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Structure and Control of Assembly of Cytoplasmic Microtubules in Normal and Transformed Cells

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Indirect immunofluorescence analyses using antibodies directed against 6S tubulin have shown an elaborate cytoplasmic microtubule complex (CMTC) in nontransformed cells in culture. The CMTC is strikingly altered in cells that have been transformed spontaneously by viruses or by chemicals. Assembly of microtubules in vitro and in vivo is markedly inhibited in the presence of elevated levels of calcium. Alteration of the surface of normal cells by brief treatment with low concentrations of trypsin initiate a rapid breakdown of cytoplasmic microtubules. Finally, a hypothesis is presented relating microtubule assembly and surface membrane modulation suggesting that calcium is the primary modulating signal.

Key words: cytoplasmic microtubule complex, calcium, normal and transformed cells, in vivo control, effects of trypsin

INTRODUCTION

It is now well established that the cytoplasm of most eukaryotic cells contains a delicate cytoskeletal fabric in the form of microfilaments and microtubules. Various morphological and experimental studies have shown that these elements play a crucial role in a wide variety of cell functions. Thus, in order to understand the molecular role of the cytoskeleton it is essential to further characterize microfilaments and microtubules and to determine factors that regulate their assembly. Excellent progress is being made in the study of actin, myosin, and related molecules in other laboratories (3, 4, 5). This report will concentrate on the structure, distribution, and regulation of assembly of cytoplasmic microtubules.

Although cytoplasmic microtubules were first identified by transmission electron microscopy (1, 2), recent progress in the use of tubulin-specific antibodies as an immunofluorescent probe has made it possible to examine microtubules by light microscopy in large populations of cells (6, 7, 9). In our own studies, we have used indirect immunofluorescence with antibodies prepared against 6S bovine brain tubulin to detect an elaborate array of Colcemid-sensitive, fluorescent filaments in the cytoplasm of many cell lines and have termed this component the cytoplasmic microtubule complex or CMTC (6, 7). Identical structures have been described in cultured cells by Weber and coworkers (8), who used similar procedures.

In this report we will further characterize the CMTC's of numerous lines of normal and transformed cells and explore the role of Ca^{++} in their regulation and their assembly and disassembly.

METHODS AND MATERIALS

Cell Cultures

The various cell lines used in this study are listed in Table I. Additional cell lines examined for tubulin immunofluorescence have been published previously (6). Cell lines BALB/c3T3, SV3T3, RT, RT-2, L_{N1-2}, LZ_{2-2}, A_{D3-2}, B_{D5-2}, $LM(TK)^-$ and RAG were grown in monolayer cultures on glass coverslips in Dulbecco's modified Eagle's Medium (GIBCO No. H-16) supplemented with 10% fetal calf serum. The CHO lines were grown in Hsu's modified McCoy's 5a Medium supplemented with 10% fetal calf serum. Cells derived from human skin fibroblasts HSF-CF and HSF and PA-2* were grown in Ham's F-10 supplemented with 10% fetal calf serum.

Efficiency of plating of normal and transformed cells in agar was carried out according to procedures described by Pollack and co-workers (10).

Immunofluorescence

Indirect immunofluorescence procedures using tubulin antibodies were precisely the same as described in a previous publication (6).

Tubulin Preparation

Fresh calf brains obtained from a local slaughterhouse were used as a source of tubulin. Tubulin was prepared by two cycles of polymerization-depolymerization as has been described by Borisy et al. (16). The repolymerization buffer consisted of 0.25 M glycerol, 0.1 M 2 (N-morpholino)-ethane sulfonic acid (MES), 1.0 mM EGTA and 0.5 mM $MgCl_2$, 1.0 mM GTP, pH 6.8 (17). The twice repolymerized tubulin (2X tubulin) was stored at $-70^{\circ}C$. Protein concentrations were determined by the Lowry procedure (35) using bovine serum albumin as a reference.

Mitochondria Preparation

Rat liver mitochondria were isolated by the methods described by Sordahl et al. (18). The final mitochondrial pellet was resuspended in 2.0 ml of 0.25 M glycerol, 0.1 M MES, 1.0 mM EGTA, 0.5 mM $MgCl_2$, and 1.0 mM GTP.

Viscometry

Measurements of the extent of tubulin polymerization were carried out using Ostwald viscometers immersed in a water bath calibrated to $30 \pm 0.01^{\circ}C$. Outflow times were measured at 5 min intervals for 60 min. Specific viscosities were calculated based on the relationship shown by Tanford (19) as described by Olmsted and Borisy (20). All measurements were done in duplicate, using different calibrated viscometers. Mitochondria were added to the tubulin preparation at 0°; 0.6 ml was pipetted into the viscometer and the initial reading taken immediately.

Calcium Determinations

Total amounts of calcium present in mitochondria were determined using the Varian Techtron Model 1200 Atomic Absorption Spectrophotometer. Complete incubation mixtures containing tubulin and mitochondria plus 3 mM sodium succinate and 1 mM

*The PA-2 human fibroblasts were a generous gift from Dr. Uta Franke at the University of California at San Diego.

potassium hydrogen phosphate (K_2HPO_4) in a final volume of 2.6 ml were incubated for varying periods of time. Following incubation the mixtures were centrifuged at 10,000 rpm in a Beckman J21 centrifuge at 0°for 15 min and the mitochondrial pellet recovered. The mitochondria were washed twice in calcium-free saline; following the final wash the mitochondria were suspended in 1 ml of 0.1 M lanthanum HCl, boiled for 5 min, and then centrifuged for 15 min at 15,000 rpm in the JA20 Rotor of the Beckman J21 centrifuge. The supernatants were taken for calcium determinations.

Cell Incubations

Cells were plated onto coverslips and allowed to grow for 40 hr prior to their removal for treatment with the calcium ionophore A23187.* Replicate coverslips were placed in petri dishes containing 5 μg/ml of A23187 that had been dissolved in dimethyl sulfoxide (DMSO). The calcium concentration of the DMEM was increased to 4.9 mM. The coverslips were incubated for 50 min and 100 min at 37°, removed and washed in phosphate buffered saline, and fixed for immunofluorescent analysis as previously described. Controls consisted of cells exposed to DMEM + DMSO; DMEM containing 4.8 mM $CaCl_2$; and DMEM + ionophore without additional calcium being added. Reversal from ionophore exposure was carried out simply by placing the coverslips into fresh media and incubating the cells for 100 min prior to fixation. All cell incubation media contained 10% fetal calf serum.

Treatment with Trypsin

In order to assess the effect of brief proteolytic treatment on the cell surface and, in turn, the effect on cytoplasmic microtubules, we exposed 3T3 cells for brief periods to varying concentrations of trypsin. Coverslips to which the 3T3 were attached were placed in petri dishes containing trypsin (2X recrystallized Sigma dissolved in Puck's saline A, balanced salt solution, Grand Island Biological Company). The cells were treated for 3.0 min at room temperature in the following concentrations of trypsin: 120 μg/ml, 60 μg/ml, 30 μg/ml, and 15 μg/ml. The coverslips were removed from the petri dishes, rinsed in phosphate-buffered saline, and immediately fixed for immunofluorescence.

