Mitotic Block Induced in HeLa Cells by Low Concentrations of Paclitaxel (Taxol) Results in Abnormal Mitotic Exit and Apoptotic Cell Death

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ABSTRACT

Paclitaxel at low concentrations (10 nm for 20 h) induces ~90% mitotic block at the metaphase/anaphase transition in HeLa cells, apparently by suppressing dynamics of spindle microtubules (M. A. Jordan et al., Proc. Natl. Acad. Sci. USA, 90: 9552–9556, 1993). It is not known, however, whether inhibition of mitosis by such low paclitaxel concentrations results in cell death. In the present work, we found that after removal of paclitaxel (10 nm-1 μM), blocked cells did not resume proliferation. Instead, cells exited mitosis abnormally within 24 h. They did not progress through anaphase or cytokinesis but entered an interphase-like state (chromatin decondensed, and an interphase-like microtubule array and nuclear membranes reformed). Many cells (~55%) contained multiple nuclei. Additional DNA synthesis and polyploidy did not occur. DNA degradation into nucleosome-sized fragments characteristic of apoptosis began during drug incubation and increased after drug removal. Cells died within 48–72 h. Incubation with paclitaxel (10 nm for 20 h) resulted in high intracellular drug accumulation (8.3 μM) and little efflux after paclitaxel removal; intracellular retention of paclitaxel may contribute to its efficacy. The results support the hypothesis that the most potent chemotherapeutic mechanism of paclitaxel is kinetic stabilization of spindle microtubule dynamics.

INTRODUCTION

Paclitaxel (Taxol) is a potent new antitumor drug that is remarkably effective against advanced ovarian carcinoma and breast carcinoma and shows promising activity against many other types of tumors (1). Paclitaxel inhibits cell proliferation and induces cell death (2–9). It appears to act by slowing or blocking progression through mitosis (3, 6, 7, 10, 11). In HeLa cells, low concentrations of paclitaxel (10 nm) induce a sustained cell cycle block at the metaphase/anaphase transition of mitosis. The mitotic spindles in such blocked cells are nearly normal morphologically and contain a normal mass of microtubules (3). Substoichiometric binding of paclitaxel to reassembled microtubules potently stabilizes microtubule dynamics (12). Together these results have suggested that mitotic block in HeLa cells by low concentrations of paclitaxel results from a potent kinetic stabilization of mitotic spindle microtubule dynamics (3, 12). However, it has generally been considered that the mitotic block induced by low concentrations of paclitaxel (or other antimitotic drugs) leads merely to a transitory slowing of the cell cycle, from which cells recover and continue proliferating. Thus, it remains to be determined whether a prolonged suppression of microtubule dynamics and mitotic progression at metaphase is sufficient to induce cell death. It is not known whether mitotic block by low concentrations of paclitaxel is an important part of the chemotherapeutic action of paclitaxel.

To examine the question of whether mitotic block by low paclitaxel concentrations induces cell death, we blocked mitosis in HeLa cells with a range of paclitaxel concentrations (3 nm-1 μM for 20 h) and then washed the cells extensively to terminate exposure to the paclitaxel. HeLa cells are advantageous for these experiments because nearly all cells (~90%) become blocked in mitosis during a 20-h incubation with paclitaxel (~10 nm). If cell death occurred, we wanted to determine its time course and characteristics. We also wanted to determine the fate of cells exposed to paclitaxel for only a limited time, thus approximating therapeutic conditions resulting from rapid administration followed by excretion of paclitaxel.

MATERIALS AND METHODS

Cell Culture. HeLa S3 cells from epithelioid carcinoma of human cervix (American Type Culture Collection, Rockville, MD) were grown in spinner culture or in monolayer in either Falcon (Becton Dickinson, Lincoln Park, NJ) T175 or Corning (Corning, NY) 225-cm² tissue culture flasks or 35-mm-well plates in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) at 37°C in 5% carbon dioxide, in the absence of antibiotics. Cells were seeded at densities (0.4–50 × 10⁶ cells/ml) such that the cells were in log phase of growth for the duration of each experiment. Two days after seeding, fresh medium plus or minus paclitaxel at the stated concentration was added. Twenty h later, the medium was replaced with paclitaxel-free medium; two additional changes of fresh medium lacking paclitaxel were added at 1-h intervals, and incubation was continued for 72 h.

Cell Proliferation and Death. Subcultures of cells for immunofluorescence microscopy and for assays of proliferation and cell death were plated at a density of ~0.5–1 × 10⁶ cells/cm² in 35-mm dishes containing no. 1 glass coverslips freshly coated with polylysine (50 μg/ml for 2 h at 37°C), followed with a rinse with water and a rinse with medium. Two days later, fresh medium containing paclitaxel (or no paclitaxel) was added. Cells were counted by hemocytometer following trypsinization to determine the number of cells at the time of paclitaxel addition; doubling time was 19 ±1 h. The cell number was similarly determined 18–20 h after paclitaxel addition and 24, 48, and 72 h after removal of paclitaxel from the medium and washing of cells in paclitaxel-free medium. The percentages of live and dead cells were determined at each time point by microscopy of cells stained with ethidium homodimer or trypan blue (dead cells) or with enzymatically cleaved calcein-AM (live cells; Molecular Probes, Inc., Eugene, OR).

