Heparin Regulates Vascular Endothelial Growth Factor165-dependent Mitogenic Activity, Tube Formation, and Its Receptor Phosphorylation of Human Endothelial Cells

COMPARISON OF THE EFFECTS OF HEPARIN AND MODIFIED HEPARINS*

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Vascular endothelial growth factor (VEGF) is a family of glycoproteins with potent angiogenic activity. We reported previously that heparin has an affinity for VEGF165, the major isoform of VEGF, whereas 2-O-desulfated heparin and 6-O-desulfated heparin have no significant affinity (Ashikari-Hada, S., Habuchi, H., Kariya, Y., Itoh, N., Reddi, A. H., and Kimata, K. (2004) J. Biol. Chem. 279, 12346–12354). In this study, we first examined the effect of heparin and modified heparins (completely desulfated N-sulfated heparin, 2-O-desulfated heparin, and 6-O-desulfated heparin) on VEGF165-dependent mitogenic activity and tube formation on type I collagen gels of human umbilical vein endothelial cells. Both were enhanced by heparin, but not by modified heparins, suggesting that both the 2-O-sulfate group of hexuronic acid and the 6-O-sulfation group of N-sulfoglucosamine in heparin/heparan sulfate are necessary for VEGF165 activity. We then examined the activation of VEGF receptor (VEGFR) to understand the mechanism. We have made several new findings: 1) heparin yielded a 1.7-fold enhancement of VEGF165-induced phosphorylation of VEGFR-2; 2) depletion of cell surface heparan sulfate by heparinase/heparitinase treatment and preferential reduction of trisulfated disaccharide units of cell surface HS by sodium chlorate treatment resulted in the reduction of such phosphorylation, suggesting the involvement of a heparin-like domain in the phosphorylation of VEGFR-2; and 3) VEGF121, an isoform without the exon 7-encoded region, which has no capacity to bind to heparin, did not show these effects. It is therefore likely that a heparin-like domain of heparan sulfate/heparin forms a complex with VEGF165 and VEGFR-2 via the exon 7-encoded region, thereby enhancing VEGF165-dependent signaling.

Heparan sulfate (HS)1 exists ubiquitously as a component of proteoglycans on cell surfaces, in extracellular matrix (ECM) and basement membranes, and has a diverse range of structures and functions (1–6). HS chains are known to interact with a variety of proteins such as heparin-binding growth factors, morphogen, ECM components, protease inhibitors, proteases, lipoprotein lipase, and various pathogens (7–11). These interactions, especially those involving cell growth factors and morphogens, have been shown to play a pivotal role in various pathophysiological phenomena involving angiogenesis as well as in tissue morphogenesis, as uncovered by recent genetic studies (7–10).

Vascular endothelial growth factor (VEGF) denotes a family of glycoproteins, currently comprised of five members in mammals (12). VEGF-A, the first molecule of the family to be identified, is a potent mitogen for endothelial cells and has been shown to be an important growth factor in initiating angiogenesis (13). Alternative splicing of the mRNA derived from a single gene gives rise to at least six different isoforms comprising 121, 145, 165, 183, 189, and 206 amino acids in humans (12, 14). VEGF165, VEGF121, and VEGF189 are the most abundantly expressed isoforms, which can be distinguished further on the basis of their affinity for heparin: VEGF121 does not bind heparin, VEGF165 has moderate affinity for heparin, and VEGF189 binds heparin strongly (14). Heparin itself is only synthesized by mast cells as serglycin but not by endothelial cells (15–18). However, HS chains on proteoglycans usually contain heparin-like domains. VEGF165 has been found sequestered in the ECM (19), and the isoform VEGF121 does not bind to HS proteoglycans (20). Endothelial cells (EC) express glypican-1, syndecan-1 and -2, and perlecain (11, 21, 22). It has been shown that cell surface HSs are required to bind VEGF165 to VEGF-R2, and for the mitogenic activity of VEGF165 (23, 24), glypican-1 is a VEGF165-binding proteoglycan that modulates VEGF165 activity (25), and syndecan-2 is essential for angiogenic sprouting during zebrafish vascular development (26). It has also been shown that VEGF binds to two tyrosine kinase receptors, VEGFR-1 (Flk-1) and VEGFR-2 (KDR/Flik-1), which are expressed on EC (14), and VEGFR-2 and VEGFR-1 are probably activated by all VEGF isoforms but fulfill somewhat different roles. Therefore, VEGF binds to multiple tyrosine kinase receptors, VEGFR-1 (Flk-1) and VEGFR-2 (KDR/Flik-1), which are expressed on EC (14), and VEGFR-2 and VEGFR-1 are probably activated by all VEGF isoforms but fulfill somewhat different roles.

