Pigment-epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization

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Pigment epithelium-derived factor (PEDF) inhibits the formation of blood vessels in the eye by inducing apotosis in actively dividing endothelial cells. The activity of PEDF equals or supersedes that of other anti-angiogenic factors, including angiostatin, endostatin and thrombospondin-1. In addition, PEDF has the potential to promote the survival of neurons and affect their differentiation. Here we show that PEDF is present in plasma at a concentration of approx. 100 nM (5 μg/ml) or twice the level required to inhibit aberrant blood-vessel growth in the eye. Thus the systemic delivery of PEDF has the potential to affect angiogenesis or neurotrophic processes throughout the body, significantly expanding the putative physiological role of the protein. A complete map of all post-translational modifications revealed that authentic plasma PEDF carries an N-terminal pyroglutamate blocking group and an *N*-linked glycan at position Asn^{266}. The pyroglutamate residue may regulate the activity of PEDF analogously to the manner in which it regulates thyrotropin-releasing hormone.

Key words: angiogenesis, collagen-binding, plasma, pyroglutamate, serpin.

INTRODUCTION

Pigment-epithelium-derived factor (PEDF) is a potent inhibitor of angiogenesis in the eye [1,2]. Inhibition of vessel formation is mediated by up-regulation of the Fas ligand on the surface of endothelial cells, with subsequent induction of apotosis in actively dividing cells [3]. Thus PEDF inhibition of vessel formation is mediated via Fas–Fas ligand activation. Initially, PEDF was identified as a neuronal differentiation factor produced by cultured human retinal pigment epithelial cells [4]. Independently, PEDF was identified as 'EPC-1' (early population doubling level cDNA-1) produced by a fibroblast-like cell line. The PEDF mRNA was induced more than 100-fold when the cells were arrested in the G_0 state [5]. More recently, however, the anti-angiogenic activity of PEDF has received much attention because blood-vessel growth in avascular compartments of the eye is a leading cause of blindness. In contrast with the induction of apotosis in endothelial cells, PEDF promoted the survival of immature cerebellar granule cells by inhibition of apoptosis [6]. A number of other studies have also demonstrated the neuronal-survival effect of PEDF (for a review, see [7]). Thus PEDF appears to both induce and inhibit apoptosis; the regulation of apoptosis by PEDF is likely to be regulated through its interaction with other proteins and/or by post-translational modifications.

PEDF was cloned from a human eye cDNA library and shown to belong to the serine-proteinase inhibitor (serpin) family [8]. This has recently been confirmed by determination of the three-dimensional structure [9]. The deduced amino acid sequence contains the consensus sequences for one *N*-linked glycosylation, and several predicted sites for phosphorylation and O-linked glycosylation. The native bovine protein has been purified from the interphotoreceptor matrix [10] and the vitreous humour [11] of bovine eyes and found to be a monomeric glycoprotein. Interestingly, the N-terminus of bovine PEDF purified from the interphotoreceptor matrix was unblocked, while the N-terminus of PEDF from bovine vitreous humour was blocked.

Authentic human PEDF purified from a natural source has not previously been biochemically characterized. Recombinant human PEDF proteins have been expressed in different systems including *Escherchia coli* [12], HEK-293 cells [1,13] and baby hamster kidney (BHK) cells [14]. Although recombinant material seems to retain the physiological functions of the native protein, analysis of PEDF produced by human fibroblasts (WI-38) and purified bovine PEDF by two-dimensional gel electrophoresis indicated that the native protein is heterogeneous in charge and is post-translationally modified [10,15]. At present, it is unclear (i) if the various expression systems produce authentic PEDF containing the native post-translational modifications or (ii) if these modifications are functionally significant.

PEDF is an important mediator of cellular functions, with the potential to induce apoptosis or cell differentiation and survival. The role of the protein has previously been considered mainly in the context of angiogenic regulation in the interphotoreceptor matrix and the vitreous and aqueous humours of the eye. In the present study we show that PEDF is found in the blood at a concentration sufficient to have functional significance. The relatively high plasma concentration of PEDF facilitated the purification and biochemical characterization of the authentic protein. The systemic delivery of this protein suggests that it may influence a wide range of physiological processes in most compartments of the body.

MATERIALS AND METHODS

Proteins

Porcine trypsin (sequence grade) was purchased from Promega. Peptide:N-glycosidase F (PNGase F) was obtained from Roche and *Pfu* (*Pyrococcus furiosus*) pyroglutamate aminopeptidase was from Takara Bio Inc., Shiga, Japan. Monoclonal antibody 1059 [anti-(human PEDF)] was obtained from Chemicon, and

Abbreviations used: BHK, baby-hamster kidney; CMM, calculated molecular mass; DTT, dithiothreitol; HRP, horseradish peroxidase; PG, immobilized pH gradient; PEDF, pigment-epithelium-derived factor; Glp, pyroglutamate; PNGase F, peptide:*N*-glycosidase F; MALDI-MS, matrix-assisted laser-desorption–ionization MS; Q-TOF, quadrupole/time-of-flight; RP-HPLC, reverse-phase HPLC; serpin, serine-proteinase inhibitor; TFA, trifluoroacetic acid.

