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EVIDENCE FOR INVOLVEMENT OF TYROSINE PHOSPHORYLATION IN TAXOL-INDUCED APOPTOSIS IN A HUMAN OVARIAN TUMOR CELL LINE

YINONG LIU,* KAPIL BHALLA,† CHARLES HILL* and DAVID G. PRIEST*‡

Departments of *Biochemistry and Molecular Biology, and †Medicine,
Medical University of South Carolina, Charleston, SC 29425, U.S.A.

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Abstract—Taxol is an antineoplastic agent with significant activity against ovarian as well as breast cancer. To investigate mechanisms by which taxol exerts its cytotoxic action, taxol-induced apoptosis, characterized by morphologic changes and internucleosomal DNA fragmentation, was examined in a human ovarian tumor cell line. Time-dependent morphologic changes, characteristic of apoptosis, were observed over the same time as the appearance of internucleosomal DNA fragmentation. The specific protein tyrosine kinase inhibitors genistein and herbimycin A, and the ATP depletion agent sodium azide, interfered with taxol-induced DNA fragmentation and clonal cell death. Based on a quantitative reverse transcription-polymerase chain reaction technique, *bcl-2α* oncogene expression was decreased in conjunction with taxol-induced DNA fragmentation, and this decrease could be blocked by genistein. These results strongly implicate protein tyrosine phosphorylation as an event that mediates apoptosis and, thus, the antitumor activity of taxol in ovarian cancer.

Key words: tyrosine kinase; taxol; apoptosis; ovarian tumor cells; clonagenic survival; *bcl-2*

Apoptosis, or programmed cell death, can be induced by a variety of anticancer drugs that act through diverse mechanisms [1]. While the detailed nature of the mechanisms underlying apoptosis remains unclear, several studies have shown that it is an active process requiring RNA and protein synthesis [2]. The biochemical hallmark of apoptosis is the activation of a calcium/magnesium-dependent endonuclease, resulting in the internucleosomal cleavage of DNA [3]. While the precise signaling pathways that lead to antitumor drug-induced apoptosis have not been elucidated, protein kinase C activation by phorbol esters has been reported to inhibit apoptosis in some cell lines but not in others [4–6]. Although not necessarily anticancer drug related, Uckun *et al.* [7] have reported protein tyrosine kinase activation associated with ionizing radiation-induced apoptosis in B-lymphocyte precursors. Recently, expression of the BCL-2 oncoprotein has been demonstrated to promote cell survival and suppress apoptosis [8]. Even though the *bcl-2* gene encodes two products, the 26 kDa BCL-2α and 22 kDa BCL-2β proteins [9], only BCL-2α appears to prevent apoptosis [10].

Taxol, an antineoplastic agent derived from the bark of the yew tree, *Taxus brevifolia*, has significant activity against ovarian as well as breast cancer [11]. Mechanistic studies have shown that taxol binds to tubulin and has at least two effects on assembly of the microtubule system: enhanced tubulin polymerization and induction of abnormal bundle formation [12]. It has been demonstrated that low intracellular ATP levels can interfere with the abnormal microtubule bundle formation induced by taxol [13], which suggested that taxol cytotoxicity could be related to a process mediated by phosphorylation.

Recently, it has been shown that taxol can induce apoptosis in human myeloid leukemia cells [14], but taxol-induced apoptosis has not been investigated as thoroughly in human solid tumor cells. To undertake such a study, DNA fragmentation and morphologic changes were examined in relation to intracellular ATP levels and in the presence of tyrosine phosphorylation inhibitors in a human ovarian cancer cell line exposed to taxol. Results implicate protein tyrosine phosphorylation in pathways associated with apoptosis induced by taxol in this system.

MATERIALS AND METHODS

Materials. Taxol was purchased from Calbiochem (La Jolla, CA). Herbimycin A, fetal bovine serum, MMLV reverse transcriptase and proteinase K were purchased from GIBCO BRL (Gaithersburg, MD). *Taq* DNA polymerase was from Perkin Elmer Cetus (Norwalk, CT). [^{3}H]dGTP was purchased from Amersham (Arlington Heights, IL). Genistein, sodium azide, guanidine thiocyanate, RPMI-1640, hematoxylin and eosin solutions, RNase A, perchloric acid, propidium iodide, diphenylamine and other reagents were purchased from the Sigma Chemical Co. (St Louis, MO).

