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Dual Cytotoxic Mechanisms of Submicromolar Taxol on Human Leukemia HL-60 Cells

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ABSTRACT. Taxol-induced mitotic block and apoptosis were investigated using taxol-sensitive human leukemia HL-60 cells at submicromolar concentrations of the drug. Cells exposed to either 20 nM taxol for 1 hr or 10 nM taxol for 12 hr were able to resume normal growth, whereas cells exposed to 60 nM taxol for 1 hr or 10 nM taxol for 24 hr failed to proliferate after drug removal. Progressive changes in the percentage of mitotic block and apoptosis induced by these four treatment protocols were monitored continuously for 3–5 days after drug removal. Cells treated with 20 nM taxol for 1 hr showed a mitotic block without a subsequent increase in apoptosis, whereas cells treated with 10 nM taxol for 12 hr showed an increase in apoptotic ratio within several hours without an increase in mitotic block. These results indicate that apoptosis does not necessarily result from mitotic block and that these two phenomena can occur independently of each other. Drug sensitivity at progressive stages of the cell cycle was also investigated. The results showed that, in addition to the cells in G_2/M phase, the cells in S phase were also sensitive to the drug, especially to a prolonged treatment. These results suggest that, in HL-60 cells, the apoptotic programs can be initiated in either the G_2/M or S phase and represent two different cytotoxic mechanisms of taxol. BIOCHEM PHARMACOL 53;11:1587–1596, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. taxol; HL-60 cells; apoptosis; mitotic block; cytotoxicity

Taxol is a diterpenoid plant product [1] that exhibits significant antitumor activity against various malignant cells such as ovarian [2, 3], breast and lung cancer cells [4], malignant melanoma [5], as well as leukemias [1, 6]. Interest in this compound stems not only from its clinical activity against poorly responsive solid tumors, but also from its unique mechanism of action [7, 8]. Unlike other antimitotic compounds such as vinblastine and colchicine that inhibit tubulin polymerization and microtubule formation, taxol enhances tubulin polymerization, stabilizes microtubules, and prevents microtubule depolymerization induced by calcium or low temperature [9, 10]. The unusual stability of microtubules in taxol-treated cells leads to mitotic block, resulting in inhibition of cell division [11, 12]. Two lines of evidence support this model of action. First, most taxol-treated cells are arrested in G_2/M phase [13, 14]. Second, cells in mitotic phase are found to be more sensitive to taxol than those in interphase [15, 16]. In addition, taxol induces nuclear fragmentation, a hallmark of apoptosis, in many cell lines [17–19]. Apoptosis occurs not only upon treatment with high concentrations of taxol, but also upon prolonged treatment with low concentrations of this drug [12, 20]. However, the detailed mechanism of its cytotoxicity remains elusive.

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It is generally believed that cytotoxicity of taxol is related to its microtubule binding ability. The relationship between the cytotoxicity of taxol and its effect on microtubule function has been studied extensively in a number of mammalian cell lines. Comparison of taxol-sensitive and resistant leukemia cell lines showed that both the formation of microtubule bundling and the generation of polyploidy correlate with taxol sensitivity [21–23]. It has been reported that human breast and lung carcinoma cells blocked in G_2/M phase by taxol may escape from mitosis, bypass cytokinesis, and re-enter the next round of DNA synthesis to form polynucleated polyploid cells, which eventually die. This pathway is the proposed mechanism of taxol cytotoxicity [24]. In contrast, it is also reported that, at the lowest effective concentration (10 nM for 24 hr), taxol blocks mitosis of HeLa cells by stabilizing spindle microtubules instead of changing the total mass of microtubules [12, 19]. Cells encountering mitotic block by this condition, however, do not resume proliferation or DNA synthesis but enter an interphase-like state, resulting in abnormal polynuclear, but not polyploid, cells [12, 19]. Although it is generally accepted that both mitotic arrest and apoptosis are major events leading to cell death, certain discrepancies regarding the mechanism of taxol-induced cytotoxicity exist in different studies. This may be due to the employment of different cells or different protocols of drug treatment.

Cells of leukemic origin, especially HL-60 cells, respond differently to taxol in several aspects. For example, micro-

tubule bundling (found in interphase cells) rather than mitotic aster formation (found in mitotic cells) appears to underlie taxol-induced cytotoxicity in these cells [21]. The HL-60 cells seem to be an exception to the finding that taxol is much more toxic to mitotic cells than to interphase cells [16]. Other investigators reported that the sensitivity of leukemic cells to taxol is a function of their susceptibility to apoptosis [25]. In this study, we determined the kinetics of both mitotic arrest and apoptosis induced by taxol, using HL-60 cells, and address a possible relationship between these two phenomena. The objective was to explore whether the mitotic block is an essential process leading to apoptosis in this cell line. Our results demonstrate that mitotic block and apoptosis can occur independently, indicating that two different mechanisms underlie cytotoxicity of taxol in human leukemia HL-60 cells.

MATERIALS AND METHODS

Cell Line and Reagents

The human promyelocytic leukemia HL-60 cell line established by Gallagher *et al.* [26] was used in this study. Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin, 10 mM HEPES, and 2 mM L-glutamine (complete medium), and maintained at 37° in a humidified incubator. Exponentially growing cells were used for all experiments. Taxol was dissolved in DMSO to make a 10-mM stock, which was then diluted as desired with complete medium. The final concentration of DMSO in medium was below 0.05% and did not exert any detectable effect on cell growth.

Cell Survival and Growth Curve

HL-60 cells, at a concentration of 5×10^5/mL, were treated with various concentrations (0–500 nM) of taxol. After 1 hr of treatment, the cells were washed, resuspended at 1×10^5/mL in a taxol-free complete medium, and incubated further for 5 days. The number of viable cells was determined every day by the trypan blue exclusion test.

In some experiments, cells were exposed to a very low concentration (10 nM) of taxol for 12 or 24 hr; they were then collected at various time intervals, and the number of viable cells was determined. At the end of each treatment, the cells were washed and incubated for 2–3 days in taxol-free medium at the same cell density. The number of viable cells was determined at each time interval as indicated.