RESULTS

The Cytoplasmic Microtubule Complex

When normal cultured fibroblasts are stained for tubulin immunofluorescence, an elaborate array of fine fluorescent filaments can be seen throughout the cytoplasm (Figs. 1 and 2). Since these filaments are stained with antitubulin and disappear in the presence of Colcemid and cold shock (Fig. 5), we safely conclude that they represent microtubules and collectively form a network we call the cytoplasmic microtubule complex (CMTC). The CMTC appear to associate with one or two cytoplasmic foci near the nucleus that correspond to the cells' centrosphere. Microtubules splay out from these foci and extend in somewhat parallel array toward the cell periphery, where they either terminate near the cell surface or bend and extend along the surface or back into the cytoplasm (Fig. 2).

Experiments involving Colcemid inhibition and reversal provide evidence that most cytoplasmic microtubules arise at the centrosome and grow toward the cell periphery. When 3T3 cells are exposed to Colcemid at a concentration of 0.06 μg/ml for 2 hr, the CMTC

*The ionophore A23187 was a generous gift from Dr. Robert Hammil of Eli Lilly Pharmaceuticals.

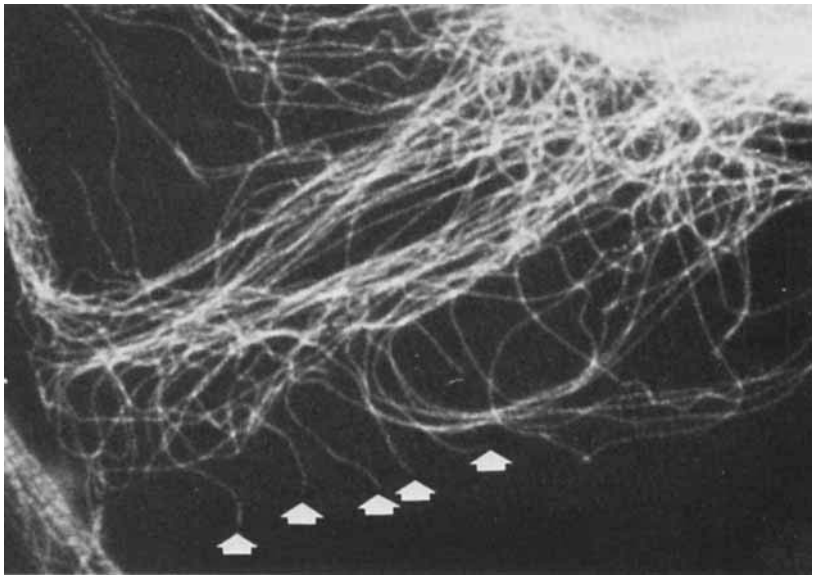


Fig. 2. Normal 3T3 cell showing microtubules that terminate near the cell surface (arrows) or extend along the cell margin.

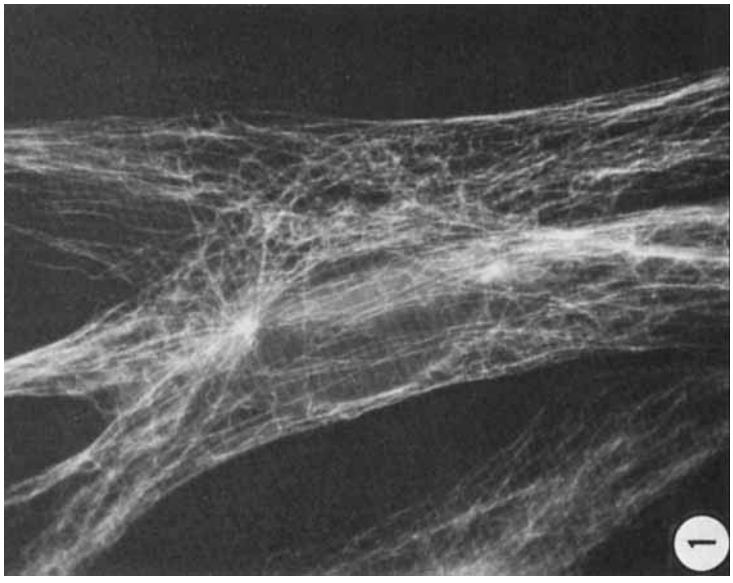


Fig. 1. Normal 3T3 cell showing extensive cytoplasm microtubules that appear to focus on one or more bright fluorescent spots presumed to be the centrosphere.

disappear, the cytoplasm displays diffuse staining, and the cells lose their fibroblast shape. If we transfer the cells to Colcemid-free media, 1 to 3 bright fluorescent spot appear near the nucleus and fluorescent filaments radiate out from the spots (Fig. 5 and 6). Within 30–60 min the complete CMTC is reformed (Figs. 6–8). Identical observations have been reported by Osborn and Weber (11). Electron microscopic examination of the small foci revealed one or more centrioles (Fig. 9) which appeared to serve as microtubule organizing centers (MTOC's). Although these experiments do not rule out the possibility that various other structures within the cytoplasm can serve as MTOC's, it does suggest that the primary organizing center is the centrosphere containing the centrioles deep within the cytoplasm.

When cells enter prophase of mitosis, the CMTC begins to disappear and is replaced by the mitotic spindle. Of course, the small foci that organized the CMTC now initiates the assembly of the mitotic spindle (Fig. 10). The details of tubulin immunofluorescence during mitosis have been given elsewhere but representative stages are shown in Figs. 10–13. It is particularly worthwhile to point out that the CMTC quickly reappears in late telophase, again in association with the centriolar regions (Fig. 13).

The CMTC in Transformed Cells

Transformed cells usually display a variety of morphological and growth related properties that distinguish them from their normal counterparts in culture. These include alteration in shape, cell surface changes, loss of density-dependent control of growth, loss

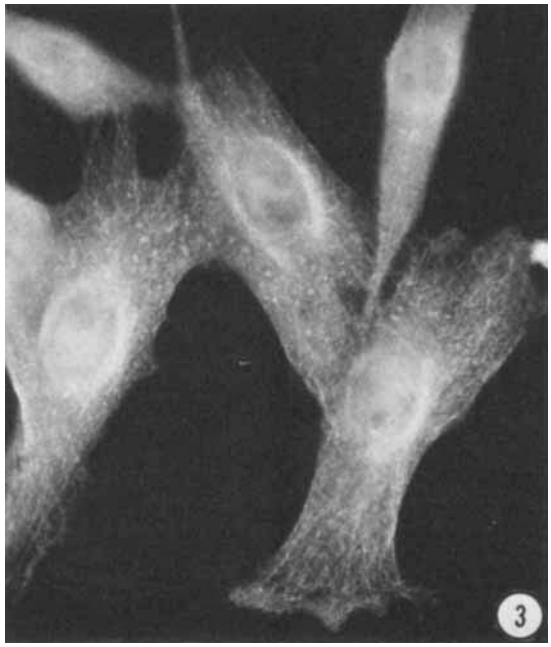


Fig. 3. Rat hepatoma induced by 9,10-dimethyl 1,2 benanthracene; Note diffuse tubulin staining and paucity of microtubules in cytoplasm.

