Review

Pigment epithelium-derived factor: a multimodal tumor inhibitor

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Abstract

Pigment epithelium-derived factor (PEDF), a noninhibitory member of the serine protease inhibitor (serpin) family, is a well-known potent endogenous inhibitor of angiogenesis. It has been known for years to be aberrantly expressed in ocular disorders, but in recent years, down-regulation has been shown to be prevalent in a range of cancers as well. This review describes the trimodal anticancer activities of this interesting protein: antiangiogenesis, apoptosis-mediated tumor suppression, and tumor cell differentiation. The key to successful antitumor therapy with this protein is the ability to synthesize the recombinant form of the protein (or its active shortened forms) and deliver at therapeutic doses or alternatively to use gene transfer technology to prolong the effect *in vivo.* Although there is a substantial amount of work carried out at the preclinical stage with this protein, more groundwork has to be done before PEDF is tested against cancer in clinical trials. [Mol Cancer Ther 2006;5(7):1641–6]

Introduction

Tumorigenesis requires the sequential acquisition of defects that allows the tumor to have the ability to grow, invade, and eventually metastasize (1). Despite the significant advances in surgical resection and neo/adjuvant chemotherapy and radiotherapy, there are a significant number of tumors that are unresponsive to aggressive treatment. The aim of targeted therapy is to attack specific pathways and mechanisms of tumor growth that are cancer specific, without affecting the host. Various targets have been studied, such as tumor angiogenesis, signaling pathways, and matrix-cell interactions.

In recent times, much research focus has been directed toward studying the role of angiogenesis in tumor development, growth, and metastasis (2). Angiogenesis, the process by which new blood vessels form from preexisting ones, is an essential step involved in allowing tumors to continue to grow in size and metastasize. Therefore, targeting the neovasculature or the signals that promote new vessel growth has become a promising approach in anticancer therapy.

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein that was first described in the late 1980s after being identified and isolated from conditioned medium of cultured primary human fetal retinal pigment epithelial cells (3). It is a noninhibitory member of the serpin (serine protease inhibitor) superfamily of proteins and its gene is highly conserved through evolution and resides on human chromosome 17p13.3 (4, 5). PEDF is widely expressed throughout fetal and adult tissues, including the adult brain (6), spinal cord (7), plasma (8), liver (9), bone (10), eye, heart, and lung (11).

Initially, PEDF was identified as an effective neurotrophic factor, with purified PEDF concentrations as low as 1 nmol/L being able to convert active Y79 retinoblastoma cells into differentiated nonproliferating neurons (12). Further studies have shown that, in fact, PEDF possesses multiple and varied biological properties, not only neurotrophic, but also neuroprotective, antitumorigenic, and potent antiangiogenic activity (13). It is its antiangiogenic action that has brought it much recent attention. PEDF has been shown to be the most potent endogenous inhibitor of angiogenesis, being more than twice as potent as angiostatin, and more than seven times as potent as endostatin (14). Studies have already shown that decreased levels of PEDF in the eye are associated with a number of ocular neovascular and neurodegenerative diseases. Furthermore, low expression of PEDF has been correlated with the increased incidence of metastasis and poorer prognosis in prostate cancer (15), pancreatic cancer (16), neuroblastomas (17), and gliomas (18). Therefore, given this correlation, PEDF may stall cancer progression as an antiangiogenic

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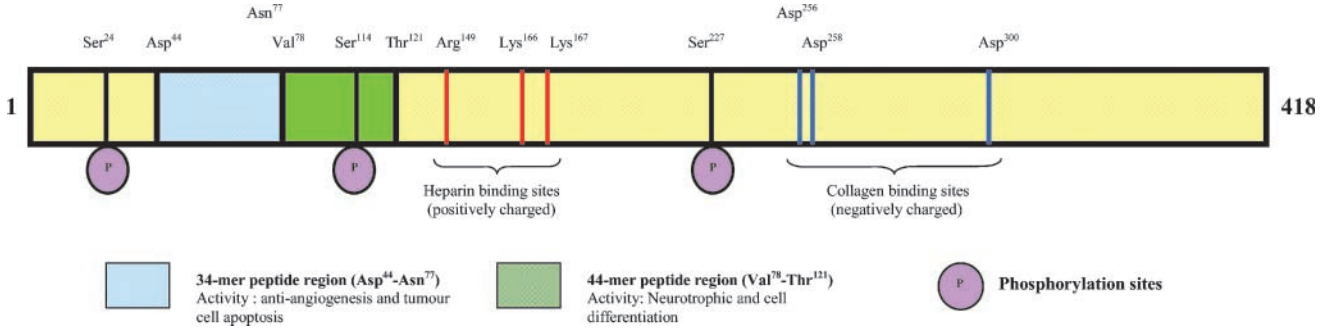

