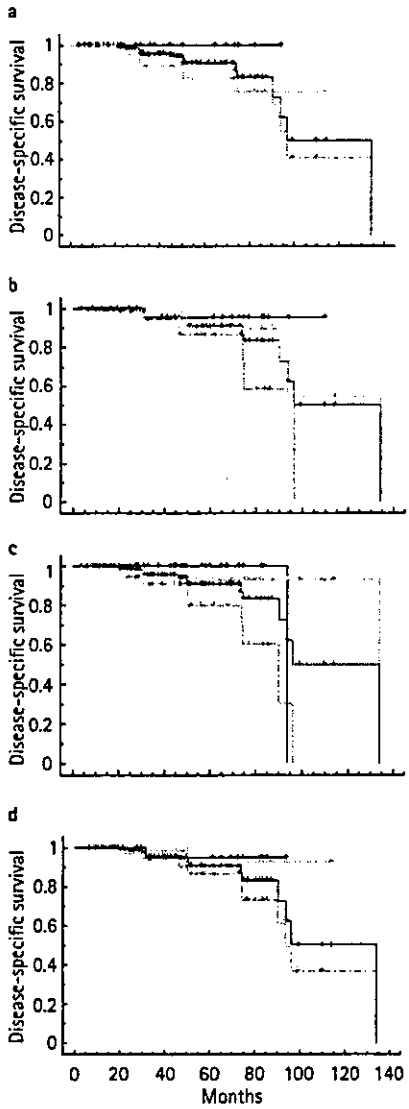
FIG. 1. DSS curves for the 128 patients treated with RP, by tumour grade (a), PSA level (b), pathological stage (c) and (d) risk groups, as determined by the Kaplan-Meier method. In each plot the solid green line is for all patients; the red dotted line, light green dashed line and light red dashed/dotted line indicate respectively; (a) well, moderately and poorly differentiated tumour (P = 0.316); (b) PSA ≤10.0, 10.1–50.0 and >50 ng/mL (P = 0.102); (c) pT2N0, pT3N0 and pN1 (P = 0.002); (d) low-risk, intermediate-risk and high-risk groups (P = 0.184).



value indicated a modest difference in DSS rates between the groups stratified by initial PSA levels ($P = 0.102$; Fig. 1b). The group with a pretreatment PSA level of ≥50.1 ng/mL had the lowest DSS rate. The DSS rates by pathological stage at 5 years for patients at stages pT2, pT3 and pN1 with any pT were 100%, 93% and 79.5%, respectively ($P = 0.002$; Fig. 1c).

There was no statistically significant difference in the DSS rate among the risk groups ($P = 0.184$) but the DSS rate 7 years after surgery was 73% in the high-risk group (Fig. 1d), and lower than in the low and intermediate risk groups (96% and 90%, respectively).

FIG. 2. DSS curves for the 75 patients treated with EBRT or NHT and EBRT alone, by tumour grade (a), PSA level (b) and risk group (c), as determined by the Kaplan-Meier method. The key is the same as for Fig. 1. (a) P = 0.079, (b) P = 0.007, (c) P = 0.002, and (d) no P value generated.

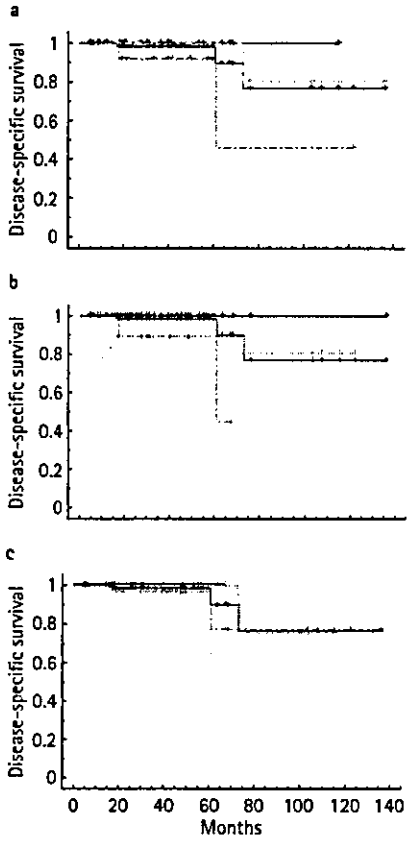


Fig. 2a–c shows the probability curves for the DSS of patients who received EBRT and NHT, and EBRT alone; three of the 75 patients died from prostate cancer during the follow-up; one had moderately differentiated and the other two poorly differentiated disease. The DSS rates at 5 and 10 years for these 75 patients were 98% and 76%, respectively. Patients with higher tumour grades had poor DSS rates ($P = 0.079$; Fig. 2a). However, paradoxically, the DSS rates were lower in patients with lower PSA levels ($P = 0.007$; Fig. 2b). Of patients with PSA levels of ≤10.0 ng/mL who died from prostate cancer, all had poorly differentiated carcinoma. The DSS rates at 5 years for both the low and intermediate risk groups was 100%; that for the high risk group was 96% (*P* not generated).

Of the 176 patients in the primary hormone therapy group, 11 died from prostate cancer; the DSS rate at 5 years for these patients was 89% (Fig. 3a–c). Patients with poorly differentiated tumours had the lowest DSS rate, although it was not statistically significant ($P = 0.309$) (Fig. 3a). There were no significant differences in DSS rates among the risk groups stratified by PSA levels ($P = 0.719$; Fig. 3b) nor among the risk groups ($P = 0.317$; Fig. 3c), but the rate at 5 years was 77% in the high risk group, which was lower than that in the low and intermediate risk groups (80% and 95%, respectively).

DISCUSSION

Therapeutic guidelines for locally advanced prostate cancer have been established in Europe [14] and the USA [15], but not yet in Japan. It remains controversial in Japan whether urologists should choose RP or radiotherapy with the aim of achieving potential cure, or use primary hormone therapy as palliative treatment. Although therapeutic guidelines have not yet been established in Japan, the therapeutic approaches to locally advanced prostate cancer assessed here were not significantly different from those set out in the European and American guidelines.

About half of all patients in Japan, Europe and the USA who were treated for locally advanced prostate cancer in the 1990s are reported to have been treated with NHT before RP [16,17]. Short-term NHT was also given to nearly half of the patients treated with RP in the present study. NHT was used to treat Japanese patients diagnosed to have more advanced disease; it can be assumed that the participating urologists preferred NHT during the present study period, supporting the hypothesis that NHT may

improve the complete cure rate. Most randomized clinical studies report that NHT significantly decreases the margin-positive rate [1,2], but randomized clinical trials have not shown that NHT improves the long-term survival rate after surgery [18,19]. Accordingly, there is serious doubt about whether NHT can be supported as a standard therapy.

