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Enhanced Ability of Heparin-Carrying Polystyrene (HCPS) to Bind to Heparin-Binding Growth Factors and to Inhibit Growth Factor–Induced Endothelial Cell Growth

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Heparin-carrying polystyrene (HCPS) consists of low-molecular-weight heparin chains enriched in trisulfated disaccharide structures linked to a polystyrene core. In this study, the interactions between HCPSs of various molecular weights and heparin-binding growth factors, $VEGF_{165}$, FGF-2, and HGF, were compared to the interactions of the same factors with native heparin, periodate-oxidized heparin (IO_4-heparin) and periodate-oxidized alkaline-degraded heparin (IO_4-LMW-heparin). The binding of each growth factor to heparin-agarose beads (heparin-beads) was more strongly inhibited by HCPSs in a molecular weight–dependent manner than by native heparin or the modified heparins, indicating a stronger interaction between HCPS and these growth factors. HCPSs also inhibit heparin-binding growth factor–induced endothelial cell growth in a molecular weight–dependent manner much more strongly than the native or modified heparins. However, HCPSs did not inhibit the mitogenic activity of $VEGF_{121}$, which has a non-heparin-binding nature. Thus, HCPSs exhibit enhanced abilities to interact with each of the heparin-binding growth factors studied and to inhibit heparin-binding growth factor–induced endothelial cell proliferation in a molecular weight–dependent manner. These effects might be ascribed to the heparin-clustering effect of HCPSs.

Key words: HCPS (heparin-carrying polystyrene), heparin, heparan sulfate, heparin-binding growth factor.

Heparin/heparan sulfates (HS) are complex polysaccharides consisting of repeating units of disaccharides. Each disaccharide unit contains a uronic acid (UA) residue (either D-glucuronic acid or L-iduronic acid) and a glucosamine residue, which is either *N*-sulfated (GlcNS) or *N*-acetylated (GlcNAc). The disaccharides may be sulfated at the C6 and/or C3-positions of the glucosamine residue and the C2-position of the UA residue (*1*). Heparin/HS are known to interact with a variety of enzymes and functional proteins including growth factors, cytokines, extracellular matrix proteins, and selectins, and to be mediated by specific domains, *i.e.* different patterns and combinations of sulfate groups on the polysaccharides (*2*). It is through these interactions that heparin/HS mediate or assist in diverse cellular responses including cell growth, differentiation, adhesion, recognition, and migration. In particular, heparin/HS have been found to function as FGF co-receptors (*3*, *4*). Prior to binding to a cell surface receptor, FGF has to bind to heparin/HS, leading to FGF dimerization, which is essential for receptor signalling (*5*).

Heparin/HS are also known to inhibit the growth of vascular smooth muscle cells (SMCs) and mesangial cells both *in vitro* and *in vivo* (*6*). The precise molecular mechanism of its anti-proliferative effect is, however, not yet understood (*7*). On the other hand, when grown in the presence of heparin-binding growth factors, heparin/HS also modulate the growth of other cell types such as vascular endothelial cells (*8*, *9*).

Heparin-carrying polystyrene (HCPS) has been described previously as a synthetic glycoconjugate that adsorbs to plastic culture plates where it possesses unique properties as a substratum for adhesion and growth of various cultured cells (*10*). On the other hand, HCPS is soluble in

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Abbreviations: HCPS, heparin-carrying polystyrene; HCMS, heparin-carrying mono-styrene, hpHCPS, highly polymerized heparin-carrying polystyrene; ppHCPS, partially polymerized HCPS; HMW-ppHCPS, high-molecular-weight–ppHCPS; MMW-ppHCPS, middle-molecular-weight–ppHCPS; LMW-ppHCPS, low-molecular-weight–ppHCPS, FGF, fibroblast growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; HS, heparan sulfate, IO_4-heparin, periodate-oxidized heparin; IO_4-LMW-heparin, periodate-oxidized low-molecular-weight–heparin, GlcNAc, *N*-acetylglucosamine; GlcNS, *N*-sulfated glucosamine; UA, uronic acid, ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum, BSA, bovine serum albumin; PBS, phosphate-buffered saline; PVLA, lactose-carrying polystyrene, PVMA, mannose-carrying polystyrene.

water and has an amphiphilic structural unit consisting of hydrophilic polysaccharides and hydrophobic polystyrene moieties. It has been estimated that the molecular size of HCPS is approximately 80–120 kDa and comprises about ten low molecular weight (LMW) heparin chains linked to its polystyrene core (*10*).

