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MECHANISM OF TAXOL-INDUCED APOPTOSIS IN HUMAN BREAST CANCER CELLS

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Objective: To investigate the mechanism by which taxol induces apoptosis in human breast cancer cells. Methods: Cell morphology, agarose gel electrophoresis, flow cytometry, video time-lapse monitor and Western blot were performed for investigating taxol-induced apoptosis in human breast cancer cells (BCap 37). Results: BCap 37 cells treated with taxol (100 nm) underwent the arrests of cell mitosis at metaphase of mitosis and induction of apoptosis. Apoptotic cells demonstrated cell shrinkage, condensation or fragmentation of chromosomes. Nuclear DNA of apoptotic cells displayed ladder bands characteristic of internucleosomal DNA fragmentation. The expression of *bcl*-2, inhibitor of apotosis, was decreased with modification, while that of bax, inducer of apoptosis, increased only at early stage of the apoptotic pathway and decreased later. Conclusion: In human breast cancer cells the induction of apoptosis by taxol was closely associated with mitotic arrest of cell cycle, and altered expressions of *bcl*-2 and bax gene possibly played an important role in regulating taxol-induced apoptosis.

Key words: Taxol, Breast neoplasm, Apoptosis.

Taxol, or Paclitaxel, originally isolated from the bark of the Pacific yew, Taxus brevifolia, is a new agent against cancer. Recently, clinical trials have demonstrated that taxol is effective against a variety of human cancers, particularly including drug- refractory ovarian cancer and metastatic breast cancer. In leukemia and ovarian cancer cell lines tested *in vitro*, taxol cytotoxicity was found to be associated with induction of apoptosis.[1,2] Although the results obtained *in vitro* and *in vivo* studies showed that taxol possessed growth inhibition and killing action upon breast cancer cells, the taxol-induced apoptosis in human breast cancer cells was not yet reported in publication. In the present study, we aimed to investigate the morphologic pattern and alteration of cell cycle and apoptosis-related gene expression induced by taxol and to provide a better understanding of the treatment of breast cancer with taxol.

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MATERIALS AND METHODS

Cell Culture and Drugs

Human breast cancer cell line (BCap 37) was grown in DMEM medium (Gibco, USA) supplemented with 10% foetal bovine serum (Hyclone, USA) and 1% antibiotic solution consisting of penicillin and streptomycin. The cells were incubated under humidified condition with 5% CO_2 at 37℃. Taxol was purchased from Calbiochem Company (USA) and dissolved in 100% dimethyl sulfoxide (DMSO) to make a stock solution. The working concentration varied from 10 nmol/L to 1000 nmol/L. The molecular weight and purity of taxol were 853.9 and 94.7% (HPLC) respectively.

Light Microscope Examination

BCap 37 cells were trypsinized and collected after treatment with taxol at indicated times. They were cytospined and fixed in methanol for 15 min at RT. The slides were stained with Wright-Giemsa.

Determination of Internucleosomal DNA Cleavage

Internucleosomal DNA fragmentation was assayed by a modification of previously described methods.[3] After treatment with taxol, a 6×10^6 of BCap 37 cells per group were harvested by trypsinization and washed twice with PBS at 4℃. The cells were suspended in 500 μl of lysis solution containing 5 mmol/L Tris-HCl, 20 mmol/L EDTA and 5% (v/v) Triton×100 for 30 min on ice. After centrifugation, supernatant was extracted by phenol/chloroform/ isomyl alcohol (25:24:1) and precipitated by pre- cold alcohol. The extraction and precipitation were performed again following RNase digestion. DNA pellets dissolved in 10 μl of TE buffer (pH 8.0) were electrophoresed in 1.5% agarose gel.

Flow Cytometry Analysis

Cell sample preparation and propidium iodide (PI) staining for flow cytometry analysis were performed according to the method described by Nicoletti et al.[4] Cell cycle distribution was determined, using a counter Epics Elite instrument (USA) with an argon laser set to excite at 488 nm. The results were analysed with Elite 4.0 and DNA multicycle software.

Time-lapse Video Phase Contrast Microscopy

BCap 37 cells in 35 mm dishes were placed on the stage of an inverted microscope. This microscope was equipped with a heated/recirculation device that maintained stage temperature at 37±0.5 ℃ and a constant through-flow of 95% air/5% CO_2. The observation and recording of cell morphology were lasted over 72 h. Time lapse video recordings were carried out at a 1:720 time lapse ratio.[5]

Western Blot Assay

Protein extraction and Western blot were performed according to the method described.[3] A 6×10^6 of BCap 37 cells each group was washed twice with cold PBS. Then, the cells were dissolved in cell lysis solution containing 2% SDS, 0.15 mol/L NaCl, 10 μmol/L Tris (pH 7.5), 1 mmol/L EDTA for 30 min on ice. Protein concentration assay was carried out using total protein diagnostic kit (Sigma, USA) and spectrometry with a wavelength at 578 nm. After heat denaturation for 5 min, 40 μg of protein per well was electrophoresically separated by 10% or 12% SDS-PAGE and transferred to PVDF membrane. Then, it is blocked in TBST buffer containing 5% nonfat milk at RT for 60 min. The membrane was incubated in primary antibody solution for 3 h, including monoclonal antibody (McAb) *bcl*-2 (1:100, Dako), polyclonal antibody bax (1:5, Santa Crutz), McAb p53 (1:50, Santa Crutz) and β-actin (1:1000, Sigma). After washed with TBST buffer for 30 min, the membrane was incubated in secondary antibody solution for 40 min, which was anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (1:1000, Amersham). Finally, the membrane was treated with enhanced chemoluminescence (ECL) and exposed to a Kodak film.

