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Thymidine phosphorylase suppresses apoptosis induced by microtubule-interfering agents

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Abstract

We investigated the ability of thymidine phosphorylase (TP) to confer cancer cells resistance to MIA (microtubule-interfering agents)-induced apoptosis. Jurkat cells were stably transfected with TP cDNA (Jurkat/TP) and the sensitivity to MIAs were examined. Jurkat/TP cells were more resistant to apoptosis induced by nocodazole, vincristine, vinblastine, paclitaxel and 2-methoxyestradiol than mock-trasfected Jurkat/CV cells. TP enzymatic activity was not required for this effect of TP.

Jurkat/TP cells showed weak phosphorylation of Bcl-2, and kinase inhibitors staurosporine and genistein attenuated not only MIA-induced Bcl-2 phosphorylation but also cytotoxicity of MIA in Jurkat/CV, but not in Jurkat/TP. MIAs diminished expression of FasL in Jurkat/TP but not in Jurkat/CV, and neutralization of FasL by anti-FasL antibody considerably attenuated the cytotoxic effect of the MIAs in Jurkat/CV, but the effect of the antibody was marginal in Jurkat/TP cells. Our study provides further evidence that TP functions in conferring resistance on cancer cells to the stress induced by MIAs. In addition, we show that TP-induced inhibition of Bcl-2 phosphorylation and suppression of FasL may contribute to the protective function of TP in cancer cells.

Keywords: Thymidine phosphorylase; Microtubule; Apoptosis; Experimental therapeutics

1. Introduction

Thymidine phosphorylase (TP) is an enzyme involved in the pyrimidine metabolism [1] and is identical to an angiogenic factor, PD-ECGF, displaying chemotactic and angiogenic activity [2]. However, the biological function of TP does not appear to be limited to the angiogenic ability since it affects clinical prognosis in some human carcinoma independent of angiogenesis. This suggests that TP may affect tumor growth by an additional mechanism. One potential function of TP may be the modulation of apoptosis as previously reported that TP conferred cytoprotection against several stresses such as hypoxia, Fas and cisplatin on cancer cell lines [3–5]. One important class of anti-tumor drugs are microtubule-interfering agents (MIAs). Through differential binding to microtubule polymers (paclitaxel, docetaxel, 2-methoxyestradiol) or tubulin monomers and dimers (nocodazole, vincristine, vinblastine), MIAs interfere with the dynamic process of microtubule assembly [6], and result in G_2/M arrest and initiation of apoptosis. The mechanism by which MIAs induce apoptosis is believed to involve activation of various kinases and phosphorylation of the anti-apoptotic Bcl-2 protein [7,8]. Treatment of several cell lines with MIAs has been shown to induce Bcl-2 phosphorylation. Induction of Bcl-2 phosphorylation appears to be specific to microtubule damage as it is not observed following treatment with other pro-apoptotic drugs [9]. Phosphorylation of Bcl-2 at serine residues results in inactivation of Bcl-2 [8]. Inactivation of Bcl-2 inhibits the dimerization of Bcl-2 and Bax thus driving the cell towards death [10]. However, the exact mechanism by which MIAs become associated with Bcl-2

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phosphorylation has not been clarified. One possibility is that it may occur via the MAPK module as it has been shown that paclitaxel activates Raf-1 kinase, which coincides with phosphorylation and inactivation of Bcl-2 [9]. However, apoptosis induced by MIAs appears to be complex and may be mediated by more than one signaling pathway.

The Fas-FasL system has also been reported to participate in the MIA-induced apoptotic process [11]. Upregulation of FasL expression with subsequent induction of cell death has been proposed to account for chemotherapy-induced apoptosis, and some correlation between Bcl-2 phosphorylation and FasL upregulation was reported although the exact mechanism is still unclear [12].

In this study, we determined the ability of TP to confer resistance to MIA-induced apoptosis in cancer cells and analysed signaling pathways that might be modulated by TP to achieve this effect. We found that TP could confer resistance to apoptosis induced by a number of MIAs, and that expression of TP suppressed the MIA-induced phosphorylation of Bcl-2 and lowered the expression level of FasL.

2. Materials and methods

2.1. Reagents and antibodies

MIAs used in the study were nocodazole, vincristine, vinblastine, 2-methoxyestradiol and paclitaxel, all of which were purchased from Sigma Chemical Co. (St. Louis, MO). Staurosporine, genistein and 2-deoxy-L-ribose were also from Sigma Chemical Co. The ERK inhibitors, PD98059 and U0126, were purchased from Calbiochem (San Diego, CA). TPI was provided by Taiho Pharmaceuticals (Saitama, Japan).

Antibodies against Bcl-2, Raf-1, phospho-ERK1/2, ERK1 and PARP were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies against FasL and actin (Ab-1) were from Transduction Labs (San Diego, CA) and Oncogene (Boston, MA), respectively. FasL-neutralizing antibody (NOK-2) was purchased from PharMingen (San Diego, CA).

2.2. Cell lines and cell culture

The human leukemic cell lines Jurkat and HL-60, and the colon carcinoma cell line HCT-15, were supplied from ATCC (Rockville, MD), and maintained in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% FBS, 100 U/ml ampicillin, 100 μg/ml streptomycin and 2 mM glutamine (Gibco BRL, Gaithersburg, MD). Jurkat cells transfected with the full-length cDNA encoding TP (Jurkat/TP), a vector control (Jurkat/CV), or with a catalytically inactive TP mutant, L148R (Leu-148 → Arg) (Jurkat/TPmu) were described previously [5].