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horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig (NIF 825) was from Amersham Biosciences.

Purification of type I collagen

Collagen was purified from calf skin collected at the local abattoir, essentially as described in [16]. Briefly, approx. 100 g (dry weight) of skin was incubated in 0.5 M acetic acid at 4 °C for 16 h. The extract was filtered through gauze and the filtrate was centrifuged at 10 000 ***g*** for 30 min. The supernatant NaCl concentration was adjusted to 5 % (w/v) and the collagen allowed to precipitate at 4 °C for 16 h. The precipitated protein was recovered by centrifugation at 10 000 ***g*** for 30 min and dissolved in 0.5 M acetic acid. The material was then dialysed against 20 mM Na_2HPO_4 and insoluble material (type I collagen) was collected by centrifugation. The pellet was dissolved in 0.5 M acetic acid, and 5 % (w/v) NaCl was subsequently added. The precipitate was recovered by centrifugation and the pellet dissolved in 0.5 M acetic acid. The material was then dialysed against water. The protein concentration was estimated by SDS/PAGE analysis [17] and Coomassie Brilliant Blue staining.

Collagen–Sepharose resin

CNBr-activated Sepharose 4B was rehydrated and washed as recommended by the manufacturer (Amersham Biosciences). The coupling of collagen was performed as described by the manufacturer using a density of 3 mg of collagen/ml of resin.

Purification of PEDF

Normal human plasma (50–100 ml) was diluted 5-fold in 20 mM Tris/HCl, pH 7.4, containing 10 mM EDTA. The diluted plasma was centrifuged at 10 000 ***g*** for 30 min and applied to a 15 ml collagen–Sepharose column pre-equilibrated in 20 mM Tris/HCl, pH 7.4. The column was washed with 20 mM Tris/HCl until the absorbance at 280 nm of the flow-through was below 0.02. Bound proteins were eluted using 4 column vol. of 20 mM Tris/HCl, pH 7.4, containing 100, 200 or 500 mM NaCl. The fractions were analysed by SDS/PAGE on 5–15 %-(w/v)-polyacrylamide gradient gels. Selected fractions were pooled and dialysed against 20 mM Tris/HCl, pH 7.4. The purification procedure was repeated using gelatin–Sepharose 4B (Amersham Biosciences) and evaluated as described above.

Concentration of PEDF in normal human plasma

The concentration of PEDF in normal human plasma was analysed by SDS/PAGE and Western blotting. Plasma from ten normal individuals was pooled and used for analysis. Human plasma (0.2–1 μl) and purified PEDF (0.5–20 ng) were subjected to SDS/PAGE under reducing conditions. The proteins were electrophoretically transferred to a PVDF membrane in 10 mM 3-(cyclohexylamino)propane-1-sulphonic acid ('Caps')/10 % (v/v) methanol, pH 11 [18]. The membrane was blocked with 5 % (w/v) skimmed milk in 20 mM Tris/HCl/150 mM NaCl, pH 7.4. PEDF was subsequently detected using a monoclonal anti-(human PEDF) antibody and an HRP-conjugated rabbit anti-mouse Ig. The membrane was developed using an enhanced chemiluminescence detection system (ECL®; Amersham Biosciences) and the intensity of the signals quantified using a Kodak Image Station 1000 equipped with a cooled charge-coupled-device ('CCD') camera.

Surface-plasmon-resonance analysis

The kinetics for PEDF binding to type I collagen was determined by surface-plasmon-resonance analysis using a BIAcore 2000 instrument (Amersham Biosciences). Purified type I collagen was immobilized on a CM5 sensor chip as previously described [19]. The resultant density was 71 fmol/mm^2. An activated and blocked flow cell was used as control for non-specific binding. PEDF in 10 mM Hepes/1 mM EGTA/1.5 mM $CaCl_2$/0.005 % (v/v) Tween 20, pH 7.4, was used as ligand at the indicated concentrations determined by amino acid analysis [20]. The obtained data were analysed using BIAevaluation software.

Two-dimensional gel electrophoresis

First-dimension isoelectric focusing was performed using the Multiphor II system with Immobiline drystrips (Amersham Biosciences). A 1 μg portion of PEDF in 125 μl of 5 M urea/2 M thiourea/2 % (w/v) CHAPS/2 % (w/v) *N*-decyl-*N*,*N*-dimethyl-3-aminopropane-1-sulphonate (Sigma)/10 mM dithiothreitol (DTT)/Bromophenol Blue and 2 % (v/v) of appropriate immobilized pH gradient (IPG) buffer was added to a strip with a non-linear pH 3–10 gradient. After overnight rehydration of the IPG strip, isoelectric focusing was performed using a voltage gradient from 200 to 3500 V for 90 min, followed by 3500 V for another 90 min. The strip was then equilibrated in 50 mM Tris/HCl/6 M urea/30 % (v/v) glycerol/2 % (w/v) SDS/5 mM DTT for 15 min, followed by another 15 min in the same buffer with 10 mM iodoacetamide substituted for DTT. Second-dimension electrophoresis was performed as described in [21], using a uniform 12.5 % (w/v) polyacrylamide gel. Silver staining was performed as described in [22].

