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TUBULIN ALTERATIONS IN TAXOL-INDUCED APOPTOSIS PARALLEL THOSE OBSERVED WITH OTHER DRUGS

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Abstract—We have described previously that during apoptosis cellular tubulin is reorganized into visible tubulin structures that correlate with apoptotic morphology. Such changes have been observed in human leukaemic cells treated with a variety of cytotoxic agents. These structures are unlike those seen in untreated non-mitotic or mitotic cells. As taxol is known to act by enhancing the polymerizaton of tubulin in the initiation and extension of microtubules (MTs), and has been shown to induce and stabilize the formation of tubulin structures in a variety of cells, we examined the involvement of tubulin in apoptosis induced by taxol. Apoptosis was induced in a human T-cell leukaemic line, CCRF-CEM, following treatment with 10 nM taxol. The morphological features typical of apoptosis were apparent in taxol-treated cells after drug addition. Immunocytochemical analysis using a monoclonal antibody to β-tubulin indicated that taxol induced visible tubulin polymerization. DNA fragmentation was detected at 10 hr post-treatment. Flow cytometric analysis of taxol-treated cells showed a time-dependent accumulation of cells in G_2/M phase with the appearance of a hypodiploid peak coincident with the detection of DNA fragmentation. Microtubule structures observed following taxol treatment were of three types. At the time of DNA fragmentation, 50% of the cells displayed tubulin structures associated with apoptotic morphology similar to those seen in apoptosis induced by treatment with methotrexate (10^{-8} M) or etoposide (17 μM). Twenty percent of the cells were arrested in mitosis at this time. These cells contained either multiple asters or disordered mitotic spindles, and did not display apoptotic morphology. The remaining cells, while normal in morphology, had extensive tubulin polymerization in the cytoplasm and around the nucleus. We examined the time–course of tubulin mRNA expression in apoptosis induced by taxol, methotrexate and etoposide. The level of tubulin mRNA displayed a transient increase after treatment, and prior to the onset of DNA fragmentation with each of the three drugs. These results suggest that during apoptosis taxol induces tubulin changes that display characteristics similar to those observed during apoptosis following treatment with drugs that do not interact directly with tubulin.

Key words: apoptosis; tubulin; taxol; chemotherapeutic agents; apoptotic tubulin structures; CCRF-CEM

Cell death can occur by either of two morphologically and biochemically distinct modes: apoptosis or necrosis [1]. Necrosis is characterized by loss of cell membrane integrity, with cell lysis and accompanying damage to surrounding tissue. Apoptosis involves the ordered breakup of the cell, with condensation of the cytoplasmic and nuclear material and, typically, fragmentation of the DNA into nucleosomal sized pieces. The cell separates into apoptotic bodies that are phagocytosed by surrounding cells, thereby minimizing tissue damage.

Recent evidence suggests that apoptosis is the likely mechanism of death in tumour cells treated with a variety of chemotherapeutic agents [2, 3]. We have observed that striking and characteristic tubulin changes occur during apoptosis in a variety of cell types and in response to treatment with different cytotoxic drugs [4]. These tubulin changes appear to occur independently of the stage of the cell cycle at which cells enter apoptosis [5]. Martin and Cotter [6] have reported the induction of apoptosis by cytotoxic drugs that disrupt MTs†, showing the formation of DNA "ladders" following treatment with colchicine or vinblastine. However, our data show that the formation of tubulin structures does occur in apoptosis induced by vincristine and colchicine, indicating that although initial depolymerization of MTs may act as a trigger for apoptosis, for the actual process of apoptosis to proceed tubulin reorganization must occur [4, 5].

Taxol is thought to act through enhancement of tubulin polymerization, both in the initiation and elongation of MTs, resulting in increased stability [7]. However, a recent study also implicates the inhibition of MT dynamics as the mechanism of disruption of mitosis at low doses [8]. Numerous reports document the formation of MT structures following treatment with taxol, usually describing the presence of multiple asters or stable MT bundles

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† Abbreviations: MTs, microtubules; MTX, methotrexate; VP-16, etoposide; MTOC, microtubule organizing centre; and SSC, 0.15 M sodium chloride + 0.015 sodium citrate.

in cells in monolayer or suspension culture [9–11]. Taxol has been described as mediating cell death via apoptosis in Chinese hamster V79 cells [12], and in human myeloid and pre-B leukaemia cells [13]. We wished to examine whether the tubulin structures observed following taxol treatment were involved in apoptosis induced by this drug, and to determine how cellular tubulin RNA levels varied during taxol-induced apoptosis. We compared the involvement of tubulin in apoptosis induced by taxol to that observed during apoptosis following treatment with MTX and VP-16, two chemotherapeutic agents that do not directly interact with tubulin. This paper presents evidence that taxol induced tubulin changes similar to those seen during apoptosis induced by other drugs, and that the MT structures seen in taxol-treated cells were not, for the most part, specific for this drug, but rather reflected the stabilization by taxol of tubulin structures typical of mitosis or apoptosis.

