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Characterization of PEDF: A Multi-Functional Serpin Family Protein

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ABSTRACT

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein that belongs to the non-inhibitory serpin family group. PEDF has been described as a natural angiogenesis inhibitor with neurotrophic and immune-modulation properties; it balances angiogenesis in the eye and blocks tumor progression. The mechanisms underlying most of these events are not completely clear; however, it appears that PEDF acts via multiple high affinity ligands and cell receptors. In this review article, we will summarize the current knowledge on the biochemical properties of PEDF and its receptors, the multimodal activities of PEDF and finally address the therapeutic potential of PEDF in treating angiogenesis-, neurodegeneration- and inflammation-related diseases. J. Cell. Biochem. 106: 769–775, 2009.

KEY WORDS: PIGMENT EPITHELIUM-DERIVED FACTOR; SERPINS; PEDF RECEPTORS; ANGIOGENESIS; DIFFERENTIATION

Serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors [Law et al., 2006]. To date, over 1,500 members of this family have been identified; these include 36 human proteins, as well as molecules in plants, fungi, bacteria, archaea and viruses. The serpins family encodes two groups of proteins. The first group comprises the predominant family of protease inhibitors in mammals and regulates cascades such as inflammation, blood coagulation and extracellular matrix remodeling [van Gent et al., 2003]. The second group represents a substantial number of serpins that are not thought to be inhibitors of specific proteases but rather perform diverse functions such as hormone transporters, molecular chaperones or tumor suppressors [Silverman et al., 2001]. Interestingly, although the function of serpins varies widely, these molecules share a common structure. All typically have three β-sheets (termed A, B, and C) and eight or nine α-helices (hA-hI). Serpins also possess an exposed region termed the reactive center loop (RCL) that in inhibitory molecules includes the specificity determining region and forms the initial interaction with the target protease.

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein that belongs to the non-inhibitory serpin group [Becerra et al., 1995]. PEDF was initially described as a biological activity in conditioned medium of cultured human fetal retinal pigment epithelial cells; and identified as a neurotrophic factor able to convert Y79 retinoblastoma tumor cells into differentiated non-proliferative neurons [Tombran-Tink and Johnson, 1989]. PEDF has also been illustrated as EPC-1 (early population doubling cDNA-1), a factor lost in senescent fibroblasts and enhancing the survival and differentiation of neurons in culture [Araki et al., 1998; Bilak et al., 1999; Cao et al., 1999; DeCoster et al., 1999]. Subsequently, PEDF was rediscovered as a potent natural inhibitor of both physiological and pathological angiogenesis [Bouck, 2002]. Decreased PEDF levels in the eye correlate with neovascular and neurodegenerative ocular conditions [Spranger et al., 2001; Ogata et al., 2005; Funatsu et al., 2006]. On the other hand, PEDF down-regulation has been linked to increased metastases and poor prognosis in many human cancer types. More recent investigations suggest that PEDF could play a role in immunomodulation [Takanohashi et al., 2006; Wang et al., 2008; Zamiri et al., 2006; Zhang et al., 2006], in the protection/

Abbreviations used: PEDF, pigment epithelium-derived factor; PEDF-R, PEDF receptor; NFκB, nuclear factor kappa-B; TSP1/2, thrombospondin-1/2; VEGF, vascular endothelium growth factor; VEGF-R1/2, VEGF receptor 1/2; ATGL, adipose triglyceride lipase.

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survival against oxidative stress [Tsao et al., 2006] and expansion of the neural stem cell niche in the brain [Ramirez-Castillejo et al., 2006]; adding per consequence new functions to this multimodal factor. The mechanisms underlying most of these events are not completely clear, however, it appears that PEDF acts via multiple high affinity ligands and cell receptors. In this review article, we will summarize the current knowledge on the biochemical properties of PEDF and its receptors, the multimodal activities of PEDF and finally address the therapeutic potential of PEDF in treating angiogenesis-, neurodegeneration- and inflammation-related diseases.

