Thymidine Phosphorylase Induces Carcinoma Cell Oxidative Stress and Promotes Secretion of Angiogenic Factors

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Abstract

Thymidine phosphorylase (TP) (E.C. 2.4.2.4), also known as platelet-derived endothelial cell growth factor, is a potent angiogenic factor. The expression of TP correlates with poor prognosis in a range of tumor types. 2-Deoxy-o-ribose-1-phosphate, a product of thymidine catabolism by TP, is a strongly reducing sugar that generates oxygen radical species during the early stages of protein glycation. We show that thymidine induces oxidative stress in TP-overexpressing carcinoma cells, promoting secretion of the stress-induced angiogenic factors vascular endothelial growth factor and interleukin-8, and inducing matrix metalloproteinase-1. Our findings outline a putative mechanism for TP-induced angiogenesis and identify novel targets for intervention.

Introduction

In 1987 a novel angiogenic factor, platelet-derived endothelial cell growth factor, was isolated (1). Five years later, platelet-derived endothelial cell growth factor was shown to be a previously characterized intracellular enzyme, TP (2). TP strongly induces neovascularization in the rat sponge model, and TP-transfected breast carcinoma cell lines exhibit accelerated growth in mouse xenografts (3). Increased TP activity promotes angiogenesis in a range of pathologies, and TP overexpression correlates with poor prognosis in many human tumor types (4). When the expression of seven angiogenic factors was quantified in primary human breast tumors, TP was found to be the sole agent correlating with vascular density (4). TP levels also predict malignancy and blood flow in ovarian cancer (4). Despite a wealth of data linking TP and angiogenic pathology, the molecular mechanisms underlying this link have thus far remained obscure. Other angiogenic factors are secreted by the carcinoma cell to act directly on endothelial cells. TP, however, is not secreted and remains within the carcinoma cells. Site-directed mutagenesis and antibody studies have proved that promotion of vessel growth by TP is dependent on the enzyme activity of TP, the catabolism of thymidine to thymine and 2dR1P (3). In this study, we obtain results suggesting that TP activity induces carcinoma cell oxidative stress, causing the tumor cell to secrete oxidative-stress-responsive angiogenic factors. TP may therefore promote angiogenesis by triggering carcinoma cell secretion of other, direct-acting, angiogenic factors.

Materials and Methods

Cell Culture. Cells were cultured in DMEM containing 25 mm glucose and 10% FCS. Experiments and pre-experiment incubations were performed in DMEM containing 5.5 mm glucose, 0.01% BSA, 45 µg/ml streptomycin, 45 µg/ml penicillin, and 40 µg/ml kanamycin (Life Technologies, Inc., Paisley, United Kingdom).

Preparation of RT112-EV and RT112-TP. The human bladder carcinoma cell line RT112 was a gift from Dr. M. Knowles (Imperial Cancer Research Fund, Leeds, United Kingdom). Two pBABE vectors were prepared (an empty vector and a vector containing full-length cDNA for human TP). The vectors were incorporated into retroviral particles, and 5 × 10⁶ semiconfluent RT112 cells were infected with 1 × 10⁶ colony-forming units of neat retroviral supernatant. The medium was changed after 4 h, and the cells were grown to confluence. They were then split and selected with a previously determined kill concentration of puromycin (1 µg/ml). Clones were picked, and their clonogenic efficiency and growth rates were determined in triplicate. An empty vector clone (RT112-EV) and a TP-overexpressing clone (RT112-TP) with similar in vitro growth rates were used in subsequent experiments.