Figure 1. Schematic diagram displaying the functional domains of PEDF.

factor and/or as a direct tumor suppressor. In this review, we will focus on the role of PEDF as a regulator of angiogenesis and tumor progression and also address the therapeutic potential of PEDF for the treatment of malignant tumors.

Biochemical Features of PEDF

The human *PEDF* gene spans ~16 kb and has been mapped to human chromosome 17p13 (11). This gene encodes a 418-amino-acid protein with a hydrophobic signal that is characteristic of secreted proteins and has molecular weight of 46.3 kDa (Fig. 1; ref. 4). However, it is the addition of a single carbohydrate side chain that increases its apparent weight to 50 kDa doublet that is seen on polyacrylamide gels. PEDF has a structural and sequence homology to members of the serpin family of serine proteinase inhibitors and contains a reactive center loop that is characteristic of this family (4). The fact that PEDF is noninhibitory toward proteases is thought to be due to differences in the reactive center loop sequence (4, 19). Furthermore, the reactive center loop in PEDF seems to be more exposed in its native state, resulting in a largely uncovered central region and potentially more rapid binding to regulatory proteins.

PEDF interacts with a wide variety of proteins, binding to glycosaminoglycans, collagens, and an 80 kDa surface protein in Y-79 retinoblastoma and cerebral granule cells (20–24). PEDF has an asymmetrical charge distribution, with a high density of basic residues concentrated on one side (positive) of the molecule and of acidic residues on the opposite side (negative; ref. 25). The varying molecular and cellular events that are triggered by PEDF indicate that there are distinct PEDF receptors that elicit divergent signals.

Negatively charged, acidic PEDF binds to collagen, lacks neurotrophic activity, and may confer antiangiogenic properties. On the other hand, three aspartates (236, 238, and 280) and the basic cluster (Arg^{126}, Lys^{127}, Arg^{129}) in mouse PEDF are critical to collagen and heparin binding (26). Mutational studies have shown that positively charged amino acids Lys^{166}, Lys^{167}, Arg^{149} are necessary for heparin binding, and negatively charged amino acids Asp^{256}, Asp^{258}, and Asp^{300} are necessary for collagen binding (Fig. 1). Furthermore, phosphorylation sites have been identified in Ser^{24}, Ser^{114}, and Ser^{227}, and variable phosphorylation states of PEDF have been shown to induce different degrees of antiangiogenic and neurotrophic activity (25).

Two functional epitopes have been identified on PEDF, a 34-mer peptide (residues 24–57) and a 44-mer peptide (residues 58–101; ref. 27). The neurotrophic function and the ability to block vascular leakage has been replicated by the 44-mer peptide that interacts with a putative 80 kDa receptor (PEDF-R^N) identified on Y-79 cells (23), cerebellar and motor neurons (21), and in neural retina (24). The 34-mer peptide, possibly via a distinct receptor (PEDF-R^A) identified on endothelial cells, induces apoptosis, blocks endothelial cell migration and corneal angiogenesis, but fails to induce Y-79 differentiation (28). Filleur et al. (27) showed *in vivo* that overexpression of the 34-mer in PC-3 prostate adenocarcinoma cell lines resulted in decreased tumor microvessel density and increased apoptosis, whereas the 44-mer lacked antiangiogenic affects but induced neuroendocrine differentiation. Hence, it may also be the differential expression of PEDF-R^N and PEDF-R^A on endothelial and tumor cells that contribute to its distinct actions.

Antitumor Activities of PEDF

There have been substantial advances made recently in the understanding of the tumor inhibitory activity of PEDF (Table 1). As previously mentioned, it is becoming increasingly clear that the ways in which PEDF exerts its antitumor effects is multiple—via (*a*) antiangiogenesis, (*b*) tumor differentiation, and (*c*) direct tumor suppression by apoptosis. These antitumor mechanisms of PEDF will now be discussed in turn.

