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Biochimica et Biophysica Acta 1760 (2006) 1465–1474
BIOCHIMICA ET BIOPHYSICA ACTA
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The vascular endothelial growth factor VEGF165 induces perlecan synthesis via VEGF receptor-2 in cultured human brain microvascular endothelial cells

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Received 3 March 2006; received in revised form 29 May 2006; accepted 20 June 2006
Available online 7 July 2006

Abstract

A member of the vascular endothelial growth factor (VEGF) family, VEGF165, regulates vascular endothelial cell functions in autocrine and paracrine fashions in microvessels. Proteoglycans are highly glycosylated poly-anionic macromolecules that influence cellular behaviors such as proliferation and migration by interacting with cytokines/growth factors. In the present study, we investigated the regulation of proteoglycan synthesis by VEGF165 in cultured human brain microvascular endothelial cells. The cells were exposed to recombinant human VEGF165, and the proteoglycans were then characterized using biochemical techniques. VEGF165 treatment increased the accumulation of proteoglycans 1.4- and 1.6-fold in the cell layer and conditioned medium, respectively. This effect resulted from the activation of VEGFR-2, and was mimicked by vammin, a VEGFR-2 ligand from snake venom but not placenta growth factor, which binds specifically to VEGFR-1. VEGF165 stimulated the production and secretion of perlecan, substituted with shorter heparan sulfate side chains, but with unaltered sulfated disaccharide composition. The perlecan secreted by VEGF165-stimulated endothelial cells may be involved in the regulation of cellular behavior during angiogenesis, in diseases of the brain microvessels, and in the maintenance of the endothelial cell monolayer.

Keywords: Vascular endothelial growth factor; Extracellular matrix; Perlecan; Proteoglycan; Heparan sulfate; Endothelial cell

1. Introduction

Proteoglycans (PGs) are macromolecules that consist of a core protein and one or more glycosaminoglycan side chains. PGs not only contribute to the formation of the extracellular matrix but also influence cellular proliferation and migration by interacting with growth factors and other ligands. For example, it has been shown that heparan sulfate PGs (HSPGs) bind heparin-binding growth factors such as fibroblast growth factor-2 (FGF-2) via their heparan sulfate chains and promote the binding of FGF-2 to the FGF-2 receptor (FGFR) [1] by the formation of an FGF-2/HSPG/FGFR ternary complex [2]. During the ternary complex formation, HSPG induces FGF-2 oligomerization that augments FGFR dimerization and signal transduction [3]. In addition, FGF-2 is protected from proteolytic degradation when complexed with the heparan sulfate chains, [4] and it retains its capacity for the long-term stimulation of endothelial cell functions such as proliferation, migration, and plasminogen activator activity [5]. The HSPG that regulates FGF-2 activity by the above mechanisms is the large extracellular matrix HSPG perlecan [6], which is one of

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doi:10.1016/j.bbagen.2006.06.010

the major HSPG produced in cultured human brain microvascular endothelial cells [7].

Vascular endothelial growth factors (VEGFs) are highly specific mitogens for vascular endothelial cells [8,9]. There are four alternative splicing isoforms of the single VEGF gene; these are designated as VEGF121, VEGF165, VEGF189, and VEGF206, and they have 121, 165, 189, and 206 amino acids, respectively. VEGF189 and VEGF206 are cell associated and act as vascular permeability factors, whereas VEGF121 and VEGF165 are secreted and induce mitogenesis [10,11]. It has been shown that VEGF165, in particular, plays a critical role in proliferation, migration, and tube formation [12]. VEGF165 mediated signal transduction occurs via receptor tyrosine kinases *fms*-like tyrosine kinase-1 (Flt-1) [13,14] and/or kinase insert domain-containing receptor (KDR) [15,16] that are designated as VEGF receptor 1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), respectively. In addition related growth factors, such as that placenta growth factor (PlGF) or Vammin and VR-1, snake venom-derived VEGFs, have been shown to selectively activate VEGFR-1 or VEGFR-2, respectively [17,18].

It has been reported that VEGF and FGF-2 interact during angiogenesis and neovascularization. Angiogenesis that is induced by either VEGF or FGF-2 is inhibited by VEGFR-2 antagonists in vivo and in vitro [19]; VEGF-neutralizing antibodies markedly reduce FGF-2-induced vascularization in the mouse cornea [20], and further, VEGFR-1-blocking antibodies and dominant-negative VEGFR-1 expression reduce the capillary morphogenesis of endothelial cells [21]. These results suggest that FGF-2 induces angiogenesis indirectly by the activation of the VEGF/VEGFR system. In fact, FGF-2 induces an increase in the expression of VEGF [20], VEGFRs, and FGFRs [22] in endothelial cells. As a result, VEGF and FGF-2 synergistically induce angiogenesis in vivo [23] and in vitro [24]. However, the role of VEGF in the activation of the FGF-2/FGFR system is not completely understood.

Among the predominant forms of VEGF, VEGF121 does not bind to heparan sulfate chains [25], while VEGF165 activity can be potentiated by HSPGs [26]. On the other hand, HSPGs may bind to VEGFRs and modulate the binding ability of VEGFRs to VEGF165 [27,28]. We hypothesized that the regulation of perlecan synthesis by VEGF165 may contribute to the activation of both the VEGF/VEGFR and FGF-2/FGFR systems, resulting in the synergistic effect of VEGF and FGF-2 on angiogenesis. In the present study, we investigated the regulation of PG synthesis by VEGF165 in cultured human brain microvascular endothelial cells.

2. Materials and methods

2.1. Materials

The human brain microvascular endothelial cells and the Western blotting blocking reagent were purchased from Dainihon Pharmaceutical Co. (Osaka, Japan). More than 95% of the cells were von Willebrand factor/Factor VIII-positive and uptake of Dil-acetylated-low density lipoprotein was confirmed by immunofluorescence. HuMedia EG-2, which is a growth medium for human endothelial cells, and bovine serum albumin (BSA) fraction V with very low amounts of endotoxin were purchased from Kurabo (Osaka, Japan) and Serologicals (Kankakee, IL, USA), respectively. Dulbecco's modified Eagle's medium (DMEM) and tissue culture dishes and plates were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) and Iwaki (Chiba, Japan), respectively. Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX, USA). [^{35}S]Na_2SO_4 (carrier-free) and Tran^{35}S-Label Metabolic Labeling Reagent, which consists of ~70% L-[^{35}S]methionine, ~15% L-[^{35}S] cysteine, and other ^{35}S-labeled compounds, were obtained from MP Biomedicals (Irvine, CA, USA). Recombinant human VEGF165 was purchased from Strathman Biotech GmbH (Hannover, Germany). The VEGF-neutralizing antibody was obtained from Immuno-Biological Laboratories (Fujioka, Japan). Human recombinant PlGF, DEAE-Sephacel, benzamidine, Tris base, dextran blue, phenylmethanesulfonyl fluoride, papain (1.7 U/mg solid), unsaturated glucuronic acid-6-*O*-sulfated *N*-acetylgalactosamine (GlcA-GalNAc(6S)), and heparan sulfate disaccharide units were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protease-free chondroitinase ABC (EC 4.2.2.4, derived from *Proteus vulgaris*), chondroitinase ACII (EC 4.2.2.5, derived from *Arthrobactor aurescens*), heparinase II (derived from *Flavobacterium heparinum*), and heparinase III (EC 4.2.2.8, derived from *F. heparinum*) were obtained from Seikagaku (Tokyo, Japan). Enhanced chemiluminescence (ECL) Western blotting detection reagents, horseradish peroxidase-linked protein A, nitrocellulose membranes (Hybond ECL), Hyperfilm ECL Quick Prep Micro mRNA purification kit, Sepharose CL-4B, Sepharose CL-6B, and PD-10 columns (disposable, Sephadex G-25M) were obtained from Amersham Biosciences (Piscataway, NJ, USA). Proteinase K (fungal) was purchased from Invitrogen (Carlsbad, CA, USA); urea, phenol red, and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Rabbit antiserum against the perlecan core protein (EY-9) was kindly provided by Dr. John Hassell (Shriners Hospital for Children, Tampa, FL, USA). Microcon YM3 (3000 MW cut-off) ultrafiltration devices were obtained from Millipore (Billerica, MA, USA); 2-aminoacridone hydrochloride was obtained from Molecular Probes (Eugene, OR, USA). Cetylpyridinium chloride and other reagents were from Nacalai Tesque (Kyoto, Japan).

