Urol Res (2002) 30: 102–111
DOI 10.1007/s00240-002-0239-4

ORIGINAL PAPER

**Takeo Nomura · Hideyuki Yamamoto
Hiromitsu Mimata · Miki Shitashige
Futoshi Shibasaki · Eishichi Miyamoto
Yoshio Nomura**

Enhancement by cyclosporin A of taxol-induced apoptosis of human urinary bladder cancer cells

Received: 1 August 2001 / Accepted: 1 February 2002 / Published online: 22 March 2002

Abstract Taxol is a microtubule-stabilizing agent which induces apoptosis in various cancer cells. In this study, we found that T24 cells derived from high grade human urinary bladder cancer were relatively resistant to taxol and that the IC_{50} value determined by a colorimetric WST-1 assay was 406.0 nM. Interestingly, cyclosporin A (CsA), an immunosuppressive drug, dramatically enhanced sensitivity to taxol, and the IC_{50} value was decreased to 47.5 nM in the presence of 1 μM CsA. KK47 cells derived from low grade human urinary bladder cancer showed high sensitivity to taxol with an IC_{50} value of 78.8 nM which decreased to 14.4 nM in the presence of 1 μM CsA. FK506, another immunosuppressive drug, also enhanced sensitivity to taxol. Furthermore, a concomitant loss of calcineurin activity was observed after the treatment of both cell lines with both CsA and FK506. Taxol induced apoptosis of the cells, as assessed by Hoechst 33258 staining and by the measurement of caspase 3 activity. Immunoblot analysis with an antibody against Bcl-2 phosphorylated at serine 70 demonstrated that taxol induced the phosphorylation of Bcl-2 with its enhancement in the presence of CsA. In addition, treatment of the cells with CsA significantly decreased the expression of Bcl-2 at both the protein and mRNA levels. These results suggest that the enhancement of taxol-induced apoptosis by immunosuppressive drugs is at least partly due to the inhibition of calcineurin activity and the loss of the antiapoptotic function of Bcl-2 via the enhancement of phosphorylation and the reduction of expression.

Keywords Bcl-2 · Bladder cancer cells · Calcineurin · Cyclosporin A · Taxol

T. Nomura · H. Mimata · Y. Nomura
Department of Urology, Oita Medical University,
Hasama-machi Oita 879-5593, Japan

H. Yamamoto (✉) · E. Miyamoto
Department of Pharmacology,
Kumamoto University School of Medicine,
2-2-1 Honjo, Kumamoto 860-0811, Japan
E-mail: hideyuki@gpo.kumamoto-u.ac.jp
Tel.: 81-96-3735076
Fax: 81-96-3735078

M. Shitashige · F. Shibasaki
Department of Molecular Cell Physiology,
The Tokyo Metropolitan Institute of Medical Science,
3-18-22, Honkomagome, Bunkyo-ku,
Tokyo 113-8613, Japan

Introduction

More than 50% of superficial urinary bladder cancers are reported to recur after endoscopic removal of an initial tumor and about 10–15% of the patients will subsequently develop muscle-invading tumors [1, 33]. Intravesical chemotherapy is performed after transurethral tumor resection to reduce the recurrence of superficial bladder cancer. Intravesical chemotherapy is a regional therapy and provides the advantages of delivering drugs at high concentrations to the urinary bladder while minimizing systemic exposure to the drugs. However, intravesical chemotherapy is not efficacious against invasive bladder cancer, because the commonly used drugs, such as thiopeta, doxorubicin and mitomycin C, have limited activity [2, 12, 23, 33].

Recent studies reported that taxol is one of the candidates for intravesical chemotherapy [3, 29, 35]. Taxol binds preferentially to microtubules at sites distinct from the binding sites of vinblastine or colchicine, and produces mitotic arrest and cell death at clinically relevant concentrations [5]. Several lines of evidence from recent studies suggest that taxol induces apoptosis through the phosphorylation of B-cell leukemia/lymphoma-2 (Bcl-2) at the G2-M phase of the cell cycle [15, 18, 19]. Bcl-2 is a 26-kDa integral membrane oncoprotein which is unique in its ability to suppress apoptosis [32]. It prevents apoptosis induced by a wide variety of stimuli, suggesting that it inhibits a crucial step in the final common pathway for apoptotic cell death. Bcl-2, which is located in part in the outer mitochondrial membrane, dimerizes with a proapoptotic molecule, Bax, and inhibits its function

[30]. The phosphorylation of Bcl-2 has been suggested to decrease the binding activity to Bax and to free Bax from the Bcl-2-Bax dimer. This is followed by the induction of apoptosis [31, 36, 44, 47]. One of the critical phosphorylation sites in Bcl-2 for the loss of antiapoptotic function has been reported to be serine 70 which lies in a loop region connecting the BH4 and BH3 regions [15, 21]. The protein kinase(s) responsible for the phosphorylation of serine 70 has not been conclusively identified [5], but one of the potential candidates appears to be c-Jun N-terminal kinase/stress-activated protein kinase (JNK) [28, 43]. Recently, the phosphorylation of Bcl-2 by the activation of the JNK pathway was reported to play a major role in vinblastine-induced apoptosis of the KB-3 human carcinoma cell line [14]. In contrast to the protein kinases, only a few papers have reported protein phosphatases of Bcl-2 [18]. Calcineurin (also called protein phosphatase 2B) is the only serine/threonine protein phosphatase under the control of Ca^{2+} and calmodulin, and plays a critical role in the regulation of various cellular functions [11, 25]. The role of calcineurin in apoptosis is still controversial [42]. It was found to dephosphorylate Bad and promote apoptosis [42]. However, we found that calcineurin bound to the BH4 region of Bcl-2 [34], and Bcl-2 has been reported to be an in vitro substrate for calcineurin [18]. Therefore, it is likely that calcineurin has an antiapoptotic action via the dephosphorylation of Bcl-2. Furthermore, the inhibition of calcineurin activity in cancer cells may enhance taxol-induced phosphorylation of Bcl-2, followed by the enhancement of the apoptosis of the cells.

In this study, we found that cyclosporin A (CsA), an immunosuppressive drug, enhanced the sensitivity to taxol of two types of human urinary bladder cancer cell lines. Since the pharmacological effects of CsA were mediated partly through the inhibition of calcineurin activity, we examined the effects of CsA on calcineurin activity, and the taxol-induced phosphorylation of Bcl-2 and activation of JNK in these cell lines. The effects of CsA on the expression of Bcl-2 were also examined.

Materials and methods

Cell culture

Two human urinary bladder cancer cell lines , T24 established by Bubenik et al. [8] and KK47 established by Taya et al. [24, 38], were used in the present study. Cells were cultured in MEM (Sigma, St. Louis, Mo.) supplemented with 10% newborn calf 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. The final concentrations of DMSO and ethanol in medium were below 0.1% and did not exert any detectable effects on cell growth.