Antiangiogenesis

Much focus on PEDF as a promising therapeutic target in cancer has stemmed from its potent antiangiogenic activity, which has shown to be more effective than any other

known endogenous angiogenic inhibitor, including angiostatin, thrombospondin-1, and endostatin (14). Moreover, PEDF inhibits endothelial cell migration even in the presence of proangiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-1, fibroblast growth factor-2, and interleukin-8 (29). Doll et al. (30) showed that PEDF plays a key role as a natural angiogenesis inhibitor, with PEDF-deficient mice exhibiting increased stromal microvessel density in several organs, including the pancreas and prostate.

The activity of PEDF is selective in that it targets only new vessel growth and spares the preexisting vasculature, which makes it an appealing candidate as an inhibitor of tumor angiogenesis (13). Although the mechanisms via which PEDF reduces neovascularization still remains to be fully elucidated, it seems that it involves endothelial cell death, through the activation of the Fas/FasL death pathway (31), and also via a disruption in the critical balance between proangiogenic and antiangiogenic factors, in particular VEGF. Cai et al. (32) recently reported that PEDF has an inhibitory effect on VEGF-induced angiogenesis in bovine retinal microvascular endothelial cells via enhancing γ-secretase-dependent cleavage of the COOH terminus of VEGF receptor-1, which consequently inhibits VEGF receptor-2–induced angiogenesis. Moreover, in a human osteosarcoma cell line, MG63, Takenaka et al. (33) showed that exogenous PEDF down-regulated VEGF expression at both the mRNA and protein levels.

The expression patterns of VEGF, a potent proangiogenic factor, and PEDF have been well characterized in the eye and it is the balance of these opposing stimuli that prevents the development of choroidal neovascularization that is involved in diabetic proliferative retinopathy and macular degeneration (34, 35). In our laboratory, this inverse correlation was also seen in epiphyseal growth plates where PEDF is highly expressed in the avascular resting and proliferative zones, whereas VEGF is more predominant in the lowermost layers of the hypertrophic zone (36). It is by careful manipulation of this balance of angiogenesis that the growth plate microenvironment switches from an angiostatic to angiogenic state during the physiologic process of endochondral ossification. Quan et al. (36) postulated that it was the expression of such potent antiangiogenic factors that significantly contributed to the inability for osteosarcoma to penetrate the avascular resting zones of the growth plate, a clinical phenomenon commonly seen in children and adolescents with metaphyseal tumors.

Therefore, based on the role of PEDF as a potent endogenously produced antiangiogenic factor, several recent studies have shown that decreased PEDF expression is associated with a higher intratumoral microvessel density and a more metastatic phenotype in several tumors, such as prostate (15) and hepatic carcinoma (16), gliomas (18), as well as lymphangiomas (37). Consequently, this has prompted further investigation into the effects of overexpressing PEDF in various tumors. Abe et al. (38) and Garcia et al. (39) both showed from *in vivo* studies that overexpression of PEDF in human malignant melanoma cell lines by stable transfection with PEDF plasmids and retroviral expression vectors, respectively, significantly