2.2. Incorporation of [^{35}S]sulfate into glycosaminoglycans (GAGs)

Human brain microvascular endothelial cells were cultured in HuMedia EG-2 in 100-mm dishes in a humid atmosphere of 5% CO_2 in air until confluent. They were transferred into 24-well culture plates at a density of 1×10^4 cells/cm^2 and cultured until confluent. After washing the cells with DMEM supplemented with 10% FBS, the medium was replaced with fresh DMEM supplemented with 10% FBS, and the cells were subsequently incubated at 37 °C for 8, 24, or 48 h with VEGF165 (25, 50, 100, 150, or 200 ng/ml) and/or VEGF-neutralizing antibody in the presence of [^{35}S] sulfate (1 MBq/ml). In another experiment, the cells were treated with PlGF (100 ng/ml) or vammin (5, 10, 25, or 50 ng/ml) for 48 h. After incubation, the conditioned medium was harvested, and solid urea was added up to a concentration of 8 M. The cell layer was washed twice with ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and harvested by scraping with a rubber policeman after extraction with an 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM *N*-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4 °C for 15 min. The medium and cell extracts were used to determine the incorporation of [^{35}S]sulfate into the GAGs by the cetylpyridinium chloride precipitation method [29], as follows. Portions of the extracts were spotted on filter papers and washed five times for 1 h in 1% cetylpyridinium chloride with 0.05 M NaCl. The radioactivity of the PGs precipitated on the dried filter paper was measured by liquid scintillation counting.

2.3. Characterization of PGs

Human brain microvascular endothelial cells cultured in 100-mm dishes were treated with VEGF165 (100 ng/ml) for 48 h in DMEM supplemented with 10% FBS in the presence of [^{35}S]sulfate (3 MBq/ml). After treatment, the cell extracts were prepared as described above and chromatographed on PD-10 columns equilibrated in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 1 M NaCl, 0.5% Triton X-100, and 50 mM Tris base to obtain high molecular mass

(>3 kDa) macromolecules. For the separation of the PGs based on their charge density differences, the macromolecules were chromatographed on a DEAE-Sephacel (5 ml of resin) column in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.1 M NaCl, 0.5% Triton X-100, and 50 mM Tris base. Any unbound radioactivity was removed from the column by washing with 30 ml of the buffer. The bound radioactivity was eluted from the column with a linear gradient of 0.25–0.7 M NaCl in urea buffer (total volume of 50 ml). The peaks in the conditioned medium containing PGs that had increased by VEGF165 treatment were pooled and concentrated by the application of the diluted samples to 0.3-ml DEAE-Sephacel minicolumns and eluting the bound radioactivity with sequential washes of 8 M urea buffer containing 3 M NaCl. The concentrated PGs were separated based on their hydrodynamic sizes by using a Sepharose CL-4B column (0.9×80 cm) in 8 M urea buffer containing 0.25 M NaCl. The void volume and the total volume were estimated by the elution positions of dextran blue and phenol red, respectively. The peaks from the conditioned medium that were eluted at a K_{av} of 0.19 were pooled because they contained PGs that had increased by VEGF165 treatment (see "Results"). These peaks, which had sufficient radioactivity for analysis, were precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol and 80 μg/ml carrier chondroitin sulfate for 2 h at −20 °C; this was repeated three times. The precipitated PGs were digested either with 1.7 U/ml chondroitinase ABC in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 mg/ml BSA and 3 mM sodium acetate at 37 °C for 4 h, with both heparinase II and heparinase III (0.03 U/ml each) in 0.2 M Tris–HCl buffer (pH 7.0) containing 10 mM calcium acetate, or with 10 μg of papain in 0.1 M acetate buffer (pH 7.0) containing 5 mM EDTA and 5 mM cysteine at 65 °C for 4 h. The digested samples were chromatographed on a Sepharose CL-6B column (0.9×80 cm) in Tris–HCl buffer (pH 7.0) with 0.2 M NaCl. The void volume and total volume were estimated by the elution positions of dextran blue and phenol red, respectively. The GAG chain sizes were estimated by comparing the experimentally determined Sepharose CL-6B elution K_{av} with a previously published curve of log M_r versus K_{av} in Sepharose CL-6B for chondroitin sulfate chains of various known M_r values [30].

2.4. Analysis of PG core proteins

Human brain microvascular endothelial cells were incubated at 37 °C for 48 h in 6 ml of fresh DMEM supplemented with 10% FBS in the presence of VEGF165 (100 ng/ml) and Tran^{35}S-Label Reagent (3 MBq/ml) in 100-mm dishes. After incubation, the radiolabeled PGs, which accumulated in the cell layer and conditioned medium, were extracted under dissociative conditions in the presence of 8 M urea. The extract was concentrated on 0.3-ml DEAE-Sephacel minicolumns and precipitated with 1.3% potassium acetate in 95% ethanol. The precipitated PGs were digested with a mixture of heparinase II and heparinase III, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 4%–12% acrylamide gradient slab gel with a 3% stacking gel according to the procedure of Laemmli [31]. The radiolabeled PG core proteins were visualized by autoradiography of the dried gel exposed to a Kodak XAR-2 film at −70 °C. For Western blot analysis, the SDS-polyacrylamide gel was equilibrated in 25 mM Tris transfer buffer (pH 9.5) with 20% methanol and transferred to a nitrocellulose membrane for 90 min by using a semidry transfer apparatus (Atoo, AE-6677). The membrane was blocked and exposed overnight to a primary antibody against perlecan (EY-9; dilution, 1:1,000) at 4 °C. After incubation of the blot with horseradish peroxidase-linked protein A, the bands to which the primary antibody had bound were visualized on Hyperfilm ECL by using an enzyme-linked chemiluminescence procedure. In another experiment, poly(A)$^+$ RNA was isolated from the cells before and after the VEGF165 (100 ng/ml) treatment for 4, 8, or 24 h; mRNA for the perlecan core protein was analyzed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), as described previously [32]. The PCR was conducted under semiquantitative conditions, which were determined by plotting signal intensities as a function of the template amount and cycle number. Oligonucleotide primers were designed on the basis of human cDNA. The sequences of the upstream and downstream primers for perlecan core cDNA were 5′-ATGAGTACATGCTGGCTGAC-3′ and 5′-CTGATACCCAG-GACTGGCTC-3′, respectively. The size of the PCR products was 559 bp. After completion of the RT-PCR, an aliquot of the reaction mixture was electrophoresed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide.