Determination of Intracellular Paclitaxel Concentration. Paclitaxel uptake into HeLa cells and retention after washing the cells with medium lacking paclitaxel were determined after incubating cells for 20 h with [3H]paclitaxel (3 nm-1 μM, 33–10,900 Ci/mol), following by washing and continued incubation for 24 h, as described above. Two methods for determining intracellular paclitaxel concentration were used that gave similar results: (a) scintillation vial monolayer culture method. HeLa cells were seeded in polylysine-coated sterile scintillation vials at 3 × 10⁵ cells/ml. Twenty-four h later, media were aspirated and replaced with 2.5 ml of medium containing [3H]paclitaxel at the desired concentration. Adherence of [3H]paclitaxel to scintillation vials was < 0.5% of total radioactivity. Following incubation with [3H]paclitaxel or following the post-washing incubation, media were aspirated, and the cells were washed quickly three times with 2.5 ml buffer (0.1 M piperrazine-N,N'-bis(2-ethane sulfonic acid, and 1 mM EGTA, 1 mM MgSO₄, pH 6.9, 37°C). Cells were lysed by adding 1 ml distilled water, and incorporated radioactivity was determined following the addition of 10 ml scintillation fluid (Beckman Ready Protein). Duplicate vials incubated under the same conditions using nonradiolabeled paclitaxel were used to determine the cell number at each time point. Total cell volume was determined by multiplying the number of cells by the volume of an average cell as measured by microscopy; and (b) suspension culture and centrifugation. Uptake and release of paclitaxel was determined on cells cultured in suspension and collected by centrifugation (3, 13). Cells were seeded at 5 × 10⁵ cells/ml; 1 day later, an equal volume of fresh media was added, followed by limiting dilution of medium and paclitaxel (10 nM). If cell death occurred, we wanted to determine its time course and characteristics. We also wanted to determine the fate of cells exposed to paclitaxel for only a limited time, thus approximating therapeutic conditions resulting from rapid administration followed by excretion of paclitaxel.
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containing paclitaxel was added. Cells were collected by centrifugation (ICN Clinical Centrifuge; setting 3 for 5 min). The total cell volume was determined by subtracting extracellular volume [determined by the addition of \(^{14}C\)-hydroxyethylmethyliminol to suspensions prior to centrifugation (13) from the total volume of the pellets of centrifuged cells]. Paclitaxel and \(^{3}H\)paclitaxel were gifts from the National Cancer Institute (\(^{3}H\)paclitaxel; NSC 125973). Data are means and SEs of four to six individual measurements from three separate experiments.

Flow Cytometry. Progression of cells through the cell cycle was based on flow cytometric determination of cellular DNA content. HeLa cells were collected by centrifugation, resuspended in PBS (Sigma Chemical Co.), and fixed in 70% ethanol (4°C for 30 min). Cells were then collected by centrifugation (ICN clinical centrifuge, 3000 \( \times g \) for 5 min), resuspended in 0.8 ml PBS, passed through a 25 gauge needle to eliminate cell clumping, and treated with RNase A (100 \( \mu \)l, 1 mg/ml) and propidium iodide (100 \( \mu \)l, 400 \( \mu \)g/ml; 30 min at 37°C). DNA content was determined using a Coulter EPICS model 541 flow cytometer equipped with an argon-ion laser tuned to 488 nm, measuring forward and orthogonal light scatter and peak and area red fluorescence. Sample size was \( \geq 10,000 \) cells.

Extraction and Agarose Gel Electrophoresis of DNA from Cells following Paclitaxel Incubation. Cells grown in suspension to a density of 1 \( \times 10^{6} \) cells/ml were incubated without or with paclitaxel (10 nm-100 nm) for 20 h, followed by washing three times at 1-h intervals with paclitaxel-free medium and continued incubation in the absence of added paclitaxel. At desired intervals, 100-ml aliquots of cells were removed from the suspension, centrifuged in a clinical centrifuge (3 min at 3000 \( \times g \)), and stored frozen (–70°C) prior to extraction of DNA for gel electrophoresis. For DNA extraction, cells were lysed in Tris buffer (0.045 M Tris-borate, 0.001 M EDTA) containing 0.1% Triton X-100, and supernatants were treated with RNase A (100 \( \mu \)g/ml), proteinase K (200 \( \mu \)g/ml), and SDS (1%); DNA was phenol-chloroform extracted and precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate (14). DNA (30 \( \mu \)g/sample) was analyzed by conventional agarose gel electrophoresis (2% agarose, 60V, approximately 5 h), using Tris acetate running buffer (0.04 M Tris-acetate, 0.001 M EDTA). A 123-bp size standard was electrophoresed in parallel. DNA was visualized by staining with ethidium bromide (1 \( \mu \)g/ml) and destaining in distilled water for \( \sim \)30 min.

Immunofluorescence Microscopy. Cells grown on coverslips were prepared for microscopy (13). Briefly, cells were fixed in 10% formalin in PBS for 10 min and then transferred to 99.6% methanol and 2 mM ethylene glycol bis(\( \beta \)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (20°C) for 10 min. Following blocking of nonspecific antibody binding using normal goat serum, fixed cells were stained for tubulin with a mouse monoclonal antibody (E7, IG1), a gift from Dr. Michael Klymkowski (University of Colorado, Boulder, CO), which is specific for \( \beta \) tubulin, followed by staining with FITC-conjugated goat antimouse immunoglobulin G (Cappell, West Chester, PA) for centrosomes with 5051, a human autoimmune anticientrosomal antisera, followed by rhodamine-conjugated goat antihuman immunoglobulin G (Cappell), and with DAPI for chromosomes. Photomicrographs were obtained using a Zeiss Photomicroscope III equipped with an epifluorescence condenser and a \( \times 40 \) objective using Kodak TMAX 400 film (Rochester, NY) developed in a Kodak TMAX developer.