In the present study, AHT after RP was most commonly used not only for treating stage pN1 disease, but also for stage pT3N0 disease, when adjuvant radiotherapy is often chosen in Europe and the USA for this stage [15,20]. Some studies report that AHT improves the progression-free survival rate [20,21] in pathological stage C patients. Conversely, Beyer *et al.* [22] stated that AHT for pathological locally advanced cancer or positive lymph nodes does not improve progression-free survival rates. Most of the previous studies were not randomized clinical studies and do not clearly identify the advantages of AHT for long-term survival. However, a recent randomized clinical trial showed that AHT for patients with positive lymph nodes improved overall survival, prostate cancer-specific survival, and clinical progression-free survival [3]. The frequent use of NHT and AHT in the present study may indicate that the participating urologists recognized the impact of staging error and determined that most clinical locally advanced prostate cancers might consist of systemic disease that would not be cured by RP alone.

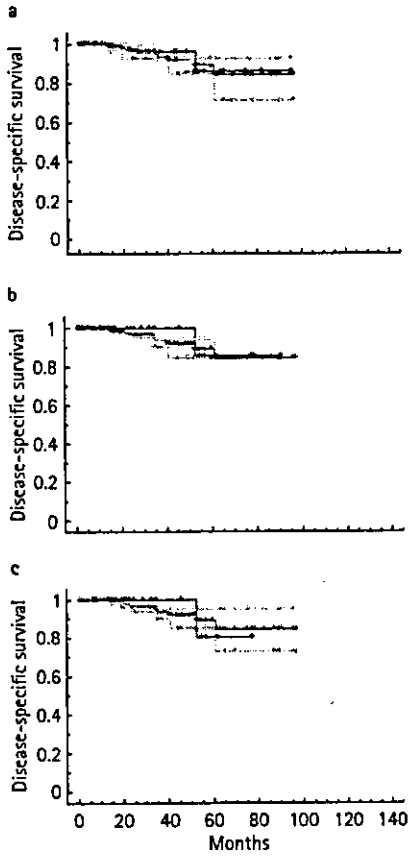
According to the guidelines [14,15] for therapeutic approaches to locally advanced prostate cancer, radiotherapy is the primary treatment option. In terms of using hormone therapy with radiotherapy, most recent reports found combined therapy to be beneficial. Although the mechanism of the interaction between radiotherapy and hormone therapy remains largely unknown, a possible additive or synergistic effect brought about by NHT has been reported [23]. The survival benefit provided by long-term AHT after radiation therapy has been shown by a randomized clinical trial [4] and meta-analysis [7] but prospective randomized trials should be undertaken to clarify which patients will not benefit at all, or benefit from short-term or only from long-term AHT [6]. In the present study, most patients received EBRT combined with hormone therapy; the results may indicate that combined therapy comprising radiotherapy and hormone therapy has been recognized as an effective treatment option by the participating urologists for the last decade.

The effectiveness of primary hormone therapy for many types of locally advanced prostate cancer was reported previously [24]. A recent report indicated that the benefits of hormone therapy alone are similar to those of other therapeutic approaches [25]. In the present study, primary hormone therapy alone tended to be used in relatively older patients or those with high PSA levels. We suggest that primary hormone therapy was chosen for relatively older patients with a higher risk of comorbidity, or for those with less chance of a complete cure.

Few randomized trials have been undertaken to evaluate the outcome of RP vs radiotherapy for locally advanced prostate cancer with no distant metastasis [5]. In the present study, the participating urologists tended to select RP more than radiation therapy for younger patients. However, most patients in this study had been diagnosed with stage pT3N0 or pN1 disease after RP and thus there may have been fewer candidates who should have been treated with RP. The choice of RP or radiotherapy for treating locally advanced prostate cancer will not become clear until well-controlled randomized studies provide sufficient evidence of long-term survival and improved QoL.

For treatment outcomes, the 5-year DSS rates after RP, radiotherapy and primary hormone therapy vary in the historical controls, at 83–92% [10,11], 50–100% [5,6], and 84–93% [9,24,25], respectively. The present results were not significantly different from these historical controls, although the characteristics of the patients were naturally biased among the reports. In the present RP group the patients with stage pT2 disease and even stage pT3 disease had longer DSS; the curves in the low- and intermediate-risk groups were satisfactory. However, the high-risk group comprising patients with a pretreatment PSA level of ≥50.1 ng/mL and/or a poorly differentiated tumour had the lowest long-term DSS, and thus probably affected the long-term DSS of the RP group. Thus, RP may also be an appropriate treatment for selected patients with either less advanced or low-grade tumours [11,12].

FIG. 3. DSS curves for the 176 patients treated with primary hormone therapy alone by tumour grade (a), PSA (b) and risk group (c), as determined by the Kaplan–Meier method. The key is the same as for Fig. 1. (a) P = 0.309, (b) P = 0.719, (c) P = 0.317.



Most of the patients who received radiotherapy concomitantly had EBRT and hormone therapy. This treatment strategy is similar to that set out in European and American guidelines but in the present study, lymph-node dissection and irradiation dose varied, so strictly the method of radiotherapy was heterogeneous. The present irradiation dose was generally lower than in other reports [4,6], although short-term DSS was not significantly different from that of the historical controls. However, this result does not necessarily suggest that even patients who are given a low irradiation dose will have a long DSS, because of the inherent bias of retrospective study, a short follow-up and the few patients. Studies involving increasing doses and combined therapy with NHT and AHT should be undertaken to clarify the effects of modern combined therapy. The

present results may also indicate the usefulness of primary hormone therapy in controlling locally advanced prostate cancer in relatively older patients with a higher risk of comorbidity, or patients with a lower possibility of complete cure.

Recent investigations indicate that different promoting factors, including genetic, epigenetic and environmental influences, might be responsible for ethnic variations in the progression of prostate cancer after its induction [26]. It has also been reported that there are large differences in prostate cancer incidence and plasma phyto-oestrogen concentration between Western and Japanese men [27]. Responses to therapy, e.g. hormone therapy, and the natural history after treatment might not be identical between ethnic groups which have considerable differences in prostate cancer incidence, plasma phyto-oestrogen concentration and racial composition, even if their standard of living, including health care, is similar. However, in the present study the primary treatments given to patients were not substantially different from those set out in European and American guidelines, and short-term DSS rates were not significantly different from those of the historical European and American controls. This might be significant, as the results were derived from an ethnic group in which prostate cancer incidence, dietary factors and racial composition differ considerably from those of Europeans and Americans. However, the results should be interpreted cautiously, because of inherent bias associated with this retrospective study. Therefore, it is necessary to undertake additional studies, including randomized controlled studies, to identify which therapeutic approach offers the best opportunity for survival and enhanced QoL for Japanese men. Further investigation may help to establish Japanese guidelines for the diagnosis and treatment of locally advanced prostate cancer.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **RP**, radical prostatectomy; **DSS**, disease-specific survival; **QoL**, quality of life; **NHT, AHT**, neoadjuvant, adjuvant hormone therapy; **EBRT**, external beam radiotherapy; **3D-CRT**, three-dimensional conformal radiotherapy.

