Cancer Chemother Pharmacol (1995) 36: 385–392

ORIGINAL ARTICLE

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Taxol cytotoxicity on human leukemia cell lines is a function of their susceptibility to programmed cell death

Received: 5 November 1993 / Accepted: 12 January 1995

Abstract Taxol is the prototype of a class of antineoplastic drugs that target microtubules. It enhances tubulin-monomer polymerization and stabilizes tubulin polymers, increasing the fraction of cells in the G2 or M phase of the cell cycle. We report that treatment of HL-60 and U937 myeloid cell lines with 1–10 μM taxol induces DNA fragmentation and the appearance of morphological features consistent with the process of apoptosis. Taxol-induced apoptosis is inhibited neither by cycloheximide nor by actinomycin D and therefore appears to be independent of new protein synthesis. Taxol causes arrest in the G2 phase of the cell cycle and affects cell viability but does not induce DNA fragmentation in the K562 erythromyeloid cell line. Protein-synthesis inhibitors, colcemid, ionomycin, and starvation, known to trigger apoptosis, proved ineffective as well. These results suggest that the antineoplastic effect of taxol is mediated in susceptible cell lines by induction of the apoptotic machinery and that K562 partial resistance may depend upon the intrinsic inability of these tumor cells to undergo apoptosis.

Key words Taxol · Apoptosis · Chemoresistance

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Abbreviations *CHX* cycloheximide · *Act D* actinomycin D · *PI* propidium iodide

Introduction

Taxol is a low-molecular-weight molecule extracted from the bark of *Taxus brevifolia* and characterized by antineoplastic activity against advanced ovarian cancer [16], breast cancer [11], and malignant melanoma [12]. Its unique mechanism of cytotoxicity consists of promoting and stabilizing the microtubule system [17, 24–26]. Unlike other antimicrotubule agents such as vinca alkaloids and colchicine, taxol promotes microtubule assembly, binding different sites for guanosine triphosphate (GTP), colchicine, podophyllotoxin, and vinblastine, and stabilizes microtubules even under conditions that normally promote disassembly [23]. Taxol has also been shown to block progression in the G2/M phase of the cell cycle in both sensitive and resistant cells [9, 21, 22]. Moreover, microtubule bundles and aster formation induced by taxol treatment occurs in human leukemia cell lines regardless of sensitivity to the drug [21]. The mechanism of its cytotoxicity in sensitive cells therefore remains elusive.

Since microtubule-disrupting agents and other antineoplastic drugs have been reported to induce a physiological mode of cell death (i.e., apoptosis) in susceptible cells [1, 2] we investigated the ability of taxol to trigger programmed cell death in susceptible and resistant human cell lines. While our experiments were in progress, induction of apoptosis by taxol treatment of human leukemia cell lines was reported by Bhalla et al. [3]. However, the molecular signals involved in the triggering of programmed cell death in neoplastic cells remain to be defined, as do the mechanisms of resistance to the drug's cytotoxic activity demonstrated in vitro in other human leukemia cell lines.

In the present study we showed that the drug-sensitive HL-60 and U937 cell lines underwent DNA

fragmentation following treatment with 1–10 μ*M* taxol for 18–24 h. DNA fragmentation was not prevented by treatment with inhibitors of mRNA and protein synthesis such as CHX and Act D. Only the presence in culture of Zn^{2+} ions enabled the complete inhibition of DNA fragmentation, demonstrating the involvement of the endonucleases. Taxol-resistant K562 cells did not show DNA fragmentation even after longer-term treatment or in the presence of higher drug concentrations. Moreover, neither protein-synthesis inhibitors, colcemid, Ca^{2+} ionophore, nor starvation could induce apoptosis in the K562 cell line. The relationship between drug resistance and apoptosis is discussed in this report.

Materials and methods

Chemicals and reagents

Taxol was kindly provided by Dr. Susan B. Horwitz (Albert Einstein College of Medicine, New York, N.Y.). Cycloheximide, ActD, ethylene glycol tetraacetic acid (EGTA), $ZnSO_4$, and ionomycin were obtained from Sigma (St. Louis, Mo.).

