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Protection against DNA damage-induced apoptosis by the angiogenic factor thymidine phosphorylase

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Abstract **Thymidine phosphorylase (TP) is involved both in pyrimidine nucleoside metabolism and in angiogenesis. TP also conferred the resistance to hypoxia-induced apoptosis of the cancer cells. In U937 cells, DNA damage-inducing agents significantly enhanced the expression of TP. Cell lines stably transfected with TP cDNA were more resistant to the DNA damage-inducing agents than the mock-transfected cells and showed augmented activity of Akt. The cytoprotective function of TP against DNA damage was independent of its enzymatic activity. The resistance to apoptosis was partially abrogated by treatment with the phosphatidyl inositol 3-kinase (PI3K) inhibitors, suggesting that the cytoprotective function of TP is mediated, at least in part, by regulation of the PI3K/Akt pathway. These findings indicate that TP expression in increased by various stress including DNA damage and that TP molecules confer resistance to DNA damage-induced apoptosis in cancer cells.**

Keywords: Thymidine phosphorylase; Apoptosis; Akt

1. Introduction

Thymidine phosphorylase (TP) is an enzyme that has unique properties. TP catalyzes the reversible conversion of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate, TP is also involved in endothelial cell chemotaxis in vitro and angiogenesis in vivo [1]. TP expression in various kinds of tumors is higher than in the adjacent non-neoplastic tissues, and numerous studies have consistently reported that the level of TP expression in cancer cells is closely associated with malignancy and/or angiogenesis [2–5]. However, in colorectal carcinomas, TP expression is an independent indicator of unfavorable prognosis irrespective of angiogenetic activity [6]. These data suggest that TP may contribute to the malignant character of cancer cells through mechanisms other than its angiogenic activity.

We previously reported that TP might enhance tumor progression by conferring resistance to hypoxia-induced apoptosis of cancer cells. We showed that 2-deoxy-D-ribose, a dephosphorylated product of 2-deoxy-D-ribose-1-phosphate, partially inhibited this apoptosis [7], suggesting that a second function of TP might be the inhibition of apoptosis induced by various stresses in tumor cells. We have also demonstrated that TP-transfected cell lines are more resistant to various apoptosis-inducing stimuli such as Fas and cisplatin [8,9]. However, the mechanisms by which TP expression is enhanced in response to stressful conditions and by which it modulates apoptosis are largely unclear.

Akt/PKB (protein kinase B) is a serine/threonine protein kinase that regulates diverse cellular processes but may play a critical role in cell survival following exposure to DNA-damage inducing agents. Akt is activated following binding to PI3K. Recently, Akt has been implicated in the inhibition of apoptosis in a variety of cell types in vitro including cancer cells. The anti-apoptotic function of Akt is reported to be mediated by its ability to phosphorylate and inactivate apoptotic regulatory molecules including BAD, caspase-9, IKK-α and the forkhead transcription factor FKHRL [10,11].

In this study, we investigated the role of TP in apoptosis induced by DNA damage as well as the biological mechanisms by which TP functions as an anti-apoptotic agent. We found that both the transcriptional activity, and the protein expression, of TP were enhanced by DNA damage-inducing agents. Moreover, we also found that TP confers cytoprotection against DNA damaging agents on tumor cells, and that TP-induced anti-apoptotic effects are at least partly mediated through regulation of the PI3K/Akt survival pathways.

2. Materials and methods

2.1. Reagents and antibodies

VP-16, cisplatin, doxorubicin, vincristine, staurosporine, vinblastine, paclitaxel, thymidine, and thymine were purchased from Sigma Chemical (St. Louis, MO). Human recombinant IFN-γ was from PeproTech (London, UK). The anti-mouse monoclonal anti-Akt and phospho-Akt from Cell Signaling (Beverly, MA) and anti-p53 (Ab-6) from Oncogene (Boston, MA).

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TPI, a specific inhibitor of TP, was synthesized by Taiho Pharmaceutical Co. Ltd. (Saitama, Japan), and described previously [12]. The PI3K inhibitors Wortmannin and LY294002 were obtained from Sigma and Calbiochem, respectively.

2.2. Plasmids

The TP promoter fragment, fused upstream of the luciferase gene (pTP-Luc1), was a generous gift from Dr. M. Ono (Kyushu University), as described previously [13]. Full-length TP cDNA was kindly provided by Dr. K. Miyazono (Tokyo University) and Dr. C-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). A *Kpn*I–*Eco*RI fragment from pPL8 that encompassed the TP coding region was cloned between the *Kpn*I and *Eco*RI restriction sites of pT7T318U (Pharmacia, Uppsala, Sweden). A *Xba*I–*Eco*RI fragment from pT7T318U was then cloned between the *Nhe*I and *Eco*RI restriction sites of expression vector pBK-RSV (Stratagene, La Jolla, CA) to make the expression vector encoding TP cDNA (RSV/TP). As a mutant, TP (L148R) which has no TP activity was prepared and ligated into same vector (RSV/L148R). Wild-type (pC53-SN3) and mutant-type (pC53-CxAN) p53 plasmids were described previously [14].

2.3. Cell culture and establishment of TP-overexpressing cell lines

The cell lines U937 (human myeloid leukemia), KB (human epidermoid carcinoma), Jurkat (human T-cell leukemia), HCT-15 (human colorectal cancer) and Saos-2 (human osteosarcoma) were obtained from the American Type Culture Collection (Rockville, MD). KB cells were maintained in (MEM). U937, Jurkat and HCT-15 were cultured in RPMI 1640, whereas Saos-2 was maintained in Dulbecco's MEM (DMEM).

The expression vector encoding TP cDNA (RSV/TP) or the vector alone (RSV) was transfected into KB cells by electroporation. As a mutant, L148R which has no TP activity was also transfected into KB cells. After selection with Geneticin, the expression of TP in each clone was determined by immunoblotting with anti-TP monoclonal antibody. TP-positive clones transfected with RSV/TP (KB/TP cells), RSV/L148R (KB/TP-mt L148R) and one clone transfected with RSV (KB/CV cells) were further analyzed [15].

