

Inhibition of Metastasis of Tumor Cells Overexpressing Thymidine Phosphorylase by 2-Deoxy-L-Ribose

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ABSTRACT

Thymidine phosphorylase (TP) catalyzes the reversible conversion of thymidine to thymine, thereby generating 2-deoxy-D-ribose-1-phosphate, which upon dephosphorylation forms 2-deoxy-D-ribose (D-dRib), a degradation product of thymidine. We have previously shown that D-dRib promotes angiogenesis and chemotaxis of endothelial cells and also confers resistance to hypoxia-induced apoptosis in some cancer cell lines. 2-Deoxy-L-ribose (L-dRib), a stereoisomer of D-dRib, can inhibit D-dRib anti-apoptotic effects and suppressed the growth of KB cells overexpressing TP (KB/TP cells) transplanted into nude mice. In this study, we examined the ability of L-dRib to suppress metastasis of KB/TP cells using two different models of metastasis. The antimetastatic effect of L-dRib was first investigated in a liver-metastasis model in nude mice inoculated with KB/TP cells. Oral administration of L-dRib for 28 days at a dose of 20 mg/kg/day significantly reduced the number of metastatic nodules in the liver and suppressed angiogenesis and enhanced apoptosis in KB/TP metastatic nodules. Next, we compared the ability of L-dRib and tegafur alone or in combination to decrease the number of metastatic nodules in organs in the abdominal cavity in nude mice receiving s.c. of KB/TP cells into their backs. L-dRib (20 mg/kg/day) was significantly ($P < 0.05$) more efficient than tegafur (100 mg/kg/day) in decreasing the number of metastatic nodules in organs in the abdominal cavity. By *in vitro* invasion assay, L-dRib also reduced the number of invading KB/TP cells. L-dRib anti-invasive activity may be mediated by its ability to suppress the enhancing effect of TP and D-dRib on both mRNA and protein expression of vascular endothelial growth factor and interleukin-8 in cultured KB cells. These findings suggest that L-dRib may be useful in a clinical setting for the suppression of metastasis of tumor cells expressing TP.

INTRODUCTION

Tumor metastasis is a complex process. Neo-vascularization is essential for both primary and metastatic tumor growth (1, 2). Various angiogenic factors that are produced by solid tumors have been identified (3–6). Angiogenesis is regulated by a balance between pro-angiogenic factors and inhibitors of angiogenesis (7). Manipulation of these factors for anti-angiogenic therapy is a new and promising avenue in cancer treatment. Anti-angiogenic therapy may be effective for a broad spectrum of tumors, may have fewer side effects, and may be less inductive to the emergence of drug resistant tumor cells (8).

We previously reported that thymidine phosphorylase (TP) is identical to platelet-derived endothelial cell growth factor and is a potent angiogenic factor that plays a key role in tumor angiogenesis (9–11). Many types of solid tumors express TP and high TP activity is correlated with microvessel density (12–15). In addition to angiogenic activity, TP can suppress hypoxia-induced apoptosis (16). TP catalyzes the reversible phosphorolysis of thymidine and other pyrimidine

2'-deoxyribonucleosides. The conversion of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate by TP activity generates a dephosphorylated product 2-deoxy-D-ribose (D-dRib). D-dRib mediates many of the biological activities of TP. Thus D-dRib displays angiogenic activity in the chorioallantoic membrane assay (17). D-dRib has also been shown to stimulate chemotaxis and tubular formation of endothelial cells and enhanced the growth of tumors by conferring resistance to hypoxia-induced apoptosis on the tumor cells (11, 18, 19). Inhibition of the functions of TP and D-dRib may, therefore, prove to be useful in the inhibition of tumor growth and metastasis of TP-expressing tumor cells.

Several novel inhibitors of TP have recently been reported (20). One of these, TP inhibitor, could suppress the effect of TP-mediated angiogenesis, tumor growth, metastasis, and resistance to hypoxia-induced apoptosis (11). However the use of these inhibitors as anti-tumor agents has a number of drawbacks because they directly inhibit TP activity. Firstly, the inhibition of TP activity could lead to increased thymidine levels in plasma that may be toxic. Nishino *et al.* have reported that patients with mitochondrial neurogastrointestinal encephalomyopathy who had very low TP activity in their plasma because of homozygous mutations in their TP genes showed pathological changes in the brain and muscle because of mitochondrial alterations (21, 22). Secondly, TP activity is required for the activation of the cytotoxic activities of other antitumor drugs such as 5'-dFUrd and tegafur (23). We have demonstrated that TP-transfected human carcinoma KB cells overexpressing TP (KB/TP cells) are more sensitive to these drugs than parental cells (24). Therefore, it will be difficult to use TP inhibitor in combination with 5'-dFUrd and its prodrugs. For these reasons, inhibition of D-dRib, the downstream mediator of TP functions, would be a more preferable target for antitumor therapy than direct inhibition of TP activity.

We have previously reported that the various effects of D-dRib could be inhibited by 2-deoxy-L-ribose (L-dRib), a stereoisomer of D-dRib. L-dRib has been shown to inhibit chemotaxis and tubulogenesis of bovine aortic endothelial cells induced by D-dRib (18), to suppress angiogenesis induced by KB/TP cells in a dorsal air sac assay, and to suppress the growth of KB/TP cells that are xenografted into nude mice. L-dRib also increases the proportion of apoptotic cells in the TP-expressing tumors (18). These results demonstrate that L-dRib could be a new type of anticancer drug that inhibits angiogenesis and induces apoptosis of tumor cells expressing TP.

Although direct inhibition of TP activity by TP inhibitor has been shown to prevent liver metastasis of tumor cells expressing TP (25), the ability of L-dRib to inhibit metastasis has not yet been explored. In this study, we, therefore, investigated the ability of L-dRib to suppress metastasis of TP-expressing tumor cells. We further investigated the ability of L-dRib to modulate the invasive activity and the production of angiogenic factors of KB/TP cells *in vitro*.

MATERIALS AND METHODS

Compounds and Reagents. Tegafur was a gift from Taiho pharmaceutical Co., Ltd. (Tokyo, Japan). L-dRib was purchased from Sigma Chemical Co. (St. Louis, MO).

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Animals and Cell Culture. Six-week old male BALB/c nude mice (CLEA Japan, Inc., Tokyo, Japan) were used. The KB-3-1 cell line, derived from human epidermoid carcinoma cells, was maintained in MEM containing 10% newborn calf serum, 100 units/ml penicillin, and 2 mM L-glutamine.

