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Glycosaminoglycans in human retinoblastoma cells: Heparan sulfate, a modulator of the pigment epithelium-derived factor-receptor interactions

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Abstract

Background: Pigment epithelium-derived factor (PEDF) has binding affinity for cell-surface receptors in retinoblastoma cells and for glycosaminoglycans. We investigated the effects of glycosaminoglycans on PEDF-receptor interactions.

Results: ^{125}I-PEDF formed complexes with protease-resistant components of medium conditioned by human retinoblastoma Y-79 cells. Using specific glycosaminoglycan degrading enzymes in spectrophotometric assays and PEDF-affinity chromatography, we detected heparin and heparan sulfate-like glycosaminoglycans in the Y-79 conditioned media, which had binding affinity for PEDF. The Y-79 conditioned media significantly enhanced the binding of ^{125}I-PEDF to Y-79 cell-surface receptors. However, enzymatic and chemical depletion of sulfated glycosaminoglycans from the Y-79 cell cultures by heparitinase and chlorate treatments decreased the degree of ^{125}I-PEDF binding to cell-surface receptors.

Conclusions: These data indicate that retinoblastoma cells secrete heparin/heparan sulfate with binding affinity for PEDF, which may be important in efficient cell-surface receptor binding.

Background

Glycosaminoglycans (GAGs) are negatively charged polysaccharides derived from an amino hexose. They are structural and functional modulators of extracellular matrices that play important roles in CNS development and repair. They exhibit both stimulatory and inhibitory influences on neurite outgrowth and survival. Evidence demonstrates the ability of heparin sulfates (HSs) to bind to growth/trophic factors and selectively regulate such factors' receptors. [1,2]. They can act as co-receptors of growth/trophic and survival factors to regulate cell behavior and/or restrict diffusion and create a relatively high local concentration of ligand.

Pigment epithelium-derived factor (PEDF) is an extracellular neuronal differentiation and survival factor for cells derived from the retina and CNS. It induces neuronal differentiation in retinoblastoma cells, protects retina neurons (including photoreceptors) from death by apoptosis and other insults, and has a morphogenetic effect on photoreceptor cells [3–6]. It also has neurotrophic effects on neurons from the cerebellum, hippocampus and spinal cord [7–12]. In the intact retina, this factor is identified as

a secreted protein associated by ionic interactions with the interphotoreceptor matrix [13,14], where GAGs are the major polyanionic components.

Biochemically, PEDF is a 50-kDa glycoprotein with structural homology to members of the serine-protease inhibitor (serpin) superfamily [3,15]. However, it has no inhibitory effects on proteases. Its neurotrophic activities are independent of its protease inhibition potential but dependent on its interaction with cell-surface receptors [9,12,16–18]. PEDF has high binding affinity for cell-surface receptors in human retinoblastoma Y-79 cells (K_d = 2.7 nM), which is mediated by interactions between a region spanning amino acid positions 78–121 of the PEDF polypeptide and the extracellular domains of the receptor protein [18]. Blockage of these interactions inhibits the PEDF neurotrophic effects. PEDF also has binding affinity for GAGs, such as, heparin, heparin- and chondroitin-sulfates, but this affinity is ~1000-fold lower than for the receptor (e.g., $K_d \cong 4$ μM for the heparin-PEDF interactions) [14,19]. The binding to GAGs is mediated by ionic interactions between an area clustered with positively charged lysines of PEDF and the negatively charged GAGs. In the PEDF spatial structure, the putative GAG binding domain is distinct from and non-overlapping with the neurotrophic active region [14,20].

Because PEDF coexists with GAGs in extracellular matrices and has binding affinity for them, it is of interest to investigate the role of GAGs on PEDF activity. Given that binding to cell-surface receptors is the first step in the biological activity of PEDF, we used human retinoblastoma Y-79 cells and their conditioned media (CM) as sources of functional PEDF receptors and extracellular matrix components, respectively, to examine the GAG content in CM and their effects on PEDF ligand-receptor interactions. The data suggest that heparan sulfate participates in the formation of a PEDF binding complex with its cell-surface receptor, and constitutes a positive modulator for the PEDF-receptor interactions.