MATERIALS AND METHODS

Morphological criteria. The morphological characteristics of apoptosis were first described by Kerr *et al.* [1]. For the purposes of this study, cells conforming to the following criteria were classified as apoptotic: shrunken cells; cells with protruding/budding cytoplasm; cells with invagination/budding of the nucleus; and misshapen cells with changes to the nucleus and cytoplasm.

We have delineated a number of tubulin structures that are typical of either untreated CCRF-CEM cells or cells undergoing apoptosis induced by treatment with any of a number of cytotoxic drugs [4, 5]. Cells were scored as normal if they presented a mildly convoluted nucleus with a single MTOC in the perinuclear space. The tubulin staining in these cells was low, with a fine network of MTs emanating from the centrosome. In untreated mitotic cells, the spindle showed extremely strong tubulin staining. The characteristic tubulin staining observed in treated cells correlated with apoptotic morphology. Treated cells with normal morphology presented with increased tubulin staining in the nucleus or cytoplasm and an enlarged or relocated MTOC. Shrunken cells and apoptotic bodies stained very strongly for tubulin. Cells with cytoplasmic protrusions displayed an enlarged or granular-staining MTOC and a parallel array of MTs at the neck of the protrusion, which sometimes contained vesicles of nuclear material. Invagination of the nucleus was usually accompanied by dense tubulin bands or parallel arrays of MTs.

Cell culture. The CCRF-CEM cell line was maintained at 37° as a suspension culture in RPMI-1640 medium supplemented with HEPES buffer, L-glutamine and 10% fetal bovine serum. Cells were placed in culture at 2×10^5 cells/mL. After a pre-equilibration period of 24 hr, the cells were exposed to various drug preparations. The concentrations of the drugs used to induce apoptosis were chosen according to the concentration that caused 90% death of the cells as determined using the tetrazolium assay [14]. The drugs were used at final concentrations of: VP-16, 17 μM; MTX, 10^{-8} M; and taxol, 10 nM. During the experiments, the trypan blue exclusion assay was used to assess membrane integrity. The controls used in each experiment were duplicate cultures with appropriate concentrations of solvent (DMSO) added. Cytocentrifuge preparations were made at various times post-treatment, and for tubulin staining the cells were fixed in PBS:1% paraformaldehyde:0.1% Triton X-100 for 20 min at room temperature prior to labelling with a monoclonal antibody to β-tubulin (Amersham Cat. No. N 357). A standard streptavidin-biotin detection system was used with immunoperoxidase. The preparations were counterstained with Giemsa. Slides without the primary antibody were prepared in each experiment to exclude the possibility of non-specific staining. Comparisons were made of slides from control and experimental populations, and for quantification, at least 300 cells were counted from at least two separate experiments. Cells were scored for tubulin staining and for the presence of apoptotic morphology.

DNA fragmentation and Northern analysis. Aliquots of cells were washed in PBS, snap-frozen in liquid nitrogen and stored at −70° prior to DNA or RNA isolation. For DNA isolation, cell pellets were resuspended in 1 × SSC/proteinase K (0.5 mg/mL, Boehringer Cat. No. 745 723), and lysed in a final concentration of 0.25% SDS. Then the cell lysates were extracted twice with phenol/chloroform: isoamyl alcohol and once with chloroform: isoamyl alcohol, and the aqueous phases were precipitated with 2 vol. of ethanol. The resultant DNAs were treated successively with RNase A (600 μg/mL, Sigma Cat. No. R4875, 37°/0.5 hr) and proteinase K (300 μg/mL, 37°/0.5 hr). The samples were then extracted with phenol/chloroform: isoamyl alcohol and chloroform: isoamyl alcohol prior to precipitation with ethanol. The DNAs were subjected to gel electrophoresis in 1.8% agarose at 50 V for 3 hr. RNA was isolated using the method of Chirgwin *et al.* [15], and Northern analysis was performed according to the method described in Maniatis *et al.* [16]. Membranes were probed with pHFβT-1 (supplied by Dr. P. Gunning, CMRF, Sydney), a virtually full-length cDNA clone specific for β-tubulin, which has the same sequence as the β-tubulin cDNA clone described by Hall *et al.* [17]. The membranes were then re-probed with an oligomer that hybridizes to 18S ribosomal RNA [18] to quantify RNA loading. The intensities of the various signals were measured on a Hoeffer GS370 densitometer.

Flow cytometric analysis. Cell samples were stained with propidium iodide (50 μg/mL) for DNA quantification, and flow cytometric analysis was performed basically as described by Pittman *et al.* [5]. Experiments were repeated three times and were analysed on a Becton Dickinson FACScan cytometer.

