Urol Res (2003) 31: 37–44
DOI 10.1007/s00240-003-0300-y

ORIGINAL PAPER

Takeo Nomura · Hiromitsu Mimata
Yusuke Takeuchi · Hideyuki Yamamoto
Eishichi Miyamoto · Yoshio Nomura

The X-linked inhibitor of apoptosis protein inhibits taxol-induced apoptosis in LNCaP cells

Received: 26 June 2002 / Accepted: 18 December 2002 / Published online: 12 February 2003

Abstract To clarify the roles of the X-linked inhibitor of apoptosis protein (XIAP), we investigated the effects of XIAP overexpression on taxol-induced cell growth arrest and apoptosis in the prostate cancer cell line (LNCaP). After the transfection of XIAP cDNA into LNCaP cells, we established clonal cell lines that overexpressed XIAP and examined the taxol effects on growth and apoptosis by 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt and flow cytometric analysis. The effects of XIAP overexpression on caspase-3 were examined by immunoblot analysis and activity assay. The interaction between XIAP and caspase-3 in LNCaP cells was examined by cotransfection with myc-XIAP and caspase-3-HA cDNAs followed by immunoprecipitation and immunoblot analysis. Large amounts of XIAP were expressed in the established cell lines. Although the growth rates were reduced in a dose- and time-dependent manner by taxol, these effects were significantly decreased in XIAP stably overexpressing cell lines. In addition, we found that taxol treatment induced the cleavage of pro-caspase-3, followed by apoptosis, and that the overexpression of XIAP inhibited apoptosis by attenuating the cleavage of pro-caspase-3 and caspase-3 activity. Interestingly, XIAP bound to pro-caspase-3 in LNCaP cells transiently cotransfected with myc-XIAP and caspase-3-HA cDNAs, and this interaction was inhibited by taxol treatment. These results suggest that the overexpression of XIAP inhibits taxol-induced apoptosis through the decrease of caspase-3 activity and inhibition of the processing of pro-caspase-3.

Keywords XIAP · Taxol · Apoptosis · LNCaP

T. Nomura (✉) · H. Mimata · Y. Nomura
Department of Urology,
Oita Medical University,
1-1 Idaigaoka, Hasama-machi,
Oita 879-5593, Japan
E-mail: TAKE@oita-med.ac.jp
Tel.: +81-97-5865893
Fax: +81-97-5865899

Y. Takeuchi · H. Yamamoto · E. Miyamoto
Department of Pharmacology,
Kumamoto University School of Medicine,
2-2-1 Monjo, Kumamoto 860-0811, Japan

Introduction

Prostate cancer is the most frequently diagnosed malignancy. Androgen withdrawal remains the only effective therapy for advanced disease. Unfortunately, androgen independence occurs within a few years in nearly all cases [4]. This remains the main obstacle to improving the survival and quality of life of patients with advanced prostate cancer. Therefore, novel therapeutic strategies targeting the molecular basis of androgen- and chemo-resistance of prostate cancer are required.

Paclitaxel (taxol), a member of the taxane family, inhibits microtubule depolymerization [17], blocks progression through the cell cycle [21], and induces apoptosis in cancer cells. However, clinically objective responses were reported in only 20–30% of cases by taxane-based combination regimens [12]. The cytotoxicity of taxol against tumor cells is reported to occur at least partly via the phosphorylation of Bcl-2, which is then inactivated, promoting apoptosis [9, 11]. Bcl-2 was suggested to be involved in the mechanism of evasion of apoptosis in a prostate cancer cell line [19]. Bcl-2 blocks the release of cytochrome-*c* from mitochondria and subsequently inhibits taxol-induced apoptosis. Caspases act as the execution molecules for apoptosis . These are activated by the release of cytochrome-*c* and destroy specific target proteins critical to cell survival. The Bcl-2 protein cannot inhibit apoptosis once the caspases are activated. The inhibitors of apoptosis proteins (IAPs), originally identified in baculoviruses, are potent inhibitors of caspases and inhibit apoptosis by a variety of lethal stimuli [5, 16]. In humans, five IAPs have been identified: X-linked IAP (XIAP), human IAP-1 (hIAP-1), hIAP-2, survivin, and

neuronal apoptosis inhibitory protein (NAIP) [7, 20, 25]. Among them, XIAP is the most potent inhibitor of caspases and apoptosis [20]. However, the exact mechanisms of XIAP in the inhibition of apoptosis have not been entirely clarified.

In the present study, we established stably XIAP overexpressing transfectants and clarified the inhibitory mechanisms of taxol-induced apoptosis in LNCaP cells.

Materials and methods

Tumor cell lines

Three human prostate cancer cell lines (LNCaP, DU145, and PC3) were cultured in RPMI 1640 (Sigma Chemical, St. Louis, Mo.) supplemented with 10% fetal bovine serum (Bio-Whittaker, Walkersville, Md.), penicillin (50 IU/ml) and streptomycin sulfate (GibcoBRL, Gland Island, N.Y.)(50 μg/ml) at 37°C in a humidified atmosphere enriched to contain 5% CO_2.