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‡ The abbreviations used are: HS, heparan sulfate; BrdUrd, bromodeoxyuridine; CDSNS, completely desulfated, N-sulfated; DMEM, Dulbecco’s modified Eagle’s medium; EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GlcNSO3, N-sulfoglucosamine; GlcUA, glucuronic acid; HB-EGF, heparin-binding EGF-like growth factor; HexUA, hexuronic acid; HSase mixture, heparitinase I, heparitinase II, and heparinase III; HSPG, heparan sulfate proteoglycans; HUVEC, human umbilical vein endothelial cells; IdU, iduronic acid; NRP, neuropilin; OD580, 0.2-desulfated; OD605, 0.6-desulfated; PBS, phosphate-buffered saline; PIO, periodate-oxidized; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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functions \textit{in vivo}, as targeted gene disruption experiments have revealed (27, 28). In addition, EC express the neuropilin (NRP)-1 and NRP-2 coreceptors, which selectively bind VEGF\textsubscript{165}, but not VEGF\textsubscript{121} (29). However, the HS structure for VEGF\textsubscript{165} binding and the detailed role of the HS binding ability of VEGF\textsubscript{165} in VEGFR binding and in the activity itself remain to be studied.

There is only one previous report indicating that heparin enhanced VEGF\textsubscript{165}-induced mitogenic activity (24). Most studies have examined \textit{125}I-VEGF binding to EC in the presence or absence of heparin and demonstrated that 0.1–10 \textmu g/ml heparin strongly potentiated VEGF\textsubscript{165} binding to its receptors such as VEGFR-2 and NRP-1 on vascular EC (23, 30–33). We have shown that dissociation constants for the interaction between VEGF\textsubscript{165} and 2-O-desulfated (2ODS) heparin or 6-O-desulfated (6ODS) heparin are only 3-fold higher than that for the interaction of VEGF\textsubscript{165} with heparin (34). The results suggest that VEGF activities are likely regulated not only by heparin, but also by 2ODS heparin and 6ODS heparin.

In this study, we first examined the effects of heparin and three differently modified heparins on VEGF\textsubscript{165}-induced mitogenic activity of human umbilical vein endothelial cells (HUVEC), whose structures have been studied previously (34). In addition, we examined the effects of heparin and modified heparins on VEGF\textsubscript{165}-induced tube formation. Our results show that heparin alone, but not modified heparins, enhanced both VEGF\textsubscript{165} activities. Next, we analyzed the phosphorylation of VEGFR-2 using the above cell culture system to gain insight into the mechanism by which heparin enhanced VEGF\textsubscript{165} activity. Our results show that heparin-induced increases in VEGF\textsubscript{165}-dependent VEGFR-2 phosphorylation. Furthermore, VEGF\textsubscript{121}, which has no heparin binding capacity, did not show these effects. Considering the different effects of heparinase treatment on the phosphorylation between VEGF\textsubscript{165} and VEGF\textsubscript{121} and also the effect of sodium chlorate treatment on VEGF\textsubscript{165}-induced phosphorylation of VEGFR-2, it is likely that heparin or HS with heparin-like domains on cell surface proteoglycans forms a complex with VEGF\textsubscript{165}, VEGF\textsubscript{121}, and other receptors, thereby enhancing VEGF\textsubscript{165}-dependent signaling. We have also shown that periodate-oxidized (PIO) heparin without antiocoagulant activity had a similar effect on the VEGF\textsubscript{165}-induced phosphorylation of VEGFR-2.