2.5. Analysis of disaccharide composition of heparan sulfate chains [33]

The PGs that had accumulated in the cell layer and conditioned medium of the cultured human brain microvascular endothelial cells exposed to VEGF165 (100 ng/ml) for 24 h were extracted under dissociative conditions in the presence of 8 M urea. The extract was concentrated on 0.3-ml DEAE-Sephacel minicolumns and precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol; the precipitation was repeated four times. The dried precipitate was digested overnight with proteinase K (800 μg/ml) in 0.1 M sodium acetate buffer (pH 7.2) at 60 °C. After inactivation of the proteinase at 100 °C for 10 min, the buffer salt and protein digests were removed by centrifuging the digests in Microcon YM3. The GAGs on the filter were recovered in 0.1 M ammonium acetate (pH 7.3) and digested at 37 °C for 18 h with a mixture of chondroitinase ABC (0.2 U/ml) and chondroitinase ACII (1 U/ml). The undigested materials (heparan sulfate chains) on the Microcon YM3 filter were recovered in 0.1 M ammonium acetate (pH 7.0) containing 0.01% BSA and digested at 37 °C for 8 h with a mixture of heparinase II and heparinase III (0.03 U/ml each). The heparan sulfate hydrolysis products were recovered using Microcon YM3 ultrafiltration devices, and they were then dried. The dried heparan sulfate samples were fluorotagged with 2-aminoacridone hydrochloride (0.1 M). The fluorotagged heparan sulfate hydrolase products were separated on separating gels (19.5% acrylamide, 0.52% *N,N′*-methylenbisacrylamide, 2.5% glycerol, 0.1 M Tris base, 90 mM boric acid, 0.05% ammonium persulfate, 0.6% agarose, and 0.1% TEMED) with a stacking gel (7.5% acrylamide, 0.2% *N,N′*-methylenbisacrylamide, 2.5% glycerol, 0.1 M Tris base, 90 mM boric acid, 0.05% ammonium persulfate, 0.6% agarose, and 0.1% TEMED). The electrophoresis was carried out in the running buffer (0.1 M Tris–borate, pH 8.3) at 4 °C. The fluorescent images were displayed on a gel documentation system (Atto, AE-6914), and the bands of heparan sulfate unsaturated disaccharides were quantitatively analyzed by NIH Image Analyses Software using the bands of unsaturated GlcA-GalNAc (6S) as standards.

2.6. Statistical analysis

Data were analyzed for statistical significance by analysis of variance and Bonferroni's multiple *t*-test, when possible. *P* values of less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. VEGF165 promotes the synthesis of PGs via VEGFR-2

Fig. 1 shows the accumulation of [^{35}S]sulfate-labeled PGs in the cell layer and conditioned medium of human brain microvascular endothelial cells after exposure to VEGF165. The growth factor, at 25 ng/ml and higher, significantly and dose-dependently increased the accumulation after a 24-h incubation period (Fig. 1A). The increase occurred after 8 and 24 h in the cell layer and conditioned medium, respectively, and longer when the cells were exposed to VEGF165 at 100 ng/ml (Fig. 1B); after 48-h incubation, the concentration of PGs increased 1.4- and 1.6-fold in the cell layer and conditioned medium, respectively. The cell number did not increase significantly by VEGF165 treatment (control, $4.22\pm0.08\times10^5$ cells/cm^2; 100 ng/ml VEGF165 for 48 h, $4.81\pm0.12\times10^5$ cells/cm^2, $n=4$ each), suggesting that VEGF165 only promotes the synthesis of PGs on a per cell basis in this cell culture system. Thus, the concentration of PGs would increase in both the cell layer and conditioned medium. Although VEGF165 is a mitogen of endothelial cells, it might not stimulate the cell growth after reaching confluence.

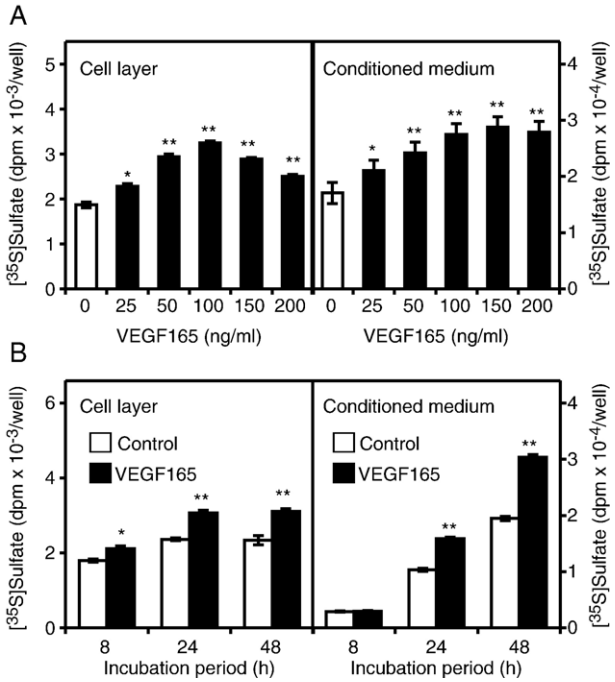

Fig. 1. (A) [^{35}S]Sulfate incorporation into the GAGs accumulated in the cell layer and conditioned medium of human brain microvascular endothelial cells after exposure to VEGF165. Confluent cultures of the cells were incubated with VEGF165 (25, 50, 100, 150, or 200 ng/ml) at 37 °C for 24 h in the presence of [^{35}S]sulfate. (B) Time course of the [^{35}S]sulfate incorporation into the GAGs accumulated in the cell layer and conditioned medium of human brain microvascular endothelial cells after exposure to VEGF165. Confluent cultures of the cells were incubated at 37 °C for 8, 24, or 48 h with VEGF165 (100 ng/ml) in the presence of [^{35}S]sulfate. Values are means±S.E. of four samples. Significantly different from the corresponding control, *$P<0.05$; **$P<0.01$.

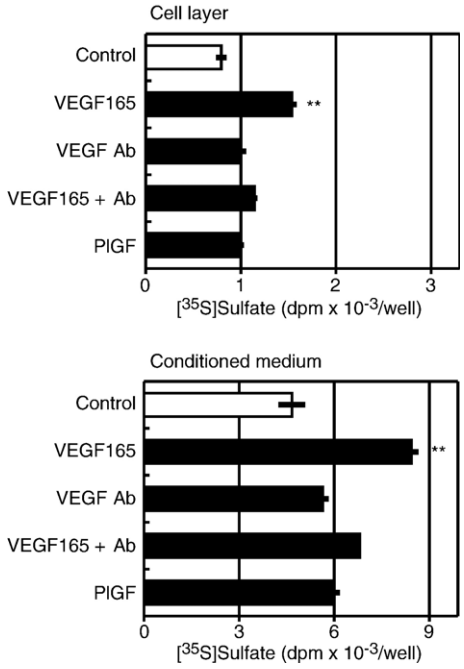

Fig. 2. [^{35}S]Sulfate incorporation into the GAGs accumulated in the cell layer and conditioned medium of human brain microvascular endothelial cells after treatment with VEGF165 and/or VEGF-neutralizing antibody or with PlGF. Confluent cultures of the cells were incubated at 37 °C for 24 h with VEGF165 (100 ng/ml) and/or VEGF-neutralizing antibody or with PlGF (100 ng/ml) in the presence of [^{35}S]sulfate. Values are means±S.E. of four samples. **Significantly different from the corresponding control, $P<0.01$.

