Suppression of Thymidine Phosphorylase-mediated Angiogenesis and Tumor Growth by 2-Deoxy-L-ribose

Hiroshi Uchimiya, Tatsuhiko Furukawa, Masahiro Okamoto, Yuichi Nakajima, Shigeto Matsushita, Ryuji Ikeda, Takenari Gotanda, Misako Haraguchi, Tomoyuki Sumizawa, Mayumi Ono, Michihiko Kuwano, Tamotsu Kanzaki, and Shin-ichi Akiyama

Departments of Cancer Chemotherapy, Institute for Cancer Research [H. U., T. F., Y. N., R. I., T. G., M. H., T. S., S.-i. A.] and Dermatology [H. U., S. M., T. K.], Kagoshima University, Faculty of Medicine, Kagoshima 890-8520, and Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, Higashi-ka, Fukuoka [M. O., K. M., K. J.]. Japan

ABSTRACT

Thymidine phosphorylase (TP), an enzyme involved in the reversible conversion of thymidine to thymine, is identical to an angiogenic factor, platelet-derived endothelial cell growth factor (PD-ECGF). Both TP and one of the TP-degradation products of thymidine 2-deoxy-ribose (dRib) display endothelial cell chemotactic activity in vitro and angiogenic activity in vivo. Recently, we demonstrated that 2-deoxy-L-ribose (lRib) could abolish the inhibitory effect of dRib on hypoxia-induced apoptosis. This suggested that lRib may be a useful inhibitor of dRib and thereby of TP functions. Therefore, we investigated the ability of lRib to inhibit the range of biological activities of TP and dRib. lRib suppressed both dRib-induced endothelial cell migration in a chemotaxis assay and endothelial tube formation induced by dRib in a collagen gel. lRib could also suppress the biological effects of TP in vivo assays of angiogenesis and tumor growth. Thus, in a corneal assay of angiogenesis, lRib inhibited angiogenesis induced by the implantation of recombinant TP. In a dorsal air sac assay of angiogenesis, lRib inhibited angiogenesis induced by the implantation of KB cells overexpressing TP (KB/TP). In a tumor growth assay, lRib treatment considerably decreased the growth rate of KB/TP cells xenografted into nude mice and also resulted in an increase in the proportion of apoptotic cells in KB/TP tumors. These findings demonstrate that TP and dRib play an important role in angiogenesis and tumor growth, and that these effects can be inhibited by lRib. Thus, lRib is a potentially useful agent for the suppression of TP-dependent angiogenesis and tumor growth.

INTRODUCTION

We have previously shown that TP1 (Enzyme Commission No. 2.4.2.4), an enzyme that is involved in pyrimidine nucleoside metabolism, is identical to PD-ECGF (1, 2). TP/PD-ECGF stimulates chemotaxis and [3H]thymidine incorporation by endothelial cells in vitro, and displays angiogenic activity in vivo (3–6). Furthermore, we have demonstrated that the enzymatic activity of TP is indispensable for its angiogenic activity (3, 6). Among the degradation products generated from thymidine by TP activity, dRib, a dephosphorylated product derived from 2-deoxy-ribose-1-phosphate, also displays chemotactic activity in vitro and angiogenic activity in vivo. These findings suggest that the biological activities of TP may be mediated by the generation of dRib (6). The biological effects of TP do not appear to be limited to angiogenic activity, because TP can affect the prognosis of human colorectal and renal cell carcinomas independently of its angiogenic activity (7, 8).

To investigate the range of biological activities of TP, we set up a model system in which KB cells were transduced with TP/PD-ECGF cDNA and thereby established a cell line, KB/TP, that overexpresses TP. These cells were then used, in conjunction with a novel specific TPI, in a number of assay systems to determine the function of TP in tumor growth, apoptosis, angiogenesis, and metastasis. TPI suppressed the growth of KB/TP cells xenografted into nude mice by increasing the proportion of apoptotic cells, and also inhibited the angiogenesis induced by KB/TP in a dorsal air sac assay (9). TPI also suppressed the liver metastasis of KB/TP cells that were injected into the spleen (10). These studies indicated that TP plays a role in tumor growth, apoptosis, and cell motility, as well as in angiogenesis.