2.4. Transient transfection and luciferase assay

All the transfection procedures were performed using LipofectAMINE reagent (Invitrogen) according to the manufacturer's protocol. Briefly, U937 cells were plated at a density of 4×10^6/well in 6-well plates. Cells were co-transfected with 5 μg of the TP-promoter-luciferase plasmid DNA and 100 ng of pRL-CMV vector as an internal control. Following induction for 18–24 h cells were treated with various stimuli including hypoxia, hyperosmolarity (150 mM NaCl), serum starvation, acidosis (pH 6.5) and DNA damage-inducing agents (described below) and were incubated for a further 18–24 h. Cells were harvested and their luciferase activity was analyzed using a Dual-Luciferase Assay according to the manufacturer's instructions (Promega, Madison, WI). Treatment of U937 cells with 50 U/ml IFN-γ was used as a positive control.

Saos-2 cells were transfected with 200 ng of the p53-expressing plasmid and 1.8 μg of the TP-promoter luciferase plasmid DNA. After 18 h incubation the cells were incubated with or without 10 μM VP-16 or UV (40 J/m^2) exposure. The cells were harvested 24 h later and luciferase activities were determined. Each procedure was performed in triplicate and the results are displayed as the means ± S.D.

2.5. Induction of DNA damage

Cells were plated at a density of 1×10^6 cells/well 24 h before treatment. VP-16, doxorubicin, and cisplatin, at the indicated doses, were added and incubated for another 36 h. For UV irradiation, culture medium was removed for the brief irradiation period and then immediately replaced. Hypoxia was induced with a Gas Pak Pouch Anaerobic System (Becton Dickinson and Company, Cockeysville, MD).

2.6. Determination of cell viability by the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to screen the relative sensitivity of cells to DNA damage-inducing agents. 1×10^4 cells in 200 μl of culture medium were inoculated into each well of a 96-well plate with various concentrations of DNA damage-inducing agents with or without 200 μM of TPI. The plates were incubated for 3 days and the MTT assay was performed as previously described [16]. Relative resistance was calculated by dividing the IC_{50} of the TP-overexpressing cells by that of the mock-transfected cells.

2.7. Measurement of apoptotic cells and cell cycle stage

Apoptotic cells were quantified by flow cytometry. Cells treated with DNA damage stimuli for 36 h were harvested and washed once with PBS. For flow cytometry, 1×10^4 cells were suspended in 100 μl PBS and mixed with 100 μl of COULTER DNA-Prep LPR (COULTER, Miami, FA) and then 2 ml of COULTER DNA-Prep Stain was added. The mixtures were incubated for 15 min at room temperature. The cell cycle stage of the cells was determined as described previously [17] using the EPICS ALTRA (Beckman Coulter, Tokyo, Japan). Apoptotic cells were estimated by determination of the proportion of the cells present that were in the sub-G_1 stage of the cell cycle.

2.8. TP activities

For radiometric assay, enzymatic activities were determined by measuring the conversion of [^{14}C] thymidine to [^{14}C] thymine as described previously [18] with slight modification. KB/TP and KB/CV cells were seeded at 1×10^6 cells/well in 12 well plates and [^{14}C] thymidine was then added in the each culture media and incubated at 37 °C for 1 h. Aliquots of media were spotted on PEI-cellulose chromatography plates. Aliquots of 10 mM thymidine and 1 mM thymine were spotted as standards. The plates were developed in a solvent system of chloroform–methanol–acetic acid 17:3:1. The nucleoside and base were identified under UV light, scraped out and analyzed for radioactivity. The ratios of radioactivity of [^{14}C] thymine to total radioactivity were determined.

2.9. Immunoblotting

Exponentially growing cells were seeded at 10^6 per 60 cm^2 dish and incubated for 36 h. Cells were harvested and resuspended in lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF]. The cytosol fractions were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and blotted onto PVDF membrane. The membrane was then incubated with appropriate primary antibody overnight at 4 °C and then with a peroxidase-linked secondary antibody for 1 h at room temperature. The membrane was developed by chemiluminescence according to the manufacturer's protocol (Amersham, Buckinghamshire, UK).

2.10. Statistical analysis

Quantitative data were expressed as the means ± S.D. Statistical comparisons were performed using the Student's t test. Differences were regarded as significant when probability values were <0.05.

3. Results

3.1. TP promoter activity is enhanced by various stresses including DNA damage-inducing agents

To determine if cellular stresses could modulate expression of the TP gene we assayed the effect of various cellular stresses on TP promoter activity using a luciferase reporter assay. The TP promoter, fused upstream of the luciferase gene (pTP-Luc1), was transfected into U937 cells. IFN-γ, which is known to enhance TP transcriptional activity [19] induced a 2.5-fold increase of the luciferase activity over the basal level. Exposure of the cell to hypoxic stress, hyperosmotic shock and simultaneous serum starvation and acidosis, also significantly enhanced the luciferase activity by 2.5, 2.7, and 3.2-fold, respectively (Fig. 1A). TP promoter activity was also enhanced 3.2-fold following treatment with thymidine but not with thymine.

We next investigated the effect on TP promoter activity of DNA damage induced by UV, cisplatin, VP-16 and doxorubicin.