2.3. Transfection of TP cDNA into HL-60 and HCT-15 cells

Establishment of stable transfectants of TP in HL-60 (HL) and HCT-15 (HCT) cells was achieved by a slight modification of the procedure previously described for Jurkat cells [5]. One TP-positive clone transfected with RSV/TP (HL/TP, HCT/TP), and one clone transfected with RSV alone (HL/CV, HCT/CV) were further analysed.

2.4. MTT assay

The cells were plated on a 96-well plate at a density of 1×10^4 per well in the presence of a range of concentrations of MIAs. The plates were incubated for the indicated number of days and then 5 μg of MTT was added to each well and the plates were incubated for an additional 3 h. The resulting formazan was dissolved with 100 μl isopropanol containing 0.3% HCl and the plates were shaken for 5 min with a plate shaker. The optical density was immediately read at 570 nm using a model 550 Micro Plate Reader (Bio-Rad, Richmond, CA). The assays were performed at least three times with triplicate samples.

2.5. Quantification of apoptotic cells

Cells were plated at a density of 3×10^5 cells per ml media, per well in a 12-well plate and treated with the indicated MIAs for 24 h, harvested and washed once with PBS. For flow cytometry 1×10^4 cells were suspended in 40 μl PBS and mixed with 50 μl of Coulter DNA-prep LRP (COULTER, Miami, FA) and then 2 ml of Coulter DNA-prep Stain was added. The mixtures were then incubated for 15 min at room temperature. The DNA content and the sub-G_1 fraction were determined as previously described [3].

2.6. RT-PCR

Using 1 μg of total RNA, first strand cDNA synthesis for mRNA was performed using a ReverTraAce kit (Toyobo, Osaka, Japan). One microliter of the resulting first strand cDNA was then used for each PCR reaction. All PCR reagents were purchased from Promega (Madison, WI). Human FasL-specific primers were as follows: forward, 5′-GGC CTG TGT CTC CTT GTG AT-3′; and reverse, 5′-TCA TCA TCT TCC CCT CCA TC-3′. Primers of β-actin for internal control were as follows: forward, 5′-CAG CTT CGG AAC AAG AGA CC-3′; and reverse 5′-GTC CGA TGA TTC CTG CTG AT-3′.

The PCR amplification mixture was adjusted to a final volume of 20 μl. Cycles employed were 94 °C for 30 s for denaturation, 58 °C for 30 s for annealing, and 72 °C for 1 min for extension. PCR products were separated on 2% agarose gels and stained with ethidium bromide.

2.7. Cytoxicity assays

Neutralizing FasL antibody-induced cytoprotection was assessed by the trypan blue dye exclusion test [13]. Briefly, Jurkat-transfected cells in a logarithmic phase of growth were suspended at a concentration of 5×10^4 cells/ml. One milliliter of cell suspension was distributed into a well of a 24-well plate, and 36 h later 10 μg/ml of NOK-2 was added. Following a further 1 h-incubation, nocodazole or paclitaxel was added and cell viability was determined 24 h later by the ability of the cells to exclude 0.4% trypan blue dyes.

2.8. Immunoblotting

Logarithmically growing cells were seeded at 10^6 per 75 cm^2 dish and incubated for 36 h. Following treatment with the indicated MIAs, the cells were harvested and resuspended in lysis buffer (10 mM Tris–HCl, pH 7.5, 400 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% NP-40, 1 mM sodium orthovanadate, 5 mM NaF, 10 mg/ml aprotinin, 1 mM DTT, 10 mg/ml leupeptin, 1 mM PMSF) for preparation of total cell lysates. Lysates containing 100 μg of protein were subjected to SDS-PAGE and blotted onto a PVDF membrane. The membrane was incubated with the primary antibody overnight at 4 °C and then with a peroxidase-linked secondary antibody for 1 h at room temperature. The blot was developed by chemiluminescence according to the manufacturer's protocol (Amersham, Buckinghamshire, UK).

2.9. Statistical analysis

Quantitative data were expressed as the mean ± S.D. Statistical comparisons were performed using one-way ANOVA or unpaired Student's *t*-test. Differences were regarded significant when the probability values were <0.05.

3. Results

3.1. Expression levels and protective effect of TP on apoptosis induced by MIAs

We examined the expression level of the TP protein in HCT-15 and HL-60 cells transfected with TP cDNA. High expression of TP was detected in HL/TP and HCT/TP cells whereas TP was not detected in the non-transfected cells (HL/CV and HCT/CV) (Fig. 1A).

The effect of TP on the sensitivity of the cells to MIA treatment was investigated using these TP-transfected cells. HL/TP and HL/CV cells were treated with a range of nocodazole concentrations from 1 to 100 ng/ml, for 72 h. Cell viability was then measured with the MTT assay. The IC_{50}s of nocodazole for HL/CV and HL/TP cells were 18.87 ± 0.94 ng/ml and 54.21 ± 2.35 ng/ml, respectively. HL/TP cells were about three-fold more resistant to the cytotoxic effect of nocodazole than HL/CV cells (Fig. 1B).

