Pigment Epithelium-derived Factor (PEDF) Shares Binding Sites in Collagen with Heparin/Heparan Sulfate Proteoglycans

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Pigment epithelium-derived factor (PEDF) is a collagen-binding protein that is abundantly distributed in various tissues, including the eye. It exhibits various biological functions, such as anti-angiogenic, neurotrophic, and neuroprotective activities. PEDF also interacts with extracellular matrix components such as collagen, heparan sulfate proteoglycans (HSPGs), and hyaluronan. The collagen-binding property has been elucidated to be important for the anti-angiogenic activity in vivo (Hosomi, et al., Biochem. Biophys. Res. Commun. 335, 756–761). Here, we investigated the collagen recognition mechanism by PEDF. We first narrowed down candidate PEDF-binding sequences by taking advantage of previously reported structural requirements in collagen. Subsequent searches for PEDF-binding sequences employing synthetic collagen-like peptides resulted in the identification of one of the critical binding sites for PEDF, human α1(I)(929–938) (IKGHRGFSGL). Further analysis revealed that the collagen recognition by PEDF is sequence- and conformation-specific, and the high affinity binding motif is KGXRG-FXGL in the triple helix. The PEDF-binding motif significantly overlapped with the heparin/HSPG-binding motif, KGHRG(F/Y). The interaction of PEDF with collagen I was specifically competed with by heparin but not by chondroitin sulfate-C or hyaluronan. The binding sequences for PEDF and heparin/HSPG also overlapped with the covalent cross-linking sites between collagen molecules. These findings imply a functional relationship between PEDF and HSPGs during angiogenesis, and the interaction of these molecules is regulated by collagen modifications.
collagen-binding site, and the basic amino acid cluster (Arg-145, Lys-146, and Arg-148) is essential for the heparin/heparan sulfate binding (23). The importance of the collagen-binding activity of PEDF has been demonstrated by a cancer cell xenograft experiment using nude mice. The collagen-binding null mutant (D299N) of PEDF lost its anti-angiogenic property, and the anti-tumor effect was totally eliminated, whereas the heparin-binding null mutant (R148A) was as active as the wild type in terms of anti-angiogenic/anti-tumor activities (25).

Collagen is the predominant ECM protein playing important roles in cell adhesion, migration, and differentiation. This protein is characterized by a unique triple helical structure constituted by long tandem repeats of Gly-X-Y triplets, in which the X and Y positions are frequently occupied by Pro and 4-hydroxyproline (Hyp (O)) residues. To date, 28 types of collagen have been identified and classified into several groups according to their immunohistochemical observations. These findings motivated us to investigate further details of the PEDF-collagen interaction to clarify the importance of this property.

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EXPERIMENTAL PROCEDURES

Prediction of PEDF-binding Sequences—An original text search program was written in Perl CGI script (Perl 5.8.1) and run on an Apache 1.3.33 HTML server. Using this program, we extracted 9–10 amino acid peptides that contain both Arg and Lys residues from the sequences of human α1(I)(776–1012) and α2(I)(776–1012) corresponding to the TCβ fragments.

Peptides—Peptide chains were constructed manually on the basis of a standard Fmoc (N-(9-fluorenylmethoxycarbonyl)-based strategy on Rink-amide resins (Novabiochem). All peptides were purified by reversed phase HPLC using a Cosmosil 5C18 AR-II column (Nacalai Tesque, Kyoto, Japan) and characterized by matrix-assisted laser desorption ionization mass spectrometry on a Bruker Autoflex II mass spectrometer. All the measured masses agreed with the theoretical values (see supplemental Table S1).

Circular Dichroism (CD) Spectrometry—To characterize the conformational status of the synthetic peptides, CD spectra of aqueous solutions of the peptides (1 mg/ml) were recorded using a Jasco J-820 spectropolarimeter as described previously (30). Prior to the measurements, the peptides were kept at 4 °C for at least 2 days. Melting temperature of the triple helix (Tm) was determined by the [θ]225 values of each peptide solution with increasing temperature at a rate of 18 °C increments/h.

Purification of Recombinant Mouse PEDF (rPEDF) and GST-PEDF—Expression and purification of rPEDF were as described previously (23). Briefly, *Escherichia coli* expressing glutathione S-transferase (GST)-PEDF fusion protein was lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 30% (v/v) glycerol containing protease inhibitors, and 0.1% Nonidet P-40. After sonication, the lysate was loaded onto a glutathione (GSH)-Sepharose 4B (GE Healthcare). For the purification of GST-PEDF, the protein was eluted using GSH buffer (50 mM Tris-HCl (pH 8.8), 10 mM GSH, 100 mM NaCl).

