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DEPOLYMERIZATION OF CELLULAR TUBULIN DURING DNA BIOSYNTHESIS INITIATED WITH GROWTH FACTORS IN 3T3 CELLS*

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ABSTRACT The results indicated that total cellular tubulin increased markedly when the cultured 3T3 cells entered DNA biosynthesis initiated by growth factors including EGF, FDGF, insulin, PDGF and vasopressin, while the polymerized microtubules remained unchanged. Such an increase in cellular tubulin was mainly unpolymerized free tubulin, being twice or thrice as much as that of control cells with a good dose-dependent manner. This was consistent with the pattern of increasing [^{3}H]TdR incorporation into cells and DNA content shown by flow cytometry. The study

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ABBREVIATIONS C = control; E = epithelial growth factor; F = fibroblast derived growth factor; GF = growth factor; I = insulin; MT = microtubules; P = platelet derived growth factor; T = Cholera toxin; V = vasopressin

of time course showed that the increase of free tubulin occured between 6 and 20 h after incubation with the growth factors, just when the cells entered DNA biosynthesis from G0/G1 phase. We suggest that the increase of free tubulin may be one of the important signals related to the regulation of the initiation of DNA biosynthesis.

KEY WORDS DNA biosynthesis; fibroblasts; flow cytometry; growth factors; microtubules; depolymerization; tubulin

The life cycle of a cell appears to dictate that MT participate in an unusual process of assembly and disassembly. MT have been implicated in multiple cellular functions and may have a crucial regulating role in the growth of eukaryotic cells[1~3]. Recent work from this laboratory has shown that disruption of the microtubule network in cultures of 3T3 cells by antitubulin agents markedly enhanced the DNA synthetic response to various GF such as E, F, I, P, V, phorbol esters and their combinations[4,5]. In addition, MT stabilized by taxol inhibited the initiation of DNA synthesis[6]. Thus the role of MT in the regulation of the trasnition of cells from G0/G1 phase into DNA synthesis has attracted considerable attention. It is of importance to understand the state of organization of MT in the regulation of cell growth. Here we report the results obtained by using the binding of [^{3}H]colchicine to tubulin to further elucidate the polymerization and synthesis of cytoplasmic tubulin in modulating the transition of 3T3 cell from G0/G1 phase into DNA biosynthesis stimulated by GF.

MATERIALS AND METHODS

Cell culture Swiss 3T3 mouse cells were subcultured in 30 mm Nunc petric dishes with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 5% CO_2 at 37℃[8]. After 6 d the cultures became confluent and quiescent at GO/G1. For the experiments, such quiescent cultures were washed twice to remove residual serum immediately before experiments with GF.

Tubulin assay The fraction of the total intracellular pool of tubulin presented as polymerized MT was measured by [^{3}H]colchicine binding[9]. Briefly, the medium was rapidly removed and replaced by MT-stabilizing medium, then the cells were harvested and sonicated at 22℃. The sonicates were centrifuged at 100 000 × *g* for 60 min at 22℃. The supernate was removed and the recovered pellet was resuspended in ice-cold phosphate buffer with 1 m*M* guanosine triphosphate and 0.5% Triton X-100 and then resonicated at 0℃ to ensure total disruption of MT. Tubulin in this solution was measured by [^{3}H]colchicine binding with DEAE-cellulose filters[10]. For the determination of total cell tubulin pool, duplicate dishes were treated as described above, except that sonication was performed at 0℃ in phosphate buffer instead of MT-stabilizing medium.

Incorporation of [^{3}H]TdR into DNA Quiescent cultures of 3T3 cells were incubated in 2 ml of 1:1 mixture of DME and Waymouth medium containing 1 μ*M*, 37 k*Bq* [^{3}H]TdR and other agents. After 40 h at 37℃, [^{3}H]TdR incorporation into acid-insoluble pools was solubilized by 30 min incubation with 0.1 *M* NaOH and 2% Na_2CO_3, and the radioactivity was assayed.

Flow cytometric assay Cells were suspended in 1 ml of saline and rapidly squirted through a 25 guage needle into 3 ml of 25% ethanol containing mithramycin 50 μg/ml and 15 m*M* $MgCl_2$. After 30 min DNA histograms were obtained with a fluorescence activated cell sorter (Becton Dickinson, CA).

Materials Bovine insulin (25.5 IU/mg/ml), T, 8Br-cAMP, colchicine, demecolcine, vinblastine sulfate and nocodazole were purchased from Sigma Chemical Co. 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Aldrich Chemical Co.. P and F were partially purified by sulfadex gel chromatography. [^{3}H]TdR (0.7 GBq/μmol), [^{3}H]colchicine (1.4 kBq/

μmol), antigens and antibodies for radioimmunoassay were purchased from New England Nuclear.

