QUANTITATIVE BIOCHEMICAL ANALYSIS OF MICROTUBULE CONTENT IN NORMAL AND TRANSFORMED 3T3 CELLS

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ABSTRACT

Microtubules in normal and transformed BALB 3T3 cells were preserved in a stabilizing medium and measured by a [^{3}H]colchicine-binding tubulin assay, and compared to total cellular tubulin measured under nonstabilizing conditions. Essentially no change in tubulin or microtubule content was seen with changes in cell density or with changes in cellular morphology at various stages of growth of normal or transformed cells or induced by dibutyryl cAMP treatment of transformed cells. Of five cell lines transformed by a variety of agents, four had a significantly higher total tubulin content than untransformed 3T3 cells and all of them had an increased microtubule content. None of the transformed lines had a lower fraction of tubulin recoverable as sedimentable microtubules compared to untransformed cells, and in three of them this fraction was significantly higher. These results establish that microtubules are present in transformed cells to at least the extent (if not greater) than in normal cells but that there are variations in the total amount of tubulin and microtubules as well as the fraction of the total tubulin present as microtubules which are not strictly correlated with transformation or cell morphology.

KEY WORDS microtubule stabilization · transformation · tubulin · colchicine

The relationship of cytoskeletal structure and, in particular, cytoplasmic microtubules to cell transformation and neoplastic cell growth recently has been the subject of much study and speculation. Some reports have stated that transformed cells, including 3T3 cells, have a decreased number of cytoplasmic microtubules (3, 9, 14) or altered microtubule patterns (5, 13), while others refute these claims by stating that the microtubule level is the same in normal and transformed cells (4, 16, 21). All of these studies, showing either differences or similarities, have depended upon the visualization of microtubules with fluorescent antibodies or electron microscopy. Because of the qualitative nature of these studies, we approached this question by applying a quantitative biochemical procedure for recovering and measuring intact sedimentable microtubules in normal and transformed BALB 3T3 cells. We have also applied this technique to examining the effect of dibutyrylcAMP (dbcAMP) on the microtubule content of a transformed cell line since it had been concluded, again on the basis of electron microscopy, that increasing the cAMP level in Chinese hamster ovary (CHO) cells increased microtubule content (18). One biochemical study of CHO cells (20) tended to support this conclusion, but our data from Kirsten sarcoma virus-transformed 3T3 cells (Ki-3T3) do not.

MATERIALS AND METHODS

Except as noted in Table I, cells in late log phase were used. All cells were derived from BALB 3T3 A-31. We used two contact-inhibited subclones of 3T3 (P3 and 714) as normal controls and cell lines transformed by various agents including Simian virus-40 (SV-3T3), Moloney sarcoma virus (Mo-3T3), Kirsten sarcoma virus

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(Ki-3T3), and 4-nitroquinoline-1-oxide (NQT-3T3). Two separate isolates of nonproducer Ki-3T3 cells were used (clones 234-21 and 1) as well as a flat revertant of Ki-3T3-1 designated as Ki-R. A description of these cell lines, their sources, and culture methods have been reported previously (11, 17).

The procedure for measuring the fraction of the total intracellular pool of tubulin present as polymerized microtubules has been described (6). Briefly, the growth medium was removed from duplicate sets of cultures in 100 × 20 mm dishes, and 0.50 ml of a microtubule-stabilizing medium, MTM, (7) consisting of 50% glycerol, 10% DMSO, 5 mM sodium phosphate, 5 mM $MgCl_2$, pH 7, was added to each dish of cells at 22°C. After 7 min, the cells were harvested, duplicate dishes were pooled, and the suspension was sonicated. The sonicate was centrifuged at 43 kG for 60 min at 22°C to sediment the intact microtubules. The supernate was removed and the recovered pellet was resuspended in 100 μl of ice-cold PM buffer (10 mM sodium phosphate, 10 mM $MgCl_2$, pH 6.95) with 1 mM guanosine triphosphate and 0.5% Triton X-100 and then resonicated at 0°C to insure total disruption of the microtubules. Tubulin in this solution was measured by the [^{3}H]colchicine-binding DEAE cellulose filter assay (2, 22) and the radioactivity recovered represented tubulin polymerized into microtubules. It was not possible to measure the free soluble tubulin in the 43 kG supernate because MTM inhibited the [^{3}H]-colchicine binding reaction, an effect also noted by Rubin and Weiss (20) using the same MTM solution. It was confirmed that this effect was due to the MTM rather than to cell components by the observation that MTM alone inhibits colchicine binding to purified bovine brain tubulin. Therefore, cell samples were prepared that were treated as described above, except that PM buffer was substituted for MTM and sonication was performed at 0°C to insure that all tubulin would be soluble. Portions of these sonicates were assayed to determine the total amount of tubulin present in the cells, thus allowing a comparison with the MTM-treated samples to yield the percent of total tubulin polymerized into microtubules. The amount of tubulin in samples was calculated from a standard curve constructed using purified bovine brain tubulin as described previously (6). In all cases, the amount of [^{3}H]colchicine bound fell within the linear portion of that curve and generally ranged between 1,000 and 10,000 cpm. All assays were performed in duplicate and deviation of duplicate values from the averages ranged between 5 and 10%. Experiments were done twice, on separate days, and the results did not vary.