Cells. The human ovarian tumor cell line, OV2008, used for these studies was derived from tissue obtained from a patient with ovarian cystadenocarcinoma [15]. Cells were grown in RPMI-1640

‡ Corresponding author: David G. Priest, Ph.D., Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. Tel. (803) 792-4321; FAX (803) 792-4322.

Table 1. Primers for PCR amplification of β-actin and *bcl-2*

Gene	Sequence	Position	Product size
β-Actin	(F) 5′-GCGGGAAATCGTGCGTGACATT-3′ (R) 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′	Spanning exons 3 and 4	254 bp
bcl-2α	(F) 5′-GACTTCGCCGAGATGTCCAGCCAG-3′ (R) 5′-CAAACTGAGCAGAGTCTTGAGAGA-3′	Spanning exons 1 and 2	335 bp
bcl-2β	(F) 5′-GACTTCGCCGAGATGTCCAGCCAG-3′ (R) 5′-CACATCACCAGATGCACCTACCCA-3′	Exon 1	275 bp

medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin/streptomycin and 2 mM glutamine at 37° in 5% CO_2. They were passaged twice weekly and exhibited a doubling time of 24 hr under these conditions.

Histologic studies. Exponentially growing OV2008 cells were exposed to 200 nM taxol in growth medium. Prior to introduction, taxol was dissolved in DMSO. Under the conditions used, the concentration of DMSO did not exceed 0.1% (v/v) in culture medium, and this level did not affect growth. After treatment, cells were trypsinized, washed twice in PBS, and resuspended in 100% fetal bovine serum. Slides prepared from these cells were fixed with 100% methanol for 20 min and stained with hematoxylin and eosin [16].

DNA fragmentation. To assess patterns of DNA cleavage, 5×10^6 cells were treated with taxol (10 nM to 1 mM) for 12–36 hr. In parallel experiments, prior to treatment with 200 nM taxol for 24 hr, cells were incubated for 60 min with (a) an intracellular ATP formation inhibitor, sodium azide (10 mM) [13], and (b) a protein tyrosine kinase inhibitor genistein (7.4 to 74 μM) [17]. In addition, cells were exposed to a second protein tyrosine kinase inhibitor, herbimycin A (1.2 to 12 μM), for 24 hr prior to taxol exposure [18]. Following treatment, cells were trypsinized, washed twice with PBS, and resuspended in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 20 mM EDTA, 0.5% (w/v) SDS and 200 μg/mL proteinase K. The suspension was incubated at 50° for 1 hr. Crude DNA preparations were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The DNA pellet was air-dried and resuspended in TE buffer that contained 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 20 μg/mL DNase-free RNase A. Following incubation for 2 hr at 37°, DNA was analysed by electrophoresis at 60 V for 90 min on a 1.8% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light.

Quantitative DNA fragmentation. Internucleosomal DNA fragmentation was quantitated by a modification of a previously described method [19]. In brief, cells (2×10^7) were treated with 200 nM taxol for 24 hr in the presence or absence of 74 μM genistein and disrupted by suspension for 20 min at 4° in 0.5 mL of a lysis buffer [50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% (v/v) Triton X-100 and 200 μg/mL proteinase K]. High molecular weight genomic DNA (pellet) was separated from low molecular weight fragmented DNA (supernatant) by centrifugation at 27,000 *g* for 20 min. The genomic DNA pellet was resuspended in 0.5 mL of lysis buffer. Following the addition of 50 μL of BSA (2 mg/mL) and 1 mL of 10% (w/v) trichloroacetic acid (TCA) to both the resuspended genomic DNA and fragmented DNA fractions, solutions were centrifuged again at 13,000 *g* (4 min) and supernatants were discarded. Precipitates were resuspended in 1 N sodium hydroxide and incubated at 37° for 1 hr to hydrolyze RNA. After another TCA precipitation and centrifugation, pelleted DNA was hydrolyzed by resuspension in 5% (w/v) perchloric acid and incubation at 90° for 15 min. Subsequently, reaction mixtures were centrifuged at 3000 *g*, and the supernatants were incubated with 2 mL of diphenylamine reagent overnight at 37° [20]. Absorbance at 600 nm was used to quantitate DNA in both preparations. Fragmented DNA, separated in the first step, was compared with the sum of DNA in both fractions (total DNA) to evaluate the fraction fragmented.

