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Taxol-Induced Apoptosis and Nuclear Translocation of Mitogen-Activated Protein (MAP) Kinase in HeLa Cells

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Taxol, a natural product with significant anti-tumor activity, stabilizes microtubules and arrests cells in the G2/M phase of the cell cycle. It has been reported that taxol has additional effects on the cell such as an increase in tyrosine phosphorylation of proteins and activation of mitogen-activated protein (MAP) kinase. This phosphorylated kinase translocates into the nucleus and phosphorylates its substrate c-jun, c-fos, ATF2, and ATF3. The MAP kinase family is comprised of key regulatory proteins that control the cellular response to both proliferation and stress signals. First examination was cytotoxicity and apoptosis-induced concentration with paclitaxel in HeLa cell. A half-maximal inhibition of cell proliferation (IC_{50}) occurred at 13 nM paclitaxel. When DNA fragmentation was analyzed by agarose gel electrophoresis, a nucleosomal ladder became evident 24 h after a taxol (50 nM) addition to the cells. In addition, an apoptotic body was detected by electron microscopy. Taxol-treated cells were arrested at the S phase at 10 nM. Treatment of 50 nM taxol activated the extracellular signal-regulated protein kinase (ERK1), and a fraction of the activated MAP kinases entered the nucleus. It was also discovered that nucleus substrates c-jun was phosphorylated and activated in the cell. The activated ERK1 could subsequently translocate into the nucleus and phosphorylate its substrate c-jun as well. This study suggests that taxol-induced apoptosis might be related with signal transduction via MAP kinases.

Keywords: Anti-tumor activity, c-jun, DNA fragmentation, MAP kinase, Taxol.

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Introduction

Paclitaxel (taxol) is a potent anti-tumor drug that is remarkably effective against advanced ovarian carcinoma and breast carcinoma, and it shows promising activity against other types of tumors (Rowinsky and Donehower, 1995). Taxol inhibits cell proliferation and induces cell death (Schiff and Horwitz, 1980; Jordan *et al.*, 1993; Bhalla *et al.*, 1993; Lopes *et al.*, 1993; Donaldson *et al.*, 1994; Liebmann *et al.*, 1994; Long and Fairchild, 1994; Milas *et al.*, 1995). It appears to act by slowing or blocking progression through mitosis (Fuchs and Johnson, 1978; Jordan *et al.*, 1993; Liebmann *et al.*, 1994; Long and Fairchild, 1994; Rieder *et al.*, 1994). Substoichiometric binding of taxol to reassembled microtubules potently stabilizes microtubule dynamics (Derry *et al.*, 1995). Previous reviews (Wyllie *et al.*, 1980; Sen and D'Incalci, 1992; Fisher 1994) have summarized the evidence that cancer chemotherapeutic agents including taxol induced programmed cell death. Prolonged exposure of cells to a low concentration of taxol induces DNA fragmentation, which is characteristic of apoptosis, and it ultimately leads to programmed cell death (Liu *et al.*, 1994). Apoptosis begins with condensation of nuclear chromatin at the nuclear periphery, following with bleb of the nuclear and cytoplasmic membranes, culminating in the fragmentation of residual nuclear structure into discrete membrane-bound apoptotic bodies (Wyllie *et al.*, 1980; Kerr *et al.*, 1987). The relationship between drug-induced apoptosis and the cell cycle has been studied (Gorczyca *et al.*, 1993). The topoisomerase inhibitors and hydroxyurea are selectively lethal to S-phase cells, nitrogen mustard preferentially kills G_1 phase cells, and both irradiation and cisplatin selectively perturb the G_2/M phase cells (Barry *et al.*, 1990; Sorecson *et al.*, 1990). Also, it was reported that p38 which was activated by the genotoxic chemotherapeuticals was concerned with inhibiting the cell cycle progression at the G_1/S phase (Molnar *et al.*, 1997). Recently, mitogen-activated protein (MAP) kinase cascades, which is well