Plasmid constructions

The pcDNA3-myc expression vector was kindly provided by R. Takahashi (RIKEN-Brain Science Institute, Wako City, Japan). Total RNAs were prepared from human urinary bladder cancer cells (T24) using TRIzol LS reagent (Life Technologies, Gaithersburg, Md.). Total RNA was reverse-transcribed into single-stranded cDNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Roche Molecular Systems, Branchburg, N.J.). The human XIAP cDNA was amplified by PCR with a sense primer: (5′-TTGAATTCATGACTTTTAACAGTTTTGAAGGAT-3′) containing an *Eco*R I site and an antisense primer (5′-TTCTCGAGTTAAGACATAAAAATTTTTTGCTTG-3′) containing a *Xho* I site. PCR was performed with a Perkin Elmer Gene Amp PCR System 2400 (Norwalk Conn.) in a 50 μl reaction volume for 32 cycles using Ex Taq (TaKaRa, Shiga, Japan). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 56°C for 45 s and polymerization at 72°C for 70 s. The PCR fragments were double digested with *Eco*R and *Xho* I , and then ligated into the *Eco*R I and *Xho* I sites of the pcDNA3-myc vector (pcDNA3-myc-XIAP). The correct sequence of a cloned fragment was confirmed by DNA sequencing. Moreover, the pcDNA3 expressing the full-length caspase-3 containing a hemagglutinin (HA) epitope was established. For the amplification of caspase-3 containing an HA epitope cDNA by PCR, we first used a sense primer: (5′-TTGAATTCATGGAGAACACTGAAAACTCAGTGG-3′) containing an *Eco*R I site and an antisense primer (5′-GTATGGGTACATGTGATAAAAATAGAGTTCTTT-3′). Using this PCR product as a template cDNA, we amplified caspase-3 cDNA with an antisense primer (5′-ATCTGGAACATCGTATGGGTACATGTGATAAAA-3′) and the same sense primer as in the first PCR. Using the second PCR product as a template cDNA, we amplified caspase-3 containing an HA epitope cDNA with an antisense primer: (5′-TTCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTA-3′) containing a *Xho* I site and the same sense primer as the first PCR. The PCR fragments were double digested with *Eco*R I and *Xho* I , and then ligated into a pcDNA3 (pcDNA3-caspase-3-HA). The correct sequence of a cloned fragment was confirmed by DNA sequencing.

Transfection of LNCaP cells

Transfection was conducted using FuGENE6 (Roche Diagnostics, Indianapolis, Ind.) as recommended by the manufacturer. Briefly, LNCaP cells were plated in a 35 mm dish at a density of 5×10^5 cells 24 h before transfection in medium supplemented with fetal bovine serum. Cells were transiently cotransfected using a total of 3 μg of plasmid DNA (1.5 μg of pcDNA3-myc-XIAP and 1 5 μg of pcDNA3-caspase-3-HA) and 8 μl of FuGENE6 per dish. After 6 h culture in serum-free medium, the medium was changed to fresh standard medium. Transfected cells were then cultured for an additional 48 h.

To obtain the stable transfectants, the pcDNA3-myc-XIAP transfected cells were cultured in the presence of 800 μg/ml geneticin sulfate.

Growth inhibition assay

The cell viability was determined with a colorimetric WST-1 assay. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was supplied by Dojin Chemical (Kumamoto, Japan). Briefly, exponentially growing cells were seeded in 96-well plates at 1×10^4 cells/well. After overnight culture, the culture medium was changed to fresh standard medium with various concentrations of taxol (Sigma)(0–1 μM) for 48 h or with 10 nM taxol for 0–72 h. After taxol treatment, 10 μl of WST-1 were added to each well. Following incubation, the absorbance was measured at 450 nm. Cell viability was expressed as a percentage of the absorbance obtained in the treated cells relative to that in the untreated control cells.

Flow cytometric analysis

For cell cycle analysis, flow cytometric analysis of propidium iodide-stained nuclei was performed. Briefly, LNCaP cells were plated at a density of 5×10^6 cells in a 60 mm dish and treated with 10 nM taxol for 0–72 h or with various concentrations of taxol (0, 1, 10, and 100 nM) for 48 h. The cells were collected by trypsinization, washed with PBS, fixed with 70% methanol, and stored at 20°C. The fixed cells were washed once with PBS, incubated with 100 μg/ml RNase A (Sigma) for 30 min at room temperature and stained with 25 μg/ml propidium iodide (Molecular Probes, Eugene, Ore.) for 30 min at room temperature. Relative DNA contents were determined by a FACScan flow cytometer (Becton Dickinson Labware, Lincoln Park, N.J.), and the sub-G1 DNA population (apoptotic cells) was calculated by CellQuest software (Becton Dickinson).

Caspase-3 activity assay

Caspase-3 activity was measured with Ac-DEVD-pNA as substrate using the Caspase-3 Colorimetric Activity Assay Kit (Chemcicon International, Temecula, Calif.). Briefly, cell lysates were prepared after various concentrations of taxol (0, 1, 10 and 100 nM) treatment for 48 h. Assays were performed in 96-well plates by incubation in a reaction buffer containing 200 μM Ac-DEVD-pNA with or without specific inhibitor (Ac-DEVD-CHO). After incubation, the absorbance at 405 nm was measured with a spectrophotometer. Caspase-3 activity was determined such that the activity in the presence of inhibitor was subtracted from that in the absence of inhibitor.

Immunoblot analysis

Samples (30 μg protein) were electrophoresed on a SDS-polyacrylamide gel by the method of Laemmli [13]. Proteins separated in SDS-polyacrylamide gel were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane by the method of Towbin et al. [26]. The PVDF membranes were incubated overnight at 4°C with T-TBS (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5 g/100 ml skim milk. The

membranes were incubated for 3 h at room temperature with monoclonal antibody to β-tubulin (1:500)(Sigma) or caspase-3 (1:1000)(R&D Systems, Minneapolis, Minn.), polyclonal antibodies to XIAP (1:1000)(R&D Systems) or HA (1:200)(Santa Cruz Biotechnology, Santa Cruz, Calif.). After washing with T-TBS, the membranes were incubated with the corresponding secondary antibodies which were conjugated with horseradish peroxidase in T-TBS for 1 h at room temperature. Immunoreactive bands were visualized with the enhanced chemiluminescence Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech, Little Chalfont, UK), and quantified by scanning densitometry using NIH Image (version 1.55).

Immunoprecipitation and immunoblot analysis

Lysates prepared from taxol-treated (100 nM, 48 h) or non-treated LNCaP cells which were cotransfected with caspase-3-HA and myc-XIAP cDNAs were subjected to immunoprecipitation with anti-myc antibody (Upstate Biotechnology, Lake Placid, N.Y.), followed by immunoblot analysis with anti-HA antibody. Briefly, the cells were transfected with both cDNAs of myc-XIAP and capase-3-HA as described above. After taxol treatment, the cells were solubilized in 200 µl of lysis buffer containing 0.25 M sucrose, pH 7.0, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 12.5 µM pepstatin A, and 7.5 µg/ml leupeptin. The insoluble material was removed by centrifugation at 14,000 rpm for 10 min. Lysates were incubated overnight at 4°C with 3 µg anti-myc antibody coupled with 10 µl of protein G Sepharose (Pharmacia Biotech, Wikstroms, Sweden). Immunoprecipitates were analyzed by immunoblotting with anti-HA antibody or anti-XIAP antibody.

