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Mechanisms by Which Tumor Cells and Monocytes Expressing the Angiogenic Factor Thymidine Phosphorylase Mediate Human Endothelial Cell Migration[1]

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ABSTRACT

The angiogenic factor thymidine phosphorylase (TP) is highly expressed in many human solid tumors, and the level of its expression is associated with tumor neovascularization, invasiveness, and metastasis and with shorter patient survival time. TP promotes endothelial cell (EC) migration *in vitro* and angiogenesis *in vivo*, and these have been linked to its enzymatic activity. The mechanism by which TP stimulates EC migration was investigated using human umbilical vein ECs (HUVECs). TP induced concentration-dependent HUVEC migration, which required a TP gradient and thymidine and which was abrogated by the TP inhibitor CIMU (5-chloro-6(1-imidazolylmethyl)uracil). The chemotactic actions of TP plus thymidine were duplicated by the TP metabolite, 2-deoxyribose-1-phosphate (dR-1-P), and 10-fold more potently by its subsequent metabolite, 2-deoxyribose (2dR). Migration induced by dR-1-P, but not 2dR, was blocked by an alkaline phosphatase inhibitor, suggesting that the actions of dR-1-P first required its conversion to 2dR. In the migration assay, [5′-^{3}H]dThd was metabolized to dR-1-P (96%) and 2dR (3.8%), and a gradient of both metabolites was maintained between the lower and upper chambers over the entire 5-h assay. TP expression in human solid tumors occurs in both tumor epithelial cells and in tumor-associated macrophages. The migration assay was adapted to use TP-transfected carcinoma cells to stimulate HUVEC migration, and they were found to induce more migration than did control vector-transfected cells. Human monocyte cells U937 and THP1, which constitutively expressed high levels of TP, also strongly induced HUVEC migration in the coculture assay. CIMU inhibited tumor-cell and monocyte-induced migration. In contrast, a neutralizing antibody to TP had no effect on cell-stimulated HUVEC migration, even though it completely blocked the migration mediated by purified TP. Thus, the intracellular actions of TP were sufficient to stimulate HUVEC chemotaxis. In contrast to purified TP, when incubated with [5′-^{3}H]-thymidine, cells expressing TP released up to 20-fold more 2dR into the medium than dR-1-P. These studies demonstrate that TP-expressing cells mediate EC migration via the intracellular metabolism of thymidine and subsequent extracellular release of 2dR, which forms a chemotactic gradient.

INTRODUCTION

The angiogenic factor PD-ECGF is identical to human TP,[3] an enzyme that catalyzes the reversible conversion of dThd and phosphate to thymine and dR-1-P (1–4). Thus TP, like PD-ECGF, was chemotactic for ECs and had angiogenic activity in several *in vivo* assays, although it did not directly stimulate EC proliferation (2–7). Studies have established a role for TP in experimental and clinical tumor angiogenesis, including transfection studies in which the *TP* gene increased the vascularization and growth of tumors growing in nude mice (6–7), and immunohistochemical studies of primary human solid tumors, in which TP was often found to be elevated in the tumors when compared with the corresponding nonneoplastic regions of the same organs (8–12). Many of the latter studies found correlations between a high level of TP expression and increased tumor microvessel density, invasion, metastasis, and/or shorter patient survival time.

The mechanism by which TP mediates angiogenesis is unknown; we have used EC migration as an *in vitro* model to address this question. No cell surface receptor for TP has been identified, indeed the protein lacks a signal sequence required for cell secretion (6). Site-directed mutagenesis studies suggested that the catalytic activity of TP was closely linked to its angiogenic actions (7, 13). One of the initial reports describing the angiogenic activity of TP indicated that 2dR could also induce the development of a vascular system in the chick chorioallantoic membrane assay, whereas the same concentration of dR-1-P was inactive (5). In contrast, it was suggested that the actions of TP could be a consequence of dR-1-P-mediated induction of cellular oxidative stress (14). In neither of these, nor in any other report, however, was the TP-dependent production of the dThd metabolites directly measured. Most studies of TP-induced angiogenesis have also used purified TP as chemoattractant, although the majority of TP is located intracellularly in human tumors. Thus, studies with purified TP cannot address two additional questions regarding the actions of TP: are the intracellular actions of TP sufficient to mediate its effects on ECs; and, if so, to what extent do one or both of the putative active metabolites of dThd efflux from the cells.

We have designed the studies in this report to address these issues by: (*a*) adapting the Boyden chamber assay such that the stimulus to migration was TP-expressing tumor cells rather than purified TP; (*b*) using [5′-^{3}H]dThd to quantitate the production and release of dThd metabolites by the cells; (*c*) using highly potent and specific inhibitors of the catalytic activity of TP and AP to determine which of the dThd metabolites is the likely active moiety mediating TP action; and (*d*) comparing the effects of cell-permeable and cell-impermeable inhibitors of TP to distinguish the intracellular from the extracellular actions of TP on HUVEC migration.

Materials and Methods

Materials. A human TP cDNA (kindly provided by C-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) was used to prepare recombinant TP. FGF-2 (basic FGF) and VEGF were from R&D Systems, as was the neutralizing anti-TP polyclonal antibody; an isotype-matched control IgG was from Sigma. The TP inhibitor, CIMU was synthesized as described previously (15). The API, 2-thiophenylphosphonic acid (16), was obtained from Dr. Vern Schramm (Albert Einstein College of Medicine, Bronx, NY).

Isolation of ECs from Human Umbilical Veins. HUVECs were isolated from umbilical cords obtained less than 5 h after delivery. After rinsing, veins were digested with collagenase (Worthington) in Medium 199 with Earle's salts (Life Technologies, Inc.) for 10 min at 37°C. Veins were flushed with warm M199 and the resulting EC suspension centrifuged for 5 min at 250 × *g*. Primary cultures of HUVEC were seeded into 25-cm^2 flasks precoated with

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[3] The abbreviations used are: EC, endothelial cell; AP, alkaline phosphatase; API, AP inhibitor; 2dR, 2-deoxyribose; dR-1-P, 2-deoxyribose-1-phosphate; CIMU, 5-chloro-6-(1-imidazolyl-methyl) uracil; FGF-2, fibroblast growth factor 2; HUVEC, human umbilical vein EC; dThd, thymidine; TP, dThd phosphorylase; VEGF, vascular endothelial growth factor; CSS, charcoal-stripped serum; PD-ECGF, platelet-derived endothelial cell growth factor.