Alkylation of cysteine residues

Approx. 90 μg of purified PEDF was alkylated in 20 mM Tris/HCl, pH 7.8, containing 6 M guanidinium chloride by adding iodoacetamide to a final concentration of 25 mM. After 3 h at 23 °C, the sample was acidified and the alkylated protein was recovered on a 2.1 mm × 220 mm Aquapore RP-300 C_8 reverse-phase (RP-)HPLC column (PerkinElmer Brownlee) connected to a Pharmacia SMART system (Amersham Biosciences). The column was developed using a 2 % solvent B/min linear gradient from 0.1 % (v/v) trifluoroacetic acid (TFA) (solvent A) to 90 % (v/v) acetonitrile/0.08 % (v/v) TFA (solvent B) at 200 μl/min. The protein was detected at 220 and 280 nm and freeze-dried.

Tryptic digestion of PEDF

The alkylated PEDF was dissolved in 50 mM NH_4HCO_3 and digested for 16 h at 37 °C using porcine trypsin at a 1:50 molar ratio. The digestion was stopped by acidification and the peptides were separated by RP-HPLC as described above, using a linear gradient of 1 % solvent B/min. Selected fractions were further purified using a C_{18} reverse-phase column (Vydac).

Electrospray quadrupole/time-of-flight (Q-TOF) mass analysis of intact deglycosylated PEDF

Approx. 20 pmol of alkylated PEDF was incubated with PNGase F (see below). Following deglycosylation, the protein was purified on a Poros 50 R1 micropurification column as described previously [23,24] and analysed by electrospray MS using a

Q-TOF mass spectrometer (Micromass) equipped with a Micromass nanospray source.

Matrix-assisted laser-desorption–ionization MS (MALDI-MS) analysis of tryptic peptides

Peptides were analysed by MALDI-MS using a Perseptive Voyager DE™ PRO mass spectrometer (Applied Biosystems). The spectra were recorded in linear or reflectron modes of operation by averaging 50 and 100 laser shots depending on the nature of the sample. The mass accuracy in the reflectron mode with external calibration was typically better than 50 p.p.m. Time-to-mass conversion was achieved by external calibration using angiotensin II (1296.68 Da) and adrenocorticotropin-(18–39)-peptide (2466.71 Da). All experiments were performed using α-cyano-4-hydroxycinnamic acid (Sigma) as the matrix. Saturated matrix solutions were prepared in 70 % (v/v) acetonitrile/0.1 % (v/v) TFA and mixed in equal volumes with peptide samples, and applied to a stainless-steel sample plate. The mixture was allowed to air-dry before being introduced into the mass spectrometer. The expected mass of peptides and proteins were calculated using General Protein/Mass Analysis for Windows ('GPMAW') software (Lighthouse data, Odense, Denmark).

N-terminal amino acid sequencing

Peptides destined for N-terminal sequence analyses were applied to a Biobrene precycled glass-fibre filter (Applied Biosystems) and subjected to automated Edman degradation in an Applied Biosystems Model 477A sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems Model 120A HPLC system.

Removal of the N-terminal blocking group

An aliquot of the RP-HPLC fraction containing the peptide of interest was freeze-dried and 0.2 munit of *Pfu* pyroglutamate aminopeptidase was added in the supplied reaction buffer. The sample was incubated at 75 °C for 4 h and purified using a μC_{18} ZipTip (Millipore). The bound material was eluted directly on to the sample plate, with the α-cyano-4-hydroxycinnamic acid matrix solution as eluant, and analysed by MALDI-MS. Similarly, intact purified PEDF (10 μg) in 70 μl of 20 mM Tris/HCl was added to 18 μl of 5 × *Pfu* pyroglutamate aminopeptidase reaction buffer and 0.5 munit of enzyme. The reaction was performed as described above and the digested material adsorbed on to a PVDF membrane using ProSorb cartridges (Applied Biosystems) and sequenced.

Deglycosylation of PEDF

A 3 μg portion of PEDF in 50 μl of 50 mM $NaHPO_4$ (pH 7.5)/50 mM DTT/0.1 % (w/v) SDS was boiled for 5 min and allowed to cool before the addition of 0.75 % (v/v) Triton X-100 and 2 units of PNGase F. The sample was incubated at 37 °C for 3 h before the reaction products were analysed by SDS/7 %-(w/v)-PAGE. The gel was stained with Coomassie Brilliant Blue. Alkylated PEDF used for MS analysis was deglycosylated for 16 h at 37 °C in phosphate buffer with 10 mM DTT only.

Monosaccharide composition analysis

The monosaccharide composition was determined by fluorophore labelling of released monosaccharides followed by PAGE of the reaction products. Briefly, PEDF (60 μg) was hydrolysed in 2 M TFA or 4 M HCl for analyses of neutral and amine sugars respectively [25]. The released monosaccharides were then labelled with 2-aminoacridone and separated by electrophoresis in a 30 % acrylamide/1.6 % *N*,*N*-methylenebisacrylamide gel using a 0.1 M Tris/borate, pH 8.3, buffer system [26]. The gels were imaged using a fluorescence camera linked to a computer, and the acquired images were analysed with the FACE software densitometer (version 2.3).