known for cell proliferation and the differentiation-inducing pathway, was reported to be associated with the apoptotic pathway (Derijard *et al.*, 1994; Kim *et al.*, 1996; Jeon *et al.*, 1998). It has been reported that taxol increases tyrosine phosphorylation of protein, including MAP kinase (Carboni *et al.*, 1993). MAP kinase families include extracellular regulated protein kinase (ERK1, ERK2), p38, which was activated by the genotoxic chemotherapeutics concerned with inhibiting the cell cycle progression at G_1/S phase (Molnar *et al.*, 1997) and stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) (Raingeaud *et al.*, 1995). It has been reported that different signaling pathways were involved in apoptosis (McConkey *et al.*, 1996: Wang *et al.*, 1998.). Among them, the MAP kinase family is known as an intermediate for the apoptotic signal pathway (Gardner and Johnson, 1996). For example, SAPK/JNK cascades were activated in apoptosis by environmental stresses such as inflammatory cytokine (Martin and Schwartz, 1997), ultraviolet C (Kyriakis *et al.*, 1994), gamma irradiation (Yu *et al.*, 1996), and DNA damaging drugs (Jimez *et al.*, 1997). It is known that this phosphorylated kinase leads to transcriptional activation of target genes, including c-jun, c-fos, ATF2 and ATF3 (Cavigelli *et al.*, 1995; van Dam *et al.*, 1995; Baek *et al.*, 1996).

In this report, we have examined the cytotoxicity and apoptitic effect of taxol in HeLa cells. In particular, potent activation of ERK1 and nuclear translocation of MAP kinases were observed after taxol treatment. The significance of these observations have been discussed.

Materials and Methods

Materials The paclitaxel (taxol) was purchased from Sigma Chemical Co. (St. Louis, USA). Anti-phosphor-ERK antibody nor an anti-phosphor-c-jun antibody was purchased from Upstate Biotechnology (Lake Placid, USA). All other chemicals and reagents were the highest grade commercially available.

Cell culture and *in vitro* cytotoxicity assay Human cervical carcinoma HeLa cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum without antibiotics nor an anti-fungal agent. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. Exponential growing cells were inoculated to 1×10^4 cells/well using 96-well plates supplemented with 100 μl RPMI 1640. After the treated cells were incubated for 24 h, 50 μl MTT (1mg/ml, Sigma) was added and the plates were incubated at 37°C for 4 h. To dissolve formazan, 150 μl DMSO was added and the plates were measured at 540 nm by spectrometery. The IC_{50} values were determined by plotting the drug concentration versus the survival ratio of the treated cells.

DNA extraction and electrophoresis Five million cells which were treated for 48 h were washed twice in a solution of 50 mM Tris·HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl and then lysed with 500 μl lysis buffer (1% Triton X-100, 50 mM Tris·HCl, pH 7.5, 20 mM EDTA). Lysates were centrifuged by 1000 $\times$ g for 10 min, and the supernatants were incubated for 3 h at 37°C with 50 μg/ml RNase A and 120μg/ml proteinase K. Then, the DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1, Sigma). After precipitation, pellets were resuspended in 30μl TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through a 1.8% agarose gel containing ethidium bromide, and DNA bands were visualized by UV light.

Flow cytometry After exposure for 24 h with compounds, the cells were harvested by 300 $\times$ g for 5 min. The pellet was treated with Cycle TEST^{TM} Plus DNA Reagent Kit (Becton Dickinson) and stained by propidium iodide (PI). A minimum of 2×10^5 cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, USA). Each of the G_1, S, G_2/M phases of the cell cycle was calculated using the RFIT program.

Electron microscopy Cells to be processed for electron microscopy were centrifuged at 400 $\times$ g, fixed with 2.5% glutaraldehyde in PBS for 2 h, washed in 0.1 M Caocodylate, pH 7.4, and then fixed with 0.1% OsO_4 in 0.1 M Caocodylate for 1 h 30 min. After being washed with 0.1 M Caocodylate, pH 7.4, the cells were dehydrated in graded ethanol. Then, the cells were impregnated with prophylene oxide and embedded in Polybed 812 (Polyscience Inc., Washington, USA). After 60°C incubation, the cells were cut, and stained with uranyl acetate and lead citrate.

