Differential Sensitivity of Taxol-induced Apoptosis in U2OS and SaOS2 Osteogenic Sarcoma Cells

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Purpose: Taxol (Paclitaxel) is a new generation of chemotherapeutic drug proven to be effective in the treatment of many cancers. In this study, to further demonstrate the differential effect of the tumor suppressor gene, p53, on the Taxol-induced apoptosis in osteogenic sarcoma cell lines, we used p53-defected SaOS2 cells and wild type p53-expressed U2OS cells.

Materials and Methods: The cell viability was measured by the XTT assay. To examine whether the differential expressions of p53, in U2OS and SaOS2 cells, were associated with Taxol-induced apoptosis, DNA fragmentation assays were performed on both cytosolic and genomic DNA. Since the cleavage of poly(ADP-ribose) polymerase (PARP) is primarily responsible for apoptosis, the cleavage of PARP, and the expression of cyclin B1, polo-like kinase, Bax, Bcl-xL, Bcl-2 in U2OS and SaOS2 cells were compared by Western blot analyses.

Results: The cell viability of the p53-defected SaOS2 cells was markedly decreased with Taxol treatment. Whereas, the cell viabilities due to 6-mercaptopurine and adriamycin were no different between the U2OS and SaOS2 cells. Treatment with Taxol induced a ladder-like pattern of DNA fragments, which is a biochemical hallmark of apoptosis, consisting of multiples of approximately 180-200 base pairs, in a dose-dependent manner in the SaOS2 cells, but insignificantly with the U2OS cells. When the cells were treated with Taxol, the 89 kDa cleavage product of PARP clearly appeared as a function of time in the SaOS2 cells, but not in the U2OS cells. The Taxol-induced apoptosis in p53-defected osteogenic sarcoma cells was associated with the PARP cleavage as a result of the increased activity of caspase 3, and the high expressions of cyclin B1 and PLK. Bax, as a proapoptotic factor, was increased in the SaOS2 cells, but the Bcl-xL and Bcl-2 were decreased when the cells were exposed to 10μM Taxol.

Conclusion: From these results, it was concluded that p53-defected SaOS2 cells are much more sensitive to Taxol-induced apoptosis than p53-expressed U2OS cells. (Cancer Research and Treatment 2003;35:148-153)

Key Words: Taxol, Apoptosis, p53, Poly (ADP-ribose) polymerase, Osteogenic sarcoma

We have also compared the activity of a number of cytotoxic drugs, including amasinc, the amasinc analogue CI-921, methotrexate, nitroprusside, denuorubicin, daunorubicin and 5-fluorouracil (2). These anticancer drugs have excellent effects on treatment, but the development of new anticancer drugs is required due to the side effects of, and resistance to, these drugs.

The antitumor effects of Taxol correlate with increased tubulin polymerization, tubulin bundling, cell cycle arrest at the G2/M phase and the inhibition of angiogenesis (3). Unlike classical antimicrotubule agents, such as colchicine and the vinca alkaloids, which induce depolymerization of microtubules, Taxol induces tubulin polymerization, and forms extremely stable, nonfunctional microtubules. Taxol has been reported to demonstrate a broad antineoplastic activity in leukemia, lung cancer, prostatic cancer, malignant melanoma, ovarian carcinoma, breast cancer, and several classically refractory tumor (4-8).

Tubulin has been synthesized on both membrane-bound and free polyribosomes, prepared from brain tissue. Tubulin, made

INTRODUCTION

The treatment of osteogenic sarcomas, by chemotherapy, has played an important role, both before and after an operation. In the past, patients with an objectively measurable disease received at least one course of chemotherapy (1). We have effectively used various chemotherapeutic agents, i.e. adriamycin, methotrexate, cisplatin or combined cyclophosphamide, vincristine and phenylalanine, in human osteogenic sarcomas.

