Distribution of Microtubule Organizing Centers in Migrating Sheets of Endothelial Cells

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ABSTRACT This study was designed to investigate the relationship between the position of the microtubule organizing center (MTOC) and the direction of migration of a sheet of endothelial cells (EC). Using immunofluorescence and phase microscopy the MTOC's of migrating EC were visualized as the cells moved into an in vitro experimental wound produced by mechanical denudation of part of a confluent monolayer culture. Although the MTOC's in nonmigrating EC were randomly positioned in relation to the nucleus, in migrating cells the position of the MTOC's changed so that 80% of the cells had the MTOC positioned in front of the nucleus toward the direction of movement of the endothelial sheet. This repositioning of the MTOC occurred within the first 4 h after wounding and was associated with the beginning of migration of EC's into the wounded area as seen by time-lapse cinemicrophotography. These studies focus attention on the MTOC as a cytoskeletal structure that may play a role in determining the direction of cell movement.

Although the intracellular mechanisms controlling and regulating cell movement are unknown, numerous studies have suggested that the distribution of the various components of the cytoskeleton, particularly the microfilaments and their associated proteins play an important role in the generation of force and in determining the direction of movement during cell migration (1–7). Several studies have also explored the role of microtubules (MT's) (8, 9) and cilia (6) in cell migration. Badley et al. (8), comparing the cytoskeleton in single migrating and stationary chick fibroblasts using immunofluorescence microscopy, concluded that the distribution of MT's does not alter significantly during the conversion from the migratory to the stationary state. There are, however, studies that show that coordinated movement in one direction requires the presence of MT's and that movement is either reduced (9), inhibited (10), or can occur only randomly (11–13) when MT's are disrupted as in colchicine-treated cells. Malech et al. (14) showed that colchicine had no effect on random migration of human neutrophils; however, activated random migration was minimally decreased and directed migration was markedly inhibited. They also showed that the position of the centriole and its associated MT's appear to be important in establishing the direction of migration of neutrophils. The distribution of MT's in cells is controlled by microtubule organizing centers (MTOC's) which are sites capable of initiating the polymerization of MT's from tubulin both in vivo and in vitro (15–24). Numerous MT's can be demonstrated to radiate in different directions throughout the cytoplasm from the MTOC's commonly found in the perinuclear region near the centrioles (25–27).

To study the role of MTOC's and MT's in cell migration we examined sheets of endothelial cells (EC's) migrating in a well-defined direction after experimental wounding of a confluent monolayer (28). Immunofluorescence microscopy with antibodies to tubulin and phase contrast microscopy of living cells were used to visualize the changes in the position of the MTOC in relation to the nucleus and the direction of movement. In nonmigrating cells the MTOC's were randomly positioned in relation to the nucleus. In migrating sheets of EC's however, the position of the MTOC's changed in the great majority of cells along the wound edge, so that they were found in front of the nucleus facing the direction of movement. These studies support the hypothesis that the MTOC's may be an important cytoskeletal structure which, together with intact MT's, play a role in determining the direction of movement.

MATERIALS AND METHODS

Cells

The harvesting and culturing of EC's obtained from fresh slaughterhouse porcine thoracic aortas has been previously described in detail (28). In brief, an enzyme dispersion method was used employing an eight-minute incubation with 0.07% collagenase, (Type II Worthington Biochemical Corp., Freehold, N. J.). The cells were grown in medium 199 containing Earl's salts, 25 mM HEPES, 0.3 mg/l L-glutamine, 20% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). For the experiments, EC subcultured from 1 to 3 times were trypsinized with 0.05% trypsin-0.02% EDTA in Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline (PBS) (Gibco Laboratories) for one minute and replated onto 22 × 40-mm glass cover slips in 60-mm dishes, and grown to confluency.

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In Vitro Wounds

Confluent cover slips of EC were mechanically wounded with a sterile flat-edged teflon spatula so as to remove all cells from ½ of each cover slip (28). The cultures were well rinsed and fresh growth media was added. The cultures were then fixed at various time intervals after wounding. In the case of time-zero wounds, the cells are fixed immediately without rinsing.