THE PEDF GENE, PROTEIN, AND EXPRESSION

After characterization of PEDF neurotrophic activity, the group of Tombran-Tink cloned and sequenced the human PEDF gene [Steele et al., 1993]. The gene spans approximately 16 kb, is divided among 8 exons and 7 introns and conserved in higher vertebrates [Xu et al., 2006]. The gene is also of interest since it maps to chromosome 17p13.1, a region containing a cluster of cancer-related genes [Steele et al., 1993; Tombran-Tink et al., 1994]. The 1.5 kb transcript has been widely detected in many fetal and adult tissues such as adult liver, testis, stomach, ovaries, prostate, eye, heart, colon, brain, and spinal cord [Tombran-Tink et al., 1996; Bilak et al., 1999; Sawant et al., 2004; Browne et al., 2006; Cheung et al., 2006]. The high resolution X-ray crystal structure of glycosylated human PEDF confirmed the familiar α/β core serpin domain and demonstrated the existence of 3 major β-sheets and 10 α-helices [Simonovic et al., 2001] (Fig. 1). However, PEDF behaves as a non-inhibitory serpin in that its neurotrophic activity does not require the serpin reactive center loop [Becerra et al., 1995]. Tombran-Tink and colleagues compared the sequence of the human PEDF protein among a wide range of species and examined its structural homology with inhibitory and non-inhibitory serpins [Tombran-Tink et al., 2005; Xu et al., 2006]. In this alignment study, they identified multiple regions evolutionary conserved. These include a leader sequence responsible for protein secretion, a single C-terminal (C-ter) glycosylation site, collagen-binding residues; and four specific conserved PEDF peptides/regions consisting of: two N-terminal (N-ter) regions, corresponding respectively to residues 40-67 and 78-95; and two C-ter regions, corresponding respectively to residues 277-301 and 384-415 in the PEDF proteic sequence (Fig. 1). In contrast to the N-ter 78-95 and C-ter 384-415 regions which show a high degree of homology with other serpins and trigger probably functions fundamental to all serpins; the N-ter 40-67 and C-ter 277-301 regions are strongly conserved across all the phyla examined and unique to the PEDF protein. The latter suggests an implication in PEDF-specific functions such as anti-angiogenic and neurotrophic actions. Investigations have also identified at position 141-151 of the human PEDF, a putative nuclear localization sequence. This information, consistent with the observation that PEDF is strongly immunolocalized in the nucleus of many mammalian cells, suggests that PEDF could migrate to the nuclear compartment to perform specific function such as regulation of the cell cycle. Indeed, this function was described by Pignolo et al. [2003] in human fibroblasts.

THE MULTIPLE FUNCTIONS OF PEDF

PEDF has been demonstrated to function as a potent and broadly acting neurotrophic factor that induce cell differentiation and protects neurons in the brain, eye, and spinal cord against a wide range of neurodegenerative insults including ischemia, axotomy, glutamate excitotoxicity and oxidative stress [Cao et al., 1999; DeCoster et al., 1999; Houenou et al., 1999; Bilak et al., 2002]. In these processes, nuclear factor kappa-B (NFκB) has been involved as the major PEDF intracellular mediator. NFκB induced the expression of anti-apoptotic genes and/or neurotrophic factors that play a key role in the control of cell survival, proliferation and death [Barnstable and Tombran-Tink, 2004]. In correlation with these findings, Smith and colleagues recently implicated NFκB in the PEDF-induced neuroendocrine differentiation of prostate cancer cells demonstrating for the first time a signaling pathway involved in PEDF anti-cancerous activities [Smith et al., 2008]. These data do not rule out the implication of other intracellular signaling cascades that may be dictated by the state of differentiation of cells exposed to PEDF, pathological signals, or other factors present in the cellular microenvironment.