Results

Complex formation between PEDF and component(s) in media conditioned by retinoblastoma cells

To determine whether PEDF interacts with component(s) in media conditioned by retinoblastoma cells (CM), we used an ultrafiltration assay. In this assay, soluble PEDF of 50-kDa is filtered through a membrane with an exclusion limit of MW 100,000, however it is retained upon formation of a complex larger than this limit [14]. The binding reactions were with a given ^{125}I-PEDF concentration and CM. Incubations were at 4°C to minimize enzymatic degradation of proteins and glycosaminglycans during the reaction. We found that 22% of ^{125}I-PEDF was retained by the membrane in the presence of concentrated CM, compared to only 4% retention in the presence of defined medium (non-conditioned medium) concentrated in an identical fashion. Specific retention, termed PEDF bound, was calculated by subtracting the retention in defined media from that in CM. Figure 1 shows that PEDF was specifically retained when mixed with soluble conditioned media and the value for PEDF bound increased proportionally to the concentration factor of the media (Fig. 1A). Interestingly, protease treatment of the CM did not abolish the binding (Fig. 1B). These observations revealed that the retained forms in the CM were PEDF complexes ≥ 100-kDa, and that the majority of these complexes were formed with soluble CM components other than proteins.

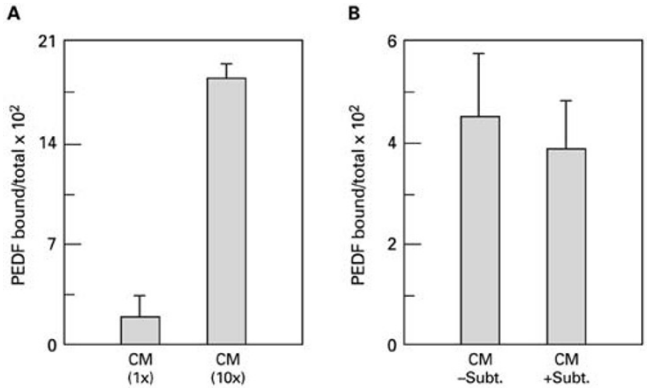

Figure 1

Complex formation between PEDF and component(s) in media conditioned by human retinoblastoma Y-79 cells. Binding reactions were performed with 0.75 nM ^{125}I-PEDF and human retinoblastoma Y-79 cell conditioned media (CM) (200 μl) at 4°C for 120 min with gentle rotation. Free and bound PEDF was separated by ultrafiltration through membrane of MWCO = 100 kDa. PEDF bound was calculated by subtracting specific ^{125}I-PEDF binding in fresh media from that in CM and is given as a ratio of the total ^{125}I-PEDF in the reaction. A) Reactions with CM (CM 1X) and 10-fold concentrated CM (CM 10X). B) Reactions with protease-treated CM. CM was concentrated 10-fold and dialyzed against 20 mM Tris pH 8, 10% glycerol. Half of the dialyzate was incubated without and the other with 0.4 μg/ml subtilisin at 37°C for 16 h (-Subt. and +Subt., respectively). The protease reaction was terminated by incubation at 75°C for 25 min to inactivate the protease. All experimental points are given as the average of duplicates. Assays were performed twice.

Heparin and HS in the conditioned media with affinity for PEDF

GAGs and polyanions in the CM were fractionated by anion-exchange column chromatography followed by PEDF-affinity column chromatography (Fig. 2). The GAG content was followed by staining with Toluidine Blue-O