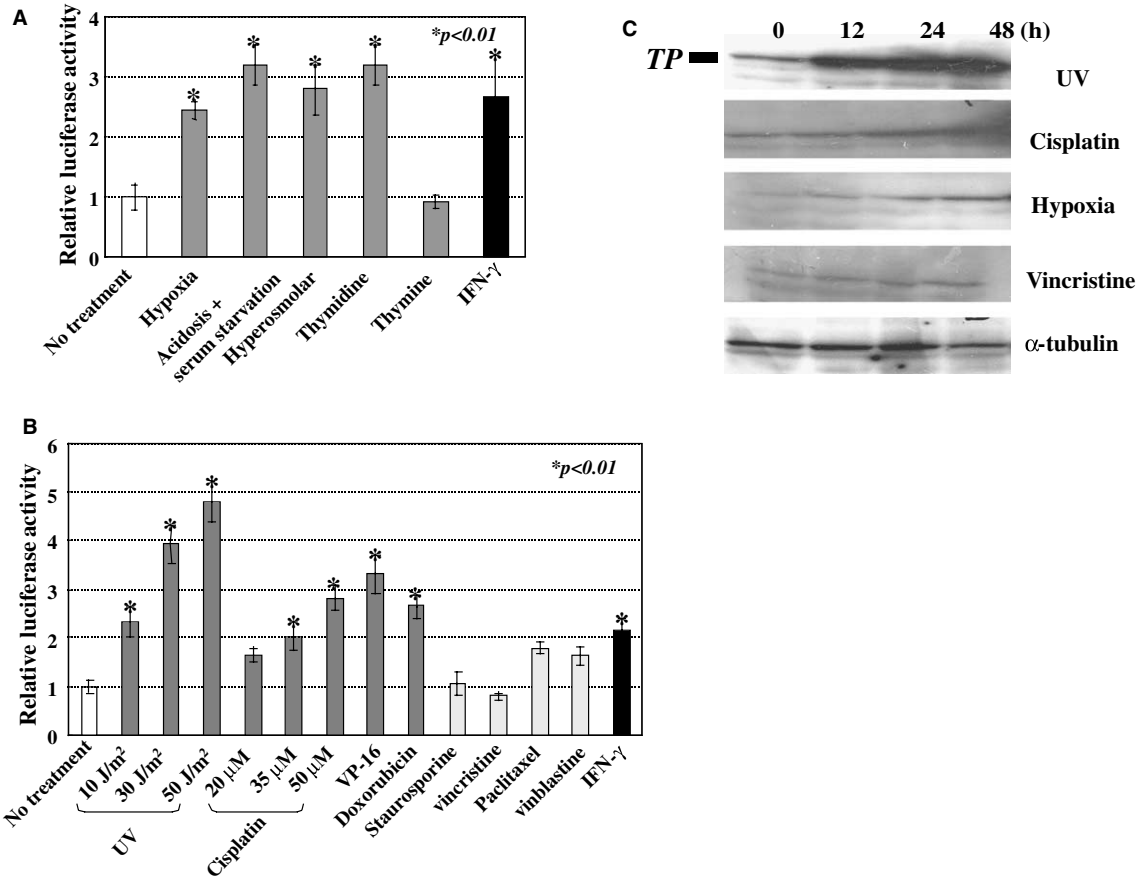

Fig. 1. Specific cellular stresses activate the transcriptional activity of the TP gene promoter. The pTP-Luc1 was co-transfected with the pRL-CMV vector into U937 cells. Following 18–24 h of induction, the cells were exposed to hypoxia, hyperosmolarity (150 mM NaCl), serum starvation and acidosis (pH 6.5), thymidine (100 μM) or thymine (5 μM) in A, or to the DNA damage-inducing agents UV, cisplatin, VP-16 (5 μM), doxorubicin (0.4 μM), vinblastine (10 ng/ml), vincristine (1 ng/ml), paclitaxel (10 μM), or staurosporine (50 ng/ml) in B as indicated. Following incubation for a further 18–24 h, cells were harvested and luciferase activity was analyzed. Treatment of U937 cells with 50 U/ml IFN-γ was used as a positive control. Each column and bar represents the means ± S.D. of three independent experiments. $^{*}p < 0.01$. (C) Protein expression levels of TP following treatment over 48 h with UV (40 J/m^2); cisplatin (20 μM) or vincristine (1 ng/ml) as indicated were assessed by immunoblotting. Cells were harvested and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Cytosolic fractions (50 μg of protein) were separated by SDS–9.4% PAGE. TP levels were determined using an anti-TP monoclonal antibody.

As shown in Fig. 1B, luciferase activity was dramatically augmented by all of these DNA damage-inducing stimuli. UV and cisplatin dose dependently upregulated the TP promoter activity. UV had the strongest effect, at a dose of 50 J/m^2, the luciferase activity was 4.7-fold higher than the basal level. 5 μM VP-16 and 4 μM doxorubicin also induced an approximate 3-fold-increase in luciferase activity. By contrast, the luciferase activity was not elevated by 50 ng/ml staurosporine, 1 ng/ml vincristine, 10 ng/ml vinblastine nor 10 μM paclitaxel. These data suggest that TP promoter activity is specifically enhanced by DNA damage-inducing agents.

3.2. TP is increased at the protein level in response to DNA damage-inducing agents

We next examined whether the cellular stresses that enhanced TP-promoter activity could also enhance TP expression at the protein level in U937 cells. Hypoxic stress was used as a positive control since this stress has been reported to augment TP protein levels. The cells were harvested, lysed in the lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF] and applied cytosol fraction on the gel. Immunoblotting analysis of TP showed a time-dependent increase of TP protein expression following exposure to UV, cisplatin and hypoxia (Fig. 1C). Vincristine and staurosporin (data not shown) induced little TP expression.

3.3. TP promoter activity is enhanced by p53

DNA damage-inducing stimuli cause cell cycle arrest or apoptosis, and one of the major executors in these pathways is p53. P53 levels are increased by these stimuli. Therefore, we investigated whether the TP-promoter activity might be influenced by p53. P53-null Saos-2 cells were co-transfected with pTP-Luc1 and pC53 [pC53-SN3 (wild-type, wt) or pC53-CxAN (point mutation, mt)], and analyzed in the TP-promoter luciferase assay. Transfection of wt-p53 alone induced a dramatic, 3.2-fold enhancement of TP-promoter activity compared to background levels (denoted as 1.0), whereas little change in TP-promoter activity was observed in mt-p53-transfected cells (Fig. 2). Treatment with 10 μM VP-16 or 40 J/m^2 UV further enhanced the TP promoter activity

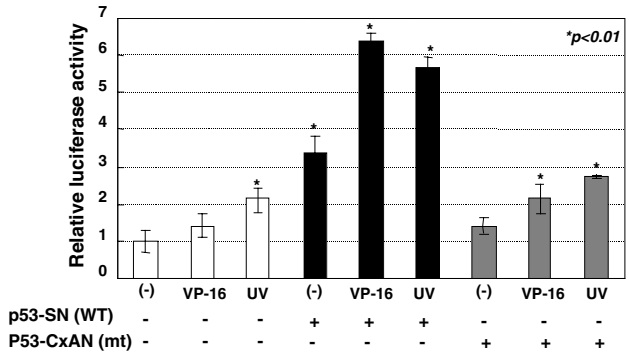

Fig. 2. The effects of p53 on TP promoter activity. P53-null Saos-2 cells were co-transfected with a wild-type (WT) or mutant (mt) p53-expressing plasmid and luciferase plasmid DNA using Lipofectamine. After 18 h, the cells were gently washed with DMEM, and subsequently incubated with serum-containing medium in the absence (−) or presence of 10 μM VP-16 or 40 J/m^2 UV. The cells were harvested 24 h later and luciferase activities were determined. All the assays were carried out in triplicate and the results are displayed as the means ± S.D.

in wt-p53-transfected cells by 6.1 and 5.3-fold, respectively. In contrast negligible induction of luciferase activity was observed following VP-16 treatment and only a 2.2-fold enhancement was observed following UV treatment of p53-null cells.