Cells and culture conditions

The human tumor cell lines HL-60, K562, and U937 were obtained from the American Type Culture Collection (Rockville, Md.). Cells were cultured at 5×10^5/ml in RPMI 1640 (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Irvine, Scotland) and kept at 37°C in a humidified atmosphere of 8% CO_2 in air.

[^{3}H]-Thymidine uptake

Cells were seeded at 1×10^5 in 96-well microtiter plates and incubated in the presence or absence of 1 μ*M* taxol. At selected intervals (4, 8, 18, 24, and 72 h), cells were exposed to 1 μ*M* [^{3}H]-thymidine (Amity, Milano) for 8 h and harvested on glass filter paper, and the radioactivity was measured in a Beckman β counter.

Gel electrophoresis and DNA fragmentation

Cells were sedimented by centrifugation at 200 *g* for 6 min, resuspended in lysis buffer [10 m*M* ethylenediaminetetraacetic acid (EDTA)/50 m*M* TRIS-HCl (pH 8) containing 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg proteinase K/ml], and incubated for 1 h at 50°C in a water bath. RNAse A (Sigma) was then added to a concentration of 0.25 mg/ml and incubation at 50°C was continued for 1 h. Lysates were then loaded onto a 2% agarose gel and electrophoresis was carried out at 6 V/cm of gel in TBE buffer (0.045 M tris-borate, 0.001 M EDTA). After electrophoresis, DNA was visualized by soaking the gel in ethidium bromide (1 μg/ml).

Flow cytometry

The DNA fluorescence intensity in the treated and control cultures was measured according to the method described by Nicoletti et al. [18]. An aliquot of the cell culture was centrifuged at 200 *g* and the cell pellet was gently resuspended in 0.4 ml hypotonic fluorochrome solution [50 μg PI/ml in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma)]. The PI fluorescence was measured using a FacStar cytofluorograph (Becton Dickinson, San Jose, Calif.). The fluorescence intensity was analyzed using a log scale.

Giemsa staining

Cell pellets were resuspended in 3:1 (v/v) methanol-acetic acid; after 30 min of incubation, cells were seeded on glass coverslips using a Pasteur pipet, air-dried overnight, and stained with 3% Giemsa solution (Merk, Darmstadt, Germany).

Clonogenic assay

Cells at a density of 5×10^5/ml were treated with 1 μ*M* taxol for 72 h. Following drug incubation, cells were washed twice and seeded in a monolayer soft-agar assay system consisting of 0.3% agar in culture medium enriched with 20% fetal bovine serum. Colonies of > 40 cells were counted with an inverted microscope at 10 days after seeding. Accurate counts were made possible by a grid carved on the wells' bottom. The number of colonies formed after taxol treatment, expressed as a percentage of the number formed by untreated cells, gives the colonogenic survival potential. At least two experiments were done for each leukemia line, and cells were seeded at progressive dilutions ranging from 5×10^2 to 5×10^4 cells/well in a 24-well plate.

Electron microscopy

Cells were centrifuged at 200 *g* and pellets were fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 4 h at 4°C. Samples were subsequently postfixed in 1% OsO_4 in 0.2 collidine buffer (pH 7.4) for 1 h at 4°C, dehydrated, and embedded in epoxy resin. Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate and observed in a Zeiss EM 10 transmission electron microscope.

Results

The human promyelocytic leukemia cell line HL-60 and the human erythromyelocytic leukemia cell line K562 have been shown to be sensitive and resistant, respectively, to taxol in clonogenic assay [22]. However, the intracellular effects of the drug in terms of aster and bundle formation occur in both cell lines, with rapid development of polyploid cells becoming evident in the taxol-resistant K562 cells [21]. To assess the reactivity to taxol of HL-60, K562, and U937 cell lines, we examined the kinetics of [^{3}H]-thymidine uptake during drug treatment and clonogenic assays. We then evaluated the DNA fragmentation, nuclear fluorescence intensity, and cellular morphology of the three human leukemia cell lines HL60, U937, and K562 cultured in the presence of 1–10 μ*M* taxol.