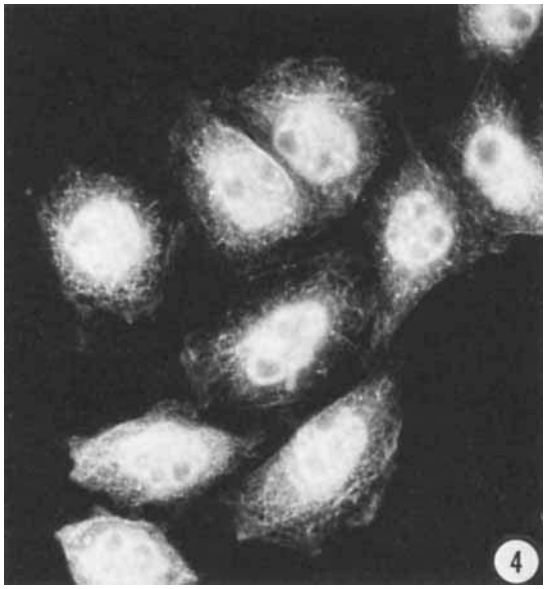


Fig. 4. Spontaneously transformed CHO cells that appear rounded and contain a greatly altered CMTC with mostly diffuse staining in the cytoplasm.

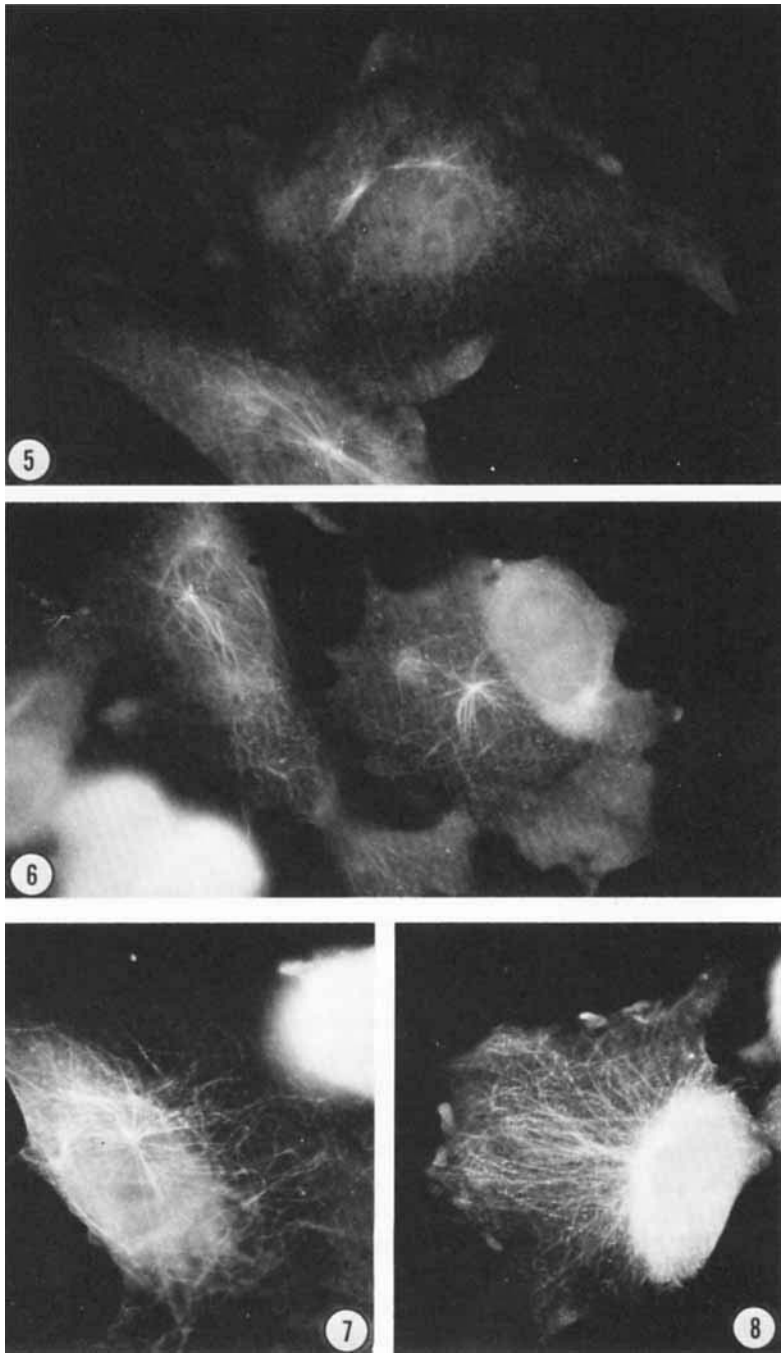
of anchorage-dependent growth, and reduced serum requirements. Although transformed cells may vary with regard to the expression of one or more of these properties, one feature – anchorage independence – appears to be a reliable indicator of tumorgenicity (14).

In our studies of tubulin immunofluorescence, we have surveyed a wide variety of transformed cells (Table I and Ref. 6) and have found that they consistently displayed a greatly altered CMTC. As shown in Figs. 3 and 4, transformed cells are usually rounded and polygonal and display a greatly altered and randomly oriented array of cytoplasmic microtubules. Using similar procedures, Edelman and Yahara (12) have recently confirmed our observations in SV3T3 cells as well as chick cells transformed by Raus sarcoma virus. We found similar alterations in the CMTC of cells that were transformed virally, chemically, or spontaneously.

Although we originally referred to the "transformed phenotype" as having a diminished CMTC, we have since found a wide range of patterns associated with the CMTC. In some cell lines such as CHO or rat hepatoma, cells in exponential growth were essentially void of cytoplasmic microtubules and the tubulin immunofluorescence was in the form of diffuse staining throughout the cytoplasm. In other lines such as SV3T3, greater numbers of cytoplasmic tubules are present but they are greatly disoriented and lack the radial pattern and parallel alignment seen in 3T3. Nevertheless, all transformed cells are easily detected by their altered tubulin immunofluorescence patterns.

Ca^{++} Regulation of Microtubule Assembly

Effects of calcium on in vitro polymerization. The degree of tubulin polymerization in vitro can be markedly inhibited by the addition of calcium as shown in Fig. 14. The extent of polymerization is inversely related to the calcium concentration; however, even at exceedingly high levels (500 μM) there was a slight increase in viscosity. When freshly prepared mitochondria (an organelle known to sequester Ca^{++}) were added to a tubulin



Figs. 5–8. Stages in the recovery of 3T3 cells after a 2 hr Colcemid treatment. Note in Fig. 5 two brightly fluorescent centers, 5 min after the cell was reincubated in fresh medium without Colcemid. After 15 min of recovery the bright centers give rise to numerous filaments (Fig. 6). Even more tubules are apparent in Fig. 7 after 30 min recovery, and after 45 min the cytoplasm again becomes filled with the CMTC.