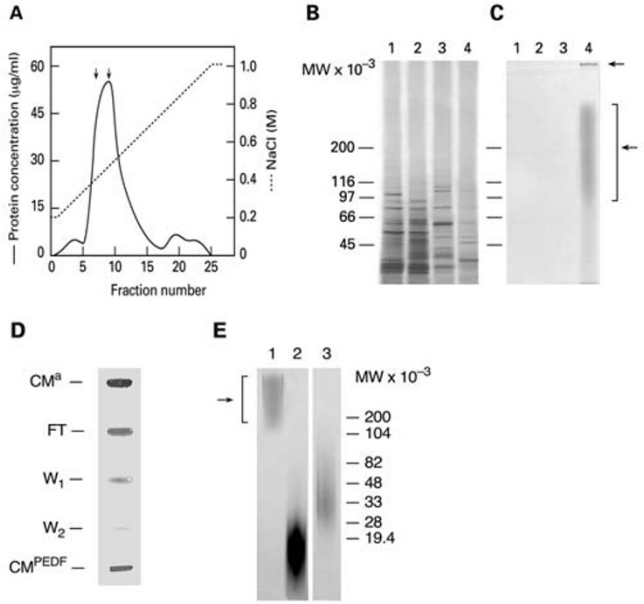

Figure 2

DEAE-Sephacel and PEDF-affinity column chromatography of media conditioned by retinoblastoma cells. GAGs and polyanions of CM were fractionated by anion-exchange column chromatography (CM^a). CM (2.5 mg protein) was applied to a DEAE-Sephacel column and the bound components were eluted with a linear gradient of 0.2–1 M NaCl. **Panel A.** *Protein profile of the DEAE-Sephacel chromatography.* Protein (--) and NaCl (-----) concentrations of eluted fractions are indicated. The load, unbound material (FT), and fractions 7 and 9 were resolved by 4–12% polyacrylamide gel electrophoresis under reducing conditions in two duplicate gels. **Panels B and C.** *SDS-PAGE analysis of the DEAE chromatography.* Gels stained with Coomassie Brilliant Blue (panel B) and with Toluidine Blue-O (panel C) are shown. Lane 1, load; lane 2, flow-through; lane 3 fraction #7; and lane 4, fraction #9. **Panels D and E.** *PEDF-affinity column chromatography.* CM^a was subjected to PEDF-affinity column chromatography. Bound GAGs were eluted with 3 M NaCl (CM^{PEDF}). Unbound material is FT and washes W_1 and W_2., A dot-blot stained with Toluidine Blue-O of fractions (as indicated) is shown in panel D. A 10–20% polyacrylamide gel (SDS-tricine) stained with Toluidine Blue-O is shown in Panel E with: lane 1, CM^{PEDF}; lane 2, 5 μg heparin of average MW 16,000; and lane 3, 5 μg HS. Migration positions of molecular-weight-standards are indicated to the right. Arrows indicate migration positions of GAGs from CM derived samples.

(Fig. 2C,2D,2E). The final fraction (CM^{PEDF}) contained components with binding affinity for PEDF that stained with Toluidine Blue-O and migrated as high molecular weight GAGs.

To determine the type of sulfated GAG in the media, we designed a spectrophotometric assay using heparinase and heparitinase, specific degrading enzymes for heparin and HS, respectively (Fig. 3). The activities of both GAG lyases reached a plateau by one hour of incubation (Figs. 3A,3C) and the degradation of GAG substrates between 0–30 μg was linear. Both CM and CM^{PEDF} contained substrates for heparinase and heparitinase (Figs. 3B,3D). The amount of GAGs was determined by comparison of the amount of Δ^4-hexuronate produced with CM samples to the standard curves with commercial GAGs. The estimated content of GAGs in CM varied between 12.4–22.7 μg/ml for heparin and between 9–10 μg/ml for HS-like molecules, among media conditioned by three different batches of Y-79 cells. The estimated GAG content in CM^{PEDF} ranged 0.2–1.8 μg/μl and 0.1–0.4 μg/μl for heparin and HS, respectively. Similar assays were followed with chondroitinase ABC, but its substrates, ΔDi4S, dermatan and ΔDi6S, in CM were below detection limits. These results demonstrated that CM and CM^{PEDF} contained heparin- and HS-like molecules, demonstrating that Y-79 cells produced GAGs with binding affinity for PEDF.

Media conditioned by retinoblastoma cells enhances the ^{125}I-PEDF binding to cell-surface receptors

We have demonstrated previously that biologically active ^{125}I-PEDF binds specifically, competitively and with high affinity to cell-surface receptors of Y-79 cells [18]. Because the reaction conditions were identical to those used for biological assays, the binding reactions were performed in the presence of media conditioned by the cells for 16 hours (CM). We investigated the effect of components of the CM, a source of extracellular matrix, on the PEDF-receptor interactions using CM and non-conditioned defined media in radioligand binding assays. Comparison of reactions in the absence and presence of CM showed that the specific PEDF-binding to Y-79 cell-surface receptors was 6.8-fold higher with conditioned medium than with defined medium (Fig. 4). Note that with the binding method used, the amount of ^{125}I-PEDF retained in CM without cells is the same to the amount of non-specific ^{125}I-PEDF binding (reactions with cells and in the presence of 50-fold molar excess of unlabeled PEDF) [18], indicating retention by PEI-treated glass-fiber filters of PEDF-receptor or GAG-PEDF-receptor complexes rather than PEDF-GAG. Similar results were obtained when the cell-bound ^{125}I-PEDF was separated by centrifugation, rather than filtration through glass-fiber filters, and comparing reactions with CM versus those with 1% BSA in PBS (data not shown). These observations showed that a component(s) secreted by retinoblastoma cells enhanced the PEDF-receptor interactions.