Materials

Paclitaxel (taxol) (Sigma) and FK506 (Calbiochem, Darmstadt, Germany) were dissolved in DMSO to make 20-mM and 1-mM stock solutions, respectively, which were then diluted as desired with water. CsA (Sigma) was dissolved in absolute ethanol to make a 1-mM stock solution, which was then diluted as desired with water. 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) was supplied by Dojin (Kumamoto, Japan) and bisbenzimide Hoechst 33258 fluorochrome trihydrochloride (Hoechst 33258) was from Calbiochem. Other chemicals used were of analytical grade. Calmodulin was purified from bovine brain [17]. A polyclonal antibody to Bcl-2 phosphorylated at serine 70 (anti-pS70-Bcl-2 antibody) was raised using a synthetic peptide {PVARTS(PO_4)PLQTPAC; Sawady, Tukuba, Japan} according to standard procedures.

Growth inhibition assay

Exponentially growing cells were seeded in 96-well plates at 1×10^4 cells/well in six replicates. After overnight culture, the culture medium was changed to fresh standard medium with or without the test reagents, and the cells were cultured for 24 h. After changing the medium to fresh standard medium without the test reagents, cells were cultured for a further 48 h. The cell viability was determined with a colorimetric WST-1 assay [22]. After 48-h culture, 10 μl of WST-1 were added to each well, and the cells were incubated for an additional 2 h. Following incubation, the absorbance at 450 nm was measured. Cell viability was expressed as a percentage of the absorbance obtained in the treated cells relative to that in the untreated control cells. The IC_{50} value is the concentration at which cell viability was inhibited by 50%.

Assay for calcineurin activity

Calcineurin activity was measured as previously described [45]. The reaction mixture contained in 25 μl: 50 mM HEPES at pH 7.5, 1 mM DTT, 0.1 mM $MnCl_2$, 1 mM $CaCl_2$, 1.5 μM calmodulin, 0.2 μM calyculin A to inhibit protein phosphatases 1 and 2A, and 0.1 mg/ml ^{32}P-labeled casein. In addition, the phosphatase activity was measured without $CaCl_2$ and calmodulin in the presence of 200 μM trifluoperazine (TFP). The mixture was incubated at 30°C for 10 min. Calcineurin activity was determined such that the activity in the presence of TFP without $CaCl_2$ and calmodulin was subtracted from that in the presence of $CaCl_2$ and calmodulin. All assays of each sample were performed in duplicate, and the activity was corrected for the protein concentration.

Detection of apoptosis by staining with Hoechst 33258

Cells were plated in triplicate and treated for 24 h with taxol in the presence and absence of CsA or FK506 in a four-well chamber slide (Becton Dickinson Labware, Franklin Lakes, N.J.). To detect apoptotic changes, the cells were stained with the DNA-binding Hoechst 33258 [26]. Briefly, the treated cells were fixed with methanol/acetic acid (4:1 v/v) for 10 min, washed twice with PBS (–), and stained for 3 min at room temperature with 1 mM Hoechst 33258 in PBS (–). The apoptotic cells were identified by the presence of nuclear condensation and fragmentation. At least 1,000 cells from randomly selected fields were counted and scored by two independent observers for the incidence of apoptotic cells using fluorescence microscopy.

Assay for caspase 3 activity

Caspase 3 activity was measured with DEVD-pNA as substrate using CPP32/caspase-3 Colorimetric Protease Assay Kit (Chemcicon International, Temecula, Calif.) according to the manufacturer's protocol. The degree of increase in caspase 3 activity was determined by comparing the results from the induced apoptosis sample with the level of the uninduced control.

SDS-PAGE and immunoblot analysis

The cells were washed twice with PBS and harvested by scraping in 500 μl of mammalian protein extraction reagent (Pierce Chemical,

Rockford, Ill.) containing 1 mM PMSF, 50 mM NaF and 1 mM Na_3VO_4. The insoluble materials were removed by centrifugation at 14,000 *g* for 10 min. Protein concentration was determined using the Bradford method with BSA as the standard [7]. An equal volume of twofold concentrated SDS sample buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 10% 2-mercaptoethanol and 0.004% bromophenol blue) was added to each lysate, which was subsequently boiled for 5 min and electrophoresed on an 8–16% SDS-polyacrylamide gel by the method of Laemmli [27]. Proteins separated in SDS-polyacrylamide gel were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane by the method of Towbin et al [40]. The PVDF membranes were incubated overnight at 4°C with T-PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, and 0.1% Tween 20) containing 5 g/100 ml skim milk or 1 g/100 ml BSA. The membranes were incubated overnight at 4°C with the monoclonal antibody to Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, Calif.) or P-glycoprotein (F4) (Neomarkers, Calif.), polyclonal antibodies to the A subunit of calcineurin (anti-calcineurin A antibody) (Chemicon International), JNK or phospho-JNK (Thr183/Tyr185) (New England Biolabs, Beverly, Mass.), in T-TBS (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5 g/100 ml skim milk. After washing with T-TBS, the membranes were incubated with the corresponding secondary antibodies which were conjugated with horseradish peroxidase (HRP) in T-PBS or T-TBS containing 5 g/100 ml skim milk for 1 h at room temperature. Immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's protocol, and quantified by scanning densitometry using NIH Image (version 1.55).

RT-PCR

Total RNA were isolated by the method of Chomczynski and Sacchi [10]. mRNA was reverse-transcribed into single-stranded cDNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Roche Molecular Systems, Branchburg, N.J.). The sequences of sense and antisense primers for amplification of Bcl-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: sense primer of Bcl-2, 5′-CACCCCTGGCATCTTCTCCTT-3′; antisense primer of Bcl-2, 5′-AGCGTCTTCAGAGACAGCCAG-3′; sense primer of GAPDH, 5′-TCCTCCAGGTATGCAGTGCCA-3′; antisense primer of GAPDH, 5′-GTTATGGTGGGCAGGTGGGTT-3′. PCR amplification was carried out with AmpliTaq Gold DNA polymerase (Roche Molecular Systems) using a TSR-300 Thermal Sequencer (Iwaki Glass, Tokyo, Japan) after denaturation of the sample at 95°C for 10 min. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 58°C and 60°C for Bcl-2 and GAPDH, respectively, for 1 min, and polymerization at 72°C for 1 min. PCR products which were labeled with [α-^{32}P]-dCTP were separated by electrophoresis in 5% polyacrylamide gel. The amount of ^{32}P incorporated into each PCR product was quantified using a Bio-Imaging analyzer (BAS2000; Fujifilm, Tokyo, Japan). In pilot experiments, the amplification curves of GAPDH and Bcl-2 cDNAs were linear from 12 to 21 cycles and from 18 to 30 cycles, respectively. We chose 16 cycles and 24 cycles for amplification of GAPDH and Bcl-2 cDNAs, respectively. In addition, we confirmed a linear relationship between the relative signal and the amount of total RNA ranging from 3.125 to 50 ng. The amount of PCR product was normalized to that of GAPDH in each sample. We repeated RT-PCR three times for each sample to confirm the reproducibility of the results.

