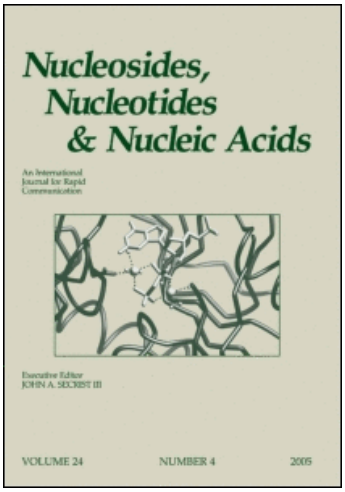

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THE ROLE OF PLATELET-DERIVED ENDOTHELIAL CELL GROWTH FACTOR/THYMIDINE PHOSPHORYLASE IN TUMOR BEHAVIOR

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□ *Platelet-derived endothelial cell growth-factor (PD-ECGF) is similar to the pyrimidine enzyme thymidine phosphorylase (TP). A high TP expression at tumor sites is correlated with tumor growth, induction of angiogenesis, and metastasis. Therefore, high TP is most likely associated with a poor prognosis. TP is not only expressed in tumor cells but also in tumor surrounding tissues, such as tumor infiltrating macrophages. TP catalyzes the conversion of thymidine to thymine and doxyribose-1-phosphate (dR-1-P). The latter in its parent form or in its sugar form, deoxyribose (dR) may play a role in the induction of angiogenesis. It may modulate cellular energy metabolism or be a substrate in a chemical reaction generating reactive oxygen species. L-deoxyribose (L-dR) and thymidine phosphorylase inhibitor (TPI) can reverse these effects. The mechanism of TP induction is not yet completely clear, but TNF, IL10 and other cytokines have been clearly shown to induce its expression. The various complex interactions of TP give it an essential role in cellular functioning and, hence, it is an ideal target in cancer therapy.*

Keywords Thymidine phosphorylase; fluoropyrimidines; angiogenesis; migration

INTRODUCTION

The platelet derived endothelial cell growth factor (PD-ECGF) is identical to the enzyme thymidine phosphorylase (TP) and participates in many pathological and nonpathological processes. TP is upregulated in many tumor types, and this upregulation has been related to a poor prognosis (reviewed by Takebayashi).[1] High TP expression at tumor-sites has been correlated to higher microvessel density (MVD), increased angiogenesis and increased metastasis.[2,3] TP expression at tumor sites is not only due to expression in tumor cells, but TP can also be expressed in tumor stroma, including infiltrating macrophages. Besides cancer, TP is also upregulated

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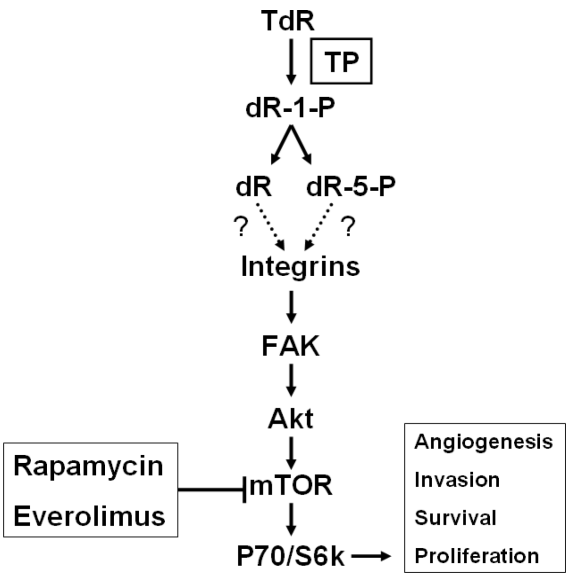

FIGURE 1 Possible pathway of activation of intracellular kinases that can signal towards angiogenesis, invasion, proliferation and survival. The question-marks indicate the gap between formation of the sugars deoxyribose (dR) or deoxyribose-5-phosphate (dR-5-P) and the activation of integrins and the downstream kinases.

in other diseases in which angiogenesis and macrophage infiltration plays a role, including rheumatoid arthritis (RA).[4]

