PEDF – A Noninhibitory Serpin with Neurotrophic Activity

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ABSTRACT The pigment epithelium-derived factor (PEDF) is a glycoprotein with a molecular weight of 50 kDa belonging to the noninhibitory serpin family. It regulates several physiological processes, such as stimulation of retinoblastoma cell differentiation into neuron cells, and facilitation of the growth and viability of photoreceptor cells and neurons of the central nervous system. Moreover, this factor protects neuronal cells against apoptosis. PEDF is not only a neurotrophic factor, but also a natural angiogenesis inhibitor. This protein, as well as its biologically active fragments, possesses significant neuroprotective, neurotrophic, and antiangiogenic capabilities. The precise molecular mechanisms underpinning the effects of PEDF are still not quite clear. However, this protein generates great interest as a promising drug for the therapy of a wide range of neurodegenerative, ophthalmological, and oncological diseases. This review is a summary of what is known today about the structural features, biochemical properties, and multimodal functions of PEDF.
KEYWORDS PEDF (pigment epithelium-derived factor), serpin, neurotrophic factor, angiogenesis.

INTRODUCTION

PEDF (Pigment Epithelium-Derived Factor) was identified first in 1989 by a group of American researchers led by L.V. Johnson. It was purified from the growth medium of a culture of retinal pigment epithelium cells extracted from a human fetus [1, 2]. This growth medium was found to stop unlimited cell division and growth in Y-79 retinoblastoma cells. Moreover, 90% of the cells acquired the morphological and biochemical characteristics of mature neurons [1]. Electrophoretic and chromatographic means allowed to purify a 50 kDa protein from the growth medium, which was responsible for the observed effects on Y-79 cells. As was shown later, human PEDF consists of a single polypeptide chain that is 418 a.a. long (Fig. 1) [3].

Practically at the same time, PEDF was extracted from a culture of human lung fibroblast-like cells (WI-38) [4]. The murine analog of PEDF, caspin, was also discovered [5]. Human PEDF and caspin cDNA are 82.7% identical, whereas the amino acid sequences of the two are 85.6% identical.

Current research shows that PEDF is expressed in almost all mammalian and avian tissues and has numerous important functions. These include a differentiating effect on embryo and tumor cells, a protective effect on mature neurons and other neural tissue cells, and antiangiogenesis activity − the ability to inhibit the formation of new blood vessels. This is why this protein is of major interest as a regulatory factor of cell and tissue growth.

PEDF PRIMARY STRUCTURE AND ITS ENCODING GENE

The human PEDF gene and the encoded protein

The *pedf* gene is localized on the short arm of chromosome 17 in its distal region − 17p13.3 [6, 7]. It is approximately 15 kbp long and includes 8 exons interspersed with 7 introns. The exon-intron contacts are all in accordance with the GC/AG consensus. The length of the exons varies from 92 to 377 bp. The promoter region of the gene is located in the 5'-flanking region, and the CAAT-box is located in the -43 position from the transcription initiation site. A cluster of Alu-repeats which includes 8 complete and 3 partial Alu-repeats is located in the 5'-terminal region of *pedf*, up to -5 kbp [8].

The *pedf* gene encodes a sequence of 418 a.a. [3]. The N-terminal region of PEDF, right after the initiatory methionine, includes a 17 a.a. stretch of hydrophobic amino acids, with a sequence characteristic of a signaling fragment that targets the protein for secretion [3, 9]. However, according to some reports, the sequence of mature PEDF secreted from producing cells begins with Asn21, and according to these data the polypeptide chain consists of 398 a.a. [10, 11]. Posttranslational modifications of blood serum PEDF include an N-terminal residue of pyroglutamic acid, which is formed via deamination of the Gln2 residue, and the canonic N-glycosylation site Asn-Leu-Thr in positions 285-287. The added polysaccharides are heterogenic and have a molecular weight of approximately 4 kDa; their presence explains the difference between the calculated

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                                                    M Q A L V L L L C I G A L L G H S S C Q 20
 21 N P A S P P E E G S P D P D S T G A L V E E E D P F F K V P V N K L A A A V S N F G Y D L Y R V R S 70
 71 S M S P T T N V L L S P L S V A T A L S A L S L G A D E R T E S I I H R A L Y Y D L I S P D I H G 120
121 T Y K E L L D T V T A P Q K N L K S A S R I V F E K K L R I K S S F V A P L E K S Y G T R P R V L T 170
171 G N P R L D L Q E I N N W V Q A Q M K G L A R S T K E I P D E I S I L L L G V A H F K G Q W V T K 220
221 F D S R K T S L E D F Y L D E E R T V R V P M M S D P K A V L R Y G L D S D L S C K I A Q L P L T G 270
271 S M S I I F F L P L K V T Q N L T L I E E S L T S E F I H D I D R E L K T V Q A V L T V P K L K L S 320
321 Y E G E V T K S L Q E M K L Q S L F D S P D F S K I T G K P I K L T Q V E H R A G F E W N E D G A G 370
371 T T P S P G L Q P A H L T F P L D Y H L N Q P F I F V L R D T D T G A L L F I G K I L D P R G P 418
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Fig. 1. Amino acid sequence of the PEDF protein. The bond Leu382-Thr383 is marked in bold red. Yellow highlights mark the serpine loop, and light blue marks the N-glycosylation site. Green – peptide responsible for the antiangiogenic function; pink – peptide responsible for the neurotrophic function. Grey – phosphorylation sites. Blue – cluster of negatively charged amino acids, brown – positively charged amino acids.

and actual masses of the factor [12]. According to 2D electrophoresis data, human PEDF secreted by pigment epithelium cells has 4 isoforms, whose isoelectric points are 6.0; 6.2; 6.4 and 6.6 [13].