0.02% (w/v) gelatin in PBS. Culture medium consisted of M199 supplemented with 20% (v/v) newborn calf serum, 5% (v/v) pooled human serum, 2 mM L-glutamine, 5 units/ml penicillin G, 5 μg/ml streptomycin sulfate, 10 units/ml heparin, and 7.5 μg/ml EC growth supplement (Sigma). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2, and the medium changed after 24 h and every 2 days thereafter until confluent. Primary cultures of HUVEC were passaged with 0.05% trypsin/0.02% EDTA, and confluent HUVEC monolayers (passages 1–4) were used in the migration assays.

Boyden Chamber Assay of Human EC Migration. Confluent HUVEC monolayers were cultured with non-growth factor-containing media for 48 h before harvesting with cell dissociation solution (Sigma). Harvested cells were suspended at 10^6/ml in M199 with 1% serum, and 10^5 cells seeded into transwell inserts (8 μm pore, Costar) precoated with 10 μg/ml fibronectin. Inserts containing HUVECs were placed into a 24-well plate containing 700 μl of M199 with 1% serum and incubated for 1 h at 37°C. In some experiments, 2% BSA or 1% CSS (Gemini Bio-Products, Woodland, CA) was substituted for the 1% serum. HUVEC migration was stimulated by addition of a purified factor (TP, VEGF, FGF-2), dThd [or its metabolites dR-1-P and 2dR (Sigma)], or cells, to the lower chamber. After 5 h, HUVECs were stained with 10 μM Cell Tracker Green (Molecular Probes) for 30 min at 37°C, and the upper membrane of the insert was swabbed to remove nonmigrated cells. Inserts were washed with PBS, fixed in formaldehyde, and mounted on microscope slides. HUVEC migration was quantitated by counting the number of cells in three random fields (×100) per insert.

In some experiments, U937 or THP1 human monocytes, human HT29 colon or MCF7 breast cancer cells, transfected with either a full-length human TP cDNA (HT29/TPneo, MCF7/TPneo) or a control vector (HT29/neo, MCF7/neo), were used as stimuli for HUVEC migration. The cell lines were stably transfected as described previously (17). Cells were seeded into 24-well plates at 2×10^5 cells per well overnight and washed twice with PBS before the addition of HUVEC-containing transwell inserts.

Assay of TP Activity and dThd Metabolites. TP activity was measured as described previously (15). Briefly, cells were lysed by sonication and were centrifuged. TP activity in the supernatants was measured in 0.2 M KH_2PO_4 (pH 7.8) containing 0.2 mM [5′-^{3}H]dThd (Moravek), and 50 μg of protein. Reactions (37°C) were stopped with ice-cold activated charcoal in 5% trichloroacetic acid. Placing the radiolabel in the deoxyribosyl group of the dThd yields a product of the TP reaction that does not bind to charcoal, whereas the substrate dThd binds tightly (>99%). After centrifugation, radioactivity in the supernatant was determined by liquid scintillation counting.

dThd metabolism was determined under the same conditions under which the HUVEC migration assays were done. The lower wells of the 24-well plates contained either purified TP or 2×10^5 tumor or monocytic cells, in 0.7 ml M199 with 1% serum; the upper wells contained 10^5 HUVECs. [5′-^{3}H]-Thymidine (5 μCi/ml) was added to the medium in the lower wells at the start of the experiment, and aliquots of medium from both the lower and upper wells were removed during the 5-h incubation at 37°. To quantitate the formation of TP metabolites, the medium was extracted with trichloroacetic acid-charcoal, as described above, and radioactivity in the supernatants determined. For further analysis of the radioactive metabolites, the medium was extracted with activated charcoal in 20 mM PIPES (pH 5.5), and then loaded on a 1-ml anion-exchange column (HiTrap Q Sepharose High Performance column; Amersham). The column was eluted with 20 mM PIPES (pH 5.5), and 0.5-ml fractions were collected. Authentic standards of 2dR and dR-1-P were also loaded onto columns, and their elution was monitored in the fractions collected using a diphenylamine reaction for reducing sugars. Radioactivity in each fraction was determined by liquid scintillation counting. To confirm the identity of the radiolabeled dThd metabolites, aliquots of the column fractions were analyzed by TLC by spotting on silica gel plates, which were developed with dichloromethane:methanol (7:1, v/v). Under these conditions, dThd, 2dR, and dR-1-P were readily separated with R_F values of 0.50, 0.35, and 0.0, respectively. Authentic standards were visualized on the TLC plates by spraying with sulfuric acid:ethanol (5:95 v/v), followed by charring.

Statistical Analyses. Means were compared with a *t* test or by ANOVA with a Tukey-Kramer multiple comparison test.

RESULTS

TP Is a Potent Chemotactic Stimulus for HUVEC Migration. Clinical and experimental data support a role for TP in angiogenesis, but the mechanism for its effects on ECs has not as yet been determined. TP was found to potently stimulate HUVEC migration in the Boyden chamber. Migration was concentration dependent, with an EC_{50} of 20 ng/ml, and the migration of cells was maximal at TP concentrations of ≥100 ng/ml (Fig. 1*A*). To further explore the nature of the chemoattractive properties of TP, migration in the Boyden chamber was examined when stimulus was added to either the lower chamber only (gradient) or in equal amounts to both the upper and lower chambers (no gradient; Fig. 1*B*). TP-stimulated migration of HUVECs was compared with known chemotactic (VEGF) and chemokinetic (FGF-2) stimuli. VEGF induced migration only in the presence of a chemotactic gradient, whereas FGF-2 induced migration in both the presence and the absence of a gradient, consistent with chemokinesis. TP-stimulated migration was observed in the presence of a gradient but not in its absence. Consistent with this observation, TP also had no effect in an assay in which migration was stimulated by scraping a confluent layer of HUVECs and monitoring the rate at which the cells filled-in the denuded area (data not shown).