Statistical evaluation

Values were expressed as means ± SE. Statistical analysis was performed using the Student's *t*-test or one way analysis of variance. Values of $P < 0.05$ were considered to be statistically significant.

Results

Expression levels of XIAP protein in LNCaP sublines

Immunoblot analysis revealed that XIAP was expressed in all human prostate cancer cell lines examined in this study. LNCaP cells expressed lower levels of XIAP than DU145 and PC3 cells (data not shown). In the following experiments, we used LNCaP cells for the overexpression of XIAP in order to study its role in apoptosis. Parental LNCaP cells (LNCaP/P) were stably transfected with myc-XIAP cDNA, and clonal cell lines (LNCaP/XIAP) which overexpressed XIAP were established. LNCaP/P transfected with the pcDNA3 vector alone (LNCaP/C1 and LNCaP/C2) were also established. As shown in Fig. 1A, XIAP protein was highly expressed in the two LNCaP/XIAP clones compared with LNCaP/P, LNCaP/C1, and LNCaP/C2.

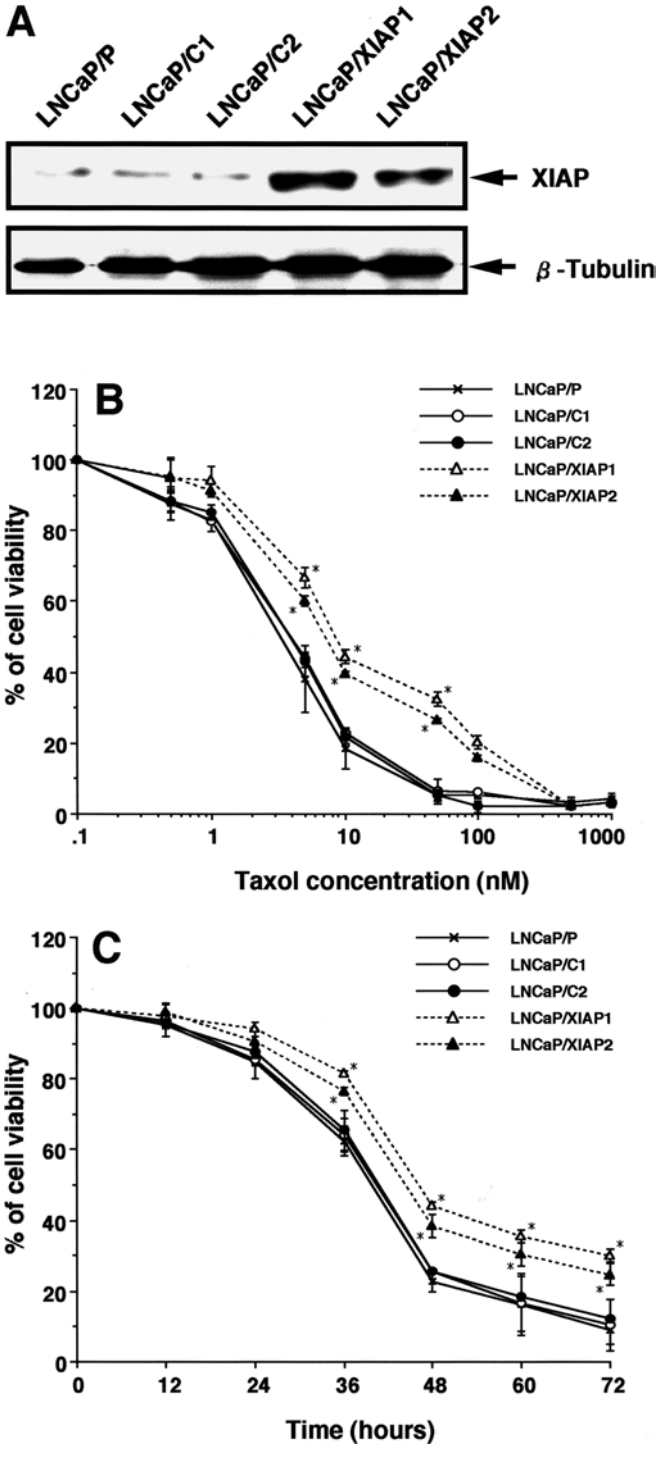


Fig. 1 Immunoblot analysis of cell lysates derived from LNCaP sublines, and cytotoxic effects of taxol on LNCaP sublines. **A** Equal amounts of protein (30 µg) extracted from LNCaP/P, LNCaP/C1, LNCaP/C2, LNCaP/XIAP1, and LNCaP/XIAP2 were used for immunoblot analysis with anti-XIAP antibody (*upper panel*) at a dilution of 1:1000 and with anti-β-tubulin antibody (*lower panel*) at a dilution of 1:500. **B**, **C** The effects of taxol on cell proliferation of LNCaP sublines were determined by WST-1 assay. A total of 1×10^4 cells from each cell lines were seeded and treated with various concentrations of **B** taxol for 48 h or with **C** 10 nM taxol. Values represent means ± SE (*bars*) from three independent experiments. An *asterisk* indicates $P < 0.05$ compared with LNCaP/P and LNCaP/C1 and 2

Effects of taxol treatment on cell growth in LNCaP sublines

We first examined the growth rates of LNCaP sublines by WST-1 assay. The exposure of LNCaP sublines to taxol within the range of clinically relevant concentrations revealed dose-response inhibition. Although the growth rates of LNCaP/P, LNCaP/C1 and LNCaP/C2 were reduced in a dose- and time-dependent manner by taxol, the anti-proliferative effects of taxol on LNCaP/XIAP1 and LNCaP/XIAP2 were significantly weaker than those on LNCaP/P, LNCaP/C1 and LNCaP/C2 (Fig. 1B, C). These results suggest that XIAP decreases the sensitivity to taxol.