Here we report the structural features of LMW heparin chains linked to a polystyrene core and the enhanced abilities of HCPS in culture medium solution (i) to interact with heparin-binding growth factors in a molecular weight–dependent manner and (ii) to inhibit the activities of heparin-binding growth factors for growth-stimulation of endothelial cells. The properties of HCPSs having various molecular weights were compared to those of native heparin and chemically modified heparins that were the actual intermediate products during the preparation of HCPS.

MATERIALS AND METHODS

Materials—Human recombinant growth factors, $VEGF_{165}$, $VEGF_{121}$, FGF-2, and HGF, as well as anti-VEGF (polyclonal, goat IgG), anti–FGF-2 (polyclonal, rabbit IgG), and anti-HGF (polyclonal, goat IgG), were purchased from R&D Systems (Minneapolis, MN). Anti–goat (rabbit) IgG (horseradish peroxidase conjugated) and peroxidase substrate (ABTS) were purchased from Bio-Rad (Hercules, CA). Heparin-beads (heparin-agarose beads, type I) were purchased from Sigma (St. Louis, MO). Other chemicals used were of the highest quality commercially available.

Preparation of Heparin-Carrying Polystyrene (HCPS)—HCPS was prepared as has been reported previously (*10*). Briefly, heparin from porcine intestine was dissolved in 0.1 M $NaIO_4$ in 0.05 M sodium acetate buffer (pH 5), and the solution was stirred at 4°C for 3 days. The unreacted $NaIO_4$ was then neutralized by the addition of glycerol, and the reaction mixture was dialyzed and lyophilized. The periodate-oxidized heparin (IO_4-heparin) produced was then degraded in alkaline solution (pH 12) at room temperature for 30 min. The degraded product was recovered after dialysis and lyophilization as periodate-oxidized, alkaline-degraded heparin (IO_4-LMW-heparin).

This product (500 mg) and 250 mg *N-p*-vinylbenzylamine were dissolved in 20 ml TEMED solution, after which 1 ml of 0.8 mM $NaCNBH_3$ was added. The reaction mixture was stirred for 24 h at room temperature, dialyzed and lyophilized to yield 533 mg white powder (heparin-carrying monostyrene; HCMS). This powder (100 mg) and 2 mg potassium peroxodisulfate were dissolved in 1 ml distilled water and polymerization was carried out at 60°C for 24 h under dried N_2 gas. The polymerization was completed under these reaction conditions and the solution was then poured into an excess amount of ethanol to obtain polymers as a precipitate. Water-soluble impurities were separated from the precipitate by ultrafiltration, and 73 mg of highly polymerized HCPS (hpHCPS) was obtained as a white powder after lyophilization.

Preparation of HCPSs with Differing Molecular Weights—Partial polymerization was carried out at 60°C for 6 h under dried N_2 gas in order to prepare HCPS with differing molecular weights. The purified, partially polymerized HCPS (ppHCPS; 0.5 g) was separated according to molecular weight on a Bio-Gel P-100 column (2 × 100 cm) equilibrated with acetate buffer (pH 7.3) containing 0.2 M NaCl and 0.1% Triton X-100 at a flow rate of 30 ml/h (Fig. 1). A gel filtration standard mixture (Nippon Bio-Rad Laboratories, Tokyo) was used to estimate the molecular weights of the fractions. The uronate content of HCPS in each fraction (3.5 ml) was determined by a carbazole assay, as described before (*10*). Fractions corresponding to HMW (high molecular weight)-ppHCPS, MMW (middle molecular weight)-ppHCPS, and LMW (low molecular weight)-ppHCPS, as shown in Fig. 1, were pooled separately and purified by chromatography on a EDAE-cellulose (1 × 5 cm) column using NH_4 acetate as the running buffer (*11*).

Compositional Analysis of Native Heparin, IO_4-Heparin, and IO_4-LMW-Heparin—Compositional analysis of native heparin, IO_4-heparin, and IO_4-LMW-heparin was performed as described previously (*12*, *13*). Briefly, the polysaccharides (0.1 mg) were treated with a mixture of heparinase (50 mU), heparitinase I (20 mU), and heparitinase II (20 mU) (Seikagaku, Tokyo) in 220 μl of 2 mM calcium acetate and 20 mM sodium acetate (pH 7.0) at 37°C for 2 h. The completeness of the digestion was confirmed by gel-filtration chromatography through tandemly arranged columns of TSK-Gel PW4000, PW3000, and PW2500 (Tosoh, Tokyo) eluted with 0.2 M NaCl, monitored by the absorbance at 230 nm and the refractive index. The disaccharide compositions of the reaction mixtures were analyzed by ion-exchange chromatography on Dionex CarboPac PA-1 (4 × 250 mm).