RESULTS

Morphologic Examination

After treated with taxol (100 nmol/L) for 48 to 96 h, most cells became shrunken, with basophilic cytoplasm and increased nuclear density. Moreover the cleavage of nuclear chromosomes usually formed intracytoplasmic nuclear bodies in different sizes. Some cells presented morphologically mitotic arrest at metaphase, such as, a disappearance of nuclear membrane, centered nuclear chromosomes and slight basophilic cytoplasm.

Examination of DNA Fragmentation

Dose-response analysis demonstrated that BCap 37 cells treated with 50 nmol/L or more in concentration of taxol for 72 h produced a typical DNA "ladder", which is the fragmentation of genomic DNA into integral multiples of 180 bp units. As showed in Figure 1, the density of DNA bands was increased with an increase of taxol concentration. To determinate minimum exposure time required for taxol to trigger apoptosis in BCap 37 cells, DNA fragments of the cells exposed to taxol at different times were

detected. Figure 2 demonstrated that BCap 37 cells exposed to taxol for only 30 min was enough to induce the cells to have DNA fragmentation and finally apoptosis.

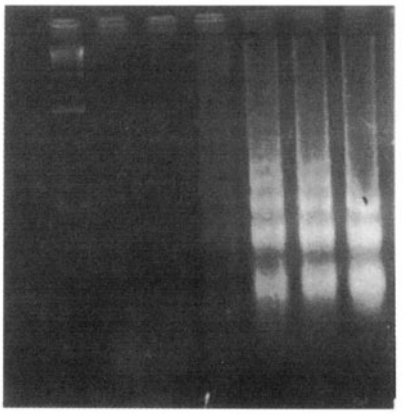

Fig. 1. Dose-response of taxol-induced internucleosomal DNA fragmentation in BCap 37 cells.

Cell Cycle Distribution Analysis

After BCap 37 cells treated with taxol (100 nmol/L) for 3 h, there was an increase of G_2/M phase percentage. The G_2/M phase percentage was 55.5% at 48 h. However, the sub-G_1 peak, which represents cellular DNA degradation, accounted for 9.2% of total cell population (Table 1).

Videomonitoring Observation

BCap 37 cells treated with taxol (100 nmol/L) gradually became round and dioptric. Nuclear chromosomes presented a ring-like arrangement, which is a characteristic of the mitotic arrest of cell cycle. Then, apoptosis was identified by the onset of cellular budding followed by cytoplasmic disruption and cell shrinkage. The relationship between the mitotic arrest and apoptosis was shown in Figure 3.

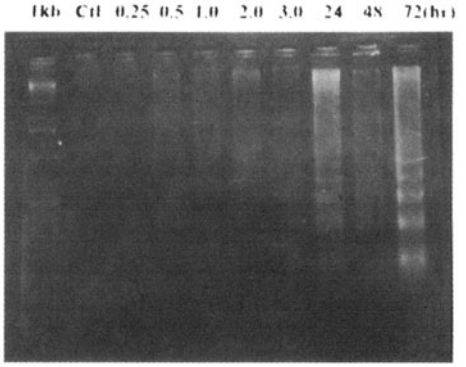

Fig. 2. Minimum exposure times required for taxol to trigger apoptosis in BCap 37 cells.

Table 1. Cell cycle distribution analysis of taxol-treated BCap 37 cells at different times (%)

Cell cycle	Control	Times (h)				
		3	12	24	48	72
Sub-G_1	0	0	1.1	1.5	7.3	9.2
G_0/G_1	51.3	56.7	44.1	18.1	11.2	28.2
S	36.0	27.9	37.8	44.4	33.3	45.2
G_2/M	12.7	15.3	18.0	37.0	55.5	26.6

Apoptosis-associated Gene Expression

Bcl-2 protein expression in BCap 37 cells was decreased at 3, 12 and 24 h after treatment with taxol. The remarked decrease of *bcl*-2 protein expression continued, and *bcl*-2 protein with retarded modification appeared at 48 h. At 72 h, *bcl*-2 protein expression was decreased below control level and the modified *bcl*-2 disappeared shown in Figure 4. At same duration, bax protein expression was increased at 3, 12 and 24 h of taxol treatment. At 48 and 72 h, bax protein expression was diminished close to control level. However, no alteration of p53 gene expression was induced in taxol-treated BCap 37 cells.

