Review

Platelet-derived endothelial cell growth factor thymidine phosphorylase in tumour growth and response to therapy

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Summary Angiogenesis plays an important role in the growth and metastasis of solid tumours. Platelet-derived endothelial cell growth factor (PD-ECGF) is known to be chemotactic for endothelial cells in vitro and angiogenic in vivo. It is also known as gliostatin, a factor promoting neuronal survival, and thymidine phosphorylase (dThdPase), which catalyses the reversible phosphorylation of thymidine to thymine and 2-deoxyribose-1-phosphate. This enzymatic activity is critical for angiogenic activity. PD-ECGF protein is highly expressed in tumours compared with most normal tissues and has been correlated with tumour growth, invasion and metastasis in clinical studies. In addition, dThdPase activity (by inference PD-ECGF) has been found to be a major determinant of the toxicity of 5-fluorouracil and its prodrugs, which are extensively studied clinically as anti-cancer agents. This review attempts to summarize recent gains in understanding the nature, location and action of PD-ECGF and its specific relevance to tumour biology.

Keywords: angiogenesis; thymidine phosphorylase; platelet-derived endothelial cell growth factor; gliostatin; 5-fluorouracil

The growth of a solid tumour from a single aberrant cell can take a few weeks or many decades. There are many stages in tumour growth from the formation of the initial foci of cells that constitute the primary tumour to the eventual spread and formation of secondary tumours at distant sites if the tumour is metastatic. One key event in this progression is the formation of a functional vasculature that can supply the tumour with oxygen and other nutrients. This allows it to grow, but also provides an avenue of dissemination. Angiogenesis is a tightly controlled process normally confined to embryogenesis, wound healing and the reproductive cycle, but is also characteristic of several disease states including rheumatoid arthritis, psoriasis and solid tumour growth. Tumours cannot grow beyond a very limited size (1–2 mm^3) unless angiogenesis occurs, as diffusion of nutrients becomes limiting (Folkman, 1986, 1990). There are many factors now known to be capable of influencing angiogenesis, and antiangiogenic strategies have been proposed to exploit this feature of the tumour environment (see Fan et al, 1995 for review). Factors promoting angiogenesis include acidic and basic fibroblast growth factor (aFGF and bFGF), vascular endothelial growth factor (VEGF), angiogenin and platelet-derived endothelial cell growth factor (PD-ECGF), many of these being found to be abnormally elevated in the tumour environment. Angiostatin, endostatin and thrombospondin are known physiological angiogenic inhibitors, and it is the balance between such opposing factors in the tumour environment that determines the development of the vascular system within the tumour. PD-ECGF is a relative newcomer to the field of angiogenesis research, and over recent years there have been significant additions to the understanding of its location and action, particularly in tumour

biology. This review summarizes the current knowledge on the structure, function, location and action of PD-ECGF within the tumour environment.

Vascularization of solid tumours is known to be poor and disordered, leading to insufficient perfusion and diffusion of nutrients to areas of the tumour. This is thought to be a major reason for the existence of hypoxia (low oxygen), glucose depletion and to a lesser extent low pH within solid tumours. It is known that microenvironmental factors, such as the above, can influence the production of angiogenic growth factors such as VEGF, and may be in part responsible, along with the action of other factors, for the observed elevations in expression of PD-ECGF (Griffiths et al, 1997). VEGF expression can be regulated by the products of activated oncogenes and mutant or deleted tumour-suppressor genes, cytokines, hormonal modulators and hypoxia (see Claffey et al, 1996 for review). PD-ECGF has many roles (Figure 1). It is known to stimulate endothelial mitogenesis and chemotaxis in vitro and promote angiogenesis. It is reported to be a 90-kDa protein dimer, first purified as the sole endothelial mitogenic activity in platelets (Miyazono et al, 1987), but since found in many other tissues and cells, including placenta and macrophages (Yoshimura et al, 1990). Levels are found to be elevated in solid tumours, rheumatoid arthritis synovium (Asai et al, 1993) and psoriatic lesions (Creamer et al, 1996). PD-ECGF is known not to bind heparin, in contrast to the other angiogenic factors aFGF, bFGF and VEGF (except splice variant 121) (Miyazono et al, 1987). When overexpressed in MCF-7 cells, PD-ECGF resulted in an increase in tumour growth and was found to be angiogenic in the rat sponge and freeze-injured skin graft models (Moghaddam et al, 1995). Gliostatin, which is a neurotrophic factor produced by quiescent astrocytes, has also been shown to be a form of PD-ECGF. It acts as an inhibitory growth regulator against all glial tumour cells and glial maturation factor-stimulated astroblasts, but not of neuronal cells. These functions have suggested that PD-ECGF may have a significant role in the development and regeneration of the nervous system and be

involved in the induction of angiogenesis for the formation of the blood–brain barrier (Asai et al, 1992a,b).

