Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores

(nocodazole/cytoplasmic microtubule complex/mitosis)

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ABSTRACT Taxol, a potent promoter of microtubule polymerization *in vitro*, induces massive assembly of free microtubules in cultured cells as visualized by immunocytochemistry and electron microscopy. The centrosomes and kinetochores largely lost their capacity to organize microtubule assembly, as became evident by the disappearance of the cytoplasmic microtubule complex and the mitotic spindle. The taxol-induced microtubules were partially resistant to nocodazole, an inhibitor of tubulin polymerization. Moreover, taxol induced microtubule assembly in cells pretreated with nocodazole. Increasing the ratio of nocodazole to taxol restored the ability of the centrosomes and kinetochores to specifically induce microtubule assembly in their immediate vicinity. The data suggest that taxol lowers the critical tubulin concentration *in vivo* as well as *in vitro* and that the organizing capacity of the microtubule-organizing centers depends on the cytoplasmic polymerization threshold.

Taxol, an experimental antitumor drug (1) isolated from *Taxus brevifolia*, was recently shown by Schiff *et al.* (2, 3) to affect microtubule assembly *in vitro* and in living cells. It essentially eliminated the initial lag phase and decreased the critical tubulin concentration to less than 0.01 mg/ml. The rate and extent of the polymerization was increased and the microtubules were relatively resistant to depolymerization by cold and CaCl_2 . In living cells (3), taxol was shown to be a potent inhibitor of HeLa and mouse fibroblast replication. The cells were blocked in the G_2 and M phases of the cell cycle. It was inferred that this was due to the stabilization of cytoplasmic microtubules. Indirect immunofluorescence was used to show that taxol-treated cells displayed bundles of microtubules radiating from a common site in addition to their cytoplasmic microtubules. These microtubules were resistant to treatment with steganacin or incubation of the cells at low temperature, both of which disintegrated microtubules in control cells. Ultrastructural observations showed that the mitotic cells contained microtubule bundles but no normal spindle. It was concluded that the inability of the cells to form a mitotic spindle in the presence of taxol could be due to the fact that the cells were unable to depolymerize their microtubule cytoskeletons (3).

We have investigated the effects of taxol on the cytoplasmic microtubule complex and the mitotic spindle in cultured cells. Our observations and conclusions differ partly from those published previously (3). Taxol apparently induces the assembly of free microtubules in the cytoplasm, not attached to the centrosomes or kinetochores. The preexisting microtubules, attached to the organizing centers, are not stabilized and disappear gradually.

MATERIAL AND METHODS

Pt K2 potaroo cells, C3H mouse 3T3 cells, and human embryonal fibroblasts were cultured in Eagle's minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum as described (4). For light microscopic immunocytochemistry, the cells were seeded on glass coverslips in sterile Petri dishes. Fixation was done as described (5) with 1% glutaraldehyde followed by permeabilization with 50% (vol/vol) ethanol containing sodium borohydride. Immunocytochemical staining was done with an affinity-purified antibody to dog brain tubulin, and the unlabeled antibody-enzyme method (peroxidase-antiperoxidase, PAP) as described (6, 7). Fixation and embedding for electron microscopy were done as described (4).

Cells were treated with taxol (100, 10, 5, 2.5, 1.25, 0.63, and 0.32 μM) and fixed after 10, 20, and 40 min and 1, 4, and 24 hr. Similar experiments were done in combination with nocodazole treatment (20, 2, and 0.2 μM) 2 hr before or 4 hr after addition of taxol. Nocodazole (4) and taxol (2) were dissolved in dimethyl sulfoxide (10 mg/ml) and diluted in tissue culture medium. At the final concentrations used the solvent had no apparent effects.

RESULTS

Taxol Induces Assembly of Free Microtubules and Blocks the Assembly of the Cytoplasmic Microtubule Complex (CMTC) and the Mitotic Spindle. Combined light microscopic immunocytochemistry with anti-tubulin antibodies and electron microscopy was used to investigate the progressive changes in the microtubule systems of cultured cells in interphase and mitosis. The first alterations of the CMTC consisted in the appearance of numerous short microtubules not attached to the centrosome and apparently formed freely in the cytoplasm (compare Fig. 1a with Fig. 1c, d, and e). This was evident within 1 hr of incubation with taxol at concentrations between 100 and 1 μM . With time the number of free microtubules increased, while the preexisting CMTC progressively faded away. Cells incubated for 4 hr with taxol had completely lost their organized CMTC and contained only short microtubules arranged in various disorganized ways (Fig. 1d). Further incubation (24 hr) resulted in increased bundling of these short microtubules (Fig. 1e). Electron microscopy (Fig. 2) confirmed the microtubular nature of the immunocytochemical staining pattern. Microtubules of normal diameter and structure were seen to be arranged in complex patterns throughout the cytoplasm. Lateral association of microtubules in bundles was a prominent feature,

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Abbreviations: CMTC, cytoplasmic microtubule complex; PAP, peroxidase-antiperoxidase; MTOC, microtubule-organizing center.