TP catalyzes the phosphorolytic cleavage of thymidine (TdR) to thymine and deoxyribose-1-phosphate (dR-1-P; Figure 1). The latter, in its parent form or in its sugar form, deoxyribose (dR) plays a role in angiogenesis. Besides this, it is often involved in the metabolism of fluoropyrimidines. This can be both by activation and degradation of anticancer compounds. Examples of compounds that can be activated are 5-FU and 5′DFUR.[5] By contrast, trifluorothymidine (TFT) can be degraded by TP to inactive TF-Thymine.[5,6]

This review focuses on the role of TP and the products formed (dR-1-P, dR, and dR-5-P) by TP enzymatic activity in activation of intracellular kinases. Besides this, regulation of TP expression and the influence of the various TP-expression locations (e.g., stromal or tumor) in tumor tissues on tumor growth and progression are discussed.

SIGNALING TOWARDS CELL MIGRATION

Angiogenesis is the formation of new vasculature from existing vasculature, a process that acts through the stimulation of migration of endothelial cells towards tumor sites. In this way, new blood vessels are formed, which can provide nutrients and thereby stimulate tumor growth. Besides

endothelial cell migration, cancer cells also have the capacity to migrate, giving a tumor the possibility to invade in surrounding tissues and to metastasize to secondary organs/tissues. For metastasis, this migration is towards blood vessels or lymph nodes. Both endothelial and cancer cell migration actions have similar characteristics in activation of intracellular kinases which stimulate cell migration. However, the migration of different cell types is stimulated by different factors.

Endothelial cell migration is activated after stimulation of the vascular endothelial cell growth factor receptor (VEGF-R),[7] the most extensively studied pro-angiogenic factor. VEGF binding to VEGF-R results in activation of downstream kinases. Evidence that TP plays a role in angiogenesis has been described by Hotchkiss,[8,9] who showed that endothelial cell migration could be stimulated by cells with a high TP expression. dR and TP addition resulted in a higher migration capacity of these endothelial cells. dR exposure of endothelial cells has been shown to result in phosphorylation of p70/S6k.[10] This phosphorylation was inhibited by addition of rapamycin, which specifically inhibits mTOR (Figure 1). Moreover, dR was able to stimulate endothelial cells in a rat aortic ring assay, which could be blocked by rapamycin.[10] This suggests a role for TP in activating the p70/S6k, thereby stimulating proliferation and cell migration. Some of our preliminary data suggest that TdR addition protected colon cancer cells from rapamycin-induced cytotoxicity. In cells that expressed TP, this protection was reversed by TPI. Therefore, TP enzymatic activity may be involved in this protection against rapamycin.

For p70/S6K activation, it is (partially) required that the focal adhesion kinase (FAK) is activated.[11] FAK is a nonreceptor protein kinase that is recruited to focal adhesions after clustering of integrins. FAK plays a central role in mediating cell attachment and cell migration. Hotchkiss et al.[9] identified focal adhesions formation and FAK phosphorylation after stimulation of endothelial cells with either TP or dR. Integrins that were involved in this activation were $\alpha 5\beta 1$ and $\alpha_V\beta 1$. VEGF is generally known to stimulate the formation of focal adhesions and to phosphorylate FAK. In contrast to TP, VEGF seems to be involved in activation of $\alpha 5\beta 3$ and $\alpha_V\beta 3$ integrins.[9]

It should be taken into account for all experiments using endothelial cells that these are isolated from various human umbilical cords, which may introduce inter-individual donor variation related to signal transduction pathway activation. Moreover, no experiments determining activation signal transduction pathways after TP stimulation in cancer cells have been reported. Activation of kinases may be completely different between endothelial cells and cancer cells. The activation of intracellular signal transduction pathways and subsequent endothelial or cancer cell responses by the enzymatic activity of TP are still not completely identified. dR-1-P is formed by TP enzymatic breakdown of TdR, but the direct interaction that results in activation of these intracellular transduction pathways remains unidentified.

MECHANISITC FEATURES OF TP RELATED CELL MIGRATION

The mechanism by which TP and dR induce neovascularization is still not completely understood. No receptor for TP has so far been identified and TP does not contain a secretion signal. Although TP is predominantly found intracellularly, some tumor cells (A431 and MKN74) appeared to release TP into conditioned medium.[12] However, it is possible that these cells did not excrete TP in the medium, but that these cells have a higher cell death induction and thereby released their intracellular content (enzymes) in the medium. In rapidly growing solid tumors, necrosis and cell lysis are common events. Therefore, TP can be detected in the medium above the cells. Also the use of conditioned medium may influence the behavior of cancer cells.