Preparation of nuclear extracts All the fractional procedures were performed at 0–4°C. The treated cells were washed twice and collected into 1 ml hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 10 μg of pepstain and leupeptin per ml, pH 7.5). The cellular suspension was homogenized and harvested at 600 $\times$ g for 5 min. The supernatant was centrifuged at 12,000 $\times$ g for 20 min to prepare the cytosol fraction. The pellets were suspended by 0.25 M sucrose containing 1.5% citric acid and passed three times through a 26 gauge needle and loaded onto 1 ml of a 0.88 M sucrose cushion containing 1.5% citric acid. After centrifugation at 900 $\times$ g for 10 min, the pellets, representing the nuclear fraction, were dissolved with hypotonic lysis buffer containing 0.5% Igepal CA-630, 0.1% deoxycholate, 0.1% Brij-35, and then centrifuged at 10,000 $\times$ g for 10 min. The purity of the nuclear fraction was determined by measuring the contaminating lactate dehydrogenase activity, a cytosol marker. Then, the nuclear fractions were confirmed by Western blot analysis.

Immunoblot analysis For a MAP kinase immunoblot assay, the prepared nuclear extracts isolated from taxol-treated HeLa cells were electrophoresed on 12% polyacrylamide gel in the presence of SDS. After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) containing 2% bovine serum albumin. Then the membrane was incubated with anti-ERK antibodies at a 1:500 dilution in TBST containing 2% bovine serum albumin for 1 h, and then washed 3 times in TBST. The blots were incubated with horseradish peroxidase conjugated anti-sheep Ig antibodies at a 1:1,000 dilution in TBST containing

2% bovine serum albumin for 1 h, and then washed 3 times in TBST, and were detected with diaminobenzidine.

Results and Discussion

***In vitro* cytotoxic effect of taxol** Prior to measurement of the cytotoxic effect of taxol on HeLa cells, we analyzed the pattern of HeLa cell growth. The cells grew exponentially over 3 days. HeLa cells were incubated for 24 h after treatment with taxol. Following appropriate incubation, live cells were analyzed by MTT assay. The IC_{50} value of taxol was 13 nM (Fig. 1). It was reported that the IC_{50} values of taxol in other cell lines such as HT-29, U373, A549, and PC-Sh cell were 2.8, 4.2, 4.1, and 7.5 nM respectively (Liebmann *et al.*, 1993).

DNA fragmentation In order to determine the apoptotic effect of taxol, we examined the apoptotic response as judged by the appearance of a DNA ladder by gel electrophoresis. The DNA ladder patterns with taxol began to appear at a 50 nM concentration (Fig. 2). This result was comparable to the exposure of 698 cells to 100 nM taxol for 24 h which produced the classical internucleosomal DNA fragmentation of apoptosis (Tang *et al.*, 1994).

Cell cycle analysis It has been known that most anti-tumor drugs which induce apoptosis cause cell cycle arrest, but the mechanism by which this occurs has not been uncovered yet. Recently, it was reported that the apoptotic signal pathway is related with the arrested phase of the cell cycle (Cortes *et al.*, 1997; Molnar *et al.*, 1997). In order to analyze the cell cycle influence by taxol, the treated cells were labeled with propidium iodide (PI) and were analyzed by flow cytometry (FACScan). The DNA content peaks of HeLa cells that were exposed at the various concentrations of taxol were measured (Fig. 3). The result showed that 1 nM taxol arrested cells in the G_2/M phase, but this shifted to the S phase when cells were treated with 5 nM and 10 nM toxol. These results indicated that taxol inhibits the cell cycle progression by arresting the G_2/M phase cell, and then later the S phase, and the taxol concentration for cell cycle arrest was lower than the DNA fragmentation-induced concentration.