FIG. 1. Light micrographs of Pt K2 cells stained with the anti-tubulin antibody and the PAP procedure. Microtubules are stained by oxidized diaminobenzidine; the nucleus is counterstained by toluidine blue. Similar results were obtained with 3T3 cells and human embryonal fibroblasts. (a) Control cells show an organized CMTC radiating from the centrosomal region. (b) Cells treated with nocodazole (20 μ M, 2 hr). Cytoplasmic microtubules have disappeared. (c, d, and e) Cells treated with taxol (5 μ M) for 1 hr (c), 4 hr (d), and 24 hr (e). The CMTC disappears and is replaced by free microtubules, which show with increased time an increased tendency to form bundles. (f) Cells treated with nocodazole (2 μ M, 26 hr) and taxol (10 μ M, 24 hr). Bundles of free microtubules are formed as in cells treated with taxol alone. (g and h) Cells treated with nocodazole (20 μ M) added 2 hr before taxol (5 μ M) fixed 1 hr (g) and 24 hr (h) after addition of taxol. Microtubule assembly induced by taxol is limited to the formation of asters radiating from the centrosomes.

FIG. 2. Electron micrographs of sections through Pt K2 cells treated with taxol. (a) Interphase ($10 \mu\text{M}$ taxol, 24 hr). Bundles of microtubules are seen; they often converge to focal points containing no centrosomes or other structures. (b) Centrosomal region, interphase ($10 \mu\text{M}$ taxol, 24 hr). Few microtubules are associated with the centrosome. Most of them pass along the centrosome. (c) Center of a microtubule aster in a mitotic cell ($10 \mu\text{M}$ taxol, 24 hr). Centrioles or osmiophilic material are not seen. (d) Kinetochore region in a mitotic cell ($10 \mu\text{M}$ taxol, 24 hr). No microtubules are seen associated with the kinetochore.

as well as radiation from focal points containing no centrosomes (Fig. 2a). Centrosomal complexes were present in normal numbers—usually one or two per cell—and showed a normal ultrastructural appearance (Fig. 2b). However, very few microtubules were seen to be linked to the centrosomes in contrast to their usual abundance in untreated cells. As is the case in cells treated with microtubule inhibitors (colchicine, nocodazole, etc.), the centrosome was often displaced from its normal perinuclear position (4). Also, the Golgi complexes and lysosomes were dispersed throughout the cytoplasm (4, 8). Taxol did, however, not induce the formation of annulate lamellae or intermediate filament bundles (9, 10).

Concomitant with the alteration of the CMTC, the shape of the cells was altered in much the same way as with colchicine, nocodazole, or any other microtubule inhibitor (4, 11, 12). Cell polarity was lost through the appearance of undulating membranes all around the cell periphery. The cytoplasm accumulated in a central dome around the nucleus. The cell periphery consisted of very thin lamellae from which microtubules were usually excluded.

Cells that entered mitosis in the presence of taxol (100 or $1 \mu\text{M}$) also showed a profoundly altered pattern of microtubule assembly, which became evident within 20–40 min after addition of the drug. During prophase the CMTC was apparently disassembled, as is the case in untreated cells. Moreover, the microtubule bundles induced by prolonged treatment with taxol were also disassembled (Fig. 3a). However, a mitotic spindle never formed. The chromosomes were arranged in a disordered fashion and did not nucleate kinetochore microtubules

(Fig. 3a). The immunocytochemical staining revealed the presence of numerous (up to 20) aster-like aggregates of short microtubules. Upon further incubation the cells behaved as if treated with colchicine or nocodazole (13). Mitotic cells were blocked for about 3 hr, whereas normal mitosis takes about 45 min. Thereafter restitution nuclei were formed. The cells flattened again on the substratum and contained a variable number of irregular nuclei. The random network of short microtubules and microtubule bundles was reconstructed in these postmitotic cells (Fig. 1e).

Ultrastructural observation confirmed the presence of multiple microtubule asters in the mitotic cells. The center of most of these asters (Fig. 2c) did not contain any detectable structure or electron-dense material. Centrosomes were present in normal numbers, usually two complexes per cell, and had a normal morphology (orthogonal centrioles surrounded by dense material). Most centrosomal complexes were located at the focus of an aster. These asters apparently did not contain more microtubules than those without centrosomes. The kinetochores were in general completely free of microtubules (Fig. 2d). The few microtubules that were occasionally seen in the vicinity of kinetochores were probably derived from nearby asters.