**EXPERIMENTAL PROCEDURES**

Materials and Cells—HUVEC were purchased from Clonetics (Walkersville, MD) and used at passages 4–7. Recombinant murine VEGF, corresponding to human VEGF\textsubscript{165}, was from Diaclon (Cedex, France) and was termed VEGF\textsubscript{165}. Rabbit polyclonal antibody against phosphorylated VEGFR-2 (Tyr-951) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary peroxidase-conjugated goat anti-rabbit antibody was from Organon Technika Corporation (Durham, NC). CD-SNS heparin, 2ODS heparin, 6ODS heparin, and PIO heparin were prepared by Seikagaku Corporation (Tokyo, Japan). PIO heparin had no binding activity for antithrombin III, and the anticoagulant activity, because GlcA-GlcNSO\textsubscript{3}SO\textsubscript{4}, the essential residue of the activity, was destroyed (35, 36). Heparitinase I (Flavobacterium heparinum) and heparitinase II (\textit{Flavobacterium heparinum}) were from Bio-Rad Laboratories (Hercules, CA). Heparitinase I was applied to 24-well culture plates and maintained at 37 °C for 1 h to allow the formation of collagen fibrils. VEGF was mixed with increasing concentrations of heparin or modified heparins in RPMI 1640 medium supplemented with 0.5% fetal bovine serum, 2 mM glutamine, and incubated for 1 h at room temperature. HUVEC dissociated by trypsinization were plated on collagen gel at a density of 5 × 10\textsuperscript{4} cells/well in EGM-2 medium (Cambrex, Walkersville, MD) for 1 h at 37 °C. After the plates were washed with RPMI 1640 medium containing 0.5% fetal bovine serum and L-glutamine, the cells were cultured in medium supplemented with 0.5% fetal bovine serum, 2 mM glutamine, 20 ng/ml VEGF, and various concentrations of heparin or modified heparins overnight. After removal of the media, 500 \mu l of collagen mixture was overlaid on the cells and incubated for 1 h at 37 °C. The media containing these factors were then added on top of the collagen gel layers and the cells were cultured further for 3 days. On the 3rd day, each well was washed with PBS, and the total length of the cells was measured in five different areas of 0.03 mm\textsuperscript{2} each using Image Gauge version 3.45 (Fuji Film Science Laboratory 99, Tokyo). Experiments were repeated independently three times, and statistical analyses were performed using Student’s t test. A p value <0.05 was considered statistically significant.

Analysis of VEGFR-2 and ERK1/2 Phosphorylation—For \textit{in vivo} phosphorylation studies, HUVEC were seeded at 1.9 × 10\textsuperscript{4} cells/well plate in EGM-2 medium containing EBM-2MV SingleQuots (Cambrex), and serum starved overnight in EGM-2 medium. The cells were then stimulated with 10 ng/ml VEGF\textsubscript{165} or VEGF\textsubscript{121} in the presence or absence of heparin for 5 min at 37 °C. The cells were lysed in cell lysis buffer containing 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Triton X-100, 1.5 mM EDTA, 1 mM Na\textsubscript{3}PO\textsubscript{4}, 25 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and 1 tablet/10 ml of protease inhibitor mixture tablets (Complete, mini, Roche Applied Science). Lysates were stirred gently and clarified by centrifugation (10,000 rpm for 30 min). Protein concentrations were measured using the QuantPro BCA assay kit (Sigma), and 30 \mu g of protein from each sample was used for analysis. For immunoblotting, cell lysates were subjected to 7.0% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blots were incubated with 10% skim milk containing PBS and probed with the primary antibody diluted in 1% skim milk containing PBS. The signal was visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Western Lightning Plus, PerkinElmer Life Sciences) according to the manufacturer’s instructions. Band density was determined using Image Gauge.

**Detection of Cell Surface/ECM HS**—The wells were digested with 10 milliunits of heparitinase I, 5 milliunits of heparitinase II, and 10 milliunits of heparitinase (HSase mixture) or 10 milliunits of chondroitinase ABC at 37 °C. After 15 min, the enzyme and released glycosaminoglycan fragments were removed. Wells were subjected to several washes with EGM-2 medium for the complete removal of enzymes and then digested with 10% chlorite as described above.

**Effect of Preferential Reduction of HS O-Sulfation by Sodium Chlorate Treatment**—Treatment with low concentrations of sodium chlorate is known to inhibit the sulfate donor synthesis of chondroitinase ABC at 37 °C. After 15 min, the enzyme and released glycosaminoglycan fragments were removed. Wells were subjected to several washes with EGM-2 medium for the complete removal of enzymes and then digested with 10% chlorite as described above.
and serum starved overnight in EGM-2 medium or in DMEM/F-12 medium containing sodium chloride as described above. The cells were then treated with or without HSase mixture for 15 min. After several washes with PBS, the cells were stimulated with VEGF for 5 min as described above. The cells were then trypanized. The supernatants containing cell surface/ECM HS were applied to a 0.3-ml DEAE-Sephacel column equilibrated with 0.1 M NaCl in 50 mM Tris-HCl, pH 7.2. The column was washed with 3 ml of the above buffer. HS fractions were eluted with 0.9 ml of 2 M NaCl in 50 mM Tris-HCl, pH 7.2, concentrated by ethanol precipitation, and treated with or without HSase mixture at 37 °C for 1 h. Unadsorbed disaccharide products were analyzed by fluorometric postcolumn high performance liquid chromatography as reported previously (39).