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Blockade of Paclitaxel-Induced Thymidine Phosphorylase Expression Can Accelerate Apoptosis in Human Prostate Cancer Cells

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ABSTRACT

Recently, survival benefit by chemotherapy using paclitaxel (PTX) and the induction of thymidine phosphorylase (TP) by PTX have been reported in several solid tumors. On the other hand, TP confers antiapoptotic effect on tumor cells through inhibition of caspase-8 activation *in vitro*. On the basis of these previous observations, we hypothesized that (*a*) TP can be induced after PTX treatment in human prostate cancer (PC) and (*b*) blockade of PTX-induced TP expression can enhance the apoptotic processes in human PC cells. PTX was used to find TP expression in all eight hormone-refractory PC cases after chemotherapy; however, cleaved caspase-8 was not expressed after chemotherapy in the six hormone-refractory PC cases with strong TP expression. In PC cell lines (PC-3, DU 145, and LNCaP), TP expression after PTX treatment was clearly up-regulated in a dose-dependent manner. Cell viability of PC cell lines treated with PTX and TP antisense was significantly reduced in a time-dependent and dose-dependent manner compared with the PTX treatment alone. Likewise, apoptotic index of PC cells treated with PTX and TP antisense was significantly increased in comparison with PTX alone. After complete blockade of PTX-induced TP translation by TP antisense transfection, cleaved form of caspase-3 and poly(ADP-ribose) polymerase was increased, and this exaggeration of apoptosis also ran parallel with caspase-8 activation in a PTX dose-dependent manner. However, in PC cell lines treated with TP antisense alone, neither caspase-3 nor poly(ADP-ribose) polymerase was cleaved despite caspase-8 activation. These results indicate that PTX-induced TP up-regulation is associated with decreased caspase-8 activation. This study is the first report showing that blockade of PTX-induced TP expression could exaggerate the processing of apoptosis in PC cells treated with PTX. Our results provide preclinical evidence that TP could be a new molecular target for enhancing the potency of PTX-mediated apoptosis in PC cells.

INTRODUCTION

Thymidine phosphorylase (TP) is identical to platelet-derived endothelial cell growth factor and functions as chemotactic and angiogenic molecules (1). TP reversibly catalyzes the phosphorylation of thymidine to thymine and 2-deoxyribose-1-phosphate. TP also promotes tumor growth and confers resistance on apoptosis independent of angiogenesis, playing a key role in the invasiveness and metastasis of TP-expressing solid tumors (2). Recently, TP has been reported to inhibit Fas-induced caspase-8 activation, which in turn leads to the loss of mitochondrial membrane potential (3), and subsequently to prevention of the cytochrome *c* release, caspase-3 activation, and finally to inhibition of apoptosis (3). On the other hand, several lines of evidence show that TP is clearly induced in established human cancer cells by taxanes such as paclitaxel (PTX; ref. 4).

PTX, first isolated from the bark of the Western yew tree (*Taxus brevifolia*), is a complex diterpene that is distinct from other antimicrotubule agents in that it binds directly to polymerized tubulin, promoting microtubule assembly and stability (rather than instability) and preventing depolymerization (5). In clinical trials in which PTX is used in chemotherapy, PTX is generating excitement for the treatment of numerous types of tumors, including several refractory tumors such as ovarian carcinoma, myeloblastic leukemia, and hormone-refractory prostate cancer (HRPC; ref. 6–8).

Induction of apoptosis appears to be the main mechanism behind the antitumor effect of PTX (9, 10). Although several proteins involved in the PTX-mediated apoptosis have been identified, the molecular pathways underlying the apoptotic processes associated with PTX are not clearly defined (11–13). Understanding how PTX induces apoptosis is crucial to the elucidation of clinical relevance of chemotherapy using PTX. Furthermore, there is no definite link between TP expression and PTX-mediated apoptosis in cases of prostate cancer (PC). This relationship should be addressed to investigate the anticancer effect of PTX. In the present study, we examined TP expression in relation to apoptosis-related protein expression by an immunohistochemical analysis using HRPC samples before and after PTX-based chemotherapy, and human PC cell lines (PC-3, DU 145, and LNCaP) *in vitro* were used to attempt to elucidate whether TP influences PTX-mediated apoptosis or has cytoprotective function against PTX.

MATERIALS AND METHODS

Tissue Samples. Tissue samples from systematic sextant needle biopsy were collected in the same HRPC patients before and after PTX-based chemotherapy (14). From a total of 32 patients, eight cases (median age, 73.5; range, 54–80 years; clinical staging, $T_4N_0M_1$ in five and $T_4N_1M_1$ in three cases), whose biopsy samples before and after chemotherapy equally contained viable cancer cells within the same target areas, were recruited. Written form of informed consent was obtained from all patients. Tosoh II tPSA assay (Tosoh Medics, South San Francisco, CA) was used to measure serum levels of prostate-specific antigen (PSA) within a week just before and after chemotherapy.

Cell Culture. The human prostate cancer cell lines PC-3, DU145, and LNCaP were obtained from the American Type of Culture Collection (Manassas, VA) and incubated in F-12K and MEM-E supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Culture media were changed every 48 hours.

Immunohistochemistry. The tissue samples were fixed in 10% buffered formalin (pH 7.0) and processed for embedding in paraffin wax. The envision-peroxidase method (Dako, Carpinteria, CA) was used to perform immunohistochemical staining. Mouse monoclonal antibody against TP (1/100 dilution; Dako), rabbit polyclonal antibodies against cleaved caspase-8 and cleaved caspae-3 (1:1,000 dilution; Sigma, St. Louis, MO), and poly(ADP-ribose) polymerase (PARP) p85 fragment (1:100 dilution; Promega Corp., Madison, WI) were used in this study. The reaction products were visualized using diaminobenzidine (Dako). Normal mouse and rabbit IgG instead of monoclonal and polyclonal antibodies, respectively, served as a negative control. A pathologist not involved in the present study evaluated the immunostaining under an experimental blind condition. The immunohistochemical staining was graded on an arbitrary scale from 0 to 2+; 0 represents negative expression (0–20% positive cells), 1+ represents weakly positive expression (20–50% positive cells), and 2+ represents strongly positive expression (50–100% positive cells). The scale was determined according to the average rate of positive cells in ten random fields of all slides.

Morpholino Antisense Oligonucleotide Transfection. A specific morpholino antisense oligonucleotide (5′-GGTCATCAAGGCTGCCATCGCTCCG-3′)

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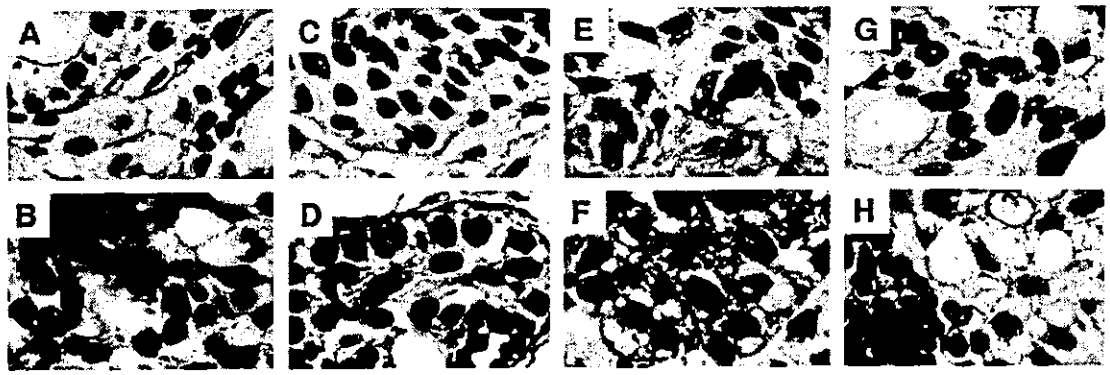