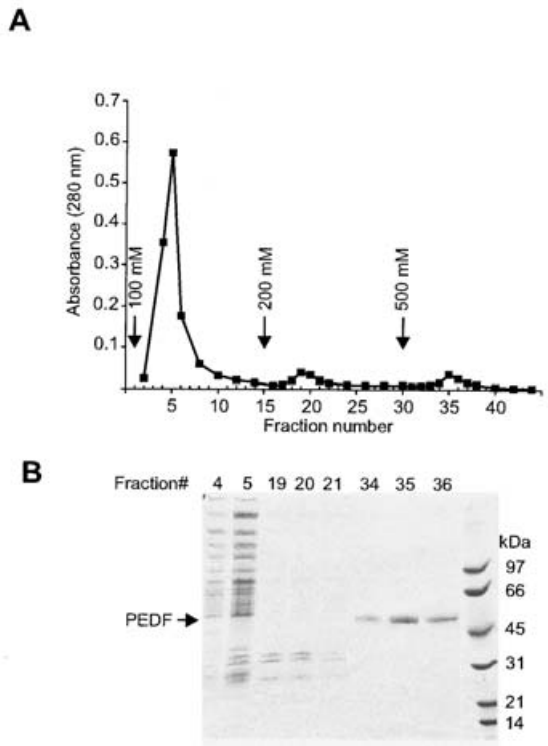

Figure 1 Purification of PEDF from human plasma

(**A**) Plasma was applied to a collagen–Sepharose column and bound proteins eluted by increasing the ionic strength as indicated by arrows. Fractions (4 ml each) were collected and the absorbance at 280 nm was recorded. (**B**) Aliquots of the collected fractions were analysed by reducing SDS/PAGE and the gel was stained with Coomassie Brilliant Blue. PEDF was eluted in the 500 mM NaCl eluate and was identified by MALDI-MS peptide mass fingerprinting. The arrow shows the position of PEDF.

RESULTS

One-step purification of PEDF from normal human plasma

In the course of studying collagen-binding plasma proteins, human plasma was applied to a collagen–Sepharose column. The column was washed, and bound proteins were eluted by applying a stepwise NaCl gradient (Figure 1A). Several proteins were eluted early in the gradient, but only a single protein was detected in the 500 mM NaCl eluate (Figure 1B). The protein was identified as PEDF by tryptic in-gel digestion followed by MALDI-MS and estimated to be more than 95 % pure by SDS/PAGE and Coomassie Brilliant Blue staining of the gel. The N-terminal sequence of purified PEDF could not be obtained by standard Edman degradation, suggesting that the N-terminus was blocked (see below). On the bais of yields from several independent preparations of the protein, we estimate the concentration of PEDF in blood to be approx. 5 μg/ml, corresponding to 100 nM. This estimate was corroborated when the concentration of PEDF in

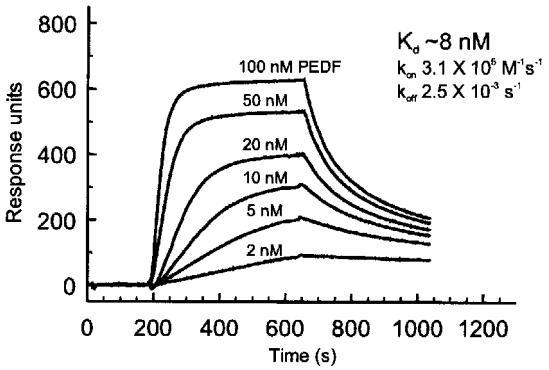

Figure 2 Surface-plasmon-resonance analysis of PEDF binding to type I collagen

Surface-plasmon-resonance analysis of PEDF binding to a bovine-type-I-collagen-coated flow cell is shown. PEDF was applied at the indicated concentrations. The response curves are corrected for non-specific binding using an empty flow cell and the data were fitted to a 1:1 binding using the Langmuir model. PEDF interacted with type I collagen in a concentration-dependent manner, and the K_d was determined to be 8 nM.

full plasma was analysed by SDS/PAGE and quantitative Western blotting (results not shown). To assess if the purification could be performed on a commercially available resin, we substituted collagen–Sepharose for gelatin–Sepharose; however, PEDF did not bind to the gelatin–Sepharose column, emphasizing that the structure of native collagen is important for binding. We conclude that PEDF can be purified from human plasma in a single step.

Interaction of PEDF with type I collagen

The PEDF–collagen type I interaction was further characterized by surface plasmon resonance analysis. Collagen was immobilized on the sensor chip and purified PEDF used as analyte. This result suggested that PEDF bound to collagen in a concentration-dependent manner (Figure 2). The dissociation constant (K_d) for the binding to collagen was determined to be 8 nM. This high affinity is a result of a very high rate of association (k_{on}) and a relatively low rate of dissociation (k_{off}) and correlates with the presence of PEDF in the 500 mM eluate from the collagen–Sepharose column (Figure 1B). It is apparent that the interaction between PEDF and type I collagen is very specific and dependent on the structure of collagen.