RESULTS

Induction of apoptosis by taxol. The human T-cell leukaemia line CCRF-CEM was treated with 10 nM taxol for various periods of time, and cells were harvested for morphological, flow cytometric and

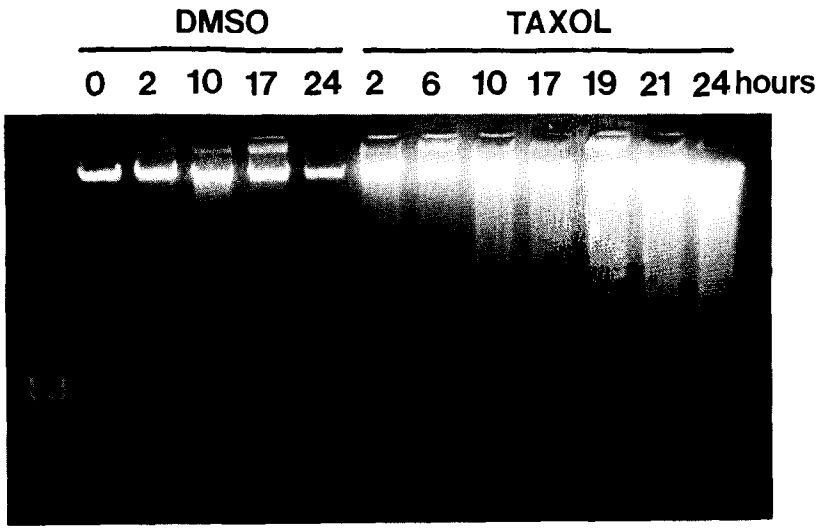

Fig. 1. Time–course of DNA fragmentation observed following taxol treatment. Apoptosis was induced in CCRF-CEM cells treated with 10 nM taxol. DNA fragmentation was first observed 10 hr post-treatment. Details of DNA isolation and electrophoresis are given in Materials and Methods.

DNA analysis. The morphological changes typical of apoptosis were visible as soon as 1 hr after treatment (see below). DNA fragmentation was observed at 10 hr post-treatment or later (Fig. 1).

Flow cytometric analysis of taxol-treated cells showed that cells accumulated in G_2/M phase following treatment, and that cell death, as evinced by the presence of a hypodiploid peak on the DNA histogram, was clearly apparent at 12 hr post-treatment (Fig. 2).

Having established that apoptosis was induced by taxol in CCRF-CEM cells, we wished to examine what effects this drug, which is known to enhance tubulin polymerization, had on the appearance and properties of the MT structures that we had observed previously to be associated with apoptosis.

Examination of tubulin-stained cytocentrifuge preparations showed that all cells, both treated and untreated, showed some tubulin positivity, which conformed to the descriptions in Materials and Methods. At 1 hr following taxol treatment approximately 40% of the cells displayed normal morphology, 6% were mitotic, 23% presented with cytoplasmic blebbing, and in 32% the nucleus was invaginated (Table 1). Of the cells with normal morphology, half displayed polymerized tubulin structures in the cytoplasm and on or around the nucleus, and all the mitotic cells had either disordered mitoses or multiple asters. At later times following taxol treatment the percentage of cells with normal interphase morphology decreased to around 25%, but of these cells an increasing proportion displayed cytoplasmic and nuclear tubulin structures. The percentage of mitotic cells in the preparations increased with time following treatment, reaching a maximum of 32% total cells at 17 hr. The numbers of cells displaying apoptotic morphology remained at roughly 50% of the population until 10 hr post-treatment.

Comparison of tubulin structures and apoptotic morphology induced by treatment with taxol and other cytotoxic drugs. We compared the tubulin structures visible in apoptotic CCRF-CEM cells following treatment with taxol with those following treatment with MTX and VP-16 at the time points at which DNA fragmentation was observed. Our previous studies [4, 5] had established that DNA fragmentation generally occurs between 48 and 72 hr post-treatment with 10^{-8} M MTX and between 4 and 6 hr post-treatment with 17 μM VP-16. It was decided to use the time points at which detectable DNA fragmentation was first observed as a basis for comparison of the tubulin structures induced by taxol and these drugs. Comparing tubulin structures in different cell populations is complicated by the fact that apoptosis is initiated and proceeds at varying rates depending on the cell type under investigation, and on the mode of action and concentration of the inducing drug. Further, in any population, apoptosis does not occur in synchrony, and the parent population may contain a proportion of cells that have been "triggered" for apoptosis by a number of endogenous factors. Such cells may rapidly complete apoptosis when challenged by a cytotoxic drug, making it difficult to assess the contribution of drug treatment to tubulin changes at early time points. By the time DNA fragmentation occurs, however, a large proportion of the cells are undergoing apoptosis, and any tubulin structures present can be attributed to the effects of the drug treatment.