Morphological Analysis of Cell Cycle Effects

After various treatments, the cells were harvested, cytocentrifuged, and stained with Wright stain for morphological examination. The cells were classified as mitotic, apoptotic, polynucleated, and interphase cells according to their nuclear morphology. Apoptotic cells were recognized by their characteristic features such as cell-volume shrinkage, chromatin condensation, and nuclear disintegration [27]. Cells containing more than 2 nuclei were designated as polynucleated cells. The percentage of mitotic, apoptotic, and polynucleated cells was determined by observing at least 200 stained cells at a magnification of 1000×. Differences between data sets were further analyzed by Student's *t*-test.

TdT End-Labeling Assay

In addition to morphological examination, induction of apoptosis was further confirmed by the TdT* end-labeling assay [28]. After treatments, 2×10^6 cells were fixed in 1% paraformaldehyde and 70% ethanol, washed with PBS, and resuspended in 50 μL of TdT reaction solution (0.2 M sodium cacodylate, 25 mM Tris-HCl, 5 mM $CoCl_2$, 0.25 mg/mL bovine serum albumin, 10 U terminal transferase and 0.5 nmol biotin-16-dUTP) (all from Boehringer Mannheim Biochemicals, Indianapolis, IN). The TdT end-labeling reaction was carried out at 37° for 30 min. The cells were then rinsed with cold PBS, resuspended in 100 μL of FITC staining buffer (2.5 μg/mL fluoresceinated avidin, 4× saline sodium citrate buffer, 0.1% Triton X-100, and 5% nonfat dry milk), and incubated in the dark for 30 min. Subsequently, the cells were rinsed in PBS containing 0.1% Triton X-100 and treated with 1 mL of PBS containing PI (5 μg/mL) and RNase A (100 μg/mL) for 30 min in the dark. The increase in fluorescence was determined by the FACScan system described below.

Cell Elutriation

Counterflow centrifugal elutriation was performed in a Beckman model J-6M centrifuge equipped with a JE-6B elutriation rotor as reported [29, 30]. Exponentially growing cells ($1–2 \times 10^8$ cells) were concentrated in 10 mL of elutriation buffer (RPMI 1640 plus 5% calf serum and antibioties) and loaded onto the elutriation system at a flow rate of 30 mL/min. After the cells settled into the chamber, fractions were collected at speeds from 3000 to 2000 rpm decreasing in steps of 100 rpm. Following elutriation, the cells were counted and recultured at a density of 5×10^5/mL for various experiments. The samples were stained by PI and subsequently analyzed by flow cytometry to determine the cell cycle phase of each fraction.

Flow Cytometry

Flow cytometry was performed on a fluorescence-activated cell sorter, FACScan (Becton-Dickinson, San Jose, CA), using CellQuest and Modfit LT software. Aliquots of 1 ×

* *Abbreviations:* FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; PI, propidium iodide; and TdT, terminal deoxynucleotidyl transferase.

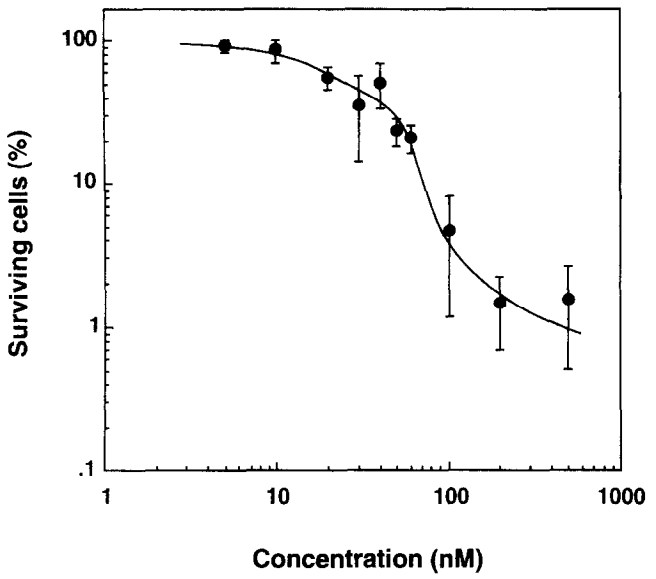

FIG. 1. Cytotoxic effect of taxol on HL-60 cells. The cells were treated with 0–500 nM taxol for 1 hr, washed and incubated in taxol-free medium, and the percent survival was determined after 72 hr. The percent survival was calculated by dividing the number of treated surviving cells by the untreated control. Data are presented as means ± SD of 8–10 experiments.

10^6 cells were fixed in 70% ethanol on ice for at least 2 hr and centrifuged. The pellets were treated with RNase (200 μg/mL) at room temperature for 30 min and then incubated with PI (10 μg/mL) for at least 10 min. The stained cells were analyzed with FACScan, and a "pulse processing" protocol was applied to filter out fluorescence from aggregated cells.

RESULTS

Effect of Taxol on HL-60 Cell Growth

Upon treating HL-60 cells with various concentrations of taxol, we found that the drug displayed a strong growth inhibiting ability in a concentration-dependent manner. The inhibition of growth occurred at very low concentrations, i.e. 10–20 nM. The survival curve (Fig. 1) indicates that the cytotoxicity of taxol varied at two concentration ranges, i.e. between 10–30 nM and >40 nM. Concentrations of ≥200 nM were found to be extremely potent, destroying most of the cells (viable cells <1% of untreated control).

To understand better the underlying mechanisms of taxol cytotoxicity at the two concentration ranges, we selected 20 and 60 nM taxol for further experiments. After a 1-hr exposure, the cells treated with 60 nM taxol stopped proliferating after removal of the drug and proceeded to die during the following 5 days. Similar exposure with 20 nM taxol caused a temporary retardation in the first 2–3 days after removal of the drug, and the number of cells increased 10- to 13-fold in 4–5 days, whereas in the same growing period the number of untreated cells increased 10- to 15-fold. This result suggests that the initial growth inhibition caused by 20 nM taxol was to a large extent reversible (Fig. 2A).