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Case #	Expression score								Serum PSA level (ng/ml)		PSA reduction rate (%)
	TP		Cleaved caspase-8		Cleaved caspase-3		Cleaved PARP		Before	After	
	Before	After	Before	After	Before	After	Before	After			
1	0	1+	0	1+	0	2+	0	2+	5.1	0.7	86.3
2	1+	1+	0	1+	0	2+	0	2+	13.2	1.5	88.6
3	1+	2+	0	0	0	2+	0	2+	3075.4	1481.4	51.8
4	1+	2+	0	0	0	2+	0	1+	580.0	67.0	88.4
5	1+	2+	0	0	0	2+	0	1+	42.6	2.9	93.2
6	2+	2+	0	0	0	2+	0	1+	69.0	2.7	96.1
7	2+	2+	0	0	0	1+	0	1+	683.0	41.6	93.9
8	2+	2+	0	0	0	1+	0	1+	17.5	1.5	91.4

Expression score
0: negative expression, 1+: weakly positive expression, 2+: strongly positive expression

Fig. 1. Representative immunostaining of TP (*A*, *B*), cleaved caspase-8 (*C*, *D*), cleaved caspase-3 (*E*, *F*), and cleaved PARP (*G*, *H*) in the prostate biopsy specimens having viable PC cells (×400) are shown. *A*, *C*, *E*, and *G*, before chemotherapy with PTX. PC cells in the tumor tissues that comprise small glandular structures are almost completely negative for TP (*A*), cleaved caspase-8 (*C*), cleaved caspase-3 (*E*), and cleaved PARP (*G*). All of these expressions were scored as 0. *B*, *D*, *F* and H, after chemotherapy with PTX. Small subsets of PC cells and stromal cells in the tumor tissues are weakly positive for TP (*B*) and cleaved caspase-8 (*D*) and are strongly positive for cleaved caspase-3 (*F*) and cleaved PARP (H). These expressions were scored as 1+, 1+, 2+ and 2+, respectively. *I*, summary of immunostaining data and PSA alteration before and after chemotherapy with PTX.

was designed complementary to the TP mRNA based on the entire cDNA sequence in the GenBank database (Genbank accession no. NM001953). Antisense containing 5 bp mismatch pairs (5′-GGTTATCAAAGCTACCGTCGCTTCG-3′) was used as a control sense oligonucleotide. For the oligonucleotide treatment of each cell line, a special delivery system following the manufacturer's protocol (Funakoshi, Tokyo, Japan) was used to transfect cells. The specificity (the degree of cross-hybridization with the entire sequence) was confirmed by Western blot analysis.

Growth Inhibition Assay. Cells treated with or without TP antisense were cultured for 24 hours in 6-well plates at a density of 5×10^5 cells/well in growth medium. Media were then changed to growth media containing PTX (1×10^{-9} to 1×10^{-7} mol/L). Cells were harvested with trypsin, and a hemocytometer was used to count them on days 2, 3, and 4. Experiments were done in quadruplicate for each time point.

MTT Assay. Cells were grown at 5×10^3 in the culture medium containing 100 μL of serum dispensed into 96-well microplates. After treatment with chemical agents, 10 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma) was added to each well, and the plates were incubated at 37°C for 4 hours. A measurement wavelength of 450 nm and a reference wavelength of 655 nm was used to read absorbance on a microplate reader (Model 3550, Bio-Rad, Richmond, CA). IC_{50} was defined as the PTX concentration that inhibited cell growth by 50% compared with control cells, according to the average cell cycle length. IC_{50} values were calculated from a linear regression analysis of plotted values.

Evaluation of Apoptosis. The APOPercentage Apoptosis Assay kit (Biocolor Ltd. United Kingdom) was used to examine alteration in the apoptosis membrane. Briefly, cells were seeded at 5×10^3 in medium containing 100 μL of serum that was dispensed into 96-well microplates. After treatment with 1×10^{-9} PTX for 48 hours, the culture medium was replaced with fresh medium containing APOPercentage Dye Label. The APOP% Dye Release Reagent was added to each well to aid cell lyses and the release of the bound dye from the apoptotic cells. A microplate colorimeter was used to measure cell-bound dye recovered in solution. A reference wavelength of 655 nm was used to estimate the apoptotic index at a measurement wavelength of 595 nm.

Preparation of Cell Lysates. After drug treatment for 48 hours, cells were harvested with 0.02% trypsin, centrifuged, and the cell pellets immediately frozen at −80°C until use. Frozen tumor cells were homogenized in lysis buffer [1% Triton X-100; 20 mmol/L Tris-HCl (pH 7.6); 0.1% SDS; 1% sodium deoxycholate], lysates were centrifuged at 15,000 × *g* for 20 minutes at 4°C, and each supernatant was used in the immunoblotting analysis. Protein concentrations were determined by the Bradford method (15).

Immunoblotting. Samples were resolved by 11% SDS-PAGE. A Bio-Rad Transblot SD was used to electrophoretically transfer proteins to nitrocellulose membranes (Millipore Corp., Bedford, MA). The membranes were immersed in 5% skim milk in 0.02 mol/L Tris-HCl, 0.4 mol/L NaCl (pH 8.0), and 0.05% Tween 20 buffer (TTBS) for 1 hour and probed with monoclonal antibody against TP, caspase-8, and β-actin or polyclonal rabbit antibody against caspase-3, PARP, and cleaved PARP. Blots were then labeled with antimouse or antirabbit antibody conjugated with peroxidase; an enhanced chemiluminescence and Western blotting was used for visualization. The software package NIH was used to quantify Image Signal intensities. The protein expression level was depicted as the relative yield to the β-actin level.

Statistical Analysis. The difference between two groups was statistically analyzed by Mann-Whitney *U* test, or the Stat View V statistical package (SAS Institute Inc., Cary, NC) was used for Student's *t* test. The *P* value of <0.05 was regarded as statistically significant.

RESULTS

Immunostaining of TP and Apoptosis-Related Proteins in HRPC Specimens. PTX on the expression of TP and apoptosis-related cleaved form of caspase-8, capase-3, and PARP was used to

immunostain eight HRPC samples and assess the effect of chemotherapy. Typical immunostaining of TP, cleaved form of caspase-8, caspase-3, and PARP before and after chemotherapy was shown in Fig. 1. PTX in four HRPC cases (cases 1, 3, 4, and 5) was used to increase TP expression after chemotherapy, whereas the remaining four cases (cases 2, 6, 7, and 8) showed no difference in TP expression before and after chemotherapy. In all eight HRPC samples, cleaved forms of caspase-3 and PARP were not detectable before chemotherapy, whereas they were detectable after chemotherapy. In six HRPC samples (cases 3–8), cleaved form of caspase-8 expression was not detectable either before or after chemotherapy. On the other hand, the remaining two HRPC samples (cases 1 and 2) showed increased expression of cleaved caspase-8 after chemotherapy. In these 2 HRPC samples, cleaved form of caspase-3 and PARP expression was scored as 2+ (strongly positive expression) after chemotherapy.

