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Role of the microtubular system in morphological organization of normal and oncogene-transfected epithelial cells

(cell polarization/cytoskeleton/microfilaments/morphogenesis)

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ABSTRACT **To understand better the role of the microtubular system in the development and maintenance of morphological organization of nonpolarized and polarized cells of the same origin we examined the effects of two microtubule-specific drugs, colcemid and taxol, on discoid cultured epithelial rat cells of the IAR-2 line and on polarized cells obtained from this line by transfection of mutated N-*ras* oncogene; morphometric, immunomorphologic, and videomicroscopic methods were used. Depolymerization of microtubules by colcemid did not cause major changes in the discoid shape of IAR cells but altered organization of actin cortex; in particular, it led to disappearance of circumferential bundle of actin microfilaments. Taxol reorganized the normal network of microtubules radiating from the perinuclear centers into numerous arrays of short microtubules not associated with any centers. Taxol-treated cells had wider circumferential bundles of microfilaments than control cells and morphometric analysis showed that their contours were closer to geometric circle than those of control or of colcemid-treated cells. These data show that function of the microtubular system is essential for maintenance of the characteristic morphological organization of discoid cells; we propose to name this function "contra-polarization." Contra-polarization is not prevented and is even promoted by taxol; this result suggests that a decentralized system of microtubules is sufficient for this function. In contrast, maintenance of polarized morphology of IAR-2 cells transfected by the N-*ras* oncogene is inhibited not only by colcemid but also by taxol and thus requires the presence of a normal centralized microtubular system.**

When the mechanisms controlling the shape and patterns of motility of cultured tissue cells are analyzed, it is important to distinguish two main types of morphological organization of these cells: (*i*) cells with polarized pseudopodial activities at the edges (cells that usually have elongated shapes and extend cytoplasmic processes) and (*ii*) nonpolarized cells (cells that usually have discoid shapes). In this paper we use the term "polarization" to describe the pattern of pseudopodial activities of the cell edge of single cells. It is well known that integrity of the microtubular system is essential for the maintenance of organization of certain types of polarized cells such as neurons and highly elongated fibroblasts (see review in ref. 1); drug-induced destruction of the microtubular system results in the inhibition of polarization of these cells. As the cells of the second group are, by definition, nonpolarized, it was usually assumed that microtubules do not play any significant role in their morphogenesis. In fact, in early studies depolymerization of microtubules was not found to lead to any changes of the shape of single discoid epitheliocytes belonging to this group (2, 3). The only exception was the finding that spreading of certain types of epitheliocytes is delayed by colcemid (4).

The aim of experiments presented in this paper was to investigate systematically the effects of two microtubule-specific drugs, colcemid and taxol, upon the morphology and organization of actin cytoskeleton of nonpolarized and polarized epithelial cells. We chose for these experiments rat IAR-2 line because single cells of this line in sparse cultures have very regular discoid shapes and a circumferential bundle of microfilaments characteristic of nonpolarized cells. We have also examined the effects of microtubule-specific drugs upon the IAR-2 cells transfected with mutated N-*ras* gene. Transfection of this oncogene results in transformation of discoid cells into polarized and elongated fibroblast-like cells (5). In this system we were able to compare the effects of colcemid and taxol upon nonpolarized and polarized cells of the same origin.

We chose colcemid and taxol for our experiments because these two drugs specifically affect the microtubular system in different ways. Colcemid prevents polymerization of tubulin and eventually induces complete depolymerization of all cytoplasmic microtubules. In contrast, taxol stabilizes polymerized microtubules; at the cellular level this excessive stabilization leads to replacement of the normal system of microtubules associated with the perinuclear centers by arrays of microtubules scattered in various zones of the cytoplasm and not associated with any centers (6). Thus, using these two drugs, we were able to compare the effects of depolymerization of the microtubular system with those of decentralization of this system.

Our experiments show that the microtubular system is important for the maintenance of both different types of morphological organization: polarized organization of transfected cells and nonpolarized organization of parent epitheliocytes. Comparison of the effects of taxol and colcemid shows that a decentralized microtubular system is sufficient for the maintenance of nonpolarized organization; however, only the normal centralized microtubular system is able to maintain a polarized organization.