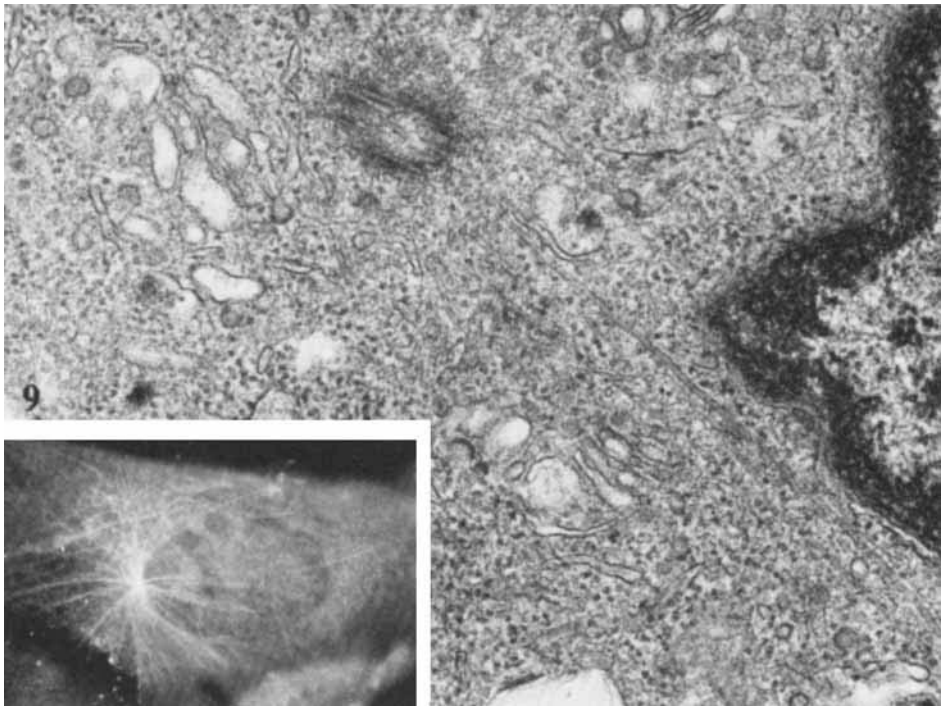


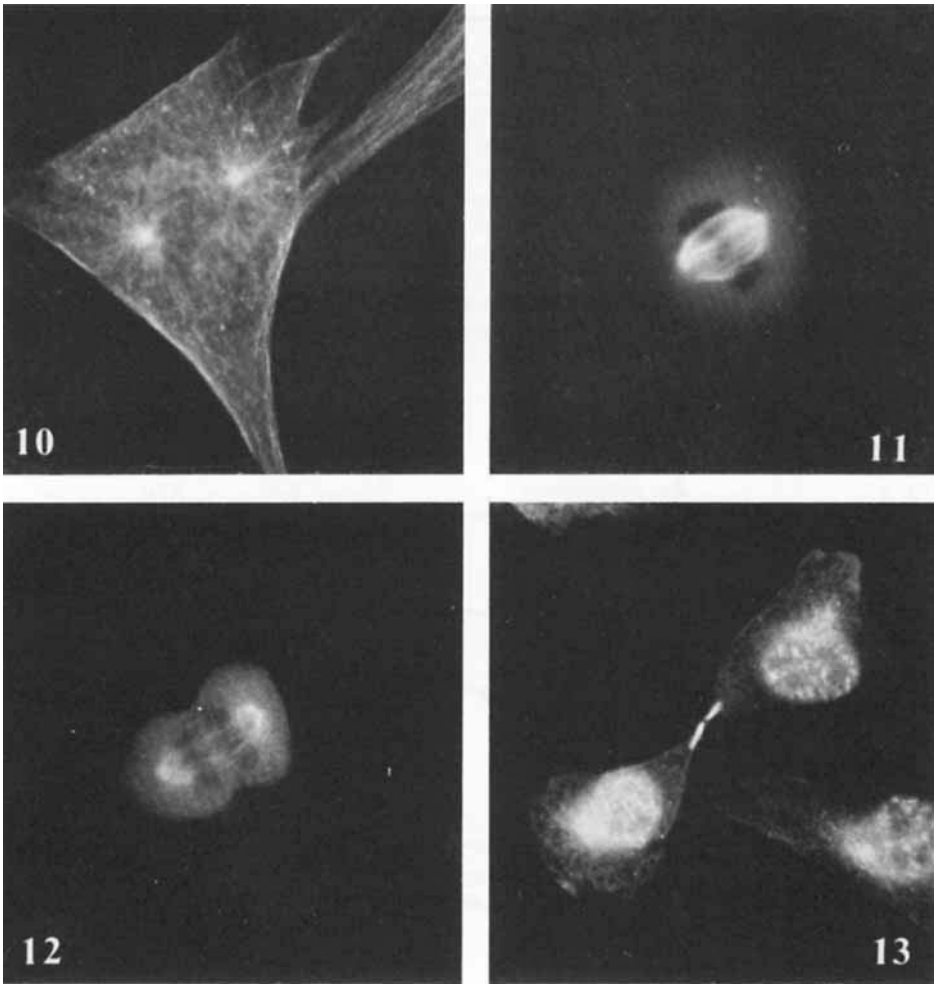
Fig. 9. Electron micrograph of glutaraldehyde–OsO_4-fixed 3T3 cell that had been treated with Colcemid for 2 hr and allowed to recover in fresh medium without Colcemid for 15 min. Note centriole, microtubule, and nucleus. Inset shows cells at similar stage after tubulin antibody staining. antibody staining.

solution containing 500 μM of $CaCl_2$, tubulin polymerization was able to proceed normally for approximately 30 min, after which there was a gradual decrease in viscosity reflecting a breakdown of microtubules (Fig. 15).

In order to demonstrate that the calcium ion was being sequestered in the mitochondria, and not just inducing an aggregation of the tubulin, we analyzed for calcium in mitochondria at the same time points at which the polymerization reaction was measured. Figure 16 shows an increasing uptake of the cation by the mitochondria, followed by a decrease. These results demonstrate that for the first 30 min a rapid removal of the cation by mitochondria occurs; however, as the energy substrate of the organelle diminishes, there is a net loss of the ion. An additional experiment was carried out in which two mitochondrial inhibitors, KCN and oligomycin, were used to prevent the active uptake of calcium (Fig. 17). The polymerization process was inhibited to the same extent as that shown in Fig. 14.

In the experiments just described, we have demonstrated that calcium levels sufficient to prevent the assembly of tubulin into microtubules can be sequestered by mitochondria. These in vitro experiments suggest that mitochondria could be indirectly involved in microtubule regulation primarily through their capacity to control the intracellular levels of the divalent cation.

