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ORIGINAL PAPER

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Involvement of mitochondrial pathway in Taxol-induced apoptosis of human T24 bladder cancer cells

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Abstract We examined a human urothelial cancer T24 cell line, which was exposed to clinically achievable concentrations of Taxol and detected the lethal effect of Taxol as measured by a cytotoxic dose-response curve. Marked nuclear condensation and the fragmentation of chromatin were observed by DAPI stain, DNA ladder formation, and flow cytometry at an LC_{90} concentration of 0.8 μg/ml Taxol, which also induced a G2/M arrest. In response to Taxol-treatment, caspase-9 activity increased at 8 h, and both caspase-2 and -3 activities were increased twofold relative to control cultures at 16 h. Moreover, treatment with the broad-spectrum caspase inhibitor (z-VAD-fmk) or the caspase-9 specific inhibitor (z-LEHD-fmk) effectively protected T24 cells against Taxol-triggered apoptosis. Furthermore, the phosphorylation of Bcl-2 and Bcl-X_L proteins in Taxol treated cells was detected at 8 h. In contrast, Taxol had no effect on the levels of Fas and FasL proteins and neither antagonistic, anti-Fas antibody affected Taxol-induced apoptosis. These results suggest that, following the phosphorylation of Bcl-2 and Bcl-X_L proteins, Taxol-induced apoptosis is induced through the mitochondria-dependent pathway in T24 cells.

Keywords Taxol · Urothelial cancer · Apoptosis · Bcl-2 · Caspases

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Introduction

Taxol (paclitaxel), one of the most clinically effective anti-neoplastic agents, has shown significant chemotherapeutic effects in the treatment of various cancers such as, urothelial cancer, metastatic breast cancer and ovarian cancer [4, 31]. Taxol is a microtubule disturbing agent that increases microtubule stability, decreases microtubule disassembly and increases mitotic arrest which precedes apoptosis [16].

Apoptosis is a morphologically and biochemically distinct form of programmed cell death that occurs in many cell types after exposure to toxic stimuli [1, 38]. Previous studies have demonstrated that aspartate-directed cysteine proteases, called caspases, play pivotal roles in the initiation and completion of this process [7, 8, 9, 20, 32, 35]. In particular, caspase-triggered cleavage destabilizes structural components of the cytoskeleton, inactivates key components of DNA repair pathways, and interrupts signal transduction pathways involved in cell survival [9]. At the same time, caspases can also activate several enzymes, such as caspase-activated deoxyribonuclease [11], gelsolin [19, 22] and kinases [9]. Consequently, this cleavage contributes to the stereotypic, morphological and biochemical changes [38] that constitute the process of apoptosis.

Recent studies have implicated Bcl-2 and its homologs as critical regulators of the cell death pathway [39]. The bcl-2 gene encodes for a 26 kDa protein, which suppresses apoptosis [39]. Likewise, its homolog bcl-X_L has revealed its structural similarity to the bacterial, pore-forming proteins that act as channels for either proteins or ions [27]. Furthermore, high intracellular levels of Bcl-2 or Bcl-X_L have been shown to inhibit apoptosis due to chemotherapeutic agents, including Taxol [26, 39]. Bax is a proapoptotic member of the Bcl-2 family, and dimerizes with itself or with Bcl-2 or Bcl-X_L [28]. The balance between Bax homodimers that favor death and Bcl-2/Bax or Bcl-X_L/Bax heterodimers that inhibit cell death is critical [39]. Recently, Taxol has been shown to activate Raf-1

and cause the phosphorylation of Bcl-2 in a number of human cancer cell lines [2]. This has been claimed to inactivate bcl-2 and correlate with Taxol-induced apoptosis [15]. In addition, some model systems suggest that the stimulation of apoptosis, due to the phosphorylation of Bcl-2 and Bcl-X_L, induces the permeabilization of the mitochondrial outer membrane. This allows the release of cytochrome *c* into the cytosol together with the adaptor molecule Apaf-1, as well as the dimerization and activation of caspase-9 proenzyme [9, 35]. Active caspase-9 subsequently activates caspase-3 [24]. Thus, we examine here whether Taxol can induce apoptosis in the bladder cancer cell line, T24. Cells were treated with Taxol and apoptosis was studied by morphological and biochemical procedures.