Additionally to this differentiation and survival action, PEDF has been described as a potent endogenous inhibitor of angiogenesis [Dawson et al., 1999]. In vitro, PEDF inhibited endothelial cells migration in a dose-dependent manner with a median effective dose (ED_{50}) of 0.4 nM. This places PEDF among the most potent natural inhibitors of angiogenesis; slightly more active than endostatine (ED_{50} of 3 nM) and thrombospondin-1 (TSP-1, ED_{50} of 0.5 nM). PEDF blocks proliferation and migration and induces apoptosis in endothelial cells activated by various angiogenic inducers, including platelet-derived growth factor, vascular endothelial growth factor (VEGF), interleukin-8 and acidic fibroblast growth factor [Dawson et al., 1999]. PEDF has also been described as the major angio-inhibitory factor in the eye where it counterbalances the pro-angiogenic effect of VEGF [Funatsu et al., 2006]. Several studies have also demonstrated that PEDF controls tumor growth. PEDF is a candidate tumor suppressor in neuroectodermal tumors and in ovarian cancer [Phillips et al., 1996; Slavc et al., 1997]. It is lost or down-regulated in most solid tumors and cancer cell lines in vitro. Furthermore, PEDF decrease has been linked to increased metastases and poor prognosis in prostate cancer [Doll et al., 2003; Halin et al., 2004], pancreatic cancer [Uehara et al., 2004], osteosarcomas [Takenaka et al., 2005; Ek et al., 2007b], breast cancer [Cai et al., 2006b], neuroblastomas [Crawford et al., 2001], melanomas [Garcia et al., 2004] and gliomas [Guan et al., 2003]; indicating the critical role of this factor in the development of many types of cancer. Recently, our group designed multiple peptides covering the N-ter region of the PEDF molecule and characterized their anti-tumor properties. Among those, we identified two epitopes, designated as the 34 mer (AA 44-77) and 44 mer (AA 78-121) peptides, that both presented significant anti-tumor effects when conditionally re-express in human prostate cancer cells [Filleur et al., 2005]. These

results were the first suggesting a potential use of PEDF-derived synthesized peptides in anti-cancer therapy.