The ability of TP to enhance cell viability following nocodazole treatment suggested that TP transfection protects cells against MIA-induced apoptosis. We therefore investigated whether the expression of TP could protect cells against apoptosis induced by a range of MIAs.

The sub-G_1 cell fraction was lower in the TP-transfected cell lines (15–18% for HL/TP; 10–15% for HCT/TP cells) compared to the levels in the respective mock transfectants (27–32% and 13–23%, respectively) (Fig. 1C).

3.2. Enzymatic activity of TP is dispensable for the cytoprotective effect of TP

We examined whether the enzymatic activity of TP is needed for the resistance to MIAs. Jurkat/TP, Jurkat cells transfected with cDNA encoding active TP, showed high levels of TP enzymatic activity. In contrast, Jurkat/Tpmu, Jurkat cells transfected with a catalytically inactive TP mutant (L148R), showed very low TP activity that were comparable to the level in the mock transfected Jurkat/CV cells (Fig. 2A).

Jurkat/TP, Jurkat/TPmu and control Jurkat/CV cells were treated with 200 ng/ml nocodazole, 5 μM vincristine, 2 μM vinblastine, 2 μM 2-methoxyestradiol, or 5 μM paclitaxel for 24 h and sub-G_1 fraction of the cells was measured. These concentrations of MIAs were chosen to induce roughly the similar proportion of sub-G_1 fraction in the cells. All of the MIAs tested increased the sub-G_1 fraction of the Jurkat cells to some extent. The proportion of the sub-G_1 fraction of Jurkat/TP was similar to that of Jurkat/TPmu ranging from 7 to 12% but significantly less than that of Jurkat/CV that ranged from 18 to 25% ($p < 0.01$) (Fig. 2B).

The similar ability of TP and the enzymatically inactive TP mutant, to protect against MIA-induced apoptosis in the Jurkat cells suggested that the TP effect might be independent of its enzymatic activity. To further investigate this possibility we tested whether inhibition of TP activity by pre-incubation of the cells for 6 h with 500 μM TPI, or with 200 μM 2-deoxy-L-ribose, might affect MIA-induced apoptosis. Neither treatment altered the percentage of apoptotic cells in the sub-G_1 fraction in either Jurkat/TP or Jurkat/CV following nocodazole treatment suggested that suppression of MIA-induced apoptosis by TP is indeed independent of its enzymatic activity (Fig. 2C).

3.3. ERK activation is suppressed in TP-overexpressing Jurkat cells but does not mediate TP-protection against MIA-induced apoptosis

Since TP enzymatic activity did not appear to play a role in MIA-induced apoptosis we investigated if we could establish the molecular mechanisms important for MIA-

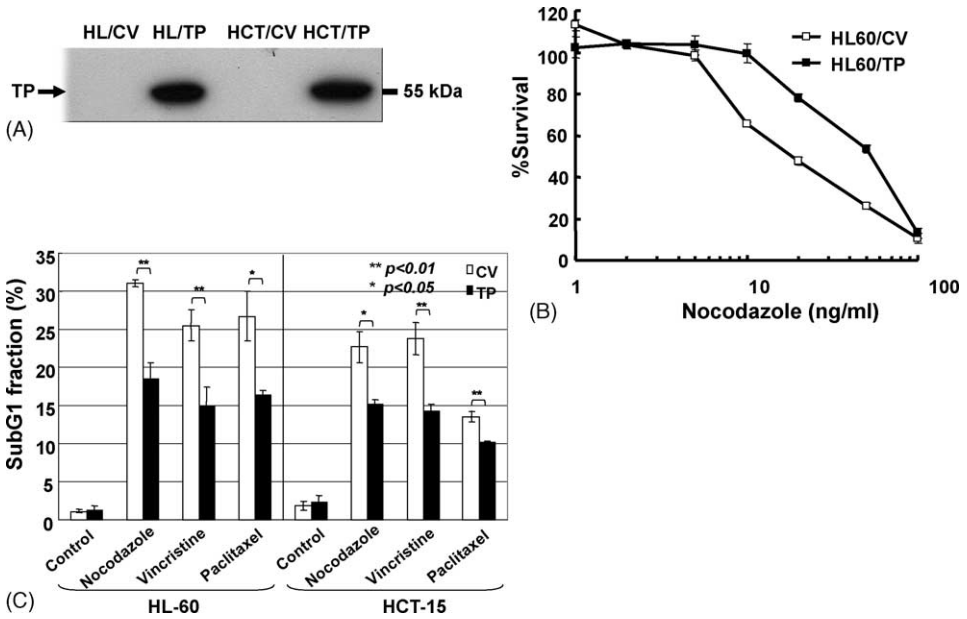

Fig. 1. Expression and cytoprotective effect of TP. (A) Cytosolic fractions (100 μg of protein) were separated by 9.4% SDS-PAGE. TP protein levels in TP-transfected HL-60 (HL/TP), HCT-15 (HCT/TP) or mock-transfected cells (HL/CV or HCT/CV) was measured by western blotting with an anti-TP monoclonal antibody. TP is indicated by an arrow. Molecular weight markers are shown at right. (B) HL/CV and HL/TP cells were incubated with the indicated concentrations of nocodazole for 72 h, and cell viability was determined with the MTT assay. Similar results were obtained in three separate experiments. (C) HL-60- and HCT-15- TP-transfected or mock-transfected cells were incubated with either 200 ng/ml nocodazole, 5 μM vincristine, 5 μM paclitaxel or buffer control for 24 h and the percentage of apoptotic cells was determined.