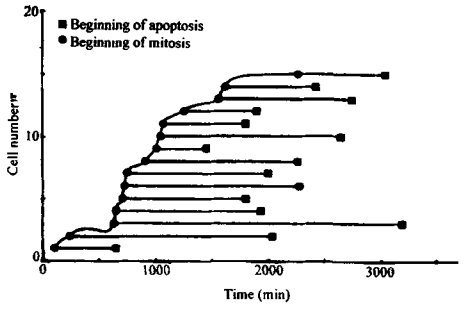

Fig. 3. Relationship between mitotic arrest and apoptosis in BCap 37 cells examined by video time-lapse microscope.

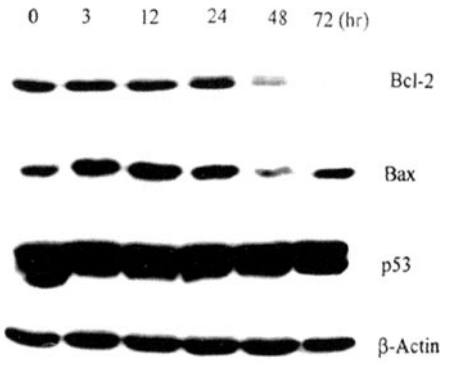

Fig. 4. Apoptosis-associated gene expression in taxol-treated BCap 37 cells at indicated times detected by Western blot. β-actin used as internal control.

DISCUSSION

Recent studies have found that taxol cytotoxicity against malignancies was related to the induction of apoptosis in addition to its anti-microtubule action. Bhalla et al. reported that taxol induced HL-60 cells into programmed cell death followed by the arrest of mitotic phase.[1] Donaldson et al. showed that the cells synchronized at G_0/G_1 or G_2/M phase, after exposure to taxol, both experienced the mitotic arrest and induction of apoptosis within 20 h, suggesting that the mitotic arrest be an important prerequisite for apoptosis, rather than the length of sustained arrest in mitosis.[6] In the present study, we found that taxol treatment resulted in BCap 37 cells arrested at the late G_2/M phase prior to the induction of apoptosis. Nevertheless, the time length when the cells treated with taxol entered the mitotic arrest and then apoptotic processes was not equal in each cell. This result suggested that the mitotic arrest of cells treated with taxol is plausible an important initiating step for taxol-induced apoptosis in BCap 37 cells, and might activate apoptosis-promoting factors leading to cell death.

Previous studies indicated that the primary intracellular action of taxol is to bind specifically to cytoplasmic microtubules. Horwitz et al. demonstrated that when cells were incubated with [^{3}H] taxol for 60 min, the specific binding reached saturated state, and then, cells were washed and cultured in taxol-free medium for 90 min, the binding of [^{3}H] taxol to microtubules was found to be almost completely reversible.[7] Our investigation found that BCap 37 cells exposed to taxol (100 nmol/L) for only 30 min, after washed twice and cultured in drug-free medium for 72 h, were still killed by apoptosis, indicating that taxol-induced mitotic arrest and apoptosis might be independent of drug concentration. This was helpful hint for developing a new clinical strategy of taxol administration, such as, external impulsive therapy, which might be suitable for treating local proliferative lesions, so that it may reduce the side effect of taxol.

Oncogene *bcl*-2 (B cell lymphoma/leukemia-2) is originally cloned from the breakpoint of a t(14:18) chromosomal translocation, codes for a 26 kd protein, it functions as a critical inhibitor of apoptosis in lymphoid and non-lymphoid tissues. In taxol-induced apoptosis of HL-60 cells, *bcl*-2 protein expression is decreased. Recently it was reported that during the process of taxol-induced apoptosis, expression of *bcl*-2 protein was not only decreased but also phosphorylation of *bcl*-2 protein happened. The phosphorylation of *bcl*-2 protein was characterized by a modification up main band in Western blotting, which was thought as an inactivated form.[8] We also found the phosphorylation modification of *bcl*-2 protein appeared in taxol-induced apoptosis of BCap 37 cells. Appearance of decreased expression and phosphorylation modification was linked with apoptotic processes, suggesting that these alterations of *bcl*-2 gene were associated with taxol-induced apoptosis of BCap 37 cells.

Bax gene is a member of *bcl*-2 gene family. Bax gene is similar to *bcl*-2 in molecular weight, but their amino acid homology is very low. Functionally, bax gene acts in opposition to *bcl*-2, which is an inducer of apoptosis. Both Yin and Hanada et al. thought that *bcl*-2 protein inhibited apoptosis by heterodimer with Bax.[9,10] It is not yet clear whether

bax gene involves in taxol-induced apoptosis. In this article, we demonstrated that increased expression of bax was seen in taxol-treated BCap 37 cells at 3, 12 and 24 h. At 48 and 72 h, bax expression was decreased close to control level. Above results indicated that in taxol-induced apoptosis, the enhancement of bax expression and diminishing of *bcl*-2 expression trigger apoptotic pathway of BCap 37 cells. In the late stage of taxol-induced apoptosis, the activation of apoptosis-associated factors other than bax plays an important role in DNA fragmentation. It was noted that p53 protein expression did not alter in taxol-induced apoptosis of BCap 37 cells.

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