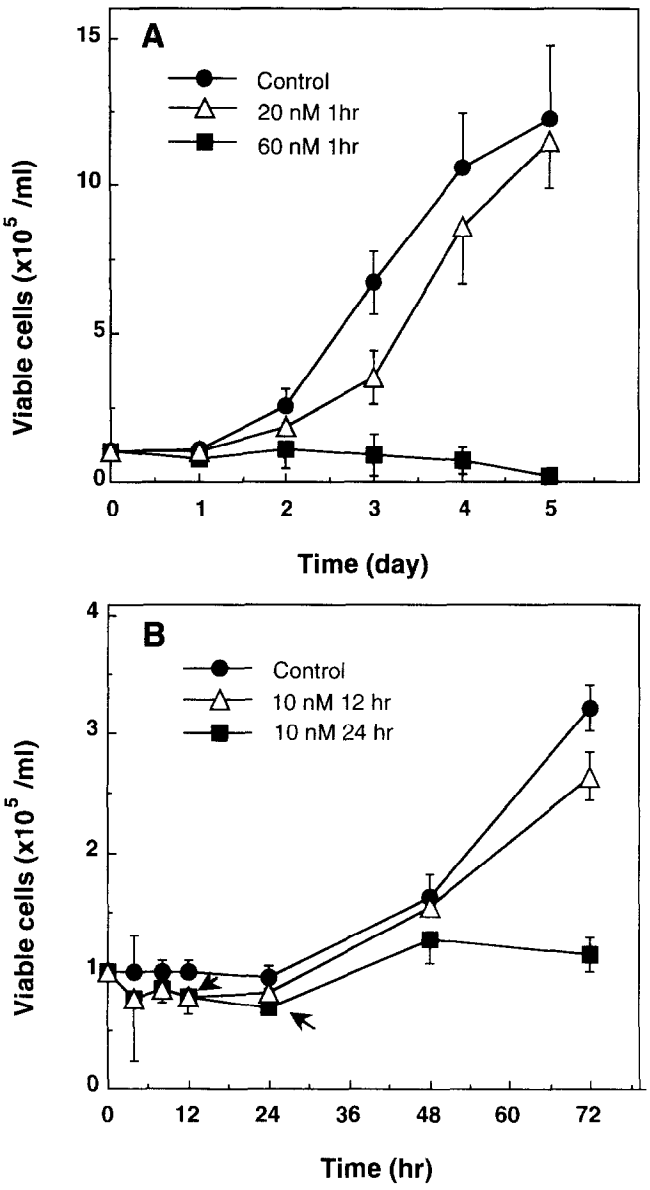

FIG. 2. Effects of four different treatments of taxol on growing HL-60 cells. The cells were exposed to 0, 20, or 60 nM taxol for 1 hr (A), and to 10 nM taxol for 12 or 24 hr (B). At the end of treatment, cells were washed and recultured in drug-free medium without changing the cell density. The number of viable cells was determined at the indicated times. The arrows shown in panel B indicate the time of taxol removal. Data are presented as means ± SD of at least 6 replicates from 2–3 experiments.

We also investigated the effect of prolonged treatment with taxol on HL-60 cells by treating the cells with 10 nM taxol for 12 or 24 hr. As shown in a representative growth curve in Fig. 2B, the prolonged treatment with taxol had a similar reversible-irreversible effect, as that of a 1-hr exposure, on cell growth. The viability of cells treated with 10 nM taxol for 12 hr decreased slightly in the presence of the drug; however, normal growth was resumed after drug removal (2- to 3-fold in 72 hr), suggesting a reversible

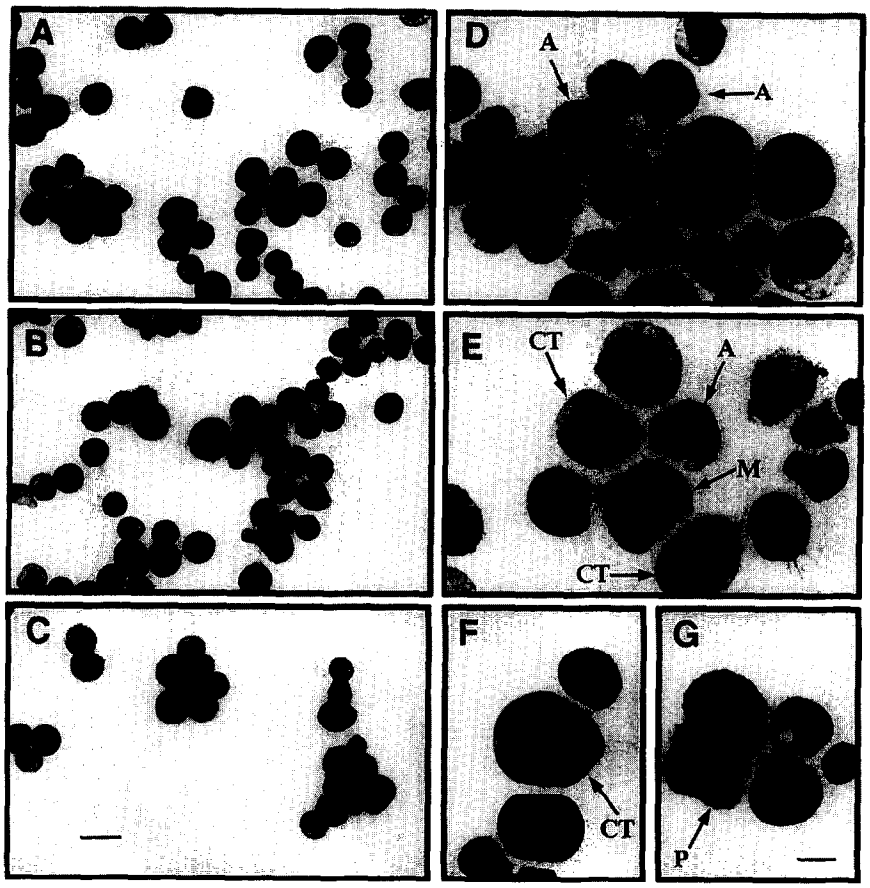

FIG. 3. Taxol-induced morphological changes of HL-60 cells (Wright stain). The cells were exposed to 0 (A), 20 (B) and 60 nM (C–G) taxol for 1 hr and incubated in taxol-free medium for 1–2 days. The cells were then harvested, cytocentrifuged, and stained for morphological examination. The arrows indicate different types of cells according to their nuclear morphology. A, M, P, and CT denote apoptotic, mitotic, polynucleated, and mitotic cells with chromosome tangling, respectively. The bars represent 10 μm in A–C and 4 μm in D–G.