Materials and methods

Drugs and reagents

Taxol and DAPI (4′, 6-diamino-2-phenylindole) were purchased from Sigma (St. Louis, Mo.). Stock solution (1 mg/ml) was prepared in ethanol and stored in the dark. z-VAD-fmk (Calbiochem, San Diego, Ca) and z-LEHD-fmk (Karmiya, Biomedical, Seattle, Wa) were prepared in 50 mM and 200 mM in DMSO stock solution, respectively. Fetal bovine serum (FBS), L-glutamine (200 mM) and penicillin/streptomycin/amphotericin B (10,000 IU/ml and 10,000 μg/ml, 25 μg/ml, respectively) mixed solutions were purchased from Gibco. Protein concentration was assayed using the Bio-Rad Laboratories (Hercules, Calif.). Anti-human Bax polyclonal antibody and anti-human Bcl-2 monoclonal antibody were purchased from DAKO (Carpinteria, Ca). Anti-human Fas monoclonal antibody, anti-human Fas-L monoclonal antibody, and anti-human Bcl-X_L polyclonal antibody were purchased from Transduction Laboratories. Anti-human cytochrome *c* monoclonal antibody (7H8.2C12)/was obtained from Pharmingen. The monoclonal anti-human Fas IgG 1 (clone ZB4) was obtained from Upstate Biotechnology (Lake Placid, N.Y.).

Cell culture and viability assay

Human bladder cell line T24 (p53 mutant) [5] was obtained from ATCC (American Type Culture Collection). Cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were split every 2 days using 0.25% (v/v) trypsin solution (Gibco). To evaluate cell viability, cells were seeded at a density of 3×10^4 cells/well in 12-well culture plates. After 24 h, the cells were treated with various concentrations (1–10 μg/ml) of drugs. Cell number was counted by the Trypan blue dye exclusion method.

Morphological assessment of cell death

T24 cells were treated with 1 μg/ml Taxol for 24 h. They were then washed with PBS and fixed in 2% paraformaldehyde for 30 min before being permeabilized with 0.1% Triton X-100/PBS for 30 min at room temperature. Nuclei were stained by incubating the cells with DAPI (1 μg/ml). Stained cells were examined under an Olympus fluorescence microscope.

Flow cytometry assay

T24 cells, subjected to different treatments, were harvested using 0.25% (v/v) trypsin and centrifugation at 2,000 *g* for 10 min. Cells (1×10^6) were suspended in citrate buffer, and first incubated in 30 μg trypsin in stock solution for 30 min at room temperature. Secondly, the cells were incubated in 0.5 ml solution B (500 μg trypsin inhibitor, 100 μg RNase in stock solution) for 20 min at room temperature, and then with 0.5 ml solution C (100 μg PI, 1.16 mg spermine tetra-hydrochloride in stock solution) for 20 min at 4°C. The apoptotic cells were evaluated by flow cytometry on a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif.). Data were analyzed using the Lysis II program.

DNA fragmentation assay

Cells treated at different concentrations and time points were collected using 0.25% (v/v) trypsin and centrifuged at 2,000 *g* for 10 min. After washing with PBS, each pellet was lysed in 800 μl lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, and 0.3% Triton X-100) and incubated for 30 min on ice to allow complete cell lysis. Cell lysates were treated with RNase (0.1 mg/ml) for 30 min at 37°C, then treated with proteinase K (0.4 mg/ml) for 1 h at 55°C. The supernatant was extracted with phenol/chloroform. The DNA was precipitated and electrophoresed on 2% agarose gels.