Effects of taxol treatment on apoptosis in LNCaP sublines

Next, we examined the apoptotic effects of taxol on LNCaP sublines by flow cytometry. LNCaP sublines were treated with 10 nM taxol and subjected to cell cycle analysis at 6, 12, 24, 48, and 72 h. In all LNCaP sublines examined, the number of cells in the G2/M phase was increased with a peak occurring at 24 h, while the number of the cells in the G1 phase was decreased. The representative DNA histograms are shown in Fig. 2A. An increase in the number of cells with hypodiploid DNA (sub-G1 population) was detected 24 h after treatment. Although the sub-G1 population was increased by taxol in a time-dependent manner in all LNCaP sublines, it was significantly decreased to 36.4% ($n=3$, $P<0.01$) and 42.8% ($n=3$, $P<0.01$) in LNCaP/XIAP1 and LNCaP/XIAP2, respectively, and from 53.6, 54.9, and 58.6% in LNCaP/P, LNCaP/C1 and LNCaP/C2, respectively, after 72 h treatment (Fig. 2B).

As the next step, we carried out dose-response studies of apoptosis using two different analyses (Fig. 3). LNCaP sublines were treated with taxol at concentrations of 1, 10 and 100 nM for 48 h. We performed flow cytometric analysis to examine the changes in the number of hypodiploid DNA containing cells (apoptotic cells). As shown in Fig. 3A, 1 nM taxol did not increase the number of cells in the sub-G1 population, whereas about 40% and 60% of both LNCaP/P and LNCaP/C1 were detected in the sub-G1 population by 10 and 100 nM taxol treatment, respectively. In contrast, only about 20% and 40% of both LNCaP/XIAP1 and LNCaP/XIAP2 were detected in the sub-G1 population by 10 and 100 nM taxol treatment, respectively.

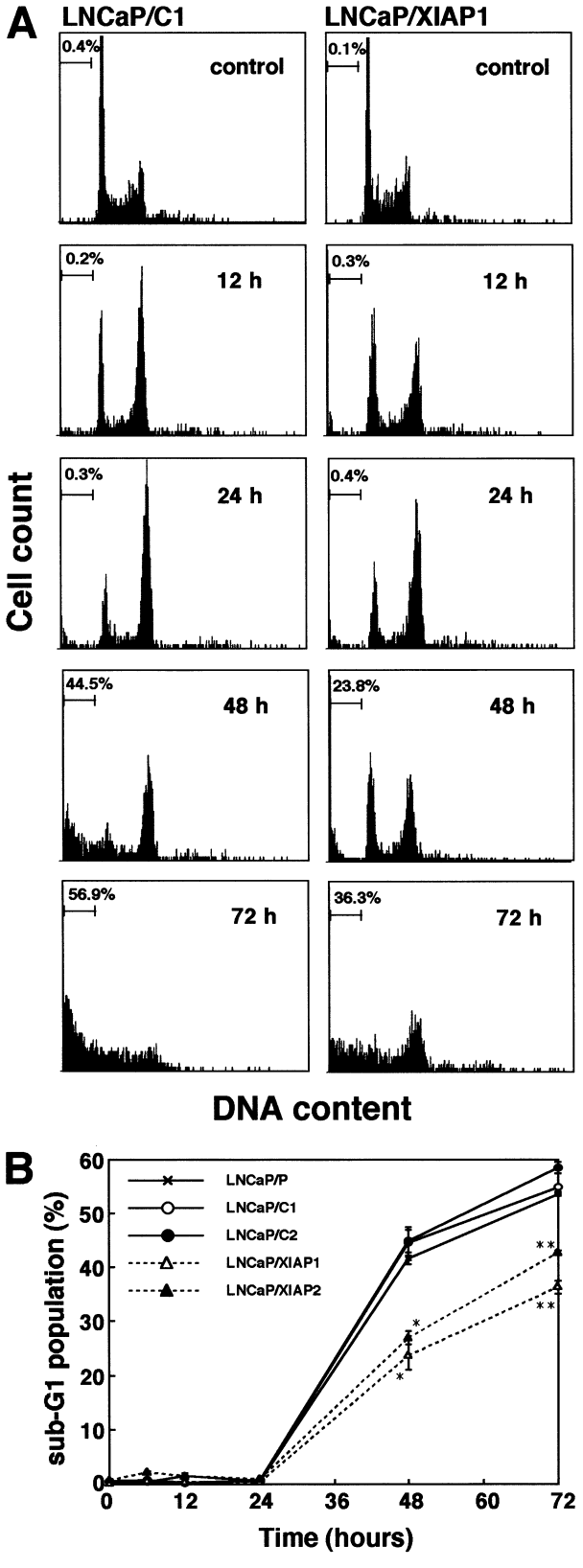


Fig. 2 Time kinetic analysis of taxol-induced G2/M arrest and apoptosis in LNCaP sublines. Cell cycle and apoptosis analysis of LNCaP sublines treated with 10 nM taxol for the indicated times were determined by flow cytometry. **A** Representative histograms of LNCaP/C1 and LNCaP/XIAP1. The percentages of apoptotic cells with sub-G1 DNA content are indicated. **B** Serial changes in the population with sub-G1 DNA content are indicated. Values represent means ± SE (*bars*) from three independent experiments. An *asterisk* indicates $P<0.05$, a *double asterisk* indicates $P<0.01$ compared with LNCaP/P and LNCaP/C1 and 2