Immunofluorescence

Methods previously described were followed (28). Cultures were fixed at 0, ⅓, ¾, 2, 3, 4, and 44 h after wounding for 5 min in methanol at −20°C followed by 2 min in acetone at −20°C, and then air-dried. The cells were then rinsed in PBS, pH 7.4, and treated with antisera to tubulin diluted 1:30 for 30 min at room temperature. This antiserum has been previously characterized and shown to be specific for tubulin (29, 30). After staining with antitubulin the cells were washed five times with PBS and incubated with a 1:10 dilution of fluorescein-labeled goat antirabbit IgG (Hyland Diagnostics, Costa Mesa, Calif.) with a fluorescein/protein molar ratio of 3.7. After washing the cover slips in PBS and mounting in 50% glycerol in PBS, the cells were viewed in a Zeiss photomicroscope II equipped with a mercury vapor lamp, epifluorescence optics, and interference filters. Photomicrographs were taken with Illford FP-4 film and developed in microphen. Some of the same wound edges were examined and photographed first with epifluorescence optics and then by phase contrast microscopy without changing the field or focus.

Recording of the MTOC Position

To quantitate the distribution of the MTOC's in migrating EC's, immunofluorescence in cells along the wound edge was examined with a × 40 objective and a × 10 ocular lens. The positions of the MTOC 's were classified relative to the wound edge as being either between the nucleus and towards the wound edge or as being between the nucleus and the rest of the monolayer away from the wound edge. The results were expressed as the percent of cells with the MTOC in one or the other orientation in the row of cells directly along the wound edge (first row) and in each of the two rows of cells immediately behind them (second and third rows). A hundred cells in each row were counted on each cover slip. At least three cover slips were counted for each time-point and standard errors of the mean were calculated.

Cinemicrophotography

To record the position of MTOC's in relation to the direction of cell movement and the position of the nucleus, living cells along the wound edge were photographed with a × 20 phase contrast objective and a × ¼ relay lens on a Nikon Inverted-microscope, Model M. The temperature was kept constant at 37°C with a recirculating hot air stage Model IN 61 A incubator (Matthias and Associates, Houston, Tex.) and the temperature recorded with a YSI Telethermometer (Yellow Springs Instrument Company, Yellow Springs, Ohio). The pH was maintained at 7.2 with a continuous stream of humidified 5%-CO_2, 95%-air flowing into a lucite filming chamber containing the cells. Filming was done at 30-s intervals with a Nikon Microflex Model CFM-A intervalometer and a Bolex H-16 M camera using plus-x reversal 7276 film that was processed commercially.

RESULTS

The position of MTOC's can be readily visualized in cultured EC's by immunofluorescence as the region from which most of the cytoplasmic microtubules emanate (Fig. 1). Comparison of immunofluorescence and phase contrast images of the same regions showed that the MTOC's could also be identified in phase-contrast micrographs as a dense region on one side of the nucleus in both fixed and living cells (Fig. 2). In the nonmigratory EC such as those present in the intact monolayer, and in the first three rows of EC's along the wound (Fig. 1 *a*) fixed immediately after wounding, the MTOC's are randomly oriented with respect to the nucleus. However, when the cells along the wound edge were examined 44 h later, after they had migrated into the wound, a change in the position of MTOC had occurred. In ~80% of the cells in the first row directly along the wound edge, the MTOC's were now located towards the wound, i.e., between the nucleus and the wound edge (Fig. 1 *b* and *c*). This orientation was not limited to the cells in the first row but was also observed in 70% of the cells in the second and third rows behind the leading edge. In 5% of the cells in the first three rows the MTOC's were located along the side of the nucleus and were neither clearly toward nor away from the wound edge. In the middle of the monolayer, well away from the wound edge, the MTOC's remained randomly distributed.