RESULTS

Effect of Heparin and Modified Heparins on VEGF-induced Angiogenic Responses—A previous study showed that heparin enhanced the ability of VEGF165 to induce HUVEC proliferation (24). We have shown that CDSNS heparin, completely desulfated N-sulfated heparin, has no affinity to VEGF165, whereas 2ODS heparin and 6ODS heparin, which are deficient in 2-O-sulfate groups and 6-O-sulfate groups, respectively, have an affinity for VEGF165, although it was weak compared with heparin (34). However, the effects of 2ODS heparin and 6ODS heparin have not been investigated. We first evaluated the biological effects of these specific O-desulfated heparins on VEGF165 activity by cell proliferation and tube formation assays. For comparison, we also examined these effects on VEGF121, an isoform without the heparin binding domain (3). Composition analysis showed that the major disaccharide components of heparin, CDSNS heparin, 2ODS heparin, and 6ODS heparin, used here were shown previously to be HexUA(2SO4)-GlcNSO3(6SO4), HexUA-GlcNSO3, HexUA-GlcNSO3(6SO4), and HexUA(2SO4)-GlcNSO3, respectively (34, 40). The VEGF-induced incorporation of BrdUrd into HUVEC was examined by culturing the cells in the presence of 10 ng/ml VEGF165 or VEGF121 with various concentrations of heparin or modified heparins for 24 h. The additional 24-h incubation with BrdUrd demonstrated that VEGF165-induced proliferation was significantly enhanced dose dependently only by heparin (Fig. 1A). Heparin at 1 and 10 μg/ml stimulated proliferation 1.4- and 1.8-fold, respectively, compared with no added heparin. None of modified heparins affected the proliferation of HUVEC, even at high concentrations. In contrast, the VEGF121-dependent incorporation of BrdUrd was not enhanced by heparin or by any modified heparins at any concentrations tested (Fig. 1B). These results indicate that both 2-O- and 6-O-sulfate residues in heparin are necessary for the enhancement of VEGF165-induced proliferation by heparin, but heparin is not involved in VEGF121-dependent proliferation.

Next, the effect of heparin or modified heparin on VEGF-induced tube formation was evaluated as an event occurring during angiogenesis. When HUVEC were cultured between two layers of a type I collagen gel in the presence of VEGF165 and various concentrations of heparin, apparent prominent tube formation was observed at higher concentrations of heparin (Fig. 2, A–D). As shown in Fig. 2H, heparin at concentrations of 1, 10, and 100 μg/ml stimulated tube formation by 1.9-, 2.6-, and 3.0-fold, respectively, compared with effects in the absence of heparin. However, three modified heparins did not affect tube formation in HUVEC even at 100 μg/ml (Fig. 2, E–H). In contrast, the tube formation induced by VEGF121 was not enhanced by heparin or three modified heparins at any concentration (data not shown).

The results showed that heparin, but neither 2ODS nor 6ODS heparin, potentiated the angiogenic activities of

VEGF165, i.e. BrdUrd incorporation and tube formation.

Investigation of the Mechanism of Heparin Effects on VEGF165 Activity—The major roles of heparin/HS in heparin-binding growth factor activity may include an increase in receptor activation by the formation of ternary complexes between heparin/HS, growth factor, and its receptor. To study the mechanisms of VEGF165 activity enhancement by heparin, the tyrosine phosphorylation of VEGFR-2 was examined by Western blot analysis using a specific antibody for phospho-VEGFR-2. VEGF is known to stimulate the autophosphorylation of VEGFR-2 (41). Treatment with VEGF165 alone for 5 min induced VEGFR-2 phosphorylation (Fig. 3A, lanes 1 and 4). The VEGF-induced VEGFR-2 phosphorylation was maximal at 5 min after stimulation (data not shown). In the presence of heparin, the VEGF165-induced phosphorylation of VEGFR-2 increased 1.7-fold (Fig. 3A, lanes 4–6, and B, gray bars). Heparin alone did not substantially affect VEGF-2 phosphorylation (Fig. 3A, lanes 1–3). Although it has been shown that heparin also interacted with its receptors (42, 43), the VEGF121-induced phosphorylation of VEGFR-2 did not increase in the presence of heparin (Fig. 3, C and D), suggesting that the heparin-enhanced phosphorylation of VEGFR-2 occurred through the interaction with VEGF165, but not with VEGF121.