Out of the reaction with TP, dR-1-P and thymine are formed (Figure 1). dR-1-P is the product from this reaction which presumably starts the angiogenic process. It is generally presumed that dR-1-P is dephosphorylated to dR. dR has been clearly shown to have pro-angiogenic effects,[10,13] but no direct evidence has been reported that dR-1-P is really converted to dR. After degradation of TdR to thymine, dR-1-P accumulation is not as high as that of thymine.[14] dR-1-P is rapidly (within minutes) metabolized to a yet unknown product. dR-1-P can either be dephosphorylated to dR, which can readily permeate the cell membrane, or be isomerized to dR-5-P by phosphopentomutase and split to glyceraldehyde-3-phosphate (G3P). G3P can enter the glycolytic pathway to yield ATP. This has been described for bacteria, but has not been fully confirmed in eukaryotic cells.

The formation of dR or dR-5-P may be cell type dependent. For example, when cells are transfected with TP causing unusually high expression of this enzyme, dR-1-P metabolism may be different. Therefore studies should be performed to determine the extent of dR and dR-5-P formation from dR-1-P. Using TP-transfected cells, dR-1-P has shown to be rapidly converted to either dR or dR-5-P. Addition of dR-5-P decreased the metabolism of dR-1-P, which indicates that dR-5-P may be formed.[3] Grierson et al.[15] used the tracer 5′-deoxy-5′-[F-18]fluorothymidine (18F-DFT) that can be converted intracellularly by TP. Since the dR-1-P group of thymidine is the labeled part of the tracer, cleavage will result in radioactive dR-1-P. This radioactive dR-1-P can easily be monitored for further action. Therefore, this tracer could be used to investigate whether dR or dR-5-P is generated to induce cell migration. Moreover, other options for the use of such a radiolabeled tracer are of interest, for example, in monitoring in vitro activity of TP.

Brown et al.[16] proposed that dR might form oxygen radicals. Both dR-5-P and dR can undergo the Schiff base reaction, during which the closed sugar-chain is opened. At the end of the described reaction, oxygen radicals are generated. However, they used very high concentrations of thymidine (0.2–1 mM) to generate these oxygen radicals. The formation of oxygen

radicals is in agreement with reports where TdR catabolism by TP increased carcinoma cell secretion of IL-8, VEGF and MMP-1. These are all known products that are generated after oxidative stress.[17,18,19]. Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates. Disturbances in the normal redox state can cause toxic effects through the production of peroxides and free radicals that damage important cellular components, such as proteins and DNA. Heme oxygenase-1 (HO-1) is an enzyme that can decrease cellular oxidative stress. A high TP expression has been found to be associated with the activation of HO-1.[20,21] HO-1 can function in detoxification, carcinogenesis and cell motility.[22] HO-1 binds to heme and oxygen, and diminishes cellular oxidative stress by reduction of the levels of pro-oxidant heme and production of the anti-oxidant bilirubin.[23] The redox environment of cytosol and mitochondria is critical to cellular performance and protection against oxidative stress. HO-1 has been found to be co-expressed in macrophages that have infiltrated in the late stage of malignant melanomas tumor tissues.[21] HO-1 is generated after the production of ROS, which is typically produced under hypoxic conditions. Under hypoxic conditions, various genes are transcribed, including hypoxia inducible factor-1 HIF-1 and the activator protein-1 (AP-1), which may regulate TP transcription (see also next section). HO-1 induction has been shown to have vasodilatory anti-inflammatory and also anti-apoptotic properties. TP has also been described to confer resistance in hypoxia-induced apoptosis.[24,25] However, the exact mechanism of TP in this has not been elucidated. Moreover, a high TP expression has also been correlated with decreased induction of apoptosis in colorectal, gastric and ovarian carcinomas.[25,26,27]

Since HO-1 is closely associated with TP gene regulation, this might be the mechanism behind the anti-apoptotic activity of TP.[28] Details of the mechanism of anti-apoptosis are still unclear, but there is clear evidence that TP may be crucial in the escape of tumor cells from various apoptotic signals such as physical stress, immune surveillance and anticancer treatments.[28] Moreover, HO-1 expression in macrophages that have infiltrated tumor sites was related to tumor growth and angiogenesis in melanomas.