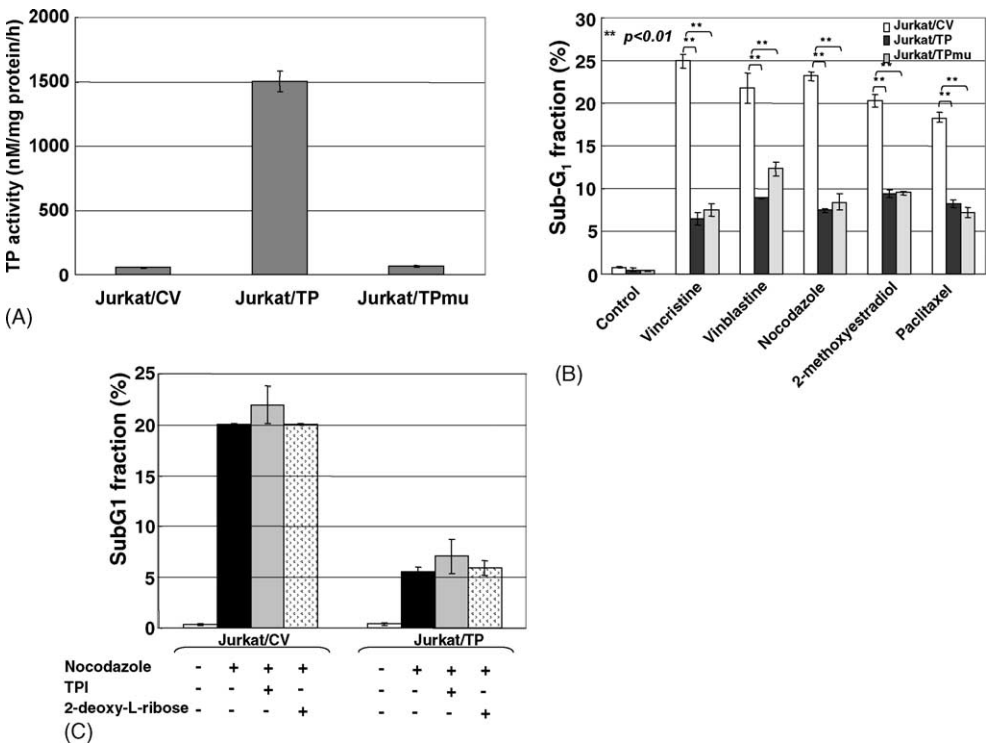

Fig. 2. Enzymatic activity and the cytoprotective effect of TP. (A) TP enzymatic activity of Jurkat/TP, Jurkat/TPmu and control Jurkat/CV cells was measured spectrophotometrically (from 200 μg protein). Each column and bar represents the mean ± S.D. of three independent experiments. Protective effect of TP on MIA-induced apoptosis. (B) Jurkat/CV, Jurkat/TP and Jurkat/TPmu were incubated in the absence (control) or presence of 5 μM vincristine, 2 μM vinblastine, 200 ng/ml nocodazole, 2 μM 2-methoxyestradiol (2-ME), or 5 μM paclitaxel for 24 h followed by flow cytometric analysis. The percentage of cells in the sub-G_1 peak is an estimate of the % of apoptotic cells. (C) Jurkat/CV and Jurkat/TP cells were treated with nocodazole for 24 h in the absence or presence of pre-treatment with TPI (500 μM) or 2-deoxy-L-ribose (200 μM) as indicated. The percentage of apoptotic cells was assayed by flow cytometry. (B–C) Each column and bar represents the mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