TP Requires dThd to Stimulate HUVEC Migration *in Vitro*. In the course of our investigation to identify which of the components of the assay system were required for the induction of EC migration by TP, we observed that activity was nearly completely lost when the 1% serum in the media was replaced with either 1% CSS (Fig. 2*A*) or 2% BSA to produce a completely defined system (Fig. 2; compare TP in *B* to SER TP in *A*). In contrast, these modifications had no effect on VEGF-mediated migration. The typical dThd concentration of serum is ~5 μM, and more than 99% of it was removed by charcoal treatment (data not shown); the medium used in the assay contains no dThd. When dThd (100 nM) was added back to wells containing TP in

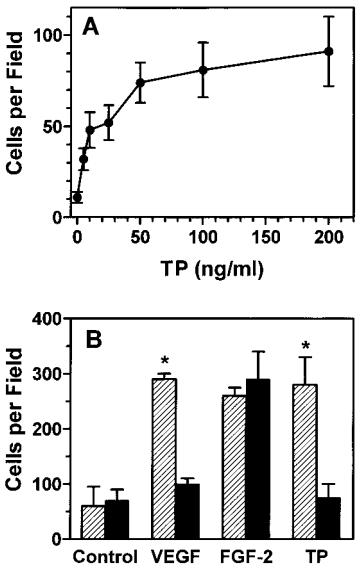

Fig. 1. TP stimulated HUVEC migration *in vitro*. HUVECs were harvested and placed into the upper well of the Boyden chamber in M199 medium with 1% serum. Control wells had media with serum alone. Migration to the underside of the transwell insert was quantitated after 5 h as described in "Materials and Methods." The data are expressed as cells per field (mean of five experiments ± SE). *A*, TP (0–200 ng/ml) was added to the lower chamber. TP concentrations of 10 ng/ml and higher were significantly different from control ($P < 0.05$). *B*, TP (100 ng/ml), VEGF (10 ng/ml), or FGF-2 (20 ng/ml) were added to either the lower chamber only (gradient, ▨), or both the upper and lower chambers (no gradient, ■). These concentrations were previously determined to elicit the maximal migration from HUVECs. *, significantly different from control and from no gradient ($P < 0.05$).

either CSS- or BSA-containing medium, the migration of HUVECs was restored and was equivalent to that of TP in unmodified serum.

When used individually in defined medium, neither dThd nor TP had chemotactic activity, indicating that TP-mediated migration was likely the result of one or more of the catalytic products of the reaction. A previous report indicated that the deoxyribose component of dThd, but not thymine, could stimulate angiogenesis in a chick chorioallantoic membrane assay (5). Therefore, we compared the response of HUVECs to increasing concentrations of dR-1-P, the direct metabolic product of TP, and the response to 2dR, which could be formed from dR-1-P via the action of ubiquitous cellular phosphatases. Both compounds were active inducers of HUVEC migration (Fig. 3*A*), although 2dR was substantially more potent (EC_{50}, ~200 nM) when compared with dR-1-P (EC_{50}, ~2 μM). For comparative purposes, we also determined whether HUVEC migration exhibited a concentration-dependent response to dThd, when assayed with TP in serum-free, defined medium. As shown in Fig. 3*B*, increasing concentrations of dThd progressively increased migration, and the concentration-response curve closely followed that of 2dR alone. Thus, the data are consistent with a model in which one or both of the TP metabolites mediate its chemotactic effects.

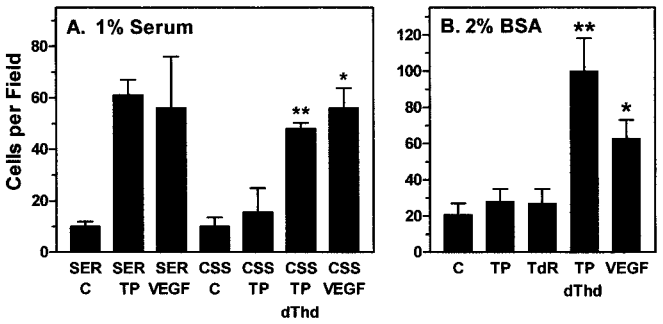

Fig. 2. TP required dThd to stimulate HUVEC migration. The Boyden chamber assay was used to investigate the effect of components of the medium on HUVEC migration. Migration to the underside of the transwell insert was quantitated as described in "Materials and Methods." The data are expressed as cells per field (mean ± SE of four experiments). *A*, assays contained M199 medium plus 1% serum (*SER C*), alone or with 100 ng/ml TP (*SER TP*) or 10 ng/ml VEGF (*SER VEGF*). In other experiments, the serum was replaced with 1% charcoal-stripped serum (CSS C), which was used in combination with TP (*CSS TP*), TP plus 100 nM thymidine (*CSS TP dThd*), or VEGF (*CSS VEGF*). *, Indicates significantly different from control (*CSS C*); **, indicates significantly different from control (*CSS C*)- and TP alone (*CSS TP*)-treated cells, $P < 0.05$. *B*, assays used serum-free M199 medium, which contained 2% BSA, and the indicated additions. *, Indicates significantly different from control (*C*); **, indicates significantly different from control-, TP-, and dThd-treated cells, $P < 0.05$.

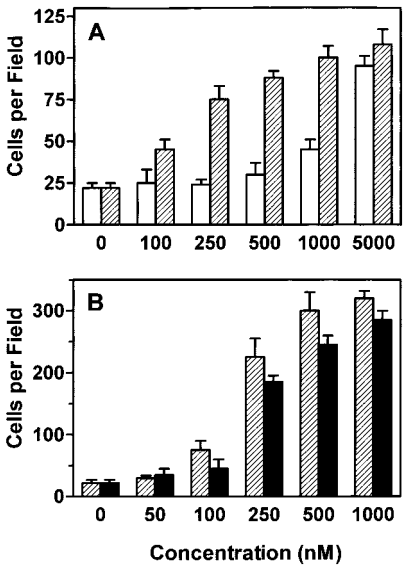

Fig. 3. The dThd metabolites dR-1-P and 2dR stimulated HUVEC migration. *A*, HUVEC migration in response to dR-1-P (□) and 2dR (▨) was determined as described in "Materials and Methods." Assays were in M199 medium with 1% serum. *B*, the effect of 2dR alone (▨) was compared with that of TP (100 ng/ml) in combination with indicated concentrations of dThd (■) on HUVEC migration in serum-free M199 medium containing 2% BSA. Migration to the underside of the transwell insert was quantitated after 5 h. The data are expressed as cells per field (mean ± SE) and are from four experiments.

Table 1 *Effect of API on HUVEC migration*

HUVECs were plated in the upper well of the Boyden chamber in 1% serum in M199, and the indicated treatments were added to the lower wells. 2dR and dR-1-P were used at 5 μM, TP at 100 ng/ml, and the API 2-thiophenylphosphonic acid was used at 10 μM.

Treatment	Cells migrated (per field)	
	− API	+ API
None	16 ± 4.3	19 ± 6.8
2dR	94 ± 11	84 ± 14
dR-1-P	93 ± 16	16 ± 3.9[a]
TP	88 ± 13	33 ± 4.3[a]

[a] Indicates significant effect of API, $P < 0.05$.