REGULATION OF TP

TP can be upregulated under influence of many reactions, including the cytokines IFN-α and tumor necrosis factor (TNF-α).[2,3] TNF and IFN release are both regulated by NFkB. When monocytes were made resistant to inhibition of NFkB by sulfasalazine (SSZ), cells lost TP expression, while wildtype cells had significant TP expression. TP activity in these cells could not be induced after exposure to IFN or TNF. TP may therefore be regulated by NFκB.

Studies of multiple soluble mediators in tumor stroma have shown that TP is often coexpressed with other inflammation-related or angiogenesis-related molecules, including VEGF, TNF-α, IL-1, HIF-2-α.[2,3] Many new anti-angiogenesis agents that are under development are directed towards either VEGF or the VEGF-receptor (VEGFR).[7] VEGF has different actions than TP in endothelial cell stimulation.[2,3] VEGF can be induced under hypoxic conditions. Besides this, TP is reportedly overexpressed in hypoxic tissues.[29] TP and VEGF have been thought to have cooperative actions, which have been partially proven by Rofstad et al.,[30] who showed that angiogenesis and spontaneous metastasis of A-07 melanomas xenografts could be inhibited by treatment with neutralizing antibodies against VEGF, IL-8, TP, or bFGF. This was especially interesting, because the inhibition of only one of these factors could not be compensated for by the others.[30]

One study showed that HIF-2α transcriptional activity is associated with more TP and that HIF-1α transcriptional activity is associated with more VEGF.[31] However, the exact mechanism of HIF-2α regulation is still unknown. Another protein that is responsive to hypoxia is ANG-1. One immunohistochemical study has shown that TP expression could be inversely related to ANG 1, angiopoietin 1.[32] Therefore, the regulation of TP in hypoxia is probably different than that of ANG-1.

Prostaglandin E2 (PGE2) is derived from arachidonic acid by cyclooxygenase. PGE2 has been shown to promote tumor growth. Furthermore, COX-2 has been shown to possess angiogenic activity. PGE2 is coexpressed with TP in squamous cell carcinoma of uterine cervix, malignant fibrous histiocytoma, and endometrial cancer.[33,34,35] Co-expression in these tumor types showed that COX might act together with VEGF and TP and thereby promote tumor growth by induction of angiogenesis. PGE2 stimulation of cells can stimulate the production of IL-10,[36] which inhibits the anti-inflammatory functions of dendritic cells (DC) and macrophages.[36] IL-10 can modulate the expression of other cytokines, soluble mediators and cell surface molecules by cells of myeloid origin, particularly macrophages and DC. IL-10 is also able to inhibit COX-2 expression, IL-6, IL-12, and TNF-α. On the other hand, TPI has shown to inhibit the production of IL-10. In this way, TPI may be able to reverse the anti-inflammatory actions of IL-10 and therewith activate an immune response against tumor cells. From our preliminary data, TP activity was partially inhibited by the stimulation of DC with PGE2 (Figure 2). After stimulation of DC with PGE2 and the addition of thymidine, IL-10 production decreased 7-fold (Table 1). IL-10 production also decreased 1.5-fold after addition of TPI, which may suggest an indirect role of TP in stimulation of IL-10 production.

TP can also be upregulated by various anticancer agents, including taxanes. The mechanism behind this upregulation is not known, but it is thought to be an indirect mechanism.[2,3] Interestingly, TP upregulation could be related to protection against drug activities.[37] Since TP has