Western blot assay

Cells were treated with Taxol for 1, 2, 4, 8, 16 or 24 h. After incubation, they were resuspended in protein lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 5 mM EDTA, 1 mM EGTA, 5% β-mercaptoethanol, 5 μg/ml leupeptin, 0.2 mM PMSF, 5 μg/ml aprotinin, 1 mM Na orthovanadate, 1 mM NaF) for 30 min at 4°C. Protein concentrations were determined by the Bradford method. For the immunoblotting of cytochrome *c*, the cells were harvested by centrifugation at 1,000 *g* for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended in five volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride), containing 250 mM sucrose and supplemented with protease inhibitors. After sitting on ice for 15 min, the cells were broken by passing 20 times through a G22 needle, then centrifuged at 10^5 g for 30 min in a tabletop ultracentrifuge (Beckman), and the cytosol fractions were used for immunoblotting. Equal amounts of total protein were applied to 15% SDS-poly-acrylamide gel for Bcl-2, Bcl-X_L, Bax, Fas, Fas-L and cytochrome *c*. The gels were transferred onto PVDF membrane (Millipore). Kaleidoscope prestained standard (Bio-Rad Laboratories) was used to determine molecular weights. The membrane was blocked with 5% non-fat dry milk in TBST buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated overnight at 4°C with specific antibody diluted in TBST. The antibodies were diluted as follows: anti-human Bax polyclonal antibody (1:1500), anti-human Bcl-2 monoclonal antibody (1:100), anti-human Bcl-X_L (1:100), anti-human Fas monoclonal antibody (1:2500), anti-human Fas-L monoclonal antibody (1:1000), anti-cytochrome *c* (1:500). The membrane was washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. Determinations were performed using enhanced chemiluminenscence kits (Amersham, ECL kits).

Determination of caspase activity

We used the colorimetric substrate Ac-YVAD-pNA, Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-IETD-pNA, Ac-LEHD-pNA (R and D) for caspase-1, -2, -3, -8, and -9 assays according to the manufacturer's protocol. Briefly, aliquots of cell lysate were prepared in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA), incubated with 200 μM substrate in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mM

EDTA, 10% glycerol) in 96-well plates at 37°C for 2 h. Absorbance of the cleaved product was measured at 405 nm in a Multiscan Ascent Microplate Reader (Labsystems).

Treatment of caspase inhibitors

The inhibition of specific caspase activity was achieved in vitro using the inhibitors z-LEHD-fmk and z-VAD-fmk which were administered at 200 μM 2 h before the addition of Taxol (0.8 μg/ml). At 24 h after the addition of Taxol, the cells were harvested and analyzed using the Trypan blue dye exclusion method.

Results

Taxol inhibited proliferation of bladder cancer T24 cells

To investigate the effect of Taxol on T24 cell proliferation, the cells were treated for 48 h in medium containing varying concentrations of Taxol. As shown in Fig. 1, Taxol inhibited the proliferation of T24 cells in a dose-dependent manner. The calculated LC_{90} value was 0.8 μg/ml which can normally be achieved in the plasma of a patient during Taxol treatment. To determine whether Taxol-induced cell death in T24 was due to apoptosis, in situ DAPI staining and a DNA fragmentation assay were performed. Typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin and cleaved nuclei, were observed in Taxol-treated T24 cells (Fig. 1B). Moreover, treatment with Taxol resulted in the degradation of chromosomal DNA into small internucleosomal fragments, as evidenced by the formation of 180–200 bp DNA ladders on agarose gels (Fig. 1C).