To obtain rPEDF, 20 units of factor Xa (GE Healthcare) in 50 mM HEPES-Na (pH 7.4), 150 mM NaCl containing 1 mM CaCl2, were added to the column and incubated at 25 °C overnight. The next day, rPEDF was eluted. The proteins were stored at −80 °C until use. Concentrations of the proteins were estimated by Bradford assay using the protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Evaluation of Inhibitory Activity of Peptides for PEDF-Collagen and Heparin-Collagen Interactions by a Collagen Fibril Formation Assay—Inhibition of rPEDF-collagen I and heparin-collagen I interactions by peptides was analyzed by a turbidimetric assay in a multiwell plate format (31). PEDF or heparin (Sigma) was preincubated with peptides to be tested in phosphate buffer (20 mM sodium phosphate (pH 7.4), 100 mM NaCl) for 10 min at 4 °C. The mixtures were added to wells of a UV-star 384-well plate (Greiner Bio-One, Frickenhausen, Germany) containing bovine dermis collagen I (Koken, Tokyo, Japan) on ice. The final concentration of collagen I was 0.1 mg/ml. The peptide concentration was calculated as the trimer form. To start the collagen fibril formation, the temperature was quickly raised to 37 °C, and the degree of fibril formation was monitored by measuring the absorbance at 313 nm on a Vient XS multiwell plate reader (DS Pharma, Osaka, Japan). % Fibril formation was defined by Equation 1,

\[
\text{%fibril formation} = \left( \frac{\Delta A - \mu_{+}}{\mu_{-} - \mu_{+}} \right) \times 100
\]  

where ΔA is the change in absorbance at 313 nm from time 0 to 120 min; μ− is the average ΔA of wells containing collagen and PEDF or heparin, and μ+ is the average change of absorbance of the collagen only in control wells on the same plate. Obtained data were analyzed using Origin version 7.5 data analysis software (OriginLab, Northampton, MA), and 50% inhibitory concentration (IC50) values were estimated from the midpoint of the inhibition curves.

Enzyme-linked Immunosorbent Assay (ELISA)—Wells of 96-well plates (Nunc, Kamstrup, Denmark) were coated with 50 μl of 10 μg/ml collagen I (bovine dermis, Koken), collagen II...
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**MMP-1 cleavage site**

α1(I) 776-835  
IAQGQHVGQLRQGGERGFOGLOGPSGEOGKQGPQGAGSEQGPGFMGPGOGLAGPGQGEGSG

α2(I) 776-835  
ILGAQGQHVGQLRQGGERGFOGLOGPSGEOGKQGPQGAOGAQGPGFMGPGOGLAGPGQGEGSG

Cluster 1

α1(I) 836-895  
REGAOGAEQGGRGGGKQDGRGETGPAQPGPAOGAQGPGFMGPGOGLAGPGQGEGSG

α2(I) 836-895  
RDGGQGQHKGQGGGKQDGRGETGPAQPGPAOGAQGPGFMGPGOGLAGPGQGEGSG

Cluster 2

Cluster 3

Cluster 4

Cluster 5

Cluster 6

Cluster 7

α1(I) 956-1012  
ASPGAPPRGPGSAGAQKGDGLNGLOFMOPGPRRTGAGPGVGPGOPGOPOPGOGPG

α2(I) 956-1012  
SVPGAPPRGPGSAGAQKGDGLNGLOMFOMOPGPRRTGAGPGVGPGOPGOPOPGOGPG

Cluster 8

**FIGURE 1. Candidate PEDF-binding sequences in the TC8 fragments of human collagen I.** Nine to 10 amino acid sequences that contain both Lys and Arg residues were searched from the TC8 fragments of human α1(I) and α2(I) chains. In the sequences, Pro residues at Y positions of Gly-X-Y repeats are assumed to be fully converted to Hyp (O) residues. The possibility of other types of modifications, such as lysyl hydroxylation and prolyl 3-hydroxylation, is ignored. Peptides satisfying the requirements are underlined. The candidate sequences were reorganized into eight clusters.

(bovine cartilage, Koken), collagen III (bovine placenta, Koken), or a synthetic peptide in 10 mM acetic acid at room temperature overnight. After washing with ELISA buffer (20 mM HEPES-Na (pH 7.4), 100 mM NaCl, 0.005% Tween 20), wells were blocked with 5 mg/ml BSA in ELISA buffer. The wells were washed with ELISA buffer three times, followed by addition of 50 μl of purified 100 nM GST-PEDF or 100 μg/ml biotinylated heparin (Calbiochem). In the peptide competition assay, 1 nM GST-PEDF was preincubated with competitor peptides for 10 min. The plate was incubated at room temperature for 90 min and then washed with ELISA buffer six times. Horseradish peroxidase (HRP)-conjugated anti-GST antibody (GE Healthcare, diluted 1:3000) or HRP-conjugated streptavidin (GE Healthcare, diluted 1:3000) was then added to ice and incubated for 30 min. After washing the wells three times, 50 μl of 0.5 mg/ml 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Wako Pure Chemical Industries, Osaka, Japan) in citrate-phosphate buffer (0.1 M citrate, 0.2 M Na2HPO4 (pH 5.0)) was added to each well. The enzymatic reaction was stopped by adding 10 μl of 10% SDS, and absorbance at 405 nm was measured.