Furthermore, we examined whether PIO heparin, which does not have anticoagulant activity, enhanced VEGF-dependent activity. Treatment with PIO heparin destroyed GlcNSO3(6SO4)-GlcNAc(6SO4)-GlcUA-GlcNSO3(5SO4,6SO4)-IdoUA(2SO4)-GlcNSO3(6SO4)-, the essential sequence for binding to antithrombin III (35, 36, 44). However, IdoUA(2SO4)-GlcNSO3(6SO4)-, an important structure for the enhancement of VEGF165 activity, was resistant to cleavage. In the presence of PIO heparin, the VEGF165-induced phosphorylation of VEGFR-2 increased 1.5-fold (Fig. 3, A, lanes 7–9, and B, striped bars), suggesting that PIO heparin has activity similar to that of heparin.

Effect of Heparin on the VEGF165-induced Phosphorylation of VEGFR-2 in Heparitinase/Heparinase-treated HUVEC—A pre-
FIG. 2. Representative micrographs of VEGF<sub>165</sub>-induced tube formation in HUVEC in the presence of heparin or modified heparins. A–G, HUVEC were stimulated for 3 days with VEGF<sub>165</sub> in the presence of 0 (A), 1 (B), 10 (C), or 100 μg/ml heparin (D), 100 μg/ml CDSNS heparin (E), 2ODS heparin (F), or 6ODS heparin (G). H, the angiogenic activity assayed by tube formation of VEGF<sub>165</sub> in the absence (open bars) or presence of 1 (striped bars), 10 (closed bars), or 100 μg/ml (gray bars) heparin or modified heparins. Tubular length of the cells was measured in five different areas of 0.03 mm<sup>2</sup> each using Image Gauge. Data are the averages of five areas. Asterisks indicate significant differences from VEGF alone as determined by t test (p < 0.05). The same results were obtained from two independent experiments. Scale bar = 50 μm.

Various study showed that the capacity of VEGF<sub>165</sub> to bind its receptors on EC was abolished by heparinase treatment and that the effect of heparinase could be reversed by the addition of heparin (23). VEGF<sub>165</sub>-induced HUVEC growth was inhibited by treatment with sodium chlorate, and this chlorate inhibition of VEGF<sub>165</sub>-induced cell growth was partially overcome by the addition of heparin (24), suggesting that HS on EC is required for the mitogenic activity of VEGF<sub>165</sub>. To determine whether cell surface HS is required for the VEGF<sub>165</sub>-induced phosphorylation of VEGFR-2, we examined VEGF<sub>165</sub>-induced receptor phosphorylation in HSase mixture-treated HUVEC. Cells were treated with or without HSase mixture as described under "Experimental Procedures." After 15 min, each well was washed and then stimulated with 10 ng/ml VEGF<sub>165</sub> in the absence or presence of heparin for 5 min at 37 °C. VEGF<sub>165</sub>-induced phosphorylation of VEGFR-2 was reduced to 75% by HSase mixture treatment (Fig. 4, A, lanes 1 and 2, and B). In contrast, digestion with HSase mixture did not affect VEGF<sub>121</sub>-induced phosphorylation of VEGFR-2 (Fig. 4, C and D), suggesting that cell surface HS regulated VEGFR-2 activation mediated by heparin-binding VEGF. Furthermore, this decrease of VEGF<sub>165</sub>-induced phosphorylation by digestion with HSase mixture was rescued by the addition of heparin (Fig. 4, A, lanes 3–6, and B). To determine how much cell surface HS was removed by digestion with HSase mixture and was also newly synthesized while stimulating the cells with VEGF, the fractions containing cell surface HS were retrieved using trypsin digestion as described under "Experimental Procedures." These fractions were treated with HSase mixture, and then unsaturated disaccharide products were analyzed. Cell surface HS (0.64 nmol as disaccharide/10<sup>6</sup> cells) were reduced to less than 5% by HSase digestion (Fig. 4G). A similar result was given by the analysis of HS fractions obtained from the cells metabolically labeled with H<sub>2</sub>SO<sub>4</sub> (data not shown).