In order to confirm that the increase in endothelial PG synthesis was directly dependent on VEGF165, the cells were treated with the growth factor in the presence or absence of the VEGF-neutralizing antibody (Fig. 2). The VEGF165-induced increase in the accumulation of [^{35}S]sulfate-labeled PGs in the cell layer and conditioned medium of human brain microvascular endothelial cells was abrogated in the presence of the antibody, indicating that the promotion of endothelial PG synthesis requires VEGF165.

Among the VEGF-related factors that were tested, PlGF, which selectively activates VEGFR-1, failed to increase [^{35}S]sulfate-labeled PG accumulation (Fig. 2), whereas vammin, which selectively activates VEGFR-2, significantly increased the accumulation in a dose-dependent manner (Fig. 3). This suggests that the promotion of endothelial PG synthesis by VEGF165 is mediated by VEGFR-2.

3.2. PGs increased by VEGF165 treatment are large HSPG molecules

Equal aliquots of [^{35}S]sulfate-labeled PGs that were extracted from the cell layer and conditioned medium of human brain microvascular endothelial cells before and after exposure to VEGF165 were separated by DEAE-Sephacel ion-exchange chromatography based on the differences in their charge densities (Fig. 4). The PGs accumulated in the conditioned medium were eluted with 0.45 M (peak I), 0.5 M (peak II), and 0.55 M (peak III) NaCl; the radioactivity of peak I was selectively increased by VEGF165 treatment. The cell layer lacked peak II but contained peak I and peak III; in this case as well, the radioactivity of peak I was increased by VEGF165 treatment. This indicates that VEGF165 promotes the synthesis of the PGs bound to peak I in human brain microvascular endothelial cell cultures.

Since VEGF165 markedly increased the radioactivity of the peak I PGs accumulated in the conditioned medium, the PGs

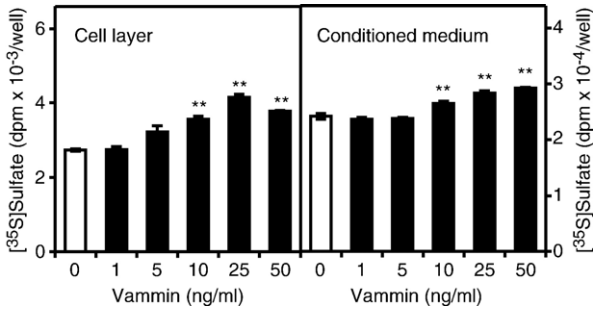

Fig. 3. [^{35}S]Sulfate incorporation into the GAGs accumulated in the cell layer and conditioned medium of human brain microvascular endothelial cells after treatment with vammin. Confluent cultures of the cells were incubated at 37 °C for 24 h with vammin (1, 5, 10, 25, or 50 ng/ml) in the presence of [^{35}S]sulfate. Values are means±S.E. of four samples. **Significantly different from the corresponding control, $P<0.01$.

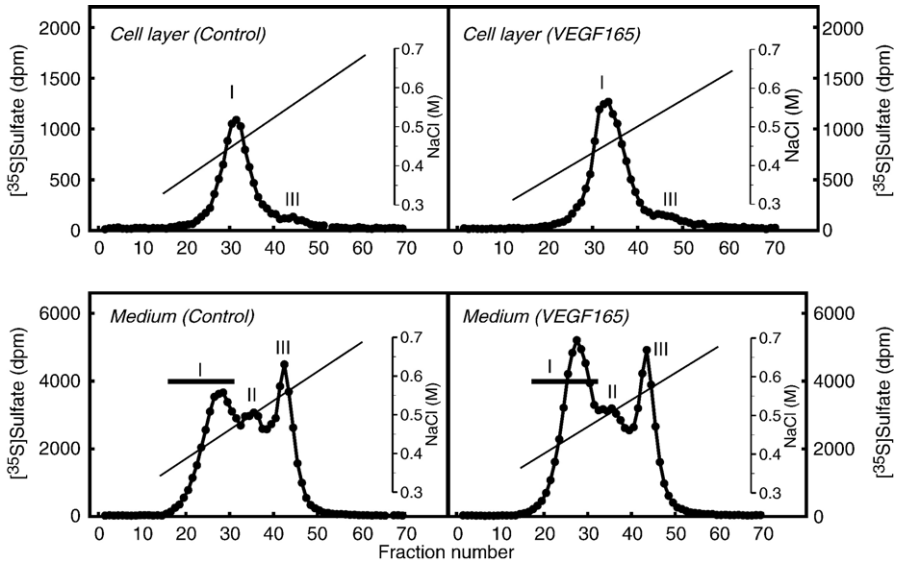

Fig. 4. DEAE-Sephacel ion-exchange chromatography of [^{35}S]sulfate-labeled PGs extracted from the cell layer and conditioned medium of human brain microvascular endothelial cells after exposure to VEGF165 by elution with a linear gradient of 0.25–0.7 M NaCl in 8 M urea buffer. Confluent cultures of the cells were incubated at 37 °C for 24 h with VEGF165 (100 ng/ml) in the presence of [^{35}S]sulfate. The horizontal bars indicate the fractions that were pooled as peak I and chromatographed on a Sepharose CL-4B column (see Fig. 5).

were further purified by Sepharose CL-4B chromatography based on their hydrodynamic sizes (Fig. 5). The peak I PGs were eluted at K_{av} values of approximately 0.19 (peak Ia) and 0.62 (peak Ib). VEGF165 specifically increased the radioactivity of peak Ia without altering the hydrodynamic sizes of the corresponding PGs.

The GAG composition of peak Ia PGs accumulated in the conditioned medium was analyzed by Sepharose CL-6B chromatography, before and after digestion with chondroitinase ABC that digests both chondroitin and dermatan sulfates, heparinase II/III, or papain (Fig. 6). The PGs bound to peak Ia were eluted at the void volume before enzyme digestion. They were resistant to digestion with chondroitinase ABC but sensitive to heparinase II/III digestion, indicating that they contained heparan sulfate chains. The lengths of the heparan sulfate chains were M_r ~68,000 and M_r ~58,000 before and after exposure to VEGF165, respectively. In three different experiments, we consistently observed this moderate shortening (15%–20%) of the heparan sulfate chains bound to peak Ia PGs.

3.3. VEGF165 induces the synthesis of perlecan core protein

In order to identify the core protein whose synthesis is induced by VEGF, human brain microvascular endothelial cells were metabolically labeled with ^{35}S-labeled amino acids, and the HSPG core proteins that were accumulated in the cell layer and conditioned medium were separated by SDS-PAGE (Fig. 7).

