments.

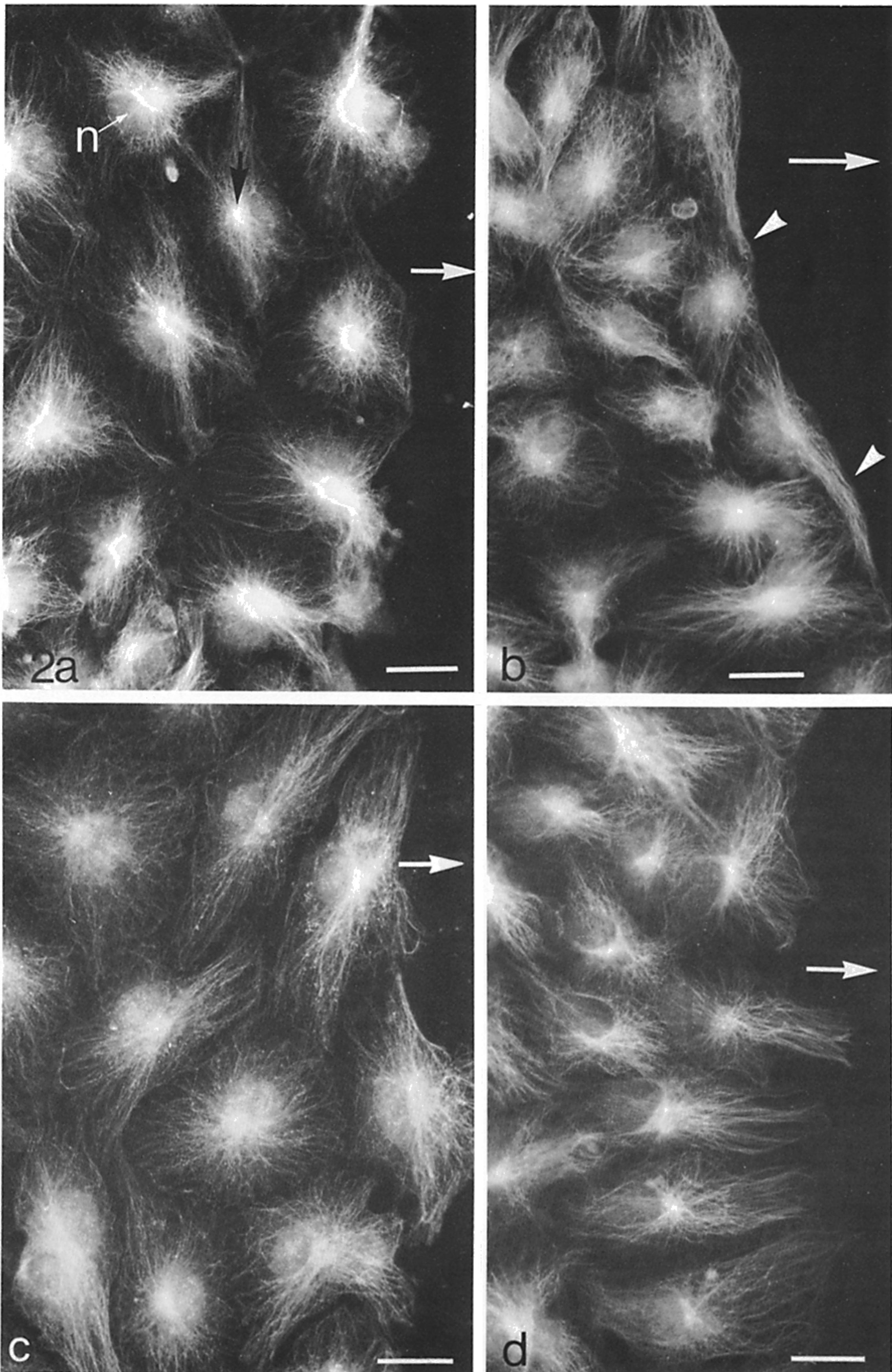

FIGURE 2 Immunofluorescent staining of EC's in wounded cultures treated with CB and then fixed and stained with antitubulin serum (*a*) 4 h, (*b*) 8 h, and (*c*) 22 h after wounding. Note that the MT's are still present after the CB treatment and that progressively more MTOC's (*short arrow*) in cells in the first row become oriented towards the wound edge. In contrast, MTOC's in the second row of EC's remain randomly distributed relative to this edge. *Arrowheads* in *b* indicate long processes of cells extending along the wound edge. In the untreated control cultures (*d*) fixed 4 h after wounding, the MTOC's in both the first and second rows are preferentially oriented toward the wound edge in the direction of movement of the EC sheet. *Large arrow* is placed perpendicular to the wound edge and indicates the direction of movement of the EC sheet after removal of CB. nucleus. *n*, Bar, 20 μm. × 6,500.