RNA isolation and RT-PCR assay of* bcl-2 *mRNA expression.* Total cellular RNA was isolated from control and taxol-treated cells by the guanidine-thiocyanate extraction method [21], and the concentration was determined spectrophotometrically at 260 nm. Total RNA was reverse transcribed with MMLV reverse transcriptase using random oligo primers. PCR amplification of resultant cDNA was carried out with specific gene primer pairs (Table 1) and 0.3 μCi [^{3}H]dGTP using *Taq* DNA polymerase. The PCR cycles were: 1 min at 95°, 1 min at 65° (55° for *bcl-2α*), and 1 min at 72°, for 30 cycles. The amount of cDNA used for PCR amplification to determine reference β-actin expression and each of the *bcl-2* genes was selected from the linear region of cDNA concentration versus PCR product plots (30 ng for β-actin, 500 ng for *bcl-2α*, and 500 ng for *bcl-2β*). The purpose was to ensure that the amount of product obtained was proportional to the amount of extract added to the PCR reaction. Half of the PCR products were used for analysis by gel electrophoresis to verify the size of each product, and the other half were centrifuged through Sephadex G50 columns to separate free labeled nucleotide from nucleic acid incorporated [^{3}H]dGTP. The housekeeping gene, β-actin, served as an internal standard. To calculate relative gene expression, a ratio was determined between the

* Abbreviation: RT-PCR, reverse transcription-polymerase chain reaction.

amount of radiolabeled PCR product from taxol-treated cells and that from control cells.

PCR primers. All PCR primers listed in Table 1 were prepared with a 380B DNA synthesizer (Applied Biosystems, Forest City, CA). All DNA sequences were obtained from GenBank or from the literature [9, 22].

Flow cytometric cell cycle analysis. Cells were treated with taxol in the presence or absence of genistein for 24 hr, as well as in the presence of genistein alone for the same time. Cells were washed twice with PBS and fixed with 70% (v/v) ethanol for 30 min on ice. Then the cells were incubated with RNase at 37° for 1 hr and stained with 50 μg/mL propidium iodide in 0.034 M sodium citrate solution. The cell cycle distribution was analysed with an Epics Elite Flow Cytometer (Coulter, Hialeah, FL).

Clonagenic assay. Clonagenicity (plating efficiency) was assayed by a modification of the method of Kovach *et al.* [23]. In brief, 350 cells in 2 mL of growth medium were seeded in 6-well tissue culture dishes. Following 24 hr of incubation at 37°, cells were treated with taxol for 6 hr in the absence or presence of the protein tyrosine kinase inhibitor genistein. The inhibitor was added 1 hr prior to addition of taxol. As a control, cells were treated with genistein alone for 7 hr. Following treatment, the medium was discarded and cells were washed twice with PBS before fresh medium was applied for another 5 days of incubation. Colonies consisting of 30 cells or more were counted with an inverted microscope. Relative cloning efficiency was assessed by the ratio of the number of colonies at a given taxol concentration compared with untreated controls.

Statistical analysis. Significant differences between values were determined by paired *t*-test analysis.

RESULTS

Morphologic changes associated with taxol-induced apoptosis in a human ovarian tumor cell line, OV2008, are shown in Fig. 1. While some cells that were synchronized in G_2/M phase after an 8-hr exposure to 200 nM taxol could be identified (Fig. 1B), few morphologic features associated with apoptosis could be seen at this time. However, cells exposed to taxol for 24 hr (Fig. 1C) exhibited several morphologic changes that were distinctly characteristic of apoptosis. These changes include: nuclear chromatin compaction, cytoplasmic condensation, and nuclear fragmentation [24]. Following a 36-hr exposure (Fig. 1D), extensive apoptotic body formation that is characteristic of later stages of apoptosis could be seen [25].