It was observed that a large HSPG core protein with a molecular mass of approximately 400 kDa had accumulated in the cell layer and conditioned medium, and the amount of HSPG core protein was increased by VEGF165 treatment. The core protein was perlecan antibody-positive, and the amount of immunostained perlecan core protein increased by VEGF165 treatment. During the treatment, the growth factor elevated the level of perlecan mRNA in a time-dependent manner (Fig. 8). These results indicate that VEGF165 induces the synthesis of perlecan in human brain microvascular endothelial cells.

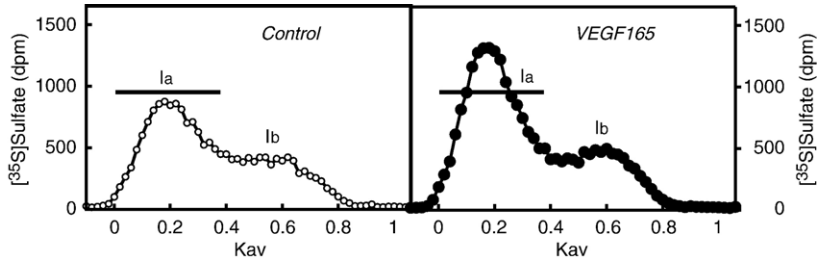

Fig. 5. Sepharose CL-4B molecular sieve chromatography of peak I PGs that were extracted from the conditioned medium of human brain microvascular endothelial cells after exposure to VEGF165 and separated by DEAE-Sephacel chromatography. The PGs were chromatographed on a Sepharose CL-4B column in 8 M urea buffer. The horizontal bars indicate the fractions that were pooled as peak Ia and chromatographed on a Sepharose CL-6B column before and after digestion with chondroitinase ABC, heparinase II/III, or papain (see Fig. 6).

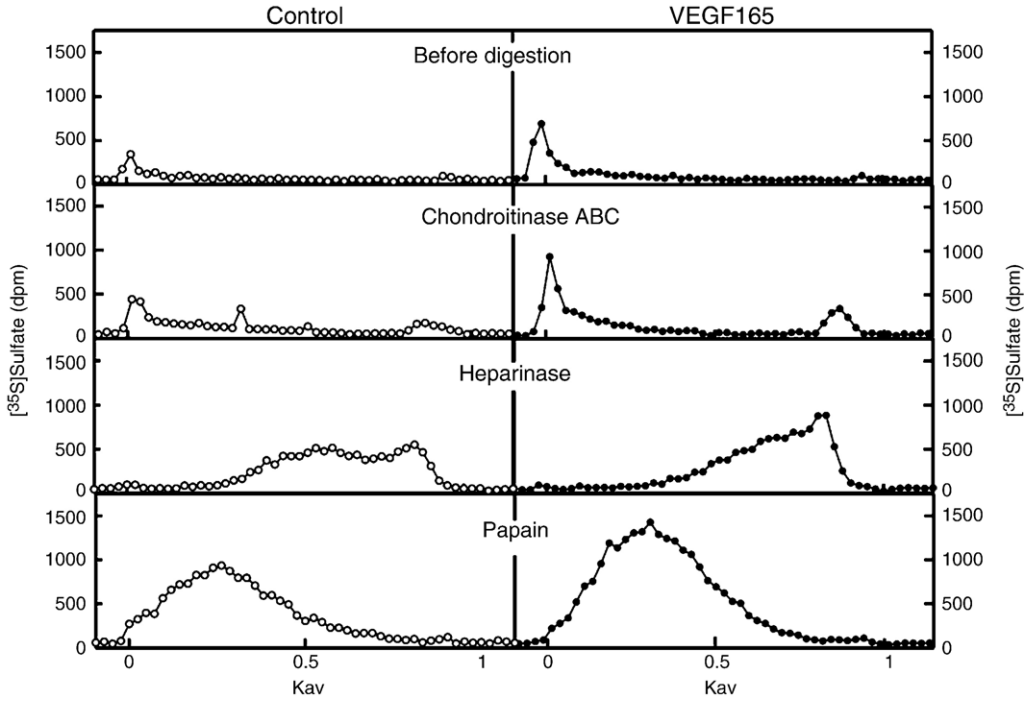

Fig. 6. Characterization of GAG chains bound to the peak Ia PGs that were isolated from the conditioned medium by DEAE-Sephacel ion-exchange chromatography and Sepharose CL-4B molecular sieve chromatography. The PGs were chromatographed on a Sepharose CL-6B column before and after digestion with chondroitinase ABC, heparinase II/III, or papain.

3.4. VEGF165 does not affect the disaccharide composition of heparan sulfate chains

The disaccharide composition of the heparan sulfate chains obtained from the PGs in the cell layer and conditioned medium of human brain microvascular endothelial cells before and after exposure to VEGF165 was analyzed by fluorophore-assisted carbohydrate electrophoresis (Fig. 9) because the growth factor may have changed the microstructure of the GAG chains during PG synthesis. The disaccharide units were detected as unsaturated GlcA/iduronic acid-*N*-acetylglucosamine (GlcA/IdoA-GlcNAc), GlcA/IdoA-*N*-sulfated glucosamine (GlcA/IdoA-GlcNS), 2-*O*-sulfated GlcA/IdoA-GlcNS (GlcA/IdoA(2S)-GlcNS), GlcA/IdoA-6-*O*-sulfated GlcNS (GlcA/IdoA-GlcNS(6S)), and GlcA/IdoA(2S)-GlcNS(6S). The percentages of these disaccharide units were not significantly changed by VEGF165 treatment, suggesting that the growth factor does not alter the microstructure of the

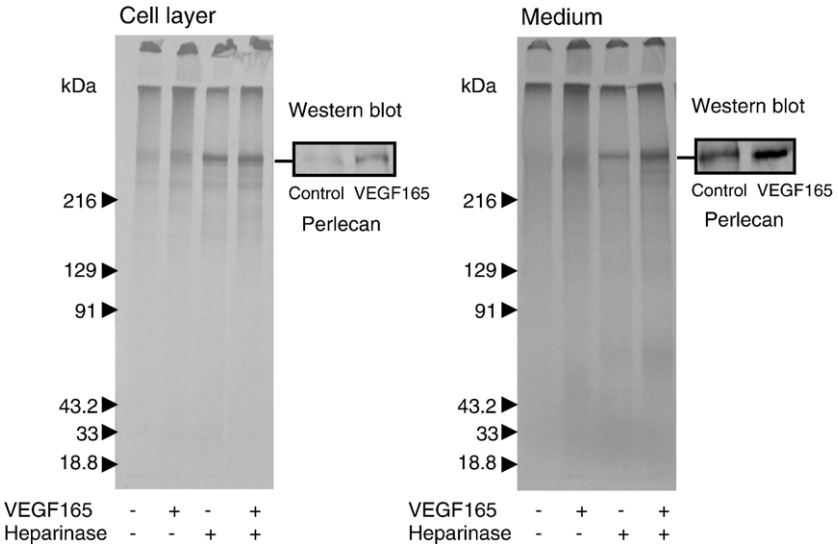

Fig. 7. SDS-PAGE of the HSPG core proteins from the cell layer and conditioned medium that were metabolically labeled with ^{35}S-labeled amino acids and identification of the HSPG core with a molecular mass of approximately 400 kDa by Western blot analysis.