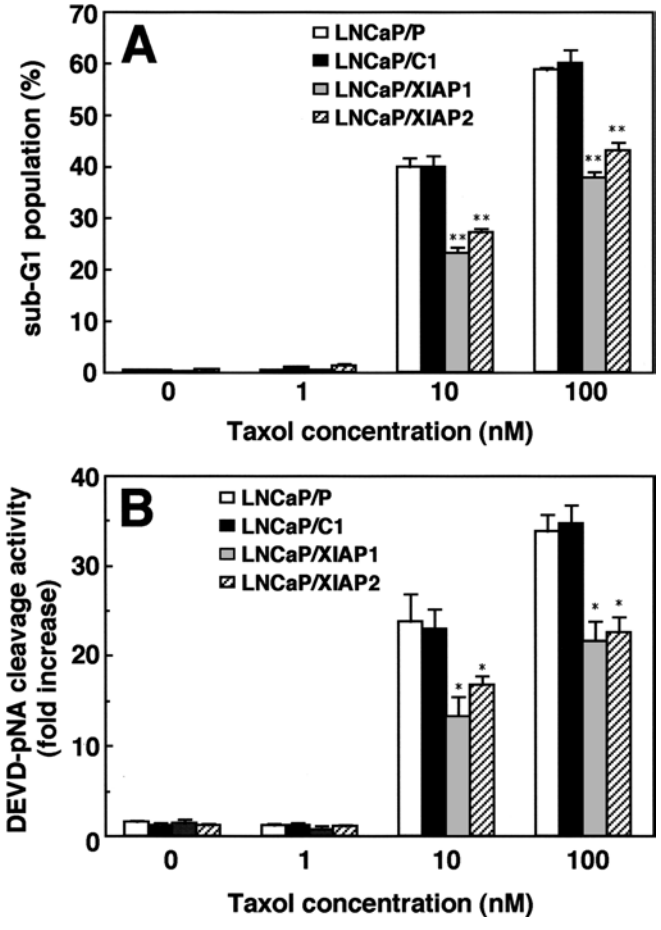


Fig. 3 Detection of apoptosis by flow cytometric analysis and by assay for caspase-3 activity in LNCaP sublines. LNCaP sublines were treated with taxol at concentrations of 1, 10 and 100 nM for 48 h. **A** The percentages of apoptotic cells with sub-G1 DNA content were determined by flow cytometry. **B** Caspase-3 activity was measured with DEVD-pNA as substrate. Values represent means ± SE (*bars*) from three independent experiments. An *asterisk* indicates $P < 0.05$, a *double asterisk* indicates $P < 0.01$ compared with LNCaP/P and LNCaP/C1

To further examine the effects of XIAP on apoptosis, we measured the caspase-3 activity for cleavage of the conjugated substrate Ac-DEVD-pNA (Fig. 3B). When we treated the cells with or without 1 nM taxol, the activity did not differ among the LNCaP sublines. The caspase-3 activities in LNCaP/P and LNCaP/C1 were increased 23.8-fold ± 3.1 ($n = 3$, $P < 0.05$) and 23.0-fold ± 2.1 ($n = 3$, $P < 0.05$), respectively, by 10 nM taxol treatment. In contrast, there was an attenuated increase in casapse-3 activity in taxol-treated LNCaP/XIAP1 and LNCaP/XIAP2. The caspase-3 activity in LNCaP/XIAP1 and LNCaP/XIAP2 were increased 13.3-fold ± 2.2 ($n = 3$, $P < 0.05$) and 16.8-fold ± 0.9 ($n = 3$, $P < 0.05$), respectively, by 10 nM taxol treatment (Fig. 3B). Treatment with 100 nM taxol increased the caspase-3 activity 33.8-fold ± 1.9 ($n = 3$, $P < 0.05$) and 34.7-fold ± 2.0 ($n = 3$, $P < 0.05$) in LNCaP/P and LNCaP/C1, respectively (Fig. 3B). In contrast, the caspase-3 activity was increased 21.7-fold ± 2.2 ($n = 3$, $P < 0.05$) and 22.6-fold ± 1.7 ($n = 3$, $P < 0.05$) in LNCaP/XIAP1 and LNCaP/XIAP2, respectively. Taken together, these results from the two different types of experiments clearly indicate that the overexpression of XIAP inhibited taxol-induced apoptosis.

Cleavage of pro-caspase-3 in LNCaP sublines by taxol treatment

In addition to the measurement of caspase-3 activity, the processing of pro-caspase-3 was evaluated by immunoblot analysis (Fig. 4). Anti-caspase-3 monoclonal antibody recognized 32 kDa pro-caspase-3 as reported. The treatment of LNCaP/P with 10 or 100 nM taxol for 48 h decreased the amount of pro-caspase-3 to 23.3% ± 0.5 ($n = 3$, $P < 0.01$) and 7.6% ± 0.2 ($n = 3$, $P < 0.01$), respectively, of the control value (Fig. 4A). However, no significant changes in the amount of pro-caspase-3 were observed by treatment with 1 nM taxol (Fig. 4A).