PEDF is a member of the noninhibitory serpine family
Based on the analysis of the primary structure of the protein and corresponding cDNA, PEDF was included into the serpine family (serine proteases inhibitors) [14]. Amino acid sequences of the factor and a1-antitrypsin are 27% identical (42% if equivalent substitutions are factored in), and 27 and 26% identical with a1-antichemotrypsin and a2-plasmin inhibitor, respectively (44 and 43% if equivalent substitutions are factored in) (Fig.2) [3]. It is known that 51 a.a. residues are necessary to form a spatial structure of the serpine type [15], in PEDF there are 39 coincidences (76%) [16]. Despite the high level of similarity with the primary and spatial structures of serine protease inhibitors, PEDF does not inhibit these enzymes [17]. This may possibly be due to the structural characteristics of the exposed reactive loop of the factor. Even though it is equal in length to other serpines (17 a.a. residues Glu366-Leu382) [18,19], its content lacks some elements that are characteristic of inhibitor serpines – Ala-rich sequences (in the 9th and 12th loop positions) and Thr in positions 8 and 14, while also possessing proline residues, which is not characteristic of serpines [16].

The structure of PEDF is similar to other serpines in that it possesses a bond that is especially prone to proteolysis in a loop exposed into the cytoplasm [14], as do other serpines. Limited proteolysis of PEDF with various serine proteases (chemotrypsin, elastase, subtilisin, etc.) reduces its molecular mass by approximately 4 kDa. It has been determined that these proteases hydrolyze the Leu382-Thr383 bond, which results in the release of a 36-a.a. residue fragment from the C-terminus. Unlike inhibitory serpines, no covalent bond is formed between the PEDF molecule and the active site

of the proteinase. PEDF with a truncated C-terminus conserves its ability to differentiate Y-79 cells into mature neurons [20].

After the release of the 36-a.a. fragment, the general ellipticity of the molecule is reduced, indicating that the molecule experiences a reduction in the a-helix structure. Unlike inhibitory serpines, this process lowers the thermal stability of the PEDF molecule. However, these conformational alterations are not widespread and involve only the C-terminal region located near the exposed loop. The functional role of the latter and the


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HUMA1AT      MPSSVSWGILLAGLCCLVPVSLAEDPQGDAAQKTDTSHHDQD--HP
             : :::    : : : :  :.   :              ..
PEDF     MQALVLLLCIGALLGHSSCQNPASPPEEGSPDPDSTGALVEEEDPFFKV

HUMA1AT      TFNKITPNLAEFAFSLYRQLAHQSNSTNIFFSPVSIATAFAMLSLGTKA
             .::.  .  .:::    : ::.::::.:::.  ::::
PEDF     PVNKLAAAVSNFGYDLYRVRSSMSPTTNVLLSPLSVATALSALSLGADE

HUMA1AT      DTHDEILEGLNFNLTEIPEAQIHEGFQELLRTLNQPDSQLQLTTGNGLF
             :  .  : ..: :  .::  .::: :.    .  :    . 
PEDF     RTESIIHRALYYDL--ISSPDIHGTYKELLDTVTAPQKNLK-SASRIVF

HUMA1AT      LSEGLKLVDKFLEDVKKLYHSEAFTVNFGDHEEAKKQINDYVEKGTQGK
             :::.  :.  . :  .  :     .          .::: :.    ::
PEDF     -EKKLRIKSSFVAPLEKSYGTRPVLT-GNPRLDLQEINNWVQAQMKGK

HUMA1AT      IVDLVKELDRDTVFALVNYIFFKGKWERPFEVKDTEDEDFHVDQVTTVK
             .  : . :  : :  :::  .  : :.  : :::  .:.  ::.
PEDF     LARSTKEIPDEISILLLGVAHFKGQWVTKFDSRKTSLEDFYLDEERTVR

HUMA1AT      VPMMKRLG-MFNIQHCKKLSSWVLLMKYLGNATAIFFLPDE--GKLQHL
             ::::   ..   ::  .      : :::::     ::
PEDF     VPMMSDPKAVLRYGLDSDLSCKIAQLPLTGSMSIIFFLPLKVTQNLTLI

HUMA1AT      ENELTHDIITKFLENEDRRSASLHLPKLSITGTYDLKSVLGQLGITKVF
             : ::.:  :       :: :.:::  .:  .  . ..   :
PEDF     EESLTSEFIHDIDRELKTVQAVLTVPKLKLSYEGEVTKSLQEMKLQSLF