Treatment of mt-p53-transfected cells with VP-16 or UV induced an enhancement of luciferase activity that was comparable to that of the mock-transfected cells being 2.4 and 2.8-fold, respectively. These data indicated that DNA damage-inducing signals could potentiate the transcriptional activity of TP at least in part through the regulation of p53, although p53-independent pathways also exist.

3.4. TP inhibits apoptosis induced by cytotoxic agents

To further analyze the role of TP in the DNA-damage process we investigated whether the increased expression of TP might confer cellular resistance to DNA damage-inducing agents. We established three TP-overexpressing cell lines, KB/TP, Jurkat/TP and HCT/TP, that show high expression of TP compared to mock-transfected cell lines, KB/CV, Jurkat/CV and HCT/CV, respectively (Fig. 3).

We used these cells to examine the effect of TP on cellular susceptibility to DNA damage. Cell viability following VP-16, doxorubicin, and cisplatin treatment was analyzed with the MTT assay (Tables 1 and 2). TP-transfected KB/TP and HCT/TP cells showed significant resistance to VP-16, doxorubicin and cisplatin treatment compared to their mock-transfected counterparts. The sensitivity to staurosporine of KB/TP

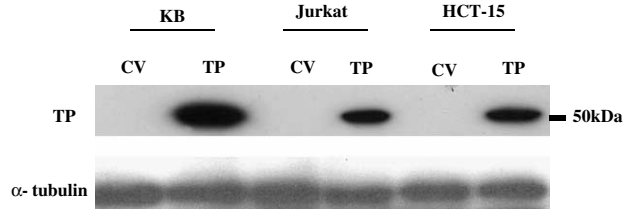

Fig. 3. TP-overexpressing cell lines. Immunoblot of TP in KB, Jurkat and HCT-15 cells transfected with TP cDNA. Cytosolic fractions (50 μg of protein) were separated by SDS–9.4% PAGE. TP levels were determined using an anti-TP monoclonal antibody.

Table 1
Effects of TP on the cytotoxicity of DNA-damaging agents

	IC_{50} (μM)		Fold resistance
	HCT/CV	HCT/TP	
VP-16	1.19 ± 0.230	16.25 ± 5.373	13.7
Doxorubicin	0.49 ± 0.028	1.69 ± 0.776	3.4
Cisplatin	8.56 ± 0.930	18.10 ± 1.220	2.1

Cell survival was determined with the MTT assay. The relative resistance of the cells was determined by division of the IC_{50} value of TP-transfected cell lines (HCT/TP) for each agent divided by the IC_{50} value of mock-transfected cell lines (HCT/CV) for the same agent. Values are the means ± S.D. of three experiments.

cells was comparable to that of KB/CV cells. We then examined whether Jurkat, KB and HCT cells transfected with TP cDNA are resistant to DNA damage-induced apoptosis. Apoptotic cells were measured by the proportion of the sub-G_1 fraction. Increased proportions of the sub-G_1 fraction were detected in both TP- and mock-transfected cell lines after treatment with 100 J/m^2 UV, 2–5 μM VP16, 4 μM doxorubicin and 2.1–50 μM cisplatin. However, the TP-transfected cell lines showed significantly lower susceptibility to DNA damage than the respective mock-transfectant (Fig. 4A–C).

In contrast, there were no significant differences in sensitivity to staurosporine or vincristine between TP- and mock-transfected cell lines. These findings indicated that TP might suppress DNA damage-induced apoptosis thereby conferring resistance to apoptosis on cancer cells.

3.5. The cytoprotective function of TP against DNA damage is independent of its enzymatic activity

We next examined whether the enzymatic activity of TP is required for its cytoprotective function. We determined if inhibition of the TP activity with TPI, a specific TP inhibitor ($Ki = 2 \times 10^{-8}$ M), could abrogate its cytoprotective effect against DNA damage-induced apoptosis. We first assayed the level of TP enzymatic activity in cellular homogenates of TP-transfected and control cells using a radiometric assay. Pre-treatment with 200 μM TPI for 12 h dramatically reduced the TP enzymatic activity in the KB/TP cells to the level lower than that in the mock-transfected cell line (Fig. 5A). TPI was not cytotoxic to the cells as it did not have any effect on the number of apoptotic cells at this concentration (data not shown). Pre-treatment with TPI did not alter the sub-G_1 fraction following the induction of DNA-damage by VP-16, UV, or VCR in either KB/CV or KB/TP cells (Fig. 5B). TPI treatment was also unable to abrogate the anti-apoptotic effect of TP in Jurkat cells or HCT cells (data not shown). The fact that the effect of TP on cellular resistance to DNA damage is unrelated to its enzymatic activity was also confirmed by MTT assay (Table 2). To confirm the finding that TP activity is not required for the cytoprotective effect, we used the KB/TPmt (L148R) cells that expressed catalytically inactive TP. The expression level of TP in KB/TP-mt (L148R) cells was similar to that in KB/TP cells but KB/TP-mt (L148R) cells had no TP activity [15]. The proportion of the sub-G_1 fraction of KB/CV cells after treatment with 100 J/m^2 UV, 2 μM VP-16, 4 μM Doxorubicin, 2.1 μM cisplatin was higher than that of KB/TP and KB/TP-mt (L148R) cells, and the proportion of the sub-G_1 fraction of KB/TP-mt (L148R) cells was comparable to that of KB/TP cells (Fig. 5C).