Table 1 [^{3}H]-Thymidine uptake — time course for 3 of 6 experiments. Data are expressed in cpm $\times 10^3$ (*Exp.* Experiment, *ND* not done)

Exp.	Cell line	4 h			8 h			18 h			24 h			48 h		
		Med[a]	Tax[b]	%[c]	Med[a]	Tax[b]	%[c]	Med[a]	Tax[b]	%[c]	Med[a]	Tax[b]	%[c]	Med[a]	Tax[b]	%[c]
1	HL60	82	76	93	94	86	91	159	57	36	76	5	7	96	2	2
	K562	211	180	85	180	114	63	158	78	49	94	80	85	129	58	45
2	HL60	135	121	90	156	147	94	190	33	17	33	7	21	87	0.5	0.6
	K562	54	54	100	131	94	72	171	54	32	150	65	43	207	30	14
3	HL60	113	103	91	132	106	80		ND		121	9	7	208	0.8	0.4
	K562	127	100	78	151	86	57	115	29	25	146	55	38	194	30	15

[a]Culture medium
[b]Taxol at 1 μM
[c][^{3}H]-Thymidine uptake of taxol-treated samples, expressed as a percentage of the control value. Cells grown in culture medium alone served as the control

Table 2 Results of the clonogenic assay. Data represent numbers of clones with more than 40 cells at day 10 of culture (*Exp.* Experiment)

Exp.	Cell line	0.5×10^3[a]			1×10^3[a]			3×10^3[a]			5×10^3[a]			10×10^3[a]		
		Med[b]	Tax[c]	%[d]	Med[b]	Tax[c]	%[d]	Med[b]	Tax[c]	%[d]	Med[b]	Tax[c]	%[d]	Med[b]	Tax[c]	%[d]
1	HL60	5	0	0	9	0	0	24	0	0	47	0	0	73	0	0
	K562	14	2	14	33	3	9	58	6	10	99	8	8	146	15	10
2	U937	31	0	0	51	0	0	113	0	0	225	0	0	406	0	0
	K562	82	6	7	110	14	13	203	32	16	395	40	10	630	78	12

[a]Treated cells seeded per well in a 24-well plate, in 0.3% soft agar
[b]Treatment with culture medium
[c]Treatment with taxol at 1 μM for 72 h prior to seeding in soft agar
[d]Number of clones expressed as a percentage of the control value. Cells grown in culture medium alone served as the control

Thymidine uptake

The percentages of cells that incorporated [^{3}H]-thymidine decreased in the HL-60, K562, and U937 cell lines with increasing duration of taxol exposure. This decrement was more pronounced in the taxol-sensitive HL-60 and U937 cells. As shown in Table 1, [^{3}H]-thymidine uptake by the HL-60 cell line ranged between 7% and 21% after 24 h of taxol treatment, whereas it varied between 85% and 38% in K562 cells. Moreover, 48 h of taxol treatment produced a dramatic decrease in [^{3}H]-thymidine incorporation in HL-60 cells and a reduction to values ranging from 14% to 45% in K562 cells.

Clonogenic assay

The clonogenic survival in soft agar of HL-60, U937, and K562 was determined following 72 h of treatment with 1 μM taxol. No colony growth was consistently observed for taxol-treated HL-60 or U937 cells after 10 days of culture. In contrast, K562 cells gave rise to a number of colonies that represented an average of 10% of the untreated control value (Table 2). It therefore seems that some degree of taxol cytotoxicity, albeit at levels lower than those observed in the sensitive HL-60 cells, coexists in the K562 cells with a partial resistance.

DNA fragmentation

The mechanism of apoptosis seems to involve the activation of endogenous endonucleases that cleave chromatin in the linkers between nucleosomes [27]. As previously reported for the HL-60 and KG-1 cell lines [3], the time course of DNA fragmentation following the exposure of HL-60 and U937 cell lines to taxol demonstrated accumulation of oligonucleosome fragments after 18 h of incubation (Fig. 1A, lane 4), which increased after 48 h (Fig. 1A, lane 6). In contrast, K562 cells did not show any DNA cleavage, even after 48 h of taxol treatment (Fig. 1B, lane 6).