Transfection of *TP/PD-ECGF* cDNA into KB-3-1 Cells. The *TP/PD-ECGF* full-length cDNA expression vector or the empty vector was transfected into KB-3-1 cells by electroporation (26). After selection with geneticin, the expression of TP in each clone was determined by immunoblot analysis using an anti-TP monoclonal antibody as described previously (27). A TP-positive clone (KB/TP cells) and a control vector-transfected clone (KB/CV cells) were further analyzed.

Experimental Liver Metastasis Model. KB/TP and KB/CV cells were harvested by brief trypsinization, washed twice with PBS, and then suspended in PBS at 10^6 cells/ml. KB/TP or KB/CV cells (10^5 cells) in 0.1 ml PBS were injected into the spleen which was exposed after transverse incision in the left flank of the anesthetized mice (six mice per group). After 1 min, the spleen was removed, and the abdominal incision was closed (25). Liver metastatic nodules were difficult to observe in this metastatic model unless the spleens were extirpated (data not shown). From one day after inoculation, mice were given p.o. L-dRib at a dose of 20 or 100 mg/kg/day every day. The same volume of physiological saline was given to the control mice. Four weeks after tumor inoculation, the number of metastatic nodules on the liver surface was counted under a stereoscopic microscope (25). The extent of the metastatic areas was evaluated from hematoxylin- and eosin-stained liver tissue sections from the left lateral liver lobe. Histological views were captured digitally. The ratio of the area of liver with metastasis to total liver area was calculated using NIH Image (US National Institute of Health) software (28, 29).

Immunohistochemical Staining of Blood Vessels in Metastatic Nodules. The liver tissues were embedded in OCT compound (Sakura Finetek, Torrance, CA), and then frozen quickly in liquid nitrogen and stored at -80°C . Cryosections were fixed in acetone for 2 min at -20°C and then incubated with 0.3% H_2O_2 in PBS for 10 min at room temperature to block endogenous peroxidase activity. After rinsing with PBS, the cryosections were stained for endothelial cells with a rat monoclonal antimouse CD31 antibody (PharMingen, San Diego, CA). Antibody binding was detected by sequential incubation with a biotin-conjugated goat antirat immunoglobulin and a streptavidin-horseradish peroxidase complex (Vector Laboratories Inc., Burlingame, CA). Color development was performed with diaminobenzidine using a substrate kit (Vector Laboratories Inc.). The cryosections were then counterstained with 0.5% methyl green. Three random microscopic fields at $\times 400$ magnification were captured for each metastasis. Histological views were captured digitally. The ratio of the vessel area to the tumor area was calculated using NIH Image software (28, 29).

TUNEL Assay and Evaluation of Apoptosis in Metastatic Nodules. Liver tissues were embedded in paraffin, and then sections were cut into 3 μm . Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) assay was performed using a commercial kit (Intergen Company, Purchase, NY). The TUNEL-positive apoptotic cells were counted in microscopic fields at $\times 200$ magnification. For all sections, apoptotic cells were counted in total areas of the metastatic nodules except the necrotic area. The apoptotic index was calculated as follows: apoptotic index (%) = TUNEL-positive cell number/total cell number $\times 100$. The evaluation was performed twice as a blind study.

Subcutaneous Tumor Model. KB/TP and KB/CV cells were suspended in PBS at 10^7 cells/ml, and 10^6 tumor cells were injected s.c. into the backs of nude mice (six mice per group; eight groups). Consistent with our other data, 100% tumorigenicity was achieved about 1 week after s.c. injection. Drug treatments were initiated from day 3 after tumor inoculation. L-dRib (20 mg/kg/day), tegafur (100 mg/kg/day), L-dRib and tegafur (20 and 100 mg/kg/day), or saline were administered p.o. every day. Mice were sacrificed on day 52, and the number of metastatic nodules on the surface of the intestines was counted (from the duodenum to the rectum).

Invasion Assay. KB/TP and KB/CV cells were cultured in 100-mm dishes to subconfluence. The growth medium was replaced with serum-free medium containing 0.01% BSA (Sigma, Poole, United Kingdom) and 3.3 mM glucose. These cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 and 1% O_2 (hypoxia group) for an additional 24 h. Control cells were placed in a standard incubator under 95% air and 5% CO_2 (normoxia group). For the invasion assay, the BD Bio Coat Matrigel Invasion Chamber (BD

Biosciences, Franklin Lakes, NJ), in which the upper and bottom chambers were separated by a Matrigel-coated membrane (8 μm pore), were used according to the protocol of the manufacturer, with some modifications. Briefly, the lower surface of the filters were precoated with collagen (1 μg) in a volume of 60 μl cold PBS and dried overnight at room temperature. The coated filters were washed with serum-free MEM medium before use. KB/TP or KB/CV cells were harvested with 0.02% trypsin containing 0.02% EDTA. Cells (5×10^4) were seeded in each of the upper chambers (24-well chambers) in 0.5 ml of serum-free medium containing 0.01% BSA and 3.3 mM glucose, and the bottom chambers contained the appropriate medium with 1% FBS as a chemoattractant. The KB/TP cells were then treated for 24 h in the absence or presence of L-dRib (10 or 100 μM). The noninvading cells on the upper surface of the filters were removed by wiping with a cotton swab. Cells at the bottom side of the membranes were fixed with ethanol, and stained with 0.5% methyl blue. The number of cells invading through the Matrigel membrane was counted in microscopic fields at $\times 200$ magnification. To minimize bias, at least 3 randomly selected fields were counted. Data were averages of triplicate determinants for each condition.

Real-time Reverse-Transcription PCR Quantification. KB/TP and KB/CV cells were cultured under the conditions described for the invasion assay. KB/TP cells were then treated for 48 h in the absence or presence of L-dRib (10 or 100 μM) or D-dRib (100 μM). KB/CV cells were treated for 48 h in the absence or presence of D-dRib (100 μM). Total RNA from the cultured cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). One