inhibitory effect of taxol under this condition. Cell viability, on the other hand, reduced dramatically upon exposure of the cells to 10 nM taxol for 24 hr. The different growth patterns in response to four different treatment protocols (i.e. 20 or 60 nM for 1 hr and 10 nM for 12 or 24 hr) may represent different cytotoxic mechanisms of taxol and were employed in the following studies.

Morphology of Taxol-Treated HL-60 Cells

To study the effect of taxol on the morphology of HL-60 cells, we treated the cells with various concentrations (0, 20, and 60 nM) of taxol for 1 hr and then reincubated the cells in drug-free medium for 1 day. Microscopic examination of the treated and untreated cells showed that taxol induced alterations in the cell morphology in a concentration-dependent manner. Whereas most of the untreated cells were normal and appeared to be in interphase (Fig. 3A), the treated cells exhibited grossly altered morphology (Fig. 3, B and C). Further analysis by nuclear staining revealed that the abnormal cells in the treated samples were undergoing apoptosis, mitotic arrest, and were polynucleated (Fig. 3, D–G).

An unusual phenomenon was found when the cells were treated with a high concentration of taxol (>40 nM). A fraction of mitotic cells exhibited unusual tangling of chromosomes, which were tightly twisted into a cross or an incomplete "8" shape as shown in panels E and F of Fig. 3. These cells were still classified as mitotic cells in the following studies. Such tangling of chromosomes was rarely found in normal mitotic cells treated with a lower concentration of taxol.

Induction of Apoptosis Detected by the TdT End-Labeling Assay

In addition to morphological examination, induction of apoptosis in HL-60 cells by the aforementioned four treatments was further confirmed by the TdT end-labeling method. In this assay, biotinylated dUTP is incorporated (by a TdT reaction) into the DNA breaks that are characteristic of apoptosis. The labeled cells were visualized by FITC-conjugated avidin and quantitated by flow cytometry. As shown in Fig. 4, DNA breaks were absent in untreated cells (Fig. 4, A and B) and in cells treated with 20 nM taxol for 1 hr (Fig. 4C); however, significant amounts of DNA breaks were evident in the cells subjected to the other three treatments (Fig. 4, D–F).

Kinetics of Mitotic Arrest and Apoptosis Induced by Brief or Prolonged Taxol Treatment

To study the relationship between mitotic block and apoptosis in the taxol-induced cytotoxic pathway, the changes in HL-60 cells treated by the four protocols were monitored for up to 72 hr. As shown in Fig. 5 (A and B),