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on rough microsomes, is incorporated into the endoplasmic reticulum membrane as it is synthesized with Taxol (9). Taxol increases the rate, and extent, of microtubule assembly, and stabilizes microtubules, both in vitro and in cells. Taxol has the ability to promote microtubule assembly in the absence of microtubule-associated proteins, rings, added GTP and organic buffers (10). Taxol-treated microtubules have depressed dissociation reactions, as determined by dilution experiments. The drug did not inhibit the binding of GTP, the hydrolysis of GTP, or GDP in our microtubule protein preparations (3). The microtubule stabilizer, Taxol, enhances the fusion between vinblastine-induced autophagosomes and lysosomes (small dense bodies), but does not affect the segregation of cytoplasm into the autophagosomes in mouse liver (11). Taxol lowers the critical tubulin concentration, both in vivo and in vitro, with the organizing capacity of the microtubule-organizing centers, depending on the cytosolic polymerization threshold (12). Although the effects of Taxol have been extensively studied, the biochemical mechanism inducing apoptosis remains largely unclear.

It has been demonstrated that the tumor suppressor p53 gene is involved in the activation of apoptosis following treatment with ionizing radiation and various chemotherapeutic drugs that contain 5-fluorouracil, etoposide and Adriamycin (13).

In this study, we explored the induction mechanism of Taxol cytotoxicity in osteogenic sarcoma p53-expressed U2OS and p53-defected SaOS2 cells. To find if the differential expressions of various markers in apoptosis were related to some function of these cytoxic mechanisms, the cytotoxicities of U2OS and SaOS2 cells were compared with apoptosis caused by Taxol.

**MATERIALS AND METHODS**

1) Materials

The human osteogenic sarcoma wild type p53-expressed U2OS and p53-defected SaOS2 cells were obtained from the American Type Culture Collection (ATCC). The DMEM (Dulbecco’s modified Eagle’s medium) was purchased from Life Technologies, Inc. (Gaithersburg, MD), FCS (fetal calf serum) from HyClone Co. Antibodies against cyclin B1, pololike kinase (PLK), Bax, Bcl-XL, Bcl-2, PARP [poly(ADP-ribose) polymerase], and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Bradford reagent, for the protein measurements, was purchased from BioRad Co. The ECL Western blot detection system was from Amersham Pharmacia Biotech.

2) Cell culture

The human osteogenic sarcoma U2OS and SaOS2 cells were grown in DMEM, with 10% heat-inactivated fetal calf serum, in a 5% CO2 incubator at 37°C.

3) Cell survival assay

To assess the cell survival, we used sodium 3’-[(phenylamino-carbonyl)] 3, 4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) as a labeling reagent, with the electrocoupling reagent, N-methylbenzopyrazine methyl sulfate. Briefly, the cells were grown in 96-well plates, then treated with Taxol for 48 hours (h), after which the XTT labeling and electrocoupling mixture was added as recommended by the manufacturer’s instructions (Boeringer Mannheim, Germany). After incubation, the cell viability was analyzed by measuring the absorbance at 492 nm.

4) Analysis of DNA fragmentation from cytosolic DNA

Cells, treated with 1-50μM Taxol, were centrifuged and harvested. After the precipitated cells had been lysed in a solution containing 5 mM Tris, pH 7.4, 20 mM EDTA and 0.5% Triton X-100, they were incubated for 1 minute (min) and then centrifuged at 15,000 g for 15 min at 4°C. The upper phase was extracted twice with 1 volume of phenol/chloroform and chloroform. The cytosolic DNA was extracted from a sample suspended with 3 M sodium acetate and ethanol and incubated for overnight at 20°C. The cytosolic fragmented DNA was precipitated with ethanol, suspended in TE buffer (10 mM Tris, pH 7.4 and 1 mM EDTA) containing 10μg/ml RNase A. The samples were reacted at 37°C for 30 min, and analyzed on 1.8% agarose gels. The characteristics of apoptosis were observed as a 200 bp stepladder due to the DNA fragmentation.