To investigate the requirement for protein synthesis in taxol-induced apoptosis, cells were treated with taxol in the presence or absence of CHX and ActD at concentrations (50 and 5 μg/ml, respectively) that have previously been shown to inhibit protein synthesis [14]. As shown in Fig. 2A, far from preventing taxol-induced

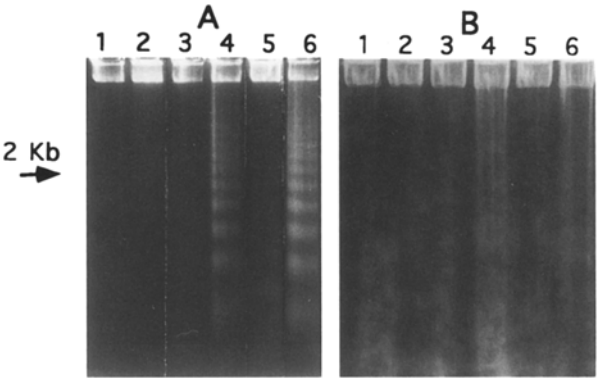

Fig. 1A, B Agarose gel electrophoresis of DNA from taxol-treated and untreated cell lines at different incubation times. **A** DNA from HL-60 cells. **B** DNA from K562 cells (*Lanes 1, 3, 5* controls, *lanes 2, 4, 6* DNA from cells incubated with 1 μ*M* taxol for 8, 18, and 48 h, respectively). The picture in **B** is overexposed to demonstrate the absence of oligonucleosome-sized bands

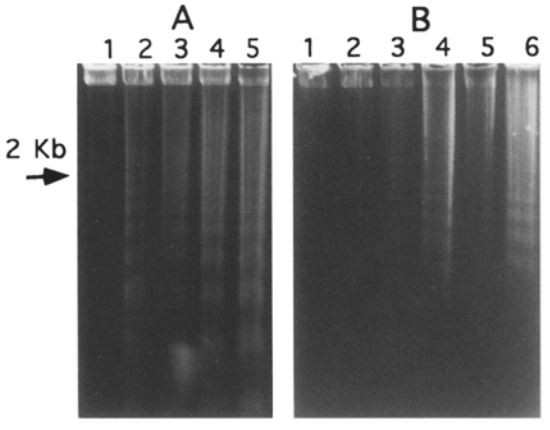

Fig. 2A, B Agarose gel electrophoresis of DNA from HL-60 cells after 24 h of incubation under different experimental conditions. **A** DNA from HL-60 cells cultured in the presence of medium alone (*lane 1*, control), 1 μ*M* taxol (*lane 2*), 50 μg cycloheximide/ml (*lane 3*), 50 μg CHX/ml and 1 μ*M* taxol (*lane 4*), and 5 μg Act D/ml (*lane 5*). **B** DNA from HL-60 cells cultured in the presence of medium alone (*lane 1*, control), 800 μ*M* $ZnSO_4$ (*lane 2*), 8 m*M* EGTA (*lane 3*), 1 μ*M* taxol (*lane 4*), $ZnSO_4$ and taxol (*lane 5*), and EGTA and taxol (*lane 6*)