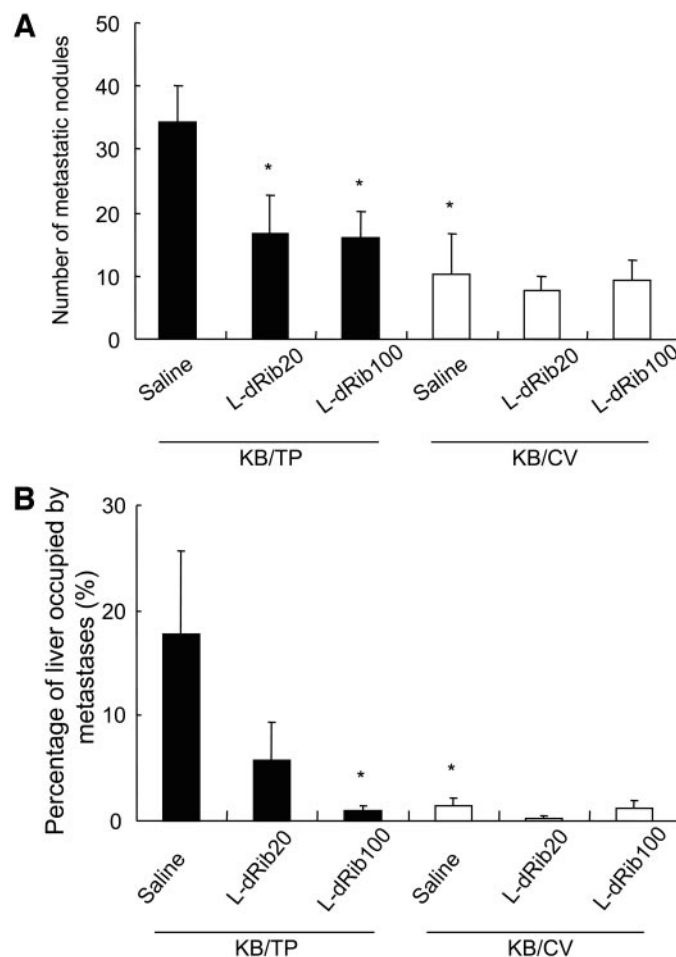


Fig. 1. The effect of 2-deoxy-L-ribose (L-dRib) on thymidine phosphorylase (TP)-mediated tumor metastases. Nude mice were intra intrasplenically inoculated with KB cells over-expressing TP (KB/TP) or the empty vector transfected cells (KB/CV) as a control. From the second day after inoculation, mice were given p.o. L-dRib [20 mg/kg/day (*L-dRib20*) or 100 mg/kg/day (*L-dRib100*)] or control saline every day as indicated. The effect of L-dRib on the numbers of metastatic nodules on the liver surface (A) and the percentage of the liver area with metastasis was assayed (B). Each column and bar represents the mean \pm SE from six mice per group. *, $P < 0.05$ versus KB/TP saline control.

microgram of RNA was reverse transcribed using a first-strand cDNA synthesis kit (ReverTra Ace α , TOYOBO, Osaka, Japan). Human angiogenic factor (VEGF and IL-8) gene expression levels were assayed by real-time reverse PCR (PRISM 7900HT, Applied Biosystems, Foster City, CA) according to the technical brochure of the company. The sets of primers and TaqMan probes were designed with a primer design software Primer Express version 1.5 (Applied Biosystems). Human GAPDH was used for normalization. Quantification of target gene expression was obtained with the comparative cycle threshold method according to the instructions of the manufacturer.

Measurement of Interleukin 8 (IL-8) and Vascular Endothelial Growth Factor (VEGF) in Conditioned Media. KB/TP and KB/CV cells were plated in 35 mm wells at 1×10^5 cells/well and cultured overnight. The growth medium was replaced with serum-free medium containing 0.01% BSA (Sigma) and 3.3 mM glucose. These cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO₂ and 1% O₂ for an additional 48 h. KB/TP cells were then treated for 48 h in the absence or presence of L-dRib (10 or 100 μ M). For some experiments KB/CV cells were treated for 48 h in the absence or presence of D-dRib (100 μ M).

IL-8 and VEGF levels in the conditioned media were quantified by using an Immunoassay Kit, (BioSource International, Camarillo, CA) according to the manufacturer's instructions. The number of cells in each well was counted with the Sysmex MICROCELL Counter F-300 (Toa, Kobe, Japan).

RESULTS

Effect of L-dRib on Liver Metastasis. The effect of L-dRib on liver metastasis of KB/TP cells was assayed in mice inoculated with KB/TP or control KB/CV cells into the spleen. Treatment with p.o. administered L-dRib at 20 or 100 mg/kg/day was started one day after inoculation of the cells and after 4 weeks the effect of L-dRib on metastasis was measured. Calculation of the mean number of KB/TP metastatic nodules in the liver indicated that administration of L-dRib at 20 and 100 mg/kg/day significantly decreased the number of metastatic nodules from 34 ± 6 (mean \pm SE) to 17 ± 6 and 16 ± 4 , respectively. The effect of L-dRib on KB/CV metastases was not

