The Effect of a Thymidine Phosphorylase Inhibitor on Angiogenesis and Apoptosis in Tumors

Shigeto Matsushita, Takao Nita, Tatsuhiko Furukawa, Tomoyuki Sumizawa, Ayako Tani, Kengo Nishimoto, Suminori Akiba, Kazutaka Miyadera, Masakazu Fukushima, Yuji Yamada, Hiroki Yoshida, Tamotsu Kanzaki, and Shin-ichi Akiyama


ABSTRACT

Thymidine phosphorylase (TP) is an enzyme involved in the reversible conversion of thymidine to thymine and is identical to an angiogenic factor, platelet-derived endothelial cell growth factor. TP is expressed at higher levels in a wide variety of solid tumors than in the adjacent nonneoplastic tissues. Patients with TP-positive colon and esophageal tumors have a poorer prognosis than those with TP-negative tumors. We have recently synthesized a new TP inhibitor (TPI), 5-chloro-6-[1-(2-iminopyrrolidinyl)] methyl] uracil hydrochloride. We investigated the effect of TPI on angiogenesis in KB cells transfected with platelet-derived endothelial cell growth factor cDNA, KB/TP, and a mock transfectant, KB/CV, using the mouse dorsal air sac assay model. We found that KB/TP cells had a higher angiogenic ability than KB/CV cells and that TPI completely suppressed angiogenesis by KB/TP. Furthermore, at a dose of 50 mg/kg/day, TPI considerably decreased the growth rate of KB/TP cells xenografted into nude mice. Microvessel density in KB/TP tumors was higher than that in KB/CV tumors, and TPI did not significantly change the density in either of the tumors. The apoptotic index in KB/TP tumors was significantly lower than that in KB/CV tumors, and TPI significantly increased the apoptotic index in KB/TP tumors but not in KB/CV tumors. These findings, taken together with previous reports, suggest that the expression of TP plays an important role in tumor growth and that TPI suppresses tumor growth by increasing the proportion of apoptotic cells and probably inhibiting angiogenesis.

INTRODUCTION

TP (EC 2.4.2.4) catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogs to their respective bases and 2-deoxyribose-1-phosphate (1-3). TP also catalyzes the transfer of deoxyribose from one deoxynucleoside to another base to form a second deoxynucleoside (4-6). In mammals, TP consists of two identical subunits with a Mr of 55,000 (7). We have previously shown that TP is identical to PD-ECGF (8, 9). TP stimulates chemotaxis and [3H]thymidine incorporation by endothelial cells in vitro and has angiogenic activity in vivo (10-13). Recently, we demonstrated that the enzymatic activity of TP is indispensable for its angiogenic activity (10, 13). Among the degradation products of thymidine by TP, 2-deoxy-d-ribose, a dephosphorylated product derived from 2-deoxy-d-ribose-1-phosphate, has chemotactic activity in vitro and angiogenic activity in vivo. These findings suggest that the enzymatic products of TP may stimulate the chemotaxis of endothelial cells and possibly other cells, causing angiogenesis (13).

Overexpression of TP in MCF-7 cells transfected with TP cDNA has no effect on their growth in vitro but confers a growth advantage on them when they are xenografted into nude mice (14). Although TP has angiogenic activity, it has effects on the prognosis of cancers independent of angiogenesis assessed by microvessel density in human colorectal carcinoma and renal cell carcinoma (15, 16). These observations suggest that TP has functions other than angiogenesis that affect tumor growth. It is very important to develop strong and selective TPIs because they may inhibit angiogenesis and suppress the progression of solid tumors. Recently, we generated a novel TPI, 5-chloro-6-[1-(2-iminopyrrolidinyl)] methyl] uracil hydrochloride (K= 2 × 10^{-8} M) that had about a 1000-fold higher inhibitor activity than 6-amino-5-chlorouracil, one of the most potent TPIs (17). In this study, we examined whether inhibition of TP activity by TPI in human epidermoid carcinoma KB cells affects tumor angiogenesis and growth.