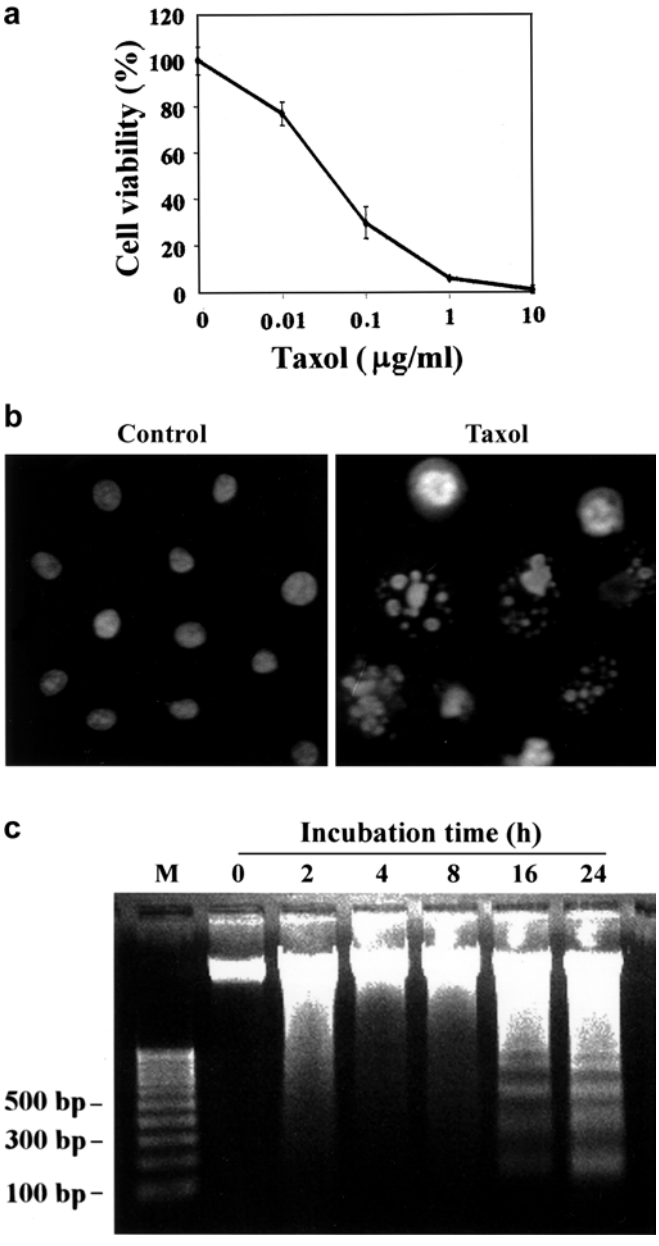

Fig. 1a–c. Taxol induced apoptotic cell death in T24 cells. **a** Cells were plated onto 12-well plates at a density of 3×10^4 cells/well. After 24 h, the cells were maintained in culture medium with 0, 0.01, 0.1, 1 or 10 μg/ml Taxol. The cell number was counted by a haemocytometer after 48 h treatment. Each *point* represents the average of six replicates from two independent experiments. **b** DAPI staining. T24 cells were grown in culture medium with or without 1 μg/ml Taxol for 24 h, then stained with 1 μg/ml DAPI and investigated under a fluorescence microscopy. **c** DNA fragmentation. T24 cells were treated with or without Taxol (0.8 μg/ml) for various periods. After incubation, total DNA was extracted and electrophoresed in 2% agarose gel. *M*, size marker (100 bp DNA ladder)

Taxol induced mitotic arrest and apoptosis

In order to quantify the kinetics of events both in apoptosis and in cell cycle phases, a flow cytometric analysis was performed. T24 cells were treated with 0.8 μg/ml Taxol for 0, 2, 4, 8, 16 or 24 h. The average percentage of apoptosis in untreated T24 cells was 2.3%. After Taxol treatment, the percentage of cells in the G0/G1 cell cycle phase decreased dramatically, while the majority of cells showed a block in G2/M phase, especially at 16 h. The percentage of apoptotic events increased to 49% at 24 h (Fig. 2). The profile indicates the induction of several hypodiploid events, typical of apoptotic cells.

Taxol induced phosphorylation of Bcl-2 and Bcl-X_L proteins

Bcl-2 family proteins (Bcl-2, Bcl-X_L, Bax etc) are important regulators of the apoptotic pathway. We evaluated protein levels of Bcl-2, Bcl-X_L, Bax in Taxol-induced apoptotic T24 cells. Taxol-induced apoptosis was associated with Bcl-2 and Bcl-X_L phosphorylation in several cell lines. Figure 3A shows that phosphorylation started to occur at 8 h in Taxol-treated T24 cells. However, Bax protein expression and phosphorylation were not modified.