Relationship between TP Expression and Serum PSA Levels before and after Chemotherapy with PTX A shown in Fig. 2, serum levels of PSA after chemotherapy were all reduced in the eight HRPC patients as compared with those before chemotherapy. Eight HRPC patients were divided into two groups based on the alteration of TP expression before and after chemotherapy with PTX; for group 1 ($n = 4$), TP expression was increased after chemotherapy, and for group 2 ($n = 4$), TP expression was not changed before and after chemotherapy. In group 1, PSA values before and after chemotherapy were 918.0 ± 731.4 and 387.6 ± 364.9, respectively, of which difference reached borderline significance ($P = 0.07$). Likewise, in group 2, PSA value before chemotherapy (203.0 ± 160.3) was significantly higher than that after chemotherapy (12.1 ± 9.8; $P = 0.07$). Furthermore, PSA reduction rate in group 2 was significantly higher (95.1 ± 0.53%) than that in group 1 (78.5 ± 9.2%; $P = 0.02$).

Effect of PTX on Cell Viability. After the treatment with PTX, cell growth of all three PC cell lines (PC-3, DU145, and LNCaP) was inhibited in a does-dependent and time-dependent manner. The PTX concentration required to completely inhibit cell growth was 1×10^{-8} mol/L in all 3 PC cell lines. If applied $>1 \times 10^{-8}$ mol/L concentration of PTX, cell counts in all of three PC cell lines were more likely to be reduced, indicating that the cell toxicity of PTX might be increased (data not shown). As shown in Fig. 3*A-C*, in the presence of TP antisense, the IC_{50} of PTX was significantly decreased ranging from 1.9×10^{-8} to 1.0×10^{-9} mol/L, 2.0×10^{-8} to 1.2×10^{-9} mol/L, and 9.0×10^{-9} to 1.0×10^{-9} mol/L for PC-3, DU145 and LNCaP, respectively. As shown in Fig. 3*D-F*, cell viability in the cells treated with 1×10^{-9} mol/L PTX alone or 1×10^{-9} mol/L PTX with TP antisense was more significantly reduced than that in the control cells ($P < 0.05$ and $P < 0.01$, respectively). However, in all 3 PC cell lines, there was no significant difference in cell viability between control and TP antisense treatment. Thus, stepwise decrease in cell viability was observed along with TP antisense treatment, PTX treatment, and combined treatment of TP antisense and PTX. In addition, the reduction of cell viability in these three PC cell lines treated with PTX + TP antisense was more time-dependent than in those cells treated with PTX alone.

Effect of PTX-Induced TP on Apoptosis. As shown in Fig. 4, we examined apoptosis using membrane alteration techniques on

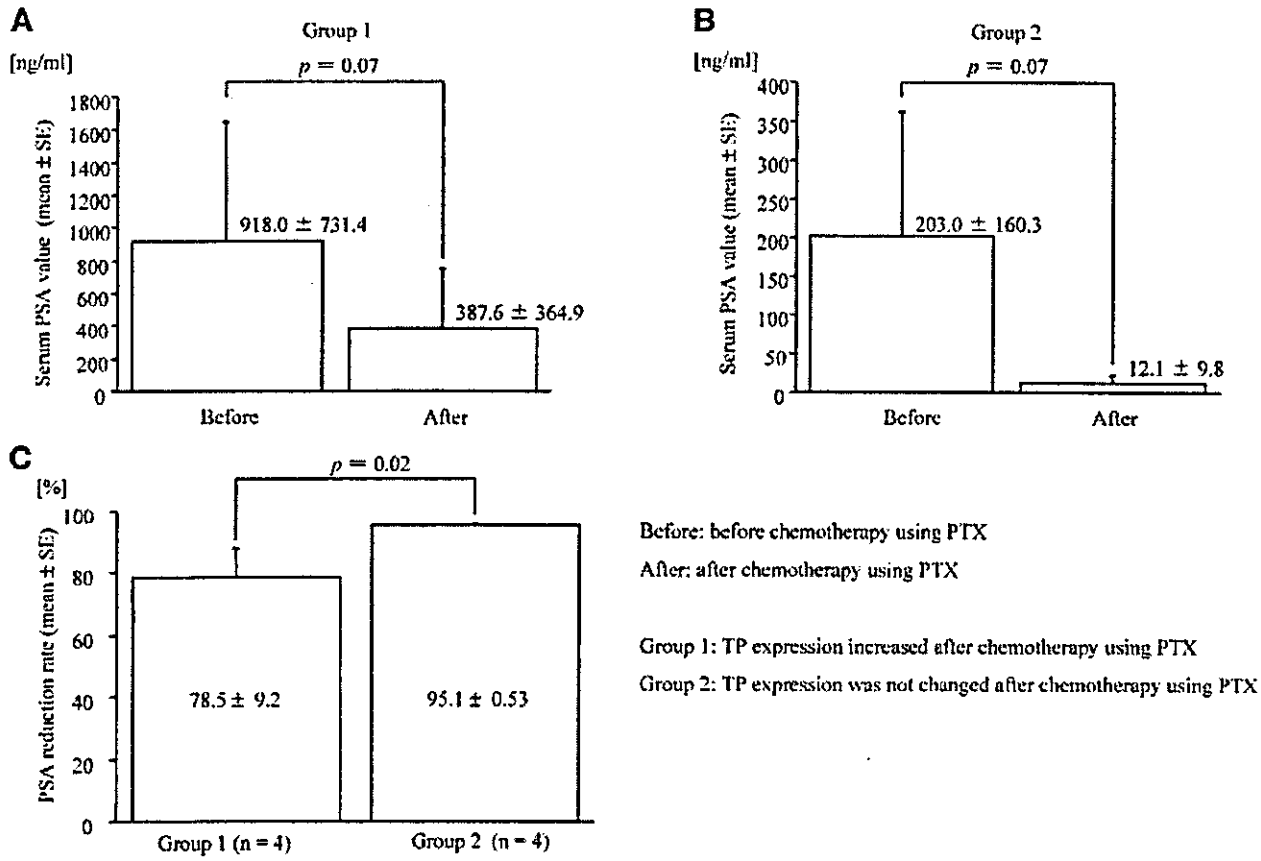

Fig. 2. Relationship of TP expression with PSA value and PSA reduction rate before and after chemotherapy with PTX in eight HRPC cases are shown. *A* and *B* show the alteration of serum PSA levels before and after chemotherapy with PTX. Group 1 includes four patients with increased TP expression after chemotherapy with PTX, whereas group 2 comprises the other four patients with TP expression being unchanged before and after chemotherapy. The difference in PSA reduction rate between Groups 1 and 2 is shown in *C*. Statistical significance was analyzed with Mann-Whitney *U* test. The values are expressed as mean ± SE.