PEDF exerts its anti-tumor effects in multiple ways [Fernandez-Garcia et al., 2006]. It directly kills tumor activated endothelium via Fas/FasL intrinsic death pathway and by blocking the synthesis of the cellular-FLICE-like inhibitory protein (c-FLIP), an endogenous dominant negative variant of the pro-apoptotic caspase-8 protein [Zaichuk et al., 2004]. PEDF also down-modulates VEGF and the matrix metalloproteinase 9 and up-regulates TSP-1/2 and Angiopoietin-2; and consequently disrupts the balance between anti- and pro-angiogenic factors [Filleur, unpublished work; Guan et al., 2004; Takenaka et al., 2005; Zhang et al., 2007]. Interestingly, Cai and collaborators recently demonstrated that PEDF inhibits angiogenesis in microvascular endothelial cells by acting through the VEGFR-1 (VEGF receptor 1), which itself can regulate angiogenesis induced by VEGFR-2 (VEGF receptor 2) -mediated signaling. More precisely, these investigators identified two mechanisms involved in PEDF inhibitory effects. PEDF enhanced the activity of the γ-secretase; resulting successively in the cleavage of the C-ter of VEGFR-1, the blockage of the translocation of VEGFR-1 intracellular C-ter domain to the nucleus and the accumulation of this domain within the cytoplasm and; inhibited the phosphorylation/activation of VEGFR-1 [Cai et al., 2006a]. Contrasting its indirect inhibitory action on tumor growth, PEDF has also been described as acting directly on tumor cells by exerting an anti-proliferative effect on these cells. PEDF induced apoptosis cell death in osteosarcoma [Takenaka et al., 2005], glioma [Zhang et al., 2007] and melanoma cells [Abe et al., 2004]. The direct injection of human recombinant PEDF into prostatic xenograft induced the necrosis of cancer cells [Doll et al., 2003]. In neuroblastoma, a better clinical outcome has been associated with an increased density of differentiated neuronal cells and Schwann cells in the stromal compartment. Crawford and colleagues have identified PEDF as the major anti-angiogenic factor secreted by Schwann cells, associated with the survival of these cells and responsible for their ability to induce the differentiation of tumor cells to a less-malignant appearance [Crawford et al., 2001]. In agreement with these data, our laboratory demonstrated that PEDF induced in prostate cancer PC3 cells the expression of chromogranin A and neuron specific enolase (NSE); two neuroendocrine differentiation specific markers, with the result of an overall lower malignancy potential in these cells [Filleur et al., 2005]. We determined that the 44 mer peptide reproduced the neuroendocrine differentiation properties of PEDF. In contrast, the 34 mer peptide curbed prostate tumor growth by inhibiting the neovessel formation in the tumor. Recently, Ek and colleagues evaluated the bioactivity of four 25 mer synthetic-derived peptides (StVOrth-1: AA 40-64, StVOrth-2: AA 78-102, StVOrth-3: AA 90-114 and StVOrth-4: AA 387-411 peptides) against the human osteosarcoma Saos2 cell line. In this study, the StVOrth-2, 3 and 4 peptides predominantly inhibited tumor cell proliferation, increased cellular adhesion to collagen type-1 and inhibited matrigel invasion, respectively. The authors also demonstrated that StVOrth-1, 2 and 3 peptides induced osteoblastic differentiation and StVOrth-3 and 4 inhibited VEGF expression. Furthermore and in agreement with our data, the StVOrth-2 and StVOrth-3 peptides were examined in vivo in an orthotopic model of human osteosarcoma. It was determined that both peptides significantly inhibited the growth of primary tumors and the development of pulmonary metastases [Ek et al., 2007a]. The totality of these studies emphasizes the multimodal potentials of PEDF in physiological and pathological conditions. However, the precise action mode and the different signaling pathways involved in PEDF function remain understudied and warrant further investigation.

A SEARCH FOR PEDF RECEPTOR(S)

The fact that PEDF presents neurotrophic/differentiation effects in the nervous system and in some tumor cells, and an apoptotic effect in endothelial cells appears contradictory and strongly suggests that PEDF could act via multiple receptors. PEDF binds with high affinity sulfated (heparin, heparin sulfate and chondroitin sulfates) and non-sulfated (hyaluronan) glycoaminoglycans, collagens and the surface of retinoblastoma cells, cerebellar granule neurons, motor neurons, prostate cancer and endothelial cells [Alberdi et al., 1999; Bilak et al., 1999; Aymerich et al., 2001; Filleur et al., 2005; Becerra et al., 2008]. Interestingly, a phospholipase-linked membrane protein was recently identified as a specific receptor for PEDF in the retinal epithelial and hepatocyte cells; suggesting one pathway by which PEDF interaction with the cell surface generates a signal [Notari et al., 2006; Chung et al., 2008].

