

Thymidine phosphorylase enhances reactive oxygen species generation and interleukin-8 expression in human cancer cells

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Received December 22, 2011; Accepted February 21, 2012

DOI: 10.3892/or.2012.1887

Abstract. Thymidine phosphorylase (TP) is an angiogenic factor that plays a pivotal role in tumor angiogenesis. Various kinds of solid tumors express TP and high TP activity is correlated with microvessel density. We have previously reported that TP enhances interleukin-8 (IL-8) expression in KB human epidermoid carcinoma cells. In this study, TP was shown to be involved in enhanced expression of IL-8 in EJ human bladder cancer cells and Yumoto human cervical cancer cells as well as KB human epidermoid carcinoma cells. The enzymatic activity of TP was required for the enhanced expression of IL-8. A degradation product of thymidine was implicated in the enhanced expression of IL-8. TP augmented reactive oxygen species (ROS) generation in KB and Yumoto cells, and the enzymatic activity of TP was again required for the generation of ROS. An antioxidant, N-acetylcysteine (NAC), attenuated the generation of ROS and IL-8 mRNA expression in KB and Yumoto cells, and H₂O₂ increased IL-8 mRNA expression in Yumoto cells, suggesting that ROS generated by TP caused the increased expression of IL-8 mRNA. Since TP also reduced cellular glutathione levels and transcription of γ -GCS in KB cells, the TP-induced augmentation of ROS

may be partially attributed to the decreased glutathione. Our findings suggest that thymidine-derived sugars enhanced ROS generation and consequently increased IL-8 expression.

Introduction

Thymidine phosphorylase (TP), an enzyme involved in pyrimidine catabolism, is identical with an angiogenic factor, platelet-derived endothelial cell growth factor (PD-ECGF) (1). TP is overexpressed in various tumors and plays an important role in angiogenesis, tumor growth, invasion and metastasis (2). The enzymatic activity of TP is required for the angiogenic effect of TP (3). A novel, specific TP inhibitor, TPI, inhibits angiogenesis induced by TP in KB/TP cells (human KB epidermoid carcinoma cells transfected with TP cDNA), as well as growth and metastasis of KB/TP cells *in vivo* (4,5).

2-Deoxy-D-ribose (DR), one of the degradation products of thymidine generated by TP activity, has both angiogenic and chemotactic activity (6). Both DR and TP inhibit a hypoxia-induced apoptotic pathway (7). These findings suggest that DR is a downstream mediator of TP function. 2-Deoxy-L-ribose, a stereoisomer of DR, inhibits the promotion of angiogenesis, tumor growth and metastasis by TP (8,9). Recent evidence suggests that DR affects endothelial cell migration through activation of the integrin downstream signaling pathway (10). Rapamycin completely abrogates DR-induced endothelial cell migration and angiogenesis, correlating with a blockade of DR-induced p70S6 kinase activation in endothelial cells (11). Thymidine-derived DR and deoxy-D-ribose 1-phosphate (DR1P) are enzymatically converted to 2-deoxy-D-ribose 5-phosphate (DR5P) (12). Bijnsdorp *et al* observed that DR1P and DR5P accumulate at high levels in TP-overexpressing cells and DR is extensively secreted by these cells (13).

Brown and Bicknell inferred that DR may be an important energy source under hypoxic conditions (14). Brown *et al* also reported that TP overexpression in cells treated with thymidine induces hemo oxygenase-1 (HO-1), a classical cellular

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Abbreviations: TP, thymidine phosphorylase; IL-8, interleukin-8; ROS, reactive oxygen species; NAC, N-acetylcysteine; HO-1, hemo oxygenase-1; DR, 2-deoxy-D-ribose; γ -GCS, γ -glutamylcysteine synthetase

Key words: thymidine phosphorylase, interleukin-8, reactive oxygen species, thymidine phosphorylase inhibitor, 2-deoxy-D-ribose

oxidative stress marker (15). Although our results (4) and many reports from other laboratories suggest that TP is pivotal for tumor progression, the molecular basis for the induction of reactive oxygen species (ROS) and angiogenic factors by TP is not completely understood.

In this study, we indicate that TP activity is required for the enhanced ROS generation and *IL-8* mRNA expression in human cancer cells and agents which inhibit TP activity are strong candidates for new anticancer drugs.

Materials and methods

Chemicals and cell culture. NAC was obtained from Sigma-Aldrich. H₂DCF-DA was obtained from Molecular Probes. KB (human epidermoid carcinoma), EJ (human bladder cancer), Yumoto (human cervical carcinoma), THP-1 (human monocyte) and MCF-7 (human breast carcinoma) cells were grown in DMEM (Nissui Seiyaku Co.) containing 10% calf serum, 2 mM glutamine and 100 U/ml of penicillin at 37°C in a 5% CO₂ humidified atmosphere. The medium was changed to fresh serum-free media before experiments.