Immunoblotting for TP. RT112-EV and RT112-TP were washed twice with ice-cold phosphate-buffered saline/2 mm EDTA, harvested, and lysed using an IKA ultraturrax homogenizer (Janké & Kunkel, Staufen, Germany). Lysates were incubated in sodium phosphate/SDS buffer (pH 7.2) containing “Complete, EDTA-free” protease inhibitors (Roche Diagnostics, Lewes, United Kingdom). Lysates were microcentrifuged for 5 min to remove particulates. The protein contents of the lysates were determined by the bicinchoninic acid protein assay (Pierce & Warriner, Chester, United Kingdom). Protein lysate (50 µg) was separated by electrophoresis on a 12% acrylamide gel and transferred to Immobilon-P membranes as described for the TP immunoblot. The membrane was probed overnight at 4°C with an anti-HO-1 monoclonal antibody PGF44C (hybridoma supernatant diluted 1:200; Ref. 3). Goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako, Ely, United Kingdom) was then added at 50 µg/ml. Proteins were visualized using the Renaissance enhanced luminol system (New England Nuclear, Hounslow, United Kingdom) and Hyperfilm (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Immunoblotting for HO-1. RT112-EV and RT112-TP were cultured in 15-cm dishes to approximately 80% confluence. The growth medium was then replaced with serum-free 5.5 mm glucose medium, and the cells were incubated for an additional 24 h. For the blot in Fig. 1B, RT112-EV and RT112-TP were treated for 16 h in the absence of thymidine or with 200, 500, or 1000 µg/ml. For Fig. 1D, RT112-TP cells were pretreated for 6 h with 0, 1.5, or 4 mm N-acetyl cysteine and then treated for 12 h with 0, 200, or 500 µg/ml thymidine in the presence of 0, 1.5, or 4 mm N-acetyl cysteine (pH 7.4). The membrane was probed overnight at 4°C with an anti-HO-1 mouse monoclonal antibody (Stressgen, Victoria, Canada) at 0.6 µg/ml and an anti-β-tubulin mouse monoclonal antibody (Sigma-Aldrich) at 0.26 µg/ml. The membrane was probed overnight at 4°C with an anti-HO-1 mouse monoclonal antibody (Stressgen, Victoria, Canada) at 0.6 µg/ml and an anti-β-tubulin mouse monoclonal antibody (Sigma-Aldrich) at 0.26 µg/ml. The membrane was probed overnight at 4°C with an anti-HO-1 mouse monoclonal antibody (Stressgen, Victoria, Canada) at 0.6 µg/ml and an anti-β-tubulin mouse monoclonal antibody (Sigma-Aldrich) at 0.26 µg/ml.
RT112-EV and RT112-TP were incubated with various concentrations of thymidine for 16 h with 0 or 200 μM thymidine in the absence or presence of 10 μM thymine. The TPI used in Fig. 2B was a gift from Dr. M. Toi (Tokyo Metropolitan Hospital, Tokyo, Japan). At the end of 16 h, the conditioned medium was collected, microcentrifuged for 5 min to remove particulates, and frozen at −80°C for future use in ELISAs. Meanwhile, the number of cells in each well was determined. One ml of trypsin was added to each well, and then a Coulter Z2 cell counter (Beckman-Coulter, High Wycombe, United Kingdom) was used to determine the number of cells in 400 μl of the trypsinized cell suspension. The concentrations of all three factors in the conditioned medium was determined by ELISA [IL-8 and VEGF kits, R&D Systems (Abingdon, United Kingdom); MMP-1 kit, Amersham Pharmacia Biotech]. The concentrations determined by ELISA were then divided by the number of cells in the corresponding wells to obtain a result in picograms of factor produced per million cells per 16 h.

Statistical Analysis of ELISA Results. Each treatment was performed in triplicate. Results from representative experiments are expressed graphically as the mean ± SD (n = 3). A two-tailed Student’s t test was used within each individual experiment to determine the significance of any changes in angiogenic factor production.

Results

In bladder carcinoma, there is a strong correlation between TP expression and tumor invasion into the bladder wall (5). We modelled TP up-regulation during the progression to invasive bladder carcinoma by producing two transfectant cell lines derived from the noninvasive bladder carcinoma cell line RT112. RT112-TP is a cell line with low TP expression similar to that of superficial bladder carcinoma. It was produced by transfecting RT112 with a control empty vector. RT112-TP is a cell line with high TP expression similar to that of invasive bladder carcinoma. It was obtained by transfecting RT112 with full-length human TP cDNA. TP immunoblotting confirmed that the TP levels of RT112-EV and RT112-TP (Fig. 1A) are similar to the TP expression of superficial and invasive bladder tumors, respectively (5). In addition, the difference in TP levels between RT112-EV and RT112-TP is comparable to that between normal breast tissue and TP-overexpressing primary human breast carcinomas (3).

Thymidine concentrations in the tumor microenvironment are raised by the hydrolysis of DNA from necrotic cells in hypoxic areas (6). We replicated this effect in vitro by supplementing cell culture medium with exogenous thymidine. Tumor cells that overexpress TP rapidly phosphorylyse thymidine, releasing thymine and 2dDR1P. Thus, when the medium is supplemented with exogenous thymidine, increased TP activity and thymidine catabolism will occur within RT112-TP, but not within RT112-EV.

RT112-EV and RT112-TP were treated with thymidine for 16 h; then cellular levels of HO-1, a classic marker of cellular oxidative stress (7), were quantified by immunoblot. Thymidine had little effect on the HO-1 expression of RT112-EV. In RT112-TP, however, thymidine up-regulated HO-1 in a dose-dependent manner (Fig. 1, B and C). To prove that cellular oxidative stress is responsible for HO-1 induction, the experiment was repeated in the presence of the antioxidant N-acetyl cysteine. Concentrations of 1.5 and 4 mM N-acetyl cysteine abrogated HO-1 induction in RT112-TP (Fig. 1D). The experiment was also repeated in the presence of excess thymine. Thymine (2.5 mM) prevented the thymidine-dependent induction of HO-1 in RT112-TP (Fig. 1E).
Next, we characterized carcinoma cell responses downstream of TP-induced stress. RT112-EV and RT112-TP were treated with thymidine for 16 h, and the IL-8 concentrations of the conditioned media were determined by ELISA. Thymidine (200 μM) increased IL-8 secretion by RT112-TP approximately 6-fold but had no effect on IL-8 production by RT112-EV (Fig. 2A). Thus, TP activity increases carcinoma cell IL-8 secretion. The Ps for IL-8 induction in three separate experiments were 0.0273, 0.0029, and 0.0028 (two-tailed t test). Excess thymine blocked IL-8 up-regulation in thymidine-treated RT112-TP cells but had little effect on the baseline level of IL-8 production. In a separate experiment, TPI prevented the induction of IL-8 in thymidine-treated RT112-TP cells (Fig. 2B).