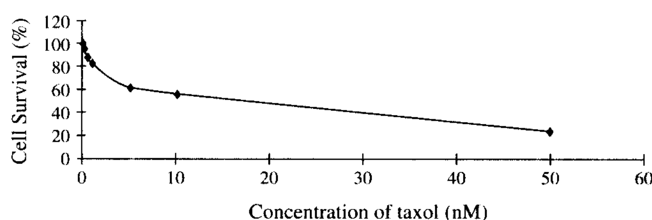

Fig. 1. Cell survival ratio of HeLa cell lines after 24 h exposure with taxol. The points (♦) represent the means of at least three replicates.

Fig. 2. DNA fragmentation extracted from HeLa cell lines treated with taxol. Lane 1, 1 Kbp ladder marker; lane 2, control; lanes 3–8, taxol 0.1, 0.5, 1, 5, 10, 50 nM.

Electron microphotography When the condensed nuclei were fixed with glutaraldehyde and examined by thin section EM, they showed a striking similarity to the nuclei of cells undergoing apoptosis. These spherical domains often appeared to be in the process of budding outwards through the nuclear envelope (Fig. 4). When nuclei were left in the extracts for long periods of time, the DNA was released into the extract as individual highly condensed 'pebbles'. That was the evidence that the domains were actually budding outwards through the nuclear envelop during apoptosis (Kerr *et al.*, 1972; 1987; Wyllie *et al.*, 1980). Given this strong resemblance in the morphological events of apoptosis, the events of chromosome condensation and blebbing that occured in the taxol-treated cell would be refered to as "morphological apoptosis".

Activation and translocation into the nucleus of MAP kinase (ERK1) It is known that the MAP kinase pathway is involved in apoptotic signal transduction (Wang *et al.*, 1998). To determine if ERKs, a member of the MAP kinase family, were activated during taxol-stimulated apoptotic response, we examined the phosphorylation of ERKs by immunoblot analysis (Fig. 5). HeLa cells were exposed with 50 nM Taxol for 10, 20, 30, and 60 min. It is known that MAP kinases are phosphorylated on both tyrosine and threonine residues in response to various stimulation (Boulton *et al.*, 1991). It was found that the activation of ERK 1 was confirmed by immunoblot analysis using anti-ERKs antibody. Taxol activated the 44 kDa ERK1 at 10, 20, and 30 min after treatment and disappeared at 60 min. There is a report that the activated ERKs were translocated into the nucleus (Chen *et al.*, 1992). To confirm this, we prepared the separation of