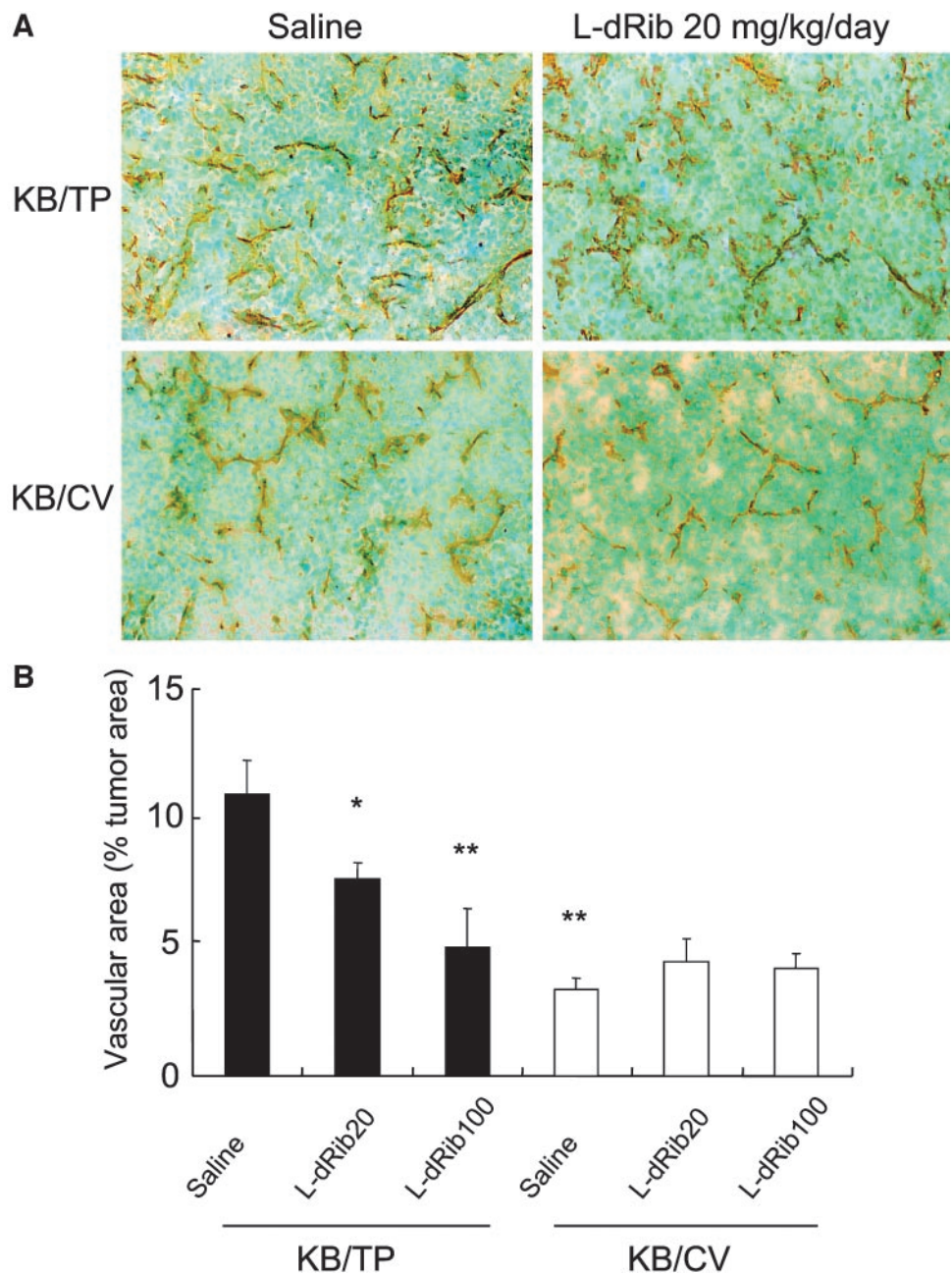


Fig. 2. The effect of L-dRib on TP-mediated tumor angiogenesis in metastatic nodules. A, endothelial cells within metastatic nodules of KB/TP or KB/CV in the liver were specifically stained with antimouse CD31 antibody and photographed ($\times 200$ magnification). The effect of L-dRib (20 mg/kg/day) or control saline administration on endothelial cells is indicated. B, the effect of L-dRib [20 mg/kg/day (*L-dRib20*) or 100 mg/kg/day (*L-dRib100*)] or control saline administration on the mean vessel area, calculated as a percentage of the tumor area is shown. Each column and bar represents the mean \pm SE from six mice per group. *, $P < 0.05$ versus KB/TP saline control. **, $P < 0.01$ versus KB/TP saline control.

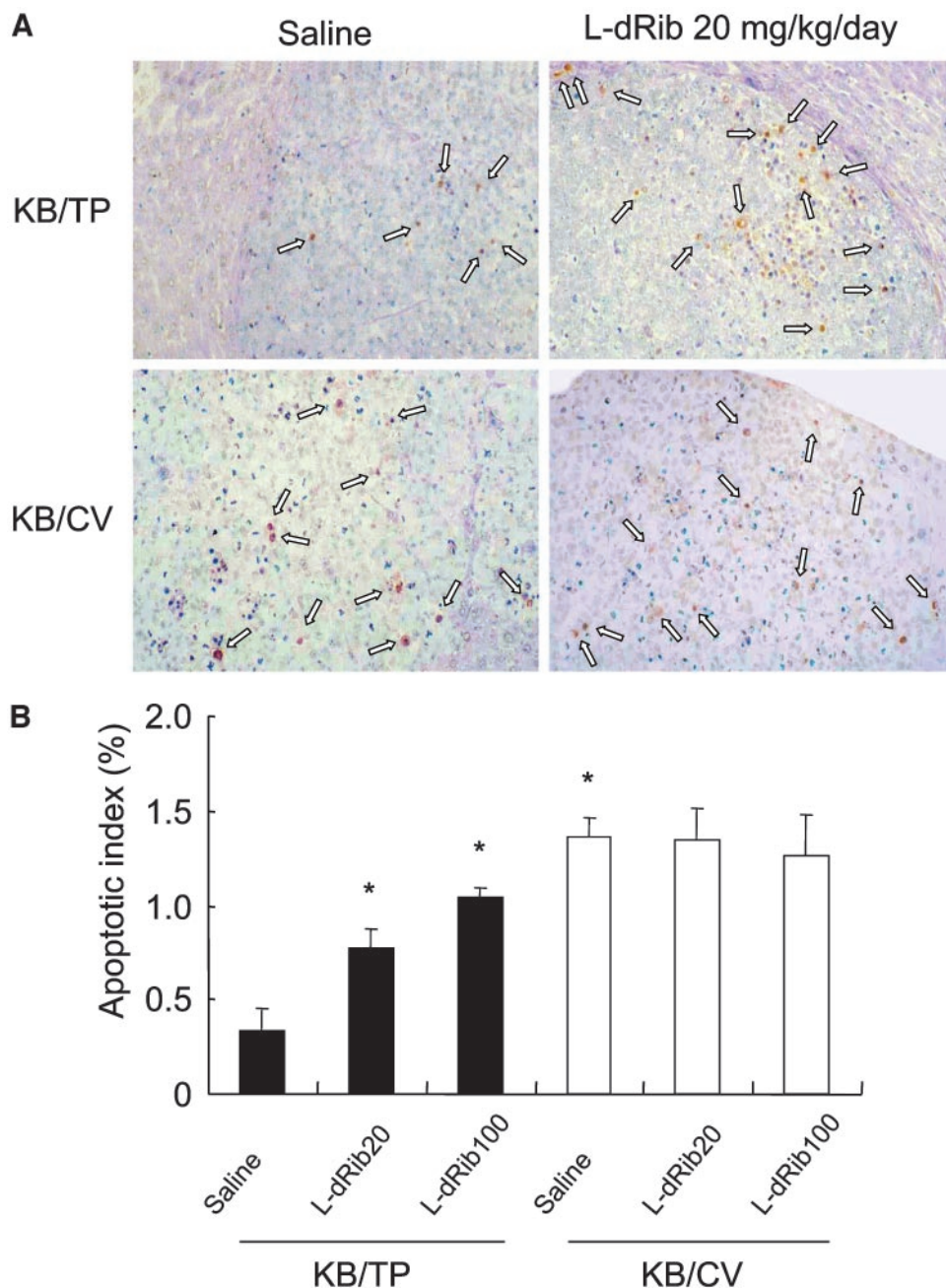


Fig. 3. The effect of L-dRib on apoptosis in metastatic nodules of murine livers. *A*, apoptotic cells in paraffin-embedded fixed sections of metastatic nodules in murine livers of mice inoculated with *KB/TP* or *KB/CV* were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) assay ($\times 200$ magnification) and are indicated by *arrows*. The effect of L-dRib (20 mg/kg/day) or control saline administration is shown. *B*, the apoptotic index, calculated as the percentage of TUNEL-positive cells, is shown for each of the indicated treatments. Each *column* and *bar* represents the mean \pm SE from six mice per group. *, $P < 0.05$ versus *KB/TP* saline control.

significant (Fig. 1A). Treatment with L-dRib at 20 or 100 mg/kg/day also significantly decreased the mean percentage of the liver area occupied by the *KB/TP* metastatic nodules from $17.9 \pm 7.7\%$ to $5.9 \pm 3.5\%$ and $0.9 \pm 0.6\%$, respectively (Fig. 1B). These data indicate that L-dRib treatment can significantly decrease both the number of metastatic nodules and the intrahepatic growth of the *KB/TP* tumors.