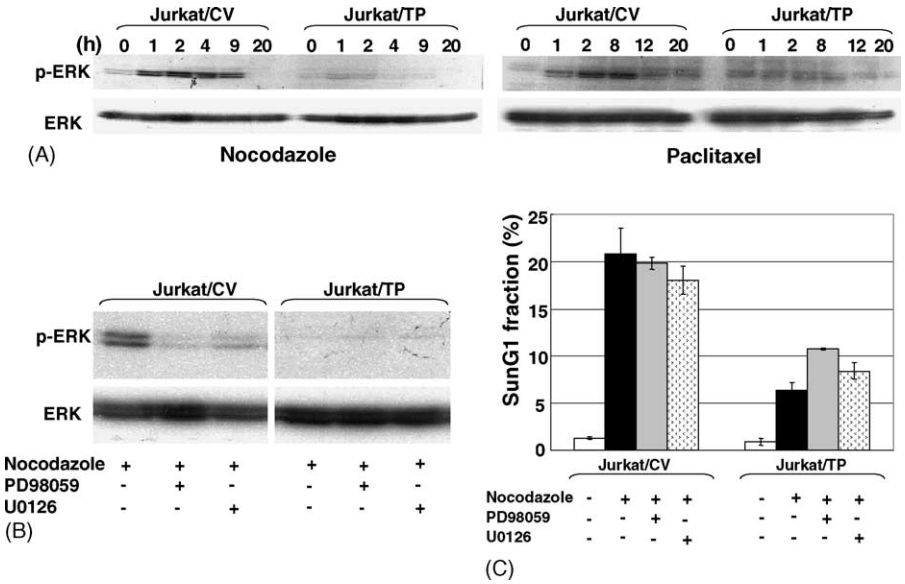

Fig. 3. ERK suppression by TP does not correlate with TP-protection against MIA-induced apoptosis. (A) Jurkat/CV and Jurkat/TP were incubated with 200 ng/ml nocodazole (Left) or 5 μM of paclitaxel (right). At the indicated times total cell lysates were immunoblotted with a phospho-specific antibody for active ERK (p-ERK). The two bands represent ERK-1 (p44) and ERK-2 (p42), respectively. As a control for total ERK levels the membranes were stripped and reprobed with an anti-ERK antibody (ERK). (B) Jurkat/CV and Jurkat/TP were incubated with 200 ng/ml nocodazole for 4 h following pre-treatment for 1 h in the absence or presence of the ERK inhibitors PD98059 or U0126. Cell lysates were subjected to immunoblotting with p-ERK and ERK specific antibodies as described in (A). (C) Jurkat/CV and /TP were treated with nocodazole for 24 h in the absence or presence of pretreatment with the ERK inhibitors PD98059 (75 μM) or U0126 (25 μM). The percentage of apopototic cells was determined by flow-cytometry as described in Fig. 2.

induced apoposis and how they might be modified by TP transfection. One molecule that plays a central role in cell cycle arrest and apoptosis, and that is activated by DNA-damage stimuli, is ERK [14]. We therefore first determined if ERK was activated following MIA-treatment of Jurkat/CV, and detectable as double bands (p42 and p44) in untreated Jurkat/CV. ERK phosphorylation was dramatically enhanced from 1 to 8 h after the addition of both nocodazole and paclitaxel (Fig. 3A) suggesting the ERK activation might be involved in MIA-induced apoptosis. The level of ERK activation increased with increasing MIA concentration (data not shown), indicating a correlation between the intensity of microtubule damage and the level of ERK activation.

We then tested the effect of TP transfection on ERK phosphorylation following MIAs treatment. In contrast to Jurkat/CV, only a slight increase in ERK phosphorylation was noted in Jurkat/TP shortly after nocodazole exposure, and thereafter only trace levels were detectable up to 24 h. Furthermore, no distinguished ERK phosphorylation was detectable in paclitaxel-treated Jurkat/TP even though the total levels of ERK proteins were almost the same in the Jurkat/TP and Jurkat/CV (Fig. 3A). No detectable difference was observed in the phosphorylation levels of other MAPK, JNK/SAPK, p38 or the expression of ATR in these cell lines (data not shown), suggesting that activation of ERK may play a specific role in MIA-induced apoptosis and the TP protective effect in Jurkat cells.

To further examine this possibility we determined if ERK inhibitors could reverse MIA-induced apoptosis or the TP protective effect against MIA-induced apoptosis. The ERK inhibitors, 75 μM PD98059 or 25 μM U0126, were added to Jurkat/CV and Jurkat/TP cells 1 h before treatment with nocodazole for 24 h. The apoptotic fraction of the cells was measured by flow cytometry. Although treatment with the ERK inhibitors suppressed ERK phosphorylation below basal levels (Fig. 3B), they did not abrogate apoptosis induced by nocodazole nor the protective effect of TP on nocodazole-induced apoptosis (Fig. 3C). To exclude the possibility that a lack of effect of the inhibitors might be due to degradation of PD98059 during the 24 h of culture we also tested the effect of addition of this inhibitor 6 h following nocodazole treatment [15]. However, this delayed treatment did not affect apoptosis in the Jurkat cells (data not shown). These data suggest that, while MIAs do induce ERK activation that is downregulated by TP transfection, the ERK activity is not directly correlated with MIA-induced apoptosis and does not play a role in the TP anti-apoptotic activity.

3.4. Involvement of Bcl-2 in TP-suppression of MIA-induced apoptosis

Since ERK activation did not appear to correlate with MIA-induced apoptosis, we examined other signaling pathways that might be modulated by TP transfection and lead to abrogation of MIA-induced apoptosis.

We determined the effect of MIAs on Bcl-2 phosphorylation and PARP cleavage in Jurkat/CV and Jurkat/TP cells. Initial levels of unphosphorylated Bcl-2 protein were

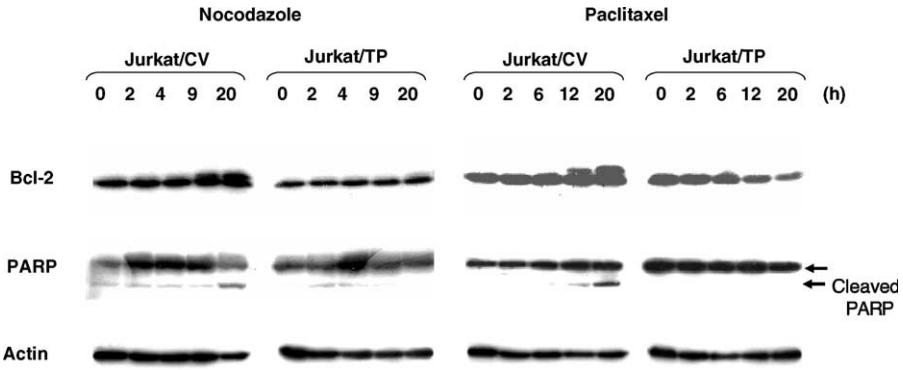

Fig. 4. Role of bcl-2 phosphorylation in TP protection against MIA-induced apoptosis. Jurkat/CV and Jurkat/TP were incubated in the presence of 200 ng/ml nocodazole (Left) or 5 μM paclitaxel (Right) for the indicated times. Total cell lysates were subjected to immunoblot analysis using specific antibodies against Bcl-2 and PARP. Phosphorylation of Bcl-2 and Raf-1 is detected by the appearance of a slower migrating band in western blot analysis. The position of phosphorylated Bcl-2 and cleaved PARP are indicated by arrows at right.

