REGULATION OF MICROTUBULE ASSEMBLY IN CULTURED FIBROBLASTS

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ABSTRACT

Microtubule assembly in diploid human skin fibroblasts was studied by \([^{3}H]\)-colchicine binding to disaggregated microtubule subunits and to total cell tubulin. Microtubule content per milligram of cell protein was critically dependent upon cell density. As cultures neared confluence, microtubules increased and total cell tubulin decreased; the percent of tubulin assembled into microtubules increased from 5.3 in sparse cultures to 58.3 in confluent cultures.

Microtubules disappeared with a half-time of 2 min in response to 0°C incubation and reformed upon rewarming. Brief treatment of intact cells with concanavalin A or cytochalasin A depolymerized microtubules to 55 or 56% of control levels. The effect of concanavalin A was prevented by α-methylmannoside. Fibroblast microtubule assembly was not significantly altered by cyclic nucleotides, ascorbate, glucose, insulin, medium calcium concentration, or calcium ionophore A23187.

Tubulin, one of the principal proteins of fibroblasts (12), can exist in cells either as a free dimer or as assembled microtubules (11). The pool of free tubulin in cultured cells is large (18, 21), suggesting that it might be a source of subunits for rapid microtubule formation. Such a role is readily understandable in relation to the mitotic spindle, which must be formed and disassembled in a short time. But the state of microtubule assembly also appears to be important in interphase cells, which contain many cytoplasmic microtubules (2). These structures appear to regulate important fibroblast processes, such as the secretion of procollagen (4), the receptor-specific uptake and degradation of low density lipoprotein (13), and the spatial distribution of cell organelles (20).

It has been suggested that drugs or intracellular mediators might act, in part, by alteration of microtubule assembly. Dibutyryl cyclic AMP appears to increase assembly of microtubules in CHO cells (21). A correlation between the ratio of cGMP: cAMP and both enzyme secretion and microtubule number has been found in polymorphonuclear leukocytes (27). Calcium, a ubiquitous intracellular messenger, increases apparent assembly of tubulin in tissue slices (5).

We have studied the effect of growth conditions and drugs on the free tubulin and microtubule content of diploid human skin fibroblasts using colchicine-binding techniques.

MATERIALS AND METHODS

Cells

Skin fibroblasts were derived from a deltoid region skin explant obtained from a 47-year-old caucasian male. The cells were grown in a humidified, 5% CO₂ incubator with Eagle’s minimum essential medium that contained 15% fetal bovine serum, 50 µg/ml streptomycin, 50 U/ml penicillin, and 1.25 µg/
bly inasmuch as no difference in [\(^1^H\)]colchicine binding to mi-
number of picomoles of [\(^1^H\)]colchicine boundcannot be directly
but colchicine did notsaturate tubulin binding sites. Hence, the
portional to the tubulin concentration under these conditions,
usinga charcoal suspension (14, 23). Colchicine binding is pro-
chicine at 37°C for 1 h. Bound tracer was separated from free,
Colchicine-binding assays were performedwith 25-pl aliquots of
m, 0.5 mM MgCl₂, 100U/ml Trasylol, 10 mM sodium
and tubulin have been published elsewhere(l4, 17). Fordeter-
Co., Pittsburgh, Pa. Glucose,
sodium L-ascorbate, and butyric acid were purchased from Fisher
yields amicrotubule subunit-containing supernate. This assay
and lipoprotein-deficient plasma (3) were prepared as previously