Transfection of TP/PD-ECGF cDNA into KB cell. TP/PD-ECGF full-length cDNA plasmid, TP/PD-ECGF mutant plasmid (L148R, Leu-148→Arg) (3) or the empty vector was transfected into KB cells by electroporation (16). After selection with geneticin, expression of TP in each clone was determined by immunoblot analysis using an anti-TP monoclonal antibody as described (17). A TP-positive clone (KB/TP cells) and a control vector-transfected clone (KB/CV cells) were used for further analyses.

Immunoblot analysis. Samples were subjected to 6 or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (18). Gel proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P transfer membrane; Millipore) using the Bio-Rad Transblot SD apparatus (19). The membrane was treated with blocking buffer containing 3% skimmed milk, 350 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 0.05% Tween-20 for 1 h and incubated with the indicated primary antibody overnight at 4°C. Following 4 washes, the membrane was incubated with a secondary antibody in the buffer for 1 h at room temperature. The membrane was then washed and developed using enhanced chemiluminescence western blotting detection system (Amersham Pharmacia). Primary antibodies against HO-1 (Santa Cruz Biotechnology), α -tubulin (Calbiochem) and β -actin (Santa Cruz Biotechnology) and HRP-conjugated secondary antibodies (Amersham Pharmacia) were used.

Reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from cells using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). RT-PCR was performed using the SuperScript One-Step RT-PCR system and gene-specific primers according to the instructions of the manufacturer (Invitrogen). Reaction mixtures containing total RNA (500 ng of each), 0.2 mM dNTPs, 0.2 μ M of each primer and an enzyme mixture composed of SuperScript II RT, Platinum *Taq* DNA

polymerase and 1X buffer with 1.2 mM MgSO₄ were maintained at 50°C for 20 min, then at 94°C for 2 min and PCR was performed as follows: 30 cycles at 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. The primers for RT-PCRs were designed based on human sequences in GenBank. The forward and reverse primers used for the amplification of *IL-8* (1-422; 422 bp; GenBank accession no. NM_000584) fragment were: 5'-ATGACTTCCAAGCTGGCCGTGG-3' and 5'-TTATGAATTCTCAGCCCTCTTC-3'; and those for GAPDH (611-885; 275 bp; GenBank accession no. NM_002046) were: 5'-AGAACATCATCCCTGCCTCTACTGG-3' and 5'-AAAGGTGGAGGAGTGGGTGTCGCTG-3'.

Real-time PCR analysis. One microgram of RNA was reverse-transcribed using a first-strand cDNA synthesis kit (ReverTra Acea; Toyobo). Quantitative real-time PCR was performed using SYBR premix Ex Taq (Takara) on the CFX96™ Real-Time PCR Detection System (Bio-Rad) according to the technical brochure of the company. Quantitative measurements were determined using the $\Delta\Delta$ Ct method and expression of GAPDH was used as the internal control. Melt curve analyses of all real-time PCR products were performed and shown to produce the sole DNA duplex. A standard curve was prepared for each target gene and PCR efficiency was determined to be in excess of 90% for all primer sets.

TP activities. Enzyme activity of TP was assayed by the spectrophotometric method. Cell lysates were incubated in a potassium phosphate buffer (pH 6.5) and 10 mM thymidine at 37°C for 1 h. The thymine formed was quantitated by the absorbance at 300 nm.

Cellular ROS measurement. ROS production was measured using H₂DCF-DA, an uncharged cell-permeable fluorescent probe. Cells were treated with H₂DCF-DA (10 μ M), then washed, re-suspended in PBS and analyzed using a fluorescence microscope (BZ-9000 Bioevo, Keyence) and FACScan (FACSCalibur, BD Biosciences) as previously described (20).

Enzyme linked immunosorbent assays (ELISA) of IL-8. IL-8 concentrations in the culture medium were determined by ELISA (R&D Systems) according to the instructions of the manufacturer.

RNA interference. TP and scramble siRNA duplexes were purchased from Sigma. The siRNA transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

Determination of cellular glutathione levels. Total glutathione levels were measured using the Total Glutathione Quantification kit (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Harvested cells were suspended in 80 μ l of 10% HCl and lysed by freezing and thawing. Twenty microliters of 5% 5-sulfosalicylic acid was added to the lysates and centrifuged at 8000 x g for 10 min at 4°C. The supernatant was used to measure glutathione levels. 5,5'-Dithiobis-(2-nitrobenzoic acid) and GSH react to generate 2-nitro-5-thiobenzoic acid. Concentrations of GSH were determined by measuring absorbance at 412 nm.