Purified PEDF is heterogeneous in charge

The theoretical pI of PEDF is 5.9. When we analysed purified PEDF by two-dimensional gel electrophoresis, the protein separated into several species in the first dimension, with apparent pI values between 5 and 6 (Figure 3). A similar two-dimensional-gel-electrophoresis signature was observed when PEDF derived from human WI-38 fibroblast-like cells [15] or bovine interphotoreceptor matrix [10] was analysed in a similar way. This suggests that PEDF carries post-translational modifications.

Identification and characterization of post-translational modifications

To determine the position and identity of these post-translational modifications, alkylated PEDF was subjected to trypsin digestion as described in the Materials and methods section. The tryptic peptides were separated by RP-HPLC (Figure 4) and thoroughly

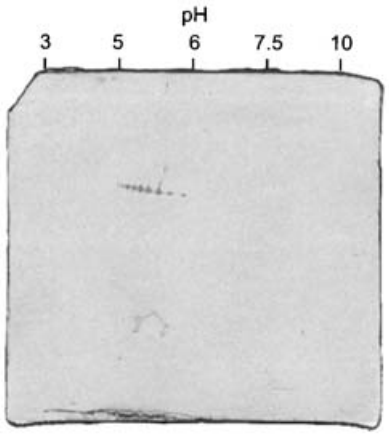

Figure 3 Two-dimensional gel electrophoresis of purified PEDF

The reduced and alkylated protein was electrophoresed on a pH 3–10 isoelectric-focusing strip in the first dimension and then applied to an SDS/12.5 %-(w/v)-polyacrylamide gel in the second dimension. After electrophoresis the protein was visualized by silver staining. Approximate pH values are indicated. PEDF migrated as several spots between pH 5 and 6, suggesting that the protein is post-translationally modified.

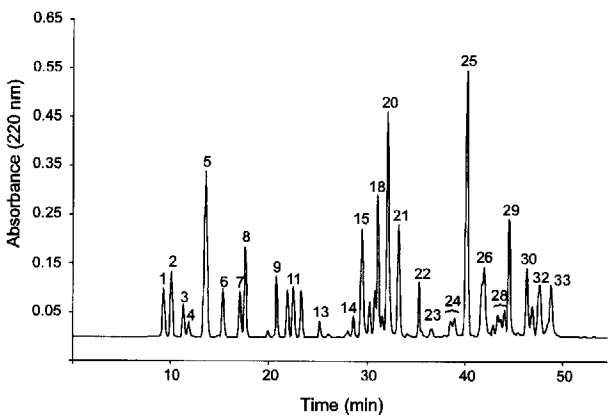

Figure 4 HPLC peptide map of tryptic PEDF peptides

Tryptic peptides were separated by RP-HPLC on Aquapore RP-300. The fractions were collected manually and characterized by Edman degradation and MALDI-MS (results not shown). The majority of the peptides displayed the expected mass (results not shown). Three contained post-translational modifications, including (i) an N-terminal peptide containing Glp in fraction 21, (ii) a peptide containing Cys^{242} in fraction 9 and (iii) the peptide containing Asn^{266} in fraction 26 (see also Table 1).

analysed by Edman degradation and MALDI-MS (results not shown). This analysis allowed us to account for 92 % of the amino acid sequence. The masses of the purified peptides were compared with the theoretical expected mass calculated on the basis of the protein sequence of PEDF. A number of peptides did not present the expected mass, suggesting the presence of post-translational modifications (Table 1).

Characterization of Cys^{242}

The cDNA sequence predicts the presence of one cysteine residue in position 242 of the mature protein (amino acid numbering is based on the identification of the mature N-terminus in this paper and assigning the first 19 residues of the cDNA sequence [8] as

Table 1 Analysis of modified PEDF peptides

Sequence*	Fraction†	Observed molecular mass (Da)‡	Calculated molecular mass (Da)	Modification
Blocked	21	3069.09	3086.38	Glp
Y^{234}*GLDSDLSCK*^{243}	9	1157.47	1100.49	Alkylation
V^{263}*TQXLTL*IEESLTSEFIHDIDR^{284}	26.1/26.2	See Figure 5		Glycosylation

* Numbering of residues is done on the basis of the determined sequence of mature PEDF with Gln^1 as the N-terminus; residues determined by Edman degradation are shown in italics.
† Fraction number refers to the numbers indicated in Figure 4.
‡ *m/z* values were determined by MALDI-MS.

signal peptide). The peptide containing Cys^{242} was recovered in fraction 9 (Figure 4). The mass was 57 Da higher than expected, a finding that is consistent with the S-carboxamidomethylation of Cys^{242}. This suggests that this residue carries a free thiol group in the native protein.

The N-terminus of PEDF is pyroglutamate (Glp)

We did not obtain an N-terminal sequence when the peptide recovered in fraction 21 (Figure 4) was analysed by Edman degradation. Since attempts to sequence the N-terminal of the purified protein were similarly unsuccessful, we hypothesized that the peptide was derived from the N-terminal of PEDF. The observed mass of the peptide was 17 Da lower than the mass predicted on the basis of the cDNA sequence. This is consistent with deamidation of an N-terminal glutamine residue and formation of the cyclic amino acid Glp. To substantiate this observation, we incubated both the collected peptide and purified PEDF with *Pfu* pyroglutamate aminopeptidase. Subsequent analysis of the peptide by MS showed that the mass was reduced from 3069.09 Da (Table 1) to 2958.3 Da, corresponding to the peptide Asn^2–Lys^{29}. In addition, N-terminal sequencing of PEDF following *Pfu* pyroglutamate aminopeptidase incubation produced the sequence Asn-Pro-Ala-Ser-Pro-Pro-Glu-Glu-Gly. We conclude that the signal peptidase cleaves the Cys–Gln bond during biosynthesis. The glutamine N-terminus is subsequently modified by the formation of the cyclic amino acid Glp.