Table 2
Effects of TP and TP activity on the cytotoxicity of DNA-damaging agents

	IC_{50} (μM)		Fold resistance	IC_{50} (μM)		Fold resistance
	KB/CV	KB/TP		KB/CV + TPI	KB/TP + TPI	
VP-16	2.45 ± 0.144	15.72 ± 4.859	6.41	2.20 ± 0.151	13.65 ± 1.229	6.20
Doxorubicin	0.25 ± 0.008	0.873 ± 0.098	3.44	0.27 ± 0.008	0.778 ± 0.10	2.96
Staurosporine	58.8 ± 4.13	56.99 ± 6.153	0.97	47.9 ± 8.440	77.37 ± 3.84	1.68

Cell survival with or without 200 μM of TPI was determined with the MTT assay. The relative resistance of the cells was determined by division of the IC_{50} value of TP-transfected cell lines (KB/TP) for each agent divided by the IC_{50} value of mock-transfected cell lines (KB/CV) for the same agent. Values are the means ± S.D. of three experiments.

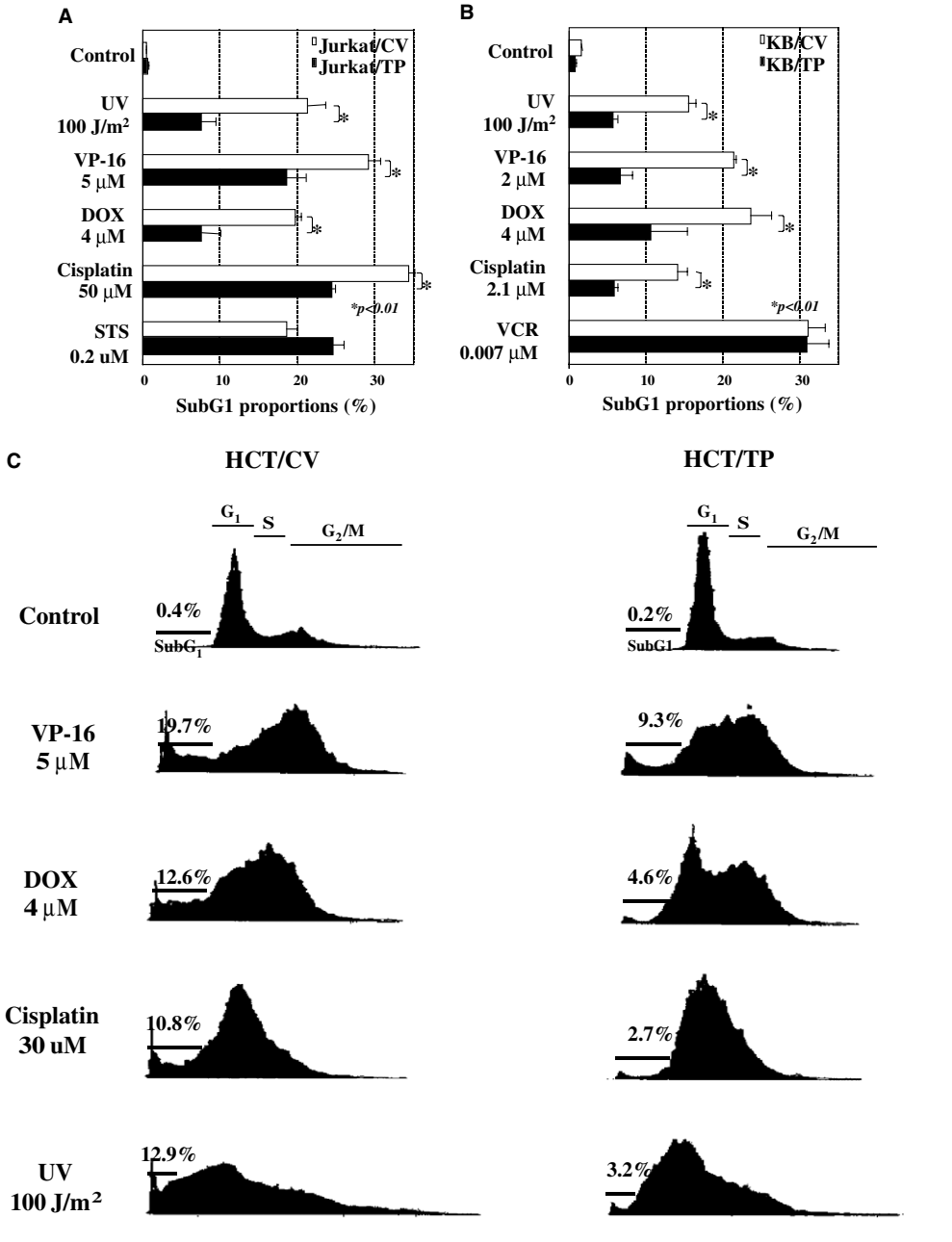

Fig. 4. The effects of DNA damage-inducing agents on the apoptotic fraction of TP-transfected cell lines. Apoptotic cells were estimated by determination of the proportion of cells in the sub-G_1 fraction. Flow cytometry of propidium iodide-stained cells was performed in the presence of the indicated doses of DNA damage-inducing agents. (A) Jurkat/CV (open bar) and Jurkat/TP (closed bar), (B) KB/CV (open bar) and KB/TP (closed bar) and (C) HCT/CV and HCT/TP cells. Each column and bar represents the means ± S.D. of three independent experiments.