5) Analysis of DNA fragmentation from genomic DNA

Cells, treated with 1-50μM Taxol, were centrifuged and harvested. After the precipitated cells had been lysed in a lysis buffer (50 mM Tris, pH 8.0 and 0.5% SDS) containing 0.5 mg/ml proteinase K and 0.15 mg/ml RNase A, they were incubated at 50°C for 1 h. The sample was extracted with phenol/chloroform, with 3 M sodium acetate and ethanol added, and incubated overnight at -20°C. The precipitated DNA was suspended in a TE buffer (10 mM Tris, pH 7.4 and 1 mM EDTA), and the amount of DNA measured by UV spectrophotometry. The genomic DNA underwent electrophoresis in a 1.8% agarose gel. After the gel was stained with ethidium bromide, apoptosis was observed.

6) Western blot analysis

The cells were lysed in 20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The total protein was determined by the Bradford method. Twenty μg of each protein sample was separated by electrophoresis on a 10% SDS-polyacrylamide gel, and then transferred to nitrocellulose filters. The blots were incubated with the following antibodies: anti-p53, anti-PLK, anti-cyclin B1, anti-Bax, anti-Bcl-XL, anti-Bcl-2, anti-PLK, anti-PARP and anti-GAPDH. The nitrocellulose membranes were then blocked with 5% non-fat dry milk, in TTBS buffer (25 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.05% Tween-20), a nd subsequently incubated with the indicated antibodies for 4 h at 25°C. The membranes were washed 3 times, for 10 min, and incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase. The membranes were washed, and then developed using a chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ), exposed to X-ray film (Kodak) and fluorography performed.
In order to evaluate the differential sensitivities of cell survival, and the potential role of several markers in Taxol induced apoptosis, various studies on p53 expressed osteogenic sarcoma U2OS and p53 defected SaOS2 cell lines were performed.

To investigate the cell viabilities of the anticancer drugs in the U2OS and SaOS2 cells, the cytotoxicities were determined, and compared, by exposing the cells to various concentrations of Taxol, 6-mercaptopurine and adriamycin. The comparative effects of Taxol, 6-mercaptopurine and adriamycin, on the viability of osteogenic sarcoma U2OS and SaOS2 cells, are shown in Fig. 1. The cells were treated for 24 h with various concentrations of each anticancer drug, and the cell viabilities measured with the XTT assay.

The cell viabilities in U2OS cells were 100, 98, 95, 90, 88 and 82%, by the treatment with 0, 5, 10, 50, 100 and 200 nM of Taxol, respectively. In contrast, the cell viabilities in the SaOS2 cells, caused by the same Taxol concentrations, were 100, 80, 50, 45, 35 and 30%, respectively. The viability of the p53-defected SaOS2 cells was significantly decreased. However, the viabilities of the cells treated by 6-mercaptopurine were no different between the U2OS and SaOS2 cells. The viabilities of the cells treated by adriamycin were slightly different between the two cell types when treated with more than 2µg/ml (Fig. 1). These results revealed that the cell viabilities expressed stronger toxicity in the p53-defected SaOS2 than in the p53-expressed U2OS cells.

To examine whether the differentiations of apoptosis caused by Taxol were induced in p53-expressed or -defected cells, DNA fragmentation assays were performed on both cytosolic and genomic DNAs. The cells were treated with 0~50µM Taxol, for 24 h, followed by isolation of the cytosolic DNA. The DNA fragments were separated on a 2% agarose gel, and detected by ethidium bromide staining. Treatment with Taxol induced a step ladder-like pattern of DNA fragments, which is a biochemical hallmark of apoptosis, consisting of multiples of approximately 180~200 base pairs, in a dose-dependent manner, in the SaOS2 cells, but this was insignificant in the U2OS cells (Fig. 2A). To confirm the large molecules of DNA fragmentation, genomic DNA was isolated from Taxol-treated cells. One µg of purified genomic DNA underwent electrophoresis in a 2% agarose gel, and the results observed. Apoptosis of the genomic DNA also induced the dose-dependent effects of Taxol on the DNA fragmentations in osteogenic sarcoma cells. The step-ladder genomic DNA fragmentation was predominantly observed with Taxol concentrations above 10µM in p53-defected SaOS2 cells (Fig. 2B).