Effects of calcium on in vivo polymerization. Indirect immunofluorescent analysis of the cytoplasmic microtubule has permitted us to examine the effects of altering the



Figs. 10–13. Stages of mitosis in 3T3 cells. During prophase (Fig. 10), the CMTC disappears and two asters appear in the cytoplasm. At metaphase, the CMTC is completely gone and a spindle is present. The chromosomes move to the pole in anaphase (Fig. 12) and the spindle breaks down. By telophase the two daughter cells are held together by a midbody and the CMTC begins to appear near each of the brightly fluorescent centrospheres.

calcium levels of intact cells. The calcium concentration was increased by exposing mouse fibroblasts to nontoxic concentrations of the ionophore A23187. Figure 18 (d and e) demonstrates the dramatic change in the CMTC following exposure to the ionophore in the presence of increased calcium concentrations. The microtubular complex is significantly reduced in size and number of tubules. There do remain, however, some microtubules. If one increases the concentration of the ionophore to 7.5 μg/ml, almost no microtubules are evident. Reversal from exposure to the ionophore at 5 μg/ml is shown in Fig. 18-f, and is fully comparable to the control cell in Fig. 18a and Fig. 18b. The increase of calcium in the absence of the ionophore showed no adverse effects on the CMTC (data not shown). Likewise, cells exposed to ionophore without added calcium showed little effect. One could, however, detect a slight diminution in the CMTC, which we postulate may be

TABLE I. Cytoplasmic Microtubule Distribution in Normal and Transformed Cells

Cell line	Transformed	Description of cells	% Normal CMTC	% Altered CMTC	EOP in Agar
3T3	–	Swiss mouse embryo fibroblast	97.8	2.2	0%
HSF	–	Human skin fibroblast	98	2.0	0%
HSF-CF	–	Human skin fibroblast from cystic fibrosis patient	97.5	2.5	0%
PA-2	–	Human skin fibroblast	95.5	4.5	–
SV3T3	+	Simian virus transformed 3T3	1.8	98.2	87%
CHO	+	Chinese hamster ovary	0.1	99.9	95%
RAG	+	Mouse renal adenocarcinoma	4.0	96.0	25%
LM(TK–)	+	Mouse L cell (BRdU resistant)	5.5	94.5	–
RT-2	+	Rat hepatoma	4.8	95.2	–
AD_{3-2}	+	Rat hepatoma	0.1	99.9	–
BD_{4-2}	+	Rat hepatoma	2.0	98.0	–
BD_{5-2}	+	Rat hepatoma	2.7	97.2	–
LN_{1-2}	+	Rat ascites tumor	0.1	99.9	–
LZ_{2-2}	+	Rat ascites tumor	8.0	92.0	–

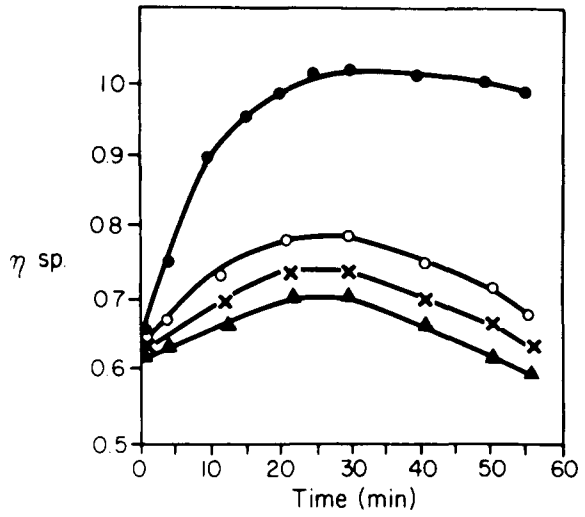


Fig. 14. Tubulin polymerization determined by viscometry. ●——Bovine brain tubulin (≈ 18 mg/ml) without added calcium. ○——Bovine brain tubulin plus 125 μM $CaCl_2$. X——Bovine brain tubulin plus 250 μM $CaCl_2$. ▲——Bovine brain tubulin plus 500 μM CaCl (34).

due to release of calcium from mitochondria initiated by the presence of the ionophore. The results of this experiment demonstrate the effect on the cytoplasmic microtubules when the intracellular level of calcium is increased.

Effects of Trypsin on the CMTC

It has been suggested that trypsin may initiate cell detachment from its substrate not because of its proteolysis on the attachment structures but rather because of changes in the cell shape (21). Since cell morphology is dependent upon the presence of micro-

tubules, we exposed cells to low concentrations of trypsin for brief periods to analyze for resultant changes in the CMTC. Figure 19 shows that the CMTC is profoundly affected by the action of this proteolytic enzyme. Figure 19 (a and b) again shows the extensive delicate CMTC. There is no perceptible change in the cell structure at the end of a 3.0 min

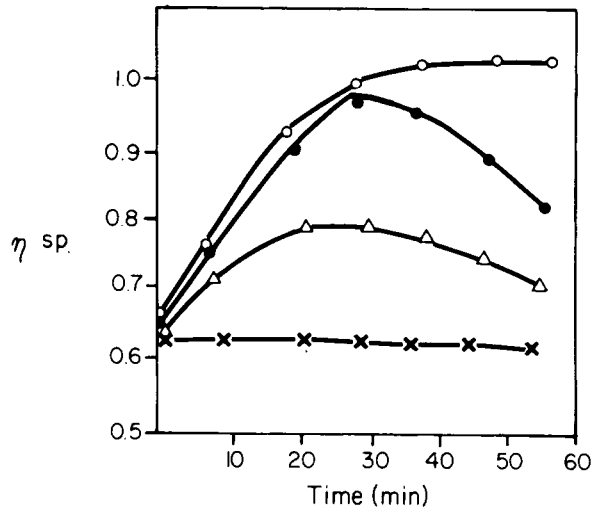


Fig. 15. Tubulin polymerization. The conditions are similar to those shown in Fig. 14, except freshly prepared rat liver mitochondria have been added. ○——Bovine brain tubulin plus rat liver mitochondria. ●——Bovine brain tubulin plus rat liver mitochondria plus 500 μM $CaCl_2$. △——Bovine brain tubulin plus 500 μM $CaCl_2$, no added mitochondria. X——Bovine brain tubulin plus 1×10^{-5} M colchicine (34).

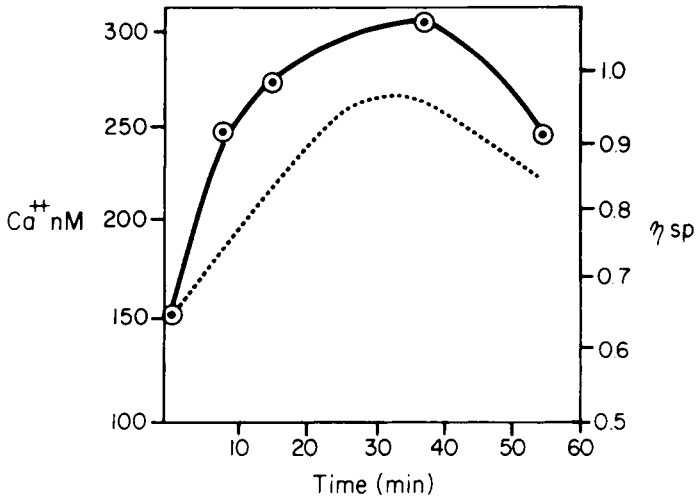


Fig. 16. Uptake of calcium by mitochondria. Mitochondria were isolated from a polymerization experiment to which calcium (500 μM) had been added. The mitochondria were washed and lysed, and the total calcium content was determined by atomic absorption spectrophotometry (solid line). Dashed line represents viscometry measurements (tubulin polymerization) in a duplicate experiment at the same time points (34).