To examine internucleosomal DNA fragmen-

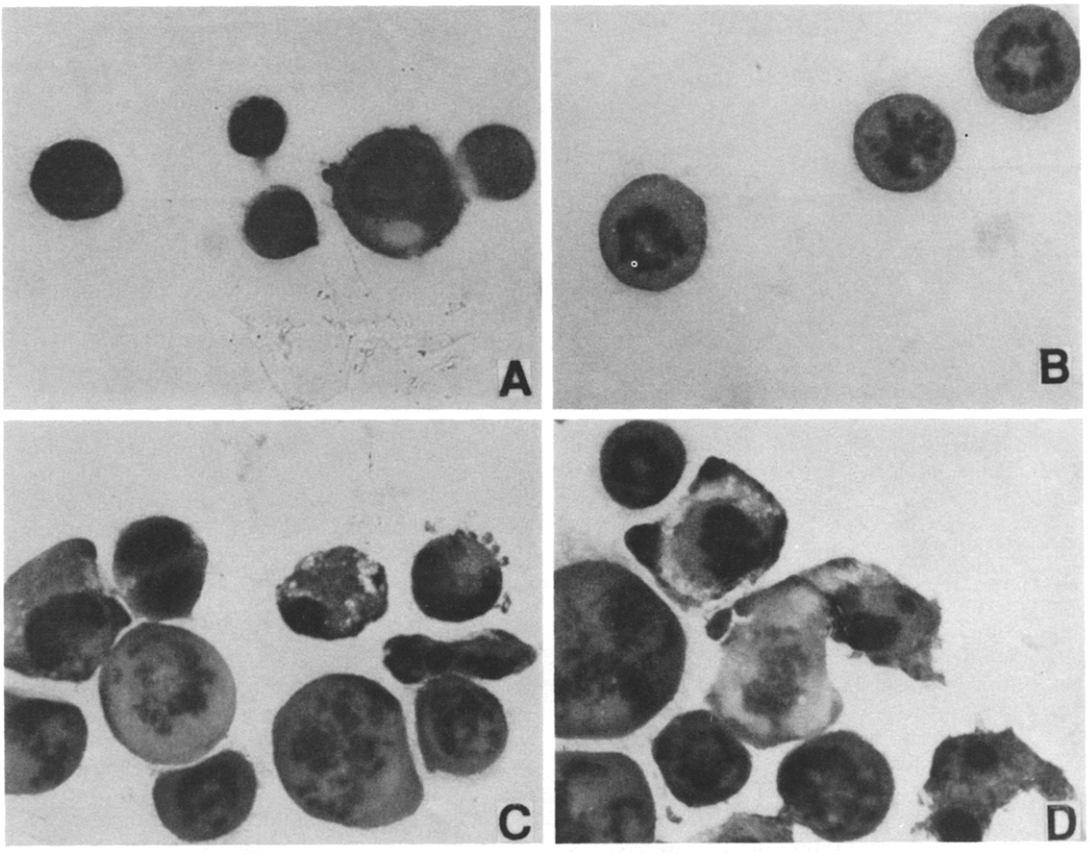

Fig. 1. Morphologic changes in OV2008 cells after exposure to taxol. Exponentially growing cells were treated with 200 nM taxol. Following 0 (A), 8 (B), 24 (C), and 36 (D) hr of exposure, cells were harvested and stained with hematoxylin and eosin.