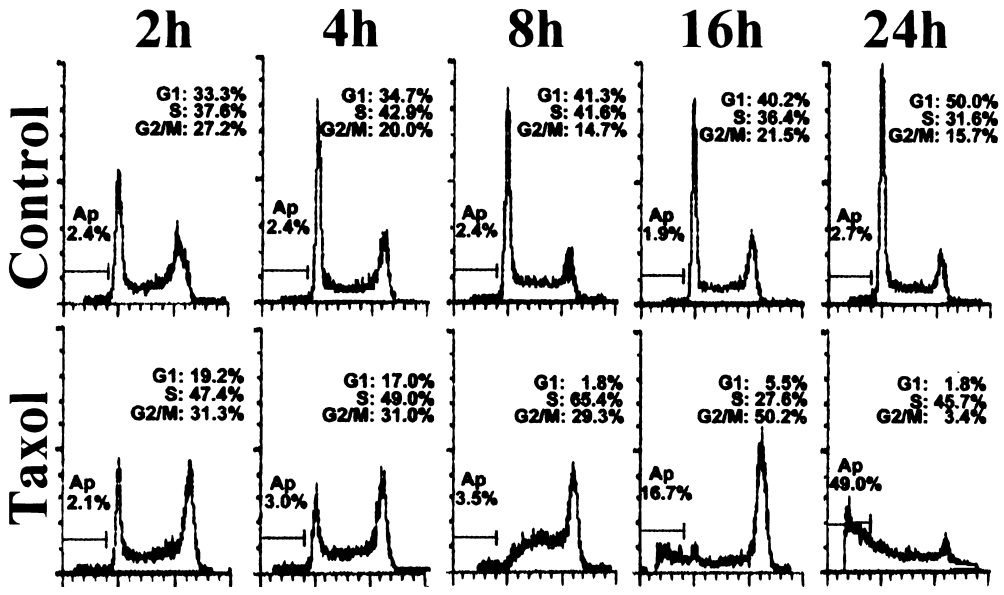

Fig. 2. Effect of Taxol on cell cycle distribution. For flow cytometry assay, T24 cells were treated with or without 0.8 μg/ml, and harvested at 2, 4, 8, 16 and 24 h. The *histograms* were generated from the analysis of propidium iodide-stained cells. The *horizontal* and *vertical axes* refer to log DNA fluorescence intensity and relative cell number, respectively. The percentage of sub-G1 cells representing apoptotic cell is shown

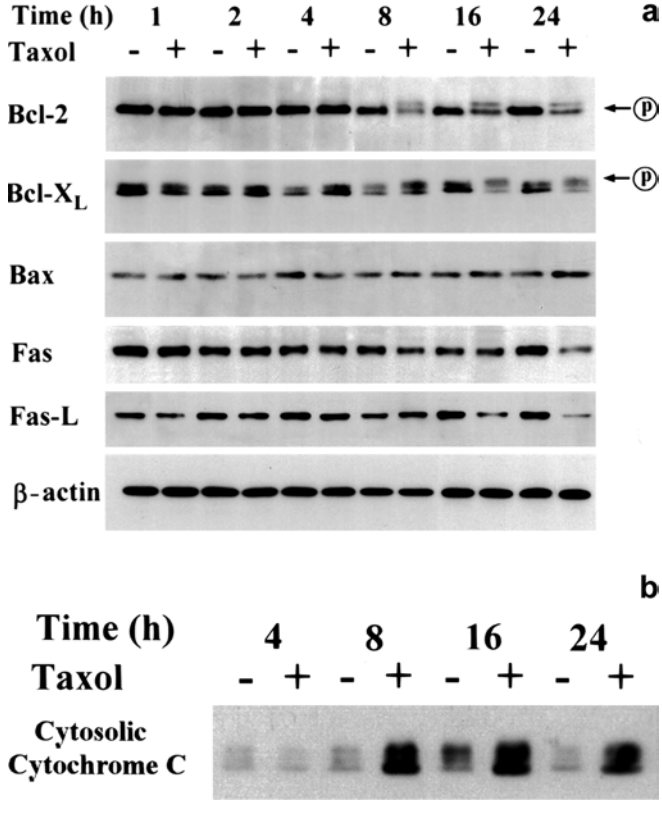

Fig. 3a,b. Modulation of Bcl-2, Bcl-X_L, Bax, Fas, FasL and cytosolic cytochrome *c* proteins by Taxol. **a** T24 cells were treated with or without 0.8 μg/ml Taxol for the indicated time periods. The expression levels of Bcl-2, Bcl-X_L, Bax, Fas and FasL were determined using specific antibodies by Western blot analysis. The *arrow* represents the phosphorylation of Bcl-2 and Bcl-X_L. **b** For cytosolic cytochrome *c* analysis, the cytosolic fractions were separated by SDS-PAGE and Western blot analysis was performed using specific antibody for cytochrome *c*

The release of cytochrome c into the cytosol during Taxol-induced apoptosis

Cytochrome *c*, which is released from mitochondria, has recently been identified as a key or important factor that can activate caspase-3 in a cell-free system. In fact, it has been reported that the levels of cytosolic cytochrome *c* are increased in response to various apoptotic stimuli such as staurosporine, etoposide, and ultraviolet-B irradiation. We therefore tested whether the levels of cytosolic cytochrome *c* increase in response to Taxol. Cytochrome *c* in the cytosol fraction increased significantly after 8 h of being challenged with Taxol, and this increase was detected for up to 24 h (Fig. 3B).