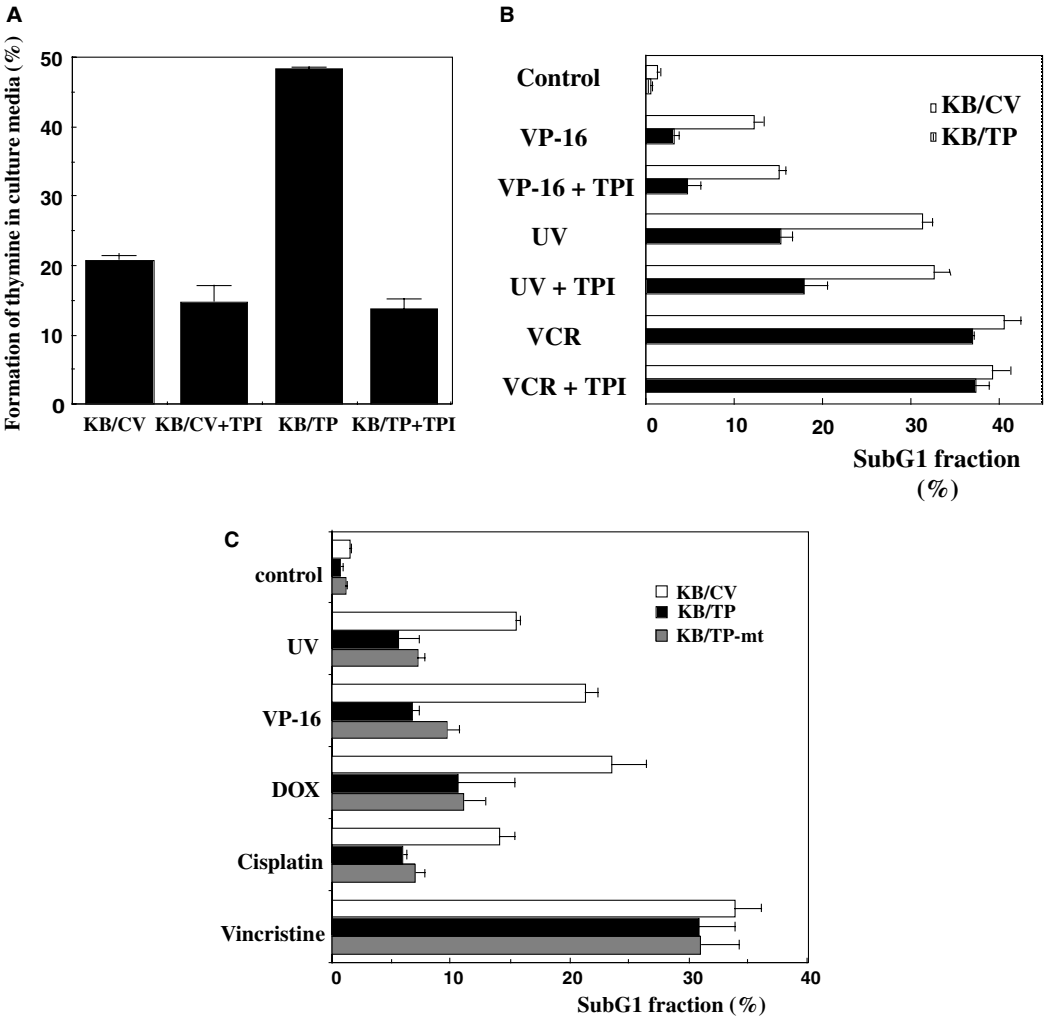

Fig. 5. The cytoprotective function of TP is independent of its enzymatic activity. (A) KB/CV cells and KB/TP cells were seeded 1×10^6 cells/well in 12 well plates and cultured with or without 200 μM of TPI under normoxic condition for 24 h. Enzymatic activities were determined by adding [^{14}C] thymidine in the culture media and measuring the conversion to [^{14}C] thymine. (B) Cells were pre-treated with TPI (500 μM) for 12 h before the addition of DNA damage-inducing stimuli. Effects of TPI on the apoptotic proportions of KB/CV (open bar) and KB/TP (closed bar). (C) KB/CV cells, KB/TP cells and KB/TP-mt (L148R) were exposed to the DNA-damage-inducing stimuli UV (100 J/m^2), cisplatin (2.1 μM), VP-16 (2 μM), doxorubicin (4 μM), VCR (0.007 μM) over 48 h. Apoptotic cells were estimated by the proportion of the sub-G_1 fraction. Flow cytometry of propidium iodide-stained cells was performed in the presence of indicated doses of DNA damaging agents.

3.6. The proportion of cells in the G_1 stage of the cell cycle is augmented in TP-overexpressing cell line

We next investigated if TP could exert its protective effect by modulation of the cell cycle. We investigated the effect of TP on the proportion of cells in the major stages of the cell cycle. Jurkat/CV and Jurkat/TP cells were UV irradiated (80 J/m^2) and the percentage of cells in the stages of the cell cycle was measured over 24 h. The Jurkat/TP cells showed a higher fraction of cells in the G1 stage than control cells in the absence of treatment (Fig. 6). Following exposure to UV, Jurkat/TP cells maintained a higher G_1 fraction of cells at both 12 and 24 h than Jurkat/CV cells. The G_1 fractions of Jurkat/CV and Jurkat/TP cells were, respectively: 52% and 58% at 6 h, 40% and 58% at 12 h, and 30% and 46% at 24 h.

3.7. Akt activation was augmented in TP-transfected cell lines

We next determined if the anti-apoptotic effect of TP might be mediated by Akt activation. KB/CV and KB/TP cells were exposed to UV at 80 J/m^2 and the activation of Akt was examined by Western blot using the anti-phosphorylated Akt antibody. A low basal level of Akt phosphorylation was detectable in both cell lines and this was enhanced as early as 30 min following UV exposure (Fig. 7A). In KB/CV cells Akt phosphorylation was maintained for up to 2 h, after which the phosphorylated Akt gradually declined and had returned to the basal level by 10 h. In contrast, the phosphorylated Akt in KB/TP was maintained at high levels for up to 10 h. The total amount of Akt was also higher in the KB/TP cells than in the KB/CV cells. We also had similar data with HCT/CV and HCT/TP cells (Fig. 7B). Similar patterns of Akt phosphorylation and protein expression were observed in the HCT-15 cells following UV exposure, and in the Jurkat cells following cisplatin treatment (data not shown). These data suggest that Akt activity tends to be augmented in TP-transfected cell lines, especially in the presence of DNA damage-inducing stimuli.

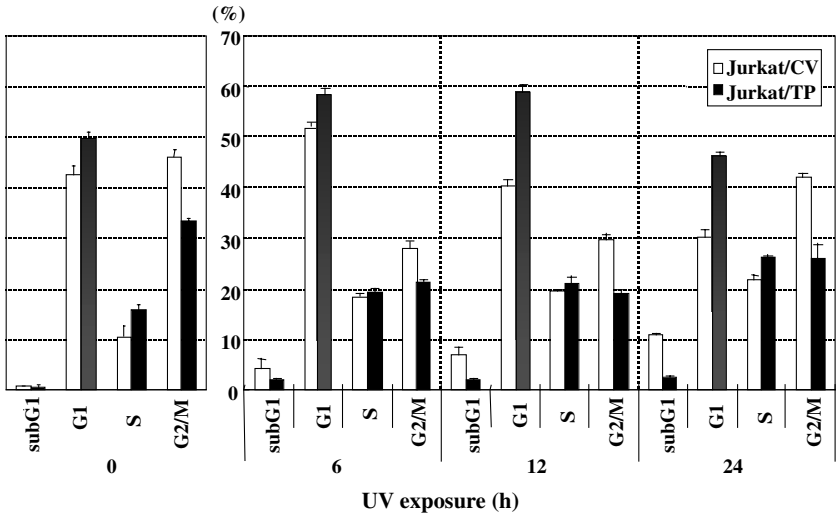

Fig. 6. G_1 proportion is augmented in TP-overexpressing cell lines. (A) Time-dependent changes in the cell cycle profile of Jurkat/CV (open bar) and Jurkat/TP (closed bar) cells after UV-exposure.