RESULTS

Experimental Strategy. We wanted to determine whether the mitotic block induced in HeLa cells by low concentrations of paclitaxel was sufficient to induce cell death or whether the blocked cells could recover after removing paclitaxel and continue proliferating. Thus, HeLa cells were incubated with paclitaxel for 20 h, a length of time that we previously determined results in maximal accumulation of cells in mitotic metaphase (3). Paclitaxel was then removed from the medium, cells were washed with paclitaxel-free medium three times at 1-h intervals, and incubation was continued for 2 to 3 days in the absence of added paclitaxel; the procedure will be referred to as "paclitaxel incubation and removal." At intervals during this procedure, we determined the cell number; the proportion of cells in interphase or mitosis; the stage of mitosis; the subcellular organization of microtubules, nuclei, and chromosomes; whether the cells were alive or dead; the DNA content; whether DNA was degraded; and the intracellular paclitaxel concentration.

Induction of Mitotic Block with Low Concentrations of Paclitaxel and Exit from Mitotic Block after Removal of Paclitaxel. Cells were incubated with paclitaxel for 20 h at concentrations ranging from 3 nm to 1 \( \mu \)M. The cells were then fixed and stained with DAPI⁴, a stain for chromatin and chromosomes, to determine the numbers of cells in mitosis and in interphase. As described previously (3), paclitaxel at concentrations \( \geq 10 \) nm blocked most cells (80–90%) in mitosis (Fig. 1). Lower concentrations of paclitaxel induced less mitotic accumulation (20% of cells were in mitosis at 3 nm paclitaxel; 34% at 6 nm paclitaxel). Mitotic block occurred specifically at the transition from metaphase to anaphase. In control populations, 2% of all cells were in late prometaphase or metaphase, whereas 0.3% of cells were in anaphase; thus, the ratio of cells in anaphase to cells in metaphase was 0.15 (Table 1). As the paclitaxel concentration was increased, cells accumulated in metaphase and did not progress to anaphase; thus, the anaphase/metaphase ratio decreased. At 10 nm paclitaxel, 89% of the cells that had accumulated in metaphase, and \(<1\% \) were in anaphase; the ratio of cells in anaphase to cells in metaphase was 0.01 (Table 1). At higher concentrations of paclitaxel (>10 nm), no cells were in anaphase.

After the 20-h incubation with paclitaxel, cells were washed three times with paclitaxel-free medium, and incubation was continued for

⁴ The abbreviation used is: DAPI, 4,6-diamino-2-phenylindole.
mitotic block with paclitaxel. To determine the cell cycle events and changes in subcellular organization caused by paclitaxel incubation and removal, we examined the subcellular organizations of microtubules, chromosomes, and nuclei by immunofluorescence microscopy (Figs. 3–6).

**Control Cells.** Control cells (Fig. 3) in interphase were flat, well spread, and uniform in size, with a fine filamentous array of microtubules radiating from the centrosomes and generally a single nucleus that was spherical or ovoid in shape. Mitotic cells contained well-organized bipolar spindles with two distinct, well-separated spindle poles (Fig. 3c; arrowheads) and few astral microtubules. At metaphase, all of the chromosomes were organized in a compact equatorial metaphase plate (Fig. 3b, arrows).

**Cells after Incubation with Paclitaxel.** Following incubation with 10 nM paclitaxel, most cells were in mitosis (Fig. 4, a–c). As

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**Table 1 Inhibition of the transition from metaphase to anaphase by low concentrations of paclitaxel**

HeLa cells were incubated with paclitaxel for 20 h; then the paclitaxel was removed from the medium ("Materials and Methods"). Following fixation of cells and staining of microtubules and chromatin ("Materials and Methods"); the numbers of cells in metaphase and in anaphase were counted by immunofluorescence microscopy. Values are mean ± SE of two to four experiments and represent counts from $\geq 40$ mitotic cells/concentration and per time point in each experiment (data for 48 h, $\geq 33$ nm, are from a single experiment).

<table>
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<th>Paclitaxel (nM)</th>
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<th>24 h after removal</th>
<th>48 h after removal</th>
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<tbody>
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<td>0</td>
<td>0.15 ± 0.04</td>
<td>0.17 ± 0.06</td>
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<tr>
<td>3</td>
<td>0.05 ± 0.03</td>
<td>0.10 ± 0.02</td>
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<td>6</td>
<td>0.02 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.11 ± 0.02</td>
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<td>10</td>
<td>0.01 ± 0.01</td>
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3 days in the absence of added drug. Twenty-four h after removal of paclitaxel (10 nM–1 μM paclitaxel, initial added concentration), most cells had exited mitosis into an apparent interphase state (as determined by decondensation of chromosomes and reformation of nuclear membrane, described further below and in Fig. 5). For example, 24 h after 10 nM paclitaxel was removed from the medium, the percentage of cells in mitosis was reduced from 87 to 26%, and 48 h after paclitaxel was removed, only 17% of the cells were in mitosis (Fig. 1). Similarly, 24 and 48 h after removal of 1 μM paclitaxel from the medium, the percentage of cells in mitosis was reduced from 84 to 43% and to 19%, respectively. Thus paclitaxel ($\geq 10$ nM for 20 h) induced complete metaphase block, and after removal of paclitaxel, the blocked cells exited mitosis.