MATERIALS AND METHODS

Growth Factors and Chemicals. [14C]Thymidine (56 mCi/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA), rat antimouse CD31 was obtained from Pharmingen (San Diego, CA), and TdT and biotin-16-dUTP (biontin-dUTP) were obtained from Boehringer Mannheim (Mannheim, Germany). TPI, a competitive inhibitor of thymidine phosphorylase (Fig. 1), was synthesized by Taiho Pharmaceutical Co. Ltd.

Animals and Cell Lines. Male 6-8-week old BALB/c mice and nude mice were used. KB/TP (human KB epidermoid carcinoma cells transfected with PD-ECGF cDNA) and KB/CV (a mock transfectant) were maintained in MEM + 10% FCS. These cells were Mycoplasma free.

Transfection of PD-ECGF cDNA into KB Cells. Full-length PD-ECGF cDNA was kindly provided by Dr. K. Miyazono and Dr. C-H. Hedin (Ludwig Institute for Cancer Research, Uppsala, Sweden). A KpnI-EcoRI fragment from pPL8 that encompassed the PD-ECGF coding region was cloned between the KpnI and EcoRI restriction sites of pT7T318U (Pharmacia, Uppsala, Sweden). A XbaI-EcoRI fragment from pT7T318U was then cloned between the Nhel and EcoRI restriction sites of expression vector pBK-RSV (Stratagene, La Jolla, CA). The expression vector encoding PD-ECGF cDNA (RSV/TP) or the vector alone (RSV) was transfected into KB cells by electroporation (18). After selection with Geneticin, the expression of PD-ECGF cDNA in each clone was determined by immunoblotting with anti-PD-ECGF/TP monoclonal antibody. One TP-positive clone transfected with RSV/TP (KB/TP cells) and one clone transfected with RSV (KB/CV cells) were further analyzed.

Preparation of Cell Lysate. Cells were lysed in 10 ml Tris-HCl (pH 7.5) by rapid freeze-thawing in liquid nitrogen. The lysates were centrifuged at 15,000 ×g for 20 min at 4°C, and the supernatants were resolved by electrophoresis (19). Protein concentrations were determined by the method of Bradford (20).

Immunoblotting. Each sample was resolved by 11% SDS-PAGE according to the method of Laemmli (21). Proteins in the gel were electrochemically transferred to a sheet of polyvinylidene difluoride membrane (Immobilon-P transfer membrane; Millipore, Bedford, MA) with Bio-Rad Transblot SD, as described previously (22). The membrane was then incubated with monoclonal

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8 The abbreviations used are: TP, thymidine phosphorylase; PD-ECGF, platelet-derived endothelial cell growth factor; TPI, TP inhibitor; TdT, terminal deoxynucleotidyl transferase; DAB, diaminobenzidine; TUNEL, TdT-mediated nick end labeling; DDW, double distilled water.
TP SUPPRESSES TP FUNCTION

Cell/tumor

<table>
<thead>
<tr>
<th>Cell/tumor</th>
<th>KB/CV</th>
<th>KB/TP</th>
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<tbody>
<tr>
<td>Protein</td>
<td>100 µg</td>
<td>10 µg</td>
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Fig. 2 Immunoblot analysis of TP in KB/CV and KB/TP cells. KB/CV and KB/TP cytolsos (100 and 10 µg of protein, respectively) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. TP levels in the cytolsos were determined using a monoclonal antibody against TP.

Effects of TPI on in Vivo Tumor Angiogenesis. To examine the effect of TPI on tumor-associated angiogenesis, we used the mouse dorsal air sac assay model (25). KB/TP and KB/CV cells were washed twice with PBS and suspended in PBS at 6.7 × 10⁵ cells/ml. A Millipore chamber (diameter, 10 mm; thickness, 2 mm; filter pore size, 0.22 µm; Millipore Co.) was filled with 150 µl (1 × 10⁶ cells) of either cell suspension or PBS and implanted s.c. into an air sac formed previously in the dorsum of 7–8 week-old male BALB/c nude mice. When the tumor size reached a volume of approximately 50–150 mm³, three different concentrations of TPI (50, 100, or 200 mg/kg/day) or vehicle alone was administered i.p. every 12 h for 22 days. Tumors were measured in two dimensions by calipers every other day, and the volume was calculated as width² × length × 0.5. The effect of TPI on tumor volume was determined using multiple regression analysis adjusting for the tumor type and the observation period.