MATERIALS AND METHODS

Cell Cultures. IAR-2 epithelial cells derived from rat liver (7) were used. The cells of IAR-2 in sparse cultures display discoid epithelial shapes; in dense cultures coherent sheets of the cells are formed. The line is nontumorigenic. C4 and C5 cell lines were obtained in collaboration with B. P. Kopnin (Oncological Scientific Center, Moscow) by infection of IAR-2 cells with amphitropic virions of pPS/hygro construction bearing human mutant N-*ras*^{Asn12} cDNA; subsequent selection was made by growing cells in the medium with hygromycin (200 μg/ml) with subsequent cloning. In the

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developed C4 and C5 cell lines the expression of exogeneous N-*ras* oncogene was confirmed by PCR analysis (I. A. Grigorian, Oncological Scientific Center, Moscow).

All cell lines were grown in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal calf serum, at 37°C in 5% CO_2. Sparse cultures (10×10^3 cells per cm^2) on glass substrate were used in the experiments at 24 hr after cell plating. Colcemid (Sigma) or taxol (kindly provided by the National Cancer Institute, Bethesda, MD) was added to culture medium at a final concentration 0.2 μg/ml or 17 μg/ml, respectively. The cells were incubated with the drug for 24 hr before examination.

Immunofluorescence Microscopy. Staining of the culture with rhodamine-labeled phalloidin (Sigma) was used to visualize actin cytoskeleton. Monoclonal mouse antibodies to tubulin DM1A (Sigma), monoclonal antibodies to vimentin (clone NT30), obtained in our laboratory (8), and antibodies to pan-keratins (clone Lu-5, Accurate Chemicals and Scientific Products) were used for indirect staining of microtubules and intermediate filaments. Fluorescein isothiocyanate-conjugated goat antibodies against mouse IgG (Sigma) were used as second antibodies. For fluorescent microscopy the cell cultures were washed in phosphate-buffered saline, extracted with 1% Triton X-100 (Sigma) in buffer M (50 mM imidazole/50 mM KCl/0.5 mM Mg Cl_2/0.1 mM MDTA/1 mM EGTA, pH 6.8), containing 4% polyethylene glycol (M_r 40,000) for 3 min at room temperature, and then fixed with 4% formaldehyde in phosphate-buffered saline. Observations