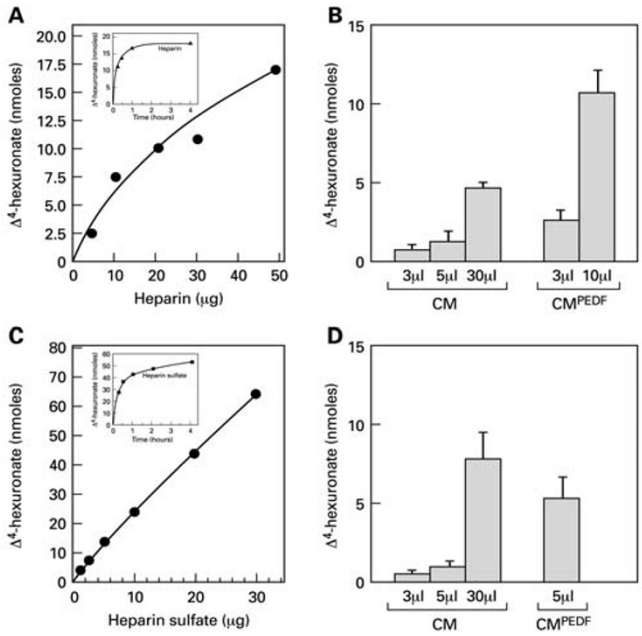

Figure 3

Spectrophotometric assays for heparin and HS-like molecules. Heparin and HS were degraded with heparinase and heparitinase, respectively. Proteins in CM concentrated 10-fold and CM^{PEDF} were digested first with subtilisin. Protease-treated CM and CM^{PEDF} were reacted with heparinase and heparitinase. The appearance of degradation products (Δ^4-hexuronate) was measured by absorbance at 235 nm (Extinction coefficient = 5500). Panels A and B show heparinase activity and panels C and D show heparitinase activity. Time course and concentration curves of enzymes using their respective substrates are shown in panels A and C. Panels B and D correspond to reactions with CM and CM^{PEDF}.

Effect of GAG lyases and chlorate on ^{125}I-PEDF binding to cell-surface receptors

To deplete the Y-79 cell cultures of HS and heparin-like GAGs, we used heparitinase and heparinase, respectively. The cultures were pretreated with each GAG lyase before using them in radioligand binding assays. The morphology and viability of the cells were not affected with the GAG lyase treatments. Figure 5A shows that specific ^{125}I-PEDF binding decreased significantly in heparitinase treated cultures compared to untreated controls, and less drastically in heparinase treated ones. Hyaluronidase treatment to deplete the cultures of hyaluronan did not have an effect on the binding. These results demonstrated that removal of heparin/HS from the cell cultures decreased the PEDF binding to receptors on Y-79 cells. Chlorate is a competitive inhibitor of ATP-sulfurylase, and inhibition of GAG sulfation in cell cultures can be achieved by pretreatment of the cultures with 30 mM sodium chlorate [21]. The effect of undersulfated GAGs on the PEDF binding to its receptor was examined. Cells pretreated with sodium chlorate did not show changes in viability or morphology; however, the treatment resulted in a decrease in the specific ^{125}I-PEDF binding to about 35% relative to untreated controls (Fig. 5B). Sodium sulfate was used to recover the loss of GAG sulfation by chlorate increasing the binding to 55% maximal with 10 mM sulfate additions. The data revealed that inhibition of sulfation of GAGs reduced the PEDF binding to cell-surface receptors of Y-79 cells, with about 20% of specific inhibition. Thus, these observations implied that HS/heparin might play a functional role in the binding of PEDF to its cell-surface receptor.

Discussion

It has been proposed that the GAG-binding property of PEDF provides the molecular basis for its association with extracellular matrices and may serve to localize PEDF activity in the retina and CNS [14,19]. However, the present results point to direct effects these polysaccharides might have on the biochemical interactions between PEDF and PEDF receptors on the surfaces of cells that respond to this neurotrophic factor. We have shown that the binding of PEDF to receptors in retinoblastoma cells is enhanced by the presence of extracellular heparin/HS-like GAGs, which can be found in the culture medium of retinoblastoma cells. The fact that the binding of PEDF to cell surfaces decreases with heparin/HS depletion, implies that heparin/HS molecules might act as cofactors for PEDF-receptor interactions. Interactions between PEDF and extracellular GAGs can also explain the complex formed by PEDF with CM even after protease treatment of the latter. The PEDF-heparin/HS complex may somehow facilitate encounters between PEDF and its receptor, *e.g.*, by inducing a conformational change in PEDF, which might accelerate the ligand-receptor interactions. In addition, the receptor may also form a complex with heparin/HS to facilitate interactions with the ligand.