VEGF production by RT112-TP was increased approximately 2-fold by 200 μM thymidine, but thymidine had no effect on the VEGF secretion of RT112-TP (Fig. 2C). The Ps for VEGF induction in two separate experiments were 0.0047 and 0.0014 (two-tailed t test). As with IL-8, excess thymine blocked the thymidine-dependent increase in VEGF secretion. Production of MMP-1 by the two transfected cell lines was also determined. As with IL-8 and VEGF, MMP-1 was induced by thymidine in RT112-TP, but not in RT112-EV (Fig. 2D). The Ps for induction of MMP-1 in two separate experiments were 0.0158 and 0.0414 (two-tailed t test). Again, this effect was abrogated by excess thymine.

**Discussion**

We have proved that the addition of thymidine to a TP-overexpressing cell line induces cellular oxidative stress. TP is known to catabolize thymidine to 2dDR1P and thymine. Thymine (2.5 mm)
abrogated the thymidine-dependent induction of cellular oxidative stress. Excess thymine will reverse thymidine catabolism and scavenge 2dDR1P. These findings therefore imply that 2dDR1P is responsible for TP-induced oxidative stress. Thus far, we have no direct proof of this, but reducing sugars are known to be capable of oxygen radical generation. For example, free radical production by glucose during hyperglycemic episodes is implicated in a number of diabetic pathologies (8, 9). This is due to the fact that reducing monosaccharides exist as a closed hemiacetal ring in equilibrium with an open chain aldehyde. The open chain aldehyde glycates proteins via a series of nonenzymatic reactions, and these reactions produce free radicals (10). Equilibrium shifts toward the open chain aldehyde if the monosaccharide’s carbon backbone is short or phosphate groups are present (10). 2dDR and 2dDR1P therefore have two of the highest proportions of sugar present in the open chain form, placing the 2dDR sugar family among the most powerful radical generators (10). For instance, 10 mM 2dDR is sufficiently pro-oxidant to trigger apoptosis in human fibroblasts (11). The 2dDR1P released from thymidine by TP is therefore a potent protein-glycating agent and generator of oxygen radicals.

Our putative mechanism for TP-driven radical production involves transition metal-catalyzed autoxidation of 2dDR1P (Fig. 3A). This process begins with the conversion of 2dDR1P to 2dDR5P by the enzyme phosphopentomutase (E.C. 5.4.2.7). 2dDR5P can then couple to an intracellular protein by nonenzymatic condensation between the sugar’s aldehyde group and a lysine residue. The resultant Schiff base then rearranges to an Amadori product. One possible Amadori product of 2dDR5P is an α-hydroxyketone. All compounds containing the α-hydroxyketone group may form an enediol intermediate (12), and in the presence of oxidized transition metal ions such as Cu(II) or Fe(III), the enediol reacts with molecular oxygen, forming a dicarbonyl and a superoxide radical (13). Superoxide dismutase then converts superoxide to hydrogen peroxide, which can enter the Fenton reaction to produce highly toxic hydroxyl radicals. By this mechanism, 2dDR1P may generate an assortment of oxygen radical species, placing the TP-overexpressing carcinoma cell under oxidative stress.

We have also proved that thymidine catabolism by TP increases carcinoma cell secretion of IL-8, VEGF, and MMP-1, three factors known to be induced by oxidative stress (14–17). IL-8 and VEGF are directly and potently angiogenic. Endothelial cell secretion of MMP-1, an interstitial collagenase, has been shown to assist angiogenesis in vivo (18), and it follows that tumor cell production of MMP-1 should facilitate blood vessel growth in the tumor microenvironment. Both thymine and TPI prevented the induction of these downstream angiogenic factors. It therefore appears likely that the production of 2dDR1P is required for these responses. Our results raise the possibility that IL-8, VEGF, and MMP-1 are responsible for the angiogenic activity of TP in vivo.

These results outline a mechanism that may link TP activity and angiogenesis (Fig. 3B). Characterizing the angiogenic pathways downstream of TP activity will allow us to uncouple angiogenesis from thymidine phosphorylase. This is of clinical relevance because although the angiogenic activity of TP is clearly undesirable, TP’s activation of prodrugs such as furtalon (5'-deoxy-5-fluorouridine) and 5-fluorouracil increases tumor-specific toxicity (19, 20). Blocking TP-induced angiogenesis downstream of TP activity could prevent the induction of blood vessel growth without affecting TP’s activation of prodrugs.

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