HUMA1AT      SNGADLSGVTEEAPLKLSKAVHKAVLTIDEKGTEAAGAMFLEAIPMSIP
             .  :.:  .:  :.::    ::.  ..:      :.   .:
PEDF     -DSPDFSKITGK-PIKLTQVEHRAGFEWNEDGAGTTPSPGLQPAHLTFP

HUMA1AT      PEVKFNKPFVFLMIEQNTKSPLFMGKVVNPTQK
             .  .: ::.::.  :  :::::..:
PEDF     LDYHLNQPFIFVLRDTDTGALLFIGKILDPRGP
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Fig. 2. Comparison of the amino acid sequences of human PEDF and α1-antitrypsin (HUMA1AT). Colons (:) depict identical amino acids, and periods depict conserved substitutions [3].

released C-terminal fragment of PEDF remain largely unstudied. Verified data demonstrate that the residues present in this region of the molecule, in conjunction with the N-terminal signaling peptide, are needed for the secretion of PEDF from the cell.

Removal of the Pro415-Pro418 and Pro373-Ala380 fragments, as well as point substitutions of Gly376, Leu377, Pro393, Phe394, or Phe396 (the first two are located in the exposed loop), either results in changes of the spatial structure of the molecule or alters its interaction with other proteins, presumably transporters. PEDF transfer from the endoplasmic reticulum into the Golgi apparatus is disrupted, which prevents PEDF secretion [16, 21].

Spatial structure of PEDF. Asymmetrical localization of positive and negative charges in the PEDF molecule

A mature molecule of the factor is a globule with a radius of <3.05 nm. Approximately 60% of PEDF amino acid residues are involved in the formation of 10 a-helices and 3 b-sheets [22]. Ser24, Ser114, and Ser227 are phosphorylation sites. The factor includes two important amino acid sequences − a 34 a.a stretch (Asp34-Asn77) and a 44 a.a. stretch (Val78-Thr121) − which are responsible for the antiangiogenic and neurothrophic interactions, respectively (Fig. 3) [23].

An interesting feature of the PEDF molecule is the presence of positively and negatively charged amino acid clusters, located on opposite sides of the globule. These clusters allow the factor to bind with structural elements of the extracellular matrix, namely glycosaminoglycans and collagen, via electrostatic bonds [24, 25].

The cluster of negatively charged residues (Asp and Glu) is located in two regions of the polypeptide chain, which are exposed on the surface of the globule, adjacent to each other: Glu41-Asp64 (residues Glu41, Glu42, Glu43, Asp44, and Asp64) and Asp256-Glu304 (residues Asp256, Asp258, Glu290, Glu291, Glu296, Asp300, and Glu304). The presence of this cluster determines PEDF affinity to type I, II, and III collagens [25]. PEDF binding to collagens, as well as to glycosaminoglycans, is weakened in high ionic strength solutions, since the bonds are electrostatic in their nature. The peptide fragments Val40-Arg67 and Phe277-Ile301, which are also involved in the formation of this cluster, are conserved from fish to mammals [16].

The cluster of positively charged amino acids, which includes 12 residues of Arg, Lys, and His, is located in the Lys 134-Lys214 region. Residues Lys134, Lys137, Lys189, Lys191, His212, and Lys214 form the "core" of the cluster, while residues Arg141, Lys146, Lys147, Arg149, Arg165, and Arg167 are located on the periphery. This local positive charge allows the binding
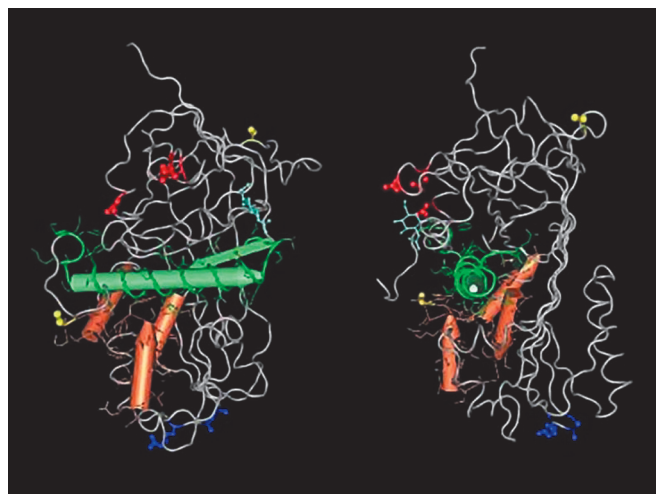

Fig. 3. 3D crystal structure of PEDF. This figure shows two projections on the vertical axis with a shift of 90°. The peptide responsible for the antiangiogenic function is marked in green, and the one responsible for the neurotrophic function is marked in orange. Red marks the negatively charged amino acids which form the collagen-binding cluster, and purple marks the positively charged amino acids which form the "core" of the glycosaminoglycan-binding cluster. Phosphorylation sites (Ser114 and Ser227) are marked in yellow, and the glycosylation site at Asn285 with the bound sugar residues is marked in light blue [23].

of PEDF to polyanionic molecules which are rich in charged sulfate groups, such as heparin, chondroitin sulfate, etc. This cluster is also involved in the interaction between PEDF and the glycosaminoglycans in the interphotoreceptor matrix of the retina [24].