Effect of L-dRib on TP-mediated Angiogenesis. The effect of L-dRib on blood vessel formation in liver metastatic nodules from *KB/TP* or *KB/CV* tumors was examined by immunohistochemical staining of endothelial cells as shown in Fig. 2A. Examination of the tissue sections at low magnification indicated that *KB/CV* nodules showed more extended necrotic and avascular areas than *KB/TP* nodules and that L-dRib at 20 mg/kg/day inhibited blood vessel formation in the *KB/TP* nodules.

The effect of L-dRib on angiogenesis was quantified by determi-

nation of the average vessel area as a percentage of the total tumor area. L-dRib treatment at 20 and 100 mg/kg/day reduced the mean vessel area in *KB/TP* metastatic nodules in the liver by 70.3% ($P < 0.05$) and 45.0% ($P < 0.01$), respectively, compared with untreated mice. L-dRib did not reduce the vessel area in *KB/CV* nodules (Fig. 2B). Thus L-dRib significantly inhibited TP-mediated angiogenesis in a dose-dependent manner.

Effect of L-dRib on TP-Mediated Resistance to Apoptosis. The effect of L-dRib on TP-mediated resistance to apoptosis was determined by measurement of apoptotic cells in metastatic nodules in the liver sections of *KB/TP* or *KB/CV* mice treated with either L-dRib or control saline. Apoptotic cells were stained with the TUNEL technique as shown in Fig. 3A and quantified as a percentage of the total cells in the metastatic nodules as shown in Fig. 3B.

The apoptotic index in *KB/TP* metastatic nodules was 4-fold lower than in *KB/CV* metastatic nodules. Treatment with L-dRib at 20 and

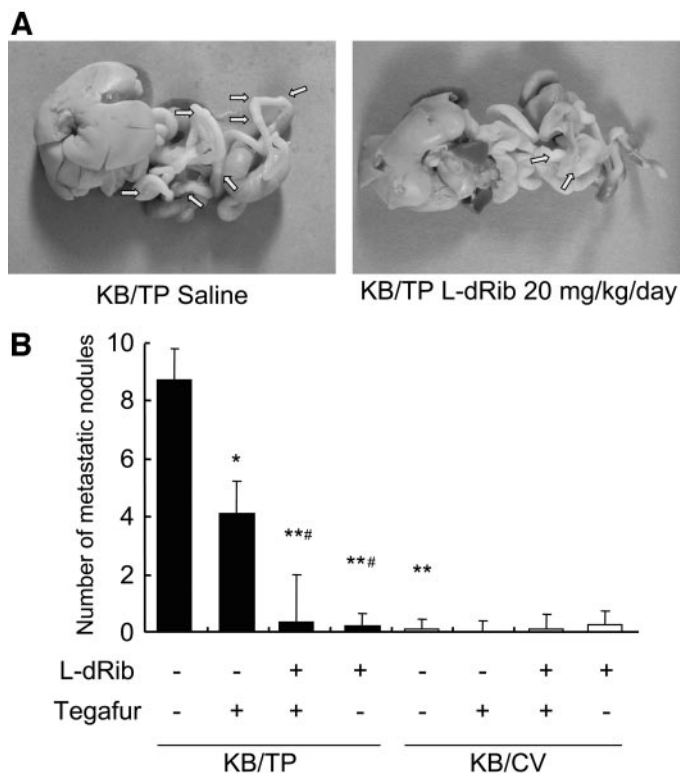


Fig. 4. Inhibition of TP-mediated tumor growth and metastases by L-dRib. *KB/TP* or the empty vector transfected cells (*KB/CV*) were injected into the shoulders of nude mice. From the fourth day after inoculation, mice were treated with either saline, L-dRib (20 mg/kg/day), tegafur (100 mg/kg/day), or L-dRib and tegafur (20 and 100 mg/kg/day), respectively every day. *A*, photographs of the metastatic nodules on the surface of intestines from *KB/TP* tumors treated with saline alone (left) or 20 mg/kg/day L-dRib (right). Arrows indicate metastatic nodules. *B*, the number of metastatic nodules on the intestinal surface from duodenum to rectum was counted using a stereoscopic microscope after the indicated treatments with L-dRib and/or tegafur. All results are the mean \pm SE from six mice per group. *, $P < 0.05$ and **, $P < 0.01$ versus *KB/TP* saline control. #, $P < 0.05$ versus *KB/TP* tumors treated with tegafur alone.

100 mg/kg/day increased the proportion of apoptotic tumor cells 2.3- and 3.1-fold respectively in *KB/TP* nodules compared with untreated nodules. However no difference in the proportion of apoptotic tumor cells was found between treated and untreated *KB/CV* nodules (Fig. 3).

Combination Chemotherapy of Tumors with L-dRib and Tegafur. The above data suggested that L-dRib might be an effective antitumor agent preferentially acting on tumors that overexpress TP. We therefore determined the ability of L-dRib to modulate blood-borne metastasis either alone, or in combination with tegafur. Tegafur is an antitumor agent that requires TP activity for its activation. Because L-dRib inhibits TP-induced downstream functions but not its enzymatic activity, tegafur should be activated in the presence of L-dRib and the combination therapy of L-dRib and tegafur should be effective against TP-expressing tumors. As a control the effect of tegafur alone was also monitored.