TP and dR-1-P Migration Was Attenuated by an API. We examined the possibility that the low potency of dR-1-P was attributable to the requirement that it first had to be converted to 2dR, which was in fact the active moiety. When the migration assay was repeated with the addition of an API to inhibit the conversion of dR-1-P to 2dR, the induction of migration by dR-1-P, but not 2dR, was blocked (Table 1). As would then be anticipated, TP-mediated migration was also inhibited by the API. The lack of effect of the API on migration induced by 2dR further showed that the API was not having a direct inhibitory or cytotoxic effect on the HUVECs. Because API was 1600-fold more potent an inhibitor of AP than of acid phosphatase (16), it was likely that an AP was present on the cells and was mediating the conversion of dR-1-P.

A Gradient of 2dR and dR-1-P Is Formed in the Presence of TP and HUVECs. We next sought to directly demonstrate the two critical components of TP action identified thus far: requirement of a chemotactic gradient, and production of an active dThd metabolite. The metabolism of [5′-^{3}H]dThd in the Boyden chamber assay was determined, using the same conditions as in the migration assays. The use of dThd in which the label was located in the 5′ position allowed for the analysis of the fate of the deoxyribose moiety of the nucleoside. [5′-^{3}H]dThd (5 μCi/ml) was added to the TP-containing medium in the lower wells at the start of the experiment; the upper wells of the chamber contained 10^5 HUVECs. Aliquots of the media from both the lower and the upper wells were removed at 1-, 3-, and 5-h intervals. dThd was first separated from its metabolites by the use of charcoal, and the metabolites were then further fractionated by anion exchange chromatography. The identity of the metabolites was confirmed by their coelution with authentic standards and by subsequent analysis by TLC.

dThd levels in the lower chamber progressively declined over 5 h, because of both its diffusion into the upper well (Fig. 4*A*) and its metabolism by TP (Fig. 4*B*). The concentration of dThd metabolites in the lower chamber were maximal by 1 h, with subsequent metabolism matched by diffusion of the metabolites into the upper chamber (Fig. 4*B*). Although the levels of dThd metabolites in the upper chamber increased linearly with time, the amount in the upper chamber was less than that in the lower chamber over the entire assay period, such that a gradient of dThd metabolites was formed and maintained in the Boyden chamber. Further analysis of the dThd metabolites found predominantly dR-1-P (95.8% of total), with the remainder 2dR (3.75%) and an additional peak of radioactivity, which constituted 0.46% of the total metabolites (Table 2). The latter radioactivity, eluted from the chromatography column midway between the 2dR and dR-1-P peaks, was neither acid nor base-labile and was not altered

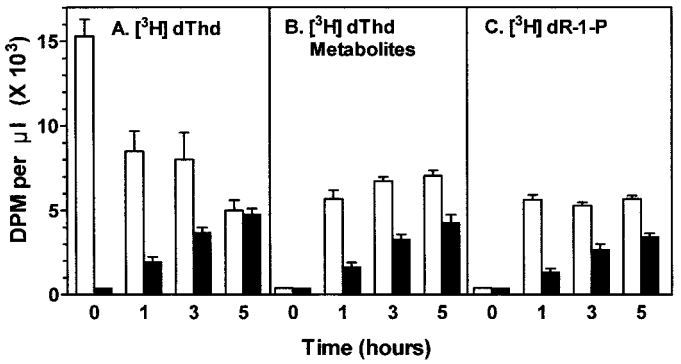

Fig. 4. Formation of a gradient of dThd metabolites by TP in Boyden chambers. The metabolism of [5′-^{3}H]dThd in the Boyden chamber assay was determined using the same conditions in which the HUVEC migration assays were carried out (see Fig. 1). [5′-^{3}H]dThd (5 μCi/ml) was added to the TP-containing medium in the lower wells at the start of the experiment; the upper wells of the chamber contained 10^5 HUVEC. Aliquots of medium from both the lower (□) and upper (■) wells were removed at the indicated times. [5′-^{3}H]dThd (*A*) was first separated from its ^{3}H-metabolites (*B*) by the use of charcoal; levels of [^{3}H]dR-1-P (*C*) were then determined after separation by anion exchange chromatography. Data are means ± SE of three experiments.

by acid phosphatase treatment. Although it was likely that this unknown was not a nucleoside nor a phosphorylated sugar, no further identification of its chemical nature was obtained. When the contributions of the TP and the HUVECs to the metabolism of [5′-^{3}H]dThd were assessed individually, the unknown metabolite was found to be formed by the HUVECs, whereas, as expected, formation of 2dR and dR-1-P were caused by the actions of the TP (Table 2). The levels of dR-1-P were maximal at 1 h in the lower well of the Boyden chamber, at which time they were 4.2-fold greater than the levels in the upper chamber (Fig. 4*C*). Although the levels of dR-1-P in the upper chamber increased linearly over time, a concentration gradient was maintained over the 5-h assay period; a similar gradient was found for 2dR (Table 2).

We had previously found that the TP inhibitor CIMU blocked TP-mediated HUVEC migration in a dose-dependent manner (15), and this was confirmed in these studies (IC_{50} of 25 μM, with complete inhibition at 100 μM; Fig. 5). At 100 μM, CIMU inhibited the metabolism of [5′-^{3}H]-thymidine by 97.5 ± 0.2% in the Boyden chamber assay. CIMU (100 μM) did not inhibit the migration of HUVECs in response to 2dR (1 μM) or VEGF (10 ng/ml), indicating that the inhibition was specific for TP (Fig. 5).

Elevated TP Expression in Cancer Cells and Monocytes Stimulated HUVEC Migration. The highest levels of TP in human tumors are found intracellularly and are located in both the tumor epithelial cells and in surrounding stromal tissue, including tumor-associated macrophages. Thus, to more appropriately analyze the mechanism by which cell-associated TP mediates EC migration, we used cell lines transfected with TP as the stimulus in the Boyden chamber assay. HUVECs were placed into the upper well of the Boyden chamber, and transfected HT29 colon or MCF7 breast cancer cells were seeded (2×10^5/well) in the lower chamber. The tumor cell lines had been transfected with either a control vector (neo) or one encoding TP (TPneo); transfection of HT29 and MCF7 cells increased TP activity 116- and 1840-fold, respectively, over the neo controls (Table 3). In addition, two human monocyte cell lines with high constitutive TP expression, THP1 and U937, were also used to stimulate HUVEC migration (Table 3).

Transfection of TP into MCF7 and HT29 cells increased HUVEC migration 2-fold over their neo-transfected counterparts (Fig. 6). THP1 and U937 monocytes also induced greater migration than did the control-transfected tumor cells; and THP1 cells, which had a higher TP expression than did U937, induced 50% more migration than did the U937. Although migration was not strictly linearly correlated with TP levels, the data indicated that cells that had a higher level of TP expression more actively stimulated HUVEC migration.