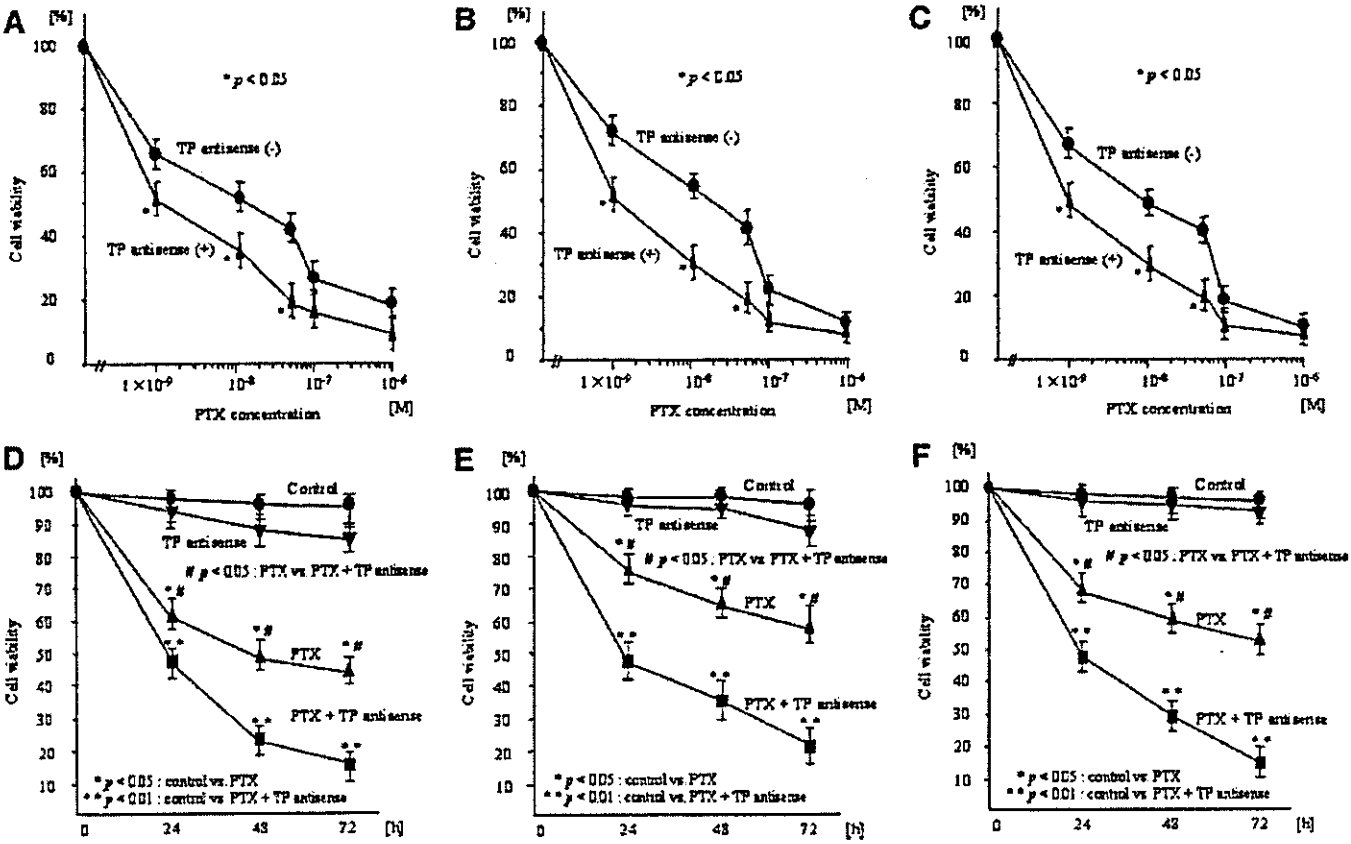

Fig. 3. Cell viability of PC cell lines treated with PTX and/or antisense TP is shown. *A*, *B*, and *C* show the dose-response curves of PC-3, DU 145, and LNCaP, respectively, and cells treated (▲) or untreated (●) with antisense oligonucleotide for TP mRNA (5'-GGTCATCAAGGCTGCCATCGCTCCG-3') after exposure of PTX at five different concentrations (1×10^{-9}, 1×10^{-8}, 5×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol/L) for 24 hours. Viable cells were measured by MTT assay and expressed as a percentage of the controls. *Asterisk* in Fig. 3*A-C* indicates the statistical difference in cell viability between TP antisense (−) and TP antisense (+) treated cell lines ($P < 0.05$). *D*, *E*, and *F* show the time-response curves of PC-3, DU145, and LNCaP cell lines, respectively. Cell viability of control nontreated PC cell lines (●), PC cell lines treated with TP antisense alone (▼), treated with 1×10^{-9} mol/L PTX alone (▲), and treated with a combination of both TP antisense and 1×10^{-9} mol/L PTX (■) for 24, 48, and 72 hours is shown. *Asterisks*, * and **, in Fig. 3*D-F* indicate the statistical difference in cell viability between control and PTX or between control and combination of PTX and TP antisense ($P < 0.05$and $P < 0.01$, respectively). # in Fig. 3*D-F* shows the statistical difference in cell viability between PTX and combination of PTX and TP antisense ($P < 0.05$). The values are expressed as mean ± SE at each point.

three PC cell lines treated with TP antisense alone or PTX of 1×10^{-9} mol/L alone, or a combination of both. Apoptotic index was standardized by that of control nontreated PC cell lines (no treatment of PTX and TP antisense) being as 1 and expressed as the arbitrary unit. In all PC cell lines, substantial stepwise increase of apoptotic index was observed along with PTX treatment alone (1.53 ± 0.12, 1.64 ± 0.14, and 1.66 ± 0.08 for PC-3, DU145 and LNCaP, respectively) and PTX treatment with TP antisense

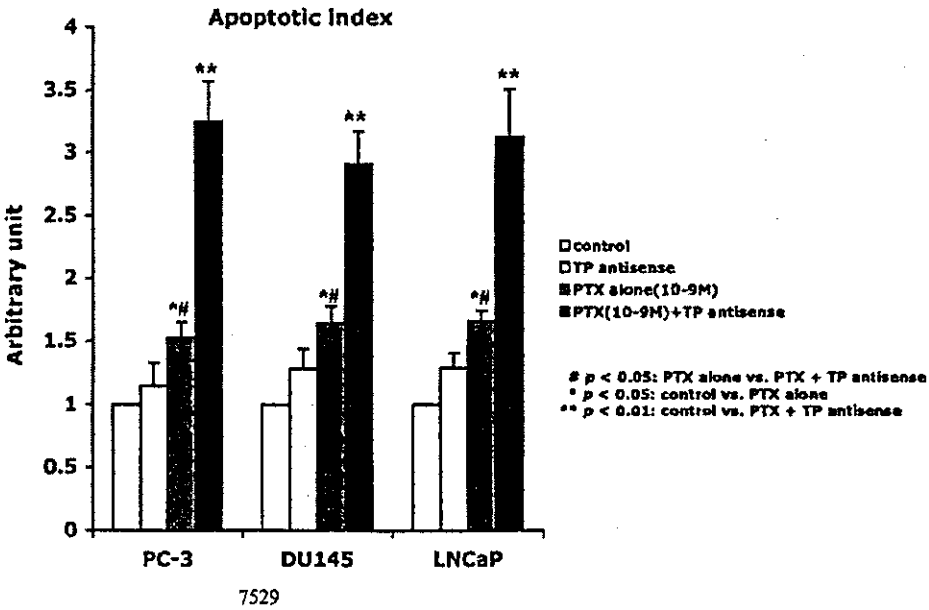

Fig. 4. Alteration of apoptotic index in PC cell lines with different treatment modalities is shown. Apoptotic indices of each group are expressed as its relative proportion to the untreated control (mean ± SE). Statistical significance was evaluated by student's *t* test. The difference in apoptotic index between PTX alone *versus* PTX + TP antisense, control *versus* PTX alone, and control *versus* PTX + TP antisense reached statistical significance ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively).