Concerning the s.c. tumor model, all mice survived to day 52. We first compared the ability of L-dRib and tegafur to reduce the number of *KB/TP* metastatic nodules in the intestine of nude mice s.c. injected with *KB/TP* cells into their backs. Oral administration of L-dRib (20 mg/kg/day) inhibited the formation of metastatic nodules as shown in the photograph in Fig. 4A. Furthermore L-dRib was more efficient in reducing the number of *KB/TP* nodules than tegafur (100 mg/kg/day) as quantified in Fig. 4B. Neither L-dRib nor tegafur had any significant effects on body weight or general condition of the mice (data not shown). Also, neither L-dRib nor tegafur reduced the metastatic nod-

ules in *KB/CV* mice in which the number of metastatic nodules was significantly lower than in *KB/TP* mice. When L-dRib and tegafur were tested in combination the effect on the number of *KB/TP* metastatic nodules was similar to that of L-dRib alone (Fig. 4B). Thus L-dRib is more effective than tegafur in the suppression of TP-mediated metastasis of *KB/TP* cells. The number and size of the metastatic nodules of *KB/TP* cells were augmented compared with those of *KB/CV* cells. The metastatic nodules of *KB/TP* cells, but not of *KB/CV* cells, invaded in subserosal muscularis, and L-dRib attenuated the invasion. The mean size of *KB/TP* tumors ($3220.0 \pm 389.3 \text{ mm}^3$) was 1.29-fold larger than that of *KB/CV* tumors ($2494.6 \pm 385.0 \text{ mm}^3$). The sizes of the *KB/TP* tumors in mice treated with L-dRib (20 mg/kg/day), tegafur (100 mg/kg/day), or L-dRib and tegafur (20 and 100 mg/kg/day), were 24, 64 or 71% of that in control mice. We could not detect macroscopic and microscopic metastasis at other organs besides intestine. The very effective reduction of metastatic nodules by L-dRib and less effective reduction of the nodules by tegafur suggests that combination chemotherapy of L-dRib and tegafur would be needed to suppress both tumor growth and metastasis.

Effect of L-dRib on TP-mediated Tumor-cell Invasion. To examine the mechanism by which L-dRib modulates *KB/TP* metastasis, we investigated the effect of L-dRib on invasion of *KB/TP* cells *in vitro* in Matrigel invasion assays. Because the invasive activity of *KB/TP* cells is higher than of *KB/CV* cells under hypoxic conditions the assay was carried out under hypoxic as well as normoxic conditions (Fig. 5). The addition of L-dRib (100 μM) to the medium significantly reduced the number of invading *KB/TP* cells under hypoxic conditions. In contrast L-dRib did not significantly reduce the number of invading *KB/CV* cells (data not shown) although the invasive activity of *KB/CV* cells was stimulated by D-dRib. Thus L-dRib selectively inhibits the invasion of *KB/TP* cells.

Effect of L-dRib on the TP-Mediated Expression of VEGF and IL-8 Genes. We next examined whether L-dRib might inhibit the invasion of *KB/TP* cells by suppressing the expression of the pro-invasive factors VEGF and IL-8 that are induced by TP and its downstream effector D-dRib in cultured *KB* cells. D-dRib increased

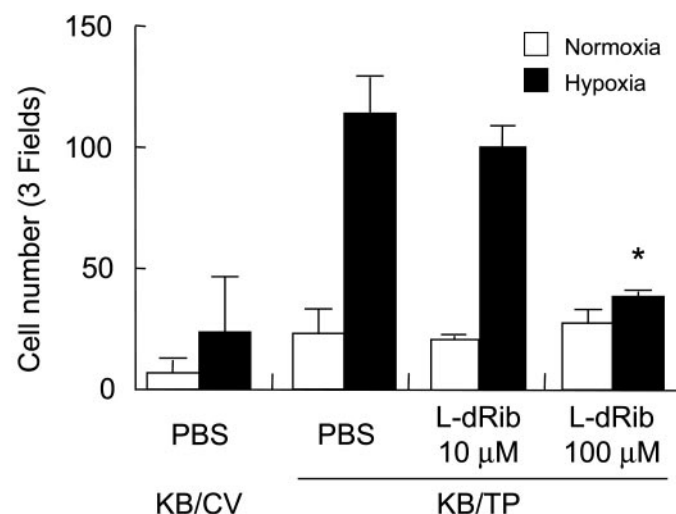


Fig. 5. Effect of L-dRib on cell invasiveness in the Matrigel basement membrane cell-invasion chamber. *KB/TP* or *KB/CV* that were cultured in serum starved medium under normoxic (open columns) or hypoxic conditions (closed columns) for 24 h were seeded on Matrigel-coated filters. Invasion was allowed to proceed for 24 h in the presence or absence of 10 μM or 100 μM L-dRib or with PBS as indicated. Cells in three randomly selected fields were counted for each treatment. The data are averages of triplicate determinants. Each bar represents the mean \pm SD. *, $P < 0.05$ versus *KB/TP* cells treated with PBS.

the expression levels of these genes in KB/CV cells only under hypoxic conditions (Fig. 6). The expression levels of *VEGF* and *IL-8* mRNAs were up-regulated in KB/TP cells under hypoxic conditions compared with KB/CV cells. In particular, the expression level of *IL-8* mRNA in KB/TP cells was more than 18-fold higher than that in KB/CV cells (Fig. 6B). L-dRib significantly decreased the expression of both *VEGF* (50% decrease) and *IL-8* mRNA (80% decrease) in KB/TP cells under hypoxic conditions.

Effect of L-dRib on TP-Mediated Secretion of VEGF and IL-8.

Because mRNA levels do not always correlate with protein levels, we further examined whether *VEGF* and *IL-8* mRNA levels correlate with the levels of these proteins secreted from KB cells. We, thus, measured VEGF and IL-8 protein levels in conditioned media from KB/TP or KB/CV cells using an ELISA assay. Although the mRNA and protein levels do not exactly correlate, the secreted protein levels of VEGF and IL-8 from KB/TP cells were higher than those from KB/CV cells (Fig. 7). Thus, TP appears to augment VEGF and IL-8 production and release from KB cells.