In the polysaccharide competition assay, heparin, chondroitin sulfate-C (Wako), and hyaluronan (Wako) were used as competitors for GST-PEDF. In this assay, final concentration of GST-PEDF was 100 nM. 20 mM HEPES-Na (pH 6.0 or 7.4), 100 mM NaCl, containing 0.005% Tween 20, was used as the ELISA buffer, and wells were blocked with 0.5% skim milk in ELISA buffer.

**Heparin Affinity Chromatography—**The interaction of peptides or rPEDF with heparin was assessed by affinity chromatography on HPLC. A TSK-gel heparin-5PW column (7.5 mm inner diameter × 75 mm, Tosoh, Tokyo, Japan) was equilibrated with 20 mM sodium phosphate buffers adjusted to pH 5.5, 6.0, 6.5, 7.0, or 7.5. Five μg of peptide was loaded onto the column and eluted with a linear gradient of NaCl (0–1000 mM).

| Peptide | Guest sequence | α-Chain and position | T_max °C |
|---------|----------------|----------------------|----------|
| C1-a    | RDGSOGAKGDR    | α1(I)(848–858)       | 61       |
| C1-b    | SOGAKGDRGET    | α1(I)(851–861)       | 63       |
| C2      | PAGKSGDRGET    | α1(I)(881–891)       | 69       |
| C3      | PQQPGKDRGET    | α1(l)(911–921)       | 69       |
| C4-a    | PQQDRGIKHKHR   | α1(l)(924–933)       | 54       |
| C4-b    | DRGIKHKHRGFS   | α1(l)(926–936)       | 42       |
| C4-c    | IKHRGFSGLO     | α1(l)(929–938)       | 47       |
| C5-a    | RDQOOGHKGER    | α2(l)(884–893)       | 64       |
| C5-b    | HKGERGYNO      | α2(l)(854–863)       | 64       |
| C6      | PAGKHGNIKRET   | α2(l)(881–891)       | 56       |
| C7-a    | PQGIRDKGEO     | α2(l)(911–921)       | 67       |
| C7-b    | DKGEQEGKIR     | α2(l)(917–927)       | 68       |
| C7-c    | EKPQGLOGILK    | α2(l)(923–933)       | 66       |
| C8-a    | PSGPAGKDGRT    | α2(l)(968–978)       | 60       |
| C8-b    | PAGKDGRTGHO    | α2(l)(971–981)       | 52       |

*A* Amino acid residues that are out of the cluster sequences were substituted with Pro or Hyp.
RESULTS

Prediction of the PEDF-binding Sequences in Collagen I—In a previous study using a residue-specific chemical modification technique, we elucidated that both Lys and Arg residues in collagen I are important for the binding to PEDF (23). The following photocross-linking experiment involving the PEDF-collagen I complex also revealed that at least one of the PEDF-binding sites is located in the C-terminal 1/4 fragments (TCB fragments) generated by matrix metalloproteinase 1 (MMP-1) digestion (32). On the basis of these findings, we first narrowed down the PEDF-binding sequences by utilizing a computer search program. We searched peptides containing both Arg and Lys from human a1(I)(776–1012) and a2(I)(776–1012) sequences corresponding to the TCB fragments. Here, we scanned peptides with a length of 9–10 amino acid residues. As shown in Fig. 1, a total of 25 sequences (11 from a1(I) chain and 14 from a2(I) chain) were selected as candidate PEDF-binding sequences. These amino acid sequences overlap significantly, and they were re-organized into eight clusters (Fig. 1).

Identification of a PEDF-binding Sequence in Collagen I—Next, we chemically synthesized peptides containing the predicted amino acid sequences in the clusters (Table 1). To maintain the triple helical structure, we employed the self-trimerizing host-guest peptide design in which guest sequences were flanked by (Gly-Pro-Hyp)n sequences with a helix-stabilizing effect (33). Here, we assumed that the homotrimeric helix could function as well as corresponding (a1(I))2(a2(I))1 heterotrimeric native sequences. Because some of the peptides containing the clustered sequences are predicted to be too long to maintain the triple helical conformation at the assay temperature of 37 °C (34), the sequences were divided into several fragments to possess four X-Y-Gly repeats with at least six-amino acid overlaps (Table 1).

The selection of PEDF-binding peptides was performed by a turbidimetric multiwell assay developed for the screening of inhibitors against collagen-binding proteins (31). This assay is based on the previous finding that PEDF retards spontaneous collagen fibril formation in vitro and that the fibril formation is restored in the presence of peptides that interfere with the PEDF-collagen interaction. Among the 15 candidate peptides, C4-c (IKHRGFGSL) was the best inhibitor of the PEDF-collagen interaction, and C4-b (DRGIKGHRGFS) also exhibited a moderate effect (Fig. 2). This result indicates that one of the PEDF-binding sites in collagen I is IKHRGFGSL located at a1(I)(929–938). Interestingly, these peptides contain the proposed heparin/HSPG-binding KGHR sequence (27).