CCRF-CEM cells were treated with taxol, MTX and VP-16 for various times, and harvested for cytocentrifuge preparations that were then stained for β-tubulin and scored for apoptotic morphology. The results are presented in Table 2. Figure 3 shows photomicrographs of cellular tubulin staining in CCRF-CEM cells using a monoclonal antibody that recognizes human β-tubulin. This antibody was indirectly labelled with immunoperoxidase.

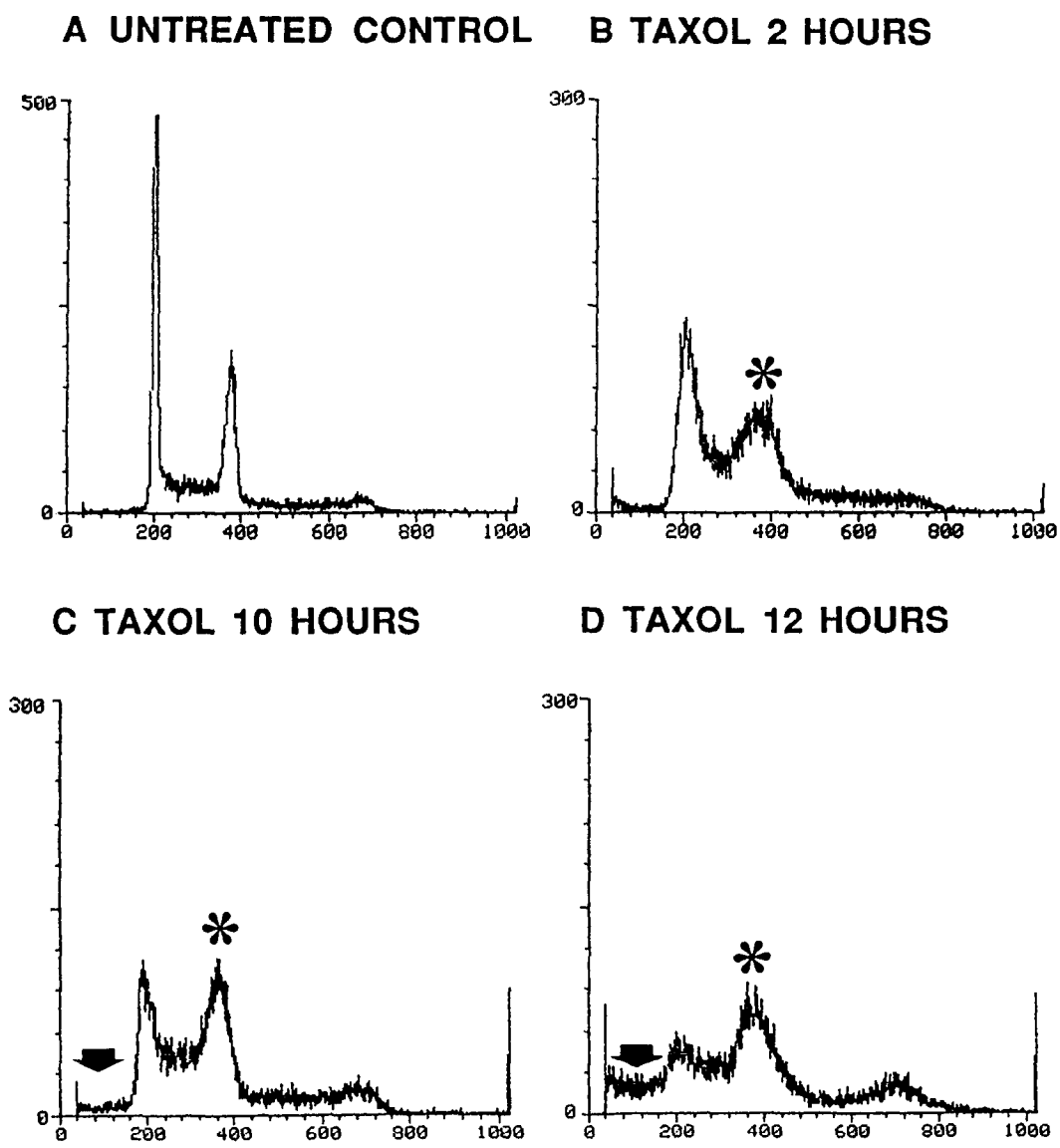

Fig. 2. DNA histograms from flow cytometric analysis of taxol-treated CCRF-CEM cells. Arrows show hypodiploid peak; asterisks indicate G_2/M block. (A) Untreated cells, (B) 2 hr post-treatment with taxol, (C) 10 hr post-treatment with taxol, and (D) 12 hr post-treatment with taxol. The ordinate (y) represents the number of events, and the abscissa (x) represents relative fluorescence (PI).