ELISA for Evaluating the Binding of HCPS to Growth Factors—To evaluate the binding of HCPSs to growth factors, an ELISA using heparin-beads was performed as reported previously (*9*). Briefly, in a 0.25 ml microtube (Nunc Inter Med, Tokyo), 100 μl of 1% (w/w) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) including 4 ng of human recombinant $VEGF_{165}$, FGF-2, or HGF, together with the indicated concentration of HCPS (or other modified heparins) were added and mixed for 30 min at room temperature. Subsequently, 50 μl of washed heparin-beads solution was added to each microtube and the solu-

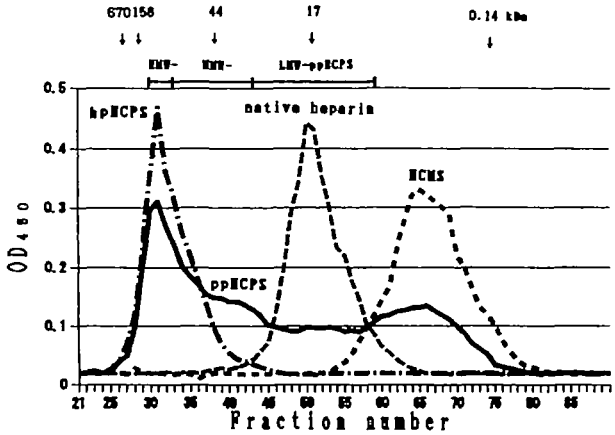

Fig 1 **Gel-filtration chromatography of HCPSs.** Molecular weights of hpHCPS, ppHCPS, and HCMS were compared to that of native heparin by gel filtration chromatography, as described in "MATERIALS AND METHODS." Fractions corresponding to HMW-ppHCPS, MMW-ppHCPS, and LMW-ppHCPS were pooled separatel and purified to examine (i) the binding ability to heparin-binding growth factors and (ii) inhibition of growth factor–induced cell growth.

tions were mixed for 30 min. The heparin-beads were then washed thoroughly by several centrifugations and 100 μl of anti–VEGF, anti-FGF-2 or anti-HGF (diluted 1:500 with BSA-PBS) was added to the beads in each tube, and mixed for 60 min at room temperature. The beads in each tube were again washed thoroughly, and 100 μl of anti-IgG horseradish peroxidase conjugate (diluted 1:1,000 in BSA-PBS) was added to the beads and mixed for 60 min at room temperature. The beads in each tube were finally washed thoroughly, and color developed by adding 100 μl of horseradish peroxidase substrate solution (Bio-Rad) and mixing for 30 min at room temperature. After centrifugation, the supernatant was transferred into the wells of a 96-well microtiter plate (Falcon, Tokyo) and the OD was read at 414 nm using an Immuno Mini Plate Reader (Nunc Inter-Med, Tokyo).

Cell Culture and Proliferation Assay—Human coronary endothelial cells were purchased from Takara Biomedical, Kyoto. The cells used in this work were between the 4th and 8th passage and grown in medium 199 (Life Technologies Oriental, Tokyo) supplemented with 10% heat-inactivated FBS, antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) and 10 ng/ml FGF-2.

To assay the inhibition of growth factor–induced coronary endothelial cell growth, cells were seeded at an initial density of 5,000 cells/well in 96-well tissue culture plates. The cells were grown for 3 days in 200 μl medium 199 supplemented with either 6 ng/ml $VEGF_{165}$, 15 ng/ml $VEGF_{121}$, 3 ng/ml FGF-2, or 20 ng/ml HGF, 10% heat inactivated FBS, the two antibiotics (as above), and the polysaccharides to be tested. To assay the growth-restoration of chlorate-treated coronary endothelial cells, the cells were seeded at an initial density of 5,000 cells/well, and grown for 3 days in DMEM lacking sulfate, but supplemented with 6 ng/ml $VEGF_{165}$, 3 ng/ml FGF-2 or 20 ng/ml HGF, 15 mM sodium chlorate, 10% dialyzed FBS, 200 U/ml penicillin G, and the polysaccharides to be tested. After incubation, the medium was removed and 100 μl fresh medium including 10 μl WST-1 reagent (cell counting kit, Dojindo, Tokyo) was added to each well. The optical densities were then read at 450 nm in an Immuno Mini plate reader (Nunc InerMed Japan).