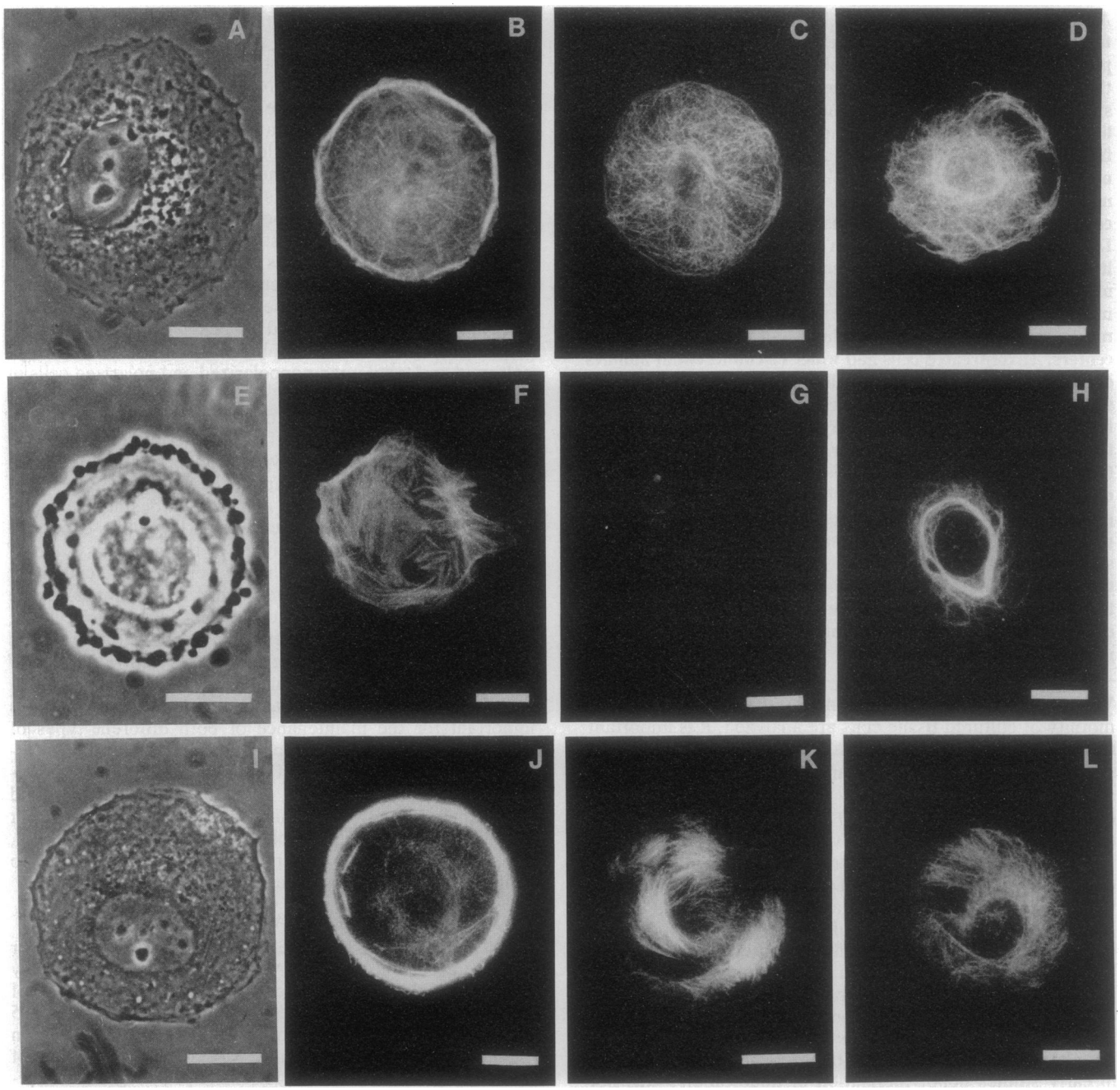

FIG. 1. Morphology and cytoskeletal organization of nontransfected IAR-2 cells treated with microtubule-specific drugs. (*A–D*) Controls. (*E–H*) Cells incubated for 24 hr with colcemid. (*I–L*) Cells incubated for 24 hr with taxol. Phase-contrast microscopy (*A*, *E*, and *I*). Fluorescence microscopy: staining with rhodamine-conjugated phalloidin for polymerized actin (*B*, *F*, and *J*), staining with mouse monoclonal antibody for tubulin to reveal microtubules (*C*, *G*, and *K*), or staining with mouse monoclonal antibody for vimentin to reveal intermediate filaments (*D*, *H*, and *L*). (Bar = 15 μm.)

