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 TP-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, T4N0M0 in five and T4N1M1 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 iPSA 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 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% CO2/95% 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 caspase-3 (1/10000 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 diamobenzidine (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’-GGTCACTCAGGCTGCATCGGCTCCG-3’)
These expressions were scored as 1/H11001,1/H11001) and are strongly positive for cleaved caspase-3 (B) and cleaved PARP (H). Cleaved caspase-8 (D), cleaved caspase-8 (E), and cleaved PARP (G) are shown. PC cells (a) were cultured for 24 hours in 6-well plates at a density of 5x10^3 cells/well. After treatment with MTT Assay 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 colorimeter. 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. The APOPercentage Apoptosis Assay kit (Bio-color Ltd. United Kingdom) was used for Student’s t test, or the Stat View V statistical package (SAS Institute Inc., Cary, NC) was used for Student’s t test. The value of P<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, caspase-3, and PARP was used to

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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.
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**

As 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 dose-dependent and time-dependent manner. The PTX concentration required to completely inhibit cell growth was 1 × 10⁻⁹ mol/L in all 3 PC cell lines. If applied 1 × 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. 3A–C, in the presence of TP antisense, the IC₅₀ 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. 3D–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...
three PC cell lines treated with TP antisense alone or PTX of $1 \times 10^{-9}$ 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 ($1.53 \pm 0.12, 1.64 \pm 0.14,$ and $1.66 \pm 0.08$ for PC-3, DU145 and LNCaP, respectively) and PTX treatment with TP antisense

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**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, DU145, 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 ($1 \times 10^{-9}, 5 \times 10^{-9}, 1 \times 10^{-8}, 5 \times 10^{-8}, 1 \times 10^{-7}$, and $1 \times 10^{-6}$ mol/L) for 24 hours. Viable cells were measured by MTT assay and expressed as a percentage of the controls. Asterisks in Fig. 3A-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 $1 \times 10^{-9}$ mol/L PTX alone (●), and treated with a combination of both TP antisense and $1 \times 10^{-9}$ mol/L PTX alone (●), and treated with a combination of both TP antisense and $1 \times 10^{-9}$ mol/L PTX alone (●), for 24, 48, and 72 hours is shown. Asterisks, # and **, in Fig. 3D-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. 3D-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.

**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).
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. 6A, 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. 6B, cleaved form of caspase-8 was expressed 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 HRPC 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

(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. 6A, 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. 6B, cleaved form of caspase-8 was expressed 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).
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.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Fukushima (Institute for Applied Oncology, Taiho Pharmaceutical Co., Ltd., Saitama, Japan) for encouraging us throughout this study.

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

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