Apart from the cytoplasm, PEDF can also be found inside the nucleus. It seems that the positively charged Lys146-Arg149 fragment can act as a nuclear localization sequence. Notably, only 10 of the studied 6,000 proteins, including PEDF, possess a C-terminal glycosylation site; all of these proteins are localized in the nucleus [16]. In its role as a nuclear protein, PEDF may possibly be involved in cell cycle regulation.

Charges on the surface of the PEDF molecule are positioned in an asymmetric fashion − the clusters of basic and acidic amino acids are on opposite sides of the globule. This allows PEDF to simultaneously interact with glycosaminoglycans and collagen. Interaction with glycosaminoglycans increases PEDF affinity towards collagen and vice versa [25]. Thus, PEDF can modulate cell adhesion processes that involve integrin-collagen interactions. Since cell adhesion is one of the key stages of angiogenesis, this may indeed be the basis for the antiangiogenic activity of the factor [25].

Phosphorylation of PEDF influences its antiangiogenic and neurotrophic activity

PEDF purified from human plasma is a phosphoprotein. There are three phosphorylation sites on the surface of the PEDF molecule - Ser24, Ser114, and Ser227. PEDF possesses and other potential phosphorylation sites; however, in normally folded molecules these sites are unavailable for kinases. In the seum factor is phosphorylated mainly by casein kinase 2 on 2 main residues, Ser24 and Ser114, but also by protein kinase A on Ser227 [26]. PEDF is functionally modulated by extracellular phosphorylation. The casein kinase 2 phosphorylated PEDF has a reduced neurotrophic activity, while its antiangiogenic activity is significantly increased. On the other hand, protein kinase A phosphorylation reduces the PEDF antiangiogenic activity but has only a slight effect on its neurotrophic activity. A fully phosphorylated factor displays both high antiangiogenic and neurotrophic activities.

Casein kinase 2 phosphorylation of Ser24 and Ser114 prevents further phosphorylation of PEDF by protein kinase A, and partial denaturation of protein restores its sensitivity to protein kinase A phosphorylation. Casein kinase 2 phosphorylation is followed by a conformational change in the PEDF molecule, thereby making Ser227 inaccessible to protein kinase A phosphorylation. On the other hand, protein kinase A phosphorylation of PEDF does not affect the conformational state of the casein kinase 2 phosphorylation sites and allows their further phosphorylation. The inhibitory effect of the casein kinase 2 phosphorylated PEDF on its protein kinase A phosphorylation may serve as a regulatory mechanism of PEDF function under conditions where the highly antiangiogenic activity of PEDF should be preserved while its neurotrophic activity should be eliminated. On the other hand, the protein kinase A phosphorylation of PEDF reduces PEDF antiangiogenic function. PEDf could be further phosphorylated by casein kinase 2, and that converts it from a poor to a very potent antiangiogenic factor that maintains its neurotrophic activity [27]. This is a novel role of extracellular phosphorylation that completely changes the nature of the physiologic activity of a circulating protein. Thus, differential phosphorylation induces variable effects of PEDF and therefore contributes to the complexity of PEDF action.

Occurrence and evolutional conservation of PEDF

The *pedf* gene is conserved and can be found in the genomes of various animal species, ranging from fish to humans (Fig. 4, 5) [16]. Through DNA-RNA hybridization methods, researchers discovered that PEDF mRNAs were present in nearly all 44 of the examined tissues of adult humans and human embryos. It is pre-

sumed that PEDF is expressed mainly by cells that have not lost their ability to divide *in vivo* [28]. In various regions of the eye and layers of the retina in human adults and embryos, antibodies to the PEDF polypeptide interacted with the cytoplasm of developing photoreceptors, the glial layer, single cells in the neuroblast layer, and pigment granules in pigment epithelium cells (starting from the 8th week of embryo development). In the organism of an adult, the antibodies interacted with the nuclei of rods (but not with those of cones), with the cytoplasm of certain cells in the nuclear and glial layers, with pigment epithelium cells, choriocapillaris of the cornea, the pupil, and ciliated epithelium [29].

The most conserved species-specific peptide fragments are the following sequences [16]. The leading N-terminal region of the factor responsible for the secretion of PEDF from the cell was conserved during the course of evolution [30]. Another highly conserved sequence, Asn285-X-Thr287, is an N-glycosylation site. Four other peptide fragments are also conserved: two of which are unique to PEDF and form a cluster of negatively charged amino acids, and two others (in positions Val78-Gly95 and Phe384-Pro415) which are similar to other serpines. It seems that the functions of the latter two are similar for all serpines.

EFFECTS OF PEDF ON CELLS AND TISSUES OF VARIOUS ORIGINS

## Differentiation effect of PEDF and its effect on metabolism

The differentiation effect of the neurotrophic PEDF factor was shown and studied on neuronal cells − both on embryonic (immature neurons, developing photoreceptors) [31,32] and tumor cells (neuroblastoma, retinoblastoma, and carcinoma) [3,33,34]. Human and bovine PEDF has a differentiation effect on *in vitro* cell cultures of immature motor neurons obtained from chicken embryo (5.5 days old) spinal cords [35]. PEDF is the main factor that controls normal morphogenesis and metabolism of photoreceptor and glial cells during the embryonic development of the retina [32, 36, 37].