We further analyzed the effects of heparin addition and heparinase/heparitinase treatment on ERK1/2 activation by VEGF<sub>165</sub> because VEGF<sub>2</sub> induces proliferation through activation of the classical ERK pathway, leading to gene transcription (12). Phosphorylation of ERK1/2 increased 2.5-fold, 5 min after stimulation by VEGF<sub>165</sub> (Fig. 4, E, lanes 1 and 2, and F); this phosphorylation was decreased markedly (to 25%) by HSase mixture digestion and restored by exogenous heparin (Fig. 4, E, lanes 3–5, and F). Digestion with chondroitinase ABC did not affect the phosphorylation of either VEGFR-2 or ERK1/2 (data not shown). These results suggested that cell surface HS was involved in the regulation of VEGF<sub>165</sub> activity.

Treatment with 15 mM sodium chlorate resulted in reduction of 2-O-sulfated and 6-O-sulfated disaccharide units of cell surface/ECM HS to 63 and 69%, respectively, which were determined by the sum of the percentages of 2-O-sulfated and 6-O-sulfated disaccharide products by HSase mixture digestion, respectively (Fig. 5A). In contrast, the slight reduction (9%) of N-sulfated disaccharide units of the HS was observed by the chlorate treatment. It should be of note that the trisulfated disaccharide component, i.e. HexUA(2SO<sub>4</sub>)-GlcNSO<sub>3</sub>(6SO<sub>4</sub>) component, was reduced to 65% by the treatment (Fig. 5A). VEGF<sub>165</sub>-induced VEGFR-2 phosphorylation also fell to 77% (Fig. 5B). These results suggest that heparin-like regions of HS, which are rich in the HexUA(2SO<sub>4</sub>)-GlcNSO<sub>3</sub>(6SO<sub>4</sub>) component, regulate VEGF<sub>165</sub>-induced phosphorylation of VEGFR-2.

**Effect of the Exon 7-encoded Region of VEGF<sub>165</sub> on VEGFR-2 Phosphorylation**—The difference between VEGF<sub>165</sub> and VEGF<sub>121</sub> molecules is the presence or absence of the region encoded by exon 7, which contains the heparin binding region. To elucidate the role of this region, VEGFR-2 phosphorylations induced by VEGF<sub>165</sub> and VEGF<sub>121</sub> were examined at the same molar concentration of 0.5 nM (corresponding to 9.5 μg/ml VEGF<sub>165</sub> and 7.0 μg/ml VEGF<sub>121</sub>). Both phosphorylations VEGFR-2 increased in a concentration-dependent manner in the range of 0.05–1.5 nM (data not shown) and were at the same level (Fig. 6, A, lanes 1 and 3, and B). However, after treatment with HSase mixture, the VEGF<sub>165</sub>-induced VEGFR-2 phosphorylation was reduced significantly (Fig. 6, A, lanes 1 and 3, and B).
This result suggests that the exon 7-encoded region may suppress VEGFR-2 phosphorylation in the absence of heparin/HS. Interestingly, VEGFR-2 phosphorylation by VEGF₁₆₅ in the presence of heparin (Fig. 6, A, lanes 2 and 6, and B) was higher than that by VEGF₁₂₁ in the presence and absence of heparin (Fig. 6, A, lanes 3, 4, and 7, and 8, respectively, and B). This result indicates that the region specific to VEGF₁₆₅ may serve as a positive regulator of the heparin/HS-enhancing effect.

DISCUSSION

We for the first time demonstrated the enhancement effect of heparin on VEGF₁₆₅-induced tube formation as well as proliferation of HUVEC. In addition, the effects of heparin on VEGF-induced phosphorylation of VEGFR-2 and activity of VEGF were evaluated by the analysis of phosphorylation of VEGFR-2. In consequence, we suggest action mechanisms for such effects of heparin. Heparin is likely to form a complex with VEGF₁₆₅.
HEPARIN-REGULATED VEGF ACTIVITY AND RECEPTOR PHOSPHORYLATION