After the cells were treated with 5µM Taxol, for 6, 12, 24 and 48 h, the expression of the p53 protein in the cells was compared, using Western blot analysis with the anti-p53 antibody. The p53 was well expressed in the untreated U2OS cells, which decreased in a time-dependent manner, but not in the SaOS2 cells. We confirmed the deletion of the p53 gene in SaOS2 cells (Fig. 3). The deletion of the p53 gene, revealed by the inhibitory effect on the cell cycle progression of Taxol and the effect of apoptosis were observed.

PARP, when used as a substrate, was cleaved by caspase 3, into two fragment molecules of 24 kDa and 89 kDa. As the cleavage of PARP by caspase 3 is primarily responsible for apoptosis, the cleavage of PARP in the U2OS and SaOS2 cells

Fig. 1. Comparative effects of Taxol, 6-mercaptopurine (6-MP) and adriamycin on the viabilities of osteogenic sarcoma U2OS and SaOS2 cells. Cells were treated with various concentrations of each anticancer drug for 24 hours, and the cell viabilities measured with the XTT assay.
Fig. 2. Dose-dependent effect of Taxol on the DNA fragmentations in osteogenic sarcoma cells. (A) Cells were treated with various concentrations of Taxol for 24 hours, followed by the isolation of the cytosolic DNA. DNA fragments were separated on a 2% agarose gel, and detected by ethidium bromide staining. (B) Osteogenic sarcoma cells were treated as in (A), followed by the isolation of the genomic DNA. 1µg of purified genomic DNA underwent electrophoresis on a 2% agarose gel, and was stained with ethidium bromide.

Fig. 3. Time-dependent effect of Taxol on the induction of p53, the cleavage of poly (ADP) ribosyl polymerase (PARP) and the expressions of cyclin B1 and polo-like kinase (PLK), in osteogenic sarcoma U2OS and SaOS2 cells. The cells were treated with 10µM Taxol, or left untreated, for the times indicated, followed by Western blot analyses with anti-p53, -PARP, -cyclin B1 and PLK antibodies. GAPDH was used as an internal standard.

was compared using Western blot analyses, with anti-PARP antibodies. When the cells were treated for 48 hour with 10µM concentration of Taxol, the 89 kDa cleavage product of PARP clearly appeared, in a time-dependent manner, in the SaOS2 cells, but not in the U2OS cells.

The antitumor effects of Taxol correlated with the increased tubulin polymerization and cell cycle arrest at the G2/M phase. We compared the expressions of polo-like kinase (PLK) and cyclin B1. These expressions were more increased in the SaOS2 than the U2OS cells. GAPDH was used as an internal standard (Fig. 3).

To investigate whether bcl-2 family members are associated with Taxol-induced apoptosis in U2OS cells, the expression levels of Bax, Bcl-xL and Bcl-2 were determined by Western blot analyses. Bax, which has a pro-apoptotic nature, was highly up-regulated in the SaOS2 compared with the U2OS cells. In contrast, Bcl-xL, which has an anti-apoptotic nature, was clearly down-regulated in the SaOS2 compared with the U2OS cells. However, Bcl-2, which is also anti-apoptotic in
nature, did not change dramatically (Fig. 4).

**DISCUSSION**

Taxol, an antimicrotubule agent, has been shown to be efficient in the treatment of ovarian and metastatic breast cancers, and in malignant melanomas (14). Taxol stabilizes microtubule formation, and continuous treatment prevents the completion of mitosis, resulting in cell cycle blockage in the G2/M phase (10).

Apoptosis is an active form of cellular suicide, and is morphologically and biochemically distinct from cell death that occurs under a variety of physiological and pathological conditions (15). Apoptosis can play a crucial part in cell proliferation, and turnover, in various cancers (16). Many of the chemotherapeutic drugs, and radiation, which cause DNA damage, are known to induce apoptosis (17). The induction of apoptosis, in Taxol-treated tumor cells, has been proposed to result from the increased nuclear DNA fragmentation.

In this paper, an increase in the cytotoxicity and apoptotic DNA fragmentation, induced by Taxol, were demonstrated, especially in the p53-defected osteogenic sarcoma cells. The cytotoxic effect of Taxol, compared with those of 6-MP and adriamycin, in p53-defected and p53-expressed cells, the p53-defected were more sensitive to the cytotoxicity induced by Taxol. The roles of p53, when treated with Taxol, were the induction of G2/M arrest, and the increase in the cytotoxicity and DNA fragmentation of the p53-defected osteogenic sarcoma cells. The p53-defected cells had a significantly increased apoptotic induced effect than the p53-expressed cells. These results were confirmed by an increase in the caspase activity caused by the PARP substrate.