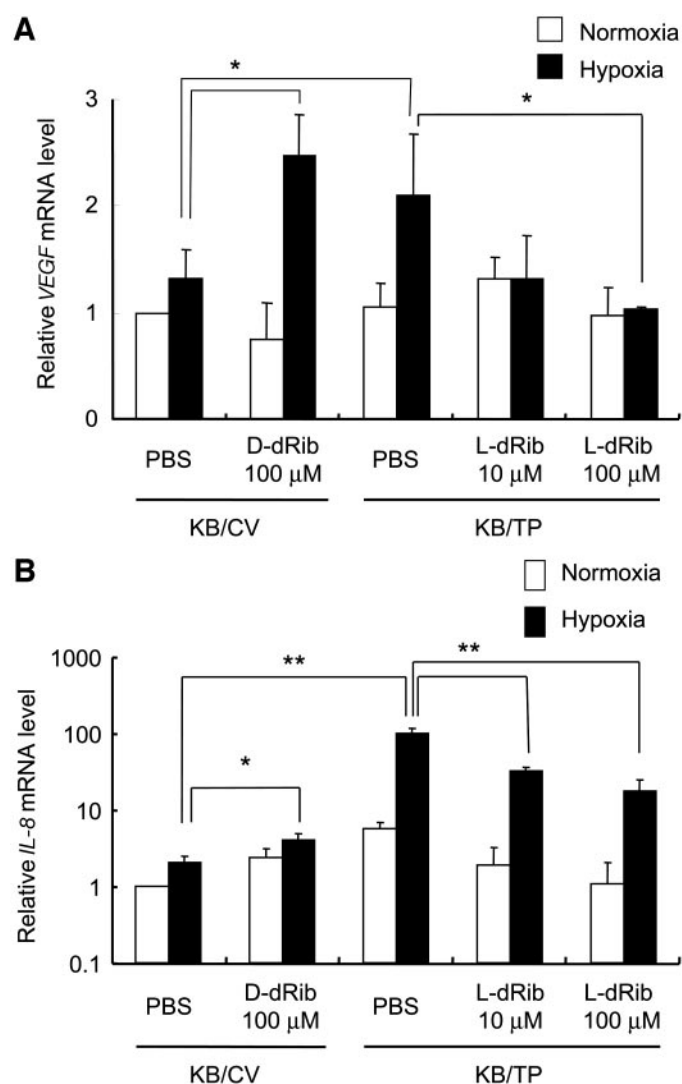


Fig. 6. The effects of D-dRib and L-dRib on the expression of angiogenic factors in cultured KB cells. KB/TP or KB/CV were cultured in serum-starved medium with or without 100 μ M D-dRib (*D-dRib 100*), 10 μ M L-dRib (*L-dRib 10*), 100 μ M L-dRib (*L-dRib 100*), or PBS under normoxic (*open columns*) or hypoxic (*closed columns*) conditions for 48 h as indicated. Relative expression levels of *VEGF* (A) and *IL-8* (B) mRNA were measured using Real-time PCR expression of the *GAPDH* gene was used to normalize the values of *VEGF* and *IL-8*. Each column and bar represents the mean \pm SD. *, $P < 0.05$. **, $P < 0.01$.

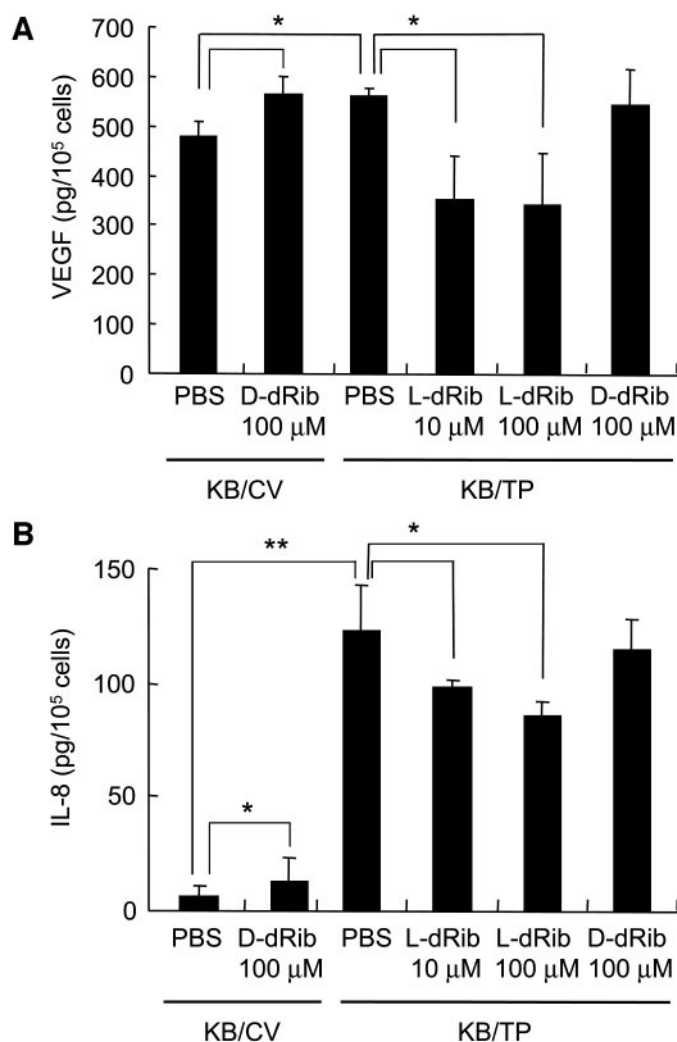


Fig. 7. Inhibition of secretion of vascular endothelial growth factor (*VEGF*) and interleukin 8 (*IL-8*) proteins from KB cells by L-dRib. *KB/TP* or *KB/CV* were cultured in serum-starved medium with or without 100 μ M D-dRib (*D-dRib 100*), 10 μ M L-dRib (*L-dRib 10*), 100 μ M L-dRib (*L-dRib 100*) or PBS under hypoxic conditions for 48 h as indicated. VEGF (A) and IL-8 (B) protein levels were measured using an ELISA assay. Each column and bar represents the mean \pm SD. *, $P < 0.05$. **, $P < 0.01$.

More importantly, treatment of KB/TP cells under hypoxic conditions with L-dRib led to a reduction in secreted protein levels of both VEGF and IL-8 (Fig. 7). Thus, inhibition of the mRNA levels for VEGF and IL-8 by L-dRib is reflected in the secreted levels of these proteins.

DISCUSSION

TP is implicated in tumor angiogenesis and metastasis (30–32). TP-mediated angiogenesis, tumor growth, and metastasis could also be inhibited by direct inhibition of TP activity by TP inhibitor (25). However, direct inhibition of TP activity by inhibitors of TP may increase plasma thymidine levels thereby causing pathological side effects (33). Thus, inhibition of D-dRib, a downstream mediator of TP function, by L-dRib will probably prove to be less toxic in a clinical setting than direct inhibition of TP activity.

L-dRib, a stereoisomer of D-dRib that has been shown to inhibit the functions of D-dRib (18), was tested for inhibition of angiogenesis and metastasis in two different mouse models in this study. In both models L-dRib inhibited angiogenesis and suppressed metastasis of tumor cells overexpressing TP. Thus, L-dRib may be an efficient antimeta-

static compound. Metastatic nodules on the surface of intestines in mice bearing s.c. KB/TP tumors were more sensitive to L-dRib than to tegafur ($P < 0.05$). Whereas the antimetastatic effects of tegafur may have been mediated by cytotoxicity (34), L-dRib at the doses used (20 mg/kg/day, 52 days) was not cytotoxic for animals. We also injected L-dRib (200 mg/kg/day) into abdominal cavities of mice for 21 days. No weight loss and no abnormal change in the organs were found (data not shown). *In vitro* experiments, the proliferation of both KB/TP and KB/CV cells was not affected when the cells were incubated in the presence of 100 μ M L-dRib for 48 and 72 h under hypoxic and also normoxic conditions (data not shown). These data suggest that L-dRib specifically inhibited TP-induced pro-metastatic effects.