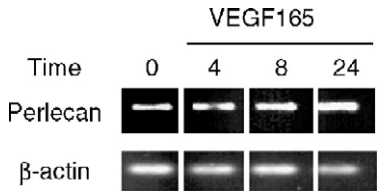

Fig. 8. Semiquantitative RT-PCR of the perlecan core protein mRNA in human brain microvascular endothelial cells after exposure to VEGF165 (100 ng/ml) for 4, 8, or 24 h.

perlecan heparan sulfate chains in human brain microvascular endothelial cells.

4. Discussion

It has been shown that in the microvessels, both endothelial cells [34] and pericytes [35] constitutively express VEGF165. Aortic smooth muscle cells also express VEGF [36], whereas aortic endothelial cells are deficient in the growth factor [34]. Under hypoxic conditions, the expression of VEGF is upregulated in both endothelial cells and pericytes [35]; as a result, endothelial cells in the microvessels are highly exposed to VEGF and angiogenesis is induced. This suggests that VEGF165 is substantially more important for microvascular endothelial cells than macrovascular endothelial cells. Since PGs are involved in the control of cellular behavior by their interaction with cytokines/growth factors, the regulation of PG synthesis in cultured human brain microvascular endothelial cells was investigated in the present study. It was demonstrated that in these cells, VEGF165 induces the synthesis of perlecan via VEGFR-2. Although the length of the heparan sulfate chains in the perlecan was slightly shorter, the growth factor did not alter their disaccharide composition. It is suggested that in microvessels, the VEGF165 that is derived from both endothelial cells and pericytes stimulates the synthesis of endothelial perlecan molecules in both autocrine and paracrine fashions not only in response to hypoxia but also constitutively. We did not determine the intracellular signaling pathway that mediates perlecan synthesis via VEGF165. In addition, it is unclear whether other types of VEGFs can regulate PG synthesis. However, it is likely that ligands for VEGFR-2, including VEGF-C [37] and VEGF-D [38], generally induce perlecan synthesis as VEGF165 does.

It has been reported that cytokines/growth factors such as transforming growth factor-β (TGF-β) [39], connective tissue growth factor (CTGF) [40], FGF-2 [41], and tumor necrosis factor-α (TNF-α) [42] regulate PG synthesis in vascular endothelial cells. However, the effect of VEGF165 on endothelial PG synthesis is different from that of these cytokines/growth factors. In particular, TGF-β promotes the synthesis of both perlecan and biglycan in dense endothelial cells, CTGF inhibits the synthesis of biglycan and newly induces that of decorin in sparse endothelial cells, FGF-2 induces endothelial biglycan synthesis during migration, and TNF-α inhibits the synthesis of both perlecan and biglycan in dense endothelial cells. Thus, it is suggested that VEGF165 is a unique growth factor that selectively induces endothelial perlecan synthesis. At the same time, it is postulated that cytokines/growth factors such as TGF-β, CTGF, FGF-2, and TNF-α cannot mediate VEGF165-induced perlecan synthesis,

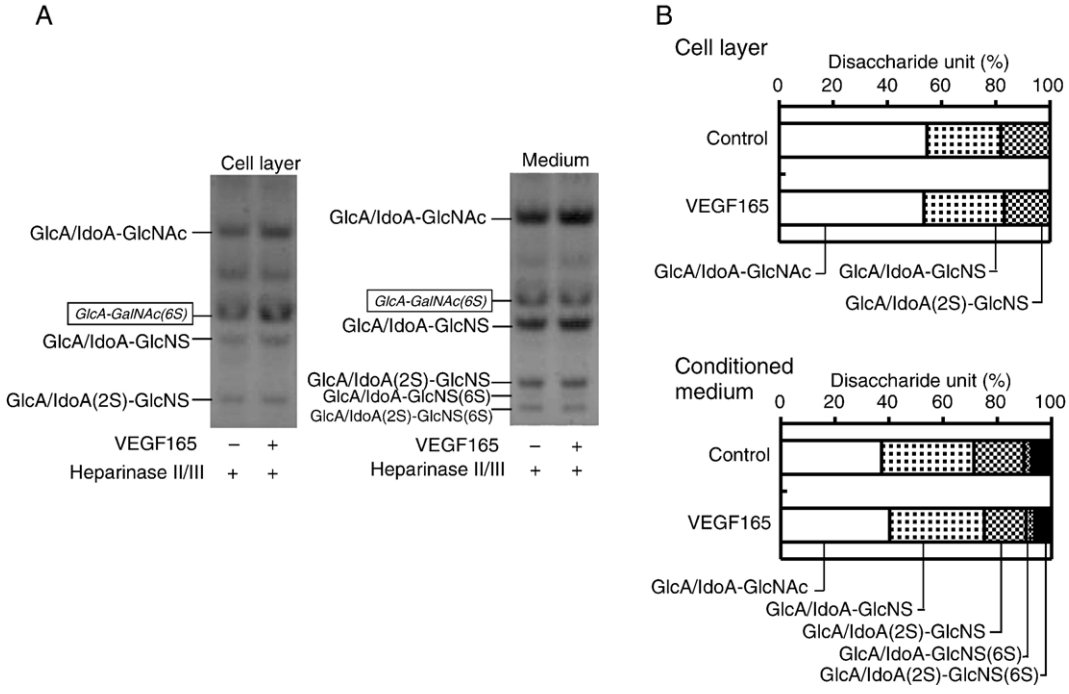

Fig. 9. Fluorophore-assisted carbohydrate electrophoresis analysis of the heparan sulfate chains in the PGs extracted from the cell layer and conditioned medium of human brain microvascular endothelial cells after exposure to VEGF165. Confluent cultures of the cells were incubated at 37 °C for 24 h with VEGF165 (100 ng/ml). (A) A representative gel image. (B) The percentage of the disaccharide units in heparan sulfate chains. Values are calculated based on the gel image.

although VEGF may induce the expression of these cytokines/growth factors [43,44] in human brain microvascular endothelial cells.

It has been shown that in general, vascular endothelial cells exhibit anticoagulant and fibrinolytic activities. In fact, human brain microvascular endothelial cells express fibrinolytic proteins, and their fibrinolytic activity is elevated in response to thrombin [45]. In addition, the heparan sulfate chains of the perlecan synthesized by endothelial cells have a heparin-like anticoagulant activity [46] and contribute to the antithrombogenic property of vascular endothelial cells [47]. However, the amount of thrombomodulin, which is an endothelial anticoagulant protein, is considerably low in the brain [48]. It is likely that the upregulation of endothelial perlecan synthesis by VEGF165, which is constitutively secreted by both endothelial cells and pericytes, partly accounts for the anticoagulant activity of the luminal surface of brain microvessels. On the other hand, in the event that the microvascular endothelial monolayer is damaged under hypoxic conditions in the microvessels, cellular events may occur as follows: (1) FGF-2 is leaked from damaged endothelial cells, and it stimulates the endothelial cells near the damaged site to reendothelialize [49]; (2) simultaneously, the expression of VEGF165 is elevated by hypoxia in both endothelial cells and pericytes [35]; (3) VEGF165 not only stimulates endothelial cell proliferation but also induces endothelial perlecan synthesis, and the newly synthesized perlecan promotes the binding of FGF-2 to FGFR in endothelial cells; (4) the FGF-2 that is activated by perlecan upregulates the expression of VEGF [20], VEGFRs, and FGFRs [22], thereby inducing a higher response of the endothelial cells to the VEGF/VEGFR and FGF-2/FGFR systems. Taking these events together, it is hypothesized that the induction of perlecan synthesis by VEGF165 may serve as a positive feedback loop to amplify the activity of both the VEGF/VEGFR and FGF-2/FGFR systems.