To determine the time at which this reorientation of MTOC's takes place we examined the wounded cultures of EC at shorter time intervals after wounding. From the results obtained (Fig. 3) it can be seen that the reorientation of the MTOC's becomes first noticeable in the cells in the first row along the wound edge as early as 20 min after wounding and that by 4 h the MTOC's in 80% of the cells faced the wound edge. The cells in the second and third rows show the same trend as those in the first row but here the reorientation of the MTOC's towards the wound edge develops more slowly. Examination of the wound edge by phase-contrast time-lapse cinemicrophotography showed that the cells spread out and began to move within the first 3 h after wounding, i.e., during the time that the MTOC's were indeed shifting their positions towards the direction of movement.

Although the MTOC's could be identified by immunofluorescence microscopy during the first few hours after wounding this was not possible in living cells at the wound edge by phase contrast microscopy. Thus direct visualization of MTOC's in living cells during the time of maximal reorientation was not carried out. Time-lapse cinemicrophotographic observations of migrating cells over a 5-h period, at later times when the cells had spread sufficiently, showed that in most cells the dense regions corresponding to the MTOC's stayed between the wound edge and the nucleus (Fig. 2 *c* and *d*) as the sheet moved forward. They also showed that the MTOC's were mobile and could change their position relative to the nucleus and the wound edge. The MTOC's could, for example, move from a position away from the wound edge to one towards it (Fig. 2 *c* and *d*, cell 2) and did not necessarily have to be between the nucleus and the wound edge as the cells in the sheet moved forward into the wounded area. This latter observation is consistent with the immunofluorescent results which showed that 15% of the first row cells and 20% of the second and third row cells have the MTOC's away from the wound edge.

DISCUSSION

Although the MTOC's are an important part of the cytoskeleton, the relationship of the MTOC's to cell migration has received little attention in experiments designed to study the role of MT's in cell migration. Our results demonstrate that although the MTOC's are randomly oriented relative to the adjacent nucleus in a confluent monolayer of nonmigrating EC's, once a wound is made the MTOC's in EC becomes preferentially located between the nucleus and the edge of the migrating sheet. We have characterized this redistribution of the MTOC's to show that it occurs within the first 4 h after wounding and is associated with the beginning of migration of the EC's into the wounded area. After the EC sheet had extended into the wound and the cells had become somewhat flatter, the MTOC's could also be identified by phase contrast microscopy and their position determined in living cells during extended periods with time-lapse cinemicrophotography. These studies confirmed the immunofluorescence results and showed that the MTOC is located in the front of the nucleus in the majority of forward moving cells. They also showed however