**Cells Do Not Proliferate after Removal of Paclitaxel ($\geq 10$ nM).** Cell numbers were determined following incubation with paclitaxel for 20 h and at 24, 48, and 72 h after removal of the drug. As shown in Fig. 2A, incubation with paclitaxel for 20 h inhibited cell proliferation in a concentration-dependent manner. Cell proliferation was inhibited in parallel with inhibition of mitosis (Fig. 1). For example, 3 nM paclitaxel induced 16% inhibition of cell proliferation and 20% mitotic block. Ten to 1000 nM paclitaxel induced >90% inhibition of cell proliferation and 80–90% mitotic block. Following removal of paclitaxel ($\geq 10$ nM), inhibition of proliferation persisted over the next 3 days. Thus, concentrations of paclitaxel that blocked mitosis also induced a sustained inhibition of proliferation after removal of the drug and exit from mitosis.

**Induction of Cell Death by Paclitaxel.** Unfixed cells were stained with calcein AM and ethidium bromide (or trypan blue) to differentiate live cells from dead cells and were counted by microscopy ("Materials and Methods"); the results are shown in Fig. 2B. Three nM paclitaxel caused very little cell killing, either during drug incubation or after removal of the drug; only 8–10% of the cells were dead 48–72 h after paclitaxel removal, as compared with 2–4% dead cells in control populations. At early times at higher paclitaxel concentrations (10 nM–1 μM), only low levels of cell killing occurred. During the 20-h incubation with drug, only 2–16% of the cells died, and 24 h after removal, 14–26% of cells were dead. However, 48–72 h after removal of paclitaxel, cell death increased; the percentage of dead cells ranged from 44–46% at 10 nM paclitaxel to 78% at 1 μM paclitaxel. Thus, incubation with paclitaxel for 20 h at concentrations that induced significant metaphase block ($\geq 10$ nM) also caused significant cell death within 2 days after removal of the drug.

**Cell Cycle Progress and Subcellular Organization following Mitotic Block with Paclitaxel.** To determine the cell cycle events and changes in subcellular organization caused by paclitaxel incubation and removal, we examined the subcellular organizations of microtubules, chromosomes, and nuclei by immunofluorescence microscopy (Figs. 3–6).
described previously (3), their spindles were either bipolar metaphase spindles that often exhibited slight abnormalities or were monopolar. Abnormal bipolar metaphase spindles had some chromosomes organized into a distinct, compact metaphase plate and some chromosomes that were near one or both of the two spindle poles, distant from the metaphase plate (Fig. 4b, arrows); monopolar spindles contained a monoaster of microtubules, with one pole or centrosome enclosed in a spherical arrangement of chromosomes (Fig. 4, b and c, *). Many interphase cells after incubation with 10 nM paclitaxel contained more than one nucleus (Fig. 7). After 20-h incubation with 10 nM paclitaxel, 36% of interphase cells were multinucleate as compared with 2% of the cells in control populations (Fig. 7, 0 and x, respectively). After incubation with 10 nM paclitaxel, the microtubules of cells in interphase resembled the network of microtubules in control cells (3); bundles of microtubules that occur at higher concentrations of paclitaxel (Fig. 4d, arrow) were not visible.

After 20 h incubation with higher concentrations of paclitaxel (33 nM-1 μM), most cells were in mitosis (Fig. 4, d-f). All spindles were monopolar or multiastral and contained only one region stained with anticentrosomal antibody (Fig. 4f), and the microtubule arrays were more brightly stained and appeared to contain more microtubules than at 10 nM paclitaxel. In contrast with the marked increase in the number of multinucleate cells observed at 10 nM paclitaxel, most interphase cells contained only a single nucleus after incubation with 33 nM-1 μM paclitaxel. Fewer than 18% of the cells became multinucleate at the higher concentrations of paclitaxel (Fig. 7). The cells contained bundles of microtubules (Fig. 4d, arrow), consistent with the 200–500% increase in the mass of polymerized microtubules that occurs at these high paclitaxel concentrations (3).

Cells after Removal of Paclitaxel. After removal of ≤6 nM paclitaxel, most cells appeared normal. They proliferated nearly normally, and a normal proportion of the cells were in metaphase (Figs. 1, 2, and 5, a and b). Interphase cells were uniform in size and generally contained only a single nucleus; spindles were normal in organization and progressed through anaphase (Table 1).

After removal of 10 nM paclitaxel, most cells exited mitosis into an apparent interphase. The chromosomes decondensed, the nuclear membranes reformed, and the microtubules underwent a significant rearrangement from mitotic astral arrays with short microtubules into interphase-like arrays with long microtubules (compare Fig. 4, a and b, with Fig. 5, c and d). However, in contrast with control cells and cells incubated with ≤6 nM paclitaxel, the interphase-like cells varied greatly in size. Most cells (55%) contained more than a single nucleus; nuclei ranged in size from just large enough to contain only one or a few chromosomes to approximately normal size (diameters between 2 μm and 19 μm; compare Fig. 5, a and b, with Fig. 5, c and d; also see Fig. 7, 0). Some cells were spread and flat and contained finely filamentous arrays of microtubules, whereas others were rounded and contained distinct circumferential bundles or baskets of microtubules that surrounded the nuclei (Fig. 5c, arrow). Cells in mitosis contained spindles that were predominantly abnormal; 77% were abnormal 24 h after drug removal, and 63% were abnormal 48 h after drug removal (Fig. 5, c and d, *).