TP Staining. Tumors embedded in paraffin were cut into 3-µm-thick sections. The sections were deparaffinized with xylene and dehydrated with 98% ethanol. Endogenous peroxidase was blocked by covering the sections with 0.3% (v/v) H₂O₂ in absolute methanol for 20 min at room temperature, and the sections were then incubated with polyclonal antibody against TP (19). Antibody binding was detected by sequential incubation with biotinylated antirabbit IgG and streptavidin-peroxidase complex. Immune complexes were visualized by incubating the sections with 0.5 mg/ml DAB and 0.03% (v/v) H₂O₂ in PBS for 3 min.

Microvessel Staining. Tumors were embedded in OCT compound (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and stored at −80°C. Cryostat sections were fixed in acetone for 10 min at 4°C and immunostained with antimouse CD31 (Pharmingen). Antibody binding was detected by sequential incubation with biotinylated antirat IgG (Vector Laboratories, Inc.) and streptavidin-peroxidase complex. Immune complexes were visualized by incubating the sections with 0.5 mg/ml DAB and 0.03% (v/v) H₂O₂ in PBS.

Microscopic analysis was done at a 400-fold magnification to obtain accurate microvessel counts that are expressed as vessels/mm².

TUNEL Staining and Evaluation of Apoptosis. TUNEL was performed according to a modification of the method described by Gavrieli et al. (27). Tumors were fixed in 10% formaldehyde in PBS, embedded in paraffin, and cut into 3-µm-thick sections. The sections were deparaffinized with xylene and dehydrated with 98% ethanol. Nuclei in the tissue sections were stripped of endogenous peroxidase by incubating with 20 µg/ml proteinase K (Life Technologies, Inc., Rockville, MD) for 20 min at room temperature, and the slides were then washed in DDW for 5 min. Endogenous peroxidase was blocked by covering the sections with 0.3% (v/v) H₂O₂ in absolute methanol for 20 min at room temperature. The sections were rinsed with DDW and immersed in TdT buffer [30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM cobalt chloride, and 0.025% (w/v) BSA]. TdT (25 units/ml) and biotin-dUTP (0.5 µM) in TdT buffer were added to cover the sections that were then incubated at 37°C for 60 min. The reaction was terminated by transferring the slides to 2× SSC. After washing with DDW, the sections were incubated with avidin and biotinylated horseradish peroxidase complex in PBS for 30 min. After washing in PBS, the immune complexes were visualized by incubating the sections with 0.5 mg/ml DAB and 0.03% (v/v) H₂O₂ in PBS for 3 min. The sections were counterstained with hematoxylin and mounted.

The apoptotic index was estimated by determining the percentage of apoptotic cells seen under a light microscope at 400-fold magnification. A minimum of 3000 cells were counted in the sections of the tumors. Positively stained tumor cells with the morphological characteristics of apoptosis were identified using standard criteria (28, 29).

Statistical Analysis. Multiple regression analysis and Student’s t test (30) were done using the statistical analysis program StatView J-4.5 (Abacus Concepts, Inc., Berkeley, CA).

Table 1 TP activity in KB cells and tumors

<table>
<thead>
<tr>
<th>Cell/tumor</th>
<th>0</th>
<th>10 µM</th>
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<tbody>
<tr>
<td>KB/CV cells</td>
<td>0.44 ± 0.04a</td>
<td>0.22 ± 0.01ab</td>
</tr>
<tr>
<td>KB/CV tumors</td>
<td>0.90 ± 0.08c</td>
<td>0.56 ± 0.06b</td>
</tr>
<tr>
<td>KB/TP cells</td>
<td>0.50 ± 1.70c</td>
<td>0.79 ± 0.07b</td>
</tr>
<tr>
<td>KB/TP tumors</td>
<td>13.65 ± 0.83d</td>
<td>0.68 ± 0.04ab</td>
</tr>
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TP activity (nmol thymine/mg protein/h) was measured with a radiometric assay as described in “Materials and Methods.” Each value represents the mean ± SE of three independent experiments. KB/TP is a subline of KB transfected with PD-ECGF cDNA. Statistical significance was determined by Student’s t test. a versus b, P < 0.005; c versus a, P < 0.0001; d versus f, P < 0.0001; e versus g, P < 0.0001; a versus e, P = 0.0057; b versus f, P = 0.0045; c versus d, P = 0.0162; e versus g, P < 0.0001; f versus h, P < 0.0001.
RESULTS