Fas-dependent pathway was not involved in Taxol-induced apoptosis

The Fas receptor and ligand system are considered to be the most common apoptotic triggers in both T lymphocytes and the majority of tumor cells. We investigated the possible role of the Fas/Fas-L system in Taxol-induced apoptosis in T24 cells. We found that the levels of Fas and Fas-L protein were not altered by Taxol (Fig. 3A). To determine whether Taxol induces apoptosis through the activation of Fas receptors at a functional level, we performed a blocking experiment using an antagonistic anti-Fas antibody (ZB4). T24 cells were cultured for 16 h in Taxol (0.8 μg/ml) or in the presence of both Taxol (0.8 μg/ml) and ZB4 (1 μg/ml). The addition of ZB4 (1 μg/ml) did not block Taxol-induced cell death and caspase-3 activity in T24 cell line at the indicated time (Fig. 4A, B).

Involvement of caspases in Taxol-induced apoptosis

To determine whether Taxol activates protease prior to apoptosis, we investigated the cleavage of specific

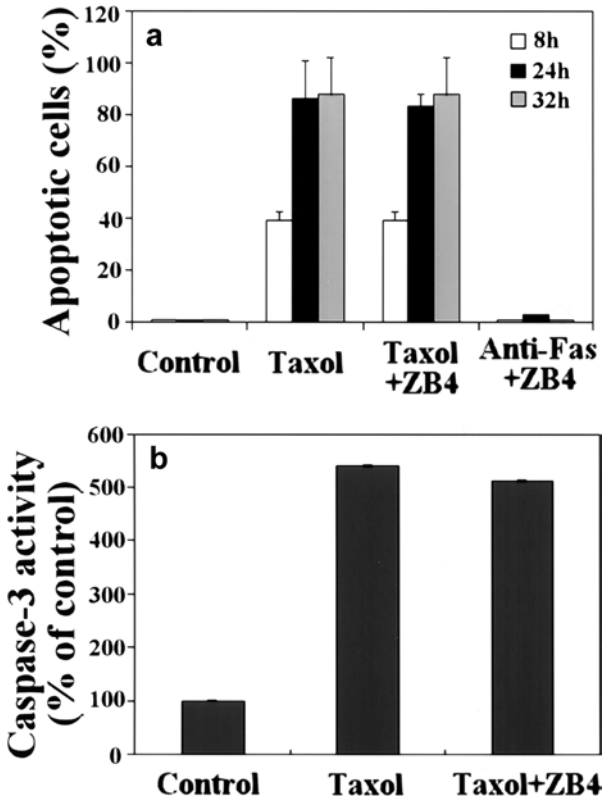

Fig. 4a,b. Antagonistic Fas antibody did not inhibit Taxol-induced caspase-3 activation and apoptosis. **a** T24 cells were incubated with 1 μg/ml ZB4 for 1 h before Taxol addition. After 8 , 24 and 32 h Taxol treatment, apoptotic cell numbers were estimated by DAPI staining. **b** ZB4 antibody 1 μg/ml was added to the cells concomitantly with 0.8 μg/ml Taxol for 16 h, cell lysates were prepared and caspase-3 activity was determined. The caspase-3 activity relative to the control is shown. This is represented as the mean ± SD of six replicates from two independent experiments

substrates for each protease (caspase-1, -2, -3, -8, -9) involved in the execution of apoptosis. As indicated in Fig. 5, there was a 1.75-fold increase in activity of caspase-9 at 8 h and over a twofold increase in the activity of caspase-2 and caspase-3 at 16 h Taxol treatment compared to the control. However, the activity of caspase-1 was not affected by Taxol (data not shown). Moreover, incubation with a broad caspase inhibitor, z-VAD-fmk, had a better effect than any other caspase inhibitor in protection against Taxol- induced cell death (Fig. 6A). In addition, recent evidence suggests that caspase-2 mediated apoptosis requires caspase-9 and that cells co-expressing caspase-2 and a dominant negative form of caspase-9 are impaired in activating a normal apoptotic response and release cytochrome c into the cytoplasm. Therefore, we pretreated cells with or without the addition of caspase-9 inhibitor to the medium before exposure to Taxol. These results showed that the caspase-9 inhibitor significantly inhibited the activities of downstream caspase-2 and -3 (Fig. 6B).