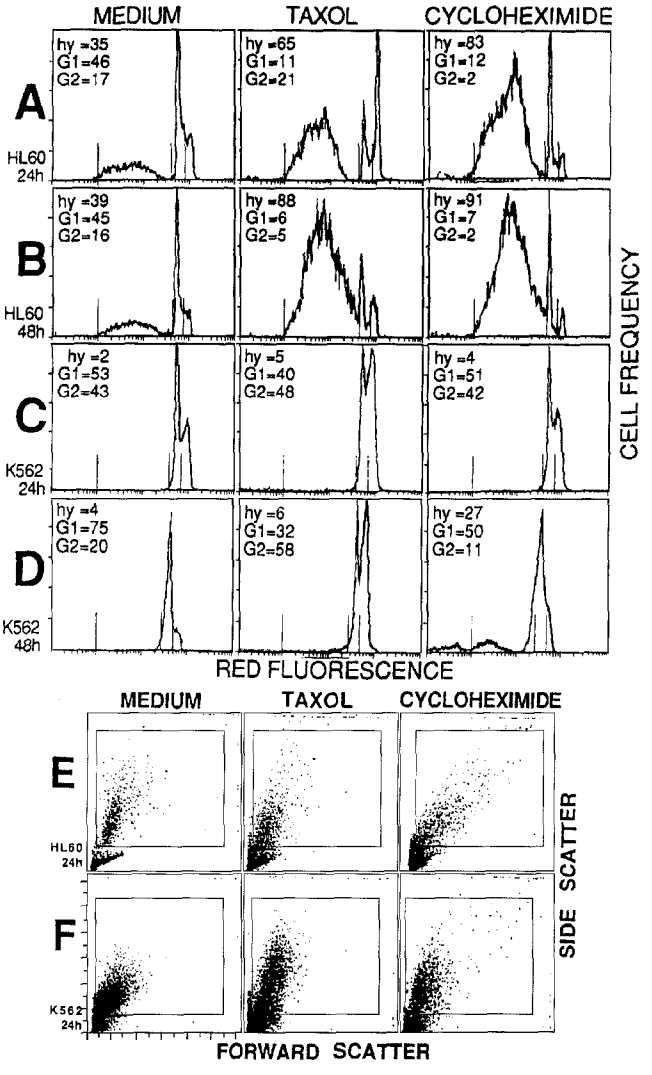

Fig. 3A–F DNA fluorescence flow-cytometric profiles of PI-stained HL-60 and K562 cells. **A, B** Histograms of HL-60 cells cultured for 24 and 48 h, respectively. **C, D** Histograms of K562 cells. *From left to right* are shown the different culture conditions: no treatment (*left*), 1 μ*M* taxol (*middle*), and 50 μg CHX/ml (*right*). (*hy* Hypodiploid — i.e. apoptotic — nuclei, *G1* nuclei in the G1 phase of the cell cycle, *G2* nuclei in the G2 phase of the cell cycle) *Numbers* represent the corresponding percentages. **E, F** Forward-scatter and side-scatter profiles of **E** HL-60 and **F** K562 cells cultured for 24 h in medium (*left*), taxol (*middle*), and CHX (*right*). From each sample, 5000 gated events (*inner squares*) were acquired for the red fluorescence analysis shown in **A** and **B**. Debris was similarly gated out in **C** and **D**

apoptosis, the presence of CHX and ActD determined DNA fragmentation in HL-60 cells (Fig. 2A, lanes 3–5). EGTA was also incapable of inhibiting apoptosis in taxol-treated cells (Fig. 2B, lane 6), suggesting that DNA fragmentation is not dependent on Ca^{2+} flux. The presence of Zn^{2+} ions, known as inhibitors of endonucleases, completely prevented DNA breakage (Fig. 2B, lane 5). We cannot exclude the possibility of high-molecular-weight DNA breakage, which may occur in cells undergoing apoptosis [19]. However, we do not favor this hypothesis, since no morphological evidence of apoptosis was found in the K562 cell cultures (see below).

Flow-cytometric analysis

To evaluate directly the percentage of fragmented nuclei, we performed flow-cytometric analysis of taxol-treated and untreated cells stained with PI in hypotonic buffer [18]. Cell debris was excluded by a gate set in SSC/FSC, and 5000 gated events were then acquired. The gates were set on untreated samples and were left unchanged for the measurement of treated ones (Fig. 3E, F). The control cultures predominantly