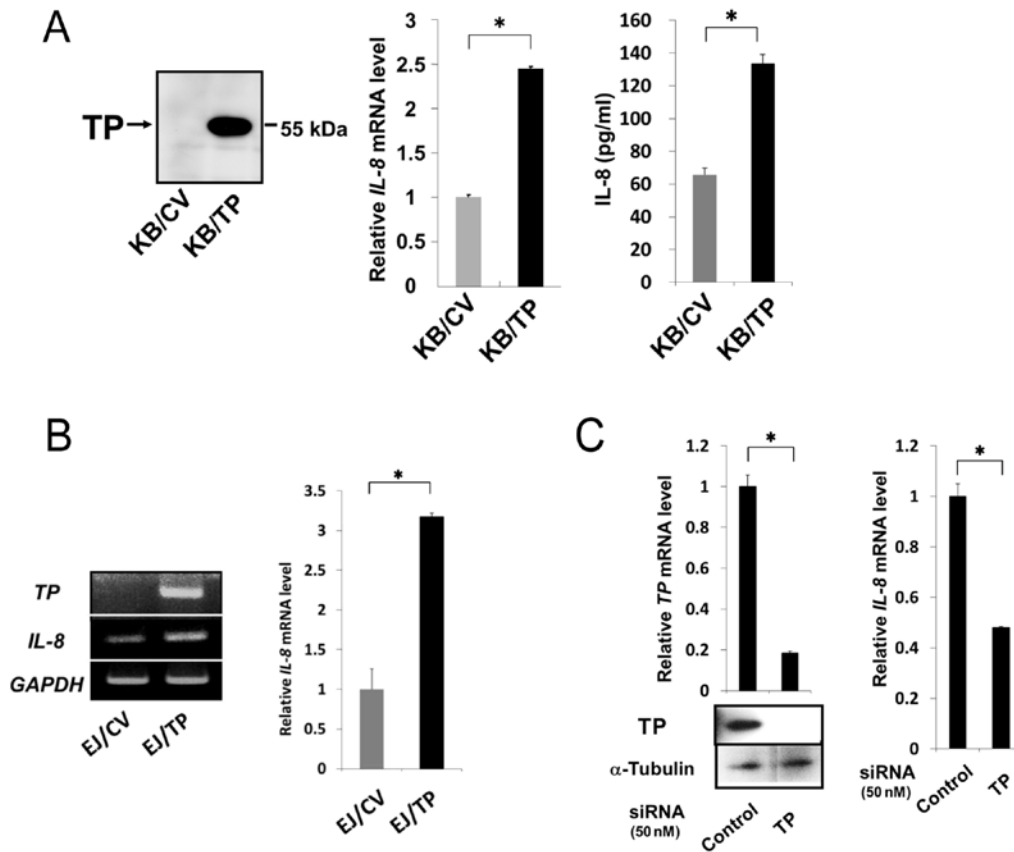


Figure 1. TP induces the expression of IL-8 in cancer cell lines. (A) Immunoblot analysis of TP in KB/CV and KB/TP cells (left panel). TP levels in the cytosol were determined using a monoclonal antibody against TP. *IL-8* mRNA levels in KB/CV and KB/TP cells were measured by real-time PCR and IL-8 protein levels secreted from the cells were detected by ELISA (right panel). (B) *IL-8* mRNA levels in human bladder cancer EJ/CV and EJ/TP cells were measured by RT-PCR (left panel) and real-time PCR (right panel) to determine the effect of TP on the expression of IL-8. (C) Effect of TP siRNA on the expression of IL-8 in human cervical cancer Yumoto cells. Yumoto cells were treated with control siRNA or TP siRNA, then the effect of the siRNAs on expression levels of TP mRNA and TP was determined by real-time PCR and immunoblotting, respectively (left panel). The levels of *IL-8* mRNA were measured using real-time PCR (right panels). Data are represented as mean \pm SD. * $P < 0.01$.

Statistical analysis. Results were statistically analyzed using the GraphPad Prism v5.0 software. Statistical analyses for all experiments including more than two groups were carried out using one-way ANOVA. Student's t-tests were used for experiments including two groups. Data are presented as the means \pm SD. The differences were considered significant at $P < 0.05$.

Results

Role of TP in the induction of IL-8. We have previously reported that expression levels of *IL-8* mRNA and protein in KB/TP cells, which overexpress TP, were higher than those in KB/CV cells that do not express TP (9). In this study, we confirmed that TP is implicated in the expression of *IL-8* in KB/TP cells (Fig. 1A) and examined whether TP is also involved in *IL-8* expression in human bladder carcinoma EJ and human cervical cancer Yumoto cells. TP mRNA was expressed at high levels in EJ/TP cells, but not detected in EJ/CV cells. The expression level of *IL-8* mRNA in EJ/TP cells was 3.3-fold higher than in EJ/CV cells (Fig. 1B), suggesting that the induction of *IL-8* by TP is not restricted in KB cells. TP siRNA efficiently downregulated expression of TP in Yumoto cells, and suppressed expression of *IL-8* mRNA in the cells, indicating

that the intrinsic TP is also implicated in the expression of *IL-8* in Yumoto cells (Fig. 1C).