Expression Level of TP. We examined the levels of TP expression in KB cells transfected with TP cDNA (KB/TP) or RSV plasmid vector (KB/CV) by immunoblot analysis using an anti-TP monoclonal antibody. KB/TP cells expressed high levels of TP, and KB/CV cells expressed TP only slightly, as shown in Fig. 2. TP activity in KB/TP cells was 114-fold higher than that in KB/CV cells \( (P < 0.0001) \), and the TP activity in both cell lines was inhibited by 10 \( \mu \text{M} \) TPI. TP activity in KB/TP cells treated with TPI was 1.6% of that in untreated KB/TP cells (Table 1). TPI could not inhibit both human and mouse uridine phosphorylase activities (data not shown).

Effect of TPI on in Vivo Angiogenesis. The inhibitory effect of TPI on angiogenesis 4 days after the implantation of a chamber containing KB/TP or KB/CV cells was analyzed using the mouse dorsal air sac assay (Figs. 3 and 4). The angiogenesis index (\( \pm \text{SE} \)) in KB/TP cells \( (4.8 \pm 0.2) \) was significantly higher than that in KB/CV cells \( (2.2 \pm 0.86; P = 0.0186) \). Furthermore, the angiogenesis index in TPI-treated KB/TP cells \( (2.0 \pm 0.95) \) was significantly lower than that in untreated KB/TP cells \( (P = 0.0203) \). The control chamber containing PBS produced no angiogenic response (data not shown). Angiogenesis was not suppressed by 6-amino-5-chlorouracil at 30 mg/kg/day (almost the molar equivalent of 50 mg TPI/kg/day) in KB/TP or KB/CV cells (data not shown).

Expression of TP in Experimental Tumors. KB/TP and KB/CV cells were xenografted into nude mice to examine the effect of TPI on tumor growth. The expression of TP in KB/TP tumors was determined by immunohistochemical staining using a polyclonal antibody against TP (Fig. 6A). The cytoplasm of almost all KB/TP tumor cells in the nonnecrotic area was strongly stained, but necrotic tumor cells were not. TP activity in experimental tumors was measured using a radioisometric assay (Table 1), and it was significantly higher in KB/TP tumors than in KB/CV tumors \( (P < 0.0001) \). TP activity in KB/TP tumors was significantly lower than that in KB/TP cells \( (P < 0.0001) \). In contrast, TP activity in KB/CV tumors was significantly higher than that in KB/CV cells \( (P = 0.0057) \).

Effects of TPI on Tumor Growth. We examined the growth of cells xenografted into nude mice and the effect of TPI on tumor growth (Fig. 5). KB/TP tumors grew significantly faster than KB/CV tumors. The mean volume of TPI-treated tumors was significantly less than in KB/TP tumors \( (P < 0.0001) \). TP activity in KB/TP tumors was significantly lower than that in KB/TP cells \( (P < 0.0001) \).

Fig. 3. The effect of TPI on TP-induced angiogenesis. Mice implanted with a chamber containing KB/TP (A and B) and KB/CV (C and D) cells were treated with vehicle (A and C) or 50 mg/kg/day TPI (B and D). TPI strongly inhibited the KB/TP-induced formation of new blood vessels (B). Magnification, \( \times 20 \).
than that of untreated tumors ($P < 0.0001$). The growth of KB/TP tumors in mice treated with 50 mg/kg TPI was inhibited by 30.8% compared to that in control mice treated with vehicle alone. Meanwhile, the growth of KB/CV tumors in mice treated with 50 mg/kg TPI was suppressed by 18.7% compared to that in control mice treated with vehicle alone. The growth of KB/TP tumors was more effectively inhibited by TPI than that of KB/CV tumors ($P < 0.0199$). Body weight loss was less than 10% in all of the mice (data not shown).