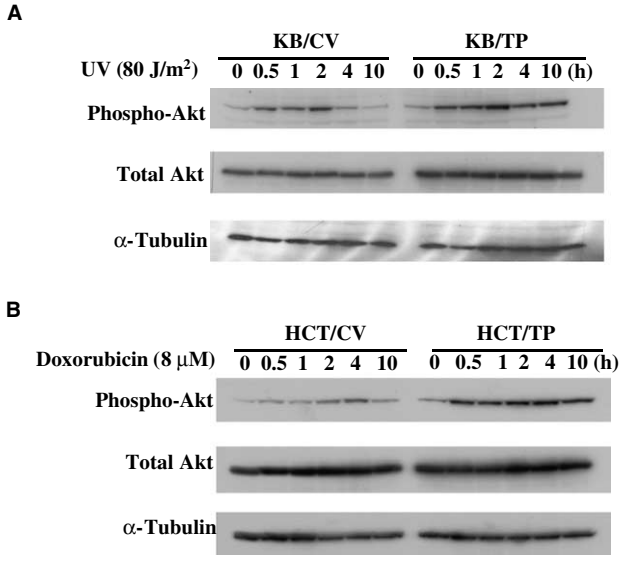

Fig. 7. Akt activation was augmented in TP-transfected cell lines. (A) KB/CV and KB/TP cells were incubated in the presence of 80 J/m^2 UV. Whole cell lysates were prepared at the indicated time. Each sample was subjected to immunoblot analysis using the anti-phosphorylated Akt and anti-Akt antibody. Immunoblotting of α-tubulin was used as a protein loading control. (B) HCT/CV and HCT/TP cells were incubated with 8 μM doxorubicin.

3.8. TP-associated cytoprotection against DNA damage is dependent on the PI3K/Akt pathway

To further analyze the role of the Akt pathway in TP-induced cytoprotection we determined the effect of inhibition of PI3K on the percentage of apoptotic cells. PI3K activity is essential for the phosphorylation of Akt. HCT and KB cells were pre-treated with the PI3K inhibitors, LY294002 and wortmannin for 1 h prior to induction of DNA damage with either UV (HCT cells) or VP-16 (KB cells) (Fig. 8A and B). As expected, the addition of 25 μM LY294002 or 500 nM wortmannin abrogated the expression of phosphorylated Akt almost completely in both cell lines. Moreover, LY294002 did not affect on the expression of either the phosphorylated ERK or the total level of ERK (Fig. 8B).

LY294002 or wortmannin treatment significantly attenuated the protective effect of TP on apoptosis in the TP-overexpressing cell lines, HCT/TP and KB/TP (Fig. 8C and D). These results indicate that PI3K activation might be required for the TP-associated cytoprotection against DNA-damaging agents.

4. Discussion

Previous studies demonstrated that TP is expressed at higher levels in a wide variety of tumors than in the adjacent non-neoplastic tissues. Considering the critical role that TP plays in tumor progression and angiogenesis, it is important to understand the cellular mechanisms by which TP expression is modulated and the molecular mechanisms by which TP exerts its effects in vivo.

TP expression has been previously shown to be upregulated by various inflammatory cytokines of monocyte/macrophage origin such as IL-1α, TNF-α and IFN-γ. This inflammation-mediated upregulation of TP might provide favorable conditions for angiogenesis and malignancy of human tumors [13,19]. However the biological role of tumor cell-derived TP is not yet clarified. We found in this study that many stimuli other than cytokines, such as hypoxia, hyperosmotic shock, acidosis and mitogen starvation, could enhance TP transcriptional activity and protein expression. These effects are consistent with other studies on the regulation of TP expression. Oxygen tension and pH have been shown to modulate TP expression in human breast tumor cells in vitro and in vivo [20].

In this study, TP promoter activity was also enhanced by thymidine, but not by thymine. This effect of thymidine and thymine on the transcriptional activity of the TP promoter mirrors previous data in which TP enzymatic activity was enhanced by thymidine and inhibited by thymine [21] and suggests a catabolic role for TP.

Of particular importance to cancer chemotherapy, we found that TP expression was influenced by DNA damage-inducing