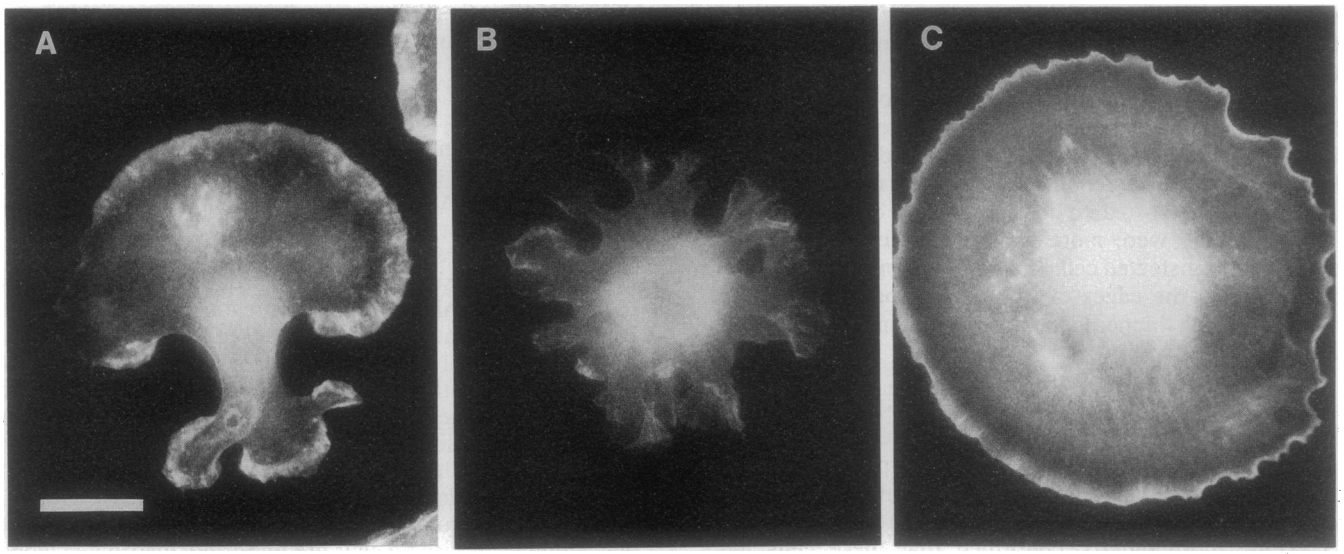

FIG. 2. Actin organization in polarized IAR-2 cells transformed by transfection of mutated N-*ras* gene. Clone C5 fluorescence microscopy after staining with rhodamine-conjugated phalloidin for polymerized actin. (*A*) Control cells. (*B*) Cells incubated with colcemid. (*C*) Cell incubated with taxol. (Bar = 15 μm.)

were made with the Aristoplane microscope (Leitz) using 63× oil-immersion objective.

Video Microscopy. Coverslips were mounted in chambers filled with growth medium and analyzed at 37°C by video-enhanced microscopy using a Zeiss Axiophot microscope equipped with differential interference contrast and Hamamatsu C2400-01 Chalnicon videocamera (Hamamatsu, Middlesex, NJ). Images were processed with an Argus 100 Hamamatsu image analysis system and recorded with the Sony U-matic VO-7630 tape recorder.

Size and Shape Measurements. Two measures of cell shape, elongation and dispersion, were determined as described by Dunn and Brown (9). Briefly, cells were washed with phosphate-buffered saline, fixed with formaldehyde (1%), and photographed using the Arisptoplane microscope (Leitz) equipped with phase-contrast optics. Outlines of cells were then drawn with the aid of a photoenlarger and entered into a PC AT computer by tracing on a digitizing tablet (Bitpad 2, Summagraphick, Seymour, CT). Each outline consisted of ≈100–200 coordinate pairs. Tracer V1.0 software (gift of A. Brown, King's College, London) was used both for entering and storing the outlines and for calculation of cell shape characteristics: elongation and dispersion. Elongation and dispersion were defined (9) as parameters that show how much the total mass of the shape extends away from its center of gravity. Elongation shows how much this extended mass can be reduced by compressing the shape to its center of gravity and can be considered as a measure of the cell's bipolarity. Dispersion describes how much the extended mass remains after compression and can be considered as a measure of cell multipolarity.

RESULTS

Morphology of Nontransfected IAR-2 Epitheliocytes. In all experiments we examined the morphology of single cells without cell–cell contacts. Untreated single IAR-2 cells were well spread and had approximately discoid shape (Fig. 1*A*). Video microscopy revealed continuous formation and retraction of flattened wide lamellipodia at the cell edges. Staining with rhodamine-conjugated phalloidin showed that almost all of these cells had narrow circumferential bundles of polymerized actin near their outer edges; thin straight actin bundles were also scattered in various parts of the cytoplasm (Fig. 1*B*). Microtubules and vimentin filaments formed the networks filling almost the whole cytoplasm; these networks were more dense near the nuclei (Fig. 1 *C* and *D*). Staining with pan-keratin antibody did not reveal any keratin filaments in sparse cultures (see also ref. 10).

IAR-2 cells treated with colcemid retained the same approximately discoid shape as control cells (Fig. 1*E*) but developed certain morphological alterations. Disappearance of circumferential actin bundles was the most characteristic of these alterations (Fig. 1*F*). These bundles were absent in >70% of colcemid-treated epitheliocytes; they were replaced by groups of short and often criss-crossed straight bundles located at the cell periphery; orientation of these bundles with regard to the cell edge varied from radial to tangential. The central part of cytoplasm surrounding the nucleus was often less spread in colcemid-treated than in control cells and looked somewhat contracted (Fig. 1*E*). The peripheral lamellas of these cells remained flattened but near the edges they often formed numerous blebs (Fig. 1*E*). The life time of an individual bleb varied from 5 to 50 sec. As expected, colcemid-treated cells had no microtubules and their vimentin filaments were collapsed around the nuclei (Fig. 1 *G* and *H*).