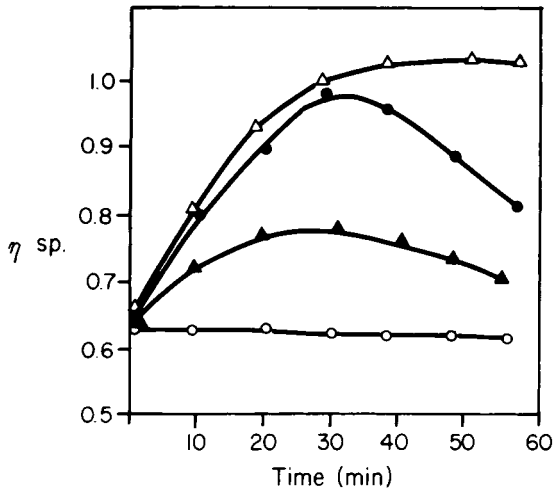


Fig. 17. Effects of mitochondrial inhibitors on tubulin polymerization. Conditions for assembly are the same as described previously. △——Tubulin plus oligomycin and KCN; the added mitochondrial inhibitors had no effect on polymerization. ●——Tubulin plus mitochondria plus $CaCl_2$ (500 μM). ▲——Tubulin plus mitochondria plus oligomycin and KCN. Mitochondria were unable to remove calcium to allow for polymerization. ○——Tubulin plus colchicine (1×10^{-5} M) (34).

exposure at a concentration of 15 μg/ml or 30 μg/ml. However, at a concentration of 60 μg/ml there is a dramatic change in the microtubular network. The structures begin to depolymerize and appear to form pools or puddles of short segments of microtubules. At the highest concentration, the microtubular network is almost entirely gone and the centrospheric region of the cell is the only brightly fluorescing area of the cell. In all cells treated at this concentration one could see the brightly fluorescing centrioles juxtaposed to the nucleus (Fig. 19f). We have done the reciprocal experiment by holding the concentration of trypsin constant and varying the exposure time up to 10 min. The lower concentration of trypsin gradually caused a diminution of the CMTC such that at 15 μg/ml at the end of 10 min the cells were visually comparable to those shown in Fig. 19e, corresponding to 60 μg/ml (data not shown). At the higher trypsin concentration the cells were rounded and many floated off the coverslip.

DISCUSSION

From morphological and experimental evidence, we expect microtubules to comprise a major component of the cytoskeleton of most eukaryotic cells. We were surprised, however, to see such an extensive array of tubules in the cytoplasm of normal cultures of fibroblasts. To what function can we attribute the elaborate CMTC? From a large source of experimental and descriptive data, we are assured that it plays a major role in maintaining cell shape and form (1, 2). Similarly, it must be involved in cell shape changes. This is particularly evident when flattened fibroblastic cells in interphase enter mitosis. The cells of most lines round up and lose their close attachment with the substrate during

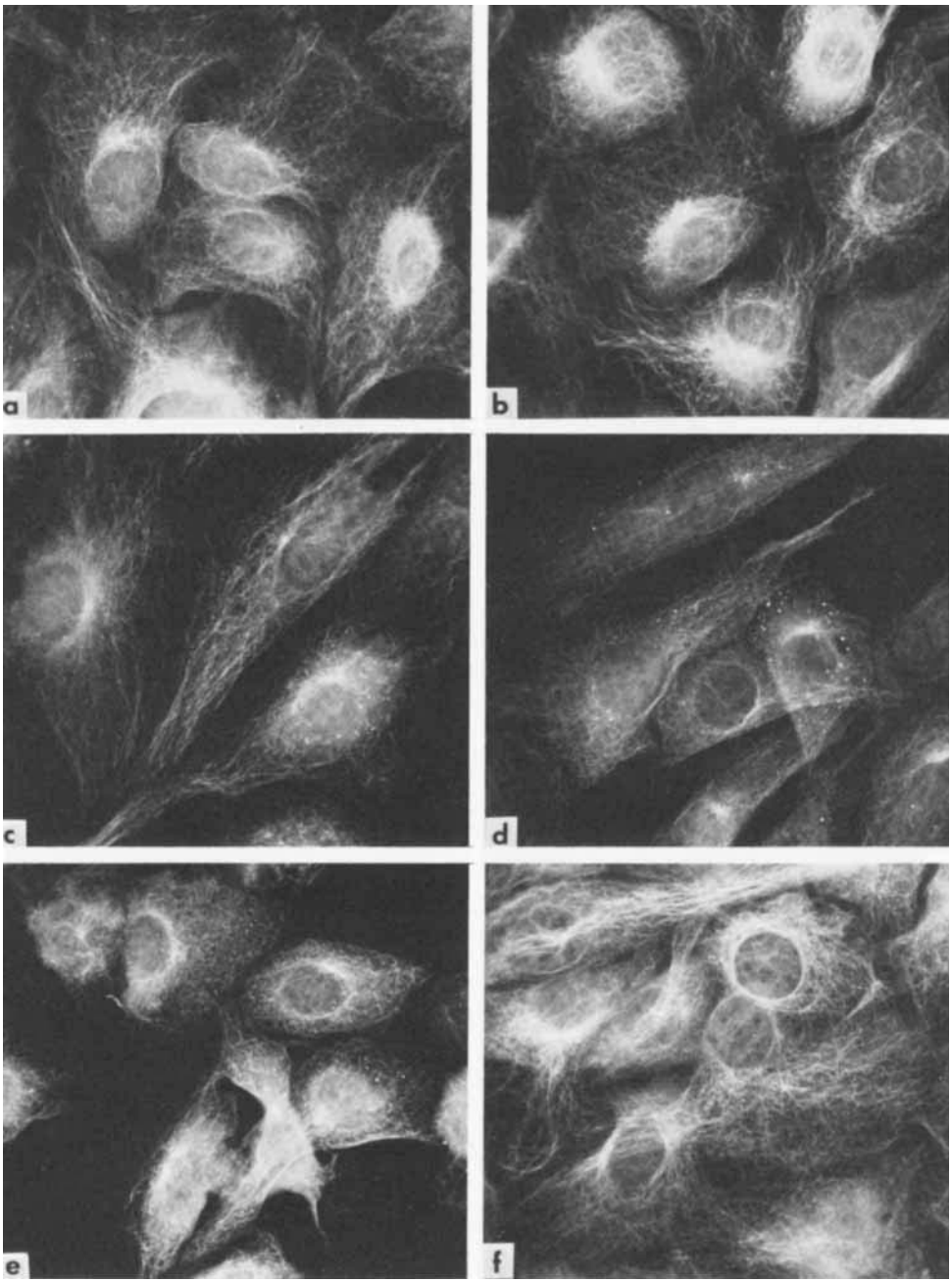


Fig. 18, Effects of increased intracellular calcium on the cytoplasmic microtubule complex of mouse 3T3 fibroblasts. a and b are control cells demonstrating the elaborate and extensive CMTC; c shows a slight decrease in the microtubular network at the end of 50 min exposure at 5.0 μg/ml of A23187; d and e show generalized breakdown of the CMTC following 100 min exposure; f shows recovery of CMTC following removal from ionophore in fresh media at the end of 50 min.