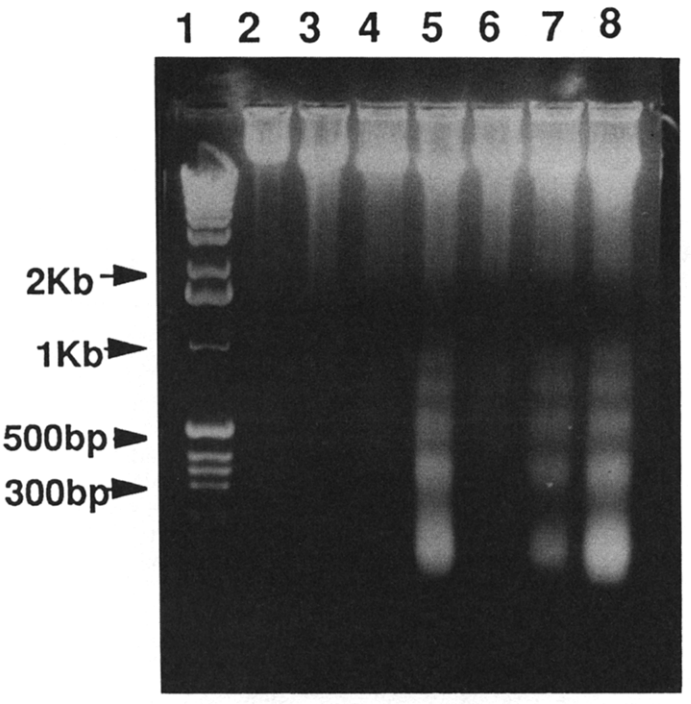

Fig. 2. Time and concentration dependence of taxol-induced DNA fragmentation in OV2008 cells. Key: 1.8% (w/v) agarose gel electrophoresis of DNA isolated from: control cells (lane 2), cells treated with 10 nM (lane 3), 100 nM (lane 4), and 1000 nM (lane 5) taxol for 24 hr, and cells treated with 200 nM taxol for 12 (lane 6), 24 (lane 7) and 36 (lane 8) hr. Lane 1 contains reference DNA standards.

tation, DNA from cells exposed to taxol was extracted and electrophoresed on agarose. It can be seen in Fig. 2 that the classic DNA laddering pattern, containing discrete fragments of 180–200 base pairs characteristic of apoptosis, was clearly evident in cells exposed to even very low levels (10 nM) of taxol for 24 hr (lane 3). As the taxol level was raised, internucleosomal DNA fragmentation became more pronounced (lanes 4 and 5). To investigate the time dependence of taxol-induced apoptosis over a period consistent with the morphologic study shown in Fig. 1, cells were exposed to 200 nM taxol for 12, 24, and 36 hr (lanes 6, 7, and 8, respectively). A very clear time-dependent increase in the intensity of the internucleosomal DNA laddering effect could be seen.

To investigate the role of tyrosine phosphorylation in taxol induction of apoptosis, specific inhibitors were tested for their ability to interfere with DNA fragmentation patterns induced by taxol. It can be seen in Fig. 3 (lane 4) that 10 mM sodium azide, an agent that depletes intracellular ATP levels [13], significantly diminished apoptosis induction by taxol when compared with cells treated with taxol alone (lane 3). Furthermore, two agents that have been characterized as specific inhibitors of protein tyrosine kinase, genistein (lanes 5 and 6) and herbimycin A (lanes 7 and 8), inhibited DNA fragmentation induction in a concentration-dependent manner, indicating involvement of protein tyrosine kinase in taxol induction of apoptosis.

Quantitative analysis of the inhibition of taxol induction of DNA fragmentation by a protein tyrosine kinase inhibitor, genistein, was undertaken and the results are shown in Fig. 4. Approximately 30% of cellular DNA was fragmented following exposure to 200 nM taxol for 24 hr. However, inclusion of genistein at a level that has been shown in other systems to inhibit cellular protein tyrosine kinase (74 μM) diminished this fragmentation nearly 10-fold ($P < 0.05$).

To determine the effects of this protein tyrosine kinase inhibitor on taxol-induced clonal cell death, colony-formation assays were conducted in the presence of taxol and genistein. Six hours was chosen as the taxol exposure time because the drug is typically administered clinically by a 6 hr infusion [26]. Genistein was added 1 hr prior to taxol exposure to assure adequate accumulation. It can be seen in Fig. 5 that the IC_{50} for taxol alone, estimated to be 103 nM, was increased approximately 3-fold (294 nM) in the presence of 74 μM genistein, the same concentration used in the DNA fragmentation studies. Genistein treatment alone, at the same concentration, inhibited colony formation by only 9%. Hence, it is clear that protein tyrosine kinase inhibitors can interfere with internucleosomal DNA fragmentation induced by taxol in these human ovarian tumor cells and that there is a concomitant resistance to the cytotoxic action of taxol.