PEDF has differentiation and antiproliferative effects on Y-79 retinoblastoma cells [1, 3]. The antitumour effect can also be observed on neuroblastoma cells. For this type of tumour, clinical prognosis is usually better when the population of cells is rich in differentiated neurons and Schwann cells, whose cultural medium has antitumor properties [38]. PEDF, which is one of the secreted products of Schwann cells, mediates the antitumor effects observed for these cultural media [33].

PEDF also has an antitumor effect on ovarian epithelial carcinoma, which is the most lethal type of can-

Fig. 4. Comparison of the amino acid sequences of PEDF proteins of different origins. 14 sequences of the factor from different biological species (human, *Homo sapiens*; chimpanzee, *Pan troglodytes*; domestic cow, *Bos taurus*; pig, *Sus scrofa*; dog, *Canis canis*; mouse, *Mus musculus*; rat, *Rattus norvegicus*; chicken, *Gallus gallus*; Western clawed frog, *Xenopus tropicalis*; African clawed frog, *Xenopus laevis*; Japanese ricefish, *Oryzias latipes*; fugu, *Takifugu rubripes*; rainbow trout, *Oncorhynchus mykiss*; zebra fish, *Danio rerio*) were analyzed using ClustalW software. Asterisks mark the positions of fully conserved residues. Colons indicate that a "strong" functional group is conserved; periods indicate that a "weak" group is conserved. The blue line marks the PEDF leader sequence. The double red line marks regions that are highly conserved in all the PEDF sequences. Dashes are used for the maximal alignment of the sequences [16].
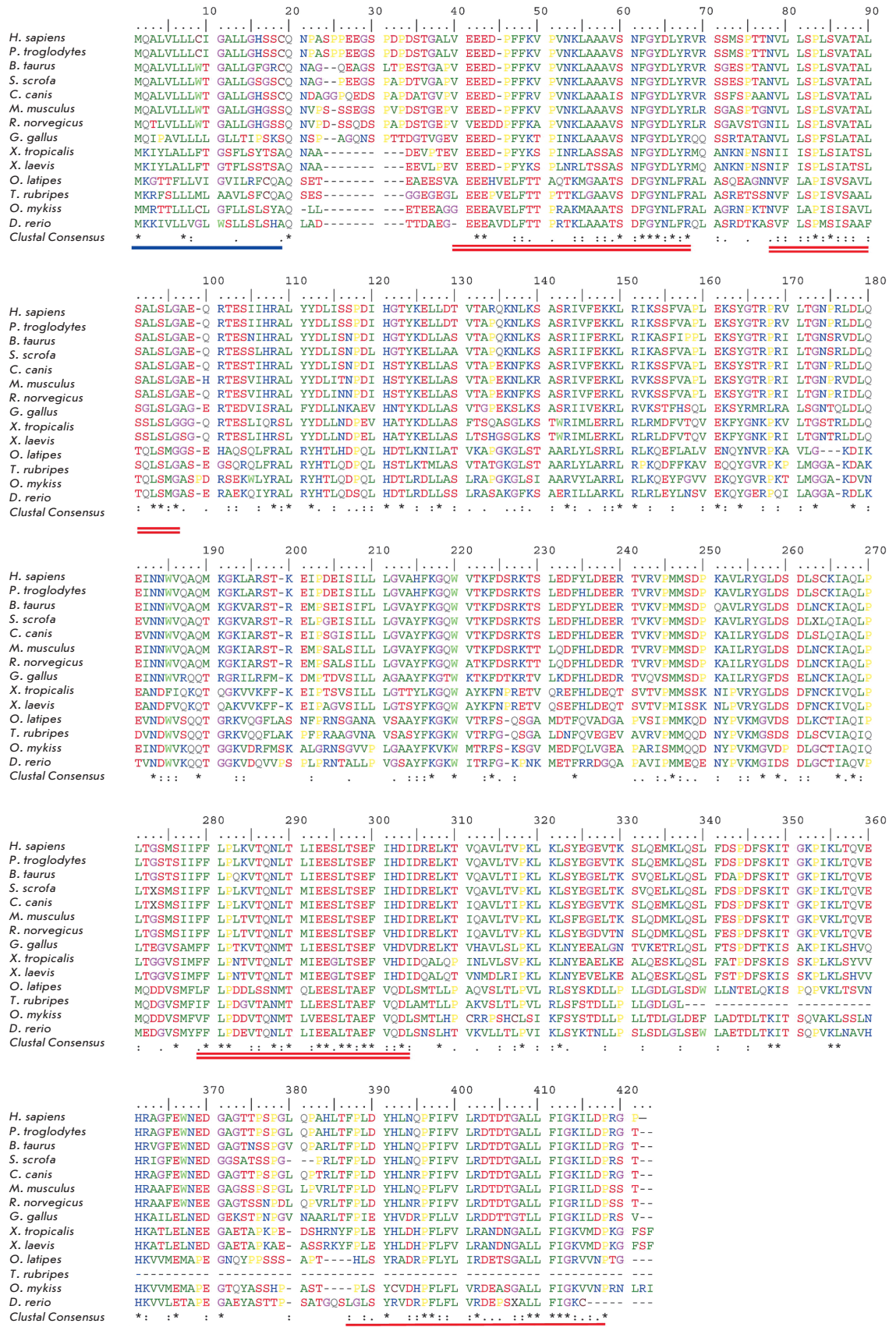
	Hs	Chimp	Bt	Pig	Dog	Mm	Rt	Chick	Xt	Xl	Fugu	Trout	Zebra
Hs	100												
Chimp	99	100											
Bt	87	88	100										
Pig	88	88	90	100									
Dog	90	90	87	88	100								
Mm	86	85	84	84	87	100							
Rt	83	83	82	82	87	93	100						
Chick	63	63	63	63	62	63	63	100					
Xt	55	55	55	56	56	56	56	55	100				
Xl	56	57	57	58	57	57	56	53	89	100			
Fugu	34	35	36	36	36	36	35	35	34	35	100		
Trout	37	37	39	38	36	37	37	39	38	38	64	100	
Zebra	40	40	41	41	41	40	39	42	41	40	56	65	100