Table 1. Antitumor activity of PEDF

Activity	Tumor type	PEDF treatment	*In vitro/in vivo*	Reference
Antiangiogenesis	Melanoma	Viral gene transfer	*In vitro/in vivo*	(39)
	Melanoma	Plasmid gene transfer	*In vitro/in vivo*	(38)
	Osteosarcoma	rPEDF	*In vitro*	(33)
	Neuroblastoma	Plasmid gene transfer	*In vitro/in vivo*	(41)
	Prostate cancer	rPEDF	*In vitro/in vivo*	(30)
	Neuroblastoma	rPEDF	*In vitro/in vivo*	(17)
	Prostate cancer	PEDF peptide (34-mer)	*In vitro/in vivo*	(27)
	Cervical cancer	Plasmid gene transfer	*In vitro/in vivo*	(47)
	Pancreatic cancer	Viral gene transfer	*In vitro/in vivo*	(40)
	Hepatocellular ca	Viral gene transfer	*In vitro/in vivo*	(48)
	Lung carcinoma	Viral gene transfer	*In vitro/in vivo*	(48)
	Mesothelioma	Viral gene transfer	*In vivo*	(49)
	Hepatocellular ca	Plasmid gene transfer	*In vitro/in vivo*	(43)
	Glioma	Plasmid gene transfer	*In vitro/in vivo*	(18)
	Wilms' tumor	rPEDF	*In vivo*	(45)
	Lung cancer	Viral gene transfer	*In vitro/in vivo*	(44)
Tumor cell differentiation	Neuroblastoma	rPEDF	*In vitro/in vivo*	(17)
	Prostate cancer	PEDF peptide (44-mer)	*In vitro*	(27)
Direct tumor suppression	Osteosarcoma	rPEDF	*In vitro*	(33)
	Melanoma	Plasmid gene transfer	*In vitro/in vivo*	(38)
	Prostate cancer	rPEDF	*In vitro/in vivo*	(30)
	Prostate cancer	PEDF peptide (34-mer)	*In vitro*	(27)

reduced intratumoral microvessel density as well as primary tumor growth and the development of metastasis. Moreover, Hase et al. (40) and Streck et al. (41) also reported similar results using virus-based expression vectors in neuroblastoma and human pancreatic cancer cell lines, respectively. Given that increased intratumoral microvessel density has shown to be associated with a more aggressive and metastatic phenotype in the majority of cancers, reduction of tumor vascularity by PEDF may prove to be a promising candidate for targeted cancer therapy.

Tumor Cell Differentiation

Another facet that PEDF may exhibit antitumor activity is in its ability to promote tumor cell differentiation. Crawford et al. (17) showed in primitive neuroblastomas that were grown s.c. in athymic mice that intratumoral injection of rPEDF resulted in tumor cell differentiation evidenced by less malignant appearing cells histologically and strong immunohistochemical staining for neurofilament, a marker for neural cell differentiation.

As mentioned above, Filleur et al. (27) showed in prostate cancer that PEDF exerts its antiangiogenic and cell differentiation ability through two functional epitopes. They showed that a 44-mer peptide induced a neuroendocrine phenotype from prostate epithelium, which was manifested by dendrite-like processes, increased neuron-specific markers, and secretion of neuropeptides. Although few studies have been done, this added ability to cause cell differentiation into less malignant phenotypes is indeed promising and warrants further investigation.

Direct Tumor Suppression

Although the bulk of the antitumor activity of PEDF is thought to be the result of potent antiangiogenesis, more recent studies have shown that PEDF also has the ability to induce tumor cell apoptosis. As previously mentioned, PEDF exerts it antiangiogenic activity in part by inducing endothelial cell death and Volpert et al. (31) showed that the mechanism by which PEDF does this is via the activation of the Fas/FasL death pathway. Similarly, Takenaka et al. (33) and Abe et al. (38) both showed in melanoma (G361) and osteosarcoma (MG63) cell lines, respectively, that PEDF administration *in vitro* caused a significant degree of cell apoptosis, which was reversed by administration of anti-FasL antibody.

Interestingly, this apoptotic activity is likely to be due to a distinct functional epitope on the PEDF protein. Filleur et al. (27) reported a >2-fold increase in prostate cancer cell death with the 34-mer peptide but not with the 44-mer. Doll et al. (30) also described *in vitro* a dose-dependent effect of rPEDF on prostate tumor cell apoptosis that was further increased (3.3-fold) in the presence of hypoxic conditions. Hence, in theory, as PEDF exerts its antiangiogenic activity, resulting in intratumoral hypoxia, the ability for PEDF to also induce direct apoptosis provides a promising and potent synergistic effect.

Production and Delivery of PEDF

Native PEDF is either purified from plasma (8) or retinal cells (4) using classic liquid chromatography or in a recombinant form, nowadays mostly using mammalian cell such as HEK 293 cells (19). Commercial supplies are also available, but the cost of PEDF is high and is not feasible for *in vivo* studies where milligram quantities of the protein may be required. For tumor therapy, the extra need for targeting PEDF to diseased site(s) presents an added challenge. In contrast, for ocular indications, delivery is usually local, precluding the need for bulk amounts of the protein. Furthermore, for ocular indications, it has been shown recently that trans-scleral movement of PEDF is possible via subconjunctival administration (42).