Statistical evaluation

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

Results

Effects of CsA and FK506 on the sensitivity to taxol of T24 and KK47 cells with WST-1 assay

In the present study, we used two human urinary bladder cancer cell lines. T24 cells and KK47 cells were derived from high and low grade cancer cells, respectively. We confirmed that inhibition of the cell proliferation of both cell lines by taxol was concentration dependent using the WST-1 assay (data not shown). We found that T24 cells were relatively resistant to taxol, and the IC_{50} value was 406.0 ± 24.6 nM ($n = 3$) (Fig. 1a). In contrast, KK47 cells were about fivefold more sensitive to taxol than T24 cells, and the IC_{50} value was 78.8 ± 7.4 nM ($n = 3$) (Fig. 1b). When we treated both cell lines with CsA in the presence of taxol, an enhancement of sensitivity to taxol was observed (Fig. 1a, b). We confirmed that the effect of CsA was concentration dependent, and

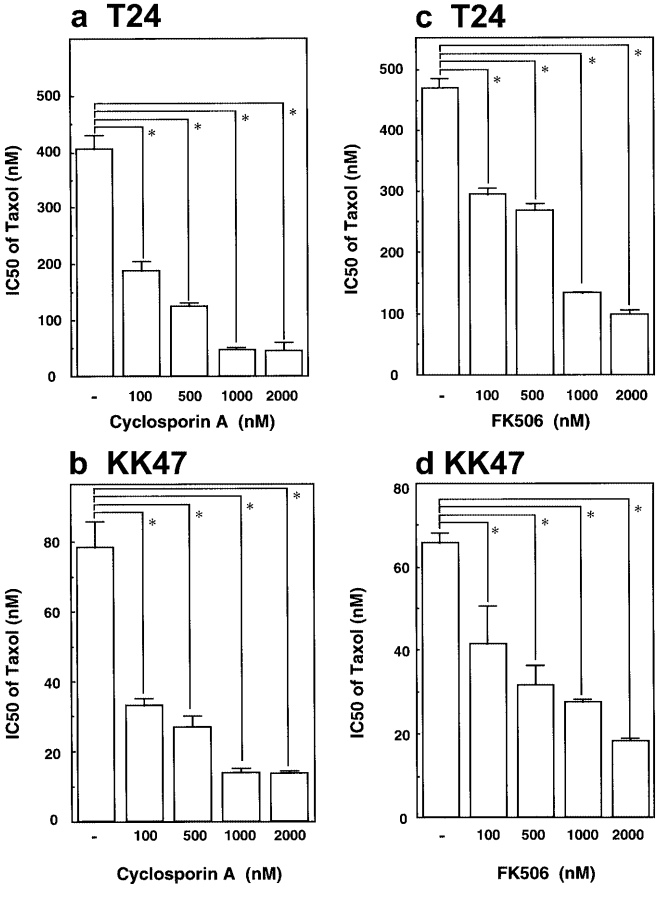


Fig. 1a–d. Determination by a WST-1 assay of the IC_{50} values of taxol in the presence of various concentrations of CsA and FK506. T24 cells (**a**, **c**) and KK47 cells (**b**, **d**) were seeded and treated with various concentrations of taxol in the presence of the indicated concentrations of CsA (**a**, **b**) and FK506 (**c**, **d**). The IC_{50} value of taxol was determined from each concentration-response curve. Values represent means ± SE (*bars*) from three independent experiments. *$p < 0.05$ compared with the control

the IC_{50} values were decreased to 47.5 ± 4.4 nM ($n = 3$) and 14.4 ± 0.8 nM ($n = 3$) in the case of T24 cells and KK47 cells, respectively, in the presence of 1 μM CsA (Fig. 1a, b). These results suggest that the effect of CsA was more pronounced in T24 cells than in KK47 cells. Because there was no significant difference in the levels of enhancement of growth inhibition between 1 and 2 μM CsA (Fig. 1a, b), 1 μM CsA was used in the following experiments. Treatment of the cells with 5 μM CsA alone for 48 h inhibited cell proliferation of T24 and KK47 cells by 58.6% and 52.0%, respectively. However, CsA alone at a lower concentration than 2 μM did not affect the cell growth of either cell line.

In addition to CsA, we examined whether FK506, another immunosuppressive drug, also enhanced the cells' sensitivity to taxol (Fig. 1c, d). The WST-1 assay clearly demonstrated that FK506 enhanced the inhibitory effects of taxol on the cell growth in a concentration-dependent manner. The IC_{50} values of T24 and KK47 cells were decreased to 135.3 ± 2.0 nM ($n = 3$) and 27.7 ± 0.7 nM ($n = 3$), respectively, in the presence of 1 μM FK506. Treatment of the cells with FK506 alone at a lower concentration than 2 μM did not affect the cell growth of either cell line (data not shown).

Inhibition of calcineurin activity by CsA and FK506

We also asked whether or not calcineurin occurred in both cell lines, and if so, whether or not the activity of calcineurin was inhibited by treating the cells with CsA and FK506 (Fig. 2). When immunoblot analysis was done with crude cell extracts of both cell lines, anti-calcineurin A antibody detected a protein with an apparent molecular mass of 61 kDa that comigrated with calcineurin A in crude extract of the human hippocampus (Fig. 2a). Although there was no difference in the amounts of calcineurin between T24 and KK47 cells, these were much less than those in the hippocampus. When we quantified the immunoreactivities and considered the amounts of protein from the hippocampus (3 μg) and from both cell lines (40 μg), the amount of calcineurin A in the hippocampus sample was more than 24 times higher than that in either cell line. These results are consistent with the reports that calcineurin is most abundant in the brain [39]. Figure 2b shows that calcineurin activity in T24 cells was almost completely lost after a 1-h treatment with CsA or FK506. When KK47 cells were treated with CsA, almost all activity was lost after 1 h (Fig. 2c). The effect of FK506 on calcineurin activity in KK47 cells was weaker than that of CsA, and the complete loss of activity was observed after 4 h of treatment (Fig. 2c). The activity was not recovered until 24 h of treatment of both cell lines with CsA. When KK47 cells were treated with FK506, the activity was gradually recovered, for unknown reasons (Fig. 2c). Immunoblot analysis showed no loss of calcineurin A protein with either treatment (Fig. 2b, c). From these results, we concluded that treatment of the cells with