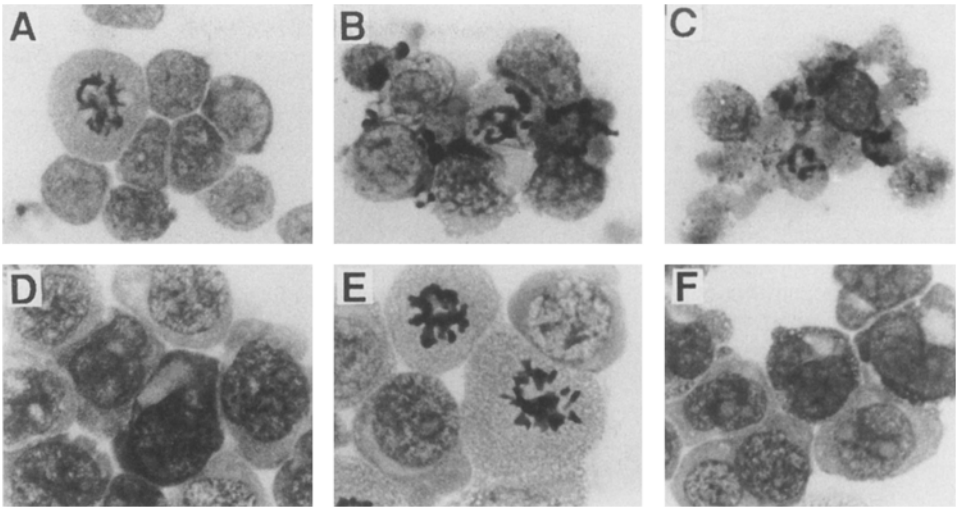

Fig. 4A–F Morphology of treated and untreated HL60 and K562 cells (Giemsa-stained cell smears, × 63). HL60 cells are shown after 24 h of incubation in the presence of **A** medium, **B** 1 μ*M* taxol, and **C** 50 μg CHX/ml. K562 cells are shown after 24 h of incubation in the presence of **D** medium, **E** 1 μ*M* taxol, and **F** 50 μg CHX/ml

contained one peak corresponding to the G1 phase of the cell cycle (Fig. 3A–D, left panels).

After 24 h of treatment with taxol, 65% of the HL-60 cells showed a reduction in fluorescence intensity (Fig. 3A, middle panel) comparable with that associated with apoptotic cell death. Moreover, the majority of the surviving cells were in the G2 phase of the cell cycle as compared with the control cultures. After 48 h of taxol treatment, 88% of the HL-60 cells were hypodiploid (Fig. 3B, middle panel), whereas only 6% of the K562 cells showed a reduction in fluorescence intensity (Fig. 3D, middle panel). Moreover, most of the surviving K562 cells were in the G2 phase. These data suggest that intracellular effects of taxol such as block in the G2 phase occur in both sensitive (HL-60) and partly resistant (K562) cell lines, but only the former line undergoes massive apoptotic cell death. The flow-cytometric data are thus consistent with the DNA electrophoretic pattern of taxol-treated cell lines.

Morphology of taxol-treated cell lines analyzed by Giemsa staining and electron microscopy

It has recently been reported that the morphological changes characteristic of apoptosis can precede or occur in the absence of internucleosomal DNA cleavage [6, 29]. To assess whether this also be the case in K562 cells undergoing taxol treatment, we analyzed both HL-60 and K562 cells by Giemsa staining and by electron microscopy at different time points following incubation with 1 μ*M* taxol.

After 8 h of incubation, most HL-60 cells retained their normal morphological appearance, with about 20% of the cells showing mitotic figures (not shown). After 24 h, 50% of the cells exhibited metaphase-like figures and apoptotic bodies (Fig. 4B). After 8 h of treatment with taxol, K562 cells were characterized by the appearance of mitotic figures. Within 24 h the majority of the cells remained viable, about 50% were blocked in metaphase, and some of them showed decreased cytoplasmic basophilia and swollen nuclei (Fig. 4E). The latter cells increased in number after 48 h of incubation with taxol, and cytolysis fragments appeared with no obviously recognizable apoptotic body (not shown): The kinetics of DNA fragmentation in HL-60 cells correlated with the appearance of the morphological changes of apoptosis and with the observed changes in cell size and fluorescence intensity. HL-60 and U937 cell lines gave overlapping results (not shown). In contrast, K562 cells developed mitotic figures due to the targeting of the microtubules by the drug, but only part of them died with the morphological characteristics of necrosis (Fig. 5).