**Number of Microvessels in Experimental Tumors.** Sections of experimental tumors were stained with antimouse CD31 antibody (Fig. 6B), and the number of vessels was counted. The average density of vessels/mm$^2$ is shown in Fig. 7. The average number of vessels ($\pm$ SE) in KB/TP tumors (51.6 $\pm$ 4.1) was significantly higher than that in KB/CV tumors (29.8 $\pm$ 1.8; $P = 0.0006$). KB/TP tumors treated with TPI contained fewer vessels (45.0 $\pm$ 1.9) than untreated KB/TP tumors, but the difference was not significant. Furthermore, the number of vessels was compared when the tumor weight ($\pm$ SE; $n = 6$) of KB/TP (226.3 $\pm$ 21.0 mg) and KB/CV (226.2 $\pm$ 15.7 mg) were similar. The average number of microvessels ($\pm$ SE; $n = 6$) in KB/TP tumors (87.2 $\pm$ 4.9) was significantly higher than that in KB/CV tumors (41.6 $\pm$ 4.9; $P < 0.0001$).

**Apoptotic Index in Experimental Tumors.** Apoptosis was quantified in situ at the cellular level by labeling fragmented DNA using the TUNEL technique (Fig. 6C). The proportion of apoptotic cells in the experimental tumors is shown in Fig. 8. The apoptotic index ($\pm$ SE) in KB/CV tumors (1.54 $\pm$ 0.12%) was significantly higher than that in KB/TP tumors (1.07 $\pm$ 0.08%; $P = 0.0052$). The apoptotic index in KB/TP tumors treated with TPI (1.36 $\pm$ 0.05%) was significantly higher than that in untreated KB/TP tumors ($P = 0.01$).

**DISCUSSION**

Angiogenesis is not only involved in tumor growth and distant metastasis but is also an important early step in carcinogenesis. It is a complex multistep process regulated by a number of angiogenic factors (31, 32). PD-ECGF was initially cloned as a novel angiogenic factor distinct from other endothelial cell growth factors (11). Previous studies demonstrated that PD-ECGF was identical to TP (8, 9) and that it stimulated chemotaxis and $[^3H]$thymidine incorporation by endothelial cells in vitro and had angiogenic activity in vivo (10–13). The expression of PD-ECGF in transformed fibroblasts in nude mice resulted in increased tumor vascularity (11). Overexpression of TP in MCF-7 cells transfected with TP cDNA had no effect on growth in vitro but conferred a growth advantage when these cells were xenografted into nude mice (14). Recent studies showed that TP is expressed in a wide variety of solid tumors (15, 16, 23, 33–36), but the role of TP in tumor proliferation was unknown.

Our study showed that in vivo, KB cells with high TP activity grew...
faster than those with low TP activity. The density of blood vessels in KB tumors with high TP activity was higher than that in KB tumors with low TP activity, suggesting that TP was involved in angiogenesis in KB tumors. Recently, we have newly synthesized a novel selective inhibitor of TP, TPI, which has a 1000-fold higher inhibitory activity than 6-amino-5-chlorouracil and does not inhibit another enzyme involved in pyrimidine nucleoside metabolism, uridine phosphorylase. We demonstrated that TPI partially suppressed the growth of TP-expressing tumors but did not significantly suppress angiogenesis in the tumors. However, in the mouse dorsal air sac assay model, TPI completely suppressed angiogenesis induced by KB/TP. Administration of TPI was started after the tumor reached a volume of 50–150 mm³ in the nude mice model, whereas it was started immediately after the implantation of the chamber in the mouse dorsal air sac assay model. Considerable experimental evidence indicates that vascular endothelial cell growth factor (37) is not necessary for growth after tumors reach a certain size, and the suppression of vascular endothelial cell growth factor has no effect on the expansion of the large tumors because of the up-regulation of other angiogenic factors such as basic fibroblast growth factor and transforming growth factor α in large tumors (38). Taken together, these findings suggest that TP might be important in the early stage of tumor angiogenesis through the remodeling of the existing vasculature (36), and another angiogenic factors might be involved in angiogenesis after tumors reach a certain size.