GENE STRUCTURE

Ishikawa et al (1989) isolated a 1.8-kb full-length cDNA sequence of PD-ECGF using poly(A)+ RNA from term placenta, predicting a protein consisting of 482 amino acid residues. Amino acid sequencing of the protein identified 389 residues giving a predicted molecular weight of 48.6–49 kDa. The cDNA sequence showed a GC rich 5' untranslated·area and the lack of a hydrophobic signal sequence. This suggested that PD-ECGF is not a classic secretory protein. However, bFGF and aFGF, which also do not have a signal, are secreted via a protein kinase C (PKC) dependent phosphorylation mechanism and PD-ECGF is known to have a putative PKC binding site (Usuki et al, 1991), but it is not known whether this is the mechanism of its secretion. Also of interest was the identification of two short internal repeats and two nucleotide binding motifs (Gly-X-Gly-X-X-Gly). Seven cysteine residues were found, indicating that PD-ECGF has at least one free thiol group and one potential glycosylation site, but it appears from comparison with the predicted M_r and the M_r obtained by SDS-gel electrophoresis that it is not glycosylated.

The PD-ECGF gene is composed of ten exons dispersed over a 4.3-kb region, with the translation start codon in exon 2 and the stop codon (within the polyadenylation signal) in exon 10. The promoter lacks a 'TATA' box and a 'CCAAT' box commonly found in eukaryotic promoters. It does however contain six copies of binding motifs of the SP-1 general transcription factor upstream of the transcription start site (Hagiwara et al, 1991).

Nucleotidylation of PD-ECGF can occur by covalent binding of serine residue(s) to the phosphate groups of nucleotides and the two nucleotide binding motifs may be involved in the reaction (Usuki et al, 1991). The physiological significance of this may be explained by the enzyme activity now attributed to PD-ECGF (below).

THYMIDINE PHOSPHORYLASE ACTIVITY

In 1992, Barton et al found PD-ECGF to have a 40% sequence similarity to thymidine phosphorylase (dThdPase) of *Escherichia coli*. Thymidine phosphorylase activity of PD-ECGF was confirmed by Usuki et al (1992) and Moghaddam et al (1992). However, although we (in this review) and many others use PD-ECGF/dThdPase interchangeably, the sequence comparison of human dThdPase to human PD-ECGF has not been reported. dThdPase is an enzyme specifically involved in the reversible dephosphorylation of thymidine to thymine and 2-deoxyribose-1-phosphate (Zimmerman and Sidenberg, 1964). The optimum pH for this activity was found to be 5.3 (Finnis et al, 1993). Site-directed mutagenesis of the PD-ECGF gene subsequently transfected into COS cells has shown that the enzymatic activity is essential to the angiogenic activity of PD-ECGF/dThdPase (Miyadera et al, 1995). This is not a phenomenon restricted solely to PD-ECGF/dThdPase: another angiogenic growth factor, angiogenin, also has enzymatic (ribonuclease) activity, which seems to be at least partially responsible for its angiogenic effect (Shapiro et al, 1989).

PD-ECGF/dThdPase may promote angiogenesis by reducing thymidine levels inhibitory to endothelial proliferation because of the use of the thymidine salvage pathway when intracellular thymidine pools are depleted (Finnis et al, 1993), or alternatively the enzymatic products of PD-ECGF/dThdPase, namely thymine
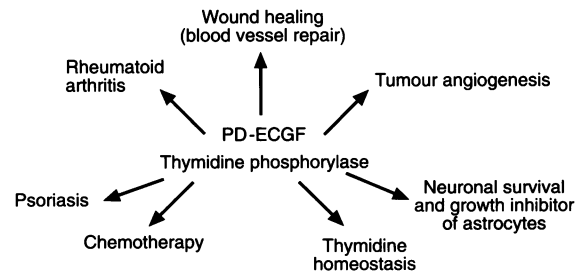