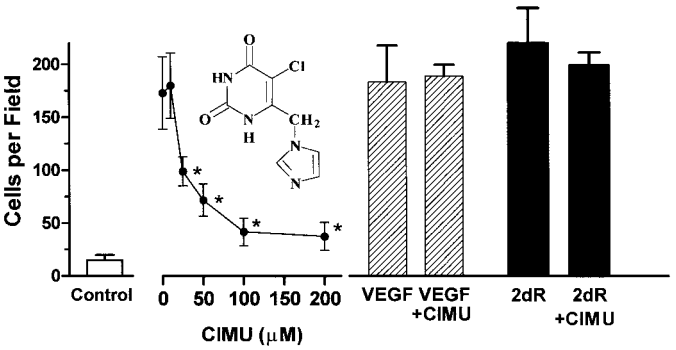

Fig. 5. The TP inhibitor CIMU selectively abrogated TP-mediated HUVEC migration. HUVEC migration assays contained, in the lower chamber, medium alone (*Control*), TP (100 ng/ml; ●), VEGF (10 ng/ml; ▨), or 2dR (1 μM; ■). The indicated concentrations of CIMU were added to the TP-containing wells; a CIMU concentration of 100 μM was added to VEGF and 2dR wells, as indicated. Migration to the underside of the transwell insert was quantitated after 5 h. The data are expressed as cells per field (means of four experiments ± SE). *, significantly different from TP alone ($P < 0.05$).

Table 2 *Analysis of [5′-^{3}H]dThd metabolites in medium in upper and lower wells of Boyden chamber at 5 h*

Analysis of the metabolism of [^{3}H]dThd was done under the same conditions in which the HUVEC migration assays were carried out (see legend to Fig. 1). The lower wells of the chambers contained either recombinant TP (100 ng/ml) or 2×10^5 TP-transfected MCF7 human breast cancer cells, TP-transfected HT29 human colon cancer cells, or THP1 human monocytic cells, in 0.7 ml of M199 medium with 1% serum; the upper wells contained 10^5 HUVECs in the same medium. [5′-^{3}H]dThd (5 μCi/ml) was added to the lower wells at the start of the experiment, and medium from either the lower or upper wells (or both) were analyzed after a 5-h incubation at 37°. To quantitate the formation of TP metabolites, the medium was first extracted with activated charcoal in 20 mM PIPES (pH 5.5), and then loaded on a 1-ml anion-exchange column, as described in "Materials and Methods." The columns were eluted with 20 mM PIPES (pH 5.5), and 0.5 ml fractions were collected, and the radioactivity in each fraction was determined by liquid scintillation counting. [^{3}H]2dR and [^{3}H]dR-1-P eluted in fractions 2 and 9, respectively, as determined with the use of authentic standards that were also loaded onto columns and monitored in the fractions using the diphenylamine reaction. Data are means ± SE of three experiments.

		[5′-^{3}H]dThd metabolite (dpm/µl)		
Treatment	Chamber	2dR	dR-1-P	Unknown
TP + HUVECs	Lower	224 ± 57	5671 ± 173	0
TP + HUVECs	Upper	133 ± 18	3446 ± 104	44 ± 2.6
TP + HUVECs	Lower + upper sum	357 (3.75%)	9117 (95.8%)	44 (0.46%)
TP + HUVECs	Lower/upper ratio	1.68	1.64	0
TP alone	Lower	290 ± 27	4595 ± 720	0
HUVECs alone	Upper	31 ± 15	11 ± 4	71 ± 11
MCF7/TP cells	Lower	339 ± 116	15 ± 10	1225 ± 76
HT29/TP cells	Lower	139 ± 21	15 ± 8	543 ± 98
THP1 cells	Lower	122 ± 26	21 ± 12	1141 ± 232

Table 3 *TP activity in cell extracts*

Cells were sonicated in ice-cold 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA, and were centrifuged. TP activity was measured in the supernatants in assays that contained 200 mM KH_2PO_4 (pH 7.8), 0.2 mM [5′-^{3}H]dThd (50 mCi/mmol), and 25–100 μg of protein, as described in "Materials and Methods." Reactions were stopped after 40 min at 37°C by the addition of 0.5 ml of ice-cold activated charcoal in 5% trichloroacetic acid, and radioactivity in the supernatant was determined by liquid scintillation counting. Values are means ± SE of at least three determinations.

Cell line	TP activity (pmol/mg/min)
HT29/neo	15 ± 4.8
HT29/TPneo	1744 ± 183
MCF7/neo	2.1 ± 1.3
MCF7/TPneo	3857 ± 175
U937	493 ± 91
THP1	2050 ± 348

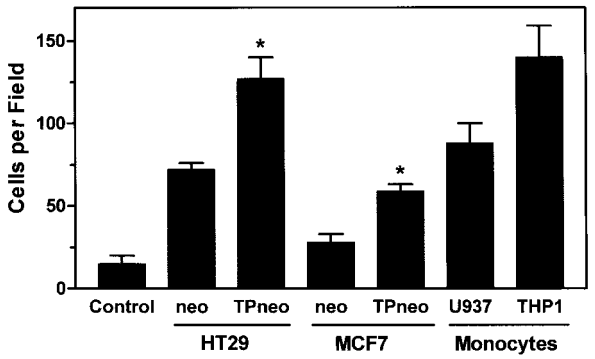

Fig. 6. Correlation between TP expression in cancer cells and monocytes and HUVEC migration. Lower wells of the Boyden chamber contained HT29 colon or MCF7 breast cancer cells, transfected with either a control vector (*neo*) or a vector encoding TP (*TPneo*), or the monocyte cell lines U937 and THP1. A control well had medium alone. Cells (2×10^5 per well) were allowed to attach overnight and were washed twice with PBS before the addition of HUVEC-containing transwell inserts to form the upper well. Migration of HUVECs was quantitated after 5 h and data expressed as cells per field (mean ± SE). ∗, significantly different from the corresponding neo-transfected cells ($P < 0.05$).

Analogous to the experiments with purified TP, the synthesis and release of [5′-^{3}H]dThd metabolites into the medium in wells containing the cell lines was examined. Metabolites accumulated in a linear manner in the conditioned media of all of the cell lines over the 5 h of the assay, and the levels in the medium were correlated with the TP activity of the cells (*i.e.*, MCF/TPneo > THP1 > HT29/TPneo > HT29/neo and MCF7neo; Fig. 7). The dThd metabolites that were released into the media by monocytes and TP-overexpressing tumor cell lines were next identified. In contrast to the experiment with purified TP, TP-expressing cells released much more 2dR than they did dR-1-P (6–20-fold higher, depending on the cell line; Table 2). Because 2dR was also found to be 10-fold more potent in inducing migration than was dR-1-P, it was likely that it was the active moiety responsible for cell-mediated migration. A large amount of the unknown deoxyribose metabolite was also released into the medium, and the possibility that it might have contributed to the chemotaxis has not been ruled out.