Although TPI can inhibit these effects of TP there are a number of drawbacks to using TPI as an antitumor or antiangiogenic agent. One particular disadvantage is that inhibition of TP activity by TPI may increase plasma thymidine levels. High plasma thymidine levels have been observed in mitochondrial neurogastrointestinal encephalomyopathy, an autosomal recessive human disease associated with depletion of skeletal muscle mt DNA in which there is a loss-of-function mutation of TP. High thymidine levels in this disease appear to impair replication or repair of mt DNA (11). Perhaps a better approach to inhibition of TP activity for clinical purposes would be to inhibit dRib, the downstream mediator of TP function, rather than to directly inhibit TP activity.

We have demonstrated recently that a stereoisomer of dRib, lRib, could suppress the effect of dRib on hypoxia-induced apoptosis (12). Thus, lRib may competitively inhibit the physiological activities of dRib and consequently the activities of TP without the adverse effect of inducing elevated thymidine levels. Therefore, in this study, we examined whether lRib could inhibit chemotaxis and tube formation induced by dRib in vitro as well as angiogenesis and tumor growth induced by TP in vivo.

MATERIALS AND METHODS

Growth Factors and Chemicals. Recombinant human TP/PD-ECGF was obtained from R&D systems (Minneapolis, MN), dRib and lRib were purchased from Sigma Chemicals Co. (St. Louis, MO).

Animals and Cell Lines. Male BALB/c mice, nude mice, and Sprague Dawley rats (6–8 weeks old) were used. KB/TP, human KB epidermoid carcinoma cells transduced with TP/PD-ECGF cDNA, and KB/CV, a mock transfectant, were maintained in MMEM containing 10% newborn calf serum. These cells were Mycoplasma free. BAE cells were obtained from Dainippon Pharmacy (Osaka, Japan) and were maintained in DMEM containing 10% FBS.

Transfection of TP/PD-ECGF cDNA into KB Cells. TP/PD-ECGF full-length cDNA was kindly provided by Dr. K. Miyazono and Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The expression

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2 To whom requests for reprints should be addressed, at Department of Cancer Chemotherapy, Institute for Cancer Research, Kagoshima University, Faculty of Medicine, Kagoshima 890-8520, Japan. Phone: 81-99-275-5490; Fax: 81-99-265-9687.

3 The abbreviations used are: TP, thymidine phosphorylase; PD-ECGF, platelet-derived endothelial cell growth factor; Ir, 2-deoxy-L-ribose; dRib, 2-deoxy-D-ribose; BAE, bovine aortic endothelial; mt DNA, mitochondrial DNA; FBS, fetal bovine serum; TPI, thymidine phosphorylase inhibitor; DAB, diaminobenzidine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; bFGF, basic fibroblast growth factor; 3-FU, 5-fluouracil.
vector encoding TP/EGF-C DNA (RSV/TP) or the vector alone (RSV) was transfected into KB cells by electroporation (13). After selection with Geneticin, the expression of TP in each clone was determined by immunoblotting with an anti-TP monoclonal antibody. One TP-positive clone transfected with RSV/TP (KB/TP), and one clone transfected with RSV alone (KB/CV) were additionally analyzed.

**Immunoblotting.** Cells were lysed in 10 mM Tris-HCl (pH 7.5) by rapid freeze-thawing in liquid nitrogen and subsequent incubation in a 37°C water bath. The lysates were centrifuged at 15,000 × g for 20 min at 4°C, and the supernatants were resolved by 11% SDS/PAGE according to the method of Laemmli (14). Protein concentrations were determined by the method of Bradford (15). Proteins in the gel were electrothermally transferred to a sheet of polyvinylidene difluoride membrane (Immobilon-P transfer membrane; Millipore, Bedford, MA) with a Bio-Rad Transblot SD apparatus (Hercules, CA) as described (16). The membrane was then incubated with a monoclonal anti-TP antibody as described previously (17) and developed using an horse-radish peroxidase-conjugated sheep antimouse IgG (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and the enhanced chemiluminescence Western blotting detection system (Amersham).

**Endothelial Cell Chemotaxis.** The chemotaxis assay using BAE cells was performed as described previously (3, 4). Briefly, modified Chemotaxicell chambers (Kurarico Co., Neyagawa, Japan) were used for the upper well. Each filter was coated with 60 μl of a 40-fold dilution of Type I collagen for BAE adhesion. BAE cells (10⁵ cells/well) were seeded into the upper wells, and dRib or bFGF was added to the lower wells at the indicated concentrations in DMEM supplemented with 1% FBS. Medium supplemented with 1% serum was added to control wells. After 6 h of incubation at 37°C, cells that migrated to the lower surface of the filter were counted using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) assay. All of the experiments were performed in triplicate.