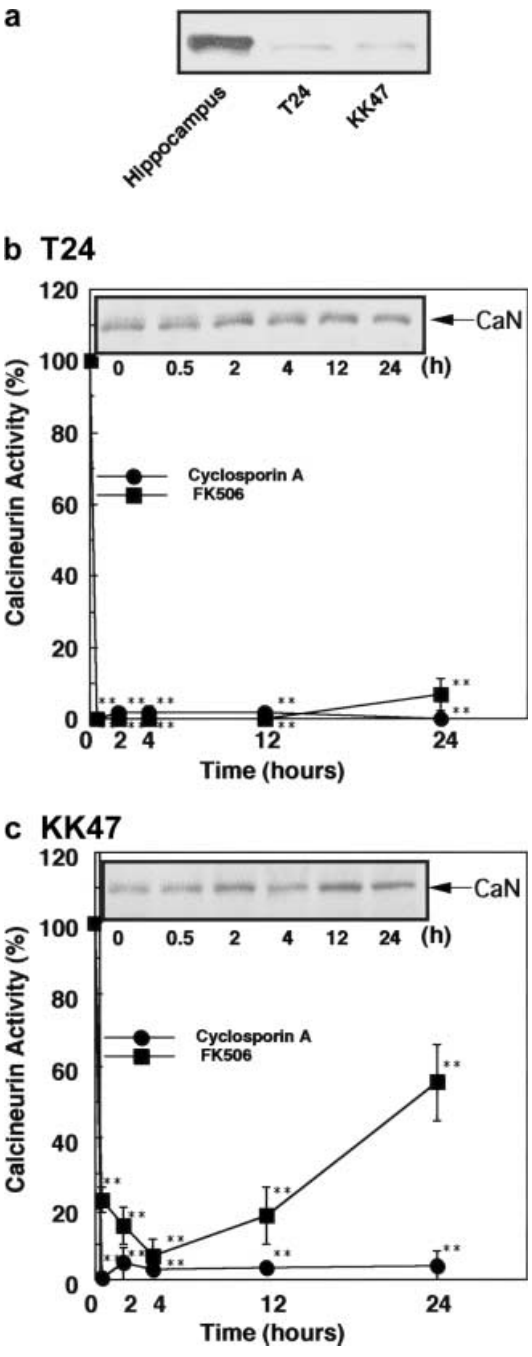


Fig. 2a–c. Immunoblot analysis of calcineurin and assay for calcineurin activity. **a** Crude extracts of human hippocampus (3 μg), T24 cells (40 μg) and KK47 cells (40 μg) were used for immunoblot analysis with anti-calcineurin A antibody at a dilution of 1:1,000. T24 (**b**) and KK47 (**c**) cells were treated with 1 μM CsA or 1 μM FK506 for the indicated times. The activity of calcineurin without CsA or FK506 was taken as 100%, and from this value, other values were calculated. Values represent means ± SE (*bars*) from three independent experiments. $^{**}p < 0.01$ compared with the control

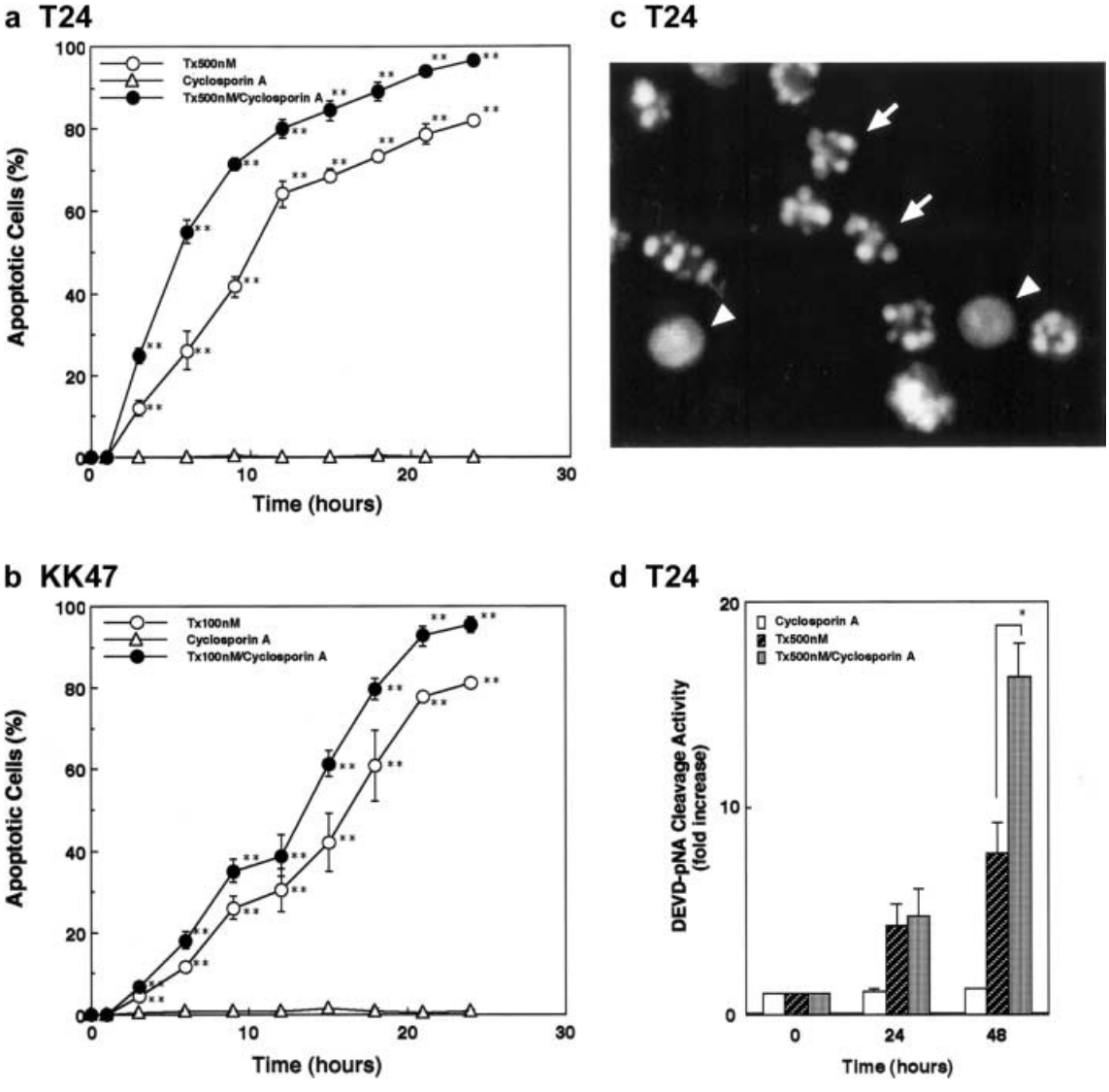
CsA and FK506 induced a large loss of calcineurin activity. Because CsA was more effective in inhibiting the activity than FK506 under our experimental conditions, CsA was used in the following experiments.