In this search of identity for high-affinity receptor(s) of PEDF, the analysis of the three-dimensional structure of PEDF provided valuable information [Simonovic et al., 2001]. Indeed, the structure analysis revealed an unusual asymmetric distribution of charged amino acid residues, producing basic and acidic regions at opposite poles of the PEDF protein. The basic region has been located mainly on helices D, E, and F and on strands 1, 2, and 3 of the β-sheet A. This region is involved in the binding of PEDF with heparin and proteoglycans. On the other hand, the acidic region, located on helices A, G, and H, on strand 6 of β-sheet B, and in the extreme N-ter of PEDF, has been demonstrated to interact directly with positively charged area of collagen [Meyer et al., 2002], to be distinct from PEDF heparin-binding region and neurotrophic active site; and to be involved in the anti-angiogenic activity of PEDF in tumor xenografts [Meyer et al., 2002; Hosomichi et al., 2005]. Analyses using residue-specific chemical modification and site-directed mutagenesis, revealed that the acidic amino acid residues on PEDF (Asp^{256}, Asp^{258}, and Asp^{300}) are critical to collagen binding, and three clustered basic amino acid residues (Lys^{146}, Lys^{147}, and Arg^{149}) are necessary for heparin binding [Meyer et al., 2002; Yasui et al., 2003]. In addition, clusters of basic residues in PEDF have been recently identified as functional regions for the binding of hyaluronan (BX_3AB_2XB and $BXBX_2BX_2B$ motifs where B represents K or R residue separated by amino acids, excluding D and E residues in the latter, but having only one E residue (A) in the former homologous motif) [Becerra et al., 2008]. More importantly, Simonovic and colleagues identified in the N-ter part of the PEDF structure a region that could bind a putative receptor. In this region, they predicted that only the exposed parts of the helices C and D and the loop 90 are important for the binding to the putative

Fig. 1. Human PEDF amino acid sequence. The secondary structure elements are based on the study performed by Simonovic et al. [2001].

receptor and for neurotrophic activity [Simonovic et al., 2001]. Our laboratory generated peptides representing the middle and lower regions of this surface and tested their ability to bind the surface of endothelial and prostate cancer cells [Filleur et al., 2005]. We demonstrated that two peptides, the 34 mer and 44 mer peptides, were both able as the whole PEDF protein to bind the surface of endothelial and prostate cancer cells confirming the importance of these regions. We also demonstrated that these peptides could not compete each other for their binding to the PEDF receptor; suggesting once again the existence of two distinct receptors for PEDF. A PEDF-R^N that could interact with the 44 mer peptide and induce a neurotrophic effect and a PEDF-R^A that could interact with the 34 mer peptide to block angiogenesis. Our hypothesis is supported by the discovery of a 80 kDa receptor on Y-79 retinoblastoma tumor cells, cerebellar and motor neurons, and in neural retina; in contrast of a 60 kDa putative receptor on endothelial cells. In agreement with our observation, the 44 mer peptide, in contrast to the 34 mer peptide, has been previously described as able to bind a 80 kDa PEDF putative receptor on the surface of motor neurons and responsible for PEDF neuroprotective action on these cells [Bilak et al., 2002].

In 2006, the human Transport Secretion Protein-2.2 (TTS-2.2)/ independent phospholipase A2 (PLA_2)ξ, a novel lipase critical for triglyceride metabolism (also known in mice as adipose triglyceride lipase -ATGL, desnutrin, and patatin-like phospholipase domain containing protein -PNPLP2), was identified as a specific receptor for PEDF (PEDF-R) in the retina and on ARPE-19 cell surfaces [Chung et al., 2008; Notari et al., 2006]. Human PEDF-R was mapped

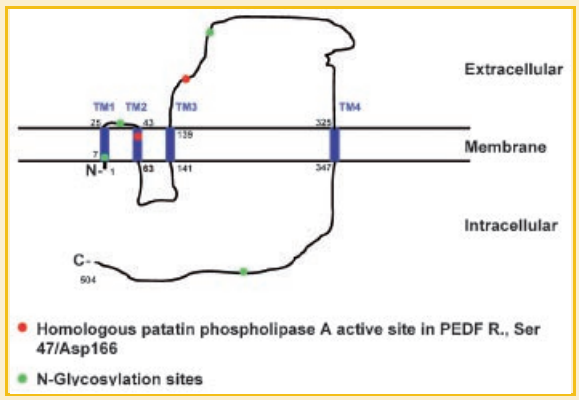

Fig. 2. Model for PEDF-R topology based on the study performed by Notari et al. [2006].