similar in both Jurkat/TP and Jurkat/CV cells and short-term treatment (4–6 h) with the MIAs did not induce Bcl-2 phosphorylation (Fig. 4). However, Bcl-2 phosphorylation was detected after 9 h of nocodazole treatment, or 12 h of paclitaxel treatment, of Jurkat/CV cells. In contrast, only weak phosphorylation of Bcl-2 could be detected following 20 h exposure of Jurkat/TP to the MIAs suggesting that TP-transfection could inhibit MIA-induced phosphorylation of Bcl-2. A similar pattern in Bcl-2 phosphorylation between Jurkat/CV and Jurkat/TP was also observed following vincristine treatment, and over-expression of TP had no effect on the expression level of other Bcl-2 related proteins such as Bax or Bcl-X_L (data not shown), suggesting that the effect of TP was specific for Bcl-2. TP-transfection inhibited PARP cleavage in paclitaxel- as well as in nocodazole-treated cells, suggesting that TP protected the cells from MIA-induced apoptosis by inhibiting the Bcl-2 phosphorylation.

3.5. Inhibition of Bcl-2 phosphorylation and cytoprotection in Jurkat/CV by staurosporine and genistein

To further investigate the involvement of Bcl-2 phosphorylation in TP-related cytoprotection, we tested the effect of inhibition of Bcl-2 phosphorylation on TP-pro-

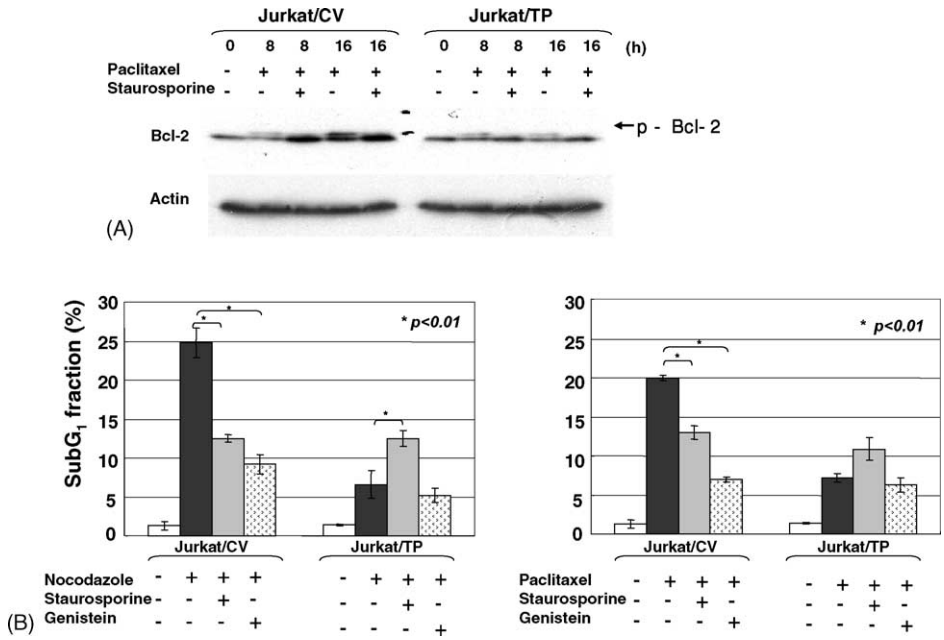

Fig. 5. Staurosporine inhibits Bcl-2 phosphorylation. (A) Jurkat/CV or Jurkat/TP cells were treated with paclitaxel (5 μM) over 16 h in the presence or absence of pretreatment with staurosporine (75 nM). Cell lysates were analysed for Bcl-2 phosphorylation over time by immunoblotting with a Bcl-2 antibody. Immunoblotting of actin was used as a control for protein loading. Phosphorylated Bcl-2 is indicated by an arrow. (B) The effect of kinase inhibitors on apoptosis in Jurkat/CV or Jurkat/TP cells was tested by treatment of the cells with 200 ng/ml nocodazole (Left) or 5 μM paclitaxel (Right) for 24 h following pre-incubation with either buffer (control) or in the absence or presence of genistein (10 μg/ml) or staurosporine (75 nM) for 1 h. The fraction of apoptotic cells were then estimated by flow cytometric determination of the percentage of cells in the sub-G_1 fraction. $^*p < 0.01$.