Taxol-treated IAR-2 cells had a discoid flattened shape; there were no blebs at the edges and no contractions of perinuclear zones (Fig. 1*I*). Circumferential actin bundles were present in all the cells; in most cells they were much wider and more intensely stained than in control cells (Fig. 1*J*). Taxol-treated cells did not contain the usual microtubular networks but had numerous closely packed groups of short microtubules not connected with one another or with any center (Fig. 1*K*). Networks of vimentin filaments filled the entire cytoplasm (Fig. 1*L*).

Morphology of N-*ras*-Transfected IAR-2 Epitheliocytes. We examined two clones of IAR-2 cells independently transfected with constructions carrying mutated N-*ras*-gene. Both clones were morphologically different from the nontransfected IAR-2 cells. Clone C5 was less changed than clone C4. Many C5 cells had fan-like shapes; their bodies had the contours of a large circle sector and there were narrow tail processes with small lamellas at the ends (Fig. 2*A*). Elongated cells without large lamellas were predominant in C4 cultures. Large or small lamellas of all these cells often had arc-like actin-rich ruffles near their outer edges (Fig. 2*A*); these ruffles contained networks of microfilaments oriented in different directions; circumferential or straight bundles of microfilaments were absent in most cells. The networks of

microtubules and vimentin filaments filling the central cytoplasm of transfected cells had the usual morphology. Both colcemid and taxol prevented formation of long cytoplasmic processes, but there were also morphological differences between the transfected cells treated with each of the drugs. Colcemid-treated cells (Fig. 2*B*) often had numerous short lamellar processes extending radially from many parts of the outer edge. Taxol-treated cells had nearly discoid contours. Their contours were more wavy than those of non-taxol-treated nontransfected cells (Fig. 2*C*). Actin-rich ruffles were formed along the edges of these cells; these ruffles were, however, much thinner than in corresponding nontransfected cells.

Morphometric Assessments of Cell Shape. We measured the indices of dispersion and elongation described by Dunn and Brown (9). The elongation index characterizes the ellipsis approximating the cell contours. This approximation is most reliable when the corresponding dispersion index is low, as was the case in experiments with nontransfected cells (Table 1). Thus, morphometry confirmed that these cells have ellipsoid contours. Colcemid did not significantly decrease either index in IAR-2 cells. Taxol induced pronounced decreases of both elongation and dispersion. In particular, dispersion indices of taxol-treated cells were significantly lower than those of control or colcemid-treated cells. Thus, the contours of taxol-treated cells became smoother and closer to geometrical circle, more than for any other cell group. Polarization in both clones of transfected cells was manifested by considerable increases of average dispersion and elongation indices as compared with corresponding indices of nontransfected cells. C4 cells had much higher elongation indices than the C5 line. Colcemid and taxol significantly decreased both indices in C4 and C5 clones as compared with the corresponding untreated cells (Table 1), indicating that polarization of these cells was microtubule-dependent. However, this dependence was only partial, as the indices of drug-treated transfected cells remained significantly higher than those of corresponding drug-treated nontransfected cells. One should stress that when the cells have high dispersion indices, one can only say that the shape of these cells is far from the ellipsoid. But one cannot conclude from the indices alone what kind of specific change had taken place. For more detailed quantitative assessment of shape changes, especially for the detailed analysis of the smoothness of cell edges, development of new methods based on the analysis of exact forms of cell contours will be essential. To summarize, measurements of dispersion and elongation indices confirm the results of qualitative visual assessment of cell shape in various examined groups.

Table 1. Morphometric characteristics of nontransfected and N-*ras*^{Asn12}-transfected IAR-2 epitheliocytes incubated with microtubule-specific drugs

Cells	Morphometric index	
	Dispersion	Elongation
Nontransfected IAR-2		
Control	0.023 ± 0.003	0.321 ± 0.035
Colcemid	0.021 ± 0.002	0.261 ± 0.024
Taxol	0.015 ± 0.002	0.223 ± 0.036
N-*ras*-transfected IAR-2 (clone C5)		
Control	0.538 ± 0.047	0.962 ± 0.098
Colcemid	0.151 ± 0.025	0.264 ± 0.168
Taxol	0.094 ± 0.024	0.281 ± 0.028
N-*ras*-transfected IAR-2 (clone C4)		
Control	0.638 ± 0.037	2.060 ± 0.117
Colcemid	0.325 ± 0.027	0.723 ± 0.052
Taxol	0.253 ± 0.022	0.770 ± 0.070