To our knowledge, this is the first report on the production of GAGs by retinoblastoma cells. We found that these cells produce HS/heparin secreted into the culturing media. The retina and malignant solid tumors also produce the sulfated GAGs [1,2,22–26]. Although HSs are mostly found as proteoglycans associated with the basal lamina or the plasma membrane, the presence of HSs in the culturing medium might be a result of shedding or release of their extracellular domains from the cell membranes as soluble components. Cell-associated HS proteoglycans can undergo regulated shedding from the membrane into the soluble extracellular matrix or culturing medium converting the membrane anchored molecules into soluble effectors [1,2]. In the conditioned media of all the tested batches of retinoblastoma cells, we detected heparin and

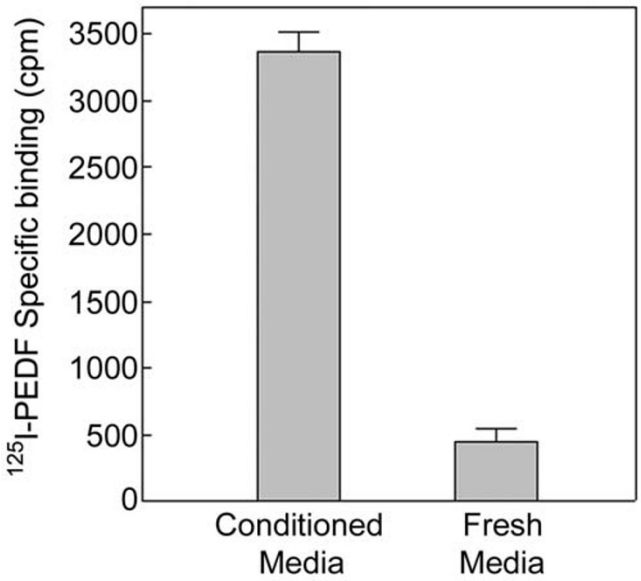

Figure 4
Media conditioned by retinoblastoma Y-79 cells enhances the ^{125}I-PEDF binding to cell-surface receptors. Binding to human retinoblastoma Y-79 cells (1.5 × 10^5 cells/ml) was performed with 0.8 nM ^{125}I-PEDF (136,000 cpm/point) in their overnight culturing media (conditioned media) or non-conditioned media (fresh media). Free and bound ligand was separated by filtration through glass fiber filters under vacuum. Non-specific binding was binding in the presence of 125-fold molar excess of unlabelled PEDF. Specific binding was calculated by subtracting the non-specific from the total binding. All experimental points are given as the average of triplicates.