Most cells also exited mitosis during the 48 h after removal of higher concentrations of paclitaxel (≥33 nM); and most of the resulting interphase-like cells (>60%) contained multiple nuclei (Fig. 7). Nuclei were highly fragmented (as many as 50/ cell) or lobulated (Fig. 5f, arrowheads). The cells were of widely varying sizes, some were flat and some were rounded, and they frequently contained dense bundles of long microtubules (Fig. 5e, arrowheads). Nearly all remaining mitoses were abnormal (77%-98%; data not shown). Thus, exit from mitotic block following removal of paclitaxel at concentrations ≥10 nM frequently resulted in formation of multinucleated interphase cells. In contrast, at lower concentrations of paclitaxel (≤6 nM), significantly fewer mitoses were abnormal, and fewer cells became multinucleate after drug removal.

A large percentage of cells (20–25%) could not be classified as either mitotic or interphase-like after removal of ≥10 nM paclitaxel. These cells appeared to be dying and apoptotic. The cells contained very dense, opaque chromatin that appeared liquid. The chromatin was present in droplets or globules of varying sizes and shapes (Fig. 6, b, d, and f). Some cells had no microtubules or any material that stained with tubulin antibody (Fig. 6a, arrow), whereas others contained tangled balls of coarse disorganized microtubules (Fig. 6a, cell...
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Fig. 4. HeLa cells incubated with paclitaxel. Cells were incubated with 10 nM paclitaxel (a-c) or 1 μM paclitaxel (d-f) for 20 h. a and d, microtubules; b and e, chromosomes and chromatin; c and f, centrosomes. a-c: 10 nM paclitaxel. All cells pictured are in mitosis, many with bipolar spindles (see spindle poles at arrowheads in c) and often with a condensed metaphase plate of chromosomes (arrowheads in b) as well as several "lagging" chromosomes (arrows in b) that have not con- gressed to the metaphase plate. Some spindles are monopolar with a ball-shaped arrangement of chromo- somes (b and c, *), d-f: 1 μM paclitaxel. Most cells are in mitosis; their spindles are monopolar with ball-shaped arrangements of condensed chromo- somes (e), and a single centrosome or spindle pole (arrowheads in f); cells in interphase have prominent bundles of microtubules (arrow in d). Bar, 10 μm.

on right) or bundles of microtubules that protruded from the cell periphery (Fig. 6, c and e).

Metaphase/Anaphase Transition. As described above, most cells exited mitosis into an interphase-like state after removal of 10 nM paclitaxel from the medium. We wanted to know whether the cells progressed into the interphase-like state by first progressing into anaphase and completing a normal mitosis or whether they entered interphase without undergoing anaphase. Thus, the numbers of cells in anaphase and metaphase were determined (Table 1). Paclitaxel concentrations of >10 nM completely prevented cell cycle progression from metaphase into anaphase; 80% of the cells were in metaphase, and no cells were in anaphase. In control populations, the ratio of cells in anaphase to cells in metaphase was 0.15 ± 0.04 (Table 1). A small number of cells entered anaphase after removal of 10 nM paclitaxel from the medium (the ratio of cells in anaphase to cells in metaphase was 0.15 ± 0.04 at 24 h after drug removal and was 0.04 ± 0.003 at 48 h after drug removal). However, no cells entered anaphase following removal of higher concentrations of paclitaxel (33–1000 nM). The results indicate that after removal of paclitaxel, most cells exit mitosis abnormally without completing anaphase and progress to an inter- phase-like state.

Cellular DNA Content. To further examine the pathway of mitotic exit following paclitaxel incubation and removal, we determined the DNA content of the cells by flow cytometry. At 20 h of incubation with paclitaxel (10 nM–1 μM), most cells were blocked in G2-M with a 4n amount of DNA (Fig. 8). Twenty-four and 48 h after removal of 10 nM paclitaxel, a few cells had divided and contained a diploid (2n) amount of DNA, whereas many cells appeared to have variable amounts of DNA, generally less than the 2n amount. With higher paclitaxel concentrations (33 nM–1 μM), cells (or parts of cells) had variable amounts of DNA, ranging from <1n to 4n, with a prominent peak of cells with <1n DNA content. Forty-eight h after removal of paclitaxel (10–1000 nM), nearly all cells or cell fragments contained <1n DNA. The 2n diploid peak did not reform. No significant peaks representing higher multiples of DNA amounts (> 4 n) were observed, i.e., cells that failed to complete mitosis and cytokinesis did not synthesize additional DNA to produce polyplloid cells with ≥ 8n DNA. The results suggest that exit from mitotic block with 10 nM paclitaxel was followed 24–48 h later by significant degradation of DNA in most cells. Exit from mitotic block with ≥33 nM paclitaxel was followed 24–48 h later by complete degradation of DNA.