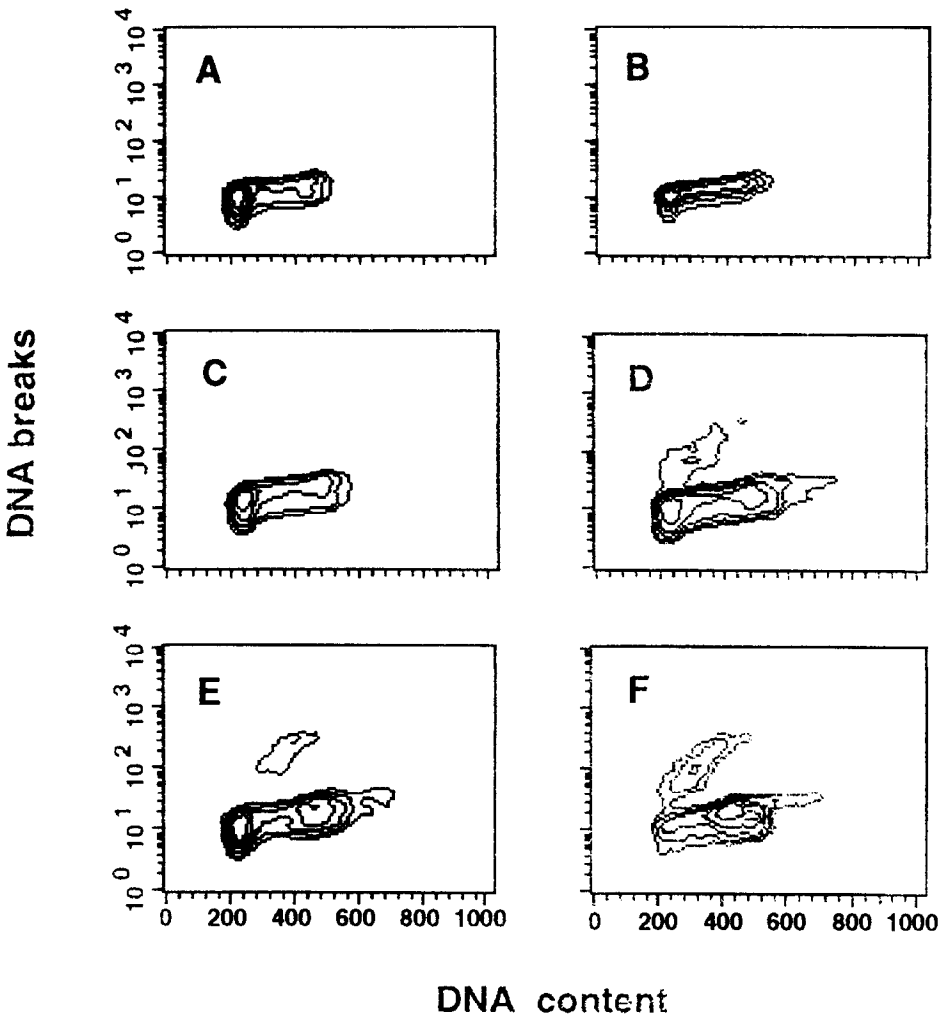

FIG. 4. Taxol-induced apoptosis in HL-60 cells detected by the TdT end-labeling assay and flow cytometry. Cells were subjected to four different treatments of taxol and labeled with biotinylated-dUTP 24 hr after treatment. The labeled cells were then detected by FITC-conjugated aviden and flow cytometry as described in Materials and Methods. (A), before treatment; (B), 0 nM for 24 hr; (C), 20 nM for 1 hr; (D), 60 nM for 1 hr; (E), 10 nM for 12 hr; and (F), 10 nM for 24 hr.

treatment with 60 nM taxol for 1 hr caused the percentage of mitosis and apoptosis in HL-60 cells to increase up to 6- and 10-fold, respectively, in 36 hr after drug removal. Similarly, the percentage of mitosis and apoptosis increased 3- and 10-fold, respectively, in cells treated with 10 nM for 24 hr during the treatment; no further increase was found after drug removal (Fig. 5, D and E). It was also found that under these treatment protocols, mitotic arrest and apoptosis were triggered almost at the same time, but formation of polynucleated cells occurred much later (Fig. 5, C and F). On the other hand, treatment with 20 nM taxol for 1 hr induced the percentage of mitotic cells to increase by about 2-fold in 24 hr and subside thereafter (Fig. 5A); concomitantly, the percentage of apoptotic cells changed in a similar pattern (Fig. 5B). This increase in mitotic ratio was significant ($P < 0.05$) compared with the untreated control using a one-tailed Student's t-test, indicating that, under this condition, a small fraction of cells is temporarily blocked in the mitotic stage but resumes a normal cell cycle later on. These data also indicate that mitotic block does not always lead to apoptosis, since no subsequent increase in apoptosis occurred after 24 hr of drug removal while the percentage of mitosis subsided.

Furthermore, when the cells were exposed to 10 nM taxol for 12 hr, no increase in the percentage of mitosis was detected in 72 hr after drug removal (Fig. 5D). Only the percentage of apoptosis increased significantly from <1% up to 7% in the presence of taxol and returned to normal 36 hr after its removal. These data indicate that treatment of 10 nM taxol for 12 hr can trigger the cell death program in the absence of mitotic block. This is direct evidence that apoptosis can occur independent of mitotic block.

Sensitivity of Cells to Taxol at the Progressive Stage of the Cell Cycle

The above results suggest that two of the four different protocols applied invoke different cytotoxic pathways in HL-60 cells. Treatment with 20 nM taxol for 1 hr induced mitotic block without a subsequent increase in apoptosis, whereas treatment with 10 nM for 12 hr induced significant apoptosis without inducing mitotic block. To determine whether this difference resulted from variations in drug sensitivity at different phases of the cell cycle, HL-60 cells were separated into progressive stages of the cell cycle by counterflow centrifugal elutriation and analyzed for taxol