DISCUSSION

Although depolymerization of microtubules by colcemid does not change significantly the nearly discoid shape of nontransfected IAR-2 epitheliocytes, organization of their actin cortex undergoes considerable alterations. In particular, the circumferential actin ring is disorganized after colcemid treatment, suggesting that microtubules are needed for maintenance of this characteristic feature of epithelioid organization. Formation of blebs at the cell edges and contraction of the central perinuclear part of the cell are probably also associated with some unidentified alterations of dynamic organization of the cortex. Thus, the maintenance of regular discoid organization in nontransfected epitheliocytes is an active microtubule-dependent process. Characteristics of discoid organization of epitheliocytes are, in many aspects, opposite to those of polarized cells. In particular, pseudopodial activities of discoid cells are uniformly distributed along the whole edge, in contrast to their restricted localization in one part of the edge in polarized cells. We propose the term "contra-polarization" for the function of microtubules that actively prevents disorganization of discoid structure.

In addition to discoid IAR-2 cells, we examined polarized cells of the same origin obtained by transfection of the original line with mutated N-*ras* oncogene. Reactions of these cells to colcemid and taxol were similar to those previously observed in the experiments with fibroblasts (1). Their polarization proved to be partially microtubule-dependent. Thus, alterations of the microtubular system induced by specific drugs significantly affect both polarized and nonpolarized cell organizations of two cell types of the same origin. That microtubular systems promote different types of morphogeneses in transfected and in nontransfected cells suggests that these systems are differently organized in the two cell types. One feature that distinguishes transfected from nontransfected cells may be the degree of centralization of the microtubular system. Our experiments show that taxol-treated epitheliocytes do not lose the ability to develop discoid morphology and circumferential bundle of microfilaments; in fact, expression of these characteristic epithelial features is somewhat enhanced by taxol in nontransfected epitheliocytes. Earlier we had found that taxol-treated fibroblasts, in contrast to colcemid-treated ones, also develop "epithelioid" characteristics (11). All of these data support the suggestion that taxol-induced decentralization of the microtubular system promotes contra-polarization. In contrast, the normal centralized organization of microtubules is essential for control of polarized organization; this control is abolished not only by colcemid but also by taxol. Epithelial Madin–Darby canine kidney cells reorganize their microtubular systems when they begin to form coherent sheets; in particular their microtubules lose association with centrosomes (12). However, in these experiments single epithelial cells before the formation of sheets did not show any morphological signs of decentralization. The same was true for discoid IAR cells in our experiments. Nevertheless, further experiments may reveal important differences in the structural and molecular organization of microtubular systems between polarized and contra-polarized cells. Specific molecular mechanisms of the control of polarization and contra-polarization by microtubular systems remain unknown. Growing evidence suggests that new actin microfilaments are formed by polymerization of actin mainly at the active cell edge and are then redistributed to various parts of the cell cortex (13). Microtubules may control these processes by directing the outward transport of some unidentified organelles needed for regulation of reorganizations of

actin. Recent data indicate that organelle transport mediated by kinesin motor molecules plays an important role in the control of fibroblast shape by microtubules (14). Certain types of organization of microtubules may direct this transport to one particular part of the edge and in this way promote polarization. In contrast, a microtubular system organized differently (e.g., taxol-decentralized microtubular arrays) may enhance the flow of these organelles to all parts of the edge and in this way promote contra-polarization. It is also possible that intermediate filaments collapsing after microtubular depolymerization may be involved in the mediation of microtubular control of cell shape (1).

Transformations of discoid epitheliocytes into polarized fibroblast-like cells induced by oncogene transfection may be due to the modifications of some microtubule-associated proteins induced by altered signal-processing systems. The same may be true for phenotypic transformations of epitheliocytes into polarized cells induced by phorbol ester (1) or by scatter factor protein (15). We suggest that these transformations are accompanied by the alterations of structural organization of microtubular system switching the shape control from polarization to contra-polarization. All of these suggestions remain to be tested.

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