The ability of L-dRib to decrease angiogenesis as well to increase the proportion of apoptotic cells in the KB/TP metastatic nodules is most likely attributable to an inhibition of TP and D-dRib pathways. We have previously demonstrated that D-dRib induces chemotaxis and tubular formation of bovine aortic endothelial cells and that these functions of D-dRib are inhibited by L-dRib (18). L-dRib was also able to suppress the prevention of hypoxia-induced apoptosis in human leukemia HL-60 cells mediated by D-dRib (19). Suppression of the enhanced levels of VEGF and IL-8 observed in KB/TP cells by L-dRib that we observed in this study was also consistent with the inhibition of TP and D-dRib pathways by L-dRib. TP has been shown to promote the secretion of angiogenic molecules such as VEGF, IL-8, and matrix metalloproteinase-1 in TP-overexpressing carcinoma cells (35). We showed that D-dRib increased the expression levels of VEGF and IL-8 in TP-negative KB/CV cells and that the expression levels of VEGF and IL-8 in KB/TP cells overexpressing TP were higher than those in KB/CV cells. These findings are also consistent with a previous report that VEGF and IL-8 are induced in TP-expressing tumor cells after the addition of thymidine (35). VEGF is angiogenic, and IL-8 not only regulates vascular endothelial cells growth, but also directly enhances matrix metalloproteinase production and the invasiveness of endothelial and tumor cells (36, 37). Although VEGF and IL-8 reportedly affected migration of various cell lines, further study is needed to confirm that L-dRib affected the invasiveness of the cells by decreasing the expression of VEGF and IL-8.

The role of oxygen in the TP and D-dRib-induced effects on VEGF and IL-8 is unclear. VEGF and IL-8 protein secretion levels are more effectively induced by hypoxia than normoxic conditions in KB/TP cells. D-dRib may be more effectively used as energy under hypoxic than normoxic conditions in tumor cells. However, the 18-fold higher induction of IL-8 under hypoxic conditions in KB/TP cells compared with control KB/CV cells cannot be explained solely by elevated levels of D-dRib. This suggests that other mechanisms for the induction of IL-8 in TP-expressing cells may exist under hypoxic conditions. Further study is needed to know whether the other degradation product(s) of thymidine by TP preferentially induce(s) IL-8.

Although L-dRib appears to modulate its effects by inhibition of D-dRib, modulated signaling pathways involved have not yet been fully elucidated. D-dRib has been implicated in a number of signaling pathways including apoptosis and integrin signaling pathways (19, 38). Because D-dRib (39), but not L-dRib (30), can enter glycolysis and provide an energy source (40), D-dRib may be an important energy source under hypoxic conditions. L-dRib inhibition of D-dRib entry into glycolysis could, thus, be detrimental to tumor growth under hypoxic conditions. Recent evidence suggests that D-dRib affects endothelial cell migration via activation of integrin downstream signaling pathways (38). Thus, L-dRib may block either the association of D-dRib to the cell surface receptor and/or D-dRib-induced downstream signaling pathways in endothelial cells.

Although the complex pathways involved in D-dRib-modulated

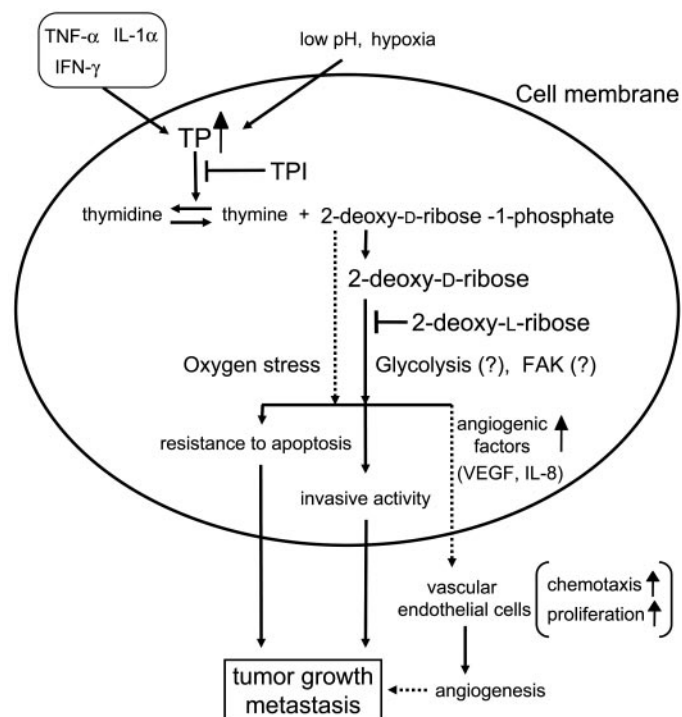


Fig. 8. Schematic representation of the role of thymidine phosphorylase (TP) and D-dRib in tumorigenesis and its inhibition by L-dRib. The expression of TP is induced by cytokines, hypoxia, or low pH in various tumor cells. TP catalyzes the reversible conversion of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate. D-dRib is produced by dephosphorylation of 2-deoxy-D-ribose-1-phosphate. D-dRib is a downstream mediator of TP and confers resistance to hypoxia-induced apoptosis, enhances chemotaxis of vascular endothelial cells, and causes angiogenesis and metastasis. 2-Deoxy-L-ribose can suppress these various biological effects of D-dRib, leading to an inhibition of tumorigenesis.

effects remain to be fully elucidated, it is clear from this study that o.p. administration of L-dRib can suppress TP-mediated metastasis. This is the first demonstration that L-dRib may be useful as an antimetastatic agent for the various solid tumors overexpressing TP. In future studies, combination chemotherapy of L-dRib with other anticancer or antiangiogenic agents may be even more effective in the suppression of the growth and metastasis of tumors overexpressing TP (Fig. 8).

In conclusion, L-dRib inhibits metastasis probably by suppressing TP-stimulated expression of angiogenic factors such as VEGF and IL-8, and L-dRib may prove to be a more efficient and less toxic agent than currently available drugs for the inhibition of metastasis of TP-expressing tumors.

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