RESULTS

Disaccharide Analysis of Native Heparin, IO_4-Heparin, and IO_4-LMW-Heparin—The complete oxidation of heparin with periodate (IO_4-heparin) results in the total loss of the unsulfated UA-containing structures (*14*). Therefore, IO_4-heparin and its alkaline-degraded form (IO_4-LMW-heparin) might be enriched in tri-sulfated disaccharide [UA(2-O-S)-GlcNS(6-O-S)] or di-sulfated disaccharide [UA(2-O-S)-GlcNS] units. As shown in Table I, the percent ratios of UA(2-O-S)-GlcNS(6-O-S) units in native heparin from porcine intestine, IO_4-heparin and IO_4-LMW-heparin were 69, 86.2, and 85.6%, respectively. Thus, IO_4-heparin and IO_4-LMW-heparin chains linked to the polystyrene core in the HCPS preparation are composed primarily of trisulfated disaccharide units.

Ability of HCPSs to Inhibit the Binding of Growth Factors to Heparin-Beads—ELISA using heparin-beads has been carried out to evaluate the interaction between HCPSs and $VEGF_{165}$, FGF-2 or HGF, as described previously (*9*). This assay measures the ability of polysaccharides, including HCPSs, native heparin and chemically modified heparins, to inhibit the binding of growth factors to heparin-beads. The amount of growth factor bound to the heparin-beads is quantified by a general ELISA procedure using a polyclonal antibody against the growth factor.

Table II shows the so-called half inhibition concentration (IC_{50}; defined as the concentration at which 50% of growth factor binding to heparin-beads is inhibited) for hpHCPS, HMW-ppHCPS, MMW-ppHCPS, LMW-ppHCPS, HCMS, native heparin, and heparin-derivatives (the intermediate products formed during the preparation of HCPS). Native heparin shows the ability to inhibit the binding of three growth factors, $VEGF_{165}$, FGF-2, and HGF, to heparin-beads with IC_{50} values of 70 ± 25, 80 ± 20 and 110 ± 30 μg/ml, respectively. IO_4-heparin, IO_4-LMW-heparin, and HCMS inhibit the binding of these growth factors to heparin-beads with IC_{50} values comparable to those of native heparin. In contrast, hpHCPS and ppHCPSs exhibit enhanced abilities to inhibit the binding of $VEGF_{165}$, FGF-2, and HGF to heparin-beads in a molecular weight-dependent manner. Especially HMW-ppHCPS inhibits the binding of $VHGF_{165}$, FGF-2, and HGF to heparin-beads with IC_{50} values of 12 ± 4, 11 ± 5, and 7 ± 2 μg/ml, respectively. On the other hand, PVLA (lactose-carrying polystyrene) and PVMA (mannose-carrying polystyrene) does not inhibit, even at a concentration of 256 μg/ml. These findings strongly suggest that HCPSs are able to interact more strongly with heparin-binding growth factors, $VEGF_{165}$, FGF-2, and HGF, than native heparin or the heparin-derivatives. The formation of highly charged and high density

TABLE I **Disaccharide composition of native heparin, IO_4-heparin, and IO_4-LMW-heparin.**

Disaccharide	Native heparin (%)	IO_4-heparin (%)	IO_4-LMW-heparin (%)
UA-GlcNAc	3.7	0	0
UA-GlcNS	2.1	0	0
UA-GlcNAc(6-O-S)	3.0	0	0
UA(2-O-S)-GlcNAc	1 8	0	0
UA-GlcNS(6-O-S)	9 2	0	0
UA(2-O-S)-GlcNS	7.8	9 9	9.7
UA(2-O-S)-GlcNAc(6-O-S)	1.4	0	0
UA(2-O-S)-GlcNS(6-O-S)	69.0	86.2	85 6
Total	98.0	96.1	95.3
Yield of disaccharide formation (%)	92.1	72.2	85.1

TABLE II. **Ability of HCPS to inhibit the binding of growth factors to heparin-beads.**

	Half inhibition concentration, IC_{50}(μg/ml)		
	$VEGF_{165}$	FGF-2	HGF
hpHCPS	14±5	15±7	8±2
HMW-ppHCPS	12±4	11±5	7±2
MMW-ppHCPS	21±8	25±7	21±6
LMW-ppHCPS	60±10	62±15	70±20
HCMS	80±12	95±30	120±30
Native heparin	70±25	80±20	110±30
IO_4-heparin	70±20	65±30	75±25
IO_4-LMW-heparin	75±30	100±35	125±25
PVLA	>256	>256	>256
PVMA	>256	>256	>256

Data represent mean ± variation of triplicate determinations.

interactive polysaccharide domains by clustering of IO_4-LMW-heparin chains associated with a polystyrene core might be a reason for such strong a binding property of HCPSs.