Determination of PEDF-binding Motif in the Collagen Triple Helix—To examine sequence and conformation dependence in the collagen recognition by PEDF, we newly prepared the following two peptides: Short is a random coil counterpart of C4-c with the shorter host Gly-Pro-Hyp repeats, and Scramble is a C4-c analog possessing a scrambled guest sequence (Table 2). The interaction of these peptides with PEDF was analyzed by the collagen fibril formation assay. As shown in Fig. 3A, Short and Scramble did not show detectable interaction with PEDF, indicating that PEDF recognizes collagen in a conformation- and sequence-specific manner. The inhibitory effect of C4-c on the PEDF-collagen interaction appeared dose-dependent, and the IC50 value was estimated to be 1.9 μM (Fig. 3, B and C). To identify the PEDF-binding motif sequence in collagen, we constructed a series of Ala mutants of C4-c (Table 2). The PEDF interacting ability of C4-c was almost completely abolished when its Arg (Scan-R) or Phe (Scan-F) was substituted with Ala. When Lys (Scan-K) or Leu (Scan-L) was replaced with Ala, the activity was decreased to some extent. However, other Ala substitutions did not significantly affect the activity of the peptides (Fig. 3D, Table 3, and supplemental Fig. S1). This result indi-
FIGURE 3. Structural requirements of PEDF-binding collagen-like peptides. A, inhibitory activities of C4-c, Short, and Scramble for PEDF-collagen interaction were evaluated in the same manner as described in Fig. 2. B and C, concentration-dependent inhibition of PEDF-collagen interaction by C4-c. B, restoration of collagen fibril formation by adding increasing concentrations of C4-c was monitored by measuring absorbance at 313 nm. C, % fibril formation at 120 min was plotted against C4-c concentrations. D, inhibitory activities of Ala-mutant peptides (30/mH9262/H11006) for PEDF-collagen interaction were evaluated by the collagen fibril formation assay. E, binding of GST-PEDF fusion protein to the collagen I-coated wells in the presence of various concentrations of competitor peptides (C4-c, Scan-R, and Short) was estimated by ELISA using HRP-conjugated anti-GST antibody. F, binding of GST-PEDF to the peptides and native collagen I was estimated by ELISA. Values are mean ± S.D. (n = 3).
TABLE 3
IC₅₀ values of C4-c and its mutant peptides for PEDF-collagen and heparin-collagen interactions

| Peptide | Collagen-PEDF IC₅₀ (µM) | Collagen-heparin IC₅₀ (µM) |
|---------|------------------------|---------------------------|
| C4-c    | 1.9                    | 5.3                       |
| Short   | >100                   | >100                      |
| Scramble| >100                   | >100                      |
| Scan-I  | 2.3                    | 7.2                       |
| Scan-K  | 24.4                   | >100                      |
| Scan-H  | 2.5                    | 16.5                      |
| Scan-R  | >100                   | >100                      |
| Scan-F  | >100                   | 25.3                      |
| Scan-S  | 3.8                    | 9.0                       |
| Scan-L  | 24.6                   | 7.7                       |

Collagen-PEDF Interaction

We further examined the effect of the series of synthetic peptides on heparin-collagen I interaction. The indispensable role of the Lys and Arg residues was confirmed because Scan-K and Scan-R lost the activity (Fig. 4D and Table 3). Scan-F was observed to be less active than the wild type C4-c in terms of inhibition of heparin-collagen I interaction (Fig. 4D), and its IC₅₀ value was estimated to be 25.3 µM (Table 3), indicating the contribution of the Phe residue in the collagen-heparin interaction. The role of the Phe residue was confirmed by comparing the activities of C4-a (QGDRGIKGHHR), C4-b (DRGIKGHRGFS), and C4-c (IKGRHGFSGL) (see supplemental Fig. S2). In addition, the IC₅₀ value for Scan-H was estimated to be 16.5 µM, suggesting the substantial contribution of the His residue in the molecular recognition of collagen by heparin (Table 3). Focusing on the contribution of the His residue, the interaction of the collagen-like peptides and heparin was further investigated using heparin-affinity chromatography. As shown in Fig. 4E, the binding affinity of C4-c to heparin decreased with an increase of pH. Conversely, the affinity of Scan-H was not affected by the change of pH. This result suggests the additional contribution of the protonated His residue to the heparin-binding to collagen. Taken together, we revised the previous conclusion and considered that the high affinity heparin-binding sequence in triple helical collagen is KGHRGF.