PEDF is heterogeneously glycosylated

PEDF was subjected to monosaccharide composition analysis as described in the Materials and methods section. Identification of *N*-acetylgalactosamine is indicative of the presence of O-linked carbohydrate; however, *N*-acetylgalactosamine was not detected when PEDF was subjected to monosaccharide composition analysis (Figure 5A). Hence PEDF is probably not O-glycosylated.

PEDF contains one potential *N*-linked glycosylation site at position Asn^{266}. Treatment with PNGase F suggested that this site is glycosylated, as a 2 kDa reduction in size was observed after

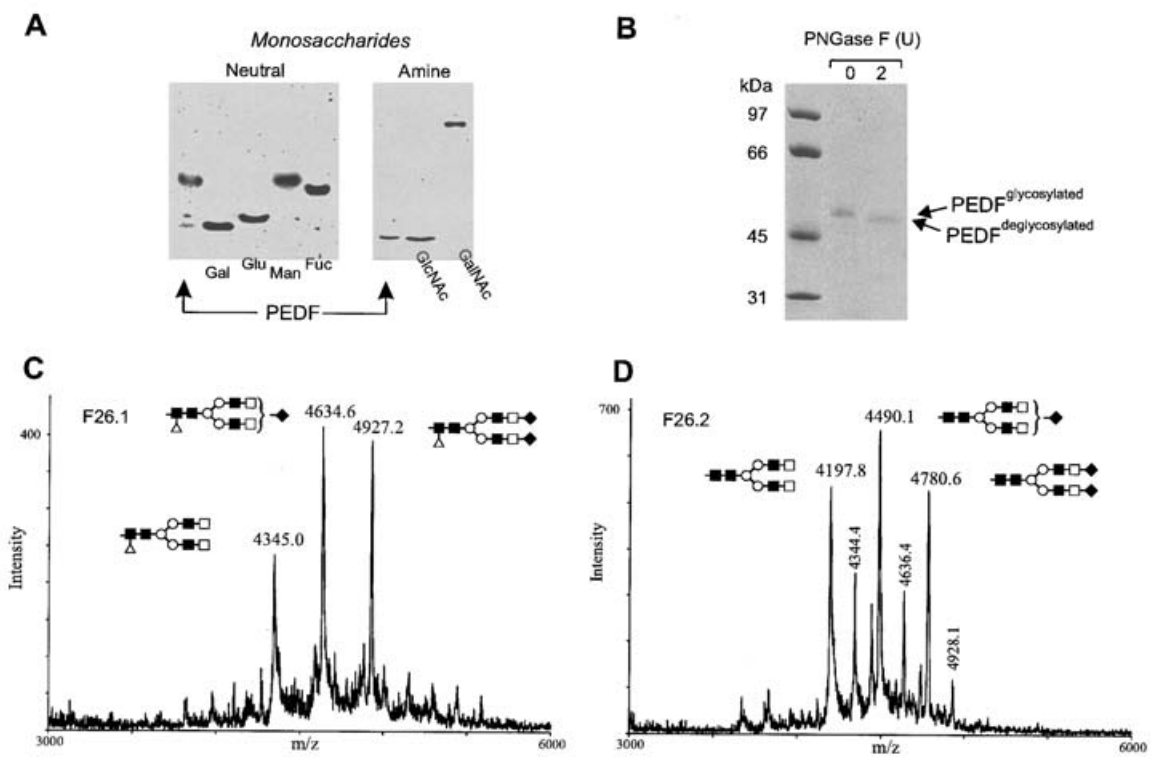

Figure 5 Composition and structure of PEDF glycans

(**A**) PEDF was first subjected to monosaccharide compositional analysis. The released monosaccharides were labelled using the fluorophore 2-aminoacridone and separated by PAGE. Known monosaccharides were included as markers. The monosaccharide analysis verified that PEDF is a glycoprotein. In addition, the absence of *N*-acetylgalactosamine suggested that the attached glycans were *N*-linked. This was supported by a decrease in molecular mass following SDS/PAGE analysis of PNGase F-digested PEDF (**B**). The only *N*-linked attachment site is Asn^{266}, and the tryptic peptide containing this residue was located in fraction 26 (see Figure 4). This fraction was further purified by RP-HPLC. Two peaks denoted F26.1 (**C**) and F26.2 (**D**) were collected and subjected to MALDI-MS in the linear mode. The deduced structures of the *N*-linked glycans are shown in the insets: ■, *N*-acetylgalactosamine; ○, mannose; □, galactose; △, fucose; ◆, sialic acid.