The Intracellular Activity of TP Was Sufficient to Mediate Chemotaxis. Although the experiment described above with TP-expressing cells suggests that the intracellular actions of TP were sufficient to mediate its effect on migration, it was possible that some of the TP was transported out of, or was otherwise released from, the cells and thereby exerted its actions extracellularly. To evaluate this possibility, we blocked the actions of TP using two approaches: with our small-molecule inhibitor of TP catalytic activity, CIMU, or with a neutralizing antibody to TP. Purified TP, TP-expressing HT29 and MCF7 cells, and THP1 monocytes were placed into the lower well of the Boyden chamber to stimulate HUVEC migration. The effect of either CIMU (100 μM), a neutralizing polyclonal antibody (100 μg/ml) against TP, or an isotype-matched control antibody (100 μg/ml) was observed on HUVEC migration (Fig. 8). The stimulation of migration mediated by the three cell lines (Fig. 8, *B*, *C*, and *D*), was significantly attenuated or abolished by CIMU but not by the TP antibody, even though the TP antibody blocked the migration induced by purified TP (Fig. 8*A*). Because the TP antibody acted only on extracellular TP, its lack of effect on cell-mediated migration sug-

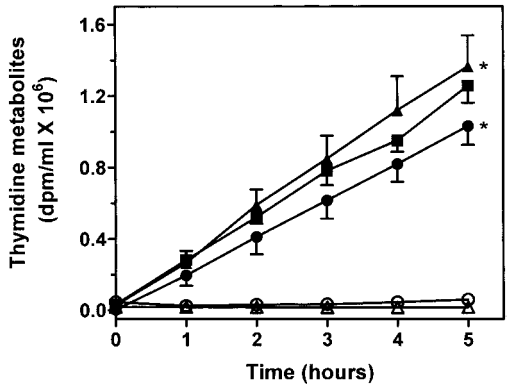

Fig. 7. Formation and release of [5′-^{3}H]dThd metabolites by TP-expressing cells. [5′-^{3}H]dThd (1 μCi/ml) was placed in the lower well of the Boyden chamber with 2×10^5 cells per well: MCF7/neo (△); HT29/neo (○); HT29/TPneo (●); Thp1 (■) and MCF7/TPneo (▲). At hourly intervals, aliquots of the media were removed and analyzed for the formation of dThd metabolites, as described in "Materials and Methods." Data are means ± SE of three experiments. ∗, significantly different from the corresponding neo-transfected cells ($P < 0.05$).

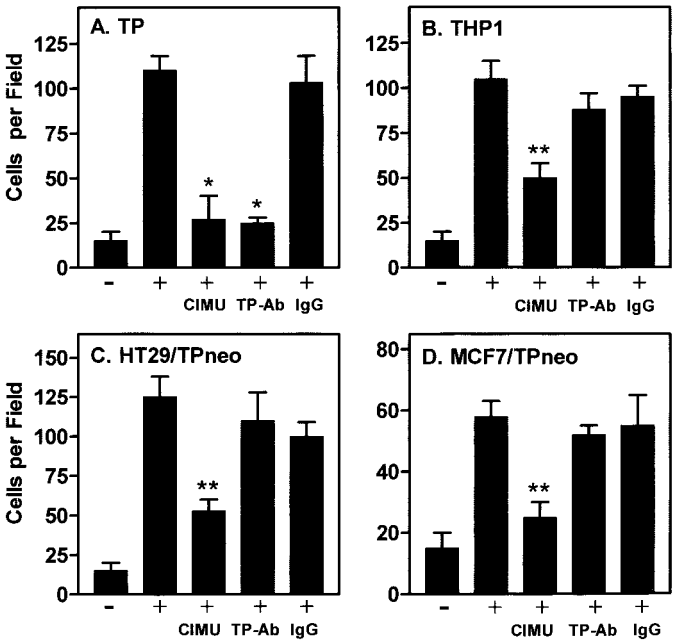

Fig. 8. CIMU, but not a neutralizing antibody against TP, attenuated cancer cell and monocyte-mediated HUVEC migration. HUVECs were plated in the upper well of the Boyden chamber in 1% serum in M199, in the absence (−) or presence (+) of the following stimulants of migration in the lower wells: purified TP (*A*); THP1 cells (*B*); HT29/TPneo cells (*C*); and MCF/TPneo cells (*D*). Also included, as indicated, were the TP inhibitor CIMU (100 μM), a TP-neutralizing polyclonal antibody (*TP-Ab*, 100 μg/ml), or an isotype-matched control antibody (*IgG*, 100 μg/ml). HUVEC migration was quantitated after 5 h, and data were expressed as cells per field (mean ± SE). ∗, significantly different from cells treated with a chemotactic stimulus alone; ∗∗, significantly different from cells treated with a chemotactic stimulus alone and from cells treated with a chemotactic stimulus plus the TP antibody ($P < 0.05$).

gested that the intracellular catalytic actions of TP were sufficient to stimulate monocyte- and tumor cell-mediated HUVEC chemotaxis.

DISCUSSION

By using a coculture assay in which TP that was expressed in tumor cells in one well stimulated the migration of ECs in a separate well, we demonstrated that the intracellular catalytic activity of TP was sufficient to induce maximal migration, and that dThd metabolites of TP were released into the medium and formed a concentration gradient between the two wells. The gradient was required for the actions of TP, consistent with a chemotactic response. The data further identified 2dR as the active moiety that mediated the chemotactic actions of TP in cells expressing high levels of the angiogenic factor. Thus, there was substantially more 2dR released from the cells than there was dR-1-P, and pure 2dR was 10-fold more potent an inducer of migration than was dR-1-P. dR-1-P, formed intracellularly, would not be predicted to be able to diffuse into the extracellular space; thus, its conversion to 2dR likely occurs as a result of the actions of an intracellular phosphatase. The abrogation of migration mediated by exogenously added dR-1-P by an inhibitor of AP suggested that the observed activity of dR-1-P may, in fact, have been attributable to its conversion to 2dR, and this was probably directly associated with an AP activity on the surface of the HUVECs. Ecto-AP activity has been found on the microvasculature in a variety of tissues, and capillary ECs express AP on their surface membrane both *in vivo* and *in vitro* (18–21). This activity could also be responsible for the conversion of any dR-1-P that occurs extracellularly, including the conversion that occurs as a consequence of cell lysis associated with necrosis. Elevated levels of cellular AP have also been found to be associated with aggressiveness in some tumor types (22), and it is possible that the role of AP in TP angiogenesis could contribute to this clinical observation.