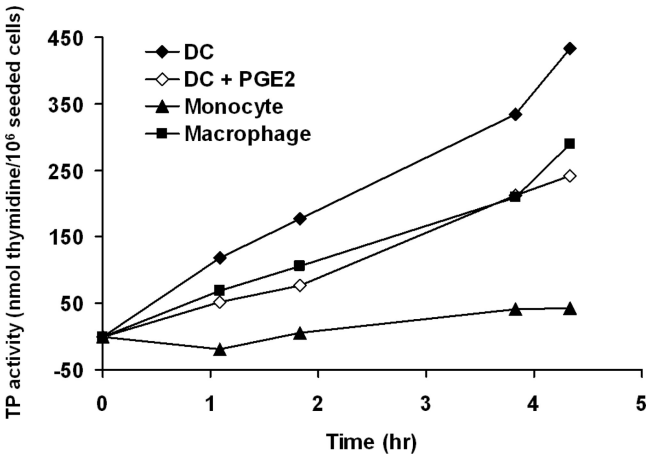

FIGURE 2 TP activity in dendritic cells (DC), monocytes and macrophages. When cells are stimulated with PGE2, TP activity in DC decreased.

anti-apoptotic properties and has been shown to protect cells against these agents, it would be interesting to study combinations of these drugs with specific inhibitors of TP, such as TPI. Moreover, since the mechanism of TP upregulation remains unclear, it would be interesting to study these aspects as well.

TP IN TUMOR-STROMA INTERACTION

Of the many performed immunohistochemical studies of TP expression in tumor tissues, differences in the locations of its expression are described. Some studies only found TP expression in tumor cells, such as breast, lung, stomach, and head and neck. Others predominantly reported TP expression in tumor surrounding tissues (mostly colorectal and endometrial cancer tissues).[2] Tumor tissues of solid tumors comprise not only cancer cells, but also nonmalignant stromal cells and infiltrated immune cells. These cells may all interact with each other, generating a microenvironment that

TABLE 1 Effect of TPI on thymidine (TdR) and Prostaglandin (PGE2) mediated cytokine release in dendritic cells

		IL-10 (pg/ml)
control	TdR	323
	TdR + TPI	212
PGE2	TdR	44
	TdR + TPI	57
TPI	TdR	190
	TdR + TPI	178

Dendritic cells were exposed to PGE2 or TPI for 7 days, after which IL-10 production was determined with addition of TdR of TdR + TPI.

can regulate tumor growth, progression, metastasis and angiogenesis.[38] Macrophages are often infiltrated in tumor sites. These so-called tumor associated macrophages (TAM) have been shown to promote cancer progression and are related to an unfavorable prognosis in preclinical and clinical investigations of various cancers.[39,40] It is unknown whether the expression of TP in tumor stroma has any clinical or prognostic significance, but macrophage infiltration has been related to an increased angiogenesis.[41] Yao et al.[42] determined TP expression in glioma tissues and correlated TP expression in infiltrated macrophages and with an increased microvessel density (MVD). This increase in angiogenesis could also be related to the macrophages, which are only able to enter tumor sites under conditions of increased MVD. TP expression in TAM has also been linked to the pathology and progression of solid tumors.[43] TAM produce inflammatory cytokines that may result in upregulation of TP in tumor cells and other tumor stroma cells. This is in contrast to one study where it was shown that high infiltration of TP-expressing macrophages in the tumor stroma was related to an improved survival in patients with colorectal cancer.[44] Therefore, the role of TP in tumor stroma tissue warrants further attention. TP expression in tumor stroma has been shown to activate anticancer drugs (5'-DFUR) via a bystander effect. In peripheral blood monocytes, TP was most prominent in activation of 5'DFUR.[45] Since TP expression was increased after differentiation of monocytes to macrophages and DC (Figure 2), this can be an advantage in killing macrophages and DC that are infiltrated in tumor tissue sites.

All immunohistochemical studies indicating that TP is a predictive factor have been retrospective and not all have shown similar results. Therefore, prospective studies with standardized methods for TP determination should take place in the future. It should also be noted that previous immunohistochemical studies were performed in various tumor tissue types at different stages, and most largely ignored the functional aspects of coexpression. Future studies should focus on these aspects as well.

CONCLUSION

TP is a unique nucleoside metabolism enzyme that can induce angiogenesis and protect against the induction of apoptosis. TP is therefore an important prognostic factor. These actions are related to the conversion of TdR to dR-1-P by TP. dR-1-P can be converted to either dR-5-P or dR, but it is not clear to what extent each product is formed. Inhibition of TP by specific inhibitors may be of interest. Since TP expression is not only found in tumor cells, but also in tumor stroma cells, future studies should focus on the role of TP in these cells and not only in cancer cells.

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