It has been reported that VEGF and FGF-2 synergistically induce angiogenesis [23,24]. These two growth factors may exhibit a synergistic effect at several stages of angiogenesis, including the invasive migration of endothelial cells [50]. VEGF independently induces the synthesis of fibrinolytic proteins such as urokinase-type plasminogen activator, tissue-type plasminogen activator, and plasminogen activator inhibitor type 1 in microvascular endothelial cells [51]; further, FGF-2 and VEGF synergistically induce the synthesis of tissue-type plasminogen activator [52]. These results suggest that the synergistic interaction between VEGF and FGF-2 can occur not only during angiogenesis but also during other cellular events. Although the mechanism of the synergism between VEGF and FGF-2 can be explained by the upregulation of VEGF [20], FGFRs, and VEGFRs by FGF-2 [22] in vascular endothelial cells, we propose an additional mechanism of the positive feedback loop composed of the VEGF/VEGFR and FGF-2/FGFR systems connected by endothelial perlecan synthesis. It has been reported that HSPGs function as extracellular storage places [53] and co-receptors [54] for the heparin-binding VEGF isoforms as well as for FGF-2; these findings support this hypothesis.

In summary, VEGF165 was found to induce perlecan synthesis via VEGFR-2 in human brain microvascular endothelial cells without inducing marked changes in the length and disaccharide composition of the heparan sulfate chains. This regulation by VEGF165 is unique and different from that induced by other growth factors/cytokines including TGF-β, CTGF, FGF-2, and TNF-α. Microvascular endothelial cells and pericytes constitutively express VEGF, indicating that the growth factor constantly upregulates endothelial perlecan synthesis to maintain the anticoagulant property of the luminal surface of the brain microvessels. On the other hand, when endothelial cells are damaged under hypoxic conditions, the newly synthesized perlecan may activate the FGF-2/FGFR system that can in turn activate the VEGF/VEGFR system, resulting in a positive feedback loop to amplify the activity of both systems. This loop may be a component of the mechanism by which VEGF and FGF-2 exhibit synergistic effects on microvascular endothelial cells.

Acknowledgments

This work was supported by the Academic Frontier Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 2005–2009; the Specific Research Fund of Hokuriku University (to T.K.); a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (to T.K.); a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to C.Y.); and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.F.).

References

[1] A. Yayon, M. Klagsbrun, J.D. Esko, P. Leder, D.M. Ornitz, Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor, Cell 64 (1991) 841–848.
[2] M. Rusnati, M. Presta, Interaction of angiogenic basic fibroblast growth factor with endothelial cell heparan sulfate proteoglycans. Biological implications in neovascularization, Int. J. Clin. Lab. Res. 26 (1996) 15–23.
[3] T. Spivak-Kroizman, M.A. Lemmon, I. Dikic, J.E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumeley, J. Schlessinger, I. Lax, Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation, Cell 79 (1994) 1015–1024.
[4] O. Saksela, D. Moscatelli, A. Sommer, D.B. Rifkin, Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation, J. Cell Biol. 107 (1988) 734–751.
[5] R. Flaumenhaft, D. Moscatelli, O. Saksela, D.B. Rifkin, Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth factor for long-term stimulation of plasminogen activator production and DNA synthesis, J. Cell. Physiol. 140 (1989) 75–81.
[6] D. Aviezer, D. Hecht, M. Safran, M. Eisinger, G. David, A. Yayon, Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis and angiogenesis, Cell 79 (1994) 1005–1013.
[7] C. Yamamoto, X. Deng, Y. Fujiwara, T. Kaji, Proteoglycans predominantly synthesized by human brain microvascular endothelial cells in culture are perlecan and biglycan, J. Health Sci. 51 (2005) 576–583.
[8] N. Ferrara, W.J. Henzel, Pituitary follicular cells secrete a novel heparin-