The assay cannot distinguish between cytoplasmic and spindle microtubules but, since a very small fraction of a culture is in mitosis at any one time, comparisons were assumed to reflect cytoplasmic microtubules. That the assay measures microtubules has been demonstrated in part by the fact that pretreatment of cells in the cold as well as with colchicine significantly reduced the fraction of tubulin present as microtubules (see Table VII in reference 6). In the present experiments, all assays were also carried out on duplicate cultures pretreated with 10 μM colchicine for 1 h to confirm depolymerization. In preliminary experiments, radioactive colchicine was added to cells during this pretreatment and it was shown that no colchicine was carried over into the assay.

Although tubulin will polymerize in vitro in the presence of glycerol or DMSO, there is a critical concentration of ~230 μg/ml which must be reached before polymerization is induced at room temperature (10). That value is for tubulin in 4 M glycerol and 0.1 M buffer and increases as the molarity of the buffer decreases so that in MTM, which contains a total of 0.01 M salt, the critical concentration may be even higher. The tubulin concentration of sonicates, however, was much lower than 230 μg/ml. The amount of total protein in sonicates ranged from 1.2 to 3.2 mg/ml. Using the values for μg tubulin per mg protein shown in Tables I and II, we calculated that the tubulin concentration in these sonicates ranged from 8 to 23 μg/ml.

While it is conceivable that the 43-kG pellet contained a small component of membrane-bound tubulin (1) or other forms of insoluble tubulin, or colchicine-insensitive microtubules, the great majority of the sedimentable tubulin in these experiments represents microtubules because pretreatment of the cells with colchicine (Tables I and II) reduced the sedimentable tubulin by 70–80%. This reduction of the fraction of tubulin present as sedimentable microtubules by pretreatment with colchicine was remarkably consistent for the parent cells and their transformants, further validating the comparison of microtubules in normal versus transformed cells which is the focus of this report. Microtubules in the normal 3T3-714 cell line and its chemical transformant NQT-3T3, however, appeared to be uniquely resistant to disruption by the standard concentration of colchicine (Table II); therefore, we concluded that these results reflected properties of the cells rather than the assay. Colchicine resistance of the microtubules in these cells correlated well with the observed resistance of 3T3-714 cells to inhibition of growth by concentrations of colchicine which inhibit growth of 3T3-P3.[1] The molar ratio of colchicine bound to purified bovine brain tubulin was 0.88, indicating that there was very little decay of colchicine binding by tubulin during the 90-min assay period. In addition, as previously shown (6), purified bovine brain tubulin added to a sample containing 3T3-P3 sonicate was quantitatively recovered in the assay, indicating that there were no inhibitory substances present and that the cell extract did not cause enhanced decay of tubulin during the incubation.

Protein was measured using Bio-Rad protein reagent (Bio-Rad Laboratories, Richmond, Calif.). The reagent

[1] Peterkofsky, B., and R. Oneson. Unpublished observations.

TABLE I

Effect of Cell Density on Microtubule and Tubulin Content of Normal and Transformed 3T3 Cells

Cells	Growth phase	Cells per dish × 10^{-6}	Tubulin present as sedimentable microtubules ($\frac{\mu g \text{ tubulin}}{\text{mg cell protein}}$)	Total tubulin ($\frac{\mu g \text{ tubulin}}{\text{mg cell protein}}$)	Fraction of tubulin present as sedimentable microtubules		Reduction by colchicine of fraction present as sedimentable microtubules
					No colchicine	Plus colchicine	
					%	%	%
3T3-P3 (untransformed)	Early log (sparse)	1.6	1.99	5.19	39	8	79
	Late log (confluent)	4.2	1.74	4.83	36	8	77
Ki-3T3-234-21	Early log	1.6	2.30	4.69	49	16	67
	Late log	4.4	2.69	5.30	51	15	71

Average of two separate determinations. Cells were cultured in 100 × 20 mm Falcon dishes and assayed as described in Materials and Methods.

TABLE II

Effect of Transformation on Microtubule and Tubulin Content of 3T3 Cells

Cell	Tubulin present as sedimentable microtubules ($\frac{\mu g \text{ tubulin}}{\text{mg cell protein}}$)	Total tubulin ($\frac{\mu g \text{ tubulin}}{\text{mg cell protein}}$)	Fraction of tubulin present as sedimentable microtubules		Reduction by colchicine of fraction present as sedimentable microtubules
			No colchicine	Plus colchicine	
			%	%	%
3T3-P3 (untransformed)*	1.84	4.83	38	9	76
SV-3T3	2.45	7.66	32	6	81
Mo-3T3	2.75	6.70	41	10	76
Ki-3T3-234-21	2.34	4.78	49	18	63
Ki-3T3-234-21 dbcAMP-treated	2.52	4.58	55	21	62
Ki-3T3-1	4.03	7.20	56	11	80
Flat revertant of Ki-3T3-1	2.50	4.55	55	16	71
3T3-714 (untransformed)	1.82	5.53	33	22	33
NQT-3T3	3.36	6.73	50	38	24

* Average of six determinations; other values are the average of two separate determinations. Cells were cultured as described in Materials and Methods. Where indicated, Ki-3T3-234-21 was grown in the presence of 0.5 mM dbcAMP as described previously (17) before analysis.

was diluted 1:4, and 0.8 ml was added to 0.2 ml of sample containing 4–10 μg of protein.