Interaction of PEDF and Heparin with Collagens I–III—We next examined the PEDF and heparin binding to native collagens I–III by ELISA. Binding of GST-PEDF fusion protein to the collagen-coated wells was detected using anti-GST antibody. As shown in Fig. 5A, the highest affinity was found to collagen I, and relatively weak binding was also observed to collagen III. Only a trace amount of PEDF bound to collagen II. This result was consistent with those reported by Kozaki et al. (1) and Meyer et al. (35). The binding of heparin to the collagens was also examined by ELISA. In terms of difference in affinity according to collagen types, the result was quite similar to that obtained using GST-PEDF (Fig. 5B). However, the result for the heparin-collagen interaction (collagen I > III > II) was different from the previous result (III > I > II) reported by San Antonio et al. (36).

PEDF-binding and Heparin-binding Sequences in Collagens I–III—On the basis of the findings described above, we further tried to identify each responsible binding sequence for PEDF and heparin in collagens I–III. We extracted Arg-Gly-Phe-containing sequences from the triple helical regions of human collagens I–III, and they were incorporated into newly synthesized triple helical host-guest peptides, Pep-1 to Pep-10. Pep-11 containing an Arg-Gly-Tyr sequence was also prepared (Table 4).

The interaction of these peptides with PEDF and heparin was examined using the similar collagen fibril formation assay (Fig. 6, A and B). Pep-1 (IKGHRGFSGLQ), Pep-2 (MKGHRGFSGLQ), and Pep-8 (LKGHRGFTGLQ) were found to effectively inhibit the PEDF-collagen interaction. Pep-9 (MKGHRGFDDRN) and Pep-10 (IKGHRFGOGN) also showed a relatively weak effect (Fig. 6A). Pep-11, which has a Tyr residue instead of the Phe residue, did not show a detectable effect on PEDF-collagen. The result confirmed the importance of the KGHRGF motif for the interaction with PEDF, as demonstrated in the Ala-scanning experiment (Fig. 3D). As shown in Fig. 6B, heparin interacted with Pep-1, Pep-2, Pep-8, Pep-9, and Pep-10, all of which possess the KGHRGF sequence. The Phe residue was revealed to be substituted by Tyr because Pep-11 (VKGHRGYQGLD) also interacted with heparin (Fig. 6B). This result indicates that the aromatic side chains, in addition to the cluster of basic amino acids in the peptides, also contribute to the interaction with heparin.

These results strongly suggest that PEDF shares binding sites in collagen with heparin/HSPG. The binding sites identified in collagens I–III are summarized in Fig. 6C.

Competition of PEDF-Collagen Interaction by Heparin—Because PEDF and heparin were elucidated to share binding sites on collagen (Fig. 6), we next examined whether the interaction of PEDF with collagen is competed with by heparin or other polysaccharides by ELISA. The binding of GST-PEDF fusion protein to a well coated with collagen I was inhibited by heparin in a dose-dependent manner (Fig. 7A). Neither chondroitin...
Collagen-PEDF Interaction

A

% Fibril formation

Heparin concentration (μM)

0.01 0.1 1

B

Absorbance (313 nm)

Time (min)

0 20 40 60 80 100 120

C

IC₅₀=5.3 μM

% Fibril formation

C4-c concentration (μM)

0.1 1 10 100

D

collagen I
heparin (+)
C4-c
Scan-I
Scan-K
Scan-H
Scan-R
Scan-F
Scan-S
Scan-L

% Fibril formation

-20 0 20 40 60 80 100 120

E

Retention time (min)

pH

5.5 6.0 6.5 7.0 7.5

C4-c
Scan-H
Collagen-PEDF Interaction

In this study, we first identified one of the PEDF-binding sequences (IKGHRGFSGL; α(1)(929–938)) from the TC fragments of collagen I employing synthetic triple helical collagen-like peptides (Fig. 2). The subsequent structure-activity relationship study has revealed that PEDF recognizes collagen in a conformation- and sequence-dependent manner, and the PEDF-binding motif is KGXRGFXGL, in which Arg and Phe are essential for the recognition and Lys and Leu are important for the high affinity binding (Fig. 3). The sequence-specific collagen recognition by PEDF implies a highly regulated biological function of the collagen binding rather than simple anchoring to ECM.

Interestingly, the PEDF-binding sequences were found to overlap with the reported heparin/HSPG-binding sequence KGHRG (27). We thus revisited collagen-heparin interaction using a series of synthetic collagen-mimetic peptides. Although Sweeney et al. (27) proposed that the dominant heparin/HSPG-binding sequence is located at α(1)(87–90), there is another KGHR sequence at α(1)(930–933). In our analysis, we detected the significant interaction of heparin with both Pep-1 (α(1)(929–933); IKGHRGFSGLQ) and Pep-2 (α(1)(86–96); MKGHRGFXGL) (Fig. 6B). We further revealed the importance of aromatic side chains of Phe or Tyr residue in addition to the basic ones of the Lys, His, and Arg of triple helical collagen for recognition by heparin, and we revised the heparin/HSPG-binding motif to be KGHRF(Y). The contribution of aromatic amino acids to the recognition of heparin is reminiscent of the antithrombin-heparin interaction (38). In that case, antithrombin has two responsible Phe residues in its heparin-binding site. We speculate that similar hydrophobic interaction should contribute to the interaction between collagen and heparin/HSPG.