Effect of CsA on taxol-induced apoptosis by Hoechst 33258 staining and by measurement of caspase 3 activity

As the next step, we examined whether or not taxol treatment induced the apoptosis of T24 and KK47 cells (Fig. 3). Using Hoechst 33258 staining, apoptotic cells displayed condensed and fragmented nuclei, while non-apoptotic cells showed nuclei which were uniformly stained (Fig. 3c). When T24 cells and KK47 cells were treated with 500 nM and 100 nM taxol, respectively, a strong induction of apoptosis was observed (Fig. 3a, b). Therefore, apoptosis was only weakly enhanced by an addition of 1 μM CsA under these conditions. The percentages of apoptotic cells were increased to 96.7% and 95.5% from 81.8% ($P < 0.0001$) and 81.1% ($P < 0.0001$) by a combination treatment of T24 (Fig. 3a) and KK47 cells (Fig. 3b), respectively, after 24-h treatment. Treatment of either T24 or KK47 cells with CsA alone (1 μM) did not induce apoptosis (Fig. 3a, b).

When we measured the activity of caspase 3 without any treatment, the activity was not different between the two cell lines (data not shown). After 24-h treatment with taxol, the caspase 3 activity in T24 and KK47 cells were increased 4.3 ± 1.0-fold ($n = 3$, $P < 0.05$) and 9.2 ± 1.2-fold ($n = 3$, $P < 0.05$), respectively. After 24-h treatment

Fig. 3. Detection of apoptosis by Hoechst 33258 staining (**a**–**c**) and by assay for caspase 3 activity (**d**). T24 cells (**a**, **c**, **d**) and KK47 cells (**b**) were treated without or with 100 nM (**b**) and 500 nM (**a**, **c**, **d**) taxol in the presence or absence of 1 μM CsA. **a**, **b** apoptotic cells were counted after Hoechst 33258 staining. Values represent means ± SE (*bars*) from three independent experiments. $^{**}p < 0.01$ compared with the control (without taxol and CsA). The representative nuclear staining in the apoptotic cells is shown in **c**. Apoptotic cells after 12 h of treatment with 500 nM taxol displayed the condensed and fragmented nuclei (*arrows*), while non-apoptotic cells showed the nuclei which were uniformly stained (*arrowheads*). **d** Caspase 3 activity was measured with DEVD-pNA as substrate. We repeated the same experiments three times with reproducible results; representative results are shown. Differences between taxol and taxol plus CsA after 48-h treatment was statistically significant ($^{*}p < 0.05$)



of KK47 cells with taxol and CsA, caspase 3 activity was increased 19.0 ± 3.0-fold ($n = 3$, $P < 0.05$). After 48-h treatment of T24 cells with taxol, caspase 3 activity was increased 7.8 ± 1.5-fold ($n = 3$, $P < 0.05$) and 16.4 ± 1.6-fold ($n = 3$, $P < 0.01$) in the absence and presence of CsA, respectively (Fig. 3d). These results indicated that CsA enhanced the apoptotic effect of taxol in addition to enhancing the inhibitory effect of taxol on cell growth.

Phosphorylation of Bcl-2 by treatment with taxol

P-glycoprotein, an ATP-dependent drug efflux pump encoded by the *MDR1* gene, is reported to be one of the targets of CsA [6, 41]. Inhibition of P-glycoprotein by CsA may be involved in the enhancement of sensitivity to taxol. However, immunoblot analysis with anti-P-glycoprotein antibody did not detect the protein in either T24 cells or KK47 cells (data not shown). Therefore, we decided to investigate whether or not the inhibition of calcineurin activity contributed to the enhancement of sensitivity to taxol. Because the effect of CsA was more pronounced in T24 cells than KK47 cells, the following experiments were conducted mainly with T24 cells. Taxol treatment has been reported to induce the phosphorylation of Bcl-2 which is well correlated with the apoptosis of various cancer cells [15, 18, 19]. It has been suggested that Bcl-2 is one of the substrates for calcineurin [18, 34]. Therefore, we intended to examine whether taxol treatment induced the phosphorylation of Bcl-2, and whether CsA enhanced the taxol-induced phosphorylation of Bcl-2. For this purpose, we prepared an antibody specific to Bcl-2 phosphorylated at serine 70 (anti-pS70-Bcl-2 antibody). Immunoblot analysis using the antibody clearly demonstrated at least three bands after taxol treatment of T24 cells (Fig. 4a). When we investigated the time course of the phosphorylation of Bcl-2 by treatment of T24 cells with 500 nM taxol, we found that it was increased within 0.5 h of treatment, with a peak occurring at 24 h followed by a decrease to 48 h (Fig. 4a). Treatment of KK47 cells with 100 nM taxol increased the phosphorylation of Bcl-2 with a similar time course to that in T24 cells, and the phosphorylation was increased 3.2-fold ($n = 3$, $P < 0.01$) after 24-h treatment (data not shown). When we treated T24 cells in combination with 500 nM taxol and 1 μM CsA, robust phosphorylation of Bcl-2 was observed. Compared with the control, taxol alone and taxol plus CsA increased the phosphorylation of Bcl-2 3.5-fold ($n = 5$, $P < 0.0001$) and 9.3-fold ($n = 5$, $P < 0.05$), respectively, after 24-h treatment (Fig. 4b). Treatment of the cells with CsA alone (1 μM) did not increase the phosphorylation of Bcl-2 in either cell line.

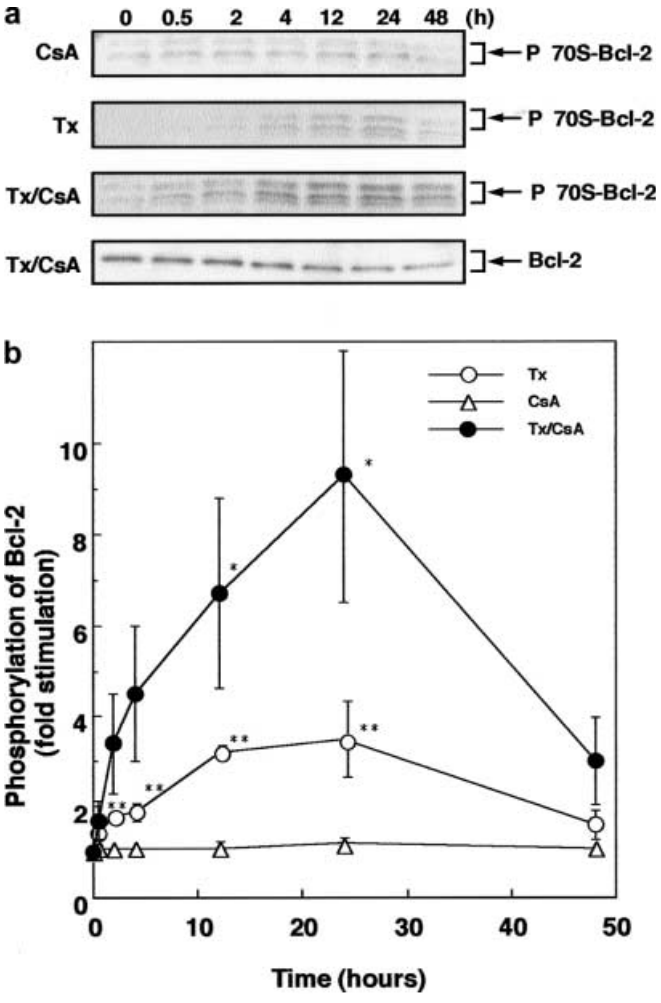


Fig. 4a, b. Serial changes in phosphorylation of Bcl-2 by treatment of T24 cells with taxol and CsA. **a** The representative autoradiographs are shown. T24 cells were treated with or without 500 nM taxol (*Tx*) in the presence or absence of 1 μM CsA for the indicated times. The cell extract (20 μg) was used for immunoblot analysis with anti-Bcl-2 antibody and anti-pS70-Bcl-2 antibody (P 70S-Bcl-2) at a dilution of 1:1,000. **b** Phosphorylation of Bcl-2 without taxol and CsA was scored as 1, and each value is shown as a multiple of stimulation. Values represent means ± SE(*bars*) from three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with the control

