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% CO_2 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/

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ml amphotericin B (Fungizone, Squibb & Sons, Princeton, N. J.). The cells were used between 5 and 30 in vitro cell doublings and were routinely plated at 500,000 cells/100-mm diameter plastic tissue culture dish. The medium (10 ml) was replaced twice weekly, and the cells were used after 7 days' growth. Saline G (19) and human low density lipoprotein (1.019–1.063 g/ml) and lipoprotein-deficient plasma (3) were prepared as previously described, except that clotting of the lipoprotein-deficient plasma with thrombin was omitted.

Reagents

Concanavalin A, dibutyryl cyclic AMP, dibutyryl cyclic GMP, GTP, cytochalasins A, B, and D, colchicine, and carbachol were purchased from Sigma-Aldrich Co., St. Louis, Mo. Glucose, sodium L-ascorbate, and butyric acid were purchased from Fisher Scientific Co., Pittsburgh, Pa. Glucagon-poor insulin was a gift of Dr. Mary Root, and compound A23187 was a gift of Dr. Robert Hamill, both of Eli Lilly Research Laboratories, Indianapolis, Ind. [^{3}H]Colchicine (4 Ci/mol) was purchased from Amersham Corp., Arlington Heights, Ill. Trasylol (aprotinin) was obtained from FBA Pharmaceuticals, Inc., New York. EGTA was purchased from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.

Tubulin and Microtubule Quantitation

Detailed methods for measurement of fibroblast microtubules and tubulin have been published elsewhere (14, 17). For determination 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 stabilization buffer containing 50% glycerol, 5% dimethyl sulfoxide, 0.5 mM $MgCl_2$, 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 $MgCl_2$, 100 U/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 inasmuch as no difference in [^{3}H]colchicine binding to microtubules was observed between cells harvested in the usual fashion and cells from replicate plates, 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 depolymerization buffer without albumin and centrifuged after 1 h on ice. Colchicine-binding assays were performed with 25-μl aliquots of tubulin in depolymerization buffer containing 1.5 μM [^{3}H]colchicine at 37°C for 1 h. Bound tracer was separated from free, using a charcoal suspension (14, 23). Colchicine binding is proportional to the tubulin concentration under these conditions, but colchicine did not saturate tubulin binding sites. Hence, the number of picomoles of [^{3}H]colchicine bound cannot be directly equated with the number of picomoles of tubulin present. The ratio of [^{3}H]colchicine bound to cell extracts of dense cells:sparse cells was constant over a fivefold range of [^{3}H]colchicine concentration.

Triplicate analyses were performed on each experimental dish and averaged. Means ± standard error of the mean for 3–12 dishes were compared with Student's *t* test.

RESULTS

Fibroblasts were plated at 5×10^5 cells per 100-mm dish and grown for up to 13 d. At intervals, some dishes were analyzed for total tubulin (microtubule subunits and free tubulin) and others for microtubule-derived tubulin. Fig. 1 demonstrates that [^{3}H]colchicine binding to depolymerized microtubules per milligram of protein was least in young cultures and progressively increased with time. At confluence (13-d cultures), microtubules per milligram of protein was 368 ± 27% more 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 out in triplicate at either 0.5×10^6 or 2.0×10^6 cells per 100-mm dish and cultured for 5 d. [^{3}H]Colchicine binding to total tubulin was 71.3 ± 4.8 pmol/mg in the low-density dishes and 47.9 ± 2.5 pmol/mg in the high-density dishes ($P < 0.01$).

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 5×10^5 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 minimum essential medium containing 0.4% lipoprotein-deficient plasma, an amount permitting the cells to remain viable but not sufficient to support growth. Half the cells then received 9% fetal bovine serum for an additional 24 h and half remained 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 per milligram of protein was up by 24%. However, microtubule-derived tubulin per milligram of protein was un-

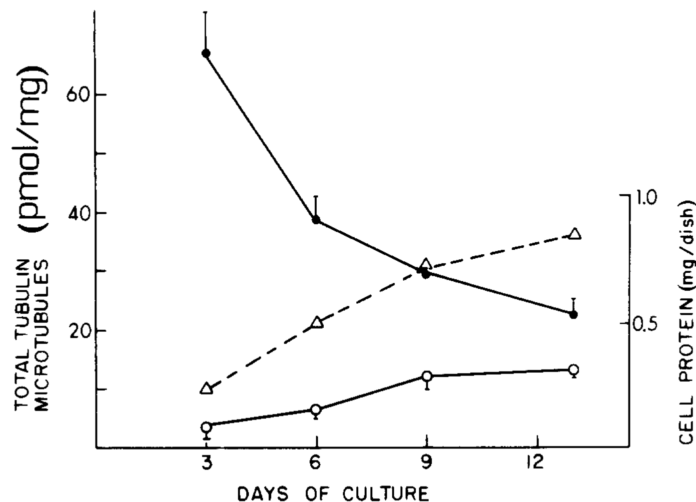

FIGURE 1 Effect of cell density on tubulin and microtubules. Human diploid skin fibroblasts were grown in stock cultures plated at 1.5×10^6 cells per 150-mm dish and replated every 7 d. At the beginning of the experiment, a stock dish ready for replating was harvested, and the cells were set out at 5×10^5 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 I
Effect of Serum on Fibroblast Microtubules

	Protein per dish	DNA per dish	DNA	Microtubules	Total tubulin
	mg	*μg*	*μg/mg protein*	*pmol/mg protein*	*pmol/mg protein*
Control (0.4% plasma)	.689 ± .05	20.5 ± 0.7	29.8	23.2 ± 4.2	75.9 ± 1.7
With 9% serum	1.06 ± .07*	32.7 ± 1.6*	30.8	23.3 ± 3.7	93.8 ± 5.8*

* $P < 0.05$ with resepct to control.