To investigate the mechanism by which genistein interferes with taxol activity, cell cycle distribution analysis was carried out (Fig. 6). Control cells were distributed over the cell cycle with approximately

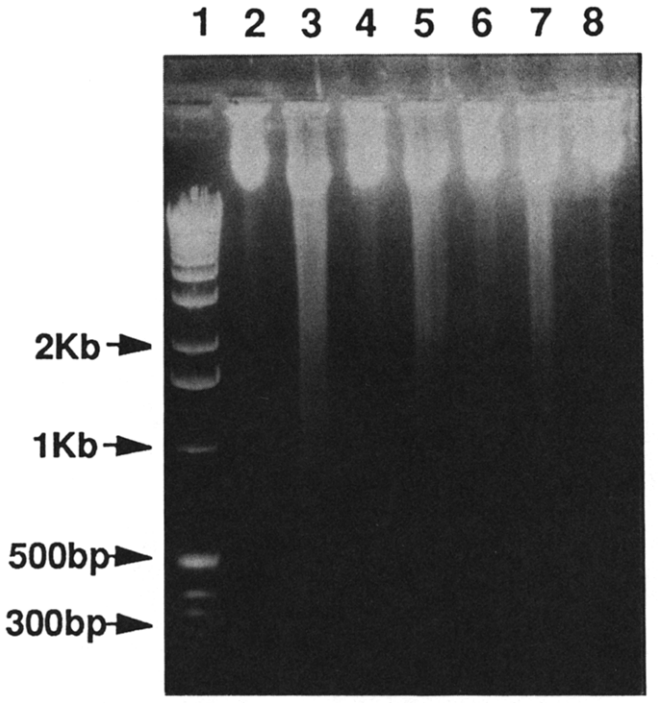

Fig. 3. Inhibition of taxol-induced apoptosis by ATP depletion and protein tyrosine kinase inhibitors. DNA was extracted from 5×10^6 OV2008 control cells (lane 2), and cells treated with 200 nM taxol alone for 24 hr (lane 3). In addition, cells were treated with 200 nM taxol and: 10 mM sodium azide (lane 4), 7.4 μM (lane 5) and 74 μM (lane 6) genistein, and 1.2 μM (lane 7) and 12 μM (lane 8) herbimycin A. DNA was analysed by electrophoresis on a 1.8% agarose gel. Lane 1 contains reference DNA standards.

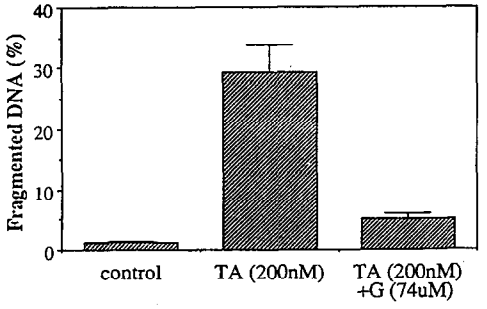

Fig. 4. Quantitative evaluation of taxol-induced DNA fragmentation inhibition caused by a protein tyrosine kinase inhibitor, genistein. OV2008 cells (2×10^7) were treated with 200 nM taxol (TA) for 24 hr in the absence or presence of 74 μM genistein (G). Genistein was added 1 hr prior to taxol. At the end of the treatment, DNA was extracted and fragmented DNA was quantitated as described in Materials and Methods. Results are expressed as mean percentage fragmented DNA ± SEM from 5 separate experiments.

40% in G_1, 20% in G_2/M and 40% in S phase. This cell cycle distribution was not altered significantly after 74 μM genistein treatment alone. In the presence of taxol, about 80% of cells were synchronized in G_2/M, and this was possibly diminished slightly by coincubation with genistein. There is also a possible modest shift into S phase caused by genistein, but no effect on the G_1 population was detected. Thus, while the cell cycle distribution was shifted dramatically to G_2/M by taxol as has been observed previously [12], genistein, at a concentration that suppresses apoptosis and clonal cell death caused by taxol, had little effect on the cell cycle distribution itself.