Activation of JNK by treatment with taxol

As the next step, we examined whether or not JNK was activated by taxol treatment, and whether or not CsA enhanced the taxol-induced activation of JNK. When immunoblot analysis was carried out with an anti-JNK antibody, the antibody recognized proteins of 46 kDa (minor) of JNK1 and 54 kDa (major) of JNK2 [14] in a crude cell extract of T24 cells. No significant changes in the amount of the two proteins were observed with either taxol or CsA treatment of either cell line (data not shown). Immunoblot analysis with the anti-phospho-JNK antibody suggested that JNK was essentially inactive as shown by the low level of phosphorylation of JNK (Fig. 5a). Treatment of T24 cells with 500 nM taxol induced the phosphorylation of JNK within 0.5 h (Fig. 5a). Furthermore, the addition of CsA enhanced the phosphorylation of JNK by taxol treatment. Immunoblot analysis showed no change in the amount of

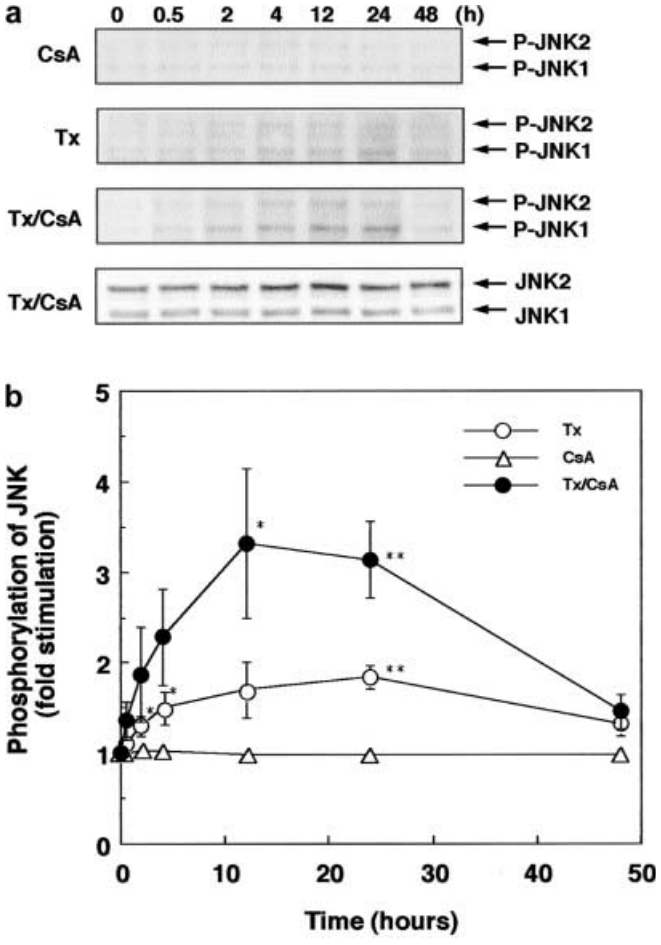


Fig. 5a, b. Serial changes in the phosphorylation of JNK by treatment of T24 cells with taxol and CsA. **a** The representative autoradiographs are shown. T24 cells were treated with or without 500 nM taxol (*Tx*) in the presence or absence of 1 μM CsA for the indicated times. The cell extract (20 μg) was used for immunoblot analysis with anti-JNK antibody and anti-phospho-JNK antibody at a dilution of 1:1,000. **b** Phosphorylation of JNK1 and JNK2 without taxol and CsA was scored as 1, and each value is shown as a fold stimulation. Values represent means ± SE (*bars*) from three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with the control

JNK protein with the combination treatment (Fig. 5a). Figure 5b summarizes the results from three independent experiments. Compared with the control, taxol alone and taxol plus CsA increased the phosphorylation of JNK 1.8-fold ($n = 3$, $P < 0.01$) and 3.3-fold ($n = 3$, $P < 0.01$), respectively, after 24-h treatment. Treatment of KK47 cells with 100 nM taxol increased the phosphorylation of JNK 1.4-fold ($n = 3$, $P < 0.05$) after 12-h treatment (data not shown). Treatment of the cells with CsA alone (1 μM) did not increase the phosphorylation of JNK in either cell line.

Effect of CsA treatment on expression of Bcl-2

When we examined the amount of Bcl-2 protein, no significant change was observed by the treatment of either cell line with taxol alone (data not shown). However, treatment of T24 cells with 1 μM CsA for 24 and 48 h decreased the amount of Bcl-2 to 76.0 ± 1.2% ($n = 3$, $P < 0.01$) and 69.0 ± 6.6% ($n = 3$, $P < 0.01$), respectively, of the control value (Fig. 6a). Up to 24-h treatment of KK47 cells with 1 μM CsA showed no significant change in the amount of Bcl-2 (data not shown). After 48-h treatment, the amount was decreased to 72.0 ± 7.0% of the control value ($n = 3$, $P < 0.05$).

As the next step, we performed the quantitative RT-PCR analysis. Figure 6b and c clearly indicate that CsA treatment of T24 cells decreased Bcl-2 mRNA in a time-dependent manner. CsA treatment of KK47 cells also decreased Bcl-2 mRNA in a time-dependent manner (data not shown). When the results were quantified, the level of Bcl-2 mRNA was decreased to 21.4 ± 0.7% ($n = 3$, $P < 0.0001$) and 34.5 ± 6.3% ($n = 3$, $P < 0.0001$) of the control values by treatment with 1 μM CsA of T24 and KK47 cells, respectively, for 24 h. These results suggest that the decrease in the amount of Bcl-2 protein by CsA treatment is due to the decrease in the amount of Bcl-2 mRNA.

Discussion

In the present study, we confirmed that taxol induced apoptosis as well as inhibition of cell growth in two cell lines. Next we found that CsA and FK506 enhanced the effects of taxol. The effects of CsA and FK506 were pronounced in T24 cells, and the sensitivity to taxol was increased more than eightfold by CsA treatment using the growth inhibition assay. To our knowledge, this is the first report on the enhancement of sensitivity to taxol by immunosuppressive drugs. As described above, taxol alone has been used for intravesical chemotherapy [3, 29, 35]. In view of the fact that CsA is not desirable for general treatment due to its immunosuppressive effect, the combination of taxol and CsA would be better for intravesical chemotherapy.