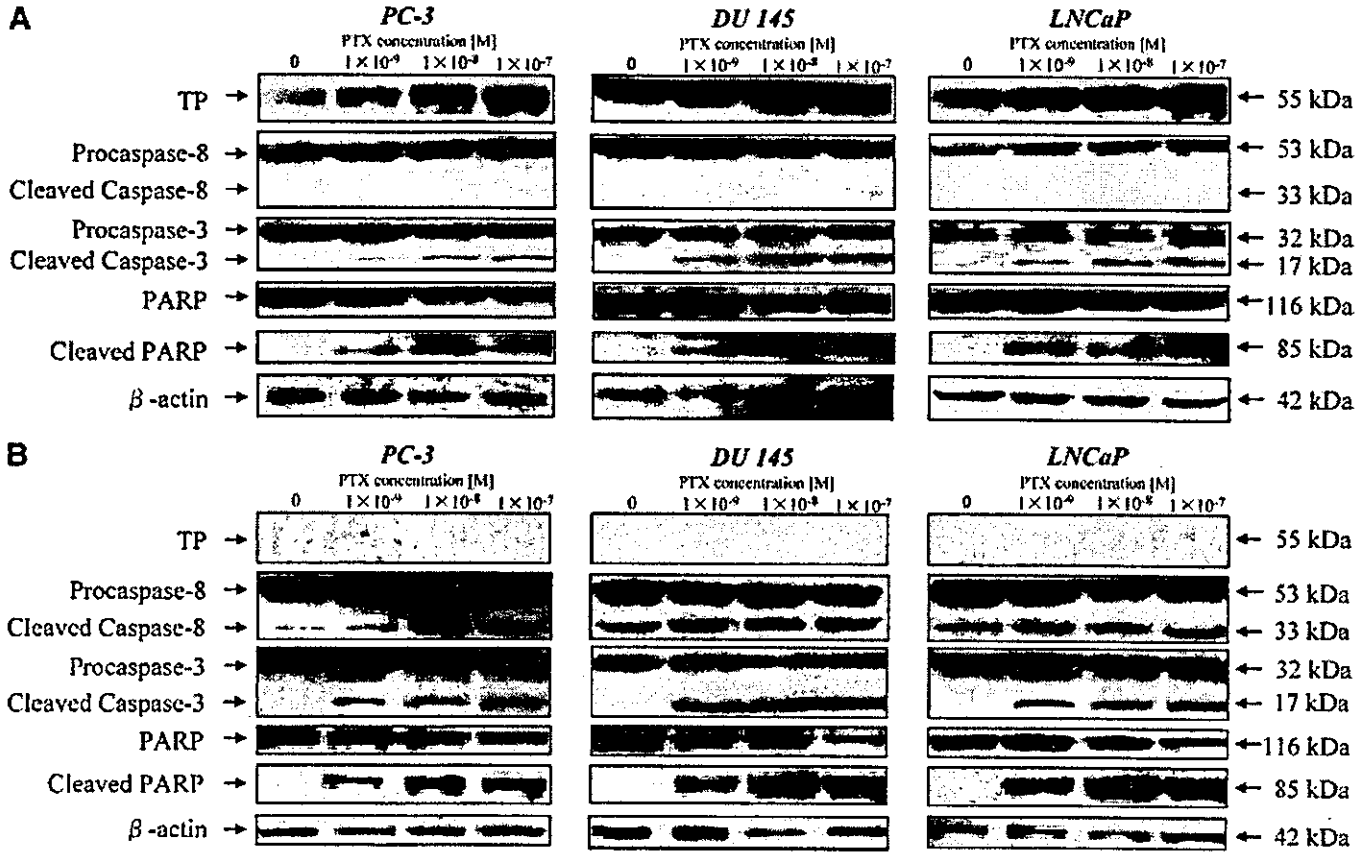

Fig. 5. Typical results of Western blotting of TP and apoptosis-related proteins in PC cell lines treated with different concentrations of PTX and/or TP antisense are shown. Protein levels of TP, procaspase-8, -3, cleaved caspase-8, -3, PARP, cleaved PARP and β-actin in PC-3, DU 145, and LNCaP cells untreated (*A*) or treated (*B*) with antisense oligonucleotide for TP mRNA. These cells were exposed to various concentrations of PTX (1×10^{-9} to 1×10^{-7} mol/L) for 48 hours. Different antibodies were used to apply total protein (100 μg) to Western blotting. The software package NIH Image was used to quantify signal intensities. Protein expression levels were depicted as their relative yield to the β-actin level.

(3.25 ± 0.32, 2.91 ± 0.26, and 3.13 ± 0.38 for PC-3, DU145 and LNCaP, respectively). Apoptotic index in PC cell lines treated with TP antisense alone was almost the same as that in the nontreated control PC cell lines.

Effect of PTX on TP Expression and Caspase-8, Caspase-3, and PARP Activation. Typical results of Western blotting were shown in Fig. 5. Western blot analysis showed that expression level of TP protein was increased dose-dependently in all of 3 PC cell lines treated with PTX alone. As shown in Fig. 6*A*, in PC-3, DU 145, and LNCaP cell lines treated with three different concentrations of PTX alone (1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L), the mean levels of TP expression were significantly higher than those in the control non-PTX-treated cells. In all of three PC cell lines treated with PTX alone, cleavage of caspase-3 and PARP, but not cleavage of caspase-8 was activated, whereas in the control non-PTX-treated cell lines none of the cleaved form of caspase-3, PARP, and caspase-8 was found. When TP expression was completely inhibited by TP antisense transfection, caspase-8 cleavage was activated in all of three PC cell lines. As shown in Fig. 6*B*, cleaved form of caspase-8 expression in PC-3, DU 145, and LNCaP cell lines treated with combination of PTX (1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L) and TP antisense transfection was significantly higher than cleaved form of caspase-8 expression in the control PC cells treated with TP antisense transfection alone. Moreover, the expression of cleaved form of caspase-3 and PARP in PC cell lines treated with a combination of PTX and TP antisense was significantly higher than the expression of those treated with PTX alone (Fig. 5).