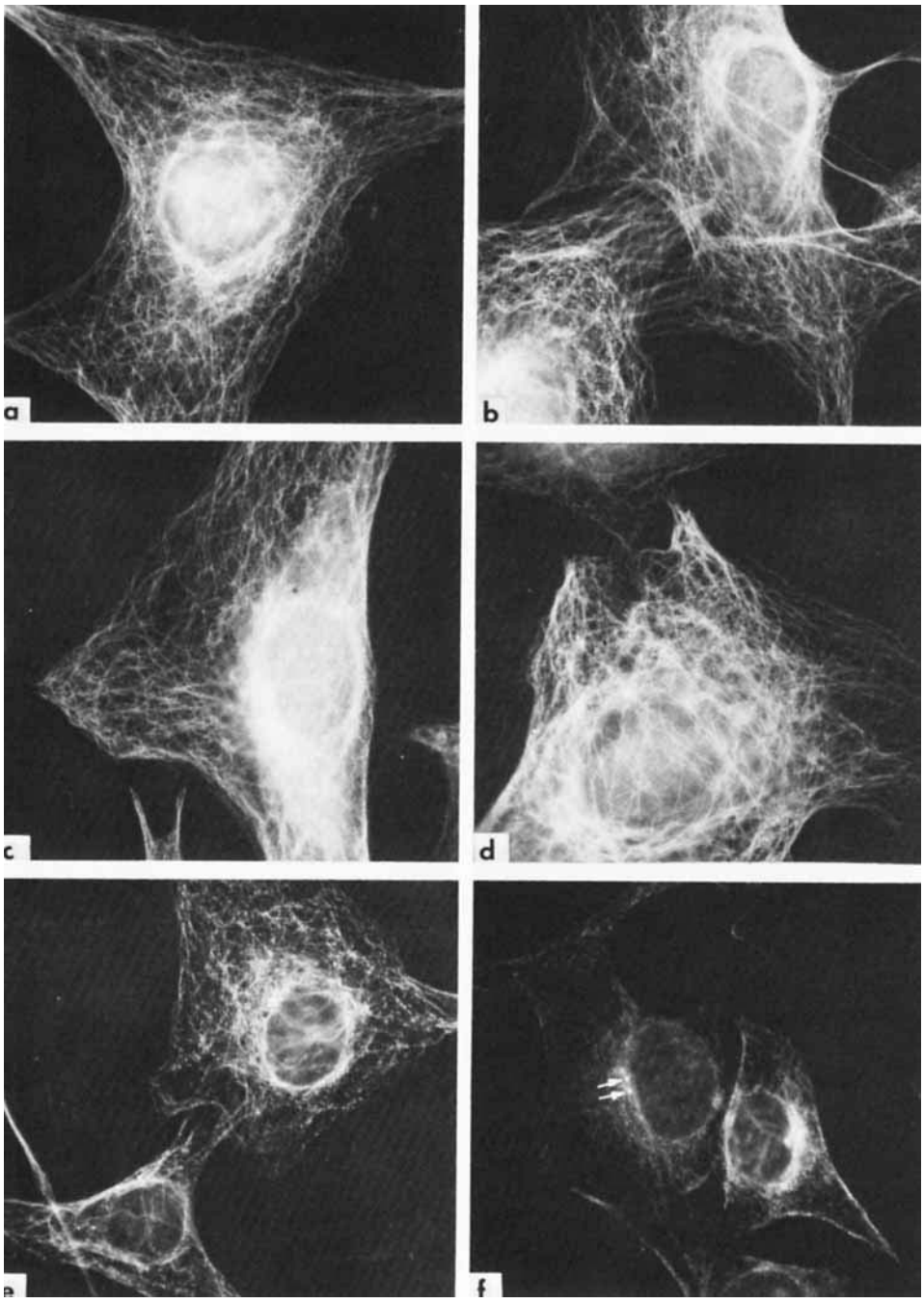


Fig. 19. Effects of trypsin on CMTC. (a and b) control mouse 3T3 fibroblasts; (c) 3.0 min exposure at 22°c to 15 μg/ml trypsin; (d) 3.0 min exposure at 22°C to 30 μg/ml trypsin; (e) 3.0 min exposure at 22°C to 60 μg/ml trypsin; (f) 3.0 min exposure at 22°C to 120 μg/ml trypsin.

metaphase and throughout mitosis. Concomitantly, the CMTC is disassembled but reappears in late telophase when the cells again become flattened and undergo major shape changes. Colcemid, cold shock, and other microtubule inhibitors also cause the cells to lose their flattened shape as well as their attachment to the substrate.

These observations all suggest that assembly as well as disassembly of microtubules, under conditions controlled by the cell, could orchestrate such fundamental morphological changes as extension and retraction of processes, rounding and flattening of cells. Clearly other mechanisms such as sliding or shearing could operate separately or in concert with assembly-disassembly to provide the force for morphological changes. At the present state of our knowledge, however, assembly-disassembly via Inoue's equilibrium model (13) appears to be the most likely mechanism for microtubule-mediated shape changes in the cytoplasm. There is little reason to conclude, however, that microtubules function alone in this capacity. The recent localization of actin, myosin, and their regulatory molecules in the cytoplasm of most eukaryotic cells (3, 4, 5), along with the capacity of these molecules to assemble and disassemble and elicit shearing forces, would imply an equally important role in cell shape changes, attachment, and motility.

Many other interesting but less well documented functions of cytoplasmic microtubules have been implied through the use of colchicine and related inhibitors. These include grieseofulvin, vinblastine and vincristine, podophyllotoxin, to name a few (22). Taking all colchicine responses collectively, it is difficult to envision a single unifying model to explain microtubule function in the cytoplasm of interphase cells. Obviously, much more must be learned about factors that regulate microtubule assembly and interactions.