Figure 1 The roles of PD-ECGF/TP

and 2-deoxyribose-1-phosphate or metabolic/catabolic products derived from them may themselves be angiogenic. Indeed, although no angiogenic activity has been ascribed to thymine, 2-deoxy-D-ribose, a dephosphorylated product of 2-deoxyribose-1-phosphate has been found to have angiogenic and chemotactic activity (Haraguchi et al, 1994). Studies on murine (liver), and human (placental and liver) PD-ECGF/dThdPase have shown inter- and intraspecies differences in substrate specificities for natural and 5-fluoropyrimidine compounds and have suggested that the hydrophobicity of the human enzymes when measured at pH 8 (not the optimum, Finnis et al, 1993) are different from their murine counterparts (Elkouni et al, 1993).

Other nucleoside phosphorylases including uridine phosphorylase (UrdPase), and purine nucleoside phosphorylase (PNP) have been identified in mammalian tissues. UrdPase catalyses the breakdown of uridine to uracil and D-ribose sugar (not thought to be angiogenic (Haraguchi et al, 1994). PNP can breakdown guanosine to guanine and D-ribose.

IN VIVO LOCALIZATION OF PD-ECGF/dThdPase

Extensive immunohistochemical studies of the location and abundance of PD-ECGF/dThdPase in normal human tissues have been carried out. Expanding on earlier work with liver, lung and placenta (Usuki et al, 1990; Yoshimura et al, 1990), Fox et al (1995) demonstrated immunohistochemical staining for PD-ECGF/dThdPase within the nuclear and cytoplasmic regions. The predominant cells staining for PD-ECGF/dThdPase were macrophages, although many other cell types were also positive; for example skin, Kupffer cells, alveolar macrophages, placental stromal cells and endothelial cells in placenta, ovary, salivary gland and brain. Lymphoid cells were found to be negative, although lymphocytes have been found to be positive in other studies (Takebayashi et al, 1996a). The lack of consistent staining in areas where normal angiogenesis would be expected (e.g. placenta) suggested a role for PD-ECGF in pathological but not physiological angiogenesis. This is consistent with the observation of high expression in macrophages, which are recruited in wound healing, the inflammatory response and in tumours. Further, platelets, also recruited in wound healing, are a substantial source of PD-ECGF/dThdPase and thus suggest a role for it in maintenance of the vasculature. PD-ECGF/dThdPase has also been suggested to have a role in differentiation of certain cell types (Yoshimura et al, 1990; Heldin et al, 1993; Fox et al, 1995).

PDECGF/dThdPase EXPRESSION IN TUMOURS

Histological analyses in a variety of human tumours, including breast (Moghaddam, 1995; Toi et al, 1995), bladder (O'Brien et al,
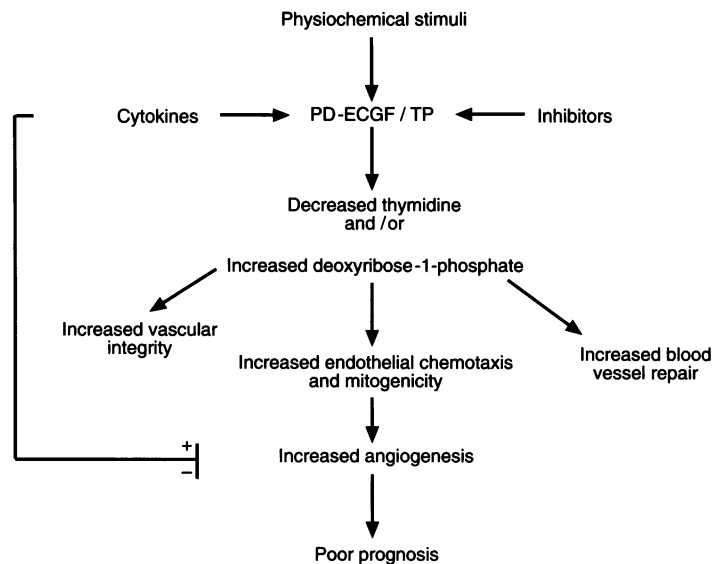
Figure 2 Schematic diagram of PD-ECGF/TP involvement in tumour growth processes

1995), ovarian (Reynolds et al, 1994) gastric (Takebayashi et al, 1996*b*; Maeda et al, 1996) oesophageal, lung, pancreas (Takebayashi et al, 1996*c*) and colorectal cancers (Takebayashi et al, 1996*a*; Takahashi et al, 1996; Saeki et al, 1996) have shown elevated PD-ECGF/dThdPase levels. It has been shown to be a prognostic indicator in colorectal and gastric cancers (Takebayashi et al, 1996 *a,b,c*). It has also been correlated with microvessel density in breast (Toi et al, 1995), colon (Takahashi et al, 1996; Takebayashi et al, 1996*c*), and gastric cancers (Takebayashi et al, 1996*b*; Maeda et al, 1996), providing evidence that it is a major contributor to the formation and maintenance of the tumour vasculature.