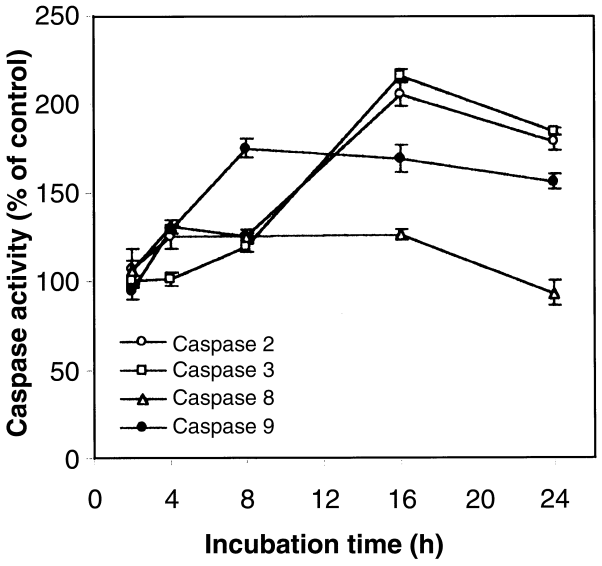

Fig. 5. Activation of caspases during Taxol-induced apoptosis in T24 cells. Cells were exposed to Taxol (0.8 μg/ml) for the indicated time periods and caspase-2, -3, -8, -9 activities were analyzed by colorimetric assay

Discussion

The findings of this study are consistent with previous reports which show that Taxol-induced apoptosis is an important mechanism of cell cytotoxicity in urothelial cancer cells. Cellular apoptosis can be demonstrated by morphological observation, DNA fragmentation and flow cytometry analysis. The mechanisms of activation of apoptosis in different physiological or pathological conditions have been studied intensively [36]. Many genes participate in the regulation of apoptosis. The activation of the caspase cascade is a central effector mechanism promoting apoptosis in response to death-inducing signals from cell surface receptors, from mitochondria, or from endoplasmic reticular stress [35]. The data presented here indicate that the exposure of T24 cells to Taxol results in the release of mitochondrial cytochrome *c* to cytosol and the subsequent activation of caspase-9, -2, -3 (>1.75-fold). Moreover, pretreatment with a broad-spectrum caspase inhibitor (z-VAD-fmk) caused an approximately 85% inhibitory effect on Taxol-induced apoptosis. Furthermore, cells pretreated with a caspase-9 inhibitor significantly inhibited caspase-2 and -3 activities during Taxol-induced apoptosis. These results suggest that caspase-9 lies upstream of caspase-2 and -3, and plays an important role in Taxol-mediated apoptosis. A previous report has suggested that Taxol induces apoptosis by activating the cytochrome *c*/Apaf-1/caspase-9 pathway [29]. Furthermore, the activation of caspase-3, -2, -9 has been demonstrated in C2-ceramide-induced apoptosis of SK-N-MC cells [18]. Some studies have raised the possibility that this anticancer drug might induce apoptosis through a death receptor and caspase-8 activation pathway [13, 14]. A recent report