TP activity is needed for the enhanced expression of IL-8. We then examined whether TP activity is required for the enhanced expression of *IL-8* in KB/TP cells (Fig. 2). KB/CV and KB/TP cells were incubated in the absence or presence of thymidine for 48 h, then levels of *IL-8* mRNA in the cells were determined by real-time PCR (Fig. 2A, left panel) and the amount of *IL-8* protein secreted from the cells was determined by ELISA (Fig. 2A, right panel). Expression of *IL-8* mRNA in KB/TP cells, but not in KB/CV cells, was considerably increased in the presence of thymidine. The secreted *IL-8* protein from KB/TP cells was also significantly increased by thymidine.

KB cells were incubated in the medium without or with 300 μ M TPI for 48 h, then TP activities in the cytosol were determined photometrically. *IL-8* mRNA levels in the cells were determined by real-time PCR. TPI at 300 μ M, which is not cytotoxic to KB/TP cells, decreased TP activity in KB/TP cells to 17% of that in untreated KB/TP cells (Fig. 2B, left panel). TPI at the same concentration significantly decreased the expression of *IL-8* mRNA in KB/TP cells, but not in KB/CV cells (Fig. 2B, right panel). Human cancer cell lines, MCF-7

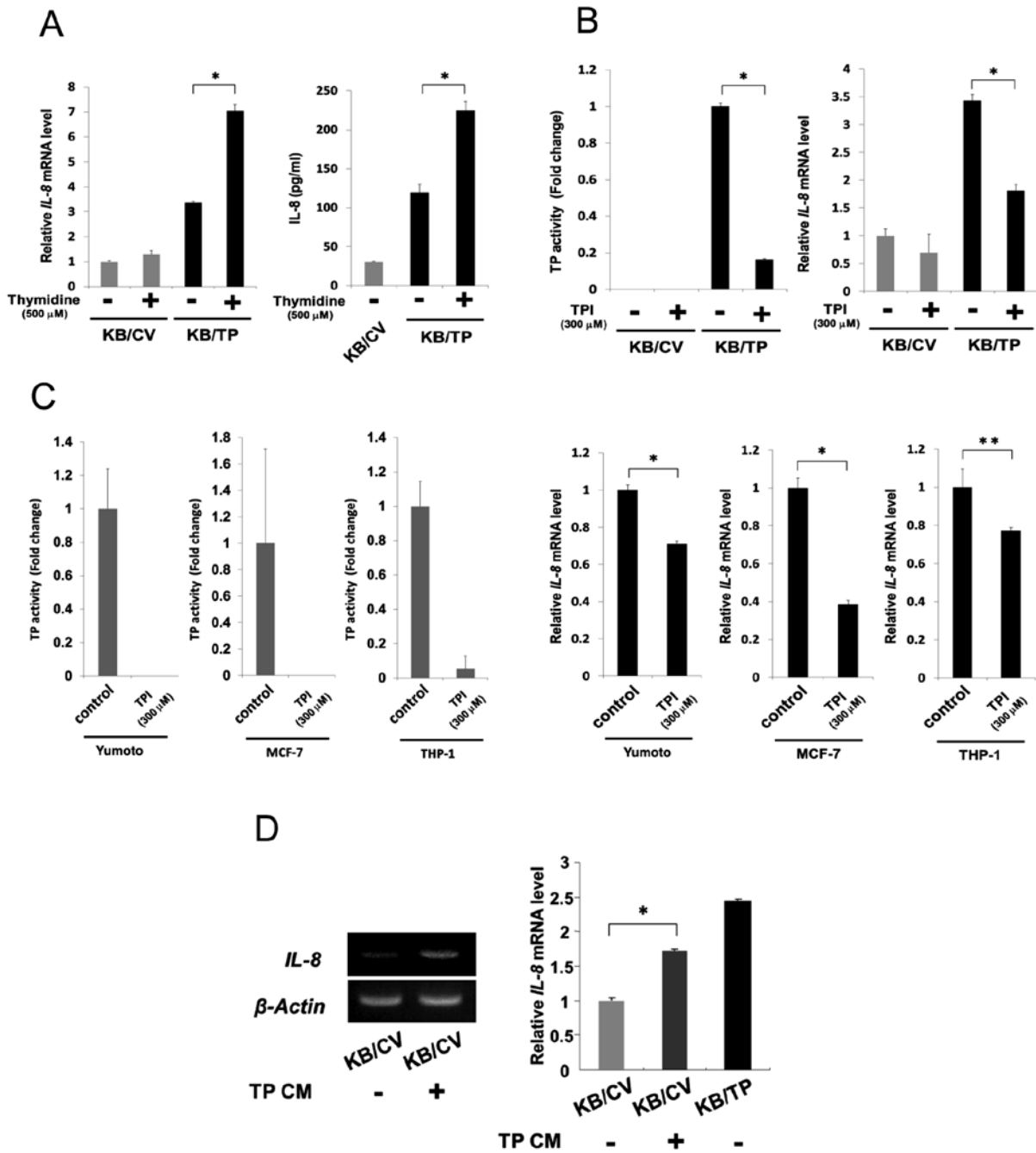


Figure 2. TP activity is required for IL-8 induction. (A) Effect of thymidine on expression of IL-8 in KB cells. KB/CV and KB/TP cells were incubated in the absence or presence of thymidine, then levels of *IL-8* mRNA in the cells were determined by real-time PCR (left panel) and the amount of IL-8 protein secreted from the cells was determined by ELISA (right panel). (B) Effect of TPI on *IL-8* expression