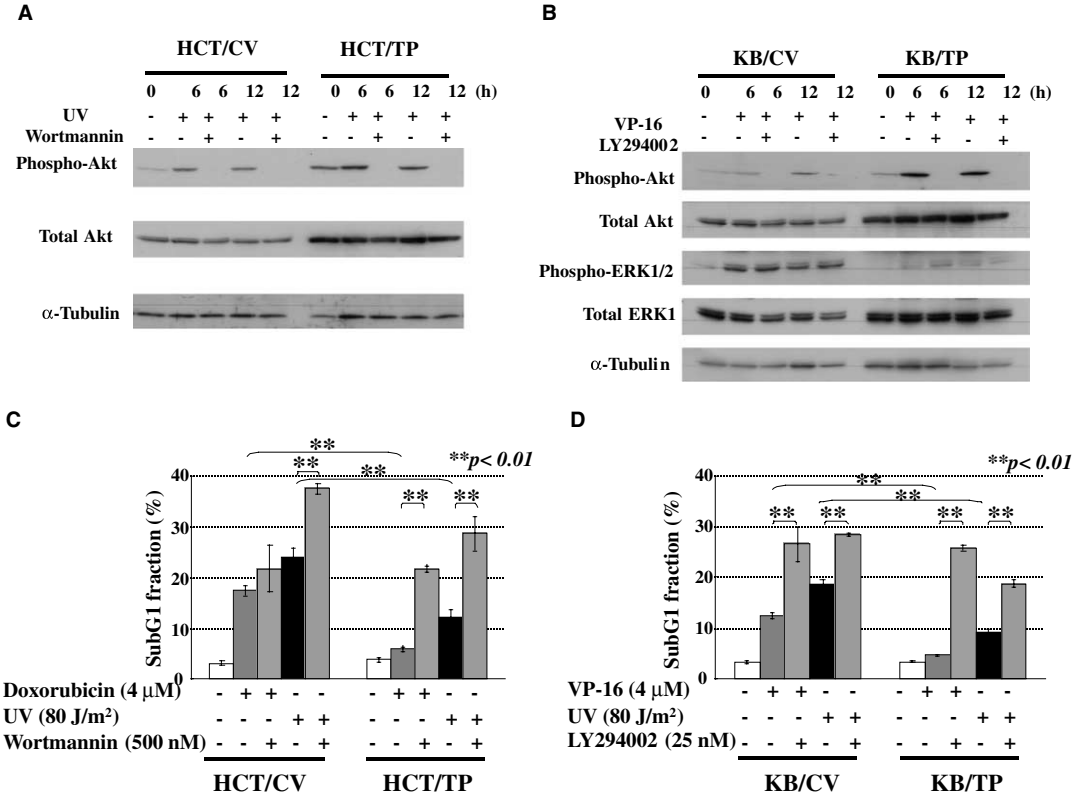

Fig. 8. TP-associated cytoprotection against DNA damage is dependent on the PI3-kinase/Akt pathway. (A) HCT cells, pre-incubated in the presence or absence of the PI3-kinase inhibitor wortmannin (500 nM), were exposed to UV (80 J/m^2). Cell lysates were obtained at 6 and 12 h following UV exposure and the level of Akt phosphorylation was assessed by immunoblotting. (B) KB cells were treated with VP-16 (10 μM) in the presence or absence of pre-treatment with the PI3-kinase inhibitor LY294002 (25 μM). Cell lysates were obtained at 6 and 12 h after VP-16 induction, and changes in the phosphorylation of ERK and Akt were assessed by immunoblotting. (C) HCT/CV and HCT/TP cells were pre-incubated with wortmannin (500 nM) for 1 h and treated with 4 μM doxorubicin or 80 J/m^2 UV. After 36 h incubation, the apoptotic fractions were estimated by analysis of the percentage of cells in the sub-G_1 fraction by flow cytometry. (D) KB/CV and KB/TP cells were preincubated with LY294002 (25 nM) for 1 h and treated with 4 μM VP-16 or 80 J/m^2 UV. After 36 h incubation, the apoptotic fractions were estimated.

agents including chemotherapeutic agents. Cisplatin, VP-16, doxorubicin and UV enhanced not only TP transcriptional activity but also its protein expression.

We have also addressed the possible molecular mechanisms by which TP induces protection against DNA damage-inducing agents. P53 plays an important role in mediating the response to stress induced by DNA damage that results in apoptosis [22] and the apoptosis inducing activity of p53 is a major contributor to its tumor suppressor function. It therefore seemed likely that p53 might play a role in modulating the TP promoter during DNA damage induced by stress. Our data do suggest some role for p53 in enhancement of TP promoter activity and this effect was enhanced following the induction of DNA damage-induced stress. Although the biological significance of the promotion of TP transcription by p53 is not clear at the present time, a strong association of TP and p53 in DNA damage and apoptotic process warrants further investigation. However, while this study indicates that DNA damage-inducing agents can potentiate TP promoter activity through a pathway that involves p53, it also indicates that there exist p53-independent pathways by which this can be achieved. Thus, transfection of mutated p53 could not potentiate TP promoter activity yet treatment with DNA damaging agents enhanced TP promoter activity in p53 mutant cells to a level that was comparable to that in the cells that do not express p53. Furthermore, TP could induce protection against apoptosis induced by DNA damage-inducing agents in all the cell lines studied irrespective of their p53 status including Jurkat cells (p53-null), KB cells (p53-wt) and HCT-15 (p53-mutated).

We have previously reported that cytoprotection of TP-overexpressing cell lines against Fas-induced apoptosis might be mediated by TP-induced suppression of the release of cytochrome *c* from the mitochondria and subsequent activation of caspase-3 and -9 [8]. TP-transfected Jurkat/TP showed a significant resistance to VP-16, Adriamycin and cisplatin treatment compared to their mock-transfected counterparts [9].

We also investigated the role of the TP enzymatic activity in modulation of the TP-protective effect against DNA damage-inducing agents. TP enzymatic activity has been shown to be important for TP-induced resistance to hypoxia-induced apoptosis in cancer cells, and the TP effect was inhibited by 2-deoxy-L-ribose, a stereoisomer of 2-deoxy-D-ribose. However recent reports suggest that the enzymatic activity of TP may not be required for the TP-induced cytoprotection against apoptosis induced by cisplatin and Fas. Inhibition of TP enzymatic activity with a specific inhibitor of TP could not inhibit TP-dependent suppression of Fas-induced apoptosis in

TP-transfected KB cells [8]. Jurkat/TPMu (L148R) cells that had no TP activity also showed comparable cytoprotective activity against VP-16, cisplatin and Adriamicin to Jurkat/TP cells [9].

In this study, we found that TP-mediated resistance to apoptosis induced by DNA damage-inducing agents was independent of the enzymatic activity of TP. This finding confirms and expands the previous results. Further study is needed to elucidate its effects independent of its enzymatic activity.

Since Akt plays an important role in cell survival, we examined the role of activation of Akt in the apoptotic process induced by DNA damage and its potential modulation by TP. Both Akt activity (phosphorylated Akt) and Akt protein were higher in TP-transfected cell lines than in the mock-transfectants, and the Akt activity was significantly abrogated by PI3K inhibitors. Our data suggest that the PI3K/Akt pathway might be implicated in the cytoprotective activity of TP against apoptosis induced by DNA damage-inducing agents. Further study is needed to elucidate whether PI3K/Akt pathway really plays an important role in the TP-induced protection against DNA damage, and to clarify the targets of TP in the PI3K/Akt pathway.

The data presented here demonstrated that expression of TP is increased in response to various stimuli including DNA damage, and that TP molecules, but not the enzymatic activity of TP confer resistance to DNA damage-induced apoptosis in cancer cells.

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