Therefore, for treatment of tumors with PEDF, smart solutions for restricting the delivery to neoplastic sites are required not only to reduce any side effects, but also to reduce the cost of therapy. One issue that will need closer analysis as PEDF becomes more commonly tested in preclinical studies against cancer is whether the protein causes side effects. Being physiologically present in plasma, it would be hard to imagine that it would exhibit cytotoxicity, but its effects on physiologic neovasculature, such as that in the menstrual cycle and wound healing, will nevertheless need addressing.

PEDF is a protein that is 418 amino acids in length, and it has already been elucidated that shorter fragments of the protein (e.g., the 34-mer and 44-mer; ref. 27) possess bioactivity on their own. Looking at natural examples such as endostatin and canstatin, antiangiogenic and anticancer peptides derived from much larger proteins, one avenue that needs looking at is whether shorter fragments of the PEDF sequence have activity. We believe that such fragments will need empirical testing in each disease model, at least at the cell culture stage, to determine whether each has potential. The fact that PEDF lacks serpin activity begs the question as to why this is so, and whether cryptic sequences that are exposed due to shorter fragments allow such activity to reappear. The real advantage of shortening peptides is that the cost of production is dramatically reduced (i.e., at the end, the patient pays less for treatment) and it opens up new avenues for enhancing pharmacokinetics and thereby pharmacodynamics of the parent protein.

Finally, as a lot of the studies that are summarized in Table 1 list gene transfer as the mode of PEDF treatment, it is very apparent that this mode of therapy may become quite commonplace for PEDF, not only in eye disorders but also in various presentations of cancer. There is an equal distribution of using viral or plasmid vectors for gene transfer. Each has its own advantages and disadvantages. Viral vectors have a high level of gene shuttling ability, but suffer from the risk of *de novo* cancer initiation via recombination within the patient cell genome. Plasmid vectors are safer to use but need to be improved upon to increase transfection efficiency.

Intratumoral injection of viral PEDF vectors has shown, as a proof of principle, that this form of therapy works (40, 43, 44). Intrapleural administration of a PEDF viral

vector significantly reduced lung metastatic burden (44), whereas cancer cells of various types overexpressing PEDF have exhibited reduced tumor growth *in vivo* (38, 39). Oxerexpressing PEDF via viral systemic transduction before tumor cell inoculation also has shown efficacy (41). On the other hand, it has already been shown that systemic administration of recombinant PEDF causes tumor regression mediated by a selective effect of the protein on the tumor as well as its vasculature (45), as does such administration of a PEDF viral vector (43).

Thus, there are some promising studies reported highlighting the therapeutic potential of PEDF, whether given as a protein or as an expressible form in a vector. Although a majority of tumors have well-established vasculatures, for instance osteosarcoma (46), better benefits of PEDF-mediated therapy will eventuate when appropriate drug delivery systems are discovered, optimized, and implemented. Thus far, mostly naked (free, unmodified) protein has been tested *in vivo*, and it is believed that with such drug delivery systems, enhanced drug pharmacokinetics and pharmacodynamics will be realized.

Summary and Future Directions

With further studies demonstrating the antitumor effects of PEDF on various different cancer types, the role of PEDF as a potential therapeutic agent is indeed promising. The real lure of PEDF, from data accumulated thus far, is that it has the attractive ability to inhibit tumor growth in more than one way, via antiangiogenesis, tumor cell differentiation, and tumor cell apoptosis. However, still relatively little is known of the overall physiologic role of PEDF in the human body, and further investigation is surely required before clinical studies are started.

Nevertheless, given that PEDF is an endogenously produced molecule that is widely expressed, the likelihood that it will produce adverse side effects, like other synthetic agents, or develop drug resistance, is substantially less. Furthermore, in light of the recent evidence that separate regions of PEDF confer different functional activity, it is highly likely that synthesis of small PEDF peptides will have important therapeutic implications in terms of specific targeted therapy. To this end, much more understanding is required of the diverse biochemical pathways that PEDF interacts with, and also the receptor(s) that it acts on. Armed with this knowledge, we may then be able to capitalize on the therapeutic potential of this versatile protein.

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