to locus 11p15.5 on chromosome 11. The inspection of the PEDF-R cDNA sequence revealed mRNA transcript of 2122 bases long with a coding capacity for a polypeptide of 504 amino acids and four N-glycosylation consensus sites (Fig. 2). A patatin-like domain that contains a GXSXG consensus sequence for serine lipases was identified from residues 10–179 on the human PEDF-R polypeptide. Hydrophobicity plots of the derived amino acid sequence anticipated up to four transmembrane domains (TM 1–4). In addition to the normal retinal pigment epithelium, PEDF-R transcript was also found as highly expressed in adipose tissue and less extensively in various organs such as prostate, testes, uterus, thymus, skin and skeletal muscle tissues. In agreement with its role as a high affinity receptor, PEDF-R was shown to be associated with the cell membrane compartment in eukaryotic cells [Chung et al., 2008] and to have a specific and high binding affinity for PEDF through its extracellular loop linking the TM domains 3 and 4. Interestingly, PEDF-R has a potent phospholipase A2 activity that liberates fatty acids suggesting that depending on the type of lipid mediators produced; PEDF/PEDF-R interaction could mediate both neuron survival/differentiation and anti-angiogenic/anti-tumorigenic activities. Valnickova et al. [2007] recently demonstrated that heparin, in contrast to other glycoaminoglycans or type I collagen, induces a conformational change in the vicinity of Lys^{198} in the PEDF protein molecule. This suggests a means by which an epitope, partially buried in the PEDF 3D structure, such as the 44 mer peptide, could become more exposed and thus binds to the receptor. In addition, the phosphorylation state of PEDF on the residues Ser^{24}, Ser^{114}, and Ser^{227} was shown to impact its biological function as neurotrophic and/or anti-angiogenic factor [Maik-Rachline and Seger, 2006] suggesting that PEDF bioactivities could be regulated at different levels. On the other hand, the absence of apparent abnormal phenotype in the eye and prostate gland in ATGL knock-out mouse, implies an incomplete phenotypic overlapping with the PEDF knock-out mice favoring once again the existence of multiple receptors for PEDF [Haemmerle et al., 2006]. These findings confirm the crucial need to validate the existence of other PEDF receptors and characterize the signaling pathways activated by the binding of PEDF on these different receptors.

PEDF AS A THERAPEUTIC AGENT

This last decade, PEDF has been described as a multi-functional protein with effective neuroprotective and anti-angiogenic activities. The fact that PEDF presents both activities makes PEDF an unusual and strong candidate for a therapeutic agent. This is indeed the case for the treatment of ocular diseases, such as age-related macular degeneration and proliferative diabetic retinopathy, where both neuroprotection and anti-angiogenic activities are essential to control the pathology. On the other hand, when multiple anti-cancer strategies are based on combined therapies, it could be very valuable to have an agent able to target simultaneously different cell populations in the tumor microenvironment. Yet, the size of PEDF may limit its utility as a therapeutic agent. This last fact emphasizes about the critical need to develop synthetic PEDF-derived peptides that retain PEDF properties. If different peptides are currently being tested, they are still in the development stage and require a closer evaluation of issues such as delivery, stability and potential toxicity. Also, of interest, is the recent implication of PEDF in inflammation. PEDF has been described as pro-inflammatory in the central nervous system [Sanagi et al., 2005; Takanohashi et al., 2006; Zhang et al., 2006; Wang et al., 2008]. In contrast, PEDF appeared anti-inflammatory in the eye where it contributes to the suppression of innate immunity in the subretinal space [Yoshida et al., 2006; Zamiri et al., 2006] insisting per consequence on the need to test the PEDF-derived synthetic peptides for their inflammatory properties.

CONCLUSION

PEDF appears as a promising therapeutic agent for neurodegeneration, angiogenesis and inflammation-related pathologies; however many questions still remain un-resolved. Are there multiple receptors for PEDF? Can these receptors have various identities and are different PEDF receptors involved in specific pathologies? Do these receptors regulated differentially or mutated in pathological tissues, such as cancers, compared to normal tissues? These specific points must be addressed prior to the initiation of any human clinical trials using PEDF active peptides.

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