The effects of taxol on the CMTC and the mitotic spindle were easily reversible. Cells treated for 4 hr with taxol ($10 \mu\text{M}$) followed by five washes with medium and further incubation without taxol reconstructed organized CMTCs and spindles within 1 hr.

Taxol Induces Microtubule Assembly in Nocodazole-Treated Cells. It has previously been shown that the microtubules of

FIG. 3. Light micrograph of mitotic Pt K2 cells stained with the anti-tubulin antibody and the PAP procedure. The chromatin is contrasted with toluidine blue. In this black and white reproduction of color slides the microtubules appear black and the chromosomes have been marked with white paint. (a) Mitotic cell treated with taxol (10 μ M, 1 hr). The mitotic spindle is replaced by multiple asters. The chromosomes have not formed kinetochore fibers. Interphase cells appeared like those in Fig. 1c. (b) Mitotic cell treated with taxol (5 μ M) and nocodazole (20 μ M) for 1 hr. Only one aster is seen, and the dark spots associated with the chromosomes represent short microtubules nucleated at the kinetochores (13). Interphase cells appeared like those in Fig. 1g.

cells pretreated with taxol (10 μ M) are resistant to cold (4°C) and steganacin (10 μ M) (3). The microtubule networks, bundles, and multiple asters induced by taxol (100 or 10 μ M; 1–24 hr) are also resistant to nocodazole (2 μ M; 1–24 hr), which induces the disappearance of microtubules in cells not treated with taxol (Fig. 1b). The reverse experiment was performed in order to see whether the resistance of these microtubules to nocodazole was due to irreversible stabilization by taxol or to assembly despite the presence of nocodazole. Cells were pretreated for 2 hr with nocodazole and further incubated with nocodazole and taxol. Taxol clearly induced microtubule assembly in nocodazole-pretreated cells. However, the pattern of microtubule assembly was dependent on the relative concentrations of taxol and nocodazole (Fig. 1f, g, and h and Table 1). High concentrations of taxol (100 μ M) with low concentrations of nocodazole (2–0.2 μ M) resulted in the formation of random microtubules and microtubule bundles as in cells not pretreated with nocodazole. When the taxol concentration was lowered and the nocodazole concentration was increased, centrosomal assembly was seen again and free microtubules diminished in number. With 5–2.5 μ M taxol and 20 μ M nocodazole, centrosomal assembly predominated and free microtubules were rarely observed. Fig. 1g and h shows that between 1 and 24 hr a small centrosomal aster grows out into a radiating network that resembles a normal CMTC. A completely normal network was, however, never restored. Pretreatment with taxol for 4–24 hr at low concentrations (2.5–5 μ M) followed by addition of high concentrations of nocodazole (20 μ M) similarly induced the resumption of centrosomal assembly.

Table 1. Prevalence of free microtubules and centrosome-associated microtubules in Pt K2 cells treated with various combinations of nocodazole and taxol

Taxol, μ M	Nocodazole, μ M			
	0	0.2	2	20
100	F	F	F	F + C
10	F	F	F	F + C
5	F	F	F + C	C
2.5	F	F	F + C	C

Cell cultures treated for 24 hr are scored as containing only free microtubules (F, as in Fig. 1c), only centrosome-associated microtubules (C, as in Fig. 1g and h), or both free and centrosome-associated microtubules (F + C).

In mitotic cells, too, increasing the ratio of nocodazole to taxol concentration resulted in strong limitation of the number of asters (one or two in most cells) and the reappearance of kinetochore-associated microtubule assembly (Fig. 3b). Normal mitoses did not follow, however.

We have previously shown that microtubule assembly in living Pt K2 cells after nocodazole has been washed away proceeds mainly by initial nucleation at the centrosomes and kinetochores (13, 14). These microtubules elongate and reconstruct radiating CMTCs and normal spindles within 30–60 min. The addition of taxol to cells treated with nocodazole thus gives rise to events that are also found after nocodazole is washed away. Reversal of nocodazole block in the presence of taxol is followed by assembly of microtubules throughout the cytoplasm in interphase cells and formation of multiple asters in mitotic cells.

DISCUSSION

Using a sensitive immunocytochemical technique for the display of intact microtubules in glutaraldehyde-fixed cells, as well as ultrastructural observations, we have obtained information on the effects of taxol on microtubule assembly in cultured cells. In summary, our data show that taxol induces spontaneous nucleation of microtubule assembly in the cytoplasm of interphase cells without spatial relation to the major microtubule-organizing centers. The preexisting CMTC is not stabilized but dissolves gradually. The organizing role of the centrosome as the main focal point is lost in the presence of taxol. However, the centrosome remains structurally intact and removal of taxol is followed by recovery of its nucleating capacity.