**Quantitative Analysis of Endothelial Cell Tube Formation on a Type I Collagen Gel.** The endothelial cell tube formation assay was performed as described previously (18–20). In brief, BAE cells were plated onto a type I collagen gel in medium containing 10% FBS. When the cells reached confluence, that medium was replaced with medium that contained 10% FBS with or without the various factors indicated, and the cells were incubated for an additional 72 h at 37°C. On day 3, phase-contrast micrographs (R5000H; Fuji Photo Film Co., Tokyo, Japan) were taken, and the total length of the tube-like structures per field was measured with a Cosmozone image analyzer (Nikon, Tokyo, Japan). Seven random fields per dish were measured, and the total tube length per field was calculated.

**Rat Corneal Assay.** The assay was done as described previously (21, 22). Poly-2-hydroxylethylmethacrylate (12%) in absolute ethanol was used as pellets. The pellets containing 200 ng of human recombinant TP/EGF-C were prepared and implanted into the corneae of male Sprague Dawley rats (250–500 g). dRib (200 ng) was added directly to the TP/EGF-C pellets. Neovascularization was observed on day 8 after implantation.

**Effects of dRib on Tumor Angiogenesis.** To examine the effect of dRib on tumor-associated angiogenesis a mouse dorsal air sac assay was used (23). KB/TP and KB/CV cells were washed twice with PBS and then suspended in PBS at 6.7 × 10⁵ cells/ml. A Millipore chamber (diameter, 10 mm; thickness, 2 mm; filter pore size, 0.45 μm; Millipore Co., Bedford, MA) was filled with 150 μl [1 × 10⁵ cells] of either cell suspension or PBS and implanted s.c. into an air sac formed previously in the dorsum of a 7–8 weeks-old male BALB/c mice by injection of a appropriate volume of air. dRib (20 or 100 mg/kg/day) or vehicle alone was administered i.p. every 12 h for 4 days. On day 4, the implanted chambers were removed from the s.c. fascia of the treated animals. The angiogenic response was assessed under a dissecting microscope by determination of the number of newly formed blood vessels >3 mm with the characteristic zigzagging pattern of tumor cell-induced new vasculature in the s.c. side of the skin area (24).

**Effect of dRib on Tumor Growth.** KB/TP and KB/CV cells were suspended in PBS at 10⁵ cells/ml and 0.1 ml was injected into the shoulders of 7–8 week-old male BALB/c nude mice. When the tumor size reached a volume of approximately 50–150 mm³, dRib (100 mg/kg/day) or vehicle alone was administered i.p. every 12 h for 24 days. Tumors were measured in two dimensions by palpating every other day, and the volume was calculated as width² × length × 0.5. The effect of dRib on tumor volume and weight was determined using multiple regression analysis adjusting for tumor type and observation period.

**TP Histochemical 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 3% (v/v) H₂O₂ in absolute methanol for 20 min at room temperature, and the sections were then incubated with a polyclonal anti-TP antibody (13). Antibody binding was detected by sequential incubation with biotinylated antirabbit IgG and a streptavidin-peroxidase complex. Immune complexes were visualized by incubating the sections with DAB using a DAB substrate kit (Vector Laboratories Inc., Burlingame, CA).

**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, San Diego, CA). Antibody binding was detected by sequential incubation with biotinylated antirat IgG (Vector Laboratories Inc.) and a streptavidin-peroxidase complex. Immune complexes were visualized by incubating the sections with DAB using a DAB substrate kit (Vector Laboratories Inc.). Microscopic analysis was done at 400-fold magnification to obtain accurate microvessel counts, which are expressed as vessels/mm².

**TUNEL Staining and Evaluation of Apoptosis.** TUNEL (25) was performed using a commercial kit (Apop Tag Plus; Oncor, Gaithersburg, MD; Ref. 26). The number of apoptotic cells positive for TUNEL staining was counted at the six regions of interest. The apoptotic index was calculated as follows: apoptotic index (%) = apoptotic cell number/total cell number × 100. A minimum of 3000 cells were counted in the sections of the tumors. The evaluation of the apoptotic cells was assessed without knowledge of the variables.