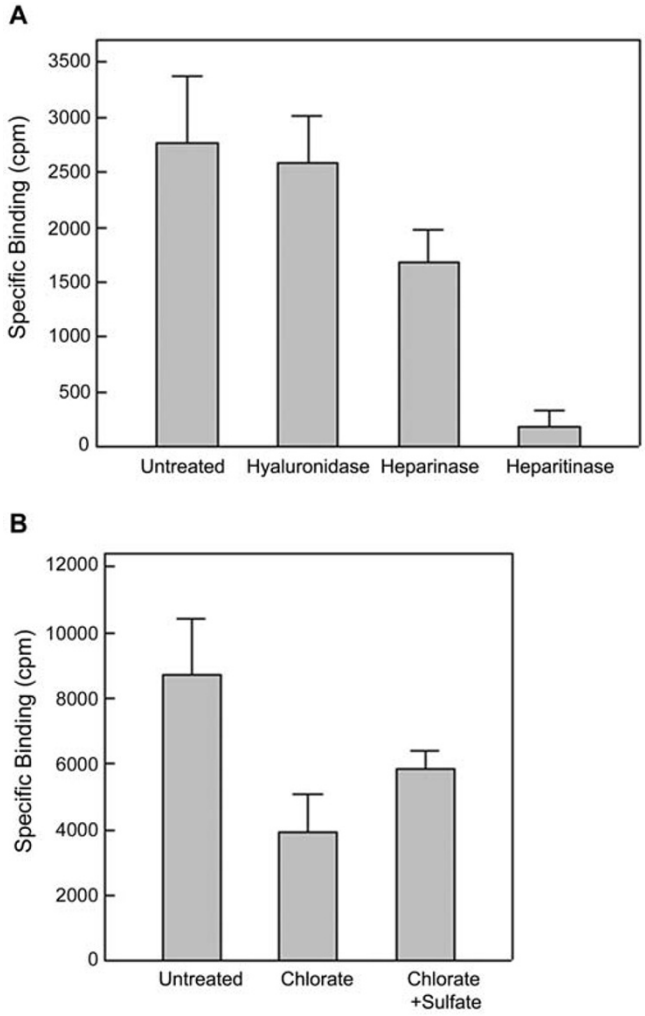

Figure 5
Effect of GAG lyases and chlorate on ^{125}I-PEDF binding to retinoblastoma Y-79 cells. Human retinoblastoma Y-79 cells (5 × 10^5 cells/ml) were cultured in serum-free media at 37°C and treated with GAG lyases or chlorate. Binding to the treated cells was performed with 2 nM ^{125}I-PEDF. Free and bound PEDF were separated by filtration through glass-fiber filters under vacuum. Non-specific binding was determined from binding reactions in the presence of 50-fold excess of unlabeled ligand. Specific binding was calculated by subtracting non-specific binding from total binding. Panel A. Cells cultured for 16 h were treated with hyaluronidase, heparinase and heparitinase at 37°C for 120 min. All experimental points are given as the average of quadruplicates. Panel B. Cells were cultured with or without 30 mM sodium chlorate and 10 mM sodium sulfate at 37°C for 16 h before the radioligand was added. The binding reactions were at 4°C for 30 min. All experimental points are given as the average of triplicates.

HS with binding affinity for PEDF. The estimated concentrations of these GAGs in the media may vary with cell density and conditioning time. However, under the conditions used, they were within the linear range of HS-PEDF complex formation (EC_{50} = 40 μg/ml) [14].

The fact that depletion of heparin/HS-like GAGs from the culturing media results in inhibition of PEDF binding to cell surface receptors points to functional roles for these GAGs such as those of positive modulators of PEDF-receptor interactions. In this regard, we observed that depletion from the Y-79 cell cultures of heparin with heparinase was lower than those depleted of HS with heparitinase, suggesting that the retinoblastoma-derived HS was more effective than the retinoblastoma-derived heparin. This observation can be explained by structural, compositional and functional differences between heparin and HS GAGs. GAG-binding proteins can be differentially sensitive to variations in GAG structure [27]. GAGs produced among different cell types have structural and

compositional differences and structural changes in GAGs are known to occur in cells undergoing morphological differentiation and/or malignant transformations [28–31]. Thus, in the native retina, or other tissue, modulation of the PEDF-receptor interactions may depend on the expression of GAGs, which occur during development and pathological conditions.

Our data offer interesting possibilities of regulation of the activity of PEDF. The ratio and amount of production of heparin and HS by cells bearing PEDF receptors may be an important mechanism to control the activity of PEDF as is the modulation of the rate of expression of PEDF receptors. The data obtained so far on the action of PEDF on different cells may have not considered the presence of these cofactors in the media. The binding of GAGs to PEDF that modulate the binding of these factors to its receptor opens also the possibility that different GAGs may modulate differently the affinity of PEDF for its receptors, by increasing or even decreasing it in some cases. Finally, the fact that the GAG and receptor binding regions are on opposite regions of the PEDF molecule, suggest the possibility of the existence of PEDF mutants or engineered variants that, having lost or decreased GAG-binding capabilities, still show high affinity for the PEDF receptor in a way not dependent upon GAGs content or composition. These possibilities are discussed under the consideration that some of them may have important implications if PEDF, or molecules derived from it, are to be used in the future as therapeutic agents.

The primary consequence of reducing the heparin/HS and its sulfation was to minimize a binding site required for PEDF activity. Although GAGs store PEDF in the extracellular matrix [14], a more direct mechanism appears necessary, namely, its participation in the binding of PEDF to its receptor. In a spatial structure of PEDF, the heparin/HS binding domain of PEDF maps to the opposite side of the neurotrophic active region [20], allowing distinct and non-overlapping interactions with heparin/HS on one side of the protein, and with the neurotrophic receptor on its opposite side. Our data suggest that the intrinsic affinity of the cell surface receptor for PEDF appears low, whereas the heparin/HS-PEDF complex is recognized with high affinity. In addition, a direct interaction between the receptor and GAGs may also be necessary. Although details of the mechanism remain to be revealed, it is clear that heparin/HS is required for the first step of the neurotrophic activity of PEDF, namely the encounters with its receptor at the cell surface. The differentiation and survival of cells in vivo may be regulated not only by the expression of PEDF and its receptor but also by the temporal and spatial expression of GAGs.

Methods

Materials

Heparin purified from bovine intestinal mucosa, chondroitin sulfates A, B and C, chondroitinase ABC, sodium chlorate, and Toluidine Blue-O were purchased from Sigma. Subtilisin was from Boehringer Mannheim. Heparitinase (E.C.4.2.2.8) and heparinase (E.C.4.2.2.7) purified from *Flavobacterium heparinum* were from ICN Biomedicals, Inc. and alternatively from Seikagaku. Heparan sulfate (HS) purified from bovine kidney was from Seikagaku, hyaluronidase (E.C.4.2.2.1) purified from *Streptomyces hyalurolyticus* from ICN Pharmaceuticals, Coomassie Brilliant Blue from BioRad, and Q-Sepharose from Pharmacia. Recombinant PEDF was purified from BHK cells containing an expression vector with human PEDF cDNA, as previously described [19].