SDS/PAGE analysis (Figure 5B). This is supported by MALDI-MS analysis of fraction 26 (Figure 4) containing the peptide encompassing Asn^{266} (results not shown). Fraction 26 was rechromatographed by RP-HPLC using different conditions, and the resulting two peaks were analysed by MALDI-MS (Figures 5C and 5D). The data obtained for fraction 26.1 ('F26.1'; Figure 5C) suggests that Asn^{266} carries a disialylated biantennary complex type glycan with core fucosylation [calculated molecular mass (CMM) 4925.99 Da]. Monosialylated (CMM 4634.73 Da) and asialylated (CMM 4343.47 Da) structures were also detected. The glycan identified in fraction 26.2 ('F26.2'; Figure 5D) is similarly a disialylated biantennary complex type, but, however, without core fucosylation (CMM 4779.85 Da). Again the monosialylated (CMM 4488.59 Da) and the asialylated (CMM 4197.33 Da) species were detected. From these data it cannot be determined whether the presence of monosialylated and asialylated species in both F26.1 and F26.2 is due to heterogeneity of the glycan structures or whether it is due to metastable decay during MALDI-MS analysis. We conclude that PEDF carries an *N*-linked glycan at position Asn^{266}. The structure is a heterogeneous biantennary complex type glycan with or without core fucosylation.

Mass of deglycosylated PEDF

The analyses of the tryptic peptides covered 92 % of the protein sequence. To ensure that all post-translational modifications were accounted for we determined the mass of alkylated and deglycosylated PEDF by nanoelectrospray MS. The mass was determined to be 44465 Da. The calculated average mass of PEDF containing the identified N-terminal Glp modification and Asp^{266} (Asn^{266} is converted into aspartic acid residue upon deglycosylation with PNGase F) is 44460 Da. A difference of 5 Da is within the experimental error, and we conclude that all post-translational modifications have been identified.

DISCUSSION

Here we show that PEDF is present in normal human plasma. This finding is supported by the fact that PEDF mRNA expression is relatively high in liver [27–29]. The protein was purified from plasma in a one-step procedure exploiting the strong affinity for type I collagen. Biacore analysis determined the K_d for this interaction to be 8 nM. Similar sensorgrams were obtained using mouse PEDF [28]. Recently, the K_d between recombinant human PEDF from BHK cells and rat type I collagen was determined to be 135 nM [30]. The difference resides in lower rates of association (10-fold) and dissociation (100-fold), as determined in the present study. Whether the difference in K_d of one order of magnitude is due to the source of collagen or PEDF is not known. This high affinity suggests that PEDF may diffuse from the blood into the extracellular matrix, where it will be immobilized by binding to type I collagen. Indeed, the presence of PEDF in a number of tissues containing type I and III collagens has been shown by the immunohistochemical staining of mouse embryos [28]. Although many tissues express PEDF mRNA [27–29], PEDF in the blood is likely to play a critical role in the regulation of angiogenesis, particularly during the latter stages after the sprouting endothelial cells roll up to form blood vessel tubes and blood circulation begins. It is conceivable that plasma PEDF functions as an angiogenesis terminator.

Interestingly, it has been shown that systemic delivery of PEDF inhibits angiogenesis in ischaemia-induced retinopathy [2]. Complete inhibition of vessel formation was obtained at a concentration of approx. 50 nM. The plasma concentration was estimated on the basis of injected recombinant PEDF [2] and did not include the murine PEDF already present in the circulation (S. V. Petersen, Z. Valnickova and J. J. Enghild, unpublished work). The systemic concentration of PEDF required to inhibit ischaemia-induced retinopathy is therefore likely to be above 50 nM. In addition it is not known whether the estimated concentration of 100 nM in human blood represents active protein or whether the antiangiogenic activity of PEDF is regulated by, e.g., limited proteolysis or protein–protein interactions. This could allow angiogenesis to take place despite the relatively high concentration of PEDF in plasma. The experiments by Stellmach et al. [2] may reflect that such a control mechanism may have been overwhelmed by systemic injection of recombinant PEDF. The estimated concentration of 100 nM in human blood is thus likely to represent a functionally significant concentration of PEDF, indicating that the circulation holds the capacity to inhibit angiogenesis in tissues throughout the body. This has recently been corroborated by the demonstration that PEDF inhibits angiogenesis in cancerous tissue *in vivo* [31]. Likewise, PEDF may be involved in wound healing. During the process of wound healing, new capillaries are formed within the granulation tissue encompassing macrophages and fibroblasts [32]. The latter cells produce the extracellular-matrix components collagen type I and collagen type III. During the final stage of healing, the majority of new blood vessels undergo apoptosis. It could be speculated, on the basis of the high affinity of PEDF for collagen and the capacity to induce apoptosis [3], that PEDF from the circulation is immobilized on the newly formed collagen, thereby facilitating the induction of apoptosis. The identification of a means to locally modulate or inhibit the antiangiogenic affect of PEDF may therefore improve the healing of wounds.