Several major observations are apparent in Table 2. First, the percentage of normal interphase cells was much lower in cells undergoing apoptosis induced by all three drugs. In addition, as mentioned above, cells with normal morphology in treated preparations often displayed microtubule structures in the cytoplasm and on or around the nucleus. Following all treatments, the percentage of cells displaying apoptotic morphology rose to roughly similar levels, i.e. between 15 and 25% of the cells displayed cytoplasmic distortions and between 37 and 45% displayed apoptotic nuclear morphology. A significant percentage of cells were mitotic at the time of observed DNA fragmentation following taxol treatment, as discussed above.

The majority of cells in untreated or DMSO-treated preparations had normal morphology with tubulin staining as described in Materials and Methods. However, in all untreated cell populations a proportion of the cells was observed to be undergoing spontaneous apoptosis. Such cells represented between 10 and 30% of the population and generally displayed cytoplasmic protrusions with MT structures in the neck of the protrusion. Normal mitoses stained strongly for tubulin (Table 2, Fig. 3A).

Treatment with taxol induced a massive reorganization of tubulin. At 10 hr post-treatment with taxol, 30% of the cells appeared morphologically normal but displayed MT bundles or networks

Table 1. Time–course of morphological changes induced by taxol treatment

Treatment		Morphology*			
		Normal		Apoptotic	
Agent	Time	Interphase	Mitotic	Protruding/vacuolated cytoplasm	Invaginated nucleus
DMSO	4 hr	81.3	4.1	14.6	0.0
Taxol	1 hr	19.0 (19.9)†	5.9‡	22.9	32.3
Taxol	2 hr	8.8 (16.5)	7.5	27.4	39.8
Taxol	6 hr	5.2 (23.5)	19.4	14.3	37.7
Taxol	10 hr	0.0 (26.8)	19.8	16.8	36.6
Taxol	17 hr	0.0 (28.4)	31.6	2.7	37.6

* At least 300 cells were scored from two separate experiments; figures represent the mean values from these experiments, expressed as a percentage.

† Figures in brackets represent percentages of cells with normal morphology but with polymerized tubulin structures in the cytoplasm and surrounding the nucleus.

‡ Mitotic cells in taxol-treated preparations displayed disordered mitotic spindles or multiple asters.

Table 2. Morphological changes observed during drug-induced apoptosis

Treatment		Morphology*			
		Normal		Apoptotic	
Agent	Time†	Interphase	Mitotic	Protruding/vacuolated cytoplasm	Invaginated nucleus
DMSO	4 hr	81.3	4.1	14.6	0.0
Taxol	10 hr	26.8	19.8‡	16.8	36.6
MTX	48 hr	31.6	1.2	22.7	44.5
VP-16	4 hr	34.2	0.0	24.3	41.5

* At least 300 cells were scored from at least two separate experiments; figures represent the mean values from these experiments, expressed as a percentage.

† Time of analysis at which DNA fragmentation was first observed following drug treatment.

‡ Mitotic cells in taxol-treated preparations displayed disordered mitotic spindles or multiple asters.

surrounding the nucleus without distortion of the nucleus or cytoplasm. In less than 5% of the cells, loops of MT were superimposed on the nucleus. Cells with cytoplasmic protrusions associated with MT structures comprised approximately 15% of the total cell population. Forty percent of the taxol-treated cells had invaginated nuclei, which stained strongly for the presence of MT bundles or bands that appeared to constrict the nucleus. Almost 20% of the cells 10 hr post-treatment with taxol were mitotic, displaying disordered mitoses or mitoses with multiple aster formation (Table 2, Fig. 3B). These mitotically arrested cells did not display the morphological features typical of apoptosis.

Treatment of CCRF-CEM cells with 10^{-8} M MTX for 48 hr or with 17 μM VP-16 for 4 hr induced apoptosis and DNA fragmentation. It appears that, at the time of DNA fragmentation induced by each drug, about 70% of the cells scored as apoptotic with tubulin structures associated with either cytoplasmic blebbing or nuclear invagination (Table 2, Fig. 3C and 3D).

The induction of apoptosis in CCRF-CEM cells by three different cytotoxic drugs, including taxol, was accompanied by the formation of similar tubulin structures whose appearance and position correlated with apoptotic morphology. Taxol-treated cells contained a high proportion of mitotically arrested cells compared with cells treated with either of the other drugs. Multiple asters in mitotically arrested cells and MT loops in cells undergoing apoptosis were only seen following treatment with taxol.