**RESULTS**

**Effect of dRib and iRib on Chemotaxis and Tubulogenesis of Endothelial Cells.** The ability of iRib to inhibit dRib-induced endothelial cell chemotaxis was examined in a chemotactic assay using BAE cells. Migration of BAE cells was enhanced by dRib in a dose-dependent manner. iRib at 100 μM dramatically inhibited the migration induced by 10 μM dRib by >25% (Fig. 1). Neither dRib or iRib at 10 μM affected the growth rate of BAE cells or exerted a cytostatic effect (data not shown). The effect of iRib on dRib-induced tubulogenesis was determined in a tubulogenesis assay in a collagen gel. dRib at 10 μM enhanced tubulogenesis by 70%. iRib (100 μM) completely inhibited the formation of tube-like structures induced by 10 μM dRib but had no effect on the basal level of tubulogenesis (Fig. 2, A and B).

**Fig. 1. Inhibition of dRib-induced chemotaxis of BAE cells by iRib.** dRib-induced motility of BAE cells was examined in a chemotaxis assay in the presence or absence of iRib as indicated. dRib enhanced the motility of BAE cells in a dose-dependent manner. The addition of iRib suppressed dRib-induced motility; bars, ± SE.
Effect of lRib on TP-induced Angiogenesis.

To investigate the effect of lRib on TP-induced angiogenesis we first used a rat corneal model of angiogenesis. For this purpose we implanted a pellet impregnated with 200 ng TP or with PBS as a negative control into rat corneas and measured angiogenesis in the absence or presence of lRib. TP clearly induced angiogenesis in the implanted corneas, whereas PBS showed no angiogenic activity. Angiogenesis induced by TP was suppressed by 200 ng of lRib (Fig. 3). Suppression of TP-induced angiogenesis by lRib suggested that lRib may indeed be a useful agent for the inhibition of TP-induced biological effects in vivo. Therefore, we additionally examined the ability of lRib to inhibit TP-induced angiogenesis in a second model system i.e., the mouse dorsal air sac assay. For this assay we implanted KB cells transfected with TP cDNA in an RSV plasmid vector (KB/TP) or the RSV plasmid vector alone (KB/CV) into a mouse dorsal air sac and monitored subsequent angiogenesis after treatment with different concentrations of lRib or saline over a period of days.

We first determined the levels of TP expression in the transfected KB cells by immunoblot analysis using an anti-TP monoclonal antibody. As expected KB/TP cells expressed high levels of TP, whereas KB/CV cells expressed very low endogenous TP levels (Fig. 4A).

Angiogenesis induced by the KB/TP implanted cells was significantly higher than that induced by the control KB/CV cells as shown in Fig. 4B and quantified in Fig. 4C. The angiogenesis index (±SE) for the KB/TP implanted cells was 4.3 ± 0.2, whereas that of KB/CV cells was 2.4 ± 0.86 (P = 0.0186). i.p. administration of lRib (20 mg/kg/day or 100 mg/kg/day) to the mice significantly reduced the angiogenesis index of the KB/TP cells to 2.5 ± 0.43 or 2.8 ± 0.31, respectively (Fig. 4, B and C). The control chamber containing PBS produced no angiogenic response (data not shown).

Effects of lRib on TP-modulated Tumor Growth.

To determine the effect of lRib on TP-modulated tumorigenesis we used a model system in which KB/TP cells were xenografted into nude mice. In this system KB/TP tumors grew significantly faster than the control KB/CV tumors. The addition of lRib at 100 mg/kg inhibited the growth of KB/TP tumors by 40% compared with tumors grown in control mice treated with vehicle alone. lRib had no effect on the KB/CV tumor growth (Fig. 5A). Furthermore the mean weight of lRib-treated KB/TP tumors was significantly less than that of untreated tumors at day 24 (P = 0.0019; Fig. 5B). The growth of KB/TP tumors was more effectively inhibited by lRib than that of KB/CV tumors. Body weight loss was <10% in all of the mice (data not shown).

To ensure that TP expression was not lost during tumor growth the expression of TP in KB/TP tumors was determined by immunohistochemical staining using an anti-TP polyclonal antibody (Fig. 5C). TP was detected in the cytoplasm of almost all of the KB/TP tumor cells, and strong staining was observed in the nonnecrotic area. Control KB/CV tumor cells were only weakly stained suggesting that TP was induced at a low level in vivo.
Effects of lRib on TP-modulated Tumor Apoptosis. We have determined previously that TP may exert an antiapoptotic effect on cells. This effect could contribute to enhanced tumor growth. To determine whether this might be a mechanism by which TP enhances tumor growth we determined the proportion of apoptotic cells in the KB/TP tumors and the effect of lRib on KB/TP tumor apoptosis. Apoptosis was quantified in situ at a cellular level by labeling fragmented DNA with the TUNEL technique. The proportion of apoptotic cells in the experimental tumors generated by implantation of KB/TP cells or control KB/CV cells is shown in Fig. 6A and quantified in Fig. 6B. The apoptotic index (± SE) in KB/TP tumors (0.46 ± 0.036%) was significantly lower than that in KB/CV (1.483 ± 0.121%) tumors (P = 0.0001) indicating that TP is exerting an antiapoptotic effect.