VEGF165 alone as determined by phosphorylation of VEGFR-2 (B). HUVEC were seeded, serum starved overnight, and then stimulated with 10 ng/ml VEGF165 for 5 min at 37 °C in DMEM/F-12 medium with (gray bars) or without (open bars) 15 mM sodium chlorate as described under “Experimental Procedures.” A, cell surface/ECM HS were retrieved using trypsin digestion and fractionated by DEAE-Sepharose chromatography. HS fractions were digested with a mixture of heparitinase I and II and heparinase. The products of unsaturated disaccharides were determined by a reversed phase ion-pair chromatography with sensitive and specific postcolumn detection. B, the cells treated with VEGF165 in DMEM/F-12 medium with (gray bars) or without (open bars) sodium chlorate as described above were subjected to gel electrophoresis, and Western blotting was performed using anti-phospho-VEGFR-2 antibody (inset). VEGFR-2 phosphorylations were quantified using Image Gauge. Each bar indicates the relative phospho-VEGFR-2, which is consistent with the results that heparin butnot2ODS heparin and 6ODS heparin could enhance VEGF165-dependent mitogenic activity, tube formation, and its receptor phosphorylation. A previous study indicated that the addition of a heparin-binding peptide present in VEGF2 inhibited VEGF165 binding to VEGFR-2 and also inhibited the VEGF-induced proliferation of EC (42), suggesting that the interaction between VEGFR-2 and HS may regulate VEGF165 activity. Furthermore, mice expressing the VEGF121 isoform exclusively via the Cre/loxP-mediated removal of exons 6 and 7, which encode the heparin binding region, had impaired postnatal myocardial angiogenesis and ultimately died of cardiac failure (45). VEGF121 alone was insufficient for normal angiogenesis, suggesting the regulatory function of the heparin binding domain of VEGF isoforms. These observations support our present results.

We have shown that heparin did not affect VEGF121-induced proliferation and tube formation. Because VEGF121 targets VEGFR-2 and VEGFR-1, but not NRP-1 (32), this behavior of VEGF121 could be explained as follows. 1) It is unlikely that the binding of VEGF121 to VEGFR-2 and VEGFR-1 would be enhanced by heparin because VEGF165 cannot bind heparin. 2) NRP-1 would enhance neither VEGF121 binding to VEGFR-2 nor its bioactivity. 3) Heparin did not directly mediate VEGFR-2 activation (Fig. 3, C and D).

A recent study showed that the heparin-binding EGF-like growth factor (HB-EGF)-induced phosphorylation of EGF receptor was enhanced by either the addition of exogenous heparin or deletion of the heparin binding domain (11 amino acid residues), indicating that the heparin binding domain suppresses the activity of the EGF-like domain of HB-EGF and serves as a negative regulator in the absence of heparin (46).
The difference between VEGF165 and VEGF121 molecules is the presence or absence of the exon 7-encoded region (44 amino acid residues), which contains the heparin binding domain. The results shown in Fig. 6 indicate that the exon 7-encoded region apparently differs in the effect of added heparin from the heparin binding domain of HB-EGF and may serve as a positive regulator of VEGFR-2 phosphorylation in the presence of heparin. In addition, considering that the region encoded by exon 7 of VEGF165 contains both heparin binding and NRP-1 binding domains (32), the physiological role of this region is not simply similar to the heparin binding domain of HB-EGF.

In contrast to the phosphorylation of VEGFR-2, VEGF165-induced ERK phosphorylation was reduced markedly to 25% by digestion with HAsase mixture. The VEGFR-2 intracellular domain is known to have six tyrosine phosphorylation sites (12). Takahashi et al. (47) have shown recently that the VEGF-VEGFR-2 signal utilizes the phospholipase Cγ-protein kinase C-ERK pathway, and phospholipase Cγ binds Tyr-1175 of VEGFR-2. In this study, we detected VEGF-induced phosphorylation of VEGFR-2 using a polyclonal antibody against phosphorylated VEGFR-2 (Tyr-951). In the case of FGFR-2-induced FGFR-1 phosphorylation, Tyr-463 and Tyr-766 in FGFR-1 exhibited distinctly different effects of heparin (48). Therefore, one can speculate that a similar situation may happen to VEGF signaling, i.e., other tyrosine phosphorylation sites such as Tyr-1175 in VEGFR-2 might be more sensitive than Tyr-951 with regard to the effects of heparin/HS on VEGF165-induced phosphorylation.