Preparation of conditioned media

Human retinoblastoma Y-79 cells (0.45–5 × 10^6 cells/ml) were cultured in defined media (MEM containing 10 mM HEPES, 1 mM Na-pyruvate, 0.1 mM non-essential amino acids, 1 mM L-glutamine, 1% penicillin/streptomycin (LifeTechnologies)) at 37°C for 16–24 h. Media exposed to these conditions is referred as CM. CM was separated from cells by centrifugation (1000 × *g* for 5 min at 4°C) and concentrated by ultrafiltration using membrane filters with MWCO = 10,000 (Amicon YM10 filters). GAGs/polyanions purification was performed as follows: concentrated CM (15 ml) was dialyzed against buffer Q (50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 6 M Urea, 0.5% CHAPS), filtered through a 0.4 micron membrane and its soluble components subjected to anion-exchange column chromatography using Q-Sepharose Fast Flow (1 ml bed volume). The column was washed with 15-column volumes of buffer Q, the bound material eluted with 1.2 M NaCl and termed CM^a. Alternatively, a DEAE-Sephacel column was used.

PEDF-affinity column chromatography

To identify components with PEDF-binding affinity, purified recombinant protein was used to prepare PEDF-affinity resin with 3 M Emphaze™ Biosupport Medium (Pierce Chemical) [18]. CM^a was dialyzed against buffer P (20 mM sodium phosphate pH 7, 150 mM NaCl, 0.5% CHAPS) and filtrated through 0.4 μm filters. The soluble dialysate was mixed with PEDF-resin (6 mg PEDF/ml resin) at a 2:1 volume-to-volume ratio and incubated at 4°C with gentle rocking for 16 h. The mixture was packed into a 10 ml Polyprep chromatography column (Bio-Rad) and washed with 10-column volumes of buffer P. The bound material was eluted with 10-column volumes of 3 M NaCl, desalted with 20 mM Tris-HCl pH 8.0, 10% glycerol, treated for protein depletion, dialyzed against deionized water, lyophilized and resuspended in deionized water. The final sample was termed CM^{PEDF}. About 100 μl of

CM^{PEDF} were obtained from 100 ml of CM. Alternatively, concentrated CM was used as starting material and protein depletion was omitted.

Radioligand binding assays

PEDF binding to cell-surface receptors was assayed using biologically active radioligand ^{125}I-PEDF and Y-79 cells [18] by a widely-used method with a mechanism of retention of receptors on polyethylenimine-treated glass-fiber filters based mainly on ionic interactions [32]. Polyethylenimine binds strongly to glass, which is negatively charged and integral membrane proteins tend to be acidic. The resultant polycationic polyethylenimine-coated glass can retain cell membranes due to their negative charges. Because binding of cell-surface receptors to polyethylenimine filters is rather insensitive to ionic strength, the ionic phenomenon is thought to be supplemented by hydrophobic forces and hydrogen binding [32]. The method used with Y-79 cells and radiolabeled PEDF has been described before in detail [18]. Briefly, cells cultured overnight in serum-deprived medium at 37°C were transferred to ice/water bath for 10 minutes before the addition of ligand. The reaction mixtures containing cell suspensions with given radioligand concentrations in untreated or treated media were incubated at 4°C for 90 min, unless indicated. The free and bound ^{125}I-PEDF were separated by filtration through glass-fiber filters and the bound radioactivity was determined in the filters using a β-scintillation counter (Beckman, model LS 3801). Nonspecific binding was calculated from reactions with a molar-excess of unlabeled ligand (≥ 50-fold) over radioligand.

Complex-formation assays

Complex formation between PEDF and CM components was assayed by a method using ultrafiltration through membranes of 100,000 MW exclusion limit [14]. Binding reactions were performed with a given concentration of ^{125}I-PEDF in defined or conditioned media, and incubations with gentle rotation at 4°C for 2 h. Free and bound ligand were separated by ultrafiltration through Microcon-100 (Amicon). The reaction mixtures were diluted 40-fold with cold 20 mM sodium phosphate pH 6.5, 20 mM NaCl, 10% glycerol and immediately ultrafiltrated, repeating twice to ensure removal of free ligand from the complexes. Each Microcon retenate cup was transferred to scintillation vials, mixed with 5 ml BioSafe II liquid scintillation solution (Research Products International) by extensive vortexing, and its radioactivity determined using a β-scintillation counter. Nonspecific binding, calculated from reactions with an excess of unlabeled ligand (100-fold) over radioligand, reached about 40% of the total binding.