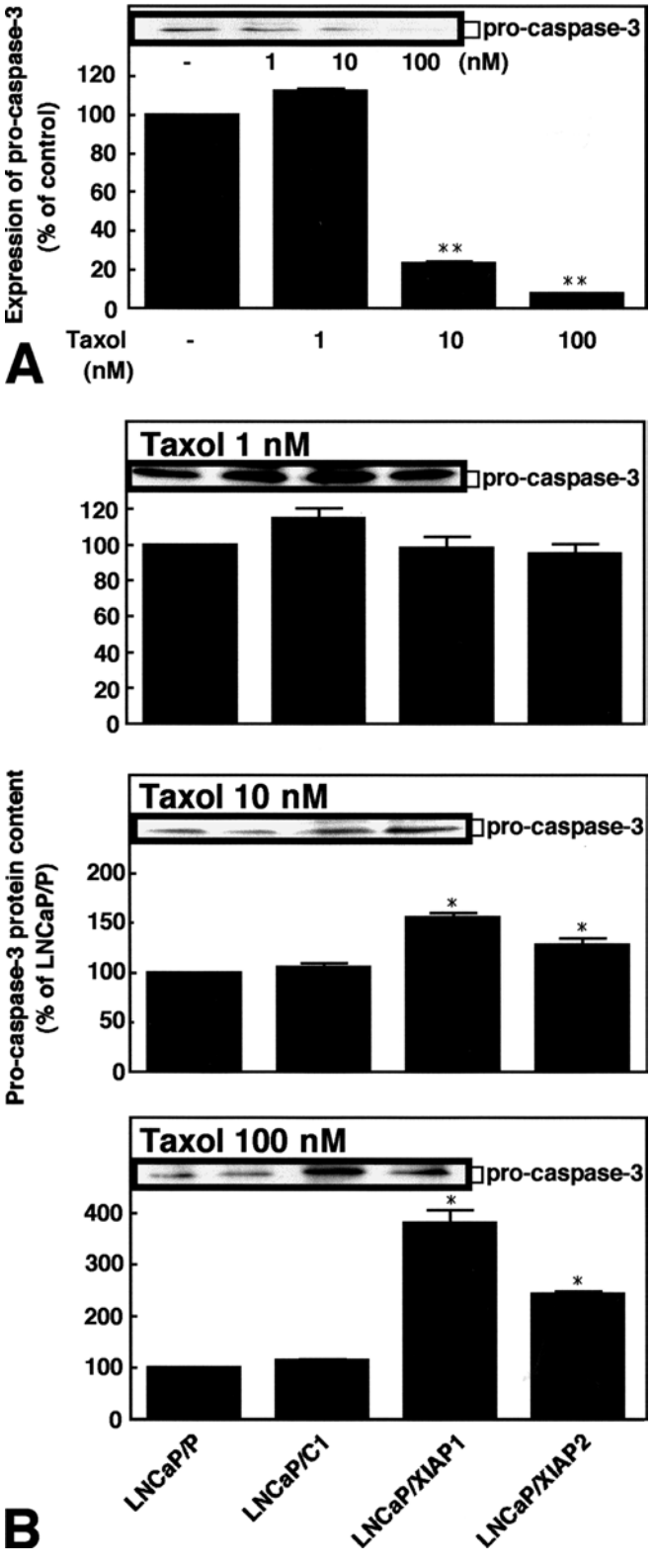
Next, we examined whether or not XIAP inhibited the processing of pro-caspase-3 in taxol treated LNCaP sublines (Fig. 4B). There were no significant differences in the amount of pro-caspase-3 by treatment with 1 nM taxol in LNCaP sublines. In contrast, the densitometric analysis indicated that the amount of pro-caspase-3 in LNCaP/XIAP1 and LNCaP/XIAP2 treated with 10 nM taxol was 156.0% ± 4.2 ($n = 3$, $P < 0.05$) and 128.1% ± 7.3 ($n = 3$, $P < 0.05$), respectively, of that in LNCaP/P (Fig. 4B). By treatment with 100 nM taxol, the amount of pro-caspase-3 in LNCaP/XIAP1 and LNCaP/XIAP2 was 381.1% ± 24.1 ($n = 3$, $P < 0.05$) and 243.1% ± 4.1 ($n = 3$, $P < 0.05$), respectively, of that in LNCaP/P (Fig. 4B). These results suggest that XIAP inhibits the processing of pro-caspase-3 in the cells.

Coexpression of myc-XIAP and caspase-3-HA

Recent studies have reported that XIAP binds to activated caspase-3 and inhibits its activity in vitro. We therefore intended to examine whether XIAP bound to pro-caspase-3. For this purpose, myc-XIAP cDNA was cotransfected with caspase-3-HA cDNA into LNCaP cells. The overexpression of myc-XIAP and caspase-3-HA was verified by immunoblot analysis using anti-XIAP antibody and anti-HA antibody (Fig. 5A).

Lysates prepared from LNCaP cells cotransfected with myc-XIAP and caspase-3-HA cDNAs were subjected to immunoprecipitation with anti-myc antibody, followed by immunoblotting with anti-HA antibody (Fig. 5B). Immunoblot analysis demonstrated the coprecipitation of caspase-3-HA with myc-XIAP (Fig. 5B). These results suggest that XIAP binds to pro-caspase-3 in the cells.

In addition, to determine whether or not taxol treatment influenced the interaction between XIAP and pro-caspase-3, we performed immunoprecipitation and immunoblot analysis of the cells overexpressing both



XIAP and caspase-3 with or without taxol treatment. Immunoblot analysis demonstrated that the interaction between XIAP and pro-caspase-3 was inhibited by 100 nM taxol treatment for 48 h (Fig. 5C). These results suggest that XIAP inhibits the activation of pro-caspase-3 by binding to it.

Fig. 4 Effects of XIAP on the cleavage of pro-caspase-3. **A** Pro-caspase-3 cleavage in LNCaP/P treated with taxol at concentrations of 1, 10 and 100 nM for 48 h was detected by immunoblot analysis. The cell extract (30 μg) was used for immunoblot analysis with anti-caspase-3 antibody at a dilution of 1:1000. The value of the control was taken as 100%, and other values were calculated from this. Representative data for immunoblotting and densitometric analyses are shown. Values represent means ± SE (*bars*) from three independent experiments. A *double asterisk* indicates $P < 0.01$ compared with the control. **B** LNCaP sublines were treated with taxol at concentrations of 1 (*upper*), 10 (*middle*) and 100 nM (*bottom*) for 48 h. The results with LNCaP/P were used as the control. The value of the control was taken as 100%, and other values were calculated from this. Representative data of immunoblotting and densitometric analyses are shown. Values represent means ± SE (*bars*) from three independent experiments. An *asterisk* indicates $P < 0.05$ compared with LNCaP/P and LNCaP/C1

Furthermore, we asked whether or not taxol treatment induced the cleavage of pro-caspase-3 in LNCaP cells overexpressing caspase-3-HA. Immunoblot analysis with anti-HA antibody revealed that cleavage of pro-caspase-3 occurred after taxol treatment (Fig. 5D).

Discussion

The prognoses of invasive and/or androgen-independent prostate cancers are still poor in spite of aggressive chemotherapy. Therefore, there is a need to identify the molecules which make the cells resistant to chemotherapy and lead to tumor progression in order to develop a novel therapy. In the present study, we considered the possibility that XIAP may be one of the candidate molecules, and investigated the effects of XIAP overexpression on taxol-induced cell growth arrest and apoptosis in LNCaP cells. We made the following observations: (1) XIAP suppressed taxol-induced apoptosis through the inhibition of caspase-3 activity in LNCaP sublines, and (2) XIAP inhibited the processing of pro-caspase-3 by taxol treatment. The interaction of XIAP with pro-caspase-3 may be involved in this inhibition. To our knowledge, this is the first report on the inhibitory roles of XIAP in taxol-induced apoptosis in LNCaP cells.