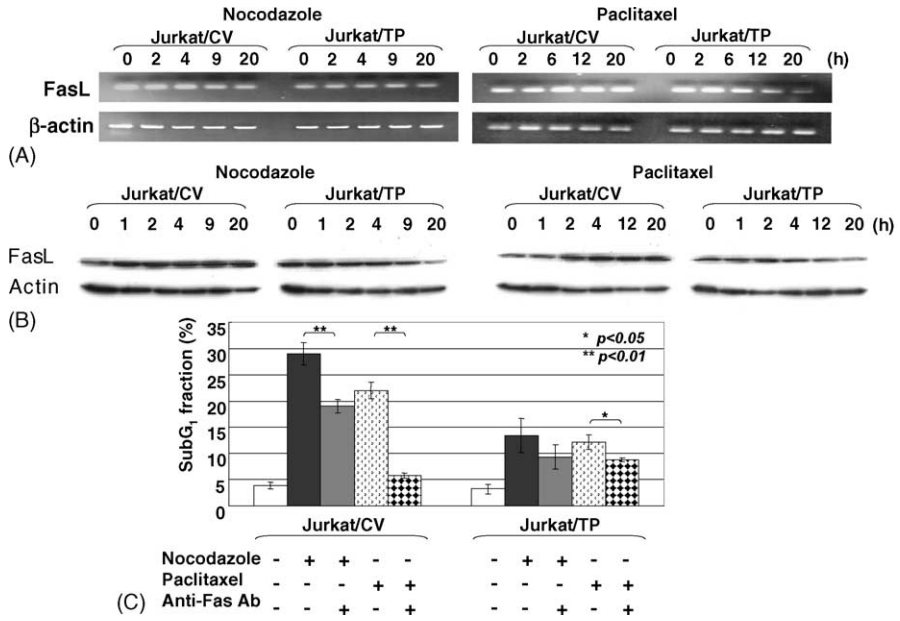

Fig. 6. Suppression of FasL by TP may play a role in TP-protection against MIA-induced apoptosis. Jurkat cells were treated with 200 ng/ml nocodazole (Left) or 5 μM paclitaxel (Right) over 20 h. (A) Total RNA was then subjected to RT-PCR using primers specific for amplification of FasL. β-actin expression was examined as an internal control to ensure RNA integrity and proper amplification. (B) Total lysates were subjected to immunoblot analysis with a FasL-specific antibody. (C) The effect of the FasL-neutralizing antibody (NOK-2) on apoptosis of Jurkat/CV and Jurkat/TP cells was measured by pre-treatment of the cells with 10 μg/ml NOK-2 for 2 h prior to incubation with 200 ng/ml nocodazole or 5 μM paclitaxel for 18 h. Cytotoxicity was then estimated by determination of the percentage of cells that had absorbed trypan blue. Each column and bar represent the mean ± S.D. of three independent experiments. $^{*}p < 0.01$.

tection against MIA-induced apoptosis. Jurkat/CV and Jurkat/TP were pre-treated with 10 μg/ml genistein or 75 nM staurosporine for 1 h prior to induction of apoptosis. These concentrations of kinase inhibitors were non-toxic to Jurkat/CV cells (data not shown). Staurosporine almost completely inhibited paclitaxel-induced Bcl-2 phosphorylation (Fig. 5A), as did genistein (data not shown). As expected, pre-treatment with staurostporine or genistein 1 h before addition of the MIAs significantly suppressed MIA-induced apoptosis ($p < 0.01$) as judged by flow cytometric analysis. In contrast, treatment of Jurkat/TP with genistein and staurosporine did not decrease the apoptotic fraction of cells. Staurosporine rather increased the apoptotic fraction (Fig. 5B). These findings indicate that the cytoprotective function of TP might, at least in part, be attributed to modification of Bcl-2 phosphorylation.

3.6. Effect of TP on the MIA-induced FasL and apoptosis

The second pro-apoptotic pathway that we investigated was the expression of FasL since treatment with MIAs has been reported to kill tumor cells in a Fas/FasL-dependent manner. Jurkat/CV expressed a basal level of FasL that was enhanced by paclitaxel at both the messenger RNA (Fig. 6A) and protein (Fig. 6B) levels. In contrast, the levels of FasL mRNA and protein in Jurkat/TP cells were suppressed following exposure to either nocodazole or paclitaxel although the initial level of FasL expression was comparable to that of Jurkat/CV. To further investigate the role of FasL expression in MIA-induced apoptosis and TP-protection against MIA-induced apoptosis, the effect of pretreatment of the cells with 10 μg/ml of the FasL-neutralizing antibody (NOK-2) was investigated. Paclitaxel-induced apoptosis was almost completely abrogated by NOK-2 in Jurkat/CV. Nocodazole-induced apoptosis was also suppressed although not to the same extent as that of paclitaxel. In contrast, the neutralizing FasL antibody only weakly abrogated the protective effect of transfected TP on apoptosis induced by paclitaxel or nocodazole. These data suggest that suppression of FasL may play a role in TP-anti-apoptotic effects (Fig. 6C). Thus, it appears that both phosphorylation of Bcl-2 and suppression of FasL level are the most likely pathways through which TP might exert its protective effect against MIA-induced apoptosis.

4. Discussion

We have previously shown that TP enzymatic activity plays a role in resistance to hypoxia-induced apoptosis in cancer cells [16]. In this study we found that TP protection against MIA-induced apoptosis is similar for both active and catalytically inactive TP, and that protection induced by active TP cannot be attenuated by inhibitors such as TPI or 2-deoxy-L-ribose that regulate TP-related angiogenic activity. The mechanism by which TP modulates apoptosis independent of its catalytic activity will remain for further study.