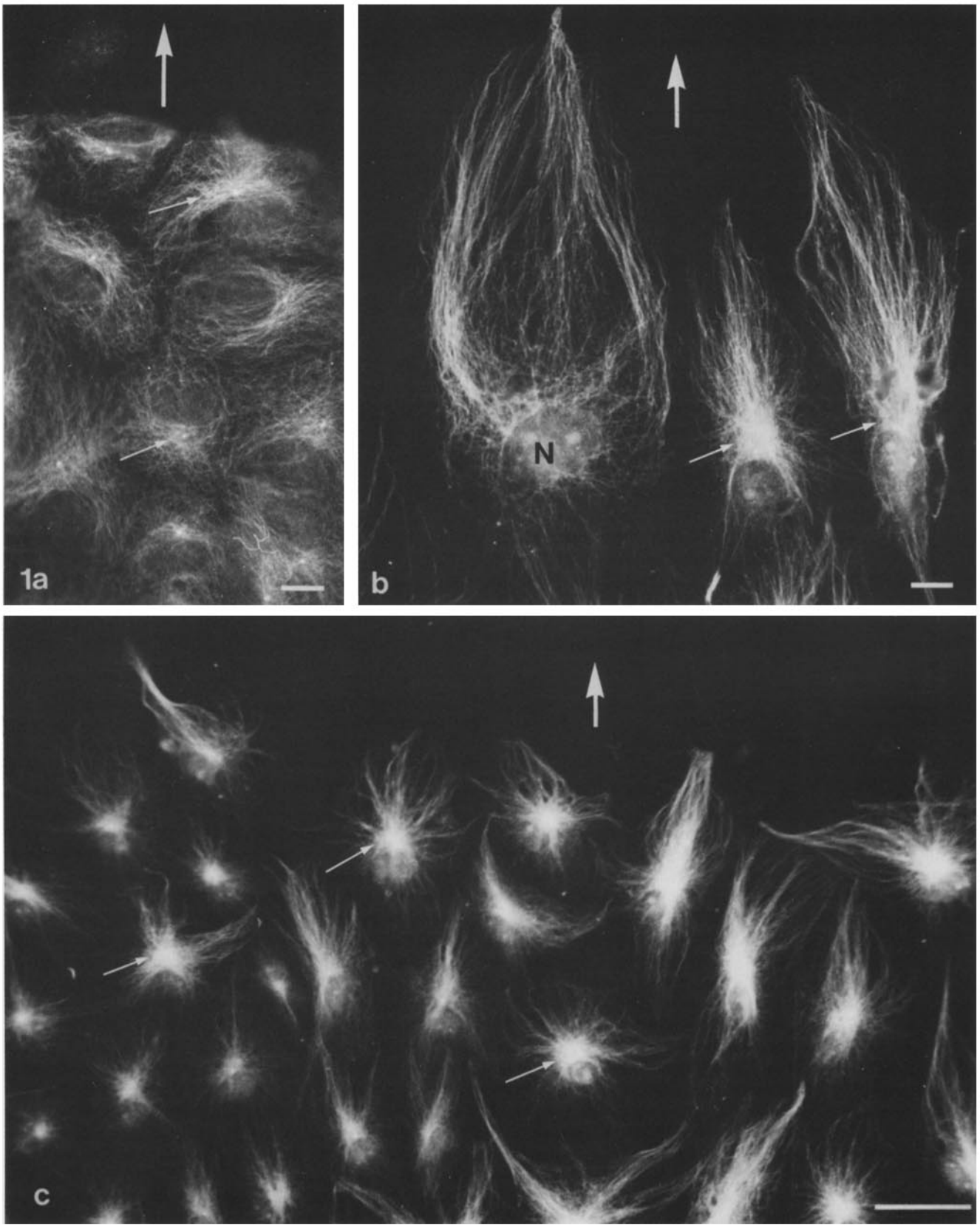

FIGURE 1 Immunofluorescent photomicrographs of EC's at the wound edge stained with antitubulin serum immediately after wounding before migration begins (*a*) and 44 h after wound (*b* and *c*). Note that the MTOC's (small arrows) which initially are randomly distributed relative to the wound edge and the position of the nuclei (*a*) become oriented so that they face the wounded area towards which the endothelial sheet is migrating (*b* and *c*). Large arrow is perpendicular to the wound edge and indicates direction of movement of EC sheet. Bar (*a*, *b*) 10 μm. Bar (*c*), 50 μm.