Fig. 5. Interspecies identity of the amino acid sequences of PEDF in percentage points. The table was generated using PSI-Blast software [16].

cers. The level of PEDF expression in ovarian tumors and their cell lines is significantly lower in comparison with normal ovarian epithelium. Exogenous PEDF inhibits the growth of both normal and tumour ovarian epithelium, while a decreased activity of endogenous PEDF accomplished through the injection of inhibitors results in increased proliferation of these cells.

The effect of PEDF on ovarian epithelial cells is mainly regulated by estrogens. Treatment of cell lines with 17β-estradiol lowers the production of PEDF and its mRNA by repressing the transcription of the *pedf* gene. Estrogen receptors play a role in this regulation. 17β-estradiol promotes the growth of normal and cancerous tumour ovarian epithelium cell lines, while simultaneous treatment with 17β-estradiol and PEDF negates the stimulating effect [34]. Some lines of ovarian carcinoma bear a deletion in the 17 chromosome at locus 17p13.3. This deletion is 15 kbp long and includes most of the *pedf* gene [39]. Proliferation of these cell lines cannot be stimulated by 17β-estradiol, which seemingly indicates that its effector region in the 5'-flanking region of *pedf* is also deleted. Epigenetic modifications, such as hypermethylation of the promoter, may act as alternative mechanisms for regulating *pedf* expression [34].

In pigment epithelium cells, PEDF stimulates the synthesis and accumulation of melanin [40]. The importance of melanin in the development of retinal neurons is suggested by the fact that, irrespective of the causative factors for albinism, all mammal albinos display impaired visual signals [41]. The factor boosts the activity of the promoter of the tyrosine kinase gene, whose product is involved in the synthesis of melanin [42].

Neuroprotective effect of PEDF
PEDF was shown to prevent apoptosis of cells in a culture of retinal neurons after H_2O_2 treatment [43]. Pretreatment with PEDF protects the pigment epithelium from the disruptions in the barrier function caused by H_2O_2. It also protects the cells from other effects of hydrogen peroxide: redistribution of the synaptic proteins occludin and N-cadherin in cell membranes of the pigment epithelium; reorganizations of actin and activation of the signaling cascade mediated by the p38/27 kDa heat chock protein [44].

The factor also has a protective effect on cultures of primary cerebellum granular cells extracted from baby rats (8 days old). It considerably slows spontaneous and serum deprivation-mediated apoptosis in this culture [45, 46]. PEDF also protects cerebellum granular cells [47], hippocampus neurons [48], and spinal motor neurons [49] from the toxic effects of glutamate. According to certain data, the main mechanism responsible for the protective effect of PEDF on granular cells is the activation of the NF-ϰB transcription factor [50].

Antiangiogenic effect of PEDF
PEDF is one of the most potent antiangiogenic factors. It inhibited vessel growth in rat corneas that were stim-

ulated with bFGF, which is a very potent angiogenic factor. The effective concentration of PEDF is much lower than that of the most potent, previously known antiangiogenic factors − angiostatin, endostatin, and thrombospondin [51]. The factor is involved in the complex and balanced control of angiogenesis, acting in opposition to angiogenic factors such as VEGF ((vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) [52−55].

Saturation of tissues with oxygen affects the concentration of PEDF. Hyperoxygenation causes the formation of retinas with decreased amounts of blood vessels, while (though) the level of PEDF expression in these conditions was shown to be high [56]. Hyperoxygenation stimulates factor production; hypoxia, on the other hand, has a repressive effect.

The mechanism responsible for the antiangiogenic effect of PEDF is currently under study. Endothelium cells display positive chemotaxis towards angiogenic factors. Thus, one of the mechanisms of PEDF action is the inhibition of the chemotaxis of endothelial cells, which form the walls of blood vessels. This effect inhibits chemotaxis towards all of the studied angiogenic factors − VEGF, PDGF, IL-8, etc. [51].