DISCUSSION

Angiogenesis is not only involved in tumor growth and in the formation of distant metastasis but is also an important early step in carcinogenesis. It is a complex multistep process regulated by a number of angiogenic factors (27, 28). PD-ECGF was initially cloned as a novel angiogenic factor distinct from other endothelial cell growth factors (4). Our previous studies demonstrated that PD-ECGF is identical to TP (1, 2). TP was shown to stimulate both chemotaxis and [3H]thymidine incorporation by endothelial cells in vitro, and that it displays angiogenic activity in vivo (3–6). A number of studies indicate a role for TP in tumorogenesis. Expression of TP/PD-ECGF in transformed fibroblasts in nude mice results in increased tumor vascularity (4). Recent studies show that TP is expressed in a wide variety of solid tumors (7, 8, 17, 29–32).

We have found that the enzymatic activity of TP is indispensable for its angiogenic activity (3, 6). Among the degradation products of thymidine engendered by TP enzymatic activity, dRib displays angiogenic activity (3). We hypothesized that lRib, a stereoisomer of dRib, may competitively inhibit the physiological activities of dRib and, thus, the biological functions of TP. We have shown recently that lRib can suppress the inhibitory effect of dRib on hypoxia-induced apoptosis (12). In this study lRib could also inhibit the chemotactic, angiogenic, and tumor-promoting activities of both dRib and TP as predicted from our hypothesis.

Our previous 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 in KB tumors with low TP activity suggesting that TP was involved in angiogenesis in KB tumors. Newly synthesized inhibitor of TP, TPI, which has 1000-fold higher inhibitory activity than 6-amino-5-chloro-...
consistent with our previous observation that IRib suppressed the resistance to apoptosis conferred by dRib (12). The finding that TP is involved in resistance to apoptosis, in addition to its angiogenic activity, may explain why KB/TP cells that overexpress TP display enhanced growth in vivo.

Our study showed that TP expression in KB/TP tumors was retained during tumor growth. However, TP was expressed at a low level in KB/CV tumors, whereas it was not detected in KB/CV cells in vitro. It has been shown that tumor necrosis factor, interleukin 1 and interferon γ up-regulate TP expression in tumor cells (34, 35). Therefore, it is likely that these cytokines may have induced a low level of TP expression in KB/CV cells in nude mice. Furthermore, the center of tumors of a certain size is known to be hypoxic. TP can be induced by hypoxia both in vitro and in vivo (36), and, thus, TP expression in KB/CV tumors may have been induced by hypoxic conditions.

Angiogenesis in human solid tumors is a risk factor for metastasis and recurrence (37). The density of microvessels in primary carcinoma lesions significantly correlates with metastasis (37). We have demonstrated previously that TP plays an important role in the invasiveness and metastasis of KB/TP cells. The inhibition of TP activity in KB/TP cells by TPI caused suppression of motility, invasion, and metastasis of the cells (10). IRib also suppressed motility of BAE cells, enhanced apoptosis of KB/TP cells, and inhibited angiogenesis by TP. It may also suppress metastasis of tumor cells that express TP.

Both IRib and TPI can inhibit the biological activities of TP including the inhibition of angiogenesis and tumor growth. However, IRib may be more useful for use as a clinical inhibitor of TP for a number of reasons. First, TPI directly inhibits TP enzymatic activity. Inactive TP can lead to high plasma thymidine levels, which can have adverse effects on replication or repair of mt DNA as evidenced by the pathology of mitochondrial neurogastrointestinal encephalomyopathy patients who suffer from loss of function mutations in the TP gene (38). IRib, which inhibits a downstream mediator of TP rather than TP activity per se, may have less adverse effects. Second, treatment of tumors with TPI in combination with 5-FU is impossible, because TP is one of the enzymes correlated with activation of 5-FU. However, IRib can be administered with 5-FU, and the combination therapy may be effective against TP-expressing tumors.

Additional study is needed to elucidate the detailed mechanism by which dRib modulates angiogenesis and suppresses apoptosis, and the mechanism of inhibition of these dRib-induced functions by IRib.

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