Since P-glycoprotein has been reported to be one of the targets of CsA [6, 41], the inhibition of P-glycoprotein by CsA may play some role in the enhancement of taxol-induced apoptosis. CsA was reported to have low potency as a calcineurin inhibitor in cells expressing high levels of P-glycoprotein [13]. The expression of P-glycoprotein was barely detected in T24 and KK47 cells at the protein level. Furthermore, we found that calcineurin occurred in both cell lines and the activities were almost completely lost after 1 h of treatment with CsA. These results suggest that CsA has a high potency as a calcineurin inhibitor in these cell lines. Therefore, we investigated additional putative mechanisms of potentiating taxol-induced apoptosis via the inhibition of calcineurin activity.

There is growing evidence that the functions of Bcl-2 are strictly regulated by reversible phosphorylation and dephosphorylation [20, 21, 36]. Taxol has been reported to activate JNK which in turn phosphorylates Bcl-2

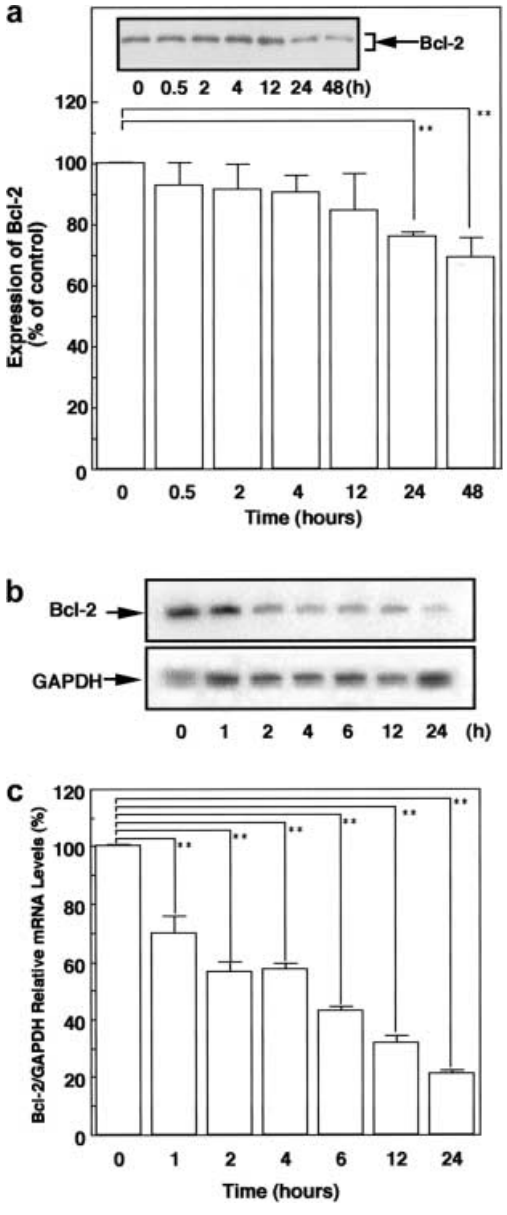


Fig. 6. Serial changes in the amount of Bcl-2 protein (**a**) and mRNA (**b**, **c**) by treatment of T24 cells with CsA. T24 cells were treated with 1 μM CsA for the indicated times. **a** The cell extract (20 μg) was used for immunoblot analysis with anti-Bcl-2 antibody at a dilution of 1:1,000. The amount of the control was taken as 100%, and other values were calculated from this value. Values represent means ± SE (*bars*) from three independent experiments. $^{**}p < 0.01$ compared with the control. **b, c** Total RNA (50 ng) prepared from the cells was subjected to a quantitative RT-PCR analysis. **b** The representative autoradiographs are shown. **c** The amount of PCR product of Bcl-2 was normalized to that of GAPDH in each sample. The value of the control was taken as 100%, and other values were calculated from this value. Values represent means ± SE (*bars*) from three independent experiments. $^{**}p < 0.01$ compared with the control

which is followed by the induction of apoptosis [5]. We confirmed that taxol treatment of T24 and KK47 cells activated JNK and induced the phosphorylation of Bcl-2. Bcl-2 has been suggested to be a substrate for calcineurin [18, 34]. It was reported that treatment of the cells with calyculin A, a specific inhibitor for protein phosphatases 1 and 2A, did not affect the phosphorylation of Bcl-2 [18]. Therefore, we considered the possibility that the inhibition of calcineurin activity may inhibit the dephosphorylation of Bcl-2. Immunoblot analysis with anti-pS70-Bcl-2 antibody indicated that a combination treatment with taxol and CsA enhanced the phosphorylation of Bcl-2. Together with other reports, these results strongly suggested that taxol treatment induced the phosphorylation of Bcl-2 via activation of the JNK pathway and that CsA enhanced the phosphorylation mainly by a decrease in the dephosphorylation of Bcl-2 via the inhibition of calcineurin activity. Since basal JNK activity was low and CsA treatment alone did not activate JNK, it is reasonable that the phosphorylation of Bcl-2 did not occur by CsA treatment alone.

Among the phosphorylation sites in Bcl-2, we focused on serine 70 based on the following, recent findings: (1) serine 70 lies in a loop region and the phosphorylation of this region impaired the antiapoptotic effect of Bcl-2 [4, 9], (2) serine 70 is one of the critical phosphorylation sites for JNK-induced apoptosis [37, 46], (3) a site-directed mutagenesis study suggests that serine 70 is one of the major phosphorylation sites by treatment with taxol [21], and (4) Bcl-2 is phosphorylated during the normal cell cycle progression. For this phosphorylation, serine 70 is the principal phosphorylation site [21, 36]. Using anti-pS70-Bcl-2 antibody, we obtained clear results on the phosphorylation of Bcl-2. These results suggest that the antibody is useful for examining the phosphorylation of Bcl-2 in cells. Anti-pS70-Bcl-2 antibody detected more than three bands. These results suggest that other sites than serine 70 were also phosphorylated by taxol treatment and that multiple phosphorylated Bcl-2 migrated at the different positions.

It is possible that phosphorylation by JNK of other proteins in addition to Bcl-2 is involved in taxol-induced apoptosis. Identification of these proteins and examination of their dephosphorylation by calcineurin may be interesting projects for future studies.

In addition to the increase in the phosphorylation of Bcl-2, we found that the content of Bcl-2 was decreased by CsA treatment at both the protein and mRNA levels. Since the decrease in the mRNA was more pronounced than that in the protein, CsA may inhibit the transcription of Bcl-2. In this context, it was reported that inhibition of calcineurin activity decreased Bcl-2 mRNA in IL-2-stimulated cells and that the overexpression of a constitutive active mutant of calcineurin induced the gene expression of Bcl-2 [16]. Taken together, calcineurin may have antiapoptotic actions at least partly via the induction of the gene expression of Bcl-2 as well as the dephosphorylation of Bcl-2.