According to other data, the reason for the antiangiogenic effect of PEDF is the apoptosis of endothelial cells [57]. A characteristic trait of the factor is its selective effect on growing vessels; it does not damage any functioning vessels [58]. The reason for such a selective effect is the mode of apoptosis induction, mediated by the Fas-ligand [59]. Angiogenic agents stimulate endothelial cells towards producing a range of anti-apoptotic molecules, which increase their viability [60], and they also stimulate Fas-receptors. Vessels that are already functioning have their endothelium cells in a state of "arrested growth," with the cells in tight contact with each other. Fas-receptor expression is also decreased [61]. These factors protect cells from the apoptotic effect of PEDF.

Nonetheless, besides PEDF having a stimulating effect on Fas/FasL expression and Fas-mediated apoptosis, there must be alternative pathways for the apoptotic activity of the factor, since Fas and FasL knockout mice are still sensitive to PEDF-mediated apoptosis [57, 62, 63]. It has been shown that PEDF can activate p38 MAPK in a culture of epithelial cells extracted from a human umbilical vein (HUVEC) by promoting their phosphorylation. Activation of p38 results in the activation of caspases 3, 8, 9 and, ultimately, in cell apoptosis [64].

It is notable that VEGF also promotes p38 phosphorylation, and this effect is potentiated during co-treatment with PEDF. One of the most important functions of VEGF is to protect endothelial cells from apoptosis in various conditions, including medium depletion. Activation of the p38 kinase by VEGF is accomplished by regulation of its transcription [65, 66] and cytoskeleton reorganization, as well as cell migration [67]. It seems likely that the threshold concentration of phosphorylated p38 MAPK needed for the proangiogenic effect to manifest is lower than the concentration needed for the activation of the apoptosis pathway. The PEDF-mediated increase in the level of activated p38 can result in concentrations of p38 that exceed the threshold concentration and, thus, cause apoptosis [64].

Even though PEDF stimulates the apoptosis of endothelial cells, it prevents the apoptosis of various cells of neural origin. PEDF can have the opposite effects on endothelial cells of differing phenotypes [68]. In these cases, it is possible that the factor's action is effected via different unrelated mechanisms.

PEDF RECEPTOR

The multiple functions of PEDF are obviously realized through different mechanisms. However, it seems likely that in most cases the interaction between PEDF and cells takes place via a ligand-receptor mechanism. The effects of the factor can be blocked by antibodies which prevent the factor from binding to the cell's surface [47,69,70].

Not long ago, researchers managed to identify a gene whose product acted as a PEDF receptor. The gene was thus named PEDF-R. This discovery was made using a two-hybrid system in retinal cells [72]. The gene is localized in region 11p15.5 in chromosome 11 and consists of 10 exons and 9 introns. The mRNA transcript of *pedf-r* is 2122 bases long. It encodes a protein molecule which consists of 504 amino acids (molecular mass of 55.315 kDa). The sequence contains 4 consensus N-glycosylation sites. When expressed in eukaryotic systems, the receptor has a mass of approximately 81 kDa, which is similar to the mass of PEDF-binding proteins on the cell's surface. The latter were identified previously in cell lines of human retinoblastoma (Y-79), in rat cerebellum granular neurons, and in cells extracted from bovine retinas [70,71].

The receptor is mainly produced in retinal pigment epithelium cells, the inner segments of photoreceptors, and neuronal cells of the retina. However, the receptor is not detected in the outer segments of rods. Apart from normal pigment epithelium cells, PEDF-R is also present in a number of other tissues and organs − in the optical nerve, uvea, retinoblastoma cells, other tumors of various origins, as well as animal brain cells that were in a state of serotonin and catecholamine depletion. The gene encoding this receptor has been evolutionarily conserved in mammals. The highest degree of similarity was observed between human PEDF-R
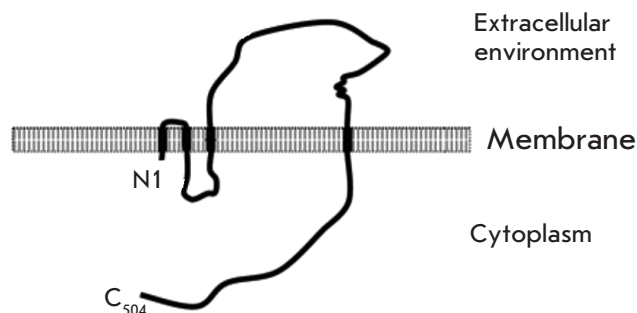
Fig. 6. Topology of PEDF-R predicted according to its amino acid sequence. The thick lines indicate the transmembrane domains [72].

and that of mice (89% identical) and rats (89% identical). Genes homologous to PEDF-R have also been found in the genomes of nematodes (*Caenorhabditis elegans*) and mosquitoes (*Anopheles gambiae*).

PEDF-R is a transmembrane protein with four transmembrane, two extracellular and three intracellular domains; the N- and C-termini of the molecule are exposed into the intracellular environment (Fig. 6) [72].