DISCUSSION

In this study, all eight HRPC cases following chemotherapy with PTX expressed TP. Because up-regulation of cleaved form of caspase-3 and PARP was clearly observed, apoptotic process was exaggerated in these HRPC tissues. In six HPRC cases with strongly positive TP expression (cases 3–8) following chemotherapy, cleaved form of caspase-8 was not expressed either before or after chemotherapy with PTX, whereas in two HRPC cases with weakly positive TP expression (cases 1 and 2), cleaved caspase-8 was expressed, and apoptosis was strongly induced after chemotherapy. These observations might indicate that (*a*) chemotherapy using PTX-induced cell apoptosis and (*b*) cleaved caspase-8 expression was associated with down-regulation of TP expression and accelerated apoptosis. Considering the potential usefulness of PSA as a biological marker in PC patients (16), as shown in Fig. 2, the PSA reduction rate in patients with increased TP expression after chemotherapy was significantly lower than that observed in patients without increased TP expression. Thus, it might be clinically plausible that antitumor effect of PTX on tumor cells was diminished by simultaneous PTX-induced TP up-regulation. Another possibility is that the other chemotherapeutic agents other than PTX affect TP expression, because our series of HRPC patients underwent combined chemotherapy including not only PTX but also estramustine phosphate and carboplatin (14). However, our preliminary data revealed that either estramustine or carboplatin did not confer any substantial effect on the expression level of TP in PC-3 cell line (data not shown). On the basis of these findings, we hypothesized that blockade of PTX-induced TP might be essential for

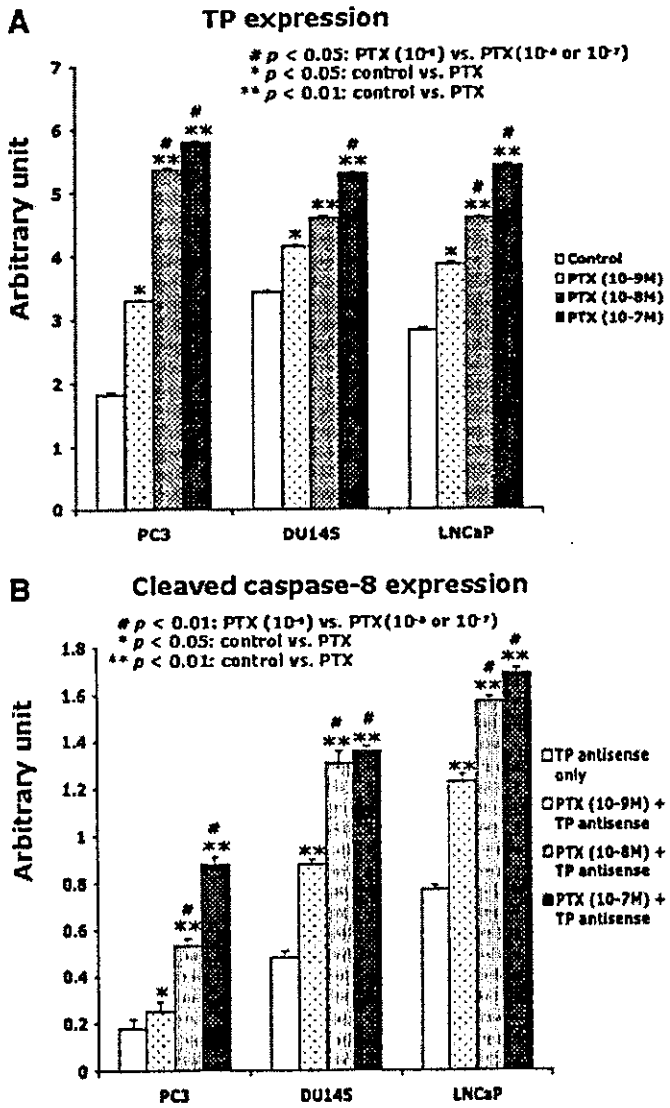

Fig. 6. TP expression after treatment with PTX (*A*), and cleaved caspase-8 expression after combined treatment with PTX and TP antisense (*B*) in PC cell lines are shown.

accelerating PTX-mediated apoptosis. As shown in Fig. 5, in PC-3, DU145, and LNCaP cell lines, PTX exposure clearly increased TP expression. The cell viability of PTX-treated PC cells with inhibition of TP translation by TP antisense transfection was significantly diminished in a time-dependent and dose-dependent manner compared with the PTX treatment alone (Fig. 3). Likewise, the apoptotic index was significantly increased in PC cells with combined treatment of PTX and TP antisense in comparison with those PC cells treated with PTX alone. However, the treatment with TP antisense alone did not make any influence on the cell viability and apoptotic index. These findings suggest that inhibition of PTX-induced TP up-regulation could confer more proapoptotic effect on PTX-treated PC cells.

Next, to verify the mechanism underlying PTX-induced apoptosis in relation to simultaneous TP expression, we focused on the molecular pathway involved in the apoptotic process. Two major apoptotic pathways are known in mammalian cells. One is the Fas-induced caspase-8 activation pathway, and the other is the mitochondrial pathway. Although these two apoptotic pathways operate independently, they converge at the level of caspase-3 activation (17). PTX-induced apoptosis has also been implicated in caspase-8 activation in breast and colon cancer cell lines (18, 19). TP has been reported to inhibit Fas-induced caspase-8 cleavage followed by the release of cytochrome *c*, the activation of caspase-3, and the apoptosis (3). However, no reports have shown a positive link between apoptotic pathways involved in PTX-induced TP expression and caspase-8 activation in PC cells. In breast and colon cancer cell lines, PTX induces proapoptotic effect on cancer cells through caspase-8 activation in addition to the activation of mitochondrial membrane potential (18, 19). However, as shown in Fig. 5, PTX-induced apoptosis in PC cell lines seems to be independent of caspase-8 activation. Thus, the mechanism underlying the antitumor effect of PTX on cancer cells appears to be potentially varied among cancer cells of different origins. On the basis of the present finding of dose-dependent TP induction by PTX treatment in PC cell lines as well as the previous report of potential inhibitory effect of TP on caspase-8 activation (3), we hypothesized that blockade of inhibitory effect of TP on caspase-8 activation could enhance the PTX-induced apoptosis in PC cells. In all three PC cell lines, after complete blockade of TP translation by TP antisense transfection, proapoptotic events such as cleaved form of caspase-3 and PARP were enhanced in a PTX dose-dependent manner. In addition, this exaggeration of apoptosis also ran parallel with the caspase-8 cleavage in a PTX dose-dependent manner. On the other hand, in all of three PC cell lines TP blockade itself did not confer any effects on the acceleration of apoptosis despite caspase-8 activation. These results suggest that cross-talk between caspase-3 activation through mitochondrial membrane potential and direct effect of caspase-8 activation pathway on cytochrome *c* release can modulate proapoptotic effect of PTX as a chemotherapeutic agent on PC cells. In turn, we can expect more antitumor apoptotic effect of PTX on PC cells with an inhibition of "adverse" effect of PTX-induced TP overexpression.

To our knowledge, this study is the first report to investigate the induction of TP expression by PTX and to present the possibility that overexpressed TP might be related to the potential decrease in caspase-8 cleavage in PC cell lines. Our results support the hypothesis that TP could be a new molecular target for enhancing the potency of PTX-mediated apoptosis in PC cells. Therefore, it is necessary to perform the clinical trial after treatment with a combination of TP antisense and PTX in the future.

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