in KB cells. KB cells were incubated in the absence or presence of 300 μ M TPI for 48 h, then TP activities in the cytosol were determined photometrically (left panel). *IL-8* mRNA levels in the cells were determined by real-time PCR (right panel). (C) Effect of TPI on the expression of *IL-8* mRNA in Yumoto, MCF-7 and THP-1 cells. TPI at 300 μ M, which was not cytotoxic to these 3 cell lines, completely inhibited TP activity in Yumoto and MCF-7 cells and decreased to 5% of the control level in THP-1 cells. (D) KB/TP cells were cultured in serum-free medium for 24 h and the medium was used as conditioned medium (TPCM). KB/CV cells were incubated in TPCM for 24 h, then expression levels of *IL-8* mRNA in KB/CV cells and TPCM-incubated KB/CV cells were determined using RT-PCR (left panel) and real-time PCR (right panel). Data are the mean \pm SD. * P <0.01, ** P <0.05.

and THP-1 as well as Yumoto, express intrinsic TP. TPI at 300 μ M, which was not cytotoxic to those cells, completely inhibited TP activity in Yumoto and MCF-7 cells and decreased it to 5% of the control levels in THP-1 cells (Fig. 2C left panel). *IL-8* mRNA expression in those cells was considerably decreased by the same concentration of TPI (Fig. 2C right panel). These results suggest that TP activity is required for the

enhanced expression of *IL-8* mRNA and thymidine-derived metabolites are involved in the enhanced expression of IL-8 in those cells.

Bijnsdorp *et al.* reported that DR, one of the thymidine-derived sugars, is extensively secreted from TP-overexpressing cells (13). When KB/CV cells were incubated in conditioned medium of KB/TP cells (TPCM), expression level