During mitosis, the taxol-induced microtubule networks and bundles shorten and are replaced by multiple asters, most of which do not contain centrosomes or other recognizable nucleating material. The microtubule-nucleating activity of the kinetochores is lost. After mitosis the microtubule networks and bundles are reconstructed. The taxol-induced microtubules are relatively resistant to nocodazole, and taxol induces microtubule assembly in the presence of nocodazole. When the ratio of nocodazole to taxol is increased, the nucleating activity of the centrosomes and kinetochores is restored to some degree and more normal radiating complexes are formed. These observations are not compatible with the assumption that taxol irreversibly stabilizes microtubules (3). Preexisting microtubule networks do in fact disassemble, and the taxol-induced microtubules

are susceptible to modulation by the mitotic cycle and by nocodazole.

Schiff *et al.* (2) have previously shown that the major effect of taxol on microtubule assembly *in vitro* is a tremendous decrease in the critical concentration of tubulin dimers. The alterations induced by taxol in living cells are entirely compatible with the assumption that, in living cells too, the major effect of taxol is to decrease the critical tubulin dimer concentration. This would indeed increase the probability of spontaneous nucleation in the cytoplasm, an extremely rare event in untreated cells (13–15). It would also explain why taxol induces microtubule assembly in the presence of tubulin polymerization inhibitors that increase the critical tubulin concentration. The induction of multiple asters in mitotic cells is also compatible with the supposed effect of taxol on the critical tubulin concentration. However, drastic rearrangements occur during this part of the cell cycle. The preexisting free cytoplasmic microtubules and microtubule bundles apparently become shorter and are rearranged into multiple asters. These multiple asters form also when the cells are first pretreated with nocodazole. We see no ready explanation for these changes, although they resemble vaguely the normal transition from interphase to mitosis. The formation of multiple asters may be due to the presence of secondary microtubule-organizing centers (MTOCs) scattered about the mitotic cytoplasm; however, we found no morphological evidence for MTOCs. Moreover, it is hard to understand why addition of nocodazole to taxol-treated cells would inhibit these secondary MTOCs while paradoxically restoring the activity of the kinetochores and centrosomes. Alternatively, the observations may indicate that during mitosis factors appear that increase the tendency of microtubules to form asters. In this context it is interesting to note that recent observations (unpublished) showed an accumulation of immunocytochemically detectable calmodulin in the centers of the taxol-induced mitotic asters. This resembles the previously demonstrated accumulation of calmodulin in the vicinity of the spindle poles in untreated cells (5, 16) and suggests that the centrosome-free asters induced by taxol may nevertheless be homologous to normal spindle poles.

Besides inducing spontaneous nucleation, taxol causes the disassembly of the organized CMTC and mitotic spindle, which has clear functional implications: loss of cell polarity and disordered organelle topography. These observations imply that the centrosomes and kinetochores have lost their capacity to function as nucleating sites, a loss that is probably not due to a direct interaction of taxol with these organelles, because the addition of nocodazole paradoxically restores their nucleating activity. The nucleating function of the MTOC is thus dependent on the critical tubulin concentration being sufficiently high to suppress spontaneous nucleation. This corroborates earlier *in vitro* observations with isolated MTOCs (centrosomes and kinetochores) (17, 18), and has implications for the mechanisms by which they may nucleate microtubule assembly.

We have recently proposed that the microtubule-nucleating capacity of the centrosomes and kinetochores may be due to these MTOCs being surrounded by a limited region in which the assembly threshold is lower than further away in the cytoplasm (13, 14). This would explain preferential nucleation of short microtubules in the vicinity of the MTOCs (13, 14). Those microtubules whose minus ends reside in the promoting environment of the MTOC and whose plus ends are peripheral would preferentially elongate and be more stable than free

microtubules. This follows from the observation that the equilibrium tubulin concentration is lower for the plus end than for the minus end (19). It is obvious that such a mechanism is extremely dependent on the cytoplasmic assembly threshold. The MTOC would not be a preferential assembly site when the critical tubulin concentration in the cytoplasm is decreased (e.g., by taxol) to the same low level as that which is supposed to exist around the MTOC, and if the taxol effect is not additive to the promoting influence of the MTOC.

Other mechanisms of organized assembly that involve the presence of seeds or templates in the MTOC, or the capping of the minus end by the MTOC (20), cannot, however, be excluded.

In conclusion, we have shown that taxol induces the assembly of free microtubules and blocks the organizing capacity of MTOCs in living cells, probably by decreasing the critical tubulin concentration. The combined use of taxol and inhibitors of polymerization, such as nocodazole, that increase the critical tubulin concentration may provide a useful experimental tool to further investigate organized microtubule assembly *in vivo*.

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