What happens to the CMTC when cells become transformed? Our observations consistently confirm that cell transformation, whether by virus, chemicals, or spontaneous stimulation, is accompanied by striking alteration in the CMTC. These alterations may lead to a highly disorganized CMTC in which the individual tubules extend in random direction to one where a CMTC is essentially absent in the cytoplasm and only diffuse fluorescence is apparent. These changes are strikingly similar to alteration in actin cables in fibroblasts as described by Pollack and co-workers (15). In the latter case, actin seems to be necessary for anchorage, and its disassembly in transformed cells is concomitant with loss of anchorage-dependent growth control of these cells (14). Since, in our own studies, alteration in CMTC appears to correlate with an increased survival of cells in agar (anchorage independence), we tentatively conclude that this aspect of transformation relates to microtubules as well as microfilaments. It is tempting to speculate that other well-known properties of transformed cells such as continuous proliferation, altered cell surface properties, and loss of density-dependent control of growth may related to the CMTC's function in surface receptor modulation. As stated previously, it is impossible to define the involvement of microtubules in cell transformation without further knowledge of the factors that regulate the assembly and interaction of microtubules within the cytoplasm.

In Vivo Control of Microtubule Assembly

Certainly one of the more tantalizing questions concerning microtubules is what are the in vivo signals that regulate the assembly and disassembly of microtubule network? Once these signals are known, perhaps some insight as to why the transformed cell has a reduced CMTC will be gained.

It is now well established that tubulin from brain preparations can undergo polymerization in vitro (16, 17, 23). Of particular interest is the observation that the ubiquitous and important divalent cation calcium has been found to be an inhibitor of the in vitro assembly process (23–28). In fact, a number of investigators have suggested that intracellular calcium may play an important role in the in vivo formation of microtubules (25, 27, 28). If calcium does play a key role in regulating the assembly of cytoplasmic microtubules, then it must act through a negative feedback type of process. The presence of unsequestered free calcium ions would either initiate disassembly of microtubules or prevent the assembly of tubulin into its supramolecular form, the microtubule. One would thus envision that any molecular process that allows either protein-bound calcium or sequestered calcium (i.e., calcium in the mitochondria or smooth endoplasmic reticulum) to be released would, in effect, act as a regulator of this portion of the cytoskeletal structure. In order to test this hypothesis, it is important a) to demonstrate that when intracellular levels of calcium are high there are reduced numbers of formed microtubules and b) to demonstrate that organelles that normally play a role in intracellular calcium levels can regulate sufficient quantities of the cation to influence the assembly of microtubules.

We have approached testing the hypothesis that calcium modulates the CMTC in two different ways. First, we have used the divalent ionophore A23187 to increase the level of intracellular calcium of intact cells, and monitored the changes in the CMTC using indirect immunofluorescence as described previously. Similar results of the effects of calcium ionophores on microtubules have been reported by Poste and Nicolson (3). Second, we have taken an organelle (the mitochondria) that is known to be a calcium sequestration site and added it to an in vitro polymerizing system in the presence of calcium. The latter experiments were designed primarily to demonstrate that inhibiting quantities of calcium could be removed using a known intracellular calcium modulator, thus allowing for microtubule formation in vitro. Both of these experimental approaches have demonstrated that calcium is clearly involved in the assembly-disassembly process, which strengthens the argument that this ion is intimately involved in controlling the formation and stabilization of microtubules.

Cell Surface Control of Microtubules and Vice Versa

Another intriguing role of the CMTC is that of cell surface modulation. According to Edelman and co-workers (12, 29) microtubules as well as microfilaments provide "surface modulating assemblies" that control the position and motility of receptors on the cell surface. Such a role would certainly expand the function of cytoplasmic microtubules beyond that of mere struts needed to hold up the tent. Functioning directly or indirectly in surface receptor modulation, they would play a major role in the cell's interaction with its immediate environment and its recognition and response to neighboring cells, a condition essential for regulation of growth control. The information provided by the experiments presented here, as well as those from a number of other investigators, warrants our postulation of the following mechanism relative to membrane modulation with calcium as the principle signal.

First we propose that calcium is the primary modulating signal and that it acts principally through its effects on cytoplasmic microtubules and perhaps microfilaments. We also believe that surface modulation and microtubule assembly (or disassembly) are intimately related such that microtubules influence what type of receptor constellation exists on the external surface, and reciprocally, that perturbations at the cell surface are

manifested by specific alteration of cytoskeletal structure. If calcium is the primary signal, as we believe it is, then events that occur at the cell surface – such as receptor binding of incoming molecules, or surface alterations via limited proteolysis – initiate a release of protein-bound calcium. One can envision, for example, transmembrane proteins with calcium-binding sites on the internal surface. When the externally exposed region of the molecule is altered – for example, by ligand binding or by proteolysis, as in the case of trypsin – a subtle change in the conformation of the molecule occurs, and the calcium-binding sites (internally disposed) are altered, effecting release of the ion. The cell reacts to such a signal initially by microtubule depolymerization. If the ion is not removed the signal cascades and a more generalized microtubule depolymerization ensues. As the microtubules depolymerize, other surface receptors could be altered in a manner analogous to changes in the surface of a tent when the support poles are shortened or removed. Because of the hydrodynamic forces exerted by the molecular components of the cell membrane, the changes would most readily appear as a rounding-up of the surface. One would predict that specific receptor constellations comprised of glycoprotein and glycolipids would no longer provide the correct configuration for ligand-receptor interaction or cell-cell recognition. The cellular response can be reversed by the three mechanisms already known for calcium sequestration, i.e., movement of the ion into the mitochondria or smooth endoplasmic reticulum, or removal to the outside via the calcium pump. An interesting correlation in this regard is the fact that transformed cells with their rounded form and diminished CMTC can be temporarily reversed to a more normal morphology by increasing the intracellular level of cAMP (6). It has been suggested that cAMP plays a stimulatory role in calcium ion removal (31). It is noteworthy that the cytoplasmic level of cAMP is quite low in transformed cells (32, 33).

We have measured the total calcium level in 3T3 fibroblasts and their virally transformed counterparts SV-3T3 and have found a somewhat higher level in the transformants (34). While this may not apply to all neoplastic cells, we find intriguing the idea that transformation in some way interferes with Ca^{++} sequestration and subsequent changes in cell morphology concomitant with this loss.

Very little information is known as to how calcium induces the depolymerization of microtubules. Weisenberg reported (at these proceedings) a specific calcium binding site on the tubulin molecule, and indicated that for reasonable stoichiometry magnesium must be present. To date, there is no information on what conformational changes occur on the tubulin molecule when Ca^{++} binds. It will be extremely interesting to quantitate changes in the molecular conformation following binding and release of calcium.

In summary, we have presented information relative to the presence of an elegant cytoplasmic microtubular network that can undergo changes that appear to be direct results of transformation. Moreover, we postulate that the changes seen in this cytoskeletal network are a result of free calcium ion in the cytosol, and predict that this ubiquitous and important divalent cation is the molecular signal that transmits changes from the surface of the cell to responding biochemical processes deep within the cell. This is accomplished principally through changes in the microtubular structure. Finally, we have described briefly what we believe is a reasonable rationale demonstrating how calcium could play the role of membrane modulation signal. While many of the details are sketchy and perhaps oversimplistic, we feel that a reasonably strong case can be made to relate the membrane surface modulation with microtubule integrity principally through the divalent cation calcium.

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