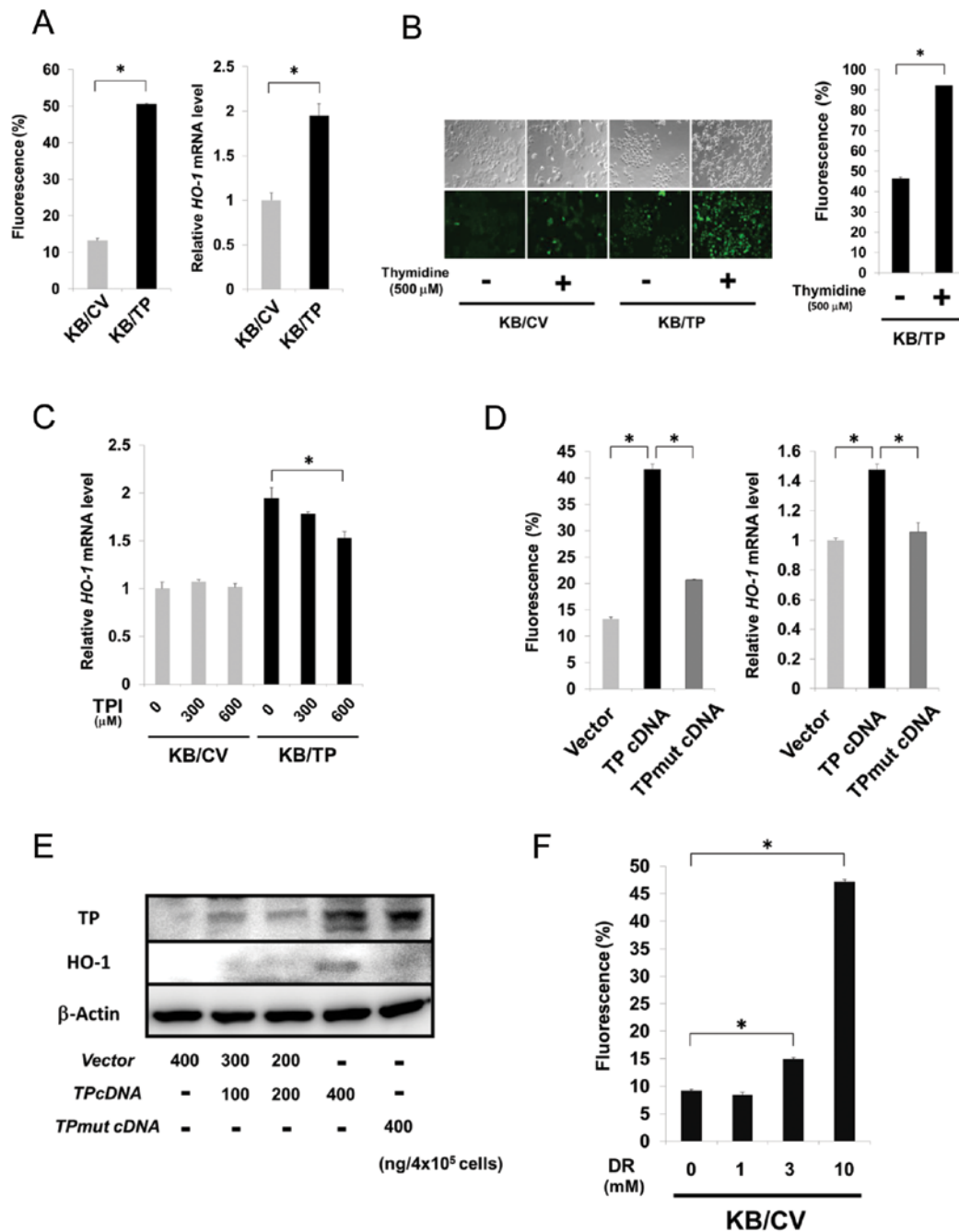


Figure 3. ROS generation by TP. (A) ROS levels in KB/CV and KB/TP cells were determined using H₂DCF-DA (left panel). HO-1 mRNA levels in the cells were measured by real-time PCR (right panel). (B) Effect of thymidine on the generation of ROS in KB/CV and KB/TP cells. The cells were incubated in the medium without or with thymidine for 24 h and ROS in the cells were measured using H₂DCF-DA. Cells expressing ROS were detected using fluorescence microscopy (left panel). ROS in the cells were quantified using FACSscan (right panel). (C) HO-1 mRNA levels in KB/CV and KB/TP cells in the absence or presence of TPI were determined by real-time PCR. (D) ROS levels in KB cells transiently transfected with empty vector, TP cDNA or TPmut cDNA (400 ng/4x10⁵ cells) were detected using H₂DCF-DA (left panel). HO-1 mRNA levels in the cells were determined by real-time PCR (right panel). (E) HO-1 expression augmented by TP in KB cells. To examine the effect of transiently expressed TP, KB cells were transfected with different amounts of TP cDNA or TPmut cDNA (400 ng/4x10⁵ cells) and the effect of TP on HO-1 expression was investigated using immunoblot analysis. (F) ROS levels in KB cells untreated or treated with DR for 24 h were detected using H₂DCF-DA (left panel). Data are the mean \pm SD. *P<0.01.

of IL-8 mRNA in KB/CV cells was significantly increased (Fig. 2D). These results suggested that DR were secreted from KB/TP cells in the medium and enhanced IL-8 mRNA expression in KB/CV cells.

ROS generation by TP. TP-overexpressing cells treated with thymidine induced expression of hemo oxygenase-1 (HO-1),

a classical marker of cellular oxidative stress (15). We firstly investigated ROS and HO-1 mRNA levels in KB/CV and KB/TP cells using the fluorescent probe H₂DCF-DA and real-time PCR, respectively. Expression levels of ROS and HO-1 mRNA in KB/TP cells were about 5- and 2-fold higher than those in KB/CV cells, respectively (Fig. 3A). ROS level in KB/TP cells was higher than that in KB/CV cells and consid-