In this study, the binding preference of PEDF to native collagen was observed as I > III > II, in agreement with that reported by other groups (Fig. 5A) (1). Similar binding preference in collagen types was also observed in the binding to heparin (Fig. 5B). This result was different from that reported by

difference in the pH sensitivity between collagen-PEDF, collagen-heparin, and PEDF-heparin interactions. As suggested from Fig. 4E, the collagen-binding affinity of heparin decreased in the basic buffer where the His side chain lost its positive charge. Conversely, the PEDF-heparin was not significantly affected by the change of pH, as examined by heparin affinity chromatography (data not shown). In addition, the PEDF-collagen interaction was similar between pH 6.0 and 7.4 (Fig. 7B, y intercept). Furthermore, the competitive effect of heparin was smaller at pH 7.4 than at pH 6.0. This observation indicates that PEDF and heparin directly compete with each other at the same sites in collagen.

DISCUSSION

sulfate-C nor hyaluronan inhibited the interaction, although both polysaccharides were also reported to interact with PEDF (20, 22). To explain this result, two different mechanisms are possible. One is the direct competition between PEDF and heparin, and the other is a negative allosteric regulation of the collagen-binding activity of PEDF by heparin because heparin was reported to induce conformational change of PEDF (37). To clarify this, we took advantage of the structural requirements of heparin-binding collagen-like peptides. A, heparin inhibits collagen fibril formation. The fibril formation was evaluated in a similar manner as described in Fig. 2. Values are mean ± S.D. (n = 3). B and C, concentration-dependent inhibition of heparin-collagen interaction by C4-c. B, restoration of collagen fibril formation by adding increasing concentrations of C4-c was monitored by measuring absorbance at 313 nm. C, % fibril formation at 120 min is plotted against C4-c concentrations. Values are mean ± S.D. (n = 3). D, inhibitory activities of Ala-scanned peptides (30 μM) for heparin-collagen interaction were evaluated by the collagen fibril formation assay. Values are mean ± S.D. (n = 3). Heparin (±) represents the data for the collagen I + heparin wells. E, involvement of the His residue in C4-c for the interaction with heparin. C4-c or Scan-H was loaded onto a heparin-affinity column equilibrated with 20 mM phosphate buffers at the indicated pH and eluted with a gradient of NaCl.
San Antonio et al. (36). Using synthetic triple helical peptides, we showed that collagens I and II have high affinity binding sequences for PEDF, α1(I)(86–97), -(929–940), and α1(II)(929–940). Similar sequences derived from type III, α1(III)(95–106) and -(938–949), showed weak binding to PEDF (Fig. 6, A and C). Collagens I–III all contain sequences that match the heparin-binding KGHRG(F/Y) motif. It should be noted again that native collagen II showed only low affinity to heparin despite the α1(II) chain containing two high affinity heparin-binding sequences. How can we explain this discrep-ant observation? The probable answer is post-translational modifications of collagen molecules. The Lys residues at α1(I) residues 87 and 930, α1(II) residues 87 and 930, and α1(III) residues 96 and 939 are known to be chemical cross-linking sites (39–41). The collagen cross-linking is a multistep process involving Lys modifications (42). Lysine hydroxylation to form a 3-hydroxylysine residue is essential in the formation of the collagen cross-links. Myllylä and co-workers (43) reported that...
ERGLKGHRG, corresponding to the PEDF-binding sequence is a good substrate for lysyl hydroxylase. Moreover, the level of Lys hydroxylation in collagen II is known to be higher than those in collagens I and III (44). Glycosylated (2-O-α-D-glucopyranosyl-O-β-D-galactopyranosyl and 2-O-β-D-galactopyranosyl) hydroxylysine residues are also more abundant in collagen II (6% of Lys residues) than in collagens I and III (0.5–1%) (45). The difference in the level of such Lys modifications should particularly affect the interaction of collagen II with heparin. We speculate that PEDF-collagen interaction is regulated similarly by Lys modifications in the PEDF-binding motif of collagens. In a previous study using a photoaffinity cross-linking technique, we detected a cross-linked product between PEDF and TCB fragment of collagen I, but we did not observe the binding to TCA region (32). This might also be accounted for by the modification of Lys-87 located in the N-terminal PEDF-binding site of collagen I. Further quantitative analysis is required to elucidate the relationship between collagen modifications and interactions with PEDF or heparin/HSPG.

Based on this study, we searched for the high affinity PEDF-binding and heparin/HSPG-binding motifs in the collagen family from NCBI data base (supplemental Table S2). Interestingly, PEDF was suggested to mainly target fibril-forming collagens.