Intracellular Paclitaxel Concentrations. Intracellular concentra- tions of paclitaxel were determined under the conditions of the ex- periments using [3H]paclitaxel (Table 2). Incubation of HeLa cells with paclitaxel (3 nM–1 μM) for 20 h resulted in high intracellular accumulation of the drug (between 2 and 241 μM; 240–830-fold higher than the paclitaxel concentration added to the medium). Pacli- taxel was taken up rapidly by the cells; half-maximal concentrations were attained within 25 min at 100 nM paclitaxel and within 1.5 h at 10 nM paclitaxel; 90% of maximal concentrations were attained within 1.3 and 6.3 h, respectively (data not shown). The intracellular paclitaxel remained at high levels twenty-four h after washing the cells to
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Fig. 5. HeLa cells 24 h after paclitaxel removal. Cells were incubated with paclitaxel for 20 h. The paclitaxel was removed from the medium, cells were washed, and incubation was continued in the absence of added paclitaxel for 24 h. a, c, and e, microtubules; b, d, and f, chromosomes and nuclei (see "Materials and Methods"). a and b, 24 h after removal of 6 nM paclitaxel from the medium. Cells resemble controls; most cells are in interphase and are mononucleate; mitoses are normal: small asterisk, metaphase; medium asterisk, anaphase; large asterisk, telophase. c and d, 24 h following removal of 10 nM paclitaxel from the medium. Cells vary greatly in size, perhaps resulting from infrequent cytokinesis (see also Fig. 8; 10 nm); most cells are in interphase, and many cells have more than one nucleus (arrowheads in d) of varying sizes. Some cells have bundles of microtubules (arrowhead in c). Most spindles are abnormal (*). e and f, 24 h following removal of 1 μM paclitaxel from the medium. Cells vary greatly in size, most cells are in interphase and have fragmented nuclei of smaller size and greater number (arrowheads in f) than after treatment with 10 nm paclitaxel. Bar, 10 μm.

remove the paclitaxel. Thus, the intracellular paclitaxel concentrations under conditions that induced cell killing remained at levels ≥4 μM after washing.

Apoptosis of HeLa Cells following Mitotic Block by Paclitaxel. Microscopic examination and flow cytometry of cells following exit from mitotic block indicated that the cells were dying by apoptosis. To further examine the mechanism of cell death and to determine whether fragmentation of DNA into nucleosome-sized pieces occurred following paclitaxel-induced mitotic block, we performed agarose gel electrophoresis on the DNA isolated from cells incubated for 20 h with paclitaxel (10–100 nm) and from cells 24–48 h after removal of paclitaxel. The results of a typical experiment are shown in Fig. 9. No DNA fragmentation was detected in control cells during the course of the experiment. Twenty h after addition of 10 nm paclitaxel, DNA fragmentation was barely detectable; however, 24–48 h after removal of 10 nm paclitaxel, substantial fragmentation of DNA into a ladder of nucleosome-sized pieces (~256 bp) occurred. Higher concentrations of paclitaxel (33–100 nm) induced clear DNA fragmentation both 20 h after drug addition and 24–48 h after drug removal.

DISCUSSION

Low concentrations of paclitaxel (~10 nm) strongly block mitosis at the metaphase/anaphase transition without enhancing microtubule polymer levels in HeLa cells, apparently by stabilizing microtubule dynamics (3). In the present study, we wanted to determine whether such a mitotic block results in a transitory slowing of the cell cycle, from which cells recover and continue proliferating, or whether the block is sufficient to induce cell death. Thus, we blocked mitosis with a broad range of paclitaxel concentrations (3 nm–1 μM for 20 h) and then washed the cells extensively to remove the extracellular paclitaxel. We found that cells did not proliferate after incubation with ≥10 nm paclitaxel and removal. In addition, significant apoptotic cell death occurred during paclitaxel incubation and 24–72 h after removal of the drug. Cell proliferation resumed, and cell killing did not occur to a significant extent at paclitaxel concentrations (<10 nm) that were too low to block mitosis. Thus mitotic block or significant mitotic slowing appears to be a critical determinant of paclitaxel-induced apoptotic cell death.
Exit from Mitosis in the Presence of Paclitaxel-stabilized Microtubules. HeLa cells exited mitosis and progressed to an interphase-like state following removal of paclitaxel from the medium. However, the exit from mitosis into interphase was abnormal; it occurred in the absence of anaphase, telophase, or cytokinesis (Table 1, Fig. 8). The cells entered into an interphase-like state: chromatin decondensed, nuclear membranes reformed, and microtubule arrays were interphase like, but nuclei were fragmented and numerous (Figs. 4, 5, and 7). It is conceivable that the multiple nuclei arise upon entry into interphase because the nuclear envelope reforms around individual chromosomes or groups of chromosomes that are maintained in a scattered array by paclitaxel-stabilized, dysfunctional spindle microtubules that can neither properly polymerize to induce anaphase spindle lengthening nor depolymerize to facilitate anaphase chromosome segregation. Similar formation of multiple nuclei occurs following mitotic slowing or block with paclitaxel in other tumor cell types, including human colon, breast, and lung carcinoma cells (6, 7), and also with other antimitotic drugs such as Vinca alkaloids (13) and dolastatin analogues (15).