In contrast, after 48 h of incubation with taxol, most HL-60 cells underwent post-apoptotic cell lysis, and many apoptotic bodies remained recognizable (not shown). To assess the susceptibility of K562 cell lines to apoptosis induced by other agents known to cause DNA fragmentation in the HL-60 cell line [13, 14], we incubated K562 cells with colcemid, CHX, and ActD. The induction of DNA fragmentation following treatment with ionomycin and incubation in medium deprived of glutamine and methionine was also investigated. Under none of these conditions was significant DNA fragmentation evident after 24 h of incubation or even following longer intervals (not shown). We hypothesize that the taxol resistance of K562 cell lines and, possibly, that of other tumor cells may be ascribed to a fault in their apoptotic machinery.

Electron microscopy was used to examine the ultrastructure of taxol-treated HL-60 and K562 cells. Cell pellets were sectioned following treatment with 1 μ*M* taxol for 24 h. Electron micrographs of treated HL-60 cells (Fig. 5A) demonstrated the presence of numerous apoptotic bodies and condensed chromatin. Despite

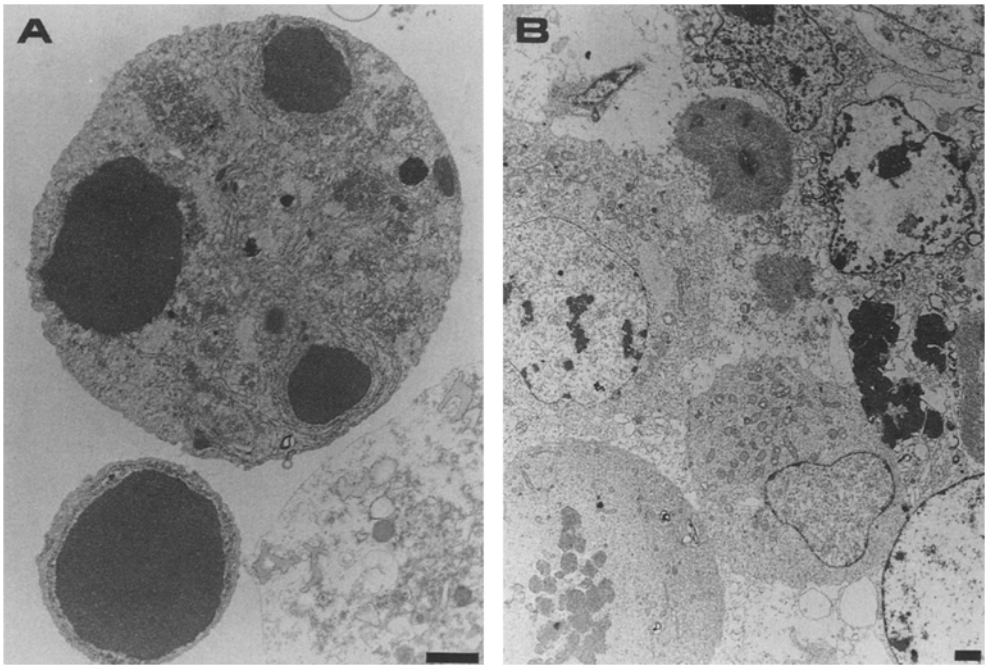

Fig. 5A, B Electron microscopy evaluation of the morphology of taxol-treated HL-60 and K562 cells. **A** Two HL-60 cells exposed to 1 μ*M* taxol for 24 h show the characteristics of apoptosis. Masses of condensed chromatin are evident. The cytoplasm of one cell is rich in rough endoplasmic reticulum cisternae. Magnification, ×9000; bar = 1 μm. **B** K562 cells treated as described above show clear nuclei and homogeneously distributed chromatin. In some of them, nucleoli are also evident. The cytoplasm is rich in organelles (rough endoplasmic reticulum, free ribosomes, and many dilated mitochondria). In the *upper part* of the figure, one cell shows condensed chromosomes, a consequence of the block in the G2 phase. Magnification, ×3500; bar = 1 μm

the complete condensation of chromatin, cytoplasmic organelles and cell membranes appeared intact. In contrast, taxol-treated K562 cells (Fig. 5B) had clear nuclei with homogeneously distributed chromatin and dilated mitochondria with necrotic figures. These results confirm that the partial cytotoxicity of taxol on K562 cells is mediated by necrosis.