A significant presence of PD-ECGF/dThdPase in invasion and metastasis has been found in ovarian (Reynolds et al, 1994), gastric (Takebayashi et al, 1996*b*) and colorectal (Takebayashi et al, 1996*c*) cancers whereas the production of PDECGF/dThdPase by infiltrating cells has been investigated in colon (Takehashi et al, 1996) and breast (Fox et al, 1996) and found to contribute to overall tumour levels.

Elevated thymidine phosphorylase enzyme activity has been reported in breast tumours (Patterson et al, 1995) and has also been detected in plasma of tumour-bearing animals and humans (Pauly et al, 1977, 1978). It is not clear whether this is due to its release from the tumour itself or production and release by host cells and tissues in response to tumour growth/proliferation.

Activity of another pyrimidine phosphorylase, UrdPase, has also been reported to be elevated in primary human tumours, including colon (Luccioni et al, 1994) and melanoma (Leyva et al, 1983). One study in human colon carcinoma has suggested PNP activity is also enhanced and suggests a positive relationship with enzyme activity and tumour invasiveness (Sanflippo et al, 1994). Thus, there may be a link between the activity of nucleoside phosphorylases generally and angiogenesis, but this remains to be established.

REGULATION OF PD-ECGF/dThdPase

In contrast with angiogenic growth factors such as VEGF, the basis of tumour-specific elevation of PD-ECGF/dThdPase levels is

currently not well understood. Cytokines or growth factors, such as interleukin (IL-1), tumour necrosis factor (TNF) bFGF, IFN-γ and IFN-α that are known to increase dThdPase activity (Eda et al, 1993*a*; Tevaerai et al, 1992) may play a role, or additionally/alternatively the influence of microenvironmental factors such as hypoxia and low pH (Griffiths et al, 1997) may be important (Figure 2). Indeed, solid tumours, rheumatoid arthritis and psoriatic conditions (all of which have been identified as conditions with elevated VEGF and PD-ECGF/dThdPase) are known to be associated with the development of hypoxia and aberrant angiogenesis. The effect of other factors that might influence PD-ECGF expression, e.g. the products of oncogenes and tumour-suppressor genes have yet to be established.

The role of PD-ECGF in vivo is still somewhat unclear. The high expression in lymphoid tissue and skin may be important for total body thymidine homeostasis (see Fox et al, 1995). As the largest source of PD-ECGF in the body is found in platelets, this strongly suggests that it has a role in maintaining the integrity of blood vessels, promoting the repair of the endothelium. The net increases in vasculature, seen when PD-ECGF is overexpressed, may be caused largely by stabilizing and maintaining the existing vasculature.

PDECGF/dThdPase IN CHEMOTHERAPY

PD-ECGF/dThdPase is known to be elevated in tumours compared with surrounding normal tissue (though absolute comparison with levels present in normally high-expressing cells, such as platelets, is difficult as most published studies have been histological). This apparent differential makes dThdPase an attractive target for chemotherapy.

5 fluorouracil (5-FU) has been extensively studied and is used routinely in the treatment of a variety of tumour types, e.g. colon, breast. dThdPase may play a role in the therapeutic application of 5-FU because it is known to catalyse the conversion of the pyrimidine antimetabolite 5-FU to 5′-fluoro-2′-deoxyuridine (5′-FdUR) by the addition of 2-deoxyribose-1-phosphate (Figure 3). This is the first step in one pathway for the metabolic activation of the 5-FU agent to deoxyribonucleotides (Iltzsch et al, 1985). Ultimately
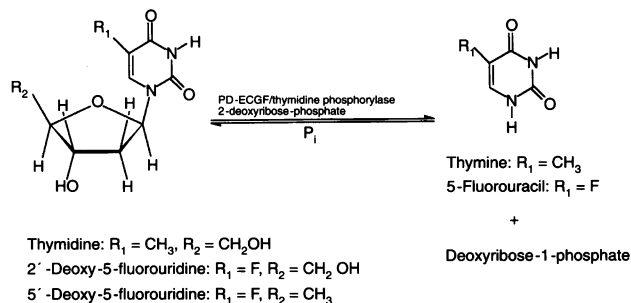
Figure 3 Reactions catalysed by PD-ECGF/TP

metabolites of these drugs can inhibit thymidylate synthase, either by restricting de novo synthesis of nucleotides, or by fraudulent incorporation into DNA, or both (Schwartz et al, 1992).