PARP is a chromatin-bound nuclear enzyme, which on activation by DNA strands breaks, and catalyzes, the successive transfer of ADP-ribose units from NAD+ to nuclear proteins (18). PARP, molecular weight 116,000 Dalton (Dal), is composed of two fragments, one is approximately 25,000 Dal, containing the amino-terminal DNA-binding domain, and the other is approximately 89,000 Dal, containing automodification and the catalytic domains (19). Apoptotic death is required to control the degradation of cellular macromolecules due to hydrolytic enzymes, therefore proteases play a central role in this process. These proteases are collectively referred to as caspases (20). The activity of PARP revealed that exogenous nicked DNA did not activate the approximately 89,000 Dal fragment, but retained its basal activity. Similar cleavage of PARP was observed after exposure of HL-60 cells to a variety of chemotherapeutic agents, including cisplatin, colcemid, 1β-D-arabino furanosylcytosine and methotrexate (15). A comparison of the apoptosis in the wild-type p53 HNE-1 to that of the p53 mutant CNE-2 cell line of a human nasopharyngeal carcinoma (NPC) has been reported (21). The above research confirmed that up-regulation of the p53 protein occurred with low-dose paclitaxel treatment, whereas high-dose paclitaxel treatment inhibited the p53 expression. The cell growth and apoptosis, induced by a high dose of the drugs, occurred independently of the p53.

In our present study, the proteolytic cleavage of PARP, due to caspase activation, was confirmed to be expressed more in the SaOS2 than in U2OS cells, and in a time-dependent manner with high-doses of Taxol. High expression of cyclin B1 and PLK were independently revealed, with p53, in the SaOS2 cells. The expression of p53 in the U2OS cells, caused by Taxol, was decreased in a time-dependent manner, but the expressions of PARP and PLK remained unchanged, whereas that of cyclin B1 was mildly increased 6 hrs after Taxol treatment. These results suggest the differential expressions of the U2OS and SaOS cells were related to the dose of Taxol.

Several genes, involved in the division of cells, and in causing cancer, can play regulatory roles in the induction of apoptosis (22). The wild type p53 has been shown to induce apoptosis when overexpressed in cultured cells. In contrast, in cases of p53 loss or mutation, p53-dependent apoptosis is lost, leading to the rapid proliferation and growth of tumors (23). In renal cell carcinomas, apoptosis is closely associated with cell proliferation, but not with the expressions of p53 and Rb proteins (24).

It is known that bcl-2 exerts a protective effect against apoptosis. Paclitaxel has been shown to down regulate the expression of bcl-2, induce bcl-2 phosphorylation and promote apoptosis, in prostatic cells. Paclitaxel induces apoptosis, and G2 arrest, in SaOS2 cells. However, a decrease in the Bcl-XL protein levels, or a change in its phosphorylation state were observed following paclitaxel treatment (25).

In our results, Bax, as a proapoptotic factor in SaOS2 cells, was increased, and Bcl-xL and Bcl-2 were decreased, when cells were exposed to 10μM Taxol.

**CONCLUSIONS**

This study explored the induction mechanism of Taxol cytotoxicity in osteogenic sarcoma p53-expressed U2OS, and p53-defected SaOS2 cells. To find if the differential expressions of the various apoptotic markers were related to some function of these cytotoxic mechanisms, the apoptosis of U2OS and SaOS2 cells, caused by Taxol, were compared. The Taxol-induced apoptosis in the p53 defected-osteogenic sarcoma cells was associated with PARP cleavage as a result of the increased caspase activity, the high expressions of cyclin B1 and PLK, and the upregulation of Bax. From these results, we concluded that p53-defected SaOS2 cells were much more sensitive to Taxol-induced apoptosis than p53-expressed U2OS cells.

**REFERENCES**


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