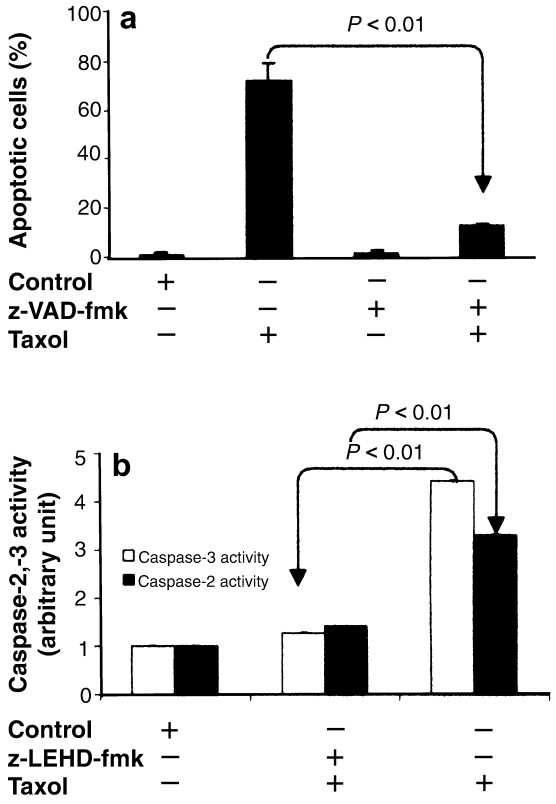

Fig. 6a,b. Inhibition of Taxol-induced apoptosis by caspase inhibitors in T24 cells. Cells were treated either with or without Taxol and with or without being pretreated with caspase inhibitor. **a** Broad-spectrum caspase inhibitor (z-VAD-fmk) 50 μM was added 1 h prior to the addition of Taxol. The data represent the mean ± SD of three independent experiments. **b** T24 cells pretreated for 2 h with caspase-9 inhibitor (z-LEHD-fmk) (200 μM) concomitantly with Taxol (0.8 μg/ml) for 16 h before harvest. Cleavage of the caspase-2, -3 colorimetric substrate was quantitated using an ELISA reader (λ: 405 nm)

demonstrated that activation of caspase-8 occurs downstream of caspase-3 in Taxol-treated MMDA-MB-468 cells [23]. However, in the present study, there was no marked increase in caspase-8 activity in Taxol-treated T24 cells. This conflicting result might reflect the genetic differences between MDA-MB-468 cells and T24 cells or it might depend on the methods used. Our subsequent experiments demonstrated that Taxol did not alter the expression of Fas or FasL proteins, and antagonistic anti-Fas antibodies failed to block the induction of caspase-3 activity and did not affect Taxol-induced apoptosis during Taxol treatment. It provided additional support for the idea that Taxol-induced apoptosis primarily involves the activation of the cytochrome *c*/caspase-9 pathway. Recent evidence has also shown that Fas ligation is not involved in apoptosis induced by etoposide in Jurkat T cells. Another study shows that non-small cell lung cancer cell lines apoptosis mediated by Taxol is independent of the Fas/FasL system despite significant activation of caspase-3 [10, 37]. Furthermore, the Taxol-induced pathway in the MCF-7 human breast cancer line is also consistent with this conclusion [23].

The question remains as to how caspase activation is achieved by Taxol treatment. The Bcl-2 family is composed of a number of genes that play critical roles in the control of mitochondrial integrity, since the loss of mitochondrial membrane potential leads to the release of intermembrane proteins (such as cytochrome *c*, AIF [33] and caspase-2, -9) into the cytosol and induces apoptotic cell death [3, 35]. Increased levels of Bax [25], decreased levels of Bcl-2 [21], or altered phosphorylation of Bcl-2 or Bcl-X_L [15, 29] have been observed in various model systems prior to the onset of apoptosis. In the present study, there were no changes in the levels of Bcl-2, Bcl-X_L or Bax proteins following Taxol treatment, indicating that the amounts and ratios of Bcl-2, Bcl-X_L and Bax were unchanged during Taxol-induced apoptosis. However, the hyperphosphorylation of Bcl-2 and Bcl-X_L proteins was observed, and the timing of appearance of Bcl-2, Bcl-X_L phosphorylation paralleled the time course of cytosolic cytochromec *c* accumulation and caspase-9 activation. The Taxol induced apoptosis has been shown to be associated with the phosphorylation of Bcl-2 and Bcl-X_L in a number of other human cell lines [6, 12, 17, 29], and the mechanism of Taxol-induced Bcl-2 and Bcl-X_L phosphorylation is correlated with the accumulation of cytosolic cytochrome *c* and the activation of caspase-9 [12, 30]. Once activated, caspase-9 can initiate a caspase cascade involving the downstream executioners, caspase-3, -6 and -7 [24, 35].

In summary, we evaluated the regulation of apoptosis related molecules in urothelial cancer T24 cells following Taxol treatment. Taxol induced the activation of upstream caspase-9 and downstream caspase-3, -2 and probably -7 to execute apoptosis in these cells. Thus, the caspase-9 zymogem plays an upstream regulatory role in the interaction of the mitochondria and downstream caspases. However, caspase-8 and the death receptor pathway (Fas and TNF-α systems) do not seem to be involved in apoptosis induced by Taxol. The Taxol-mediated phosphorylation of Bcl-2 and Bcl-X_L proteins is the critical process which disturbs the mitochondrial membrane permeability and activates the downstream caspases which induce cells to undergo apoptosis. Additional studies are needed to learn how Taxol is connected to the mitochondrial pathway and to investigate the relationship between protein kinase and Bcl-2 and Bcl-X_L phosphorylation.

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