Time–course of tubulin involvement in apoptosis following treatment with taxol. To ascertain whether the appearance of the MT structures observed during apoptosis induced by the three drugs used in this study required *de novo* synthesis of tubulin, we measured levels of β-tubulin mRNA at various times following treatment. Comparison of tubulin mRNA levels in drug- and DMSO-treated cells was

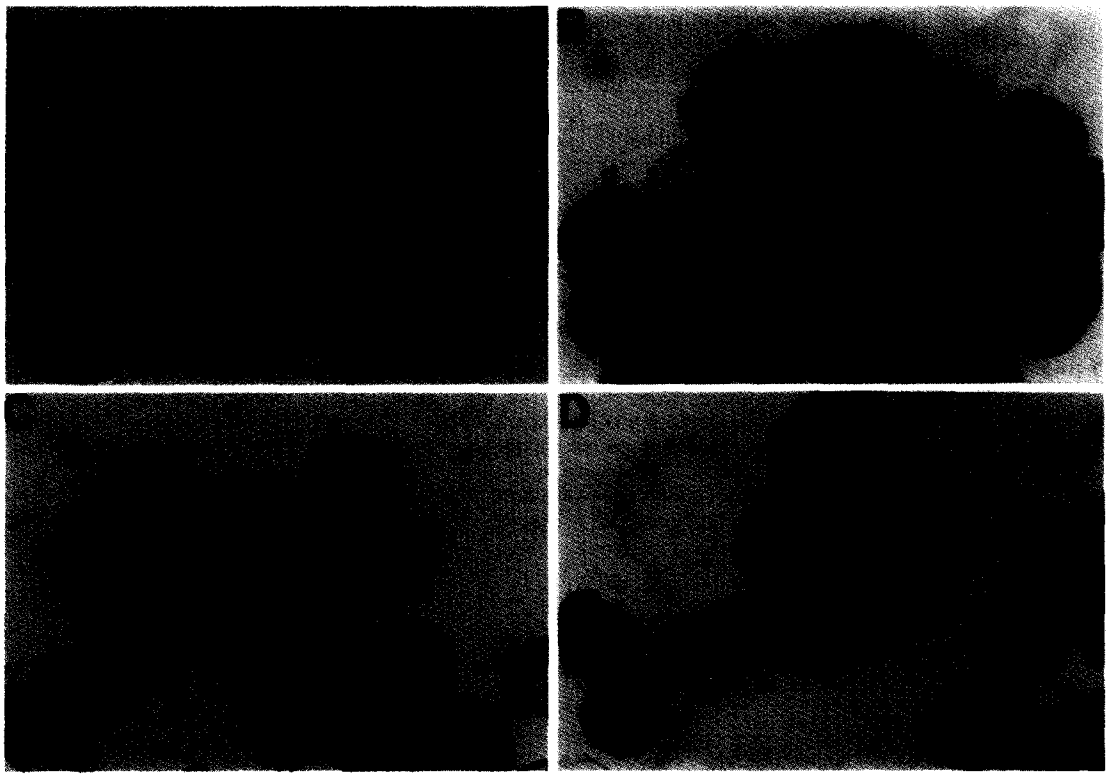

Fig. 3. Tubulin staining of CCRF-CEM cells, untreated and following drug treatment. The photomicrographs of cytocentrifuge preparations (magnification: 100×) show tubulin-stained CCRF-CEM cells that were: (A) Untreated with (a) strong tubulin staining in dividing cells, (b) tubulin staining of MTOC in an interphase cell, and (c) tubulin structures in cytoplasmic bleb in a cell undergoing spontaneous apoptosis; (B) Treated with 10 nM taxol for 10 hr with (a) invagination of the nucleus with staining of dense polymerized bands or parallel arrays of tubulin, (b) protruding or budding cytoplasm with strong tubulin staining, (c) multiple asters or disordered mitoses strongly stained for tubulin, and (d) loop structure strongly stained for tubulin; (C) Treated with 17 μM VP-16 for 4 hr with (a) invagination of the nucleus with staining of dense polymerized bands or parallel arrays of tubulin, and (b) protruding or budding cytoplasm with strong tubulin staining; and (D) Treated with 10^{-8} M MTX for 48 hr with (a) invagination of the nucleus with staining of dense polymerized bands or parallel arrays of tubulin, and (b) protruding or budding cytoplasm with strong tubulin staining.

performed at each time point to correct for possible fluctuations in the levels of tubulin message due to the cell cycle status of the different cell populations. Northern analysis of RNA isolated following treatment with taxol, VP-16 and MTX showed similar increases in tubulin RNA levels at time points prior to the detection of DNA fragmentation following these treatments. These results are summarized graphically in Fig. 4. Figure 5 shows a representative Northern blot for VP-16-treated cells. The probe used to detect β-tubulin hybridizes to both 2.8 and 1.8 kb messages, which have been reported in CCRF-CEM cells [19].

Following treatment with all three drugs, the levels of both tubulin messages demonstrated a transient rise of a similar magnitude and at a relatively early time in the apoptotic process. In addition, tubulin protein levels have been shown to increase in cells undergoing apoptosis induced by these treatments, as measured by flow cytometry or western analysis ([5],*).

* Manuscript in preparation.