In some human tumor cell lines such as K562 leukemia, HCT116 colon carcinoma, and A2780 ovarian carcinoma cells, exit from paclitaxel-induced mitotic block into interphase is accompanied by continued rounds of DNA synthesis (4, 6, 16). This has been suggested to contribute to the cytotoxicity of paclitaxel as a result of gene dosage effects of tetraploidy or octaploidy (6). However, no further DNA synthesis occurred in HeLa cells following exit from paclitaxel-blocked mitosis, suggesting that continued DNA synthesis cannot be responsible for cell killing in HeLa cells.

Apoptosis Is a Consequence of Mitotic Block and Abnormal Mitotic Exit. Apoptosis is a form of programmed cell death that has been postulated to result from aberrant cell cycle control and to be a consequence of conflicting growth regulatory signals that ultimately lead to an unsuccessful attempt to traverse the cell cycle (17, 18). The biochemical events leading to apoptosis are complex and appear to vary among cell types (19). It is known that drugs that interact with microtubules can induce apoptosis at relatively high drug concentrations. For example, paclitaxel at concentrations of ≥60 nM induces apoptosis in human leukemia cells, HeLa cells, and human prostate cancer cells (5, 8, 20, 21). Vinblastine and colchicine at concentrations that depolymerize microtubules (250 nM and 2 μM, respectively) also induce apoptosis in human leukemia cells (22). However, it was not previously known whether apoptosis occurs at low concentrations.
Table 2. Paclitaxel accumulates intracellularly and is retained after removal from the medium.

<table>
<thead>
<tr>
<th>Paclitaxel added to medium at time 0 (µM)</th>
<th>Paclitaxel in cells at 20 h (µM)</th>
<th>Fold uptake</th>
<th>Paclitaxel 24 h after washing (µM)</th>
<th>Retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003</td>
<td>1.7 ± 0.2</td>
<td>570</td>
<td>0.7 ± 0.04</td>
<td>42</td>
</tr>
<tr>
<td>0.01</td>
<td>8.3 ± 2.1</td>
<td>830</td>
<td>4.0 ± 0.7</td>
<td>48</td>
</tr>
<tr>
<td>0.1</td>
<td>71.5 ± 6.2</td>
<td>720</td>
<td>27.0 ± 6.7</td>
<td>38</td>
</tr>
<tr>
<td>1.0</td>
<td>241.0 ± 27.0</td>
<td>240</td>
<td>38.6 ± 9.9</td>
<td>16</td>
</tr>
</tbody>
</table>

HeLa cells that had been blocked at the metaphase/anaphase transition by paclitaxel (~10 µM) did not proliferate after removal of the drug. Instead, most of the cells underwent degradation characteristic of apoptosis. The nuclear contents of many cells became condensed, opaque, fragmented, and almost liquid in appearance during the first 24 h following paclitaxel removal (Figs. 5 and 6). Agarose gel electrophoresis indicated that the DNA was fragmented into nucleosome-sized fragments characteristic of apoptosis during the initial 20-h drug incubation, and fragmentation continued after paclitaxel removal (Fig. 9). Flow cytometry indicated that significant DNA degradation occurred during the first 24 h after removal of the drug (Fig. 8). Microtubules became tangled and formed disorganized balls or bundles that protruded from the cell periphery. In some cells, microtubules were degraded and disappeared after paclitaxel removal (Fig. 6). Additional recent evidence indicates that the initial nicking of DNA can occur as little as 30 min after mitotic block induced by 200 nM paclitaxel in HeLa cells (21).

The importance of our findings is that apoptosis occurs at very low taxol concentrations that do not increase the mass of microtubules in cells and do not induce formation of microtubule bundles or greatly distort the organization of mitotic spindles. Thus, apoptotic cell death at low paclitaxel concentrations appears to result from a mitotic block induced by suppression of spindle microtubule dynamics. Exit into an interphase-like state from paclitaxel-blocked mitosis occurred in the absence of anaphase or cytokinesis (Table 1, Fig. 8). Thus, paclitaxel-induced mitotic block resulted in a significant disintegration or asynchrony of cell cycle events that triggered apoptotic cell death. The critical cell cycle events that are perturbed by paclitaxel are unknown, but they might involve, for example, the prolonged influence of elevated levels of cyclin B and p34<sup>cdc2</sup> kinase activity (8, 25, 26). Alternatively, biochemical events associated with anaphase, telophase, and cytokinesis may occur while spindle pole separation and cytokinesis are mechanically blocked by paclitaxel-stabilized spindle microtubules (27, 28). Additional evidence suggests that the tumor suppressor protein p53 may be prevented from exerting its activity in HeLa cell nuclei as a result of abnormal retention in the cytoplasm of HeLa cells (29, 30); thus, it is conceivable that paclitaxel-induced perturbation of mitotic exit and nuclear reformation may permit access of p53 to the chromatin and result in cell death. Cell death also might involve the continued abnormal presence or activity of some of the numerous proteins that are normally transported to the midzone region of the spindle to form the "telophase disc" or "intercellular bridge" that has been postulated to function critically in cytokinesis (31–33).
Microtubule Dynamics in Mitosis and the Action of Paclitaxel.