does not distinguish microtubules from possible intracellular

crotubule subunits and free tubulin) and others

Reagents
Concanavalin A, dibutyryl cyclic AMP, dibutyryl cyclic GMP,

Tubulin and Microtubule Quantitation
Detailed methods for measurement of fibroblast microtubules
and tubulin have been published elsewhere (14, 17). For deter-
mination of microtubules, cells from a single 100-mm dish were
washed six times at room temperature in 0.15 M NaCl, drained,
and quickly rinsed with 1.0 ml of microtubule-poor stabilization buffer
containing 50% glycerol, 5% dimethyl sulfoxide, 0.5 mM MgCl₂,
0.5 mM EGTA, 0.5 mM GTP, 100 U/ml Trasylol, and 10 mM
sodium phosphate, pH 6.95. The cells were quickly scraped into
residual stabilization buffer (0.3 ml) and homogenized. A small
amount was removed for the measurement of protein (9) and
DNA (25), and the homogenate was centrifuged at 130,000 g for
10 min in a Beckman Airfuge (Beckman Instruments, Inc.,
Spinco Div., Palo Alto, Calif.). The microtubule pellet was gently
rinsed with stabilization buffer, drained, and homogenized with
0.15 ml of depolymerization buffer containing 0.25 M sucrose,
0.5 mM GTP, 0.5 mM MgCl₂, 100U/ml Trasylol, 10 mM sodium
phosphate, pH 6.95, and 0.2% bovine albumin. The microtubules
were placed on ice for 1 h, and the suspension was recentrifuged
to yield a microtubule subunit-containing supernate. This assay
does not distinguish microtubules from possible intracellular
tubulin aggregates sedimentable at 100,000 g. Brief exposure
(<1 min) of intact cells to microtubule stabilization buffer before
homogenization did not appear to influence microtubule assembly
as much as no difference in [\(^1^H\)]colchicine binding to mi-
crotubules was observed between cells harvested in the usual
fashion and cells from replicates, which were allowed to
stand for 3 min at room temperature in stabilization buffer before
homogenization.

For measurement of total tubulin (microtubule subunits and
free tubulin), cells were homogenized directly in depolymeriza-
to yield amicrotubule subunit-containing supernate. This assay
does not distinguish microtubules from possible intracellular
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fashion and cells from replicates, which were allowed to
stand for 3 min at room temperature in stabilization buffer before
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In order that both increases and decreases in
microtubule content could occur and be detected in the
experiments that are described below, studies were performed on cells plated at 5 × 10⁶ per
100-mm dish and were harvested after 7 d in culture, unless otherwise stated.

Effect of Serum (Table I)
Fibroblasts were kept for 24 h in Eagle's mini-
num essential medium containing 0.4% lipopro-
tein-deficient plasma, an amount permitting the
cells to remain viable but not sufficient to support
growth. Half the cells then received 9% fetal bo-
vine serum for an additional 24 h and half re-
mained in the serum-deficient medium. Table I
shows that 24 h of serum treatment increased the
protein and DNA contents per dish by 54 and
60%, respectively. Tubulin increased more than
total protein, and total tubulin was 34 ± 3% more than
that of sparse 3-d cultures. The percent of tubulin in
microtubule form rose with increasing time in
culture: 5.3 ± 3.7 at day 3; 16.7 ± 4.0 at day 6;
41.4 ± 7.7 at day 9; and 58.3 ± 4.3 at day 13. This
does not appear to be the result of the duration of
growth in culture after plating. Fibroblasts were set
in triplicate at either 0.5 × 10⁶ or 2.0 × 10⁶
cells per 100-mm dish and cultured for 5 d.

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shows that 24 h of serum treatment increased the
protein and DNA contents per dish by 54 and
60%, respectively. Tubulin increased more than
total protein, and total tubulin per milligram of
protein was up by 24%. However, microtubule-
derived tubulin per milligram of protein was un-

R. E. OSTLUND, JR., J. T. LEUNG, AND S. V. HAJEK Fibroblast Microtubules 387
Effect of Cold

Figure 1  Effect of cell density on tubulin and microtubules. Human diploid skin fibroblasts were grown in stock cultures plated at 1.5 x 10⁶ cells per 150-mm dish and replated every 7 d. At the beginning of the experiment, a stock dish ready for replating was harvested, and the cells were set out at 5 x 10⁶ cells per 100-mm dish and grown for up to 13 d. At intervals, triplicate dishes were analyzed for either total tubulin or microtubule-derived tubulin. Medium was replaced every 3 or 4 d, and dishes for analysis were harvested 3 or 4 d after the last medium change to minimize any effect of cell feeding. Bars indicate one standard error of the mean. Total tubulin, ●. Microtubules, ○. Cell protein, △.

Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein per dish</th>
<th>DNA per dish</th>
<th>DNA/Mg protein</th>
<th>Microtubules</th>
<th>Total tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.4% plasma)</td>
<td>689 ± 0.05</td>
<td>20.5 ± 0.7</td>
<td>29.8</td>
<td>23.2 ± 4.2</td>
<td>75.9 ± 1.7</td>
</tr>
<tr>
<td>With 9% serum</td>
<td>1.06 ± 0.07*</td>
<td>32.7 ± 1.6*</td>
<td>30.8</td>
<td>23.3 ± 3.7</td>
<td>93.8 ± 5.8*</td>
</tr>
</tbody>
</table>

* P < 0.05 with respect to control.

Fibroblasts plated at 10⁶ per 100-mm dish were grown in Eagle's minimum essential medium containing 15% fetal bovine serum with medium exchanges every 3 d. On day 9, the medium was removed, the cells were washed with saline G, and 10 ml of minimum essential medium containing 0.4% lipoprotein-deficient human plasma was replaced. The cells were incubated for 24 h, after which dishes received either 1 ml of fetal bovine serum (final concentration 9%) or no addition, followed by a further 24-h incubation. The cells were then harvested. Results are from four dishes per experimental condition.

Effect of Drugs (Table II)

Colchicine (10⁻⁶ M) disrupted more than 97% of the microtubules after a 3-h incubation, as was expected. However, concanavalin A and cytochalasin A also significantly reduced the cell microtubule content. At 300 μg/ml, concanavalin A reduced the microtubules to 56% of the expected level, and the effect was completely prevented by 0.1 M α-methylmannoside. Cytochalasin A (10 μM) also significantly reduced the microtubule content—to 55% of the expected level. This effect changed by serum treatment. Thus, the induction of cell division in quiescent cells by the addition of serum caused a selective accumulation of free tubulin.

Effect of Cold

Fig. 2 demonstrates that microtubules rapidly disassembled on exposure to a temperature of 0°C. After 2 min, only 47 ± 7% of the microtubules remained, and at 60 min the microtubules were reduced to 2.5% of control levels. When dishes incubated for 60 min at 0°C were rewarmed at 37°C, microtubules reformed, although more slowly than the cold-induced depolymerization. At 30 min of rewarming, the microtubule content was 58% of the original value (Fig. 2).
FIGURE 2 Effect of temperature on fibroblast microtubule content. Cells grown under standard conditions were removed from the CO2 incubator and washed with saline at room temperature. In one experiment, dishes were then incubated for the indicated times at 0°C with saline in air, after which microtubule content was determined (O). In another experiment, dishes were incubated at 0°C for 1 h and then rewarmed at 37°C for the indicated times before microtubule quantitation (C). Each point is the mean of three culture dishes.

was not seen with cytochalasin B or D, however. Neither concanavalin A nor cytochalasin A had any effect on total cell tubulin content (95.1 ± 4.3 and 110 ± 12% of control, respectively). Carbachol, ascorbic acid, cyclic nucleotides, glucose, insulin, and low density lipoprotein had no significant effect on microtubule content.

Effect of Calcium (Table III)

Fibroblasts were grown in Eagle’s minimum essential medium containing 15% fetal bovine serum (total calcium content, 2.0 mM as determined by atomic absorption spectroscopy). The addition of 1 mM EGTA (effectively reducing the calcium content to 1 mM) or 4.0 mM extra calcium (elevating the calcium content to 6.0 mM) for 1 h had no effect on microtubule mass (Table III). Cells treated with unmeasurable calcium levels by the addition of 1 mM EGTA in divalent cation-free saline began to detach from the dish in a few minutes but demonstrated no change in microtubule content when compared with controls in growth medium. Previous data have shown, however, that removal of established cell lines from monolayers by trypsin is associated with decreased numbers of microtubules (6).