Fibroblasts plated at 10^5 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.

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).

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

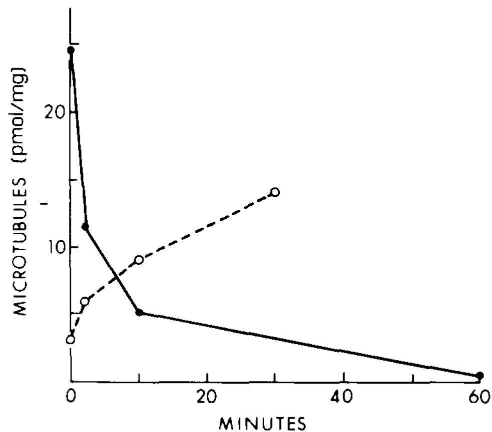

FIGURE 2 Effect of temperature on fibroblast microtubule content. Cells grown under standard conditions were removed from the CO_2 incubator and washed with saline G at room temperature. In one experiment, dishes were then incubated for the indicated times at 0°C with saline G in air, after which microtubule content was determined (●). 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 (○). 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 G 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 II

Effect of Drugs on Fibroblast Microtubules

Drugs	Incubation period	Microtubule-derived tubulin
	min	*% of control**
Colchicine 10^{-6} M	180	<3.0‡
Concanavalin A		
50 μg/ml	60	79.8 ± 9.3
100 μg/ml	60	77.3 ± 2.9§
300 μg/ml	60	56.2 ± 6.9‡
300 μg/ml with 0.1 M α-methylmannoside	60	107.0 ± 12.0
0.1 M α-methylmannoside alone	60	101.0 ± 18.0
Cytochalasin A‖ 10 μM	60	54.9 ± 5.9‡
Cytochalasin B‖ 10 μM	60	85.8 ± 9.7
Cytochalasin D‖ 10 μM	60	96.6 ± 8.8
Sodium L-ascorbate		
10 mM	30	99.4 ± 8.4
1 mM	30	92.4 ± 11.0
Carbachol 10^{-3} M	30	100.0 ± 9.2
Dibutyryl cyclic AMP		
1.0 mM	60	112.0 ± 6.9
10.0 mM	60	112.0 ± 4.5
Dibutyryl cyclic GMP 10 mM	60	97.0 ± 11.0
Sodium butyrate 10 mM	60	87.2 ± 8.4
Glucose 19 mM¶	24 h	93.7 ± 17.0
Insulin 0.2 μg/ml	60	110.0 ± 9.3
Low density lipoprotein (LDL)** 500 μg/ml	10	109.0 ± 11.0

* 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. [^{3}H]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.

TABLE III
Effect of Calcium on Cell Microtubule Content

	Microtubule-derived tubulin
	*% of control**
Eagle's minimum essential medium containing 15% fetal bovine serum (2.0 mM Ca)	100 ± 11.1
with 1.0 mM EGTA	108 ± 10.7
with 4.0 mM $CaCl_2$	114 ± 13.5
with 10 μM A23187	101 ± 5.6
with 0.3 μM A23187	104 ± 20.6
Divalent cation-free saline G with 1 mM EGTA	102 ± 5.4

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 [^{3}H]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.

cultures). However, total tubulin (including both free tubulin and that assembled into microtubules) on day 13 had declined to only 34 ± 3% of the day-3 value. The percent of tubulin assembled into microtubules rose tenfold from 5.3 ± 3.7 at day 3 to 58.3 ± 4.3 at day 13.

One variable between confluent and sparse cultures is the rate of cell growth. The cytoplasmic microtubular network of dividing cells disappears and is replaced by the mitotic spindle (2). Dividing cells might be expected to thereby undergo a net change in microtubule content. However, when quiescent fibroblasts maintained in 0.4% plasma were stimulated to grow by the addition of 9% serum (Table I), the microtubule content per milligram of cell protein did not change despite a 60% increase in DNA per culture dish, which indicated that cell division had occurred. It is difficult, therefore, to attribute the poor polymerization of tubulin in sparse cultures entirely to rapid cell growth. It seems likely that cell-to-cell contact or a humeral signal at confluence may increase the assembly of microtubules. Although cAMP may 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 be 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 [^{3}H]-colchicine binding to tubulin was not altered by concanavalin A. Concanavalin A has been reported to increase microtubule assembly in human polymorphonuclear leukocytes by four- to sevenfold (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), carbachol (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.

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