TABLE III

Comparison of the Effect of Cytochalasin B (CB) on MTOC Reorientation in the 1st and 2nd Row of EC's Along the Wound Edge

Time after wounding	Controls No CB 4 h	4 h	6 h	8 h	22 h	Controls No CB 22 h
	X* SE	X SE	X SE	X SE	X SE	X SE
Toward						
1st	78 ± 1.0	36 ± 0.7	48 ± 2.5	62 ± 3.0	62 ± 3.5	74 ± 0.88
2nd	65 ± 2.1	38 ± 0.5	37 ± 1.0	34 ± 0.5	37 ± 0.5	68 ± 0.88
Away						
1st	14 ± 2.3	31 ± 1.3	23 ± 1.5	10 ± 1.5	8 ± 1.5	11 ± 2.03
2nd	20 ± 1.8	24 ± 1.5	24 ± 2.0	28 ± 0.5	23 ± 2.0	10 ± 2.33
Middle						
1st	8 ± 2.5	33 ± 1.2	29 ± 1.0	28 ± 1.5	30 ± 2.0	15 ± 1.86
2nd	15 ± 2.3	38 ± 1.2	30 ± 1.0	38 ± 1.0	40 ± 1.5	22 ± 1.53

* Mean of three experiments.

TABLE IV

Distribution of MTOC'S After Release from Cytochalasin B (CB) Treatment in EC'S in the First and Second Row along the Wound Edge

Time after release from CB treatment	0 h	3 h	5 h
	X* SE	X SE	X SE
Toward			
1st	60.0 ± 0.5	64.0 ± 1.8	71.0 ± 3.5
2nd	38.0 ± 2.0	49.0 ± 1.9	58.0 ± 2.0
Away			
1st	17.0 ± 1.5	13.0 ± 0.9	7.0 ± 2.2
2nd	22.0 ± 1.0	18.0 ± 2.3	15.0 ± 1.7
Middle			
1st	23.0 ± 2.0	23.0 ± 1.2	22.0 ± 1.9
2nd	40.0 ± 3.0	33.0 ± 1.9	27.0 ± 1.8

* Mean of three experiments.

reoriented towards the wound, only 37% of the cells in the second row had their MTOC's similarly oriented.

REVERSAL OF CB TREATMENT: To determine whether the effect of CB can be reversed, the EC cultures treated with CB for 22 h after wounding were incubated in fresh growth medium after removal of CB. Observations of these cultures by time-lapse cinemicrography showed that the cells at the wound edge started their forward migration and acquired normal morphology within the first hour after removal of CB.

The immunofluorescent labeling with antiserum to tubulin showed that the MTOCs in the ECs at the wound edge remained in front of the nucleus. Over a 5-h period, this number increased and ~71% of the EC in the first row had their MTOC's oriented toward the wound edge (Table IV). In the second row of cells, the MTOC's that had failed to reorient after 22 h in CB began to reorient 3 h after removal of CB, indicating that the failure of reorientation in the second row during CB treatment was reversible. While only 38% of the cells had their MTOC's oriented toward the wound edge immediately after CB removal, this number had increased to 58% after 5 h in normal growth medium.

DISCUSSION

A number of studies using different cell types has strengthened the hypothesis that the centrosome region may be involved in controlling the direction of cell migration. We have previously reported that MTOC's became preferentially oriented towards the leading edge of porcine thoracic aortic EC migrating as a sheet (6). Similarly, we have observed the reorientation of MTOC's in the direction of cell movement in primary cultures of porcine thoracic aortic smooth muscle cells and in retinal pigment epithelial cells using the experimental wound model (unpublished observations). It has also been shown recently that a similar reorientation of MTOC's together with the associated Golgi apparatus occurs in sheets of migrating fibroblasts (NRK cells) (9). In addition, electron microscopy of single migrating 3T3 fibroblasts in which the front part of the cell was located by the phagokinetic track has indicated that in the great majority of cells the centriole faces in the direction of the leading edge (7). In a completely different system, i.e., human neutrophils migrating in response to a chemical gradient, it has been shown that the centriole is also positioned behind the leading edge ahead of the nucleus (8). Moreover, in single moving polarized mouse embryo fibroblasts a colcemid-sensitive system of MT determines the direction in which the leading edge is located and thus the direction of cell movement (15). Thus, it may not be a coincidence that higher plant cells (16) and microplasts of animal cells (17) that lack a centriole are unable to locomote. In comparison, cytoplasts obtained by enucleation with CB, which do contain centrioles and associated MT's (18), can move in a manner comparable to that of normal nucleated cells (19). Observations such as these led Albrecht-Buehler to conclude that directional movement of whole cells may require a supervising mechanism that confers certain coordination and strategy on different components of the cytoplasm (17).

It is interesting that ultrastructural studies of migrating 3T3 fibroblasts have shown that one centriole of a pair is preferentially oriented perpendicular, and the other parallel, to the substrate (7). This observation indicates that centrioles can maintain a certain well-defined orientation with respect to the direction of movement and the plane of the substrate. Theoretical considerations have therefore led to the suggestion that centrioles may be sensors for locating the direction of signal sources from the environment (20) or may act as navigational devices like a pair of gyroscopes (21). It would indeed seem appropriate to have such devices at the front end of a moving cell.

In this paper, using C and CB as agents to disrupt the MT's and MF's, respectively, and to stop cell migration, we have shown, first, that the observed reorientation of MTOC's towards the leading edge in migrating EC's (6) is independent of cell migration and secondly, that a C-sensitive mechanism is responsible for this reorganization.