RESULTS

Effect of antitubulin agents on MT A dose-dependent decrease of the fraction of cellular tubulin in the polymerized MT occurred after exposure of 3T3 cells to antitubulin agents, such as nocodazole (Fig 1). We

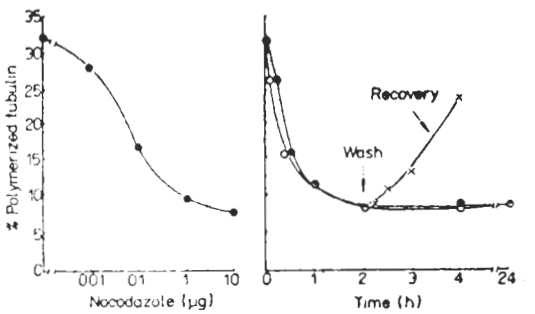

Fig 1. Depolymerization of cytoplasmic microtubules (MT) by nocodazole (●) and demecolcine (○) and polymerization recovered after washing off nocodazole.

assayed the cellular total tubulin and MT tubulin showing in Fig 1 that half of MT was depolymerized in 15 min, and almost all of the antitubulin-sensitive tubulin was depolymerized from MT in 1–2 h. After having washed thoroughly with phosphate buffer, the polymerization of MT was recovered in 2 h(Fig 1). Therefore, the exposure of 3T3 cells to antitubulin agents lasted 1–2 h in the following experiments while the exposure of cells to growth fators was for 20–24 h.

Effect of various GF on the tubulin synthesis and polymerization of MT GF such as I(1 μg/ml), E (5 ng/ml), V (20 ng/ml), F (5 μg/ml) and P (3.5 μg/ml) were able to initiate the synthesis of cytoplasmic free tubulin 1–3 times more than the control cells while they had no effect on the polymerized MT (Fig 2). Hence the proportion of polymerized tubulin was decreased due to the enhancement of cellular free tubulin, rather than the de-

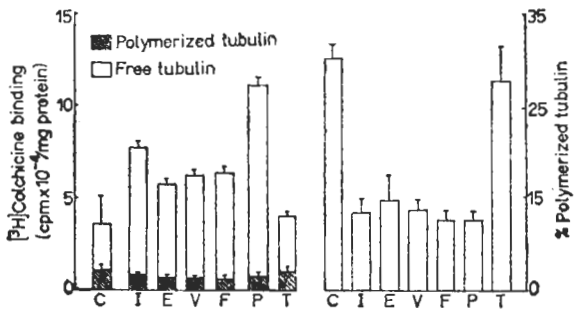

Fig 2. Tubulin assay of total cellular tubulin and MT tubulin under the effect of various growth factors. C = control; I = insulin 1 μg/ml; E = epithelial growth factor 5 ng/ml; V = vasopressin 20 ng/ml; F = fibroblast derived growth factor 5 μg/ml; P = platelet derived growth factor 3.5 μg/ml; T = cholera toxin 100 ng/ml. The symbols and concentrations are the same in the following figures.

polymerization of MT. T (100 ng/ml) had no effect on either free tubulin or MT tubulin and it did not enhance the effect of GF on cellular tubulin synthesis (Fig 3).

Relationship between increasing cellular tubulin synthesis and DNA synthesis initiated by GF There was a good dose-dependent

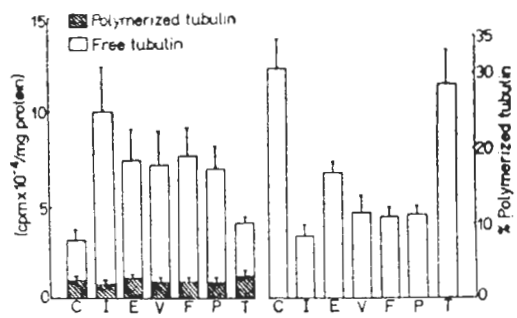

Fig 3. Tubulin assay to total cellular tubulin and MT tubulin under the effect of growth factors combined with T.

fashion of cellular tubulin synthesis under the effect of I 0.1–10 μg/ml, and I plus T 100 ng/ml. At the dose of I at 0.3 μg/ml, the total tubulin in the cells increased rapidly and achieved the plateau over 1 μg/ml, while the MT tubulin remained unchanged (Fig 4). The decreased ratio of polymerized tubulin total tubulin was consistent with the extent of increasing [^{3}H]TdR incorporation into 3T3 cells,

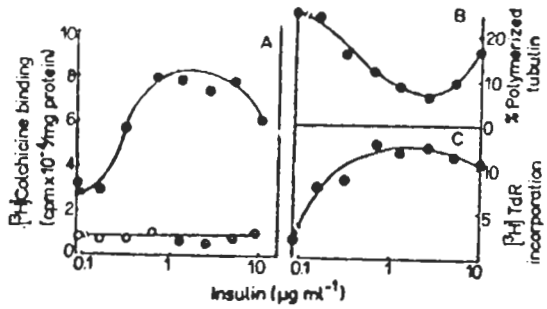

Fig 4. Concentration-response curves of A) Cellular total and MT tubulin (● , ○); B) percentage of MT tubulin; C) [³H]TdR incorporation under the effects of I plus T.