Enzymatic digestion treatments

The presence of GAGs was assayed using specific GAG lyases, *i.e.*, the presence of heparin, HS, and chondroitin sulfates with heparinase, heparitinase, and chondroitin ABC respectively. The amount of GAGs was determined by the amount of Δ^4-hexuronate produced after the eliminative cleavage of each substrate by the corresponding GAG lyase. Samples were depleted of proteins by protease treatment to avoid interference in absorbance readings of the product. For heparinase and heparitinase reactions, samples were treated with 5 milliunits of each enzyme in 150 μl of 0.1 M sodium acetate and 1 mM $CaCl_2$, pH 7 and incubations at 37°C for various time periods. The reactions were stopped by the addition of 1 ml of 0.06 M HCl. The soluble material was separated by centrifugation (3000 × *g*, 10 min) and assayed for absorbance at 235 nm to measure the concentration of product Δ^4-hexuronate (Molar extinction coefficient = 5500; [33]). For the chondroitinase ABC reactions, each chondroitin sulfates A, B and C substrate (1 mg each) and concentrated CM, were incubated with 0.12 units of chondroitinase ABC in 1 ml of 50 mM Tris-HCl pH 8.0, 60 mM sodium acetate, and 0.02% BSA at 37°C. At various time periods, aliquots of 0.1 ml were removed and mixed with 0.9 ml of 45 mM KCl pH 1.8 to stop the reaction. Insoluble material was removed by centrifugation (1000 × *g*, 10 min) and the supernatant assayed for absorbance of Δ^4-hexuronate at 232 nm. For protein depletion, CM was mixed with subtilisin at 0.4 μg/ml in 20 mM Tris-HCl pH 8.0, 10% glycerol and incubated at 37°C for 16 h. Subtilisin was heat-inactivated at 75°C for 25 min. The protein concentration after the reaction was less than 0.1% of the starting material.

To deplete cell cultures of GAGs, Y-79 cells in defined serum-free medium (as above) at a density of 1.25×10^5 cells/ml were cultured in 96-well culture plates (150 μl/well) and incubated at 37°C in a 5% CO_2 environment for 16 h. Hyaluronidase (>1TRU/μl), heparinase (1 mu/μl) or heparitinase (1 mu/μl) were each added to various wells and incubated at 37°C in a 5% CO_2 environment for 1 h.

Chlorate treatment of cell cultures

To prevent sulfation of GAGs in cell cultures, we used a method previously described [33]. Y-79 cells (1.25×10^5 cells/ml) were cultured in 48-well plates (300 μl/well) in defined serum-free medium with or without 30 mM sodium chlorate and 10 mM sodium sulfate at 37°C in a 5% CO_2 environment for 24 h.

GAG detection assays

GAGs and proteins resolved by SDS-polyacrylamide gel electrophoresis in Tricine/SDS buffer, as instructed by manufacturer (Novex), were detected with specific stains. For GAG detection, the gels were incubated sequentially

in 0.2% Toluidine Blue-O in ethanol-water-acetic acid (50:49:1) for 30 minutes, in ethanol-water-acetic acid (50:49:1) for 1 h, and in H_2O for 16 h. [34]. For protein detection, the gels were sequentially incubated in 0.5% Coomassie Brilliant Blue in 50% methanol/10% acetic acid and in 10% methanol/10% isopropanol. GAG detection was also performed by the Toluidine Blue-O precipitation and dot-blot method as described previously [34]. Briefly, 200 μl of sample were applied to each well of a 48-well manifold (Life Technologies) onto a PVDF membrane without vacuum. Then, 5 μl of 0.2% Toluidine blue-O were added to each well and vacuum was applied. The membrane was removed, washed twice with destaining solution (ethanol-water-acetic acid, 50:49:1) for 5 min and air-dried.

Other methods

The protein concentration was determined using BioRad Protein Assay (BioRad).

Abbreviations

PEDF, pigment epithelium-derived factor; GAG, glycosaminoglycan; HS, heparan sulfate; CM, media conditioned by retinoblastoma cells.

Authors' contributions

EMA participated in the design, assay development and carried out the complex-formation, GAG detection and receptor binding assays. JEW carried out the purification and characterization of GAGs, the enzymatic and chemical treatments of cell cultures for receptor binding studies, and drafted the manuscript. SPB conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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