The results of the C experiments show that either the MT's themselves or some other system, such as that of the intermediate filaments or the Golgi apparatus, which may depend on

MT's for their distribution and function, is necessary for the observed reorientation of MTOC in EC's. A number of studies have shown that MT's and IF's may in fact be closely associated (22, 23) and that the disruption of MT's with C can result in a rearrangement of IF's as well as in the depolymerization of MT's (24). Previous studies have shown that the migration of centrioles in other systems, for example during the formation of the mitotic spindle (2) or during the formation of syncytia in virus-infected BHK cells (24), is sensitive to C. Thus, it is not surprising that the movement and reorientation of the centrioles and MTOC's in the EC is also sensitive to C and requires MT's. That MT's rather than IF's may be involved in the reorientation mechanism is suggested by other experiments. For example, the collapse of the IF system by the intracellular microinjection of vimentin-specific antibody does not interfere with the locomotion of rat fibroblastoid cells (25) and it is possible to disrupt IF's without greatly disturbing cell polarity or altering the distribution of either the MF's or MT's (26).

It is difficult to come to specific conclusion from reviewing several previous studies on the effect of colchicine or C on cell migration. In fibroblasts, C-sensitive structures were found to be essential for directional translocation (15) and in its presence both cell motility and the persistence of direction of motion were inhibited or reduced (27, 28, 29). Similarly, colchicine has been shown to inhibit the movement of BHK-21 cells (30) and to cause a shift from gliding to ameboid movement in peritoneal macrophages (31). A loss of directional, but not random movement has also been observed during chemotaxis in neutrophils (8, 32) and lymphoblasts (33) after treatment with colchicine and other antitubulins (8, 32), leading to the suggestion that MT's are essential for direction finding. These results, however, are difficult to reconcile with those of Zigmond et al. (34) who found that in colchicine-treated neutrophils migrating in response to a chemotactic gradient neither the morphological nor the behavioral polarity was dependent upon the presence of MT.

When movement of cells in a sheet joined by junctions (instead of cells moving individually) is examined, again conflicting results have been obtained. Vasiliev et al. have reported that the rate of migration of epithelial sheets into an in vitro wound, in contrast to that of fibroblasts, (28) was insensitive to C (35). It was suggested that in this case the polarization of the cells at the wound edge results from the firmness of local cell-cell contacts and the efficiency of contact inhibition rather than from the C-sensitive system composed of MT's.

Similarly, colchicine and C had no effect on the extension of the flattened leading edge of cells at the margin of a spreading epithelial sheet in culture (36), which led to the suggestion that MT's are not involved in the locomotion of the marginal cells. The migration of corneal epithelium into wounded areas in organ culture likewise is not inhibited by colchicine (37). In contrast, Seldon et al. (38) have shown that colchicine inhibited cell migration at wound edge in cultures of EC's in a system similar to ours. Our results show that in EC sheets, where cells are linked to each other by junctions, cell migration is inhibited by C and moreover, a C-sensitive mechanism is responsible for the orientation of the MTOC's towards the free edge of the cell sheets.

CB has been previously used to inhibit the migration (39, 40) of different cell types. At low concentrations, although CB exerts only a mild distorting effect on the MF bundles (41), it inhibits cell movement because it retards actin assembly (42). The experiments with CB showed that in our system the reorientation of MTOC's towards the wound edge still occurred even when the cell migration was inhibited. This suggests that the migration itself was neither the cause nor the signal for the reorientation. Instead, the signal may come from the absence of cell-to-cell contact along the front of the EC's at the wound edge. The second row of cells did not show a similar reorientation of MTOC in the presence of CB. However, once the sheet begins to move after CB is washed out the MTOC's in cells of the second row also reorient. One possibility is that this may be due to a reduction in cell contacts between the EC's in the first and second row after cells in the first row begin to translocate.

CB may have some minor inhibitory effect on the mechanism of reorientation. This is suggested by the fact that the number of EC's showing MTOC reorientation increases more slowly after wounding in CB-treated cultures. In addition, the extent of MTOC reorientation in the cells of the first and second rows in CB-treated cultures was less than that observed in untreated ones.

From the results presented, we suggest that the following general steps may be required to induce directional migration in EC's and in other cell types. Once the cell receives a signal to migrate in a given direction, intracellular processes are activated to orient the MTOC and structures associated with it (such as the Golgi apparatus) toward the direction of movement. MT's or components dependent on MT's are required for this process. After the MTOC has turned in the direction of intended movement, the mechanism responsible for moving the cell forward is activated. This last step is sensitive to CB and probably requires MF's. In the presence of CB, the two steps were dissociated, i.e., the MTOC's could turn towards the stimulus but the cell was unable to move forward because its system of MF was disorganized. Since the reorientation of MTOC's precedes cell translocation, this reorientation could be causally involved in starting cells off in a particular direction.

In summary, it appears that directed cell migration requires at least three steps—an initiation step, which we have not studied, an MTOC reorientation step to induce directionality, and a force-generating step for the actual translocation of the cell.

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