Previous results have strongly suggested that sensitive suppression of microtubule dynamics by paclitaxel is responsible for the mitotic block induced in HeLa cells at low concentrations of the drug (3). Microtubules are intrinsically dynamic polymers (23, 24). Their dynamics speed up 10–100-fold upon entry into mitosis and undergo complex changes during mitosis (34). During prometaphase, microtubules make vast growing and shortening excursions, functioning to “find” and attach chromosomes to the forming spindle (35). During metaphase, the chromosomes attached to the microtubules oscillate back and forth in the spindle equatorial region in concert with growing and shortening of the microtubules (36). In addition, tubulin is continually added to the microtubule ends at the chromosomes and lost from the microtubule ends at the spindle poles in a balanced fashion (37). During anaphase, microtubules attached to chromosomes shorten at the same time that other microtubules in the spindle midzone lengthen. Thus, it is becoming clear that microtubule dynamics are critical for the progress of cells through mitosis, in particular, for the transition from metaphase to anaphase.

We previously found that in addition to paclitaxel, low concentrations of other drugs, such as vinblastine, colchicine, and nocodazole, which potently suppress microtubule dynamics in the test tube and in cells (24, 38–41), also block mitosis in HeLa cells (13, 42). Mitotic block occurs specifically at the transition from metaphase to anaphase without gross distortion of the mitotic spindle and without changes in the mass of assembled microtubules. The transition from metaphase to anaphase is an important cell cycle checkpoint that prevents cells from completing mitosis until the spindle is fully assembled and the chromosomes are properly poised for separation (43). Additional studies have indicated the importance of tension that may be produced by dynamic microtubules in mitosis. Rieder et al. (11) found that low concentrations of paclitaxel added to rodent cells in mitotic metaphase delayed anaphase by as long as 7 h, with no detectable alterations in spindle morphology. Li and Nicklas (44) found that tension on sister chromatids is required for the metaphase/anaphase transition. Together, the data suggest that successful transition from metaphase into anaphase may involve tension between sister chromatids that is dependent, at least in part, upon dynamic microtubules.

Paclitaxel stimulates microtubule polymerization by binding directly to tubulin along the length of the microtubule (reviewed in Ref. 24). There is a paclitaxel binding site on each molecule of tubulin in microtubules, and the powerful ability of paclitaxel to increase microtubule polymerization is associated with nearly stoichiometric binding of paclitaxel to tubulin in microtubules (45). However, recent studies from our laboratory indicate that binding of only a small number of paclitaxel molecules to a microtubule (1 molecule of paclitaxel/600 molecules of tubulin) can stabilize its dynamics (12).

Intracellular Paclitaxel Concentrations Associated with Mitotic Block, Abnormal Mitotic Exit, and Apoptosis. Interestingly, extensive quantities of paclitaxel accumulated in the HeLa cells during drug incubation, and a large proportion of the accumulated paclitaxel was retained intracellularly following the washing procedure. For example, at 10 nM ³H]paclitaxel, the intracellular paclitaxel concentration was ~8.3 μM after 20 h of incubation (an 830-fold accumulation), and 48% of the accumulated drug was retained after washing. Determinations of the intracellular microtubule polymer mass at 10 nM paclitaxel (3) indicate that approximately 8 μM tubulin is present in microtubule polymer in these cells. Since each tubulin molecule in microtubules contains one paclitaxel binding site (45, 46), these data suggest that most or all of the paclitaxel may be bound to microtubules.

At 100 nM and 1 μM paclitaxel, the intracellular paclitaxel concentration after 20 h incubation was ~72 μM and 241 μM, respectively, which was reduced to 27 μM and 39 μM, respectively, 1 day after washout. Measurements of microtubule polymer mass after 20 h paclitaxel incubation indicated that tubulin in microtubule polymer was ~20 and ~30 μM, respectively (3). Thus, during the initial 20-h incubation, the amount of intracellular paclitaxel far exceeded the intracellular microtubule binding capacity. The aqueous solubility of paclitaxel is low (~35 μM; Refs. 47 and 48). Thus, at paclitaxel concentrations ≥100 nM, the drug must be compartmentalized into nonaqueous phases or bound to other organelles. The paclitaxel concentrations remained high after washout, but they were reduced to levels that approximated the intracellular concentration of tubulin in microtubule polymer form. An important implication of these results is that the high degree of paclitaxel retention in cells may contribute significantly to the cytotoxicity and antitumor activity of the drug. In similar experiments performed with vinblastine, we found that, in contrast to paclitaxel, most of the vinblastine effluxed from the cells after washing, and cell death did not occur.³

In summary, paclitaxel at low concentrations inhibits mitotic progression in HeLa cells without increasing the mass of microtubules, apparently by potently suppressing spindle microtubule dynamics. HeLa cells do not recover from mitotic block with low concentrations of paclitaxel (10 nM). Instead, mitotic block is followed by an abnormal exit from mitosis into an interphase-like state with no accompanying cytokinesis. The mitotic block appears to be sufficient to inhibit further cell proliferation and to induce death by apoptosis. Cell killing does not occur at paclitaxel concentrations that are too low to induce mitotic block. Thus, mitotic block appears to be a critical determinant of paclitaxel-induced cell death in HeLa cells. The important underlying mechanism responsible for the potent ability of paclitaxel to inhibit cell proliferation and kill tumor cells may be the kinetic stabilization of spindle microtubule dynamics rather than excessive polymerization of spindle microtubules. The results suggest that therapeutic administration of paclitaxel at low concentrations for prolonged durations may effectively inhibit tumor growth.

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Mitotic Block Induced in HeLa Cells by Low Concentrations of Paclitaxel (Taxol) Results in Abnormal Mitotic Exit and Apoptotic Cell Death


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