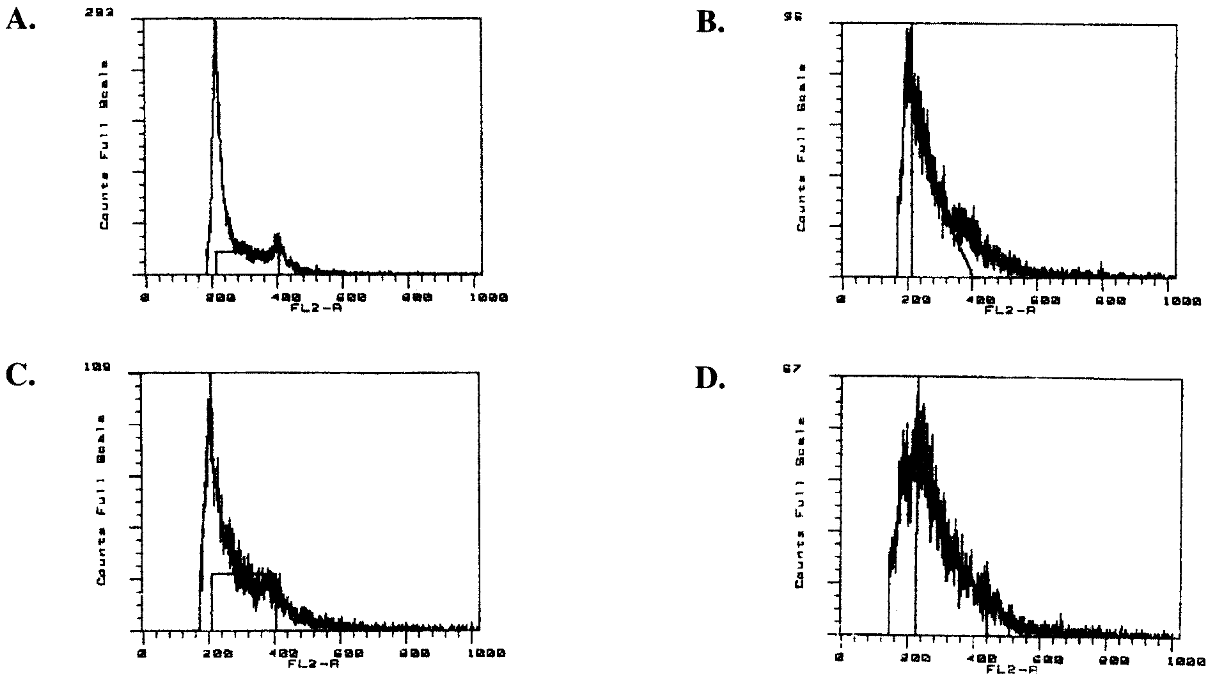

Fig. 3. Cell cycle analysis of HeLa cells exposured to taxol by flow cytometry. **A.** Untreated cells (G_1, 58.1%; S, 36.6%; G_2 + M, 5.2%). **B.** 1 nM exposed cells (G_1, 28.6%; S, 55.6%; G_2 + M, 15.9%). **C.** 5 nM exposed cells (G_1, 44.0%; S, 53.8%; G_2 + M, 2.2%). **D.** 10 nM exposed cells (G_1, 13.7%; S, 81.2%; G2 + M, 5.1%).

Fig. 4. Electron micrographs (× 4,000) of HeLa cell treated with 50 nM taxol for 24 h (B–D); taxol-free control (A). Cell morphological change occurred from B to D.

nuclei from the cells exposed with Taxol for 10, 30, and 60 min and examined this by immunoblot analysis (Fig. 6). Our result showed that ERK 1 (44 kDa) appeared to be in the nuclear fraction at 10 and 20 min, but it disappeared at 30 min. As shown in Fig. 6, ERK 1 was phosphorylated and translocated into the nucleus by Taxol at the same condition of inducing apoptosis. Accordingly, ERK 1 may be involved in the signal transduction pathway of Taxol-induced apoptosis.

c-jun phosphorylation To investigate the nuclear target phosphorylated by Taxol, cytosol from control and Taxol-treated HeLa cells were examined for the activity of transcriptional factor phospho-c-jun by immunoblot

Fig. 5. Immunoblot analysis of phospho-MAP Kinase. The starved HeLa cells were treated with taxol for 10, 20, 30, 60 min (lanes 2–5); serum-free cellular fraction (lane 1).

Fig. 6. Immunoblot analysis of phospho-MAP Kinase translocation to the nucleus. The starved HeLa cells were treated with taxol for 10, 20, 30, 60 min (lanes 2–5); serum-free nuclear fraction (lane 1).

Fig. 7. Immunoblotting assay of phospho-c-jun from the HeLa cell treated with 50 nM taxol for 10, 20, 30 and 60 min (lanes 2–5); serum-free cellular fraction (lane 1).

analysis. Crude extract obtained from control cells revealed a low level of phospho-c-jun activity, whereas the level of 10 and 20 min in taxol-treated HeLa cells were comparatively higher (Fig. 7). Treatment of several cell lines with H_2O_2 primarily evoked an activation of ERKs while JNKs were stimulated only moderately (Guyton *et al.*, 1996).

In summary, taxol at an effective dose has an anti-tumor activity against cervical cancer cells and induces the apoptotic response. It was found that taxol initially induced the G2/M phase arrests, and later S phase arrests. Also, the results imply that the activation and nuclear translocation of ERK 1 by taxol treatment may be related to apoptotic signal transduction, and suggest that c-Jun might be a downstream effector responding to the treatment with Taxol.

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