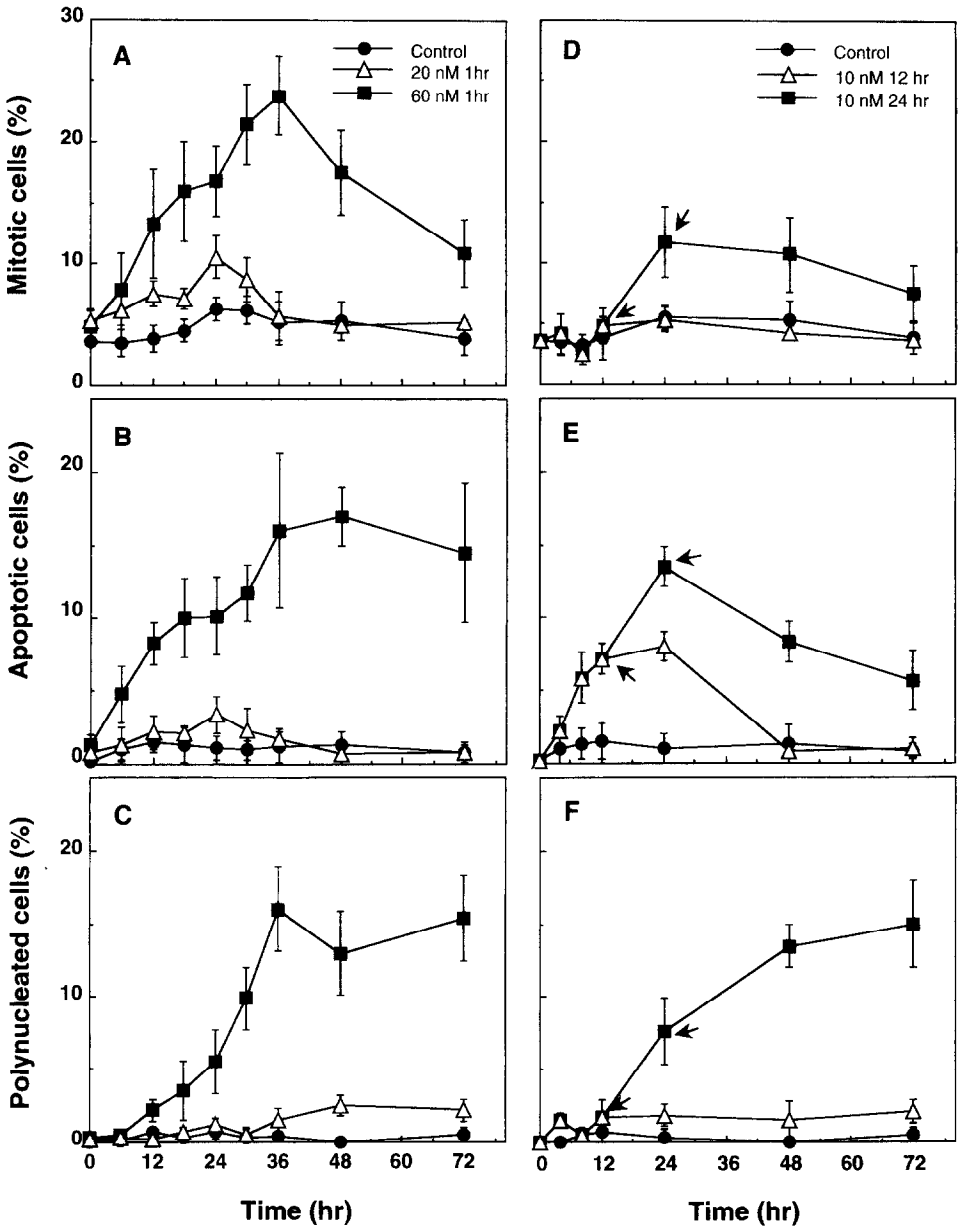

FIG. 5. Kinetics of mitotic arrest, apoptosis, and polynucleation of HL-60 cells after four different treatments of taxol. The cells were exposed to 20 or 60 nM taxol for 1 hr (A–C), and to 10 nM taxol for 12 or 24 hr (D–F). The cells were then allowed to grow in taxol-free medium, and the progressive changes in percentage of mitosis (A, D), apoptosis (B, E), and polynucleation (C, F) were determined at the indicated times. The arrows in D–F indicate the time of taxol removal. Data are presented as means ± SD from a representative experiment with triplicate determinations.

sensitivity. Sorting of populations at incremental stages of the cell cycle was verified by FACS analysis of DNA content in parallel with drug treatment. Representative flow cytometry profiles of DNA content in cells from unfractionated, G_1, S, and G_2/M phases (fractions 0, 4, 6, and 9, respectively) are shown in Fig. 6A. Equal numbers of cells from each fraction were exposed to either 20 nM taxol for 1 hr or 10 nM for 12 hr. At 72 hr post-exposure, the number of viable cells was determined and percent survival was calculated. Figure 6B demonstrates that, under both treatments, taxol sensitivity gradually increased as cells progressed from G_1 to G_2/M phases. However, treatment with 20 nM taxol for 1 hr only slightly inhibited the growth of cells in G_1 and S phases. Only cells in the late G_2/M phase (fractions 8 and 9) were sensitive to this treatment. In contrast, cells in both S and G_2/M phases were sensitive to treatment with 10 nM taxol for 12 hr. Maximum difference in drug sensitivity of cells treated with these two protocols occurred from the S phase to early G_2/M phase. This result indicates that, in addition to M phase, the cells in S phase were also sensitive to taxol, especially to a prolonged exposure of the drug.

This raises the question of whether increased sensitivity to taxol in S phase results from a transition of cells from S to M phase during the prolonged taxol treatment, and whether the triggering of apoptosis is still occurring at M phase. To address this question, each fraction of cells was exposed to increasing levels of taxol for 1 hr, and the IC_{50} values were determined from the concentration-response curve of each population. Three different phase-specific sensitivities to 1-hr treatment of taxol were found (Fig. 7). Cells in both G_2/M phase (fraction 8–9) and S phase (fraction 5–6) were highly sensitive to taxol with IC_{50} values of 15–20 and 20–30 nM, respectively, while cells in