The activation of ERK in MIA-treated cells, as well as the lack of correlation between ERK activation and MIA-induced apoptosis, is consistent with previous reports [17,18]. We showed here MIA-induced ERK activation was considerably suppressed in Jurkat/TP although it did not appear to play a role in the TP-modulated protective effect. Thus, although suppression of ERK phosphorylation may not be important for protection against apoptosis it may play other roles in TP function.

We have shown that TP can also modulate phosphorylation of the anti-apoptotic factor Bcl-2 and subsequent downstream cleavage of PARP and activation of caspases. This finding is consistent with previous data that Bcl-2 phosphorylation may play a role in MIA-induced apoptosis and in cancer cell resistance to MIAs [19,20]. Human leukemic, breast, and prostate cancers exposed to paclitaxel express a phosphorylated form of Bcl-2 preceding Bcl-2 inactivation and PARP cleavage. It has also been shown that phosphorylated Bcl-2 loses its ability to interact with Bax in paclitaxel-treated cancer cells [21]. The fact that the Bcl-2 protein has a long half-life and that its regulation by protein expression is limited, are suggestive that phosphorylation of Bcl-2 plays a critical role in Bcl-2 function. Therefore the delay in, and suppression of, Bcl-2 phosphorylation by TP-overexpression observed here is likely to play a role in protection against MIA-induced apoptosis although the mechanism by which TP suppresses Bcl-2 phosphorylation remains to be established.

The biological relevance of MIA-induced Bcl-2 phosphorylation for MIA-induced cytotoxicity is not fully elucidated. A recent report demonstrated that phosphorylation of Bcl-2 occurs only in cells blocked at the G_2/M phase following MIA treatment, suggesting that Bcl-2 phosphorylation merely represents a consequence of mitotic arrest rather than a causative factor of MIA-induced cytotoxicity [22]. In contrast, another report suggested that the high susceptibility of cells to apoptosis induced by microtubule agents during G_2/M can be directly attributed to the phosphorylation of Bcl-2 [7]. Lowering the threshold for apoptosis at the G_2/M phase of the cell cycle might ensure the elimination of cells with aberrant chromosomal segregation.

Both genistein and staurosporine inhibited Bcl-2 phosphorylation and MIA-induced apoptosis in Jurkat/CV although they inhibit different kinases. Interestingly, TP-induced cytoprotection against MIAs was partly attenuated by staurosporine which inhibits a greater range of kinases than genistein. Although the suppression of MIA-induced apoptosis by staurosporine may be attributed, at least in part, to the inhibition of Bcl-2 phosphorylation via inhibition of kinases that directly phosphorylate Bcl-2, staurosporine may also inhibit other kinases that play a role in cell survival pathways such as the PI3-kinase/Akt pathway [23,24]. We are currently investigating if the anti-apoptotic effect of TP transfection might also affect this pathway.

As well as demonstrating that TP suppressed phosphorylation of Bcl-2, this study also showed that FasL expression following MIA exposure was suppressed in Jurkat/TP cells at both the mRNA and the protein level. This finding is consistent with a potential role for enhanced FasL expression in contributing to paclitaxel-induced apoptosis in several cancer cell lines [25,26]. Since Fas is ubiquitously expressed in most cancer cells, induction of FasL, whose expression is more restricted, would be an amplification signal for tumor apoptosis. Recent studies have suggested that stresses induced by ultraviolet rays, chemotherapeutic agents and irradiation activate the Fas pathway resulting in cell death not only by increasing Fas expression, but also by affecting intracellular signalling molecules activated upon Fas ligation. Numerous drug-resistant cell lines were also found to be resistant to Fas-mediated apoptosis [27], and sustained suppression of FasL expression was one of the characteristics of cisplatin-resistant cell lines [28]. The exact role of FasL in tumor-cell apoptosis, and the potential relationship between FasL and Bcl-2 phosphorylation is not yet clear in our study. It has been suggested that phosphorylation of Bcl-2 induces FasL and apoptosis by inducing the release of calcineurin and subsequent nuclear traslocation of NFAT and enhanced expression of FasL [29]. Although paclitaxel is consistently reported to induce apoptosis via FasL upregulation and Bcl-2 phosphorylation, suppression of FasL has not been proved as a mechanism regulating paclitaxel-resistance. It is therefore conceivable that TP-induced suppression of Bcl-2 could be responsible for the suppression of FasL. We used the sub-G_1 fraction as the only measure of apoptosis in this study. Other methods besides the measurement of sub-G_1 fraction will detect the cells in different stages of apoptosis, and reliability of our data about apoptosis might be intensified. In summary, our study has provided further evidence that TP functions in conferring resistance on various cancer cells to MIAs. In addition we have shown that TP may contribute to the progression of tumors irrespective of its enzymatic activity. Clearly further study is required to elucidate the exact molecular mechanism by which TP regulates these biological functions. However this study has explored the effect of TP on signaling pathways implicated in MIA-induced apoptosis and has shown that TP-induced inhibition of Bcl-2 phosphorylation and suppression of FasL may contribute to the protective function of TP against MIA-induced cytotoxicity in Jurkat cells.

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