DISCUSSION

The principal factor regulating the assembly of normal fibroblast microtubules appears to be cell density or something closely associated with it. Fig. 1 shows that microtubule levels per milligram of cell protein at day 13 of culture (confluence) were 368 ± 27% of the levels at day 3 (sparse

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Incubation period min</th>
<th>% of control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine 10⁻⁶ M</td>
<td>180</td>
<td>&lt;3.0‡</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>60</td>
<td>79.8 ± 9.3</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>60</td>
<td>77.3 ± 2.9§</td>
</tr>
<tr>
<td>300 µg/ml</td>
<td>60</td>
<td>56.2 ± 6.9‡</td>
</tr>
<tr>
<td>300 µg/ml + 0.1 M α-methylmannoside</td>
<td>60</td>
<td>107.0 ± 12.0</td>
</tr>
<tr>
<td>0.1 M α-methylmannoside alone</td>
<td>60</td>
<td>101.0 ± 18.0</td>
</tr>
<tr>
<td>Cytochalasin Aij 10 µM</td>
<td>60</td>
<td>54.9 ± 5.9§</td>
</tr>
<tr>
<td>Cytochalasin Bj 10 µM</td>
<td>60</td>
<td>85.8 ± 9.7§</td>
</tr>
<tr>
<td>Cytochalasin Dij 10 µM</td>
<td>60</td>
<td>96.6 ± 8.8§</td>
</tr>
<tr>
<td>Sodium L-ascorbate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>30</td>
<td>99.4 ± 8.4</td>
</tr>
<tr>
<td>1 mM</td>
<td>30</td>
<td>92.4 ± 11.0</td>
</tr>
<tr>
<td>Carbachol 10⁻³ M</td>
<td>30</td>
<td>100.0 ± 9.2</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 mM</td>
<td>60</td>
<td>112.0 ± 6.9</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>60</td>
<td>112.0 ± 4.5</td>
</tr>
<tr>
<td>Dibutyryl cyclic GMP</td>
<td>60</td>
<td>97.0 ± 11.0</td>
</tr>
<tr>
<td>10 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium butyrate 10 mM</td>
<td>60</td>
<td>87.2 ± 8.4</td>
</tr>
<tr>
<td>Glucose 19 mM</td>
<td>24 h</td>
<td>93.7 ± 17.0</td>
</tr>
<tr>
<td>Insulin 0.2 µg/ml</td>
<td>60</td>
<td>110.0 ± 9.3</td>
</tr>
<tr>
<td>Low density lipoprotein (LDL)** 500 µg/ml</td>
<td>10</td>
<td>109.0 ± 11.0</td>
</tr>
</tbody>
</table>

* The microtubule content ± SE of 4–12 100-mm dishes of treated fibroblasts is expressed as percent of the mean microtubule content of an equal number of simultaneous controls. [³H]colchicine binding of controls ranged from 10.4 to 37.4 pmol/mg.

‡ P < 0.02
§ P < 0.05
¶ Both treated and control dishes contained 0.05% ethanol or dimethyl sulfoxide. The solvent alone at this level had no effect on microtubule content.

¶ Control dishes contained 5.6 mM glucose.

** On day 7, cells were washed, the medium was replaced with 2 ml of MEM containing 5% lipoprotein-deficient human plasma, and the cells were incubated for 24 h to induce LDL receptors. LDL was then added to the cells from a concentrated stock.

R. E. OSTLUND, JR., J. T. LEUNG, AND S. V. HAJEK Fibroblast Microtubules

389
TABLE III

Effect of Calcium on Cell Microtubule Content

<table>
<thead>
<tr>
<th>Condition</th>
<th>Microtubule-derived tubulin</th>
<th>% of control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eagle's minimum essential medium containing 15% fetal bovine serum (2.0 mCa)</td>
<td>100 ± 11.1</td>
<td></td>
</tr>
<tr>
<td>with 1.0 mM EGTA</td>
<td>108 ± 10.7</td>
<td></td>
</tr>
<tr>
<td>with 4.0 mM CaCl₂</td>
<td>114 ± 13.5</td>
<td></td>
</tr>
<tr>
<td>with 10 μM A23187</td>
<td>101 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>with 0.3 μM A23187</td>
<td>104 ± 20.6</td>
<td></td>
</tr>
<tr>
<td>Divalent cation-free saline G with 1 mM EGTA</td>
<td>102 ± 5.4</td>
<td></td>
</tr>
</tbody>
</table>

Fibroblasts were grown for 7 d in Eagle's minimum essential medium containing 15% fetal bovine serum (total calcium concentration, 2.0 mM). The medium was then changed to give the concentrations of additives listed, and the cells were incubated for 1 h (30 min in the case of cells receiving compound A23187) at 37°C before harvest. Cells receiving divalent cation-free saline G that contained EGTA partially detached from the dish over 5 min and were completely removed by vigorous pipetting and sedimented for assay at that time. The range of [3H]colchicine binding to control microtubule subunits was 12.4-26.3 pmol/mg in five experiments.