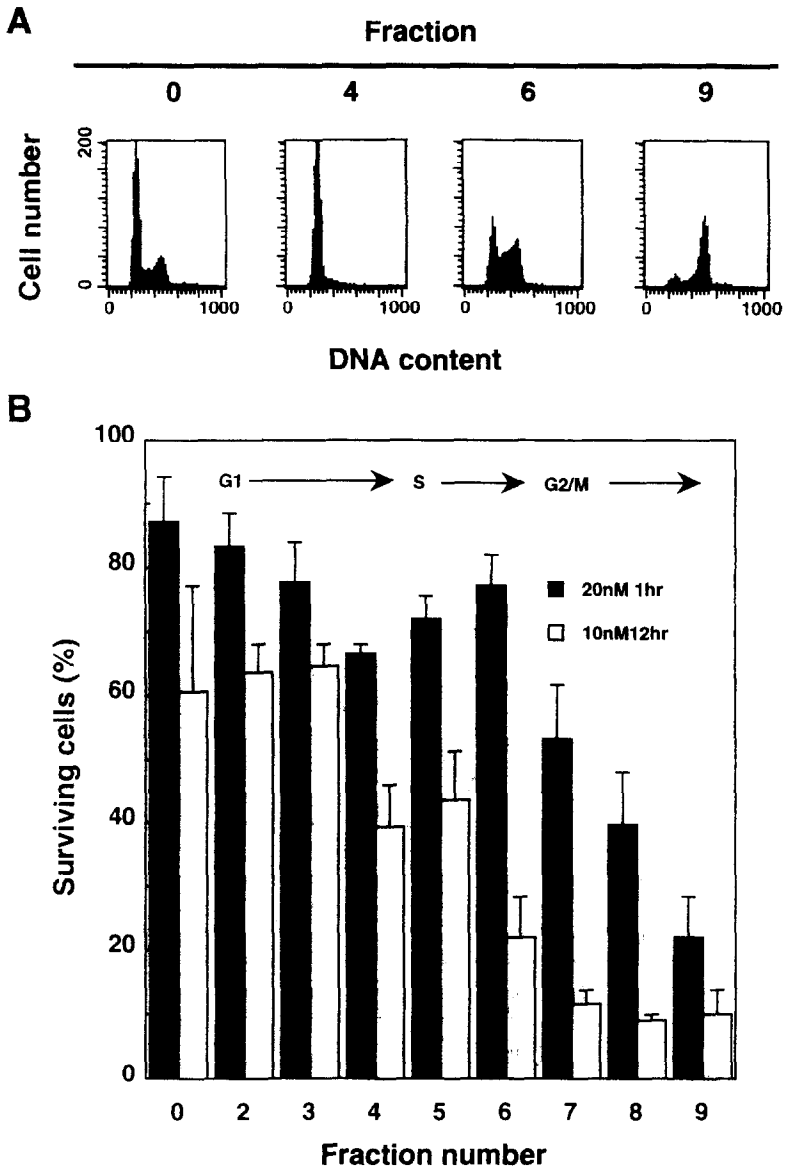

FIG. 6. Comparison of the cytotoxic effects of two different treatments of taxol at progressive stages of the cell cycle. Proliferating HL-60 cells were separated by centrifugal elutriation into 9 fractions corresponding to progressive stages of the cell cycle. An aliquot from each fraction was fixed and stained with PI, and the relative DNA content of each fraction was determined by flow cytometry. Flow cytometry profiles for cell fractions 0, 4, 6, and 9 are shown (A). Fractionated cells were exposed to 20 nM taxol for 1 hr or to 10 nM taxol for 12 hr, and the number of viable cells was determined 72 hr after treatment. The percentage of surviving cells was determined by dividing the number of surviving cells by the corresponding untreated control of each fraction. Fraction 0 represents unfractionated cells. Data are presented as means ± SD of 4–6 replicates from 2–3 experiments.

G_1 phase were most resistant to taxol treatment with an IC_{50} of 40–70 nM. It should be noted that, although the IC_{50} curve showed a steady decrease at progressive stages of the cell cycle, the cells in S/G_2 boundary seemed more resistant than their S phase counterparts. Taken together, these data indicate that a brief exposure to a higher concentration of taxol as well as prolonged exposure to a lower concentration of taxol is enough to induce apoptosis in S phase.

DISCUSSION

Although both mitotic arrest and apoptosis induced by taxol have been observed in many different mammalian cells, their relation to the cytotoxic effect of taxol still remains controversial. It has been suggested that cytotoxicity of taxol results from the abnormal exit of mitotically blocked cells into interphase, accompanied by continued rounds of DNA synthesis and consequently cell death due to gene dosage effects of polyploidy [11, 16, 24, 31], and that taxol-induced prolonged cell cycle arrest in G_2/M may also increase the susceptibility of cells to DNA fragmentation associated with apoptosis [32]. However, some investigators found that taxol-induced apoptosis may not result from mitotic block. Kung *et al.* [33] proposed that the cytotoxic effects of cell cycle phase-specific agents do not result directly from their overt biological effects but rather from a dissociation of temporary cell cycle events. Donaldson *et al.* [34] suggested that mitotic block alone is insufficient for taxol-induced apoptosis to occur and that the drug initiates apoptosis via a $p34^{cdc2}$ kinase pathway. Recent studies showed that overexpression of *bcl-2* gene product $p26^{bcl2}$ significantly inhibits taxol-induced apoptosis but not mitotic arrest [35]. Taxol-induced apoptosis can be directly modulated by agents that affect the activities of protein kinases, and these effects are not necessarily mediated by modulations of taxol-induced mitotic arrest [36].

In the present study, four lines of evidence suggest that taxol-induced mitotic block and apoptosis may occur inde-

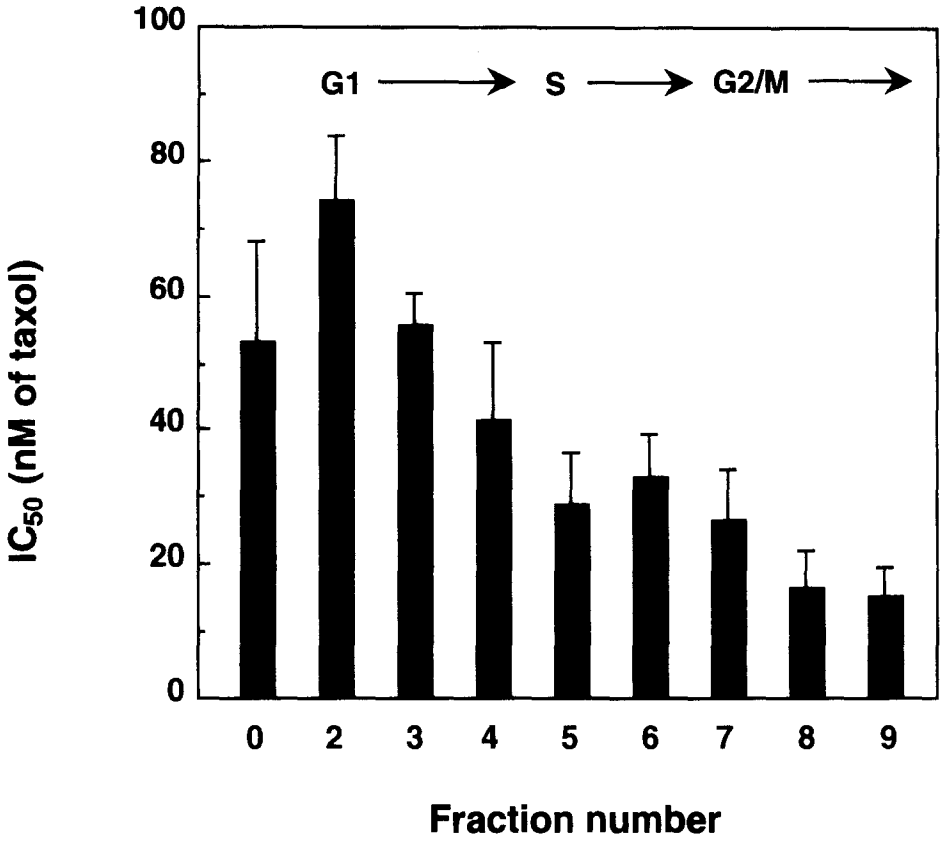

FIG. 7. IC_{50} of taxol on HL-60 cells at progressive stages of the cell cycle. The cells were separated by centrifugal elutriation into 9 fractions corresponding to progressive stages of the cell cycle. Cells from each fraction were exposed to various concentrations of taxol, and the IC_{50} values were determined. Fraction 0 represents unfractionated cells. Data are presented as means ± SD of 4–6 replicates from 2–3 experiments.