DISCUSSION

The fact that taxol, as well as vincristine [4, 6], induces apoptosis, indicates that any perturbation in the dynamics of MT assembly, whether to decrease or increase the critical tubulin concentration for polymerization, is able to trigger apoptosis. Following taxol treatment, the tubulin structures that we associate with apoptosis, as well as structures apparently unique to taxol-treated cells, were observed. The main effect of taxol is assumed to be to decrease the critical tubulin concentration [20], i.e. to lower the concentration of tubulin dimers at which MT formation occurs. As proposed by De Brabander *et al.* [21], this property would lower the critical tubulin concentration in the cytoplasm to below that which normally exists around the MTOCs, so that they cease to operate as specific sites for the assembly of MTs. Thus, in the presence of taxol, processes involving MT assembly would proceed, but with nucleation occurring at random. This would explain the presence of multiple asters in cells undergoing mitosis when treated with taxol, and also

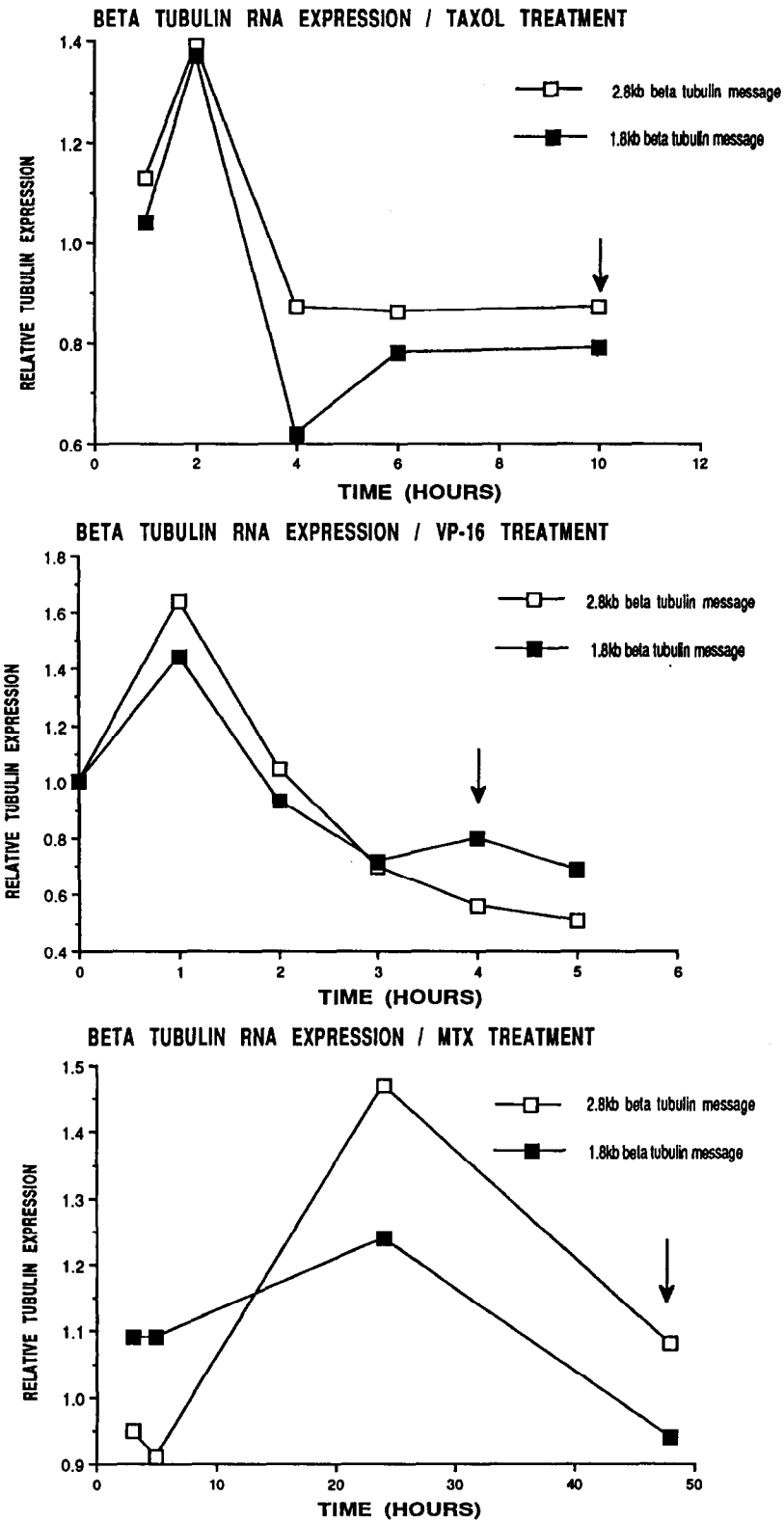

Fig. 4. Changes in β-tubulin RNA expression during apoptosis following drug treatment in CCRF-CEM cells. Graphs show changes in β-tubulin RNA expression during apoptosis induced by 10 nM taxol (top panel), 17 μM VP-16 (middle panel) and 10^{-8} MTX (bottom panel). Arrows indicate time that DNA fragmentation was first observed. Points represent the average values obtained from at least three experiments and are normalized to expression in DMSO-treated control preparations. Loading has been quantified with respect to 18S ribosomal RNA signal.