Discussion

Recent reports have demonstrated that a variety of anticancer drugs can induce cell death with the characteristics of apoptosis in susceptible cells [10]. Several microtubule-disrupting drugs, such as colchicine and vinblastine, induce apoptotic cell death in HL-60 cells [13]. In the present report we confirm that the cytotoxicity of the antimicrotubule agent taxol is related to its ability to trigger apoptosis. Moreover, we show that taxol resistance is not related to its inability to target microtubules or to block cell-cycle progression in the G2 phase but to the intrinsic inability of the resistant cells to undergo apoptosis. In this study we examined the kinetics of [^{3}H]-thymidine uptake, clonogenic assays, DNA fragmentation, and morphological modifications in resistant and sensitive cell lines following taxol treatment.

The occurrence of the morphological and molecular features of apoptosis in the HL-60 and U937 cell lines becomes significant after 18 h. [^{3}H]-Thymidine uptake is also inhibited after 18 h of incubation, with almost complete loss of incorporation being noted after 48 h. At this time point, Giemsa smears show that over 90% of cells are dead. Zn^{2+} ions are capable of inhibiting DNA fragmentation induced by taxol in HL-60 cells but do not prevent the appearance of apoptotic bodies. As previously shown in other experimental systems [14, 20], CHX and ActD do not inhibit DNA fragmentation, confirming that HL-60 cells represent the prototype of apoptosis-"primed" cells [28], which require no new protein synthesis but only the activation of effectors of the apoptotic machinery.

How are the biochemical effects of taxol coupled with the triggering of cell death? It appears that dynamic reorganization of the cytoskeleton is essential for multiple cellular functions such as motility, cell shape, intracellular transport, and signaling from the cell membrane to the nucleus [22]. Taxol has been shown to affect the cytoskeleton during interphase as well as to block the cell cycle in G2. As an increase in free tubulin has been found to represent a mitogenic signal [7], an increase in tubulin polymerization and /or a decrease in free tubulin might represent a signal coupled with the activation of the cell-death program. Alternatively, a block in the G2 phase of the cell cycle may be a signal toward the suicide pathway. The partial resistance of K562 cells to the toxic effect of taxol may be due to a break of the linkage between cell-cycle arrest in G2 and apoptosis or to a fault in the apoptotic machinery itself.

Inhibition of apoptosis has been related to the expression of several oncogenes [5]. Moreover, recent

evidence suggests that elevated levels of ABL tyrosine kinase activity in K562 cells prevent apoptosis induced by cytotoxic drugs [15]. After 48 h of taxol treatment, only 14%–45% of the K562 cells incorporate [^{3}H]-thymidine. Clonogenic assays confirmed that K562 cells are only partially taxol-resistant since only a maximum of 15% of the cells developed colonies in soft agar after 72 h of treatment. Electron microscopy demonstrated that the cytotoxic action exerted by taxol on a fraction of the K562 cells occurred with the morphological characteristics of necrosis. Apoptotic figures were not present in any of the microscope fields studied, whereas they were massively present in the taxol-treated HL-60 cultures. The partial taxol cytotoxicity may therefore be explained by the ability of K562 cells to undergo necrosis rather than apoptosis.

The K562 cell line has been shown to be resistant to different apoptosis inducers such as colchicine [13], protein-synthesis inhibitors [14], and ET 18 OME (the ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine) [8]. Moreover, human killer lymphocytes are not capable of causing DNA fragmentation in the K562 cell line [4]. We do not exclude, however, that as yet undefined stimuli might be capable of triggering apoptosis in K562 cells [10]. Most antineoplastic drugs, even those acting with different biochemical targets, are in fact capable of activating an endogenous program of cell death in sensitive cells. The capability of tumor cells to undergo apoptosis is therefore of central importance to our understanding of cancer resistance beyond the classic mechanisms of drug inactivation, drug efflux, or receptor modulation. However, necrosis and other, as yet undefined modes of cell death may contribute to drug cytotoxicity.

Acknowledgements We are grateful to Prof. Maria Elisabetta Cosulich for fruitful discussions and the critical review of this manuscript. This work was supported by the AIRC (Italian Association for Cancer Research), by the Italian Ministry of University, and by the Italian National Research Council (CNR, target project ACRO).

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