The question remains as to the metabolite responsible for the actions of purified TP. The inhibitory effect of the API strongly suggests that 2dR, derived from dR-1-P, mediated the effect of TP. We did not detect a significant decrease in 2dR levels when an API was used to block dR-1-P-mediated migration (data not shown); however, our analytical methods may not have been sufficiently sensitive to detect a relatively small change. It is also possible that a localized concentration of 2dR, not detectable in the bulk media, may be sufficient to initiate cell migration. Additionally, 2dR that is derived from dR-1-P at the cell surface may be simultaneously metabolized and/or internalized as it was produced, resulting in no net formation of extracellular 2dR. The mechanism by which 2dR might be exerting a chemotactic effect on ECs is unknown. We have recently found, however, that 2dR can activate signaling pathways involved in HUVEC migration, including effects on integrins and the tyrosine phosphorylation of focal adhesion kinase (23).

The TP protein lacks a hydrophobic signal sequence and is secreted slowly, if at all, from cells (6, 24). This is consistent with our data that demonstrated that efflux of TP from the cells was not required for it to exert its full angiogenic activity. Although TP is found in both the cytoplasm and the nucleus, one clinicopathological study of TP overexpression in head and neck tumors found that angiogenesis was correlated with nuclear, but not cytoplasmic, TP levels; thus, the subcellular distribution of TP might also influence its angiogenic activity *in vivo* (25). Studies that examined TP expression in human colorectal, breast, and other cancers by immunohistochemistry found that TP was frequently highly expressed in the normal infiltrating cells in the tumor, including tumor-associated macrophages and other stromal cells, and that this occurred both in conjunction with expression in the tumor cells and, in some cases, in the absence of expression in the tumor neoplastic epithelial cells (10, 12, 26–29). This is noteworthy because it is consistent with the observation that macrophages derived from tumor stroma were capable of inducing an angiogenic response (30). The two monocyte cell lines that we studied had constitutive levels of TP activity that were higher than 10 different colon carcinoma cell lines (data not shown), consistent with *in vivo* observations (10, 12, 26–29). It has been reported that TP activity in 32 human colon carcinoma biopsies averaged 3350 pmol/min/mg (12); thus, the levels of TP activity in the THP1 monocytes and the two TP-transfected cell lines used in this study (2050, 1744, and 3857 pmol/mg/min, respectively) were representative of the activity seen in tumors *in vivo*. TP expression can be further induced in both tumor cells and monocytes by inflammatory cytokines, including IFN and tumor necrosis factor α (31–34). Our migration data support the suggestion that cells expressing high levels of TP *in vivo*, whether they are tumor cells or stromal tissue, can contribute to the neovascularization process. TP has also been shown to be identical to gliostatin, a factor found in central nervous system glial cells; both inhibited glial cell proliferation and had neurotrophic actions on cortical neurons (*i.e.*, increased neuronal survival and neurite outgrowth; Ref. 35). The reported ED_{50} for the neurotrophic actions of gliostatin was identical to our observed effect on ECs, suggesting that a similar mechanism of action could be operational.

In conclusion, we have provided direct evidence that the angiogenic actions of TP are likely caused by the intracellular synthesis, extracellular release, and formation of a concentration and chemotactic gradient of the dThd metabolite, 2dR.

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REFERENCES

1. Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M., Akiyama S-I, Fukui, K., Ishizawa, M., and Yamada, Y. Angiogenic factor. Nature (Lond.), *356:* 668, 1992.
2. Moghaddam, A., and Bicknell, R. Expression of PD-ECGF factor in *E. coli* and confirmation of its thymidine phosphorylase activity. Biochemistry, *31:* 12141–12146, 1992.
3. Sumizawa, T., Furukawa, T., Haraguchi, M., Yoshimura, A., Takeysu, A., Ishizawa, M., Yamada, Y., and Akiyama, S-I. Thymidine phosphorylase activity associated with platelet-derived endothelial cell growth factor. J. Biochem., *114:* 9–14, 1993.
4. Finnis, C., Dodsworth, N., Pollitt, C. E., Carr, G., and Sleep, D. Thymidine phosphorylase activity of platelet-derived endothelial cell growth factor is responsible for endothelial cell mitogenicity. Eur. J. Biochem., *212:* 201–210, 1993.
5. Haraguchi, M., Miyadera, K., Uemura, K., Sumizawa, T., Furukawa, T., Yamada, K., and Akiyama, S-I. Angiogenic activity of enzymes. Nature (Lond.), *368:* 198–199, 1994.
6. Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Usuki, K., Takaku, F., Risau, W., and Heldin, C-H. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature (Lond.), *338:* 557–562, 1989.
7. Moghaddam, A., Zhang, H-T, Fan, T-P. D., Hu, D-E., Lees, V. C., Turley, H., Fox, S. B., Gatter, K. C., Harris, A. L., and Bicknell, R. Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc. Nat. Acad. Sci. USA, *92:* 998–1002, 1995.
8. Luccioni, C., Beaumatin, J., Bardot, V., and Lefrancois, D. Pyrimidine nucleotide metabolism in human colon carcinomas: comparison of normal tissues, primary tumors and xenografts. Int. J. Cancer, *58:* 517–522, 1994.
9. Takabayashi, Y., Akiyama, S., Akiba, S., Yamada, K., Miyadera, K., Sumizawa, T., Yamada, Y., Murata, F., and Aikou, T. Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phosphorylase, in human colorectal cancer. J. Natl. Cancer Inst. (Bethesda), *88:* 1110–1117, 1996.
10. Takahashi, Y., Bucana, C. D., Liu, W., Yoneda, J., Kitadai, Y., Cleary, K. R., and Ellis, L. M. Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis: role of infiltrating cells. J. Natl. Canc. Inst. (Bethesda), *88:* 1146–1151, 1996.
11. Relf, M., LeJeune, S., Scott, P. A. E., Fox, S., Smith, K., Leek, R., Moghaddam, A., Whitehouse, R., Bicknell, R., and Harris, A. L. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor β-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relations to angiogenesis. Cancer Res., *57:* 963–969, 1997.