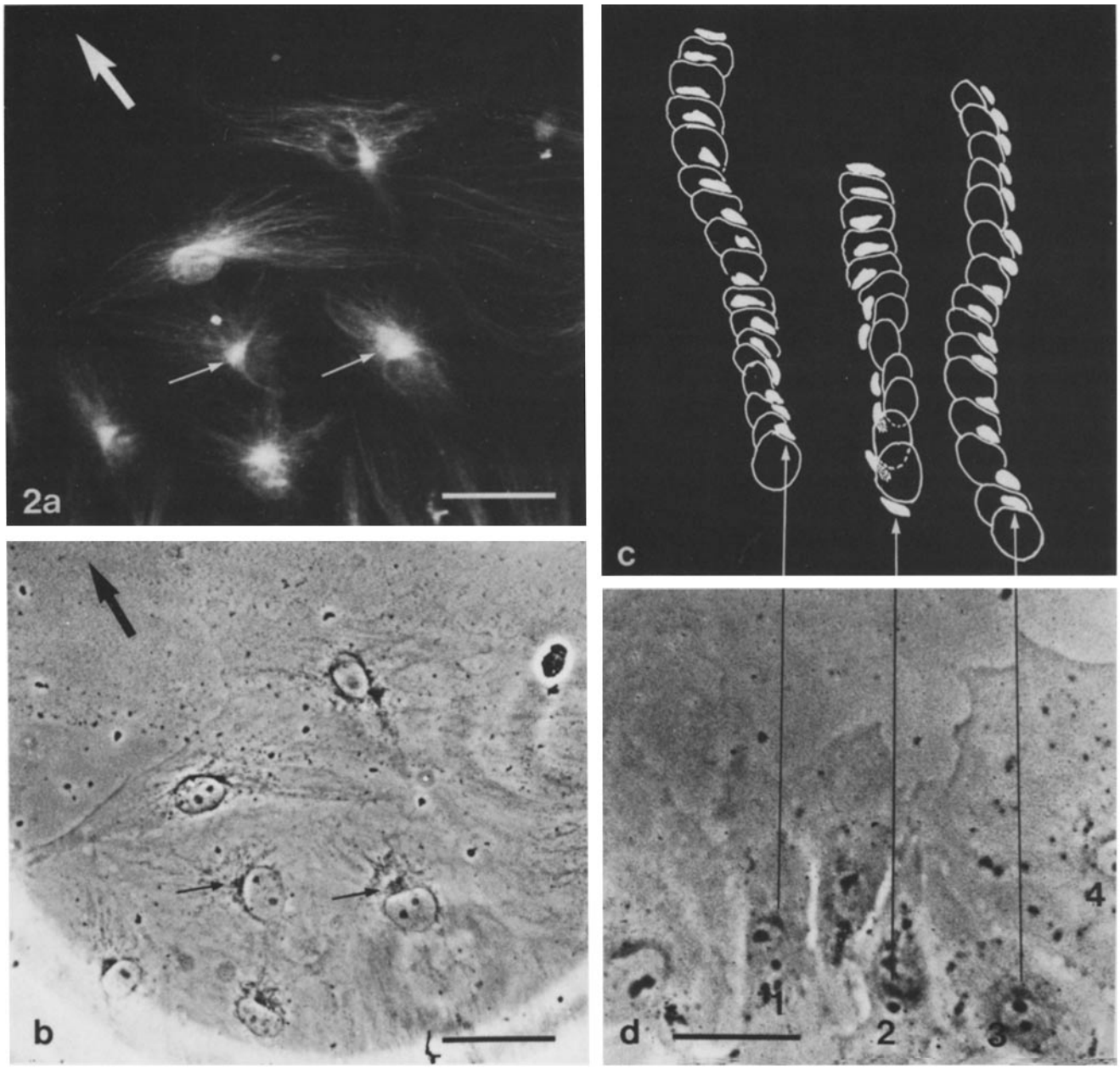

FIGURE 2 Fluorescent (*a*) and phase (*b*) photomicrographs of the same field of migrating cells at 4 h indicating that the dark perinuclear region seen in phase contrast optics ([*b*] small arrows) corresponds to the brightly labeled MTOC seen in fluorescence ([*a*] small arrows). The light region at the base of (*b*) is due to a reference mark used to locate cells. Large arrow is perpendicular to wound edge and indicates direction of movement of EC sheet. Tracings taken from three representative cells in time-lapse photomicrographs of the wound edge over a 5-h period (*c*) showing the relative positions of MTOC's, nuclei and the direction of sheet movement (arrows). The frame from which the first tracing was made is shown in (*d*) and the cells traced in (*c*) are indicated by arrows. In (*c*) the white circle represents the nucleus of the cell and the adjacent white area, the perinuclear MTOC seen as a dark region in phase contrast microscopy (*d*). The tracings indicate that during the 5-h period the MTOC in cell *1* remained between the nucleus and the wound edge as the cell migrated in the first row in a direction perpendicular to the wound edge. The MTOC in cell *2* moved from the back to the front of the cell relative to the wound edge in this migrating second row cell. The MTOC in cell 3 moved from the front of the nucleus to the side in this cell which initially was located in the second row and then migrated into the first row when cell 4 moved to the right. Bar (*a*, *b*, *d*), 50 μm.

that some EC, especially those in the second or third rows may advance forward while the MTOC is located behind or on the side of the nucleus and finally that the MTOC's can change their positions considerably within a 5-h period. Further time-lapse cinemicrophotographic studies are required over longer time-periods to study these patterns of redistribution of MTOC's in living cells. These studies would also allow us to examine the influence of cell-cell interactions on MTOC redistribution. One possibility is that the cells in which the MTOC is not in the front may have their direction of movement determined by adjacent cells in the advancing sheet.