pendently in HL-60 cells. First, mitotic block without a subsequent increase in apoptosis was observed upon treatment with 20 nM taxol for 1 hr. On the other hand, apoptosis was found to occur in the absence of mitotic block upon treatment with 10 nM taxol for 12 hr. These two observations point to the fact that mitotic block and apoptosis can occur independently. Second, both mitotic block and apoptosis occurred simultaneously when cells were treated with 60 nM taxol for 1 hr and 10 nM for 24 hr. These results also imply that the two events may proceed via different pathways since no sequential relationship was found. Third, the cells in S phase were found to be more sensitive to prolonged treatment (10 nM for 12 hr) than brief treatment (20 nM for 1 hr) with taxol. The cell death triggered in S phase may be due to the increase in apoptosis by prolonged treatment (10 nM for 12 hr). Finally, we found that the cells in S phase were also sensitive to brief treatment with taxol (IC_{50} = 20–30 nM). This rules out the possibility that increasing taxol-sensitivity of the cells in S phase results from transition of cells from S to M phase during the prolonged treatment in which the onset of apoptosis is still triggered in M phase. Thus, taxol-induced apoptosis can be initiated in the S phase as well as in the M phase. Therefore, the cytotoxic effect of taxol on HL-60 cells may be exerted mainly via two different pathways: cell death via mitotic block in G_2/M phase or direct induction of apoptosis in S phase.

Our finding that G_2/M is not the only taxol-sensitive point in the cell cycle and that mitotic block is not a prerequisite for taxol-induced cell death is consistent with those of Donaldson *et al.* [15, 34]. The results can also explain the unusual behavior of HL-60 cells where interphase cells (formation of microtubule bundling) were more sensitive than mitotic cells (formation of multiple asters) to prolonged exposure to taxol [21]. It is reported that mitotic block induced by a low concentration of taxol (10 nM for 20 hr) results in abnormal mitotic exit and apoptotic cell death in HeLa cells [19]. This finding, however, does not contradict our results, since a different cell line and a different taxol treatment period were employed.

It has been reported that living cells are able to accumulate large amounts of taxol, and the drug release may extend over hours [37]. Therefore, brief exposure to a high concentration of taxol may resemble prolonged incubation with relatively small amounts of the drug. For example, Jordan *et al.* [19] reported that incubation of 10 nM taxol for 20 hr resulted in high intracellular drug accumulation (8.3 μM) and little efflux after taxol removal (cells retained 48% of taxol 24 hr after washing). Drug retention in cells depends on concentration and duration of taxol treatment. Of the four different treatment protocols employed in this study, the responses of the cells treated with 60 nM taxol for 1 hr and those treated with 10 nM for 24 hr were similar in that both treatments resulted in irreversible cell death. It is possible that, several hours after taxol was removed from the medium, intracellular taxol concentrations were at similar levels in these two treatments. The other two treatments, i.e. 20 nM for 1 hr and 10 nM for 12 hr, were

relatively mild and thus the residual drug would elicit no significant response, and the treated cells therefore could resume normal growth after drug removal.

The effect of residual drug can also explain why different treatments could turn on different mechanisms of cytotoxicity. The brief taxol treatment may induce the cell death program via two different pathways. One is by targeting the cells in G_2/M phase and arresting them transiently in mitosis. The cells would then either proceed toward apoptosis directly or escape from mitosis without cytokinesis, continue the next round of DNA synthesis, and eventually die of polyploidy several days later. On the other hand, cells in stages other than G_2/M may be subjected to long-term exposure of residual drug even after removal of the drug, a condition similar to prolonged treatment at low concentrations of taxol. The onset of apoptosis could then be triggered within several hours of exposure, mainly in S phase since this phase is highly sensitive to prolonged exposure of taxol. In addition to brief treatment, the residual drug effect may also play a role in the other two prolonged treatments. It is conceivable that longer exposure (24 hr) may result in higher intracellular drug accumulation and little efflux after removal as compared with shorter exposure (12 hr). These effects may be high enough to turn on profound apoptosis in both G_2/M and S phases. The cell death program triggered in G_2/M phase and S phase can thus be referred to as the chronic and acute mechanisms of cytotoxicity, respectively.

Formation of polynucleated cells at later stages remains unclear. It has been reported that these kinds of cells were formed not only in the chronic but also in the acute mechanism of taxol cytotoxicity. Previous reports show that taxol-sensitive HL-60 cells could hardly form polyploid polynucleated cells, but many polynucleated polyploid cells were formed when another resistant cell line, K562, was used [22]. The authors suggested that the polynucleated cells were more resistant to taxol, and this characteristic might attribute to the hypersensitivity of HL-60 cells to taxol [23]. Using HL-60 cells, we found that significant amounts of polynucleated cells were formed but at a later stage. In a highly toxic condition (>100 nM), most of the surviving cells found on the second or the third day of drug removal were mitotic or polynucleated (data not shown). These findings support the notion that polynucleation follows mitotic arrest and contributes to delayed cell death in the chronic mechanism of cytotoxicity. The formation of polynucleated cells in the acute mechanism needs further investigation.

In conclusion, we found that apoptosis need not result from mitotic arrest, though they often occur concomitantly. Apoptosis can be triggered in either G_2/M or S phase, which reflects the chronic and the acute cytotoxicity mechanisms of taxol in the human myeloid leukemia HL-60 cells. Taxol arrests cells in mitosis through a chronic mechanism. The cells then bypass cytokinesis, become polyploid polynucleated cells, and progress to cell death several days after drug removal. In addition, taxol also affects cells in S phase via the acute mechanism, and turns on their death program within several hours. The chronic and the acute mechanisms may operate independently under mild treatment, but simultaneously under stronger treatment.

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