12. van Triest, B., Pinedo, H. M., Blaauwgeers, J. L. G., van Diest, P. J., Schoenmakers, P. S., Voorn, D. A., Smid, K., Koekman, K., Hoitsma, H. F. W., and Peters, G. F. Prognostic role of thymidylate synthase, thymidine phosphorylase/platelet-derived endothelial cell growth factor, and proliferation markers in colorectal tumors. Clin. Cancer Res., *6:* 1063–1072, 2000.
13. Miyadera, K., Sumizawa, T., Haraguchi, M., Yoshida, H., Konstanty, W., Yamada, Y., and Akiyama, S. Role of thymidine phosphorylase activity in the angiogenic effect of platelet-derived endothelial cell growth factor/thymidine phosphorylase. Cancer Res., *55:* 1687–1690, 1995.
14. Brown, N. S., Jones, A., Fujiyama, C., Harris, A. L., and Bicknell, R. Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors. Cancer Res., *60:* 6298–6302, 2000.
15. Klein, R. S., Lenzi, M., Lim, T. H., Hotchkiss, K. A., Wilson, P., and Schwartz, E. L. Novel 6-substituted uracil analogs as inhibitors of the angiogenic actions of thymidine phosphorylase. Biochem. Pharmacol., *62:* 1257–1263, 2001.
16. Myers, J. K., Antonelli, S. M., and Widlanski, T. S. Motifs for metallophosphatase inhibition. J. Am. Chem. Soc., *119:* 3163–3164, 1997.
17. Schwartz, E. L., Baptiste, N., Wadler, S., and Makower, D. Thymidine phosphorylase mediates the sensitivity of human colon carcinoma cells to 5-fluorouracil. J. Biol. Chem., *270:* 19073–19077, 1995.
18. Low, M. G., and Finean, J. B. Release of alkaline phosphatase from membranes by a phosphatidyl inositol-specific phospholipase C. Biochem. J., *167:* 281–284, 1977.
19. Higgins, J. C., and Eady, R. A. Human dermal microvasculature: II. Enzyme histochemical and cytochemical study. Br. J. Dermatol., *104:* 521–529, 1981.
20. Roberts, R. L., and Sandra, A. Apical-basal membrane polarity of membrane phosphatases in isolated capillary endothelium: alteration in ultrastructural localisation under culture conditions. J. Anat., *182:* 339–347, 1993.
21. Calhau, C., Martel, F., Pinheiro-Silva, S., Pinheiro, H., Soares-da-Silva, P., Hipolito-Reis, C., and Azevedo, I. Modulation of insulin transport in rat brain microvessel endothelial cells by an ecto-phosphatase activity. J. Cell. Biochem., *84:* 389–400, 2002.
22. Manivel, J., Jessurun, J., Wick, M., and Dehner, L. Placental alkaline phosphatase immunoreactivity in testicular germ-cell neoplasms. Am. J. Surg. Pathol., *11:* 21–29, 1987.
23. Hotchkiss, K. A., Ashton, A. W., and Schwartz, E. L. Endothelial cell migration stimulated by thymidine phosphorylase is mediated via the integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$. Proc. Am. Assoc. Cancer Res., *43:* 31, 2002.
24. Usuki, K., Heldin, N-E., Miyazono, K., Ishikawa, F., Takaku, F., Westermark, B., and Heldin, C-H. Production of platelet-derived endothelial cell growth factor by normal and transformed human cells in culture. Proc. Natl. Acad. Sci. USA, *86:* 7427–7431, 1989.
25. Koukourakis, M. I., Giatromanolaki, A., Fountzilas, G., Siviridis, E., Gatter, K. C., and Harris, A. L. Angiogenesis, thymidine phosphorylase, and resistance of squamous cell head and neck cancer to cytotoxic and radiation therapy. Clin. Cancer Res., *6:* 381–389, 2000.
26. Koukourakis, M. I., Giatromanolaki, A., Kakolyris, S., O'Byrn, K. J., Apostolikas, N., Skarlatos, J., Gatter, K. C., and Harris, A. L. Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small cell lung cancer: impact on tumor neoangiogenesis and survival. Br. J. Cancer, *77:* 1696–1703, 1998.
27. Lee, A. H. S., Dublin, E. A., and Bobrow, L. G. Angiogenesis and expression of thymidine phosphorylase by inflammatory cells in ductal carcinoma *in situ* of the breast. J. Pathol., *187:* 285–290, 1999.
28. Engels, K., Fox, S. B., Whitehous, R. M., Gatter, K. C., and Harris, A. L. Up-regulation of thymidine phosphorylase expression is associated with a discrete pattern of angiogenesis in ductal carcinomas *in situ* of the breast. J. Pathol., *182:* 414–420, 1997.
29. Takahasi, Y., Bucana, C. D., Akagi, Y., Liu, W., Cleary, K. R., Mai, M., and Ellis, L. M. Significance of platelet-derived endothelial cell growth factor in the angiogenesis of human gastric cancer. Clin. Cancer Res., *4:* 429–434, 1998.
30. Polverini, P. J., Cotran, R. S., Gimbrone, M. A., and Unanue, E. M. Activated macrophages induce vascular proliferation. Nature (Lond.), *269:* 804–806, 1977.
31. Eda, H., Fujimoto, K., Watanabe, S., Ura, M., Hino, A., Tanaka, Y., Wada, K., and Ishitsuka, H. Cytokines induce thymidine phosphorylase in tumor cells and make them more susceptible to 5′-deoxy-5-fluorouridine. Cancer Chemother. Pharmacol., *32:* 333–338, 1993.
32. Schwartz, E. L., Wan, E., Wang, F., and Baptiste, N. Regulation of expression of thymidine phosphorylase/platelet-derived endothelial cell growth factor in human colon carcinoma cells. Cancer Res., *58:* 1551–1557, 1998.
33. Goto, H., Kohno, K., Sone, S., Akiyama, S., Kuwano, M., Ono, M. Interferon γ-dependent induction of thymidine phosphorylase/platelet-derived endothelial growth factor through γ-activated sequence-like element in human macrophages. Cancer Res., *61:* 469–473, 2001.
34. Zhu, G. H., Lenzi, M., and Schwartz, E. L. The Sp1 transcription factor contributes to the constitutive and tumor necrosis factor-induced expression of the angiogenic factor thymidine phosphorylase in human colon carcinoma cells. Oncogene, *21:* 8477–8485, 2002.
35. Asai, K., Nakanishi, K., Isobe, I., Eksioplu, Y. Z., Hirano, A., Hama, K., Miyamoto, T., and Kato, T. Neurotrophic action of gliostatin on cortical neurons. Identity of gliostatin and platelet-derived endothelial cell growth factor. J. Biol. Chem., *267:* 20311–20316, 1992.

Mechanisms by Which Tumor Cells and Monocytes Expressing the Angiogenic Factor Thymidine Phosphorylase Mediate Human Endothelial Cell Migration

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