binding growth factor specific for vascular endothelial cells, Biochem. Biophys. Res. Commun. 161 (1989) 851–858.
[9] D. Gospodarowicz, J.A. Abraham, J. Schilling, Isolation and characterization of a vascular endothelial mitogen produced by pituitary-derived folliculo stellate cells, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 7311–7315.
[10] K.A. Houck, N. Ferrara, J. Winer, G. Cachianes, B. Li, D.W. Leung, The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA, Mol. Endocrinol. 5 (1991) 1806–1814.
[11] N. Ferrara, K.A. Houck, L.B. Jakeman, J. Winer, D.W. Leung, The vascular endothelial growth factor family of polypeptides, J. Cell. Biochem. 47 (1991) 211–218.
[12] P. Carmeliet, Mechanisms of angiogenesis and arteriogenesis, Nat. Med. 6 (2000) 389–395.
[13] M. Shibuya, S. Yamaguchi, A. Yamane, T. Ikeda, A. Tojo, H. Matsuchime, M. Sato, Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family, Oncogene 5 (1990) 519–524.
[14] C. de Vries, J.A. Escobedo, H. Ueno, K. Houck, N. Ferrara, L.T. Williams, The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor, Science 255 (1992) 989–991.
[15] B.I. Terman, M. Dougher-Vermazen, M.E. Carrion, D. Dimitrov, D.C. Amellino, D. Gospodarowicz, P. Bolen, Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor, Biochem. Biophys. Res. Commun. 187 (1992) 1579–1586.
[16] T.P. Quinn, K.G. Peters, C. de Vries, N. Ferrara, L.T. Williams, Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7533–7537.
[17] J.E. Park, H. Chen, J. Winer, K. Houck, N. Ferrara, Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR, J. Biol. Chem. 269 (1994) 25646–25654.
[18] Y. Yamazaki, K. Takani, H. Atoda, T. Morita, Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2), J. Biol. Chem. 278 (2003) 51985–51988.
[19] J.C. Tille, J. Wood, S.J. Mandriota, C. Schnell, S. Ferrari, J. Mestan, Z. Zhu, L. Witte, M.S. Pepper, Vascular endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth factor-induced angiogenesis in vivo and in vitro, J. Pharmacol. Exp. Ther. 299 (2001) 1073–1085.
[20] G. Seghezzi, S. Patel, C.J. Ren, A. Gualandris, G. Pintucci, E.S. Robbins, R.L. Shapiro, A.C. Galloway, D.B. Rifkin, P. Mignatti, Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis, J. Cell Biol. 141 (1998) 1659–1673.
[21] S. Kanda, Y. Miyata, H. Kanetake, Fibroblast growth factor-2-mediated capillary morphogenesis of endothelial cells requires signals via Flt-1/vascular endothelial growth factor receptor-1: possible involvement of c-Akt, J. Biol. Chem. 279 (2004) 4007–4016.
[22] C. Gabler, A. Plath-Gabler, G.J. Killian, B. Berisha, D. Schams, Expression pattern of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) system members in bovine corpus luteum endothelial cells during treatment with FGF-2, VEGF or estradiol, Reprod. Domest. Anim. 39 (2004) 321–327.
[23] T. Asahara, C. Bauters, L.P. Zheng, S. Takeshita, S. Bunting, N. Ferrara, J.F. Symes, J.M. Isner, Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo, Circulation 92 (1995) 365–371.
[24] M.S. Pepper, N. Ferrara, L. Orci, R. Montesano, Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro, Biochem. Biophys. Res. Commun. 189 (1992) 824–831.
[25] . J.E. Park, G.A. Keller, N. Ferrara, Vascular endothelial growth factor (VEGF) isoforms- differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF, Mol. Biol. Cell 4 (1993) 1317–1326.
[26] H. Gitay-Goren, S. Stoker, I. Vlodavsky, G. Neufeld, The binding of vascular endothelial growth factor to its receptor is dependent on cell-associated heparin-like molecules, J. Biol. Chem. 267 (1992) 6093–6098.
[27] M.K. Chiang, J.G. Flanagan, Interactions between the Flk-1 receptor, vascular endothelial growth factor, and cell surface proteoglycan identified with a soluble receptor reagent, Growth Factors 12 (1995) 1–10.
[28] A.M. Dougher, H. Wasserstrom, L. Torley, L. Shridaran, P. Westdock, R.E. Hileman, J.R. Fromm, R. Anderberg, S. Lyman, R.J. Linhardt, J. Kaplan, B.I. Terman, Identification of a heparin binding peptide on the extracellular domain of the KDR VEGF receptor, Growth Factors 14 (1997) 257–268.
[29] Å. Wasteson, K. Uthne, B. Westermark, A novel assay for the biosynthesis of sulphated polysaccharide and its application to studies on the effects of somatomedin on cultured cells, Biochem. J. 136 (1973) 1069–1074.
[30] Å. Wasteson, A method for the determination of the molecular weight and molecular-weight distribution of chondroitin sulfate, J. Chromatogr. 59 (1971) 87–97.
[31] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[32] C. Yamamoto, T. Wakata, Y. Fujiwara, T. Kaji, Induction of synthesis of a large heparan sulfate proteoglycan, perlecan, by thrombin in cultured human coronary smooth muscle cells, Biochim. Biophys. Acta 1722 (2005) 92–102.
[33] A.H.K. Plaas, L.A. West, R.J. Midura, Keratan sulfate disaccharide composition determined by FACE analysis of keratanase II and endo-β-galactosidase digestion products, Glycobiology 11 (2001) 779–790.
[34] A. Ladoux, C. Frelin, Expression of vascular endothelial growth factor by cultured endothelial cells from brain microvessels, Biochem. Biophys. Res. Commun. 194 (1993) 799–803.
[35] M. Nomura, S. Yamagishi, S. Harada, Y. Hayashi, T. Yamashima, J. Yamashita, H. Yamamoto, Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes, J. Biol. Chem. 270 (1995) 28316–28324.
[36] N. Ferrara, J. Winer, T. Burton, Aortic smooth muscle cells express and secrete vascular endothelial growth factor, Growth Factors 5 (1991) 141–148.
[37] V. Joukov, K. Pajusola, A. Kaipainen, D. Chilov, I. Lahtinen, E. Kukk, O. Saksela, N. Kalkkinen, K. Alitalo, A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt-4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases, EMBO J. 15 (1996) 290–298.
[38] M.G. Achen, M. Jeltsch, E. Kukk, T. Makinen, A. Vitali, A.F. Wilks, K. Alitalo, S.A. Stacker, Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk-1) and VEGF receptor 3 (Flt-4), Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 548–553.
[39] T. Kaji, A. Yamada, S. Miyajima, C. Yamamoto, Y. Fujiwara, T.N. Wight, M.G. Kinsella, Cell density-dependent regulation of proteoglycan synthesis by transforming growth factor-β_1 in cultured bovine aortic endothelial cells, J. Biol. Chem. 275 (2000) 1463–1470.
[40] T. Kaji, C. Yamamoto, M. Oh-i, T. Nishida, M. Takigawa, Differential regulation of biglycan and decorin synthesis by connective tissue growth factor in cultured vascular endothelial cells, Biochem. Biophys. Res. Commun. 322 (2004) 22–28.
[41] M.G. Kinsella, C.K. Tsoi, H.T. Järveläinen, T.N. Wight, Selective expression and processing of biglycan during migration of bovine aortic endothelial cells. The role of endogenous basic fibroblast growth factor, J. Biol. Chem. 272 (1997) 318–325.
[42] S. Ramasamy, D.W. Lipke, C.J. McClain, B. Hennig, Tumor necrosis factor reduces proteoglycan synthesis in cultured endothelial cells, J. Cell. Physiol. 162 (1995) 119–126.
[43] K. Suzuma, K. Naruse, I. Suzuma, N. Takahara, K. Ueki, L.P. Aiello, G.L. King, Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-Akt-dependent pathways in retinal vascular cells, J. Biol. Chem. 275 (2000) 40725–40731.
[44] Z.D. Li, J.P. Bork, B. Krueger, E. Patsenker, A. Schulze-Krebs, E.G. Hahn, D. Schuppan, VEGF induces proliferation, migration, and TGF-β1 expression in mouse glomerular endothelial cells via mitogen-activated

protein kinase and phosphatidylinositol 3-kinase, Biochem. Biophys. Res. Commun. 334 (2005) 1049–1060.
[45] C. Yamamoto, M. Sugato, Y. Fujiwara, T. Kaji, Selective promotion of plasminogen activator inhibitor-1 secretion by activation of proteinase-activated receptor-1 in cultured human brain microvascular pericytes: comparison with endothelial cells, Biol. Pharm. Bull. 28 (2005) 208–211.
[46] G. Mertens, J.J. Cassiman, H. Ven den Berghe, J. Vermylen, G. David, Cell surface heparan sulfate proteoglycans from human vascular endothelial cells, J. Biol. Chem. 267 (1992) 20435–20443.
[47] J.A. Marcum, R.D. Rosenberg, Heparin-like molecules with anticoagulant activity are synthesized by cultured endothelial cells, Biochem. Biophys. Res. Commun. 126 (1985) 365–372.
[48] H. Ishii, H.H. Salem, C.E. Bell, E.A. Laposata, P.W. Majerus, Thrombomodulin, an endothelial anticoagulant protein, is absent from the human brain, Blood 67 (1986) 362–365.
[49] Y. Sato, D.B. Rifkin, Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis, J. Cell Biol. 107 (1988) 1199–1205.
[50] Q. Yan, Y. Li, A. Hendrickson, E.H. Sage, Regulation of retinal capillary cells by basic fibroblast growth factor, vascular endothelial growth factor, and hypoxia, In Vitro Cell Dev. Biol. Anim. 37 (2001) 45–49.
[51] M.S. Pepper, N. Ferrara, L. Orci, R. Montesano, Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells, Biochem. Biophys. Res. Commun. 181 (1991) 902–906.
[52] M.S. Pepper, C. Rosnoblet, C. Di Sanza, E.K. Kruithof, Synergistic induction of t-PA by vascular endothelial growth factor and basic fibroblast growth factor and localization of t-PA to Weibel–Palade bodies in bovine microvascular endothelial cells, Thromb. Haemost. 86 (2001) 702–709.
[53] J.E. Park, G.A. Keller, N. Ferrara, The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF, Mol. Biol. Cell 4 (1993) 1317–1326.
[54] H. Gitay-Goren, S. Soker, I. Vlodavsky, G. Neufeld, The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules, J. Biol. Chem. 267 (1992) 6093–6098.