It is not clear from the above studies whether the reorientation of the MTOC's that we have observed determines the direction of cell movement or whether the movement of the

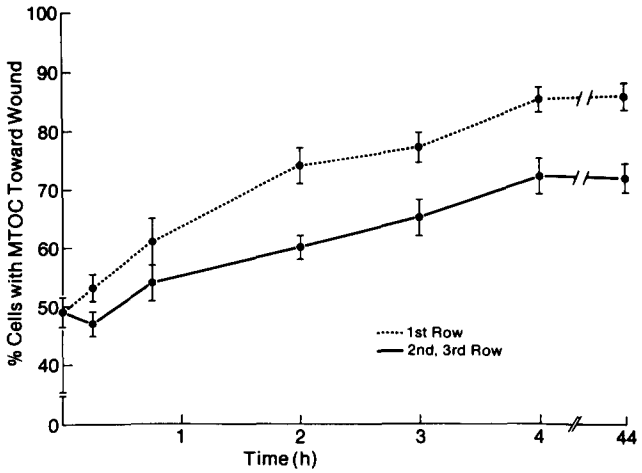

FIGURE 3 Graph showing the percent of migrating EC with MTOC's oriented towards the wound edge in the first row of cells along the edge and in the second and third rows of cells at various times after wounding. Note that gradually increasing numbers of cells show a reorientation of the MTOC towards the wound as the cells migrate forward into the wound during the first 4 h.

cell causes a reorientation of the MTOC's in a preferred direction. The electron microscopic study on neutrophil migration in a chemotactic chamber (14) showed that after reversal of a chemoattractant gradient there was relocation of the centriole toward the new attractant. In addition, the increase of microfilaments in the side of the cell toward the chemoattractant, the formation of pseudopods, and the migration was not generally seen before relocation of the centriole. These observations were carried out by maintaining the neutrophils in a fixed orientation toward the chemoattractant which allowed pseudopod penetration but prevented migration. Thus this study and our present work provide support for the idea that the MTOC and its associated MT's and centrioles play a role in establishing the direction of cell migration. If this were true the MT's emanating from the MTOC's could stabilize the cytoplasm as it spreads to cover new areas of substrate.

Several mechanisms can be envisioned by which the observed reorientation of the MTOC's could take place. It could be brought about for example by a rotation of the nucleus. The ease with which centriole-intermediate filament-nuclear complexes can be isolated indicates that the centrioler area is closely linked to the nucleus, probably by means of intermediate filaments, (31) in a number of cell types. If this were the case in EC's, a rotation of the nucleus could lead to the observed rotation of the MTOC's. It is also possible that the individual cells rotate so as to bring their MTOC-containing region towards the direction of movement of the entire EC sheet. This however seems unlikely because it would require the breaking and reformation of a large number of junctions that are known to interconnect sheets of endothelial cells (32). Moreover time-lapse cinemicrophotography observations of individual cells that were changing their direction of movement within the sheet as well as the position of the MTOC's did not show this type of rotation. Finally, it is also possible that the highly extended cytoskeleton may have to break down at least partially to allow for the rotation and would then have to reassemble after its completion.

The results of our studies indicate that the MTOC may be an important cytoskeletal structure involved in the regulation of the directionality of cell migration. Studies to disrupt MT's and microfilaments are being planned to observe the orientation of the MTOC in cells in which migration is inhibited. These studies should contribute to our understanding of the mechanism of the reorientation and the significance of the observed phenomena.

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