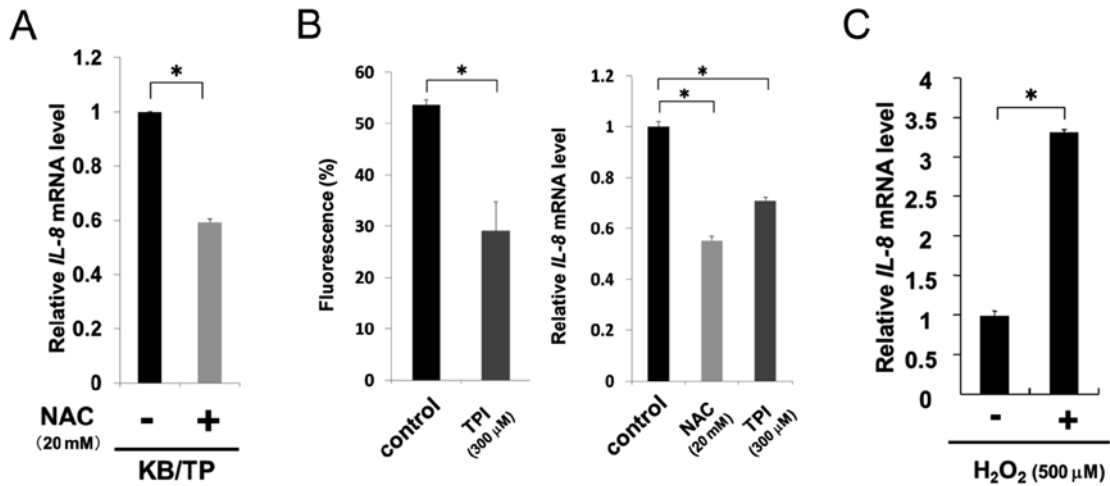


Figure 4. TP-induced ROS results in increased expression of *IL-8*. (A) Effect of N-acetylcysteine (NAC) on *IL-8* mRNA expression in KB/TP cells. Expression levels of *IL-8* mRNA were measured using real-time PCR. (B) Effect of TPI and NAC on *IL-8* mRNA expression and ROS generation in Yumoto cells. Cells were treated with TPI or NAC for 48 h, then the levels of ROS and *IL-8* mRNA in the cells were measured using H₂DCF-DA (left panel) and real-time PCR (right panel), respectively. (C) Effect of H₂O₂ on *IL-8* mRNA expression in Yumoto cells. Yumoto cells were incubated in the medium without or with 500 μM H₂O₂ for 24 h, then *IL-8* mRNA levels were determined by real-time PCR. Data are the mean ± SD. *P<0.01.

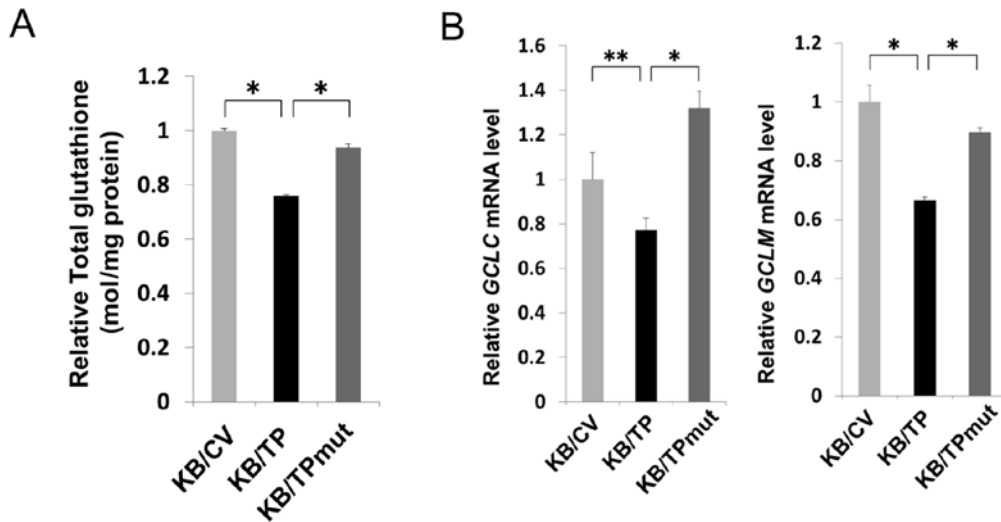


Figure 5. TP decreases the levels of cellular glutathione. (A) The glutathione levels in KB/CV, KB/TP and KB/TPmut cells were determined using the Total Glutathione Quantification kit. (B) mRNA levels of the heavy catalytic (GCLC) and the light regulatory (GCLM) subunits of γ -glutamylcysteine synthetase in KB/CV and KB/TP cells measured by real-time PCR. Data are the mean ± SD. *P<0.01, **P<0.05.

rably increased when thymidine was added in the medium (Fig. 3B). Expression level of *HO-1* mRNA in KB/TP cells, but not in KB/CV cells, was decreased by TPI (Fig. 3C). We then examined ROS levels in KB cells transiently transfected with empty vector, *TP* cDNA or *TPmut* cDNA (400 ng/4x10⁵ cells) using H₂DCF-DA (Fig. 3D, left panel). *HO-1* mRNA and protein levels in the cells were also determined by real-time PCR (Fig. 3D, right panel) and immunoblot analysis (Fig. 3E), respectively. ROS and HO-1 levels in KB cells transiently transfected with *TP* cDNA were higher than those in KB cells transfected with empty vector or *TPmut* cDNA that codes mutant TP lacking the enzymatic activity. ROS level in KB/CV cells was dose-dependently increased by DR (Fig. 3F). These results indicated that transiently expressed TP enhanced

ROS generation and the enzymatic activity of TP is needed for the enhanced generation of ROS.