HSPGs are located at the ECM (i.e. perlecan and collagen XVIII) and at cell membrane (i.e. syndecan and glypican) and play critical roles in higher order regulation of cells, including angiogenesis (46). Previously, Meyer et al. (35) predicted that PEDF and heparin should compete with each other at basic clusters on collagen molecules because the collagen-recognition site in PEDF contains an acidic amino acid cluster. In this study, we experimentally confirmed that heparin competes with PEDF in the interaction with collagen. The dissociation constants of PEDF and HSPG (syndecan) to collagen were similar and they were estimated to be 140 nM (35) and 150 nM (36), respectively. We thus speculate that the competition between PEDF and heparin/HSPG plays a role in the anti-angiogenic activity of PEDF.

Angiogenesis is a complex event involving pro-angiogenic factors (i.e. VEGF and FGF), ECM components, and matrix metalloproteinases. Signaling from ECM receptors such as integrin and syndecan is known to be important for the process. Syndecan, the dominant membrane-type HSPG, is the collagen receptor that regulates cell attachment and migration (47). Overexpression of syndecan induced neovascularization and promoted cancer cell growth (48). Additionally, collagen-mimetic peptides containing the heparin/HSPG-binding motif (KGHRGF, 1(I)(87–91) and 1(I)(930–935)) are reported to reduce tube formation of human umbilical vein endothelial cells on collagen matrix in vitro (27). Taking this information into consideration, we speculate that PEDF competes with membrane HSPG binding to collagen, resulting in the reduction of the signal inputs essential for angiogenesis.

Another mechanism is also possible for the anti-angiogenic function of PEDF. FGF functions as an angiogenic factor as a complex with heparan sulfate (49, 50). Yang et al. (51) elucidated that perlecan, a soluble HSPG, facilitates FGF accumulation to collagen fibrils and promotes cell growth. Not only FGF but also various pro-angiogenic factors, including VEGF, IL-8, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) and heparin-binding epidermal growth factor-like growth factors (HB-EGF) have affinity to heparan sulfate (for review see Ref. 52). PEDF was previously reported to inhibit angiogenesis primed by FGF, VEGF, or IL-8 (9). Therefore, PEDF may inhibit the accumulation of pro-angiogenic factors complexed with HSPGs to collagen fibrils.

Further study to unravel the entangled interactions among PEDF, HSPGs, pro-angiogenic factors, and collagen is needed to clarify the regulation of angiogenic processes. The collagen-like peptides containing the PEDF- or heparin/HSPG-binding motif sequences that we found in this study should prove useful in future studies.
Collagen-PEDF Interaction

Acknowledgments—We are grateful to Dr. Shinichi Asada, Kenya Kubota, Tomomi Tanaka, Eriko Ikeda, Yuuki Washida, Ayano Seki, Saori Kurihara, and Mitsuki Ohashi for help in peptide synthesis and characterization. We thank Dr. Norihisa Yasui and Prof. Kouki Kitagawa for valuable discussions.