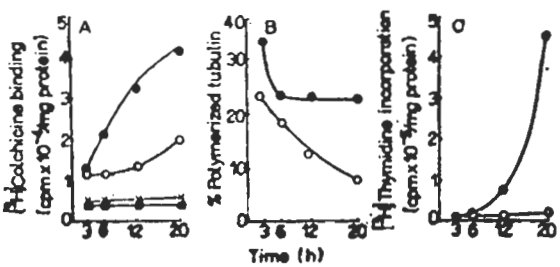

Fig 5. Assay of A) total cellular tubulin(control: ○ , E, I, V: ●) and MT tubulin(control: × , E, I, V: ●);B)% polymerized tubulin (control: ● , E, I, V: ○);C) [³H]TdR incorporation (control: ○ , E, I, V: ●).

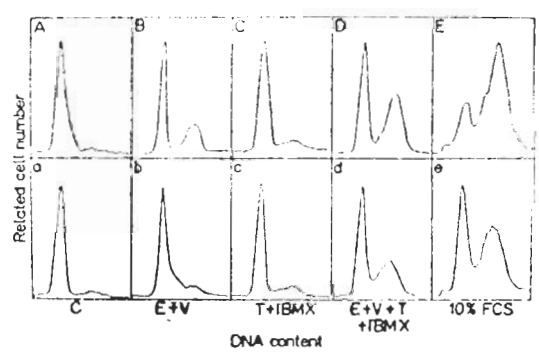

Fig 6. Flow cytometric DNA histograms showing the effect of nocodazole 2.5 μ*M* on the progress from G1 to S phase in Swiss 3T3 cells stimulated for 40 h by E plus V and both combined with T plus IBMX 92 m*M*. DNA in the right peak (G 2) is equivalent to twice the DNA in the left peak (G 1). A-E: addition of nocodazole at the same time as the incubation with growth factors; a-e: addition of nocodazole 20 h after the growth factors. FCS: fetal calf serum.

The time course of the effect of GF including E (5 ng/ml), I(1 μg/ml) and V (20 ng/ml) on the cellular tubulin synthesis showed that such an enhanced effect occurred during the period of 6–20 h after exposure of cells to the combination (Fig 5). It was wholly consistent with the synergistic effect of antitubulin agents on DNA synthesis stimulated by GF or interplay of antitubulin agents with cAMP occurred within 20 h (Fig 6).

DISCUSSION

The recent results indicate that the state of organization of MT may act as a signal in the regulation of cells moving from G 0/G1 phase into DNA synthesis. The disruption of MT with various antitubulin agents markedly potentiate the DNA biosynthesis of 3T3 cells[4,5]. The results reported here showed that there was a marked increase in free tubulin during DNA biosynthesis stimulated by GF and their combinations. Thus, there was a marked decrease in the ratio of polymerized tubulin to total cellular tubulin due to the increased free tubulin rather than the depolymerization of MT tubulin. Therefore, there is a definite relationship between the depolymerization of tubulin and DNA biosynthesis. Suprisingly, the key point is not related to the process of depolymerization itself, but the amount of free tubulin instead. Both GF and antitubulin agents produced more free tubulin in the cell. However, GF created more free tubulin while holding the MT tubulin level at a stable horizon, while cell volume increased with the stimulation by GF, when antitubulin agents increased the amount of free tubulin by the depolymerization of MT, but kept the total cellular tubulin at the same level. The results meant that the increase in free tubulin was important for the cell growth stimulated by GF.

It has been reported that the increase in free tubulin could creat a kind of feedback inhibition of transcrpit efficiency of tuublin-mRNA, thus causing the lowered production as

well as the inhibited polymerization of MT tubulin[13]. Eichhorn et al. reported an increase in freetubulin in the transformed Balb 3T3 cells by the [^{3}H] colchicine binding assay. It is plausible that increase in free tubulin followed by a decrease in depolymerized MT is a necessary condition for the cell growth either in untransformed or in transformed cultured cells.

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生长因子诱导 3T3 细胞 DNA 合成时微管蛋白失聚合态

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提要 在各种生长因子诱导下，3T3 细胞从 G0/G1 相进入 DNA 合成相时，伴随着失聚合态的游离微管蛋白的大量增加，而聚合态微管蛋白含量则维持不变。这种增加随着生长因子浓度的增加而增长，最多可三倍于对照细胞。游离微管蛋白量的增加还与[^{3}H]TdR 的掺入和单位细胞 DNA 含量的增加相一致。作用发生在给予生长因子后 6-20 h。可见，这种微管蛋白的增加并维持在失聚合状态是 DNA 合成始动的重要信号之一。

关键词 脱氧核糖核酸复制；纤维母细胞；流式细胞光度计；生长因子；微管；失聚合态；微管蛋白