Recent reports showed that survivin, one of the IAPs, was prominently expressed in prostate cancer [1, 27]. Although survivin was originally described as a protein that could inhibit cell death, it functions primarily in the regulation of cell division [22]. The ability of survivin to inhibit the caspase-3 activity in vitro has been debated, and conflicting results were reported [2, 25]. In contrast, XIAP is the most potent inhibitor of caspases among the human IAP family of proteins [20]. XIAP was reported to be highly expressed in some human cancers, such as ovarian epithelial cancer [14] and non-small cell lung cancer [10], and may be involved in chemo-resistance. Moreover, some reports have demonstrated that XIAP is expressed in several prostate cancer cells at the level of both RNA and protein [15]. XIAP may be involved in

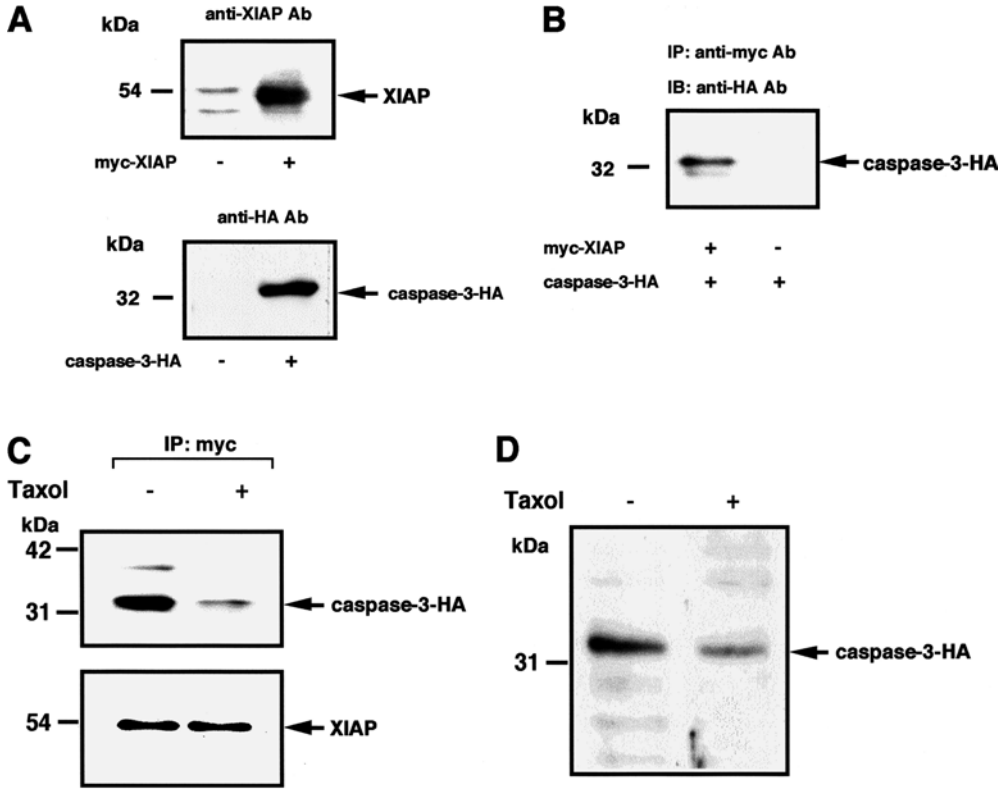


Fig. 5 Coexpression of myc-XIAP and caspase-3-HA, and the interaction of caspase-3 with XIAP in LNCaP cells with or without taxol treatment. **A** Cell lysates from LNCaP cells cotransfected with pcDNA3-myc-XIAP (myc-XIAP) and pcDNA3-caspase-3-HA (caspase-3-HA) were subjected to immunoblot analysis with anti-XIAP antibody at a dilution of 1:1000 (*upper panel*) or anti-HA antibody at a dilution of 1:200 (*lower panel*). **B** Lysates prepared from LNCaP cells cotransfected with pcDNA3-myc-XIAP or pcDNA3 and pcDNA3-caspase-3-HA were subjected to immunoprecipitation with anti-myc antibody, followed by immunoblot analysis with anti-HA antibody. **C** Cell lysates from LNCaP cells cotransfected with pcDNA3-myc-XIAP and pcDNA3-caspase-3-HA, treated with or without 100 nM taxol, were subjected to immunoprecipitation with anti-myc antibody. Immunoprecipitates were analyzed by immunoblotting with anti-HA antibody (*upper panel*) or with anti-XIAP antibody (*lower panel*). **D** Cleavage of caspase-3-HA overexpressed in LNCaP cells with or without taxol treatment was detected by immunoblot analysis, as described above

prostate cancer cell resistance to the induction of apoptosis . Therefore, we focused on the role of XIAP in taxol-induced apoptosis in LNCaP cells.

It has been reported that XIAP inhibits pro-caspase-9 cleavage [7, 8] and the activity of processed caspase-9 fragments [3]. Direct inhibition of caspase-3 [7] and caspase-7 [24] by XIAP have also been reported. Although recent studies with constitutively active mutants of caspase-3 indicated that XIAP promotes the degradation of the active form of caspase-3 [23], the effects of apoptosis inducing agents such as taxol on the interaction of XIAP with caspase-3 have not been reported.