TP-induced ROS results in increased expression of IL-8. To examine whether TP-induced ROS augmented *IL-8* expression, we assessed the levels of *IL-8* in KB/TP cells treated with an antioxidant, NAC. *IL-8* mRNA expression in KB/TP cells was considerably decreased by NAC, suggesting that ROS is involved in the enhanced expression of *IL-8* mRNA in these cells (Fig. 4A). TPI significantly suppressed the levels of ROS and *IL-8* mRNA in Yumoto cells (Fig. 4B). NAC also decreased *IL-8* mRNA expression in Yumoto cells (Fig. 4B, right panel). H₂O₂ at 500 μM increased *IL-8* mRNA levels up to 3.3-fold in Yumoto cells (Fig. 4C). These results suggest that

TP is involved in the generation of ROS in Yumoto cells which express intrinsic TP and ROS cause the enhanced *IL-8* mRNA expression in those cells.

TP decreases the levels of cellular glutathione. DR decreased cellular glutathione level in various types of cells (16,17). We examined the glutathione levels in KB/CV, KB/TP and KB/TPmut cells. Glutathione levels in KB/TP cells were about 20-30% lower than those in KB/CV and KB/TPmut cells (Fig. 5A). γ -glutamylcysteine synthetase (γ -GCS) is the first rate-limiting enzyme of glutathione synthesis. The enzyme consists of two subunits, a heavy catalytic subunit (GCLC) and a light regulatory subunit (GCLM). Both *GCLC* and *GCLM* mRNAs in KB/TP cells were significantly decreased compared with those in KB/CV and KB/TPmut cells (Fig. 5B). These results suggested that TP activity is involved in the decreased expression of γ -GCS and the lowered level of cellular glutathione in KB/TP cells.

Discussion

TP is expressed in various malignant tumors and plays a pivotal role in angiogenesis, tumor growth, invasion and metastasis of TP-expressing tumors (2). DR, one of the thymidine-derived sugars, has similar functions to TP. We have previously suggested that DR is a downstream mediator of TP function (2,6).

Brown *et al* observed that thymidine upregulated HO-1 in a dose-dependent manner in human bladder carcinoma RT112-TP cells with high TP expression (15). Since cellular oxidative stress is responsible for HO-1 induction, they suggested that TP induced cellular oxidative stress in the cells. In this study, we directly measured ROS levels in KB cells and indicated that TP enhanced ROS generation. TP activity was required for the enhanced generation of ROS and DR also enhanced ROS generation. High concentrations of DR cause ROS generation and lowered intracellular glutathione levels in various cells (21,22).

The level of cellular glutathione in KB/TP cells was significantly lower than that in KB/CV and KB/TPmut cells (Fig. 5A). Transcription of γ -GCS in KB/TP cells was also attenuated compared with those in KB/CV and KB/TPmut cells (Fig. 5B). Glutathione is considered as a main intracellular defense against oxidative stress. Decreased glutathione levels in KB/TP cells may be in part implicated in the augmented ROS in the cells.

Brown *et al* suggested that thymidine catabolism by TP increased carcinoma cell secretion of angiogenic factors induced by oxidative stress (15). We observed that TP augmented the expression of *IL-8* mRNA in human cancer KB, EJ and Yumoto cells. TP activity was again needed to enhance *IL-8* mRNA expression and DR increased *IL-8* mRNA expression in KB/CV cells that do not express TP. Furthermore, NAC suppressed the increased expression of *IL-8* mRNA as well as the augmented generation of ROS. These findings indicate that ROS induced by TP enhanced the *IL-8* mRNA expression. ROS was previously suggested to stimulate cell growth by direct activation of certain redox sensitive transcription factors such as NF- κ B (23). The *IL-8* promoter region contains binding sites for the transcription

factors, AP-1 (-126 to -120 bp), NF- κ B (-80 to -71 bp) and NF-IL-6 (-94 to -81 bp) (24). ROS may activate NF- κ B, which is supposed to bind the *IL-8* promoter and to enhance *IL-8* gene transcription.

In conclusion, our study demonstrated that the enzymatic activity of TP is required for the enhanced ROS generation and *IL-8* expression by TP. DR, one of the thymidine-derived sugars, enhanced the generation of ROS in TP-negative KB/CV cells. NAC suppressed the enhanced *IL-8* mRNA expression in KB/TP cells. The level of cellular glutathione was decreased in TP-overexpressing cells. Decreased glutathione levels in the TP-overexpressing cells may be in part implicated in the augmented ROS generation in the cells, since glutathione is considered as a main intracellular defense against oxidative stress. These findings suggest that ROS generated by thymidine-derived sugars enhance the expression of *IL-8*. TPI inhibited the enzymatic activity of TP and attenuated the TP-induced ROS generation and *IL-8* mRNA expression in human cancer cells. The results support the notion that compounds that inhibit TP activity, such as TPI, are good candidates for new progressive anticancer agents.

Further study of the molecular mechanisms for the generation of ROS by thymidine-derived sugars and for the enhanced *IL-8* expression by ROS will contribute to our understanding of the roles of TP in the malignant progression of tumors.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Research Support Foundation of The University of Tokushima and TAIHO Pharmaceutical Co., Ltd.

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