PEDF-R can bind PEDF with a Kd ~ 3.03 ± 0.716 nM both in solution, as well as after immobilization of one of the proteins. It retains this ability even after denaturation and refolding. Posttranslational modifications, including N-glycosylation, are not required for the factor to bind to the receptor − PEDF-R expressed in bacteria interacts with PEDF with a Kd nearly identical to that observed under normal conditions. The factor-binding domain of PEDF-R has been identified as Gln250-Arg383 and is called 12c. This fragment was shown to be highly similar to type I rat collagen. It seems likely, however, that this is not the only fragment that plays a role in PEDF binding, since the factor's affinity towards a full-sized receptor is higher. The dissociation constant of the latter is nearly two orders of magnitude larger than that of the 12c fragment ((Kd.12c ~ 134 nM).

The factor-receptor interaction involves the Val78-Thr121 fragment of the PEDF molecule. A synthetic analog (44-a. a. residues long) retains the ability to bind to the receptor [70].

Apart from the ability to bind PEDF, PEDF-R also manifests phospholipase activity and has been classified as a member of the calcium-independent, A_2 phospholipase family. Another feature of the PEDF-R sequence is two spatially adjacent patatin-like fragments (Asn39-Ala54 and Ser158-Tyr177) that form a Ser47/Asp166 catalytic dyad, which is present in the active sites of patatin B2 and human cytosolic A_2 phospholipases [73].

A_2 Phospholipases release lysophospholipids, which play a role in cell signaling and affect the development

and functioning of all mammalian organ systems [74]. A_2 phospholipases also release biologically active fatty acids from the cell's membrane. These acids act as messengers or as precursors of eicosanoids, which mediate signal transduction [75]. Arachidonic and docosahexaenoic acids, which are abundant in the membranes of cells in the retina and the central nervous system, can influence the viability of retinal and central nervous system cells [76, 77] and demonstrate antitumor and antiangiogenic effects, similar to PEDF [78,79].

PEDF-R can hydrolyze arachidoinoyl-*sn*-glycerin-3-phosphocholine, thus releasing arachidonic acid [80]. Even though there is currently no data on the release of docosahexaenoic acid by PEDF-R, it has been shown that PEDF in subnanomolar concentrations can activate the synthesis and release of its derivatives from the ARPE-19 human cell line [81, 82]. These data indicate that PEDF can facilitate the survival of retinal cells by influencing the lipid signaling pathways that are connected with PEDF-R. Interaction of PEDF with its receptor, PEDF-R, results in the release of fatty acids and lysophospholipids from cell membranes. These entities act both para- and autocrinally, by raising the viability and differentiation potential of neuronal cells, or by causing the death of tumor and endothelial cells [72].

PEDF AS A SUBSTRATE OF MMP-2 AND MMP-9

As previously mentioned, regulation of angiogenesis is very dependent on the quantitative ratio between the differentiation factors VEGF and PEDF. It has been shown that increasing the concentrations of exogenous PEDF in a model of choroidal neovascularization stimulates the production of VEGF by endothelial cells. In turn, increased concentrations of VEGF induce the production of matrix metalloproteases MMP-2 and -9 (or the corresponding gelatinases A and B). The activated proteinases hydrolyze the components of the extracellular matrix and the PEDF previously bound to these components. However, they do not influence VEGF. Thus, increased concentrations of PEDF result in elevated inactivation of this factor by MMP, which increases the VEGF/PEDF ratio and stimulates angiogenesis.

The region of PEDF most accessible for proteolysis by MMP-2 and -9 is the exposed loop that contains the Leu382-Thr383 bond. However, apart from this bond, the PEDF sequence also has several sites for MMP-2 and -9 proteolysis, which are partially or completely "masked" by a tertiary structure. These sites are less accessible to the activity of matrix metalloproteinases. It is possible that the activity of MMP-2 and/or -9 results in a gradual loss of its tertiary structure by the PEDF molecule, making the inner bonds more accessible to metalloproteinases. Complete proteolysis creates

a multitude of peptide fragments, which manifest no antiangiogenic or neurotrophic activity even though they retain the ability to bind components of the extracellular matrix. Thus, matrix metalloproteinases can inactivate PEDF through the same mechanism as the one effected by inhibitory serpines (such as antitrypsin and antithrombin III). They affect the reactive site of the loop and are thought to form a stable inactive complex with the factor molecule (although the existence of such a complex has yet to be proven). However, there is still a possibility that the loss of biological activities by PEDF is achieved by the complete gradual proteolysis of the protein [83].

CONCLUSION

The PEDF protein has been the subject of intensive studies for several years, and the amount of PEDF-related research increases with every year. It has been shown that the protein regulates a wide range of processes in the cells and tissues of various eukaryotic organisms, including in those of humans. PEDF is a key player in high-level intracellular interactions, though it is possible that some of its functions in the metabolic pathways of the cell have yet to be identified. The antiproliferative, antiangiogenic, protective, differentiative and other activities of PEDF are very promising not only from the viewpoint of basic scientific research, but also for the use of this protein in the treatment of various diseases, such as neurodegenerative damage to the optical and nervous systems and cancerous processes. ●

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