To study the effects of XIAP on taxol-induced phenomena, we established stable transfectants which overexpressed XIAP in LNCaP cells. The XIAP expression level was least in LNCaP/P among the cell lines tested (Fig. 1A). These features of LNCaP/P were suitable for the purposes of analyzing the role of XIAP. When we treated LNCaP sublines with taxol, concentration- and time-dependent decreases in cell viability were observed. In contrast, XIAP overexpressing transfectants showed less sensitivity to taxol (Figs. 1B, C). These results suggest that the inhibition of apoptosis by the overexpression of XIAP causes resistance to taxol in LNCaP cells. In accordance with these results, XIAP significantly inhibited the activity of caspase-3 after taxol treatment (Fig. 3B). Overexpression of XIAP also inhibited the processing of pro-caspase-3 (Fig. 4B). The inhibition of caspase-9 activity by XIAP may play some roles in the decrease in the processing of pro-caspase-3 as reported [3]. It has been reported that XIAP mainly binds to active caspase-3, but also partially to the unprocessed pro-caspase-3 [6]. We confirmed that the caspase-9 activity in LNCaP sublines was increased by taxol treatment, but XIAP overexpression did not significantly inhibit caspase-9 activity under our assay conditions (data not shown). Therefore, we considered the possibility that XIAP may inhibit the processing of pro-caspase-3 by interacting with it, in addition to the inhibiting caspase-9 activity. We transiently overexpressed both XIAP and caspase-3, and examined the interaction by immunoprecipitation and immunoblot analysis. The immunoreactive band of caspase-3-HA after immunoprecipitation revealed a strong interaction between XIAP and pro-caspase-3. Thus the immunoreactivity was diminished by treatment with taxol (Fig. 5C). The cleavage of overexpressed caspase-3-HA

in LNCaP with taxol treatment revealed a slight decrease in the immunoreactive band of caspase-3-HA (Fig. 5D). This might be due to the excessive intracellular expression of pro-caspase-3. Together with the results indicating that caspase-9 activity was not efficiently inhibited by XIAP in LNCaP sublines, our findings suggested that XIAP inhibited the processing of pro-caspase-3 via the interaction with pro-caspase-3 in addition to the inhibition of capase-3 activity.

XIAP has been reported to be a substrate for several caspases, and is cleaved during apoptosis induced by TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand) [8, 18]. In this study, the relevance of the cleavage of XIAP during taxol-induced apoptosis in cells transiently overexpressing both XIAP and caspase3 is unknown. In addition, the cleavage of endogenous XIAP during taxol-induced apoptosis was not investigated. These should be addressed in future studies.

In summary, we have shown that XIAP plays an important role in the regulation of taxol-induced apoptosis in LNCaP cells. In addition to its direct inhibitory effect on caspase-3 activity, XIAP may inhibit the activation of pro-caspase-3 by binding to it. It is tempting to speculate that XIAP is related to tumor progression and chemo-resistance. Identification of the inhibitors of XIAP may lead to interesting new developments for therapy against prostate cancer.

Acknowledgements We gratefully acknowledge Ms. N. Hamamatsu and Ms. Y. Ayaki (Oita Medical University) for their excellent technical assistance in the experiment and Drs. H. Saya and Y. Ohta (Kumamoto University School of Medicine) for their technical support and critical comments on the manuscript.

References

1. Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 3: 917
2. Banks DP, Plescia J, Altieri DC (2000) Survivin does not inhibit caspase-3 activity. Blood 96: 4002
3. Datta R, Oki E, Endo K, Biedermann V, Ren J, Kufe D (2000) XIAP regulates DNA damage-induced apoptosis downstream of caspase-9 cleavage. J Biol Chem 275: 31733
4. Denis L, Murphy GP (1993) Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. Cancer 72: 3888
5. Deveraux QL, Reed JC (1999) IAP family proteinssuppressors of apoptosis. Genes Dev 13: 239
6. Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388: 300
7. Deveraux QL, Roy N, Stennicke HR, Arsdale TV, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome-*c* by direct inhibition of distinct caspases. EMBO J 17: 2215
8. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J 19: 5242
9. Fang G, Chang BS, Kim CN, Perkins C, Thompson CB, Bhalla KN (1998) "Loop" domain is necessary for taxol-induced mobility shift and phosphorylation of Bcl-2 as well as for inhibiting taxol-induced cytosolic accumulation of cytochrome c and apoptosis. Cancer Res 58: 3202
10. Ferreira CG, van der Valk P, Span SW, Ludwig I, Smit EF, Kruyt FAE, Pinedo HM, van Tinteren H, Giaccone G (2001) Expression of X-linked inhibitor of apoptosis as a novel prognostic marker in radically resected non-small cell lung cancer patients. Clin Cancer Res 7: 2468
11. Haldar S, Chintapalli J, Croce CM (1996) Taxol induces *bcl*-2 phosphorylation and death of prostate cancer cells. Cancer Res 56: 1253
12. Hudes GR, Nathan F, Khater C, Haas N, Cornfield M, Giantonio B, Greenberg R, Gomella L, Litwin S, Ross E, Roethke S, McAleer C (1997) Phase II trial of 96-hour paclitaxel plus oral estramustine phosphate in metastatic hormone-refractory prostate cancer. J Clin Oncol 15: 3156
13. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680
14. Li J, Feng Q, Kim JM, Schneiderman D, Liston P, Li M, Vanderhyden B, Faught W, Fung MFK, Senterman M, Korneluk RG, Tsang BK (2001) Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. Endocrinology 142: 370
15. McEleny KR, Watson RWG, Coffey RNT, O'Neill AJ, Fitzpatrick JM (2002) Inhibitor of apoptosis proteins in prostate cancer cell lines. Prostate 51: 133
16. Miller LK (1999) An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol 9: 323
17. Moos JP, Fitzpatrick FA (1998) Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. Cell Growth Differ 9: 687
18. Nesterov A, Lu X, Johnson M, Miller GJ, Ivashchenko Y, Kraft AS (2001) Elevated Akt activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. J Biol Chem 276: 10767
19. Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS, Buttyan R (1995) Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res 55: 4438
20. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 16: 6914
21. Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci U S A 77: 1561
22. Shankar SL, Mani S, Norman O, Kandimalla ER, Agrawal S, Shafit-Zagardo B (2001) Survivin inhibition induces human neural tumor cell death through caspase-independent and dependent pathways. J Neurochem 97: 426
23. Suzuki Y, Nakabayashi Y, Takahashi R (2001) Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci U S A 98: 8662
24. Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem 273: 7787
25. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC (1998) IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 58: 5315
26. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350
27. Xing N, Qian J, Bostwick D, Bergstralh E, Young CY (2001) Neuroendocrine cells in human prostate over-express the anti-apoptosis protein survivin. Prostate 48: 7