REFERENCES

1. Kozaki, K., Miyaishi, O., Koiwai, O., Yasui, Y., Kashiwai, A., Nishikawa, Y., Shimizu, S., and Saga, S. (1998) J. Biol. Chem. 273, 15125–15130
2. Doggett, D. L., Rotenberg, M. O., Pignolo, R. J., Phillips, P. D., and Cristofalo, V. J. (1992) Mech. Ageing Dev. 65, 239–255
3. Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Fariss, R. N., Wu, Y. Q., Montuenga, L. M., Wong, P., and Becerra, S. P. (1996) J. Biol. Chem. 271, 1275–1283
4. Alberdi, E., Hyde, C. C., and Becerra, S. P. (1998) J. Biol. Chem. 273, 13043–13051
5. Oh, J. H., Lee, H. S., Park, S. H., Ryu, H. S., and Min, C. K. (2010) J. Biol. Chem. 285, 33310–33320
6. Konson, A., Pradeep, S., D’Acunto, C. W., and Seger, R. (2011) J. Biol. Chem. 286, 2775–2794
7. Petersen, S. V., Valnickova, Z., and Enghild, J. J. (2003) Biochem. J. 374, 199–206
8. Filleur, S., Nelius, T., Montuenga, L. M., Wong, P., and Boucher, B. A. (2004) Exp. Eye Res. 78, 223–234
9. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W., and Bouch, N. P. (1999) Science 285, 245–248
10. Abe, R., Shimizu, T., Yamagishi, S., Shibaki, A., Amano, S., Inagaki, Y., Watanabe, H., Sugawara, H., Nakamura, H., Takeuchi, M., Imaizumi, T., and Shimizu, H. (2004) Am. J. Pathol. 164, 1225–1232
11. Garcia, M., Fernandez-Garcia, N. I., Rivas, V., Carretero, M., Escamez, M. J., Gonzalez-Martin, A., Medrano, E. E., Volpert, O., Jorcano, J. L., Jimenez, B., Larcher, F., and Del Rio, A. (2004) Cancer Res. 64, 5632–5642
12. Matsuzato, K., Ishikawa, H., Nishimura, D., Hamaoka, K., Nakao, K., and Eguchi, K. (2004) Hepatology 40, 259–259
13. Wang, L., Schmitz, V., Perez-Mediavilla, A., Izal, I., Prieto, J., and Qian, C. (2003) Mol. Ther. 8, 72–79
14. Cai, J., Jiang, W. G., Grant, M. B., and Boulton, M. (2006) J. Biol. Chem. 281, 3604–3613
15. Notari, L., Ballard, V., Aroca-Aguilar, J. D., Ball, N., Heredia, R., Meyer, C., Notario, P. M., Saravanamuthu, S., Nueda, M. L., Sanchez-Sanchez, F., Escobedo, J., Laborda, J., and Becerra, S. P. (2006) J. Biol. Chem. 281, 38022–38037
16. Bernard, A., Gao-Li, I., Franco, C. A., Bouceba, T., Huet, A., and Li, Z. (2009) J. Biol. Chem. 284, 10480–10490
17. Konson, A., Pradeep, S., D’Acunto, C. W., and Seger, R. (2011) J. Biol. Chem. 286, 3540–3551
18. Notari, L., Arakaki, N., Mueller, D., Meier, S., Amaral, J., and Becerra, S. P. (2010) FEBS J. 277, 2192–2205
19. Baba, H., Yonemitsu, Y., Nakano, T., Onimaru, M., Miyazaki, M., Ikeda, Y., Sumiyoshi, S., Ueda, Y., Hasegawa, M., Yoshino, I., Maehara, Y., and Suezushi, K. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 1938–1944
20. Alberdi, E., Hyde, C. C., and Becerra, S. P. (1998) Biochemistry 37, 10643–10652
21. Alberdi, E. M., Weldon, J. E., and Becerra, S. P. (2003) BMC Biochem. 4, 1–9
22. Becerra, S. P., Perez-Mediavilla, L. A., Weldon, J. E., Locatelli-Hoops, S., Senanayake, P., Notario, L., Notario, V., and Hollyfield, J. G. (2008) J. Biol. Chem. 283, 33310–33320
23. Kasai, N., Morii, T., Morito, D., Matsushita, O., Kourai, H., Nagata, K., and Koide, T. (2003) Biochemistry 42, 3160–3167
24. Simonovic, M., Gutton, P. G., and Volz, K. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 11131–11135
25. Hosomichi, I., Yasui, N., Koide, T., Soma, K., and Morita, I. (2005) Biochem. Biophys. Res. Commun. 335, 756–761
26. Bridge, C. G., Morton, L. F., Peachey, A. R., Tuckwell, D. S., Farndale, R. W., and Barnes, M. J. (2000) J. Biol. Chem. 275, 35–40
27. Sweeney, S. M., Guy, C. A., Fields, G. B., and San Antonio, J. D. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 7275–7280
28. Gandhi, N. S., and Mancera, R. L. (2008) Chem. Biol. Drug Des. 72, 455–482
29. Koda, J. E., Rapraeger, A., and Bernfield, M. (1985) J. Biol. Chem. 260, 8157–8162
30. Koide, T., Nishikawa, Y., and Takahara, Y. (2004) Bioorg. Med. Chem. Lett. 14, 125–128
31. Okano-Kosugi, H., Matsushita, O., Asada, S., Herr, A. B., Kitagawa, K., and Koide, T. (2009) Anal. Chem. 81, 125–131
32. Notari, L., Baladron, V., Aroca-Aguilar, J. D., Balko, N., Heredia, R., Meyer, C., Notario, P. M., Saravanamuthu, S., Nueda, M. L., Sanchez-Sanchez, F., Escobedo, J., Laborda, J., and Becerra, S. P. (2006) J. Biol. Chem. 281, 38022–38037
33. Bernard, A., Gao-Li, I., Franco, C. A., Bouceba, T., Huet, A., and Li, Z. (2009) J. Biol. Chem. 284, 10480–10490
34. Konson, A., Pradeep, S., D’Acunto, C. W., and Seger, R. (2011) J. Biol. Chem. 286, 3540–3551
35. Notari, L., Arakaki, N., Mueller, D., Meier, S., Amaral, J., and Becerra, S. P. (2010) FEBS J. 277, 2192–2205
36. Baba, H., Yonemitsu, Y., Nakano, T., Onimaru, M., Miyazaki, M., Ikeda, Y., Sumiyoshi, S., Ueda, Y., Hasegawa, M., Yoshino, I., Maehara, Y., and Suezushi, K. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 1938–1944
37. Alberdi, E., Hyde, C. C., and Becerra, S. P. (1998) Biochemistry 37, 10643–10652
38. Alberdi, E. M., Weldon, J. E., and Becerra, S. P. (2003) BMC Biochem. 4, 1–9