If 2-deoxyribose-1-phosphate is the molecule responsible for PD-ECGF/dThdPase-stimulated angiogenesis, then 5-FU fulfils a dual role: it can disrupt DNA synthesis by the production of 5′-FdUR and in doing so also removes potentially angiogenic molecules from the tumour environment. In theory, this would lend its use to more aggressive and metastatic diseases, often characterized by a more angiogenic phenotype.

Fujimoto et al (1985) also showed that dThdPase was important in the conversion of 5′-deoxy-5-fluorouridine (5′-DFUR) (an analogue of thymidine and a prodrug of 5-FU) to 5-FU, which was later confirmed by studies with cells overexpressing dThdPase; cells with a 90-fold increase in dThdPase activity showed a 165-fold increase in sensitivity to 5′-DFUR, whereas in this study sensitivity to 5-FU itself was not affected (Patterson et al, 1995).

In vitro studies have demonstrated the potentiation of 5-FU and 5′-DFUR toxicity by IFN-α, as it can elevate dThdPase levels (Schwartz et al, 1995 and Tevaerai et al, 1992 respectively). Clinical trials of patients with metastatic colorectal carcinoma treated with 5-FU and IFN-α have shown an increase in response rates over controls treated with 5-FU alone (Wadler et al, 1989). These effects are presumed to be the result of increases in dThdPase expression owing to the action of IFN-α causing enhanced metabolic activation of 5-FU. However, in this context, the use of IFN-α presents some incongruities. It is reported to be an inhibitor of angiogenesis, so it would seem that the antiangiogenic effect of IFN-α together with its ability to induce dThdPase and thereby increase the activation of 5FU may be more important than the potentially proangiogenic effect of the elevated dThdPase in the outcome of clinical treatment.

Interestingly, UrdPase is also capable of converting 5′-DFUR to 5-FU and, like dThdPase, is inducible by TNF-α, IL-1α and IFN-γ (Eda et al, 1993b). This suggests both a functional and a co-regulatory relationship between dThdPase and UrdPase.

Another avenue for chemotherapy targeted at dThdPase may be through the inhibition of thymidine phosphorylase enzyme activity and therefore its angiogenic properties. It has been shown that a dThdPase inhibitor (6-amino-5-chlorouracil) inhibits the angiogenic activity of purified dThdPase in vitro (Miyadera et al, 1995). Inhibition at nanomolar concentrations has been reported for related enzymes such as UrdPase (Naguib et al, 1993). Known inhibitors of dThdPase are less potent than those for UrdPase, the most effective being 6-aminothymine (Woodman et al, 1980) and 6-amino-5-bromouracil (Desgranges et al, 1982). Alternative substrates for dThdPase have also been considered as inhibitors

(Desgranges et al, 1983), and may be of further use in antiangiogenic therapy of cancer.

SUMMARY

The study of PD-ECGF/dThdPase is now a very active area in in vitro, in vivo and clinical research. It is one of the few angiogenic growth factors that, in addition to being overexpressed in the tumour environment, also provides a large scope for chemotherapeutic exploitation, combining antiangiogenic and antiproliferative treatment regimens. However, the presence of PD-ECGF/dThdPase in other tissues and blood would make the development of specifically tumour-targeted therapy preferable. Very little is known about its gene expression, although emerging evidence suggests that both cytokine/growth factor and physicochemical/microenvironmental stimuli can influence protein levels and activity. The absolute reliance on the enzymatic nature of PD-ECGF/dThdPase for its angiogenic properties is unique, and the possible importance of UrdPase and PNP, enzymes with similar functions, remains to be determined. This has led to questions about the specific role of PD-ECGF in tumour angiogenesis and angiogenesis in other disease states, along with exactly how it stimulates endothelial mitogenesis and chemotaxis.

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