RESULTS AND DISCUSSION

To examine any effect of cell density on microtubule content, we studied normal and Ki-3T3 transformed cells in early and late log phases of growth. One previous study of CHO cells suggested that there were density-dependent differences in the fraction of tubulin present as sedimentable microtubules (20). The data in Table I show that, in both normal and transformed cells, there was no significant density-dependent difference in either microtubule content or fraction of tubulin recoverable as sedimentable microtubules. All subsequent studies were carried out on cells in late log phase.

Both normal 3T3 and Ki-3T3 cells undergo major morphological changes as cultures progress from nonconfluent early log phase to nearly confluent late log phase. The normal cells, in particular, cease growth, lose their amorphous shape and cell processes and become flatter, contiguous and assume a mosaic-like pattern. The absence of any real difference in microtubule content or fraction of tubulin present as microtubules between early and late log phases of each line (Table I) demonstrates that major changes in cell shape can take place without any quantitative change in microtubules. Further, in these 3T3 cells, the presence of contact inhibition appears not to be associated with any quantitative change in microtubules.

Two untransformed subclones of 3T3 cells (P3

and 714) had identical microtubule contents and similar levels of total cellular tubulin and fractions of tubulin present as sedimentable microtubules (Table II). All five transformed cell lines (SV-3T3, Mo-3T3, Ki-3T3 clones 234-21 and 1, and NQT-3T3) had an increased content of microtubules (column 1) although the increase was marked only in Ki-3T3-1 and NQT-3T3. One of the Ki-3T3 lines (clone 1) had more microtubules than the other and, in fact, had the highest microtubule content of all cells tested. All of the transformed cells except Ki-3T3 clone 234-21 had increased total cellular tubulin (column 2) and all but the SV-40 transformant had an increased fraction of tubulin present as sedimentable microtubules (column 3, no colchicine). It has previously been reported that an SV-40 transformant of BALB-3T3 had a slightly higher tubulin content than untransformed 3T3 (23). The 4-nitroquinoline-1-oxide transformation of 3T3 clone 714 led to increases in all three parameters. Further, a revertant of Ki-3T3 clone 1 exhibiting a more normal flat shape had reduced tubulin and microtubule contents which were similar to those of untransformed cells, but no change in the fraction of tubulin in microtubules. This was the only instance in which a morphological change was associated with a change in tubulin or microtubule content. Overall, transformation of 3T3 cells resulted in an increased content of microtubules and the fraction of tubulin in microtubules as well as total cellular tubulin. These results may be viewed as representing the content of the various components per unit volume of cell, since the levels are expressed on the basis of protein and protein content is a reasonable measure of cell volume (8). When the data were calculated on the basis of cell numbers, however, transformed cells also showed 10–60% increases in total tubulin content per cell compared to the untransformed clones P3 and 714.

Treatment of transformed cells with dbcAMP causes them to change their morphology and characteristics so as to more closely resemble the normal untransformed parent cell lines (13). The Kirsten sarcoma virus-transformed cells used here are more rounded than 3T3 cells and have markedly refractile cell bodies with a few spindly processes. DbcAMP treatment returns Ki-3T3 to flat, elongated cells with prominent nuclei very similar in morphology to the parent 3T3 cells (17). To examine the relationship of microtubules to these dbcAMP-induced changes, we studied control and dbcAMP-treated Ki-3T3-234-21 cells in parallel. As shown in Table II, there was little difference between the two in microtubule content, total tubulin, fraction of tubulin present as sedimentable microtubules, or reduction of this fraction by colchicine treatment. This constitutes another example of a major morphologic change unaccompanied by any quantitative changes in the microtubular system. It has been reported that an increase in the number of microtubules per unit volume of cytoplasm accompanied and probably caused the morphological change induced in CHO cells by dbcAMP (18) and that this treatment increased the fraction of tubulin in microtubules by at least 25% (20). Our results on 3T3 cells showed neither of these changes. Since dbcAMP treatment increases the intracellular level of cAMP (12), our results suggest that increasing the intracellular cAMP has no significant quantitative effect on the microtubular system in 3T3 cells. These results do not support a recent hypothesis of a central role for cAMP and the microtubular system in the transformation of cells from normal to malignant (18). The general conclusion to be drawn from the data reported here is that, in BALB cells, transformation may be associated with a quantitative change in the microtubular system, but this change is quite different than that previously suggested (3, 5, 9, 14, 15, 19).

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