*Controls received Eagle's minimum essential medium containing 15% fetal bovine serum. All controls were concurrent with the experimental dishes.

be the signal in some cells inasmuch as it may rise at confluence (15), the reported lack of change in cAMP content at confluence in normal human skin fibroblasts (7) and the lack of a response of normal fibroblasts to exogenous cyclic nucleotides (Table II) suggest that some other agent is the mediator of density-dependent microtubule assembly.

Tubulin preferentially accumulated in sparse and rapidly growing fibroblast cultures (Fig. 1). Likewise, Table I (last column) demonstrates that growth induced in quiescent fibroblasts close to confluence by the addition of serum was accompanied by a selective accumulation of free tubulin (total tubulin per milligram of cell protein increased by 24%). These changes are similar to, but much less striking than, the threefold increase in total tubulin per milligram of cell protein (22) or per microgram of DNA (16) in lymphocytes stimulated with phytohemagglutinin.

Fibroblast microtubules were very labile with exposure to cold (Fig. 2). Over half the microtubules disassembled after 2 min of 4°C temperature, and essentially none remained after 60 min. Rewarming the cells resulted in assembly of tubulin. It is apparent that fibroblast microtubules are capable of rapid assembly and disassembly.

Cytochalasin A (10 μM) dissociated microtubules into subunits such that only 54.9 ± 6% of the expected amount remained after 1 h (Table II). Cytochalasins B and D had no effect on microtubules. This is consistent with data presented by Himes et al. (8) showing that the in vitro polymerization of brain tubulin is inhibited by cytochalasin A but not by cytochalasin B. The effect is thought to be the result of a reaction of cytochalasin A with sulfhydryl groups on tubulin. Whereas those authors demonstrated that preincubation of tubulin with cytochalasin A in concentrations higher than those we employed inhibited colchicine binding to tubulin, we found no inhibition with addition of 10 μM cytochalasin A to our assays. The total tubulin content of cytochalasin A-treated cells was not altered, indicating a redistribution of tubulin from microtubules to a free form. The ability of cytochalasin A to depolymerize microtubules in living cells should be considered when the effects of the compound on cell filaments are studied.

Concanavalin A, 300 μg/ml for 1 h, was found to depolymerize fibroblast microtubules to 56 ± 7% of the control levels (Table II). The effect was completely prevented by the addition of 0.1 M α-
methylmannoside, a competitive inhibitor of lectin binding to cell surface glycoproteins. Total [³H]-
oculine binding to tubulin was not altered by concanavalin A. Concanavalin A has been re-
port to increase microtubule assembly in human polymorphonuclear leukocytes by four- to seven-
fold (10). It appears that Concanavalin A can exert opposite effects on microtubule assembly through
membrane binding in these two cell systems. Fibroblast microtubules were resistant to a variety of
drugs and agents thought to influence or to be dependent upon microtubule function, including ascorbate (26),
carbocاح (10), cyclic nucleotides (1, 24, 27), glucose and insulin (16), and the receptor-
specific uptake of low density lipoprotein (13). Likewise, manipulation of intracellular free calcium
concentration with ionophore A23187 or extracellular calcium failed to influence the state of
microtubule assembly (Table III).

These results indicate that microtubules from normal skin fibroblasts are relatively resistant to
many common drugs and alterations of culture conditions, and that cell density-related factors are
the primary determinants of tubulin assembly.

Supported by grants from the St. Louis Diabetic Children's Welfare Fund and the National Institutes of
Health.

Received for publication 22 January 1979, and in revised form 2 November 1979.

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R. E. OSLUND, JR., J. T. LEUNG, AND S. V. HAJEK

Fibroblast Microtubules 391