Purified plasma PEDF appeared to be heterogenous in charge when analysed by two-dimensional gel electrophoresis, suggesting the presence of post-translational modifications. A thorough analysis of tryptic peptides derived from human PEDF allowed us to determine the molecular structure of these modifications. Treatment of PEDF with *Pfu* pyroglutamate aminopeptidase revealed that the N-terminus is glutamine, which is post-translationally modified to Glp [33] (Figure 6A). This finding suggests that the signal peptide of PEDF is composed of the first 19 residues of the polypeptide encoded by the cDNA [8]. When PEDF was expressed in BHK cells, the N-terminus of the secreted protein was determined to be Asn^{2} [14]; however, when HEK-293 cells were transfected with a plasmid containing the entire open reading frame for PEDF, the N-terminus of the expressed material was blocked [13]. Whether this is due to the formation of Glp was not determined. In addition, it is noteworthy that native PEDF purified from bovine interphotoreceptor matrix is unblocked (sequence: Asp-Ala-Gly-. . . -Phe-Arg-Val) [10], whereas, PEDF purified from bovine vitreous humour is blocked [11]. It should be noted that a sequence conflict exists for the first residue (Asp) and the second last residue (Arg) sequenced by Wu et al. [10]. These residues are also deposited as asparagine and lysine respectively, in the SWISSPROT database (accession number Q95121). Likewise, the N-terminus of PEDF purified from a murine adenocarcinoma cell line was blocked [28]. Alignment of the primary sequence of human, murine and bovine PEDF shows that these species contain Gln^{1} (Figure 6B). On the basis of these observations, we propose that both murine and bovine PEDF are post-translationally modified to contain an N-terminal Glp group, as detected in native human PEDF.

The absence of the post-translational Glp modification on PEDF in the interphotoreceptor matrix of the bovine eye suggests that an active process is removing the Glp residue after secretion,

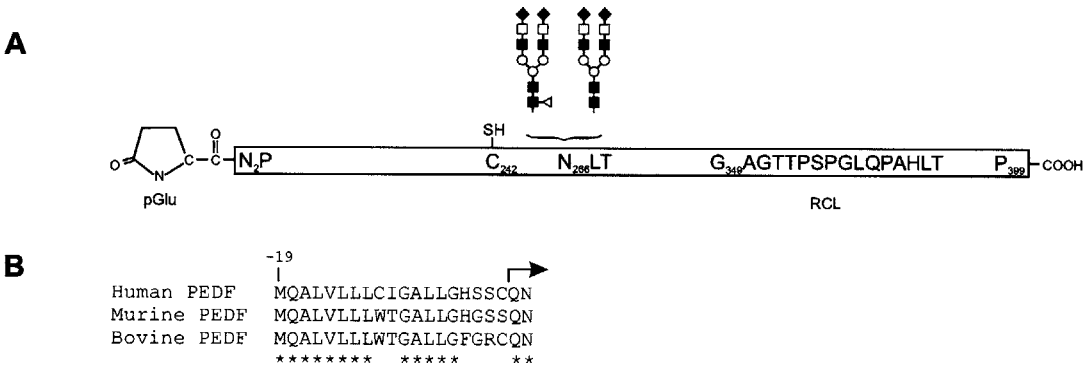

Figure 6 Schematic diagram depicting the post-translational modifications of PEDF

(**A**) The open bar represents mature PEDF, including the identified post-translational modifications. The reactive centre loop (RCL) of the serpin structure is indicated. Plasma PEDF contains two post-translational modifications: (i) the N-terminus is a Glp; (ii) the Asn^{266} residue is glycosylated. In addition, the thiol group of Cys^{242} is free. (**B**) Alignment of the N-terminal region of human (P36955), murine (P97298) and bovine (Q95121) PEDF. Identical residues are indicated (*). The arrow designates the origin of the mature N-terminus as detected in human PEDF. All species contain the Gln-Asn pair, indicating that murine and bovine PEDF may contain an N-terminal Glp modification.

thereby exposing the preceding asparagine (or aspartic acid) as the new unblocked N-terminus of the protein. The physiological implication of this observation is not clear, but we speculate that it may regulate the activity of PEDF. The presence of Glp has been proposed to be important for the generation of hydrogen bonds between N- and C-terminal parts, such as has been observed in onconase [34] and luteinizing-hormone-releasing hormone [35]. Thus the removal of Glp may modify the higher-order structure of PEDF. Interestingly, the crystal structure of recombinant PEDF from BHK cells (i.e. PEDF without Glp) shows that the first 15 residues of the N-terminus are unstructured [9]. This finding may be a consequence of the absence of a stabilizing hydrogen bond between the missing Glp residue and the C-terminal region. Recent data shows that PEDF treatment of phenotypically different endothelial cells generates opposite response [36]. As discussed by the authors, one of the possible explanations for this finding could be the differential expression of a protease that modulates the activity of PEDF. Such a protease may be either membrane-bound or secreted and belong to the family of pyroglutamyl peptidases (EC 3.4.19.3–3.4.19.6) [37].

We have shown that PEDF is present in the circulation at a concentration that is likely to allow for the functional activity of PEDF throughout the body. Eye homoeostasis has been the major focus of recent PEDF research; however, our current observations expand the physiological scenarios in which PEDF may function to include wound healing, ischaemia–reperfusion injury and cancer metastasis. The one-step purification procedure supports the use of native PEDF to address this question. We also show that the N-terminus of PEDF is post-translationally modified to a cyclic Glp residue and suggest that this modification may be present in murine and bovine PEDF as well.

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