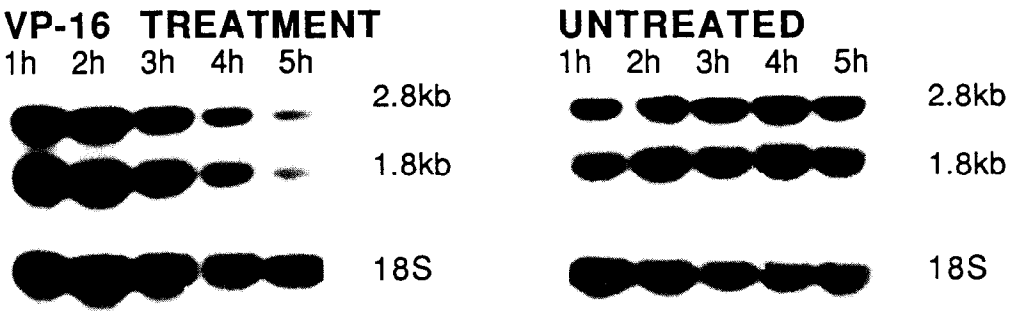

Fig. 5. Northern blot of total RNA isolated from CCRF-CEM cells treated with 17 μM VP-16. Blots were prepared and probed as described in Materials and Methods. Upper panel shows signals from the two β-tubulin species present in these cells; lower panel shows signal from reprobing with an oligomer to detect 18S ribosomal RNA.

the somewhat haphazard formation of loops that otherwise may have been associated with the nuclear invagination characteristic of apoptosis. So, the structures that seem to be typical of taxol effects on MT are, very likely, a result of the decrease in critical tubulin concentration and stabilization of MTs in structures that are typical of either mitosis or apoptosis in all cells.

Rowinsky *et al.* [9] examined the effects of taxol treatment on four different leukaemia cell lines. They described two major cell populations, those containing MT "bundles," which often disrupted cell shape, and mitotic cells containing multiple asters. Whilst this paper made no attempt to analyse the morphological changes associated with taxol-induced MT bundles, in all likelihood these changes are those associated with apoptosis induced by the drug. Like these authors, we also noted that both types of structure were independent, i.e. did not occur in the same cell. Our observations on the stages of the cell cycle at which cells can enter apoptosis suggest that mitotic cells do not undergo the morphological changes typical of apoptosis [5]. However, it has been reported that cells arrested in mitosis and subsequently treated with agents that cause apoptosis do undergo DNA fragmentation [22].

The control of tubulin expression following treatment with agents that alter the level of polymerized tubulin within the cell appears to operate through a co-translational mechanism. The translation of the first four amino acids of the β-tubulin molecule is integral to a mechanism that leads to the destruction of the tubulin message if the cellular tubulin dimer content exceeds a certain level [23, 24]. An increase in tubulin mRNA synthesis has been reported following taxol treatment [25], presumably in response to the fall in tubulin dimer content produced by the enhanced tubulin polymerization observed with this drug. This taxol-induced increase in tubulin mRNA levels was abrogated by the displacement of tubulin mRNAs from polysomes with the inhibitors puromycin and pactamycin [25], indicating that the autoregulatory mechanism operates on polysome-bound tubulin mRNA.

We have observed a rise in tubulin mRNA levels similar to that induced by taxol in apoptosis induced by two agents that have no intrinsic effect on tubulin dimer levels. While we have yet to establish the mechanism of this tubulin mRNA induction, it is conceivable that the polymerization of tubulin into the structures that we have observed to be associated with apoptosis causes a fall in the level of tubulin dimers to the extent where the autoregulatory mechanism directs an increase in tubulin mRNA synthesis. Alternatively, the critical levels of tubulin dimers may be altered by other mechanisms during the initiation or progression of apoptosis.

Our evidence suggests that the observed increase in tubulin mRNA synthesis during apoptosis is followed by a rise in the levels of tubulin protein ([5],*). This occurs despite the general decrease in total cellular protein levels observed in apoptosis [26].

It thus appears that there is a sequence of events involving tubulin expression and reorganization that follows cytotoxic drug addition and the onset of apoptosis in CCRF-CEM cells, and that taxol initiates a sequence similar to that observed with agents with very different modes of action. These results lend support to the notion that while there may be many events that trigger apoptosis, there seems to be a common pathway by which the process proceeds, and that this pathway involves reorganization of cellular tubulin.

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