The *bcl*-2 oncogene has been implicated in the apoptotic processes [8]. To evaluate the effect of taxol and the protein tyrosine kinase inhibitor on expression of the *bcl*-2 oncogene, a quantitative RT-PCR technique was developed. The technique is an adaptation of the approach used by Danenberg and coworkers [27] and is based on evaluation of radiolabeled PCR products prepared with specific primers using cDNA from reverse transcribed total cellular RNA as a template. The labeled products were referenced to expression of the β-actin gene. It can be seen in Fig. 7 that *bcl*-2α, but not *bcl*-2β, expression, was suppressed significantly when cells were treated with taxol and that this suppression was entirely reversed by genistein. Hence, a protein tyrosine phosphorylation is apparently involved in *bcl*-2α expression in this system, and this is likely an important factor in the induction of apoptosis by taxol.

DISCUSSION

A wide spectrum of anticancer drugs has now

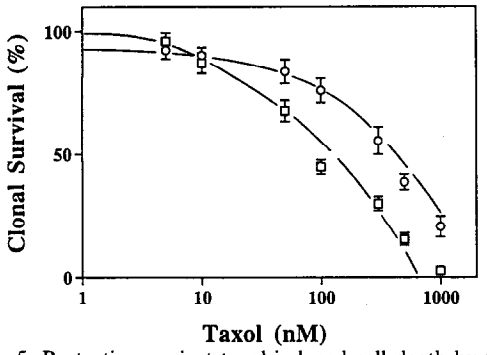

Fig. 5. Protection against taxol-induced cell death by the protein tyrosine kinase inhibitor genistein. OV2008 cells (350) were seeded in 6-well tissue culture plates and were allowed to grow for 24 hr prior to exposure to taxol for 6 hr in the absence (□) or presence (○) of 74 μM genistein. Genistein was added 1 hr prior to taxol. After taxol and the inhibitor were removed, cells were permitted to grow for an additional 5 days before colonies consisting of 30 cells or more were counted. Clonal formation efficiency in 74 μM genistein alone represents the initial condition in the taxol + genistein curve (○). Values are the means ± SEM from five separate experiments.

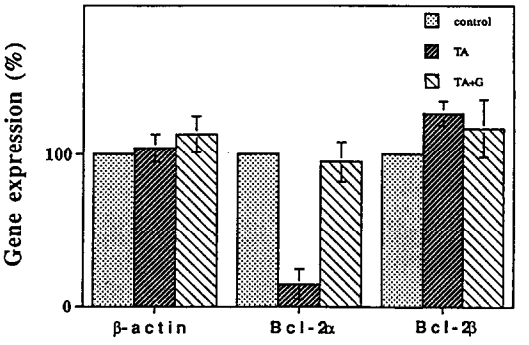

Fig. 7. Effect of taxol and the protein tyrosine kinase inhibitor genistein on *bcl-2* gene expression. OV 2008 cells (2×10^7) were treated with 200 nM taxol for 6 hr in the absence or presence of 74 μM genistein. Total cellular RNA was isolated, and the concentration was estimated spectrophotometrically. RNA was reverse transcribed with MMLV reverse transcriptase. PCR amplification was performed using cDNA template, primer pairs specific for each gene (see Table 1). Incorporation of [^{3}H]dGTP was estimated by passage of PCR products over Sephadex G50 columns and counting in a scintillation counter. Values are the means ± SEM from five separate experiments.

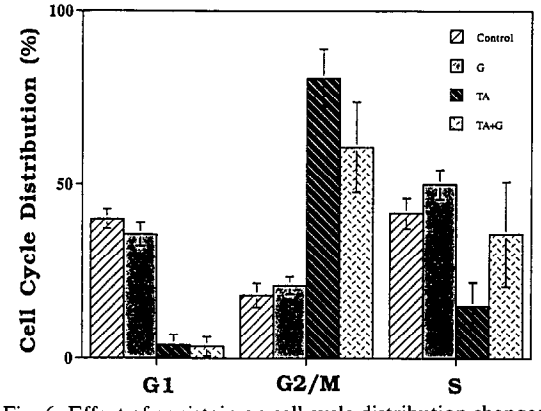

Fig. 6. Effect of genistein on cell cycle distribution changes induced by taxol. Cells (1×10^7) were treated with 200 nM taxol for 24 hr in the presence or absence of 74 μM genistein, and 74 μM genistein alone. Cells were washed, fixed with ethanol, and stained with propidium iodide solution before being subjected to flow cytometric analysis. Values are the mean ± SEM from three separate experiments.