In the nude mice model, TPI did not inhibit angiogenesis but still had an inhibitory effect on tumor growth, suggesting that TP has some role(s) in tumor growth other than angiogenesis, and we recently proposed another role for TP in the progression of solid tumors besides angiogenesis. TP conferred resistance to apoptosis induced by hypoxia, and the degradation products of thymidine were involved in the resistance (39). The proportion of apoptotic cells assessed by the TUNEL assay in KB/TP tumors was significantly lower than in KB/CV tumors, and TPI abolished the difference. This suggests that TP conferred resistance to apoptosis. The finding that TP can confer resistance to apoptosis, in addition to its angiogenic activity, may explain why breast carcinoma cells that overexpressed TP had increased growth in vivo without increased vessel density (14). In accordance with this, patients with TP-positive tumors have a poorer prognosis than those with TP-negative tumors, whereas microvessel density is not a significant prognostic factor in colorectal and renal cell carcinomas (15, 16).

Fig. 7. Analysis of the number of microvessels in the tumors. TPI-untreated KB/TP tumors contained significantly more vessels than TPI-untreated KB/CV tumors. The number of microvessels in the TPI-untreated KB/TP tumors was not significantly different from that in TPI-treated KB/TP tumors. Statistical significance was determined by Student’s t test (mean ± SE; n = 6). *, P = 0.0006.

Fig. 8. Apoptotic indices in the tumors. The proportion of apoptotic cells in TPI-untreated KB/TP tumors was significantly higher than that in KB/TP tumors. The proportion of apoptotic cells in TPI-treated KB/TP tumors was significantly higher than that in TPI-untreated KB/TP tumors. Statistical significance was determined by Student’s t test (mean ± SE; n = 6). *, P = 0.0052; **, P = 0.01.

Our study showed that TP activity in KB/TP tumors was significantly lower than in KB/TP cells in vitro (P < 0.0001). The considerable extent of necrosis in KB/TP tumors may be related to the low TP activity in the tumors. In contrast, TP activity in KB/CV tumors was significantly higher than in KB/CV cells in vitro (P = 0.0057), although the necrotic lesions were also found in KB/CV tumors. It has been shown that tumor necrosis factor α, interleukin 1, and IFN-γ up-regulate TP expression in tumor cells (40, 41), and these cytokines may have induced TP expression in KB/CV cells in nude mice. Furthermore, the center of tumors with a certain size is supposed to be hypoxic. Griffiths et al. (42) reported that hypoxia regulated and influenced the levels of expression of PD-ECGF/TP in vitro and in vivo. Therefore, TP may have been induced by hypoxic conditions in KB/CV tumors.

Angiogenesis is also important for metastasized cells to grow in their target lesions (43). The density of microvessels in primary carcinoma lesions significantly correlates with metastasis (43). We have previously demonstrated that TPI alone or TPI in combination with 5-trifluorothymidine has antimetastatic activity in mice bearing an experimental liver metastasis of TMK-1 gastric cancer or a spontaneous liver metastasis of Co-3 colon cancer (17). Furthermore, we have also shown in this study that TPI could completely inhibit the early events of angiogenesis in the mouse dorsal air sac assay model. TPI may be valuable in the therapy of some patients with locoregional recurrent and metastatic TP-expressing tumors.

In summary, we have demonstrated that the newly synthesized inhibitor of TP, TPI, competitively inhibits TP activity, inhibits angiogenesis induced by TP, increases the proportion of apoptotic cancer cells in TP-positive tumors, and suppresses the growth of the tumors. Whereas the precise mechanisms for these phenomena remain to be elucidated, these findings suggest the possibility that TPI alone or in combination with other anticancer agents may suppress the locoregional recurrence and metastasis of TP-expressing tumors.

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REFERENCES


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