In a previous study, we measured the dissociation constants (KD) for the interaction of VEGF165 with 2ODS heparin/VEGF165 and that with 6ODS heparin (6ODS heparin/VEGF165); KD values were 524 and 592 nM, respectively, and were 3-fold that for heparin/VEGF165 (165 nM) (34). CD-SNS heparin did not interact with VEGF165 (data not shown). Although this result indicated weak affinity of both 2ODS heparin and 6ODS heparin for VEGF165, neither 2ODS heparin nor 6ODS heparin enhanced the VEGF165-dependent proliferation or proliferation formation at any of the concentrations tested. Heparin interactions with receptors for VEGF165 such as VEGFR-2 and NRP-1, are also known (30, 33), although we have not yet examined how sulfate groups in heparin are involved in binding to these VEGF165 receptors. VEGF-2, which is characterized by seven immunoglobulin-like domains within its extracellular region, interacts directly with heparin/HS, possibly through the hexapeptide sequence RKKKRR between the sixth and seventh Ig-like domains (42). NRP-1, which is a coreceptor for VEGF165, binds to heparin via its b1b2 domain (43). These results suggest that heparin contributes, at least in part, to the easier formation of a complex between VEGF165 and its receptors. The 2-O-sulfate groups in heparin/HS, which are essential for binding to FGF-2, and the 6-O-sulfate group, which is essential for binding to the FGF in addition to the 2-O-sulfate groups, were required to regulate FGF-2 activity by heparin/HS (49). If this is also the case for the regulation of VEGF165 activity, 2ODS heparin and 6ODS heparin may lack the structure required for binding to VEGF165 receptors, such as VEGFR-2 and NRP-1, or for formation of the complex, which could explain that 2ODS heparin and 6ODS heparin failed in enhancing effects. Such an observation also suggests that these modified heparins may inhibit VEGF165 activities because they have an affinity for VEGF165 and compete with its binding to cell surface HS proteoglycan. However, neither of the modified heparins inhibited VEGF165 activities (Figs. 1 and 2), suggesting that 2ODS heparin and 6ODS heparin have a weak affinity for VEGF165 compared with the heparin-like domain of cell surface HSPG, and/or VEGF165 associated with these modified heparins have a weak affinity for VEGFRs compared with VEGF165 associated with the heparin-like domain of cell surface HSPG. We have shown that KD values of 2ODS heparin/VEGF165 and 6ODS heparin/VEGF165 were even higher than that of 2ODS heparin/FGF-2 (340 nM) (34). Such weak affinity may have little effect on the biological activity.

In this study, we have demonstrated the stimulatory effect of heparin on VEGF165-induced phosphorylation of VEGFR-2, consistent with the observation that growth stimulatory signals of VEGFs are transduced, to a major extent, via VEGFR-2 (50). VEGFR-2 and NRP-1, but not VEGFR-1, have been detected as labeled complexes by cross-linking analysis of VEGF165 binding to the surface of HUVEC (32). We have shown that VEGF121-dependent VEGFR-2 phosphorylation in HUVEC was unaffected by the addition of heparin and by digestion with HAsase mixture. Therefore, in HUVEC, VEGF-2 activation may play a central role in the signal pathway controlling VEGF165-induced activities.

Here we propose an action mechanism of VEGF165 activity enhancement by heparin considering the reported roles of NRP-1 (33, 51, 52), as shown in Fig. 7. In the absence of heparin, both VEGF165 binding to VEGFR-2 and VEGF165-dependent phosphorylation of VEGFR-2 are regulated by cell surface HSPG via binding to the heparin-like domain. In the presence of heparin, VEGF165-VEGFR-2, and NRP-1 form a complex through the intermediary molecule of heparin. As a result, VEGF165-induced signaling processes, such as the phosphorylation of VEGFR-2, are increased by heparin. This mechanism is also suggested by the following results: Heparin enhances VEGF165 binding to VEGFR-2 (51), the affinity of VEGF165 for NRP-1 is also enhanced by the addition of heparin (52), NRP-1 in turn enhances VEGF165 binding to VEGFR-2 and its bioactivity (33), heparin induces the binding of NRP-1 to VEGF165, and in consequence, heparin may regulate VEGF165 activity selectively via NRP-1. We should also consider another mechanism of VEGF165 activity enhancement by heparin: VEGF165 stability is increased by complexing with heparin.3

There are a number of studies demonstrating the importance of heparin/HS for the activation of growth factor binding to its receptor via complex formation (4). Heparin fragments containing more than 20 monosaccharide units were superior potentiators of VEGF165 binding to VEGFR-2 and NRP-1 (53). Now that we have a heparin-derived octasaccharide library consisting of octasaccharide (HexUA-GlcNSO3)4 with a variety of sulfate positions and degrees (27), we can develop heparin-derived oligosaccharide libraries with a variety of sizes, sulfate posi-

We can also develop the oligosaccharide without anticoagulant. In addition, PIO heparin may be substituted for heparin because the heparin-binding proteins and degrees using similar techniques and elucidate the structure(s) important for the regulation of VEGF activity. In discussions.

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