been shown to activate a common pathway of cell death termed apoptosis [1, 28–30]. In contrast to protein kinase C, protein tyrosine kinase, which plays a pivotal role in the initiation of various signaling cascades that regulate cell function and proliferation [31–33], has not been implicated previously in induction of apoptosis by antitumor agents. However, Uckun *et al.* [7] have reported that ionizing radiation stimulates tyrosine phosphorylation of multiple substrates in human B-lymphocyte precursors and that inhibition of protein tyrosine kinase activity retards radiation-induced DNA fragmentation in this system. The demonstration in this report that protein tyrosine kinase inhibitors can interfere with taxol-induced apoptosis not only implicates a process mediated by tyrosine phosphorylation, but suggests that there may be a common pathway for anticancer drug- and radiation-induced apoptosis.

Horwitz and coworkers [13] have demonstrated in a mouse macrophage-like cell line (J774.2) that depleted ATP levels inhibit taxol induction of abnormal microtubule bundle formation. Likewise, in our human ovarian tumor cell system, the presence of sodium azide, which has the effect of lowering intracellular ATP levels, interfered with induction of apoptosis by taxol. In addition to causing an intracellular energy deficiency, ATP depletion would be anticipated to retard phosphorylation reactions such as those catalyzed by protein tyrosine kinases. Hence, these ATP depletion results are consistent with a role for protein tyrosine kinase activity in both taxol-induced abnormal microtubule bundle formation in murine lymphocytes and taxol-induced apoptosis in this human ovarian tumor cell line.

In non-drug-treated cells, sensitivity to the lethal effects of ionizing radiation is cell cycle dependent [34]. Cells are most sensitive in M phase and least in S phase. Likewise, it has also been proposed that cells lethally damaged by anticancer drugs continue to progress through the cell cycle and eventually destroy themselves by apoptosis at the G_2/M transition [35]. Further, it has been suggested that during M phase an endonuclease can react more readily with DNA as the nuclear membrane is broken [36]. Results reported here are consistent with this concept. When cell cycle distribution analysis was performed, 80% of cells were in G_2/M phase after

treatment with taxol, and genistein caused only a marginal shift from G_2/M into S phase. Although it has been reported that genistein itself leads to arrest of cells in G_2 phase in other systems [37], treatment with genistein at the concentration used to interfere with apoptosis did not change the cell cycle distribution in this cell line. Hence, the protection offered by the protein tyrosine kinase inhibitor does not appear to be a direct effect on cell cycle kinetics. Rather, it is apparently related to protection from DNA cleavage itself through some other mode of action.

Decreased expression of the *bcl*-2 oncogene has been correlated with induction of apoptosis by anticancer drugs [14]. Furthermore, *bcl*-2α mRNA expression is suppressed by taxol (see Fig. 7) in a manner that is blocked by genistein. Hence, it is possible that mediation of apoptosis by phosphorylation is related to *bcl*-2α down-regulation. It has been proposed that effects of BCL-2 on apoptosis occur primarily during M phase. This is based on the observation that antibodies to BCL-2 accumulate extensively on chromatin only during the absence of the nuclear membrane [36]. Thus, when *bcl*-2α down-regulation is reversed by preventing tyrosine phosphorylation, the protection from apoptosis could occur primarily during M phase without a requirement that phosphorylation have a direct impact on cell cycle kinetics.

In summary, results reported here demonstrate that taxol can induce internucleosomal DNA fragmentation or apoptosis in a human ovarian tumor cell line. This induction requires adequate intracellular ATP levels and protein tyrosine kinase activity. There was a decrease in *bcl*-2α gene expression before DNA fragmentation in taxol-treated cells that can be blocked by the protein tyrosine kinase inhibitor genistein. Future studies will attempt to determine the generality of a role for protein tyrosine kinase in apoptosis.

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