Acknowledgements We gratefully acknowledge Ms. N. Hamamatsu for her excellent technical assistance in the experiment. This work was supported in part by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan; by a

Research Grant from Human Frontier Science Program (H. Y. and E. M.).

References

1. American Cancer Society (1996) Estimated new cancer cases and deaths by sex for all sites. http://www.cancer.org
2. Atray B, Girgin C, Kefi A, Cikili N (2000) The best management of superficial bladder tumours: comparing TUR alone versus TUR combined with intravesical chemotherapy modalities? Int Urol Nephrol 32: 53
3. Au JLS, Kalns J, Gan Y, Wientjes MG (1997) Pharmacologic effects of paclitaxel in human bladder tumors. Cancer Chemother Pharmacol 41: 69
4. Basu A, Haldar, S (1998) Microtubule-damaging drugs triggered Bcl2 phosphorylation-requirement of phosphorylation on both serine-70 and serine-87 residues of Bcl2 protein. Int J Oncol 13: 659
5. Blagosklonny MV, Fojo T (1999) Molecular effects of paclitaxel: myths and reality (a critical review). Int J Cancer 83: 151
6. Botling J, Liminga G, Larsson R, Nygren P, Nilsson K (1994) Development of vincristine resistance and increased sensitivity to cyclosporin A and verapamil in the human U-937 lymphoma cell line without overexpression of the 170-kDa P-glycoprotein. Int J Cancer 58: 269
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248
8. Bubenik J, Baresova M, Viklicky V, Jakoubkova J, Sainerova H, Donner J (1973) Established cell line of urinary bladder carcinoma (T24) containing tumours-specific antigen. Int J Cancer 11: 765
9. Chang BS, Minn AJ, Muchmore SW, Fesik SW, Thompson CB (1997) Identification of a novel regulatory domain in Bcl-xL and Bcl-2. EMBO J 16: 968
10. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156
11. Cohen P (1989) The structure and regulation of protein phosphatases. Annu Rev Biochem 58: 453
12. Cookson MS, Herr HW, Zhang ZF, Soloway S, Sogani PC, Fair WR (1997) The treated natural history of high risk superficial bladder cancer: 15-year outcome. J Urol 158: 62
13. Fakata KL, Elmquist WF, Swanson SA, Vorce RL, Prince C, Stemmer PM (1998) Cyclosporin A has low potency as a calcineurin inhibitor in cells expressing high levels of P-glycoprotein, Life Sci 62: 2441
14. Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA, Chambers TC (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-X_L is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. J Biol Chem 275: 29980
15. 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
16. Gomez J, Martinez-AC, Gonzalez A, Garcia A, Rebollo A (1998) The Bcl-2 gene is differentially regulated by IL-2 and IL-4: role of the transcription factor NF-AT. Oncogene 17: 1235
17. Gopalakrishna R, Anderson WB (1982) Ca^{2+} -induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. Biochem Biophys Res Commun 104: 830
18. Haldar S, Jena N, Croce CM (1995) Inactivation of Bcl-2 by phosphorylation. Proc Natl Acad Sci U S A 92: 4507
19. Haldar S, Chintapalli J, Croce CM (1996) Taxol induces *bcl*-2 phosphorylation and death of prostate cancer cells. Cancer Res 56: 1253
20. Haldar S, Basu A, Croce CM (1997) Bcl-2 is the guardian of microtubule integrity. Cancer Res 57: 229
21. Haldar S, Basu A, Croce CM (1998) Serine-70 is one of the critical sites for drug-induced Bcl2 phosphorylation in cancer cells. Cancer Res 58: 1609
22. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He PG (1993) A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull 41: 1118
23. Kamat AM, Lamm DL (2000) Intravesical therapy for bladder cancer. Urology 55: 161
24. Kimiya K, Naito S, Soejima T, Sakamoto N, Kotoh S, Kumazawa J, Tsuruo T (1992) Establishment and characterization of doxorubicin-resistant human bladder cancer cell line, KK47/ADM. J Urol 148: 441
25. Klee CB, Ren H, Wang X (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin, J Biol Chem 273: 13367
26. Kobayashi T, Consoli U, Andreeff M, Shiku H, Deisseroth AB, Zhang W (1995) Activation of $p21^{WAF1/Cip1}$ expression by a temperature-sensitive mutant of human p53 does not lead to apoptosis. Oncogene 11: 2311
27. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680
28. Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC, Arkinstall S (1997) Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J Biol Chem 272: 25238
29. Nativ O, Aronson M, Medalia O, Moldavsky T, Sabo E, Ringel I, Kravtsov V (1997) Anti-neoplastic activity of paclitaxel on experimental superficial bladder cancer: in vivo and in vitro studies. Int J Cancer 70: 297
30. Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. Cell 74: 609
31. Poommipanit PB, Chen B, Oltvai ZN (1999) Interleukin-3 induces the phosphorylation of a distinct fraction of Bcl-2. J Biol Chem 274: 1033
32. Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol 124: 1
33. Richie JP (1992) Intravesical chemotherapy. Treatment selection, techniques, and results. Urol Clin North Am 19: 521
34. Shibasaki F, Kondo E, Akagi T, McKeon F (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature 386: 728
35. Song D, Wientjes MG, Au JLS (1997) Bladder tissue pharmacokinetics of intravesical taxol. Cancer Chemother Pharmacol 40: 285
36. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-Chung YS, Longo D L (1998) Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol Cell Biol 18: 3509
37. Srivastava RK, Mi QS, Hardwick JM, Longo DL (1999) Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. Proc Natl Acad Sci U S A 96: 3775
38. Taya T, Kobayashi T, Tsukahara K, Uchibayashi T, Naito K, Hisazumi H, Kuroda K (1977) In vitro culture of malignant tumor tissue from the human urinary tract. Jpn J Urol 68: 1003
39. Stemmer P, Klee CB (1991) Serine/threonine phosphatases in the nervous system. Curr Opin Neurobiol 1: 53
40. 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
41. Twentyman PR (1992) Cyclosporins as drug resistance modifiers. Biochem Pharmacol 43: 109
42. Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC (1999) Ca^{2+} -induced apoptosis through calcineurin dephosphorylation of BAD. Science 284: 339

43. Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J (1998) Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 273: 4928
44. White, E (1996) Life, death, and the pursuit of apoptosis. Genes Dev 10: 1
45. Yamamoto H, Hasegawa M, Ono T, Tashima K, Ihara Y, Miyamoto E (1995) Dephosphorylation of fetal-tau and paired helical filaments-tau by protein phosphatases 1 and 2A and calcineurin. J Biochem 118: 1224
46. Yamamoto K, Ichijo H, Korsmeyer SJ (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. Mol Cell Biol 19: 8469
47. Yang E, Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood 88: 386