Ability of HCPSs to Inhibit $VEGF_{165}$-, FGF-2-, and HGF-Induced Cell Growth—The HCPSs were tested for their ability to inhibit $VEGF_{165}$-, FGF-2-, or HGF-induced human coronary endothelial cell growth. In Fig. 2, it can be seen that endothelial cells do not grow well in medium 199 supplemented with 10% heat-inactivated FBS without the addition of a growth factor such as $VEGF_{165}$, FGF-2, or HGF. Native heparin, IO_4-heparin, and IO_4-LMW-heparin at low concentrations (8–32 μg/ml) do not inhibit $VEGF_{165}$-, FGF-2-, or HGF-induced cell growth, whereas at higher concentrations (above 64 μg/ml), these polysaccharides inhibit growth factor–induced cell growths. In contrast, hpHCPS showed a stronger inhibitory effect on these growth factor–induced cell growths, even at a concentration of 8 μg/ml. As shown in Fig. 3, ppHCPSs also strongly inhibit $VEGF_{165}$, FGF-2, and HGF induced endothelial cell growth in a molecular weight–dependent manner. Thus, the abilities of HCPSs to inhibit heparin-binding growth factor–induced cell growth are correlated with their molecular weights.

Sodium chlorate, a potent inhibitor of sulfate adenylyltransferase, reduces the sulfation of various carbohydrates such as glycosaminoglycans (*11*, *15*). It has been reported that chlorate interferes with the ability of FGF-2 and $VEGF_{165}$ to stimulate the growth of endothelial cells, and that inhibition of growth factor–mediated cell growth by chlorate can be overcome by exogenous native heparin (*11*). We have designed a mitogenic assay to evaluate whether HCPS is (as in the case of heparin) able to restore the mitogenic activity of growth factors in chlorate-treated human coronary endothelial cells. Coronary endothelial cells incu-

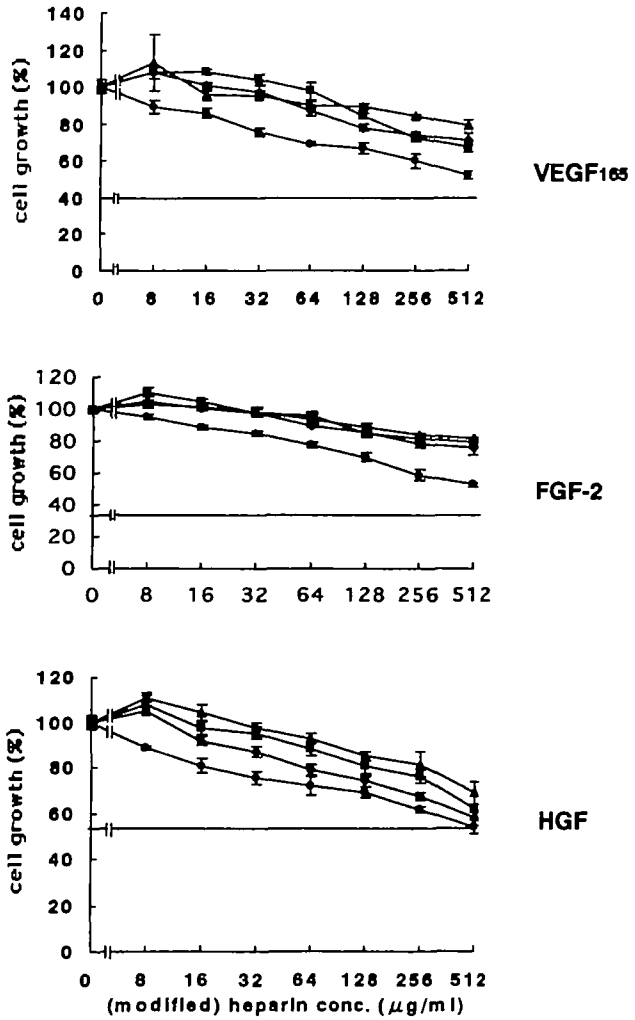

Fig 2. **Inhibition of heparin-binding growth factor–induced coronary endothelial cell proliferation by various heparinoids.** Human coronary endothelial cells were grown in the presence of the indicated concentrations of hpHCPS (●), native heparin (■), IO_4-heparin (▲), or IO_4-LMW-heparin (◆). The growth of cells incubated with either $VEGF_{165}$, FGF-2, or HGF in the absence of any heparinoids was defined as a 100% growth, and the growth data were calculated as percentages. The straight line in each panel represents the level of cell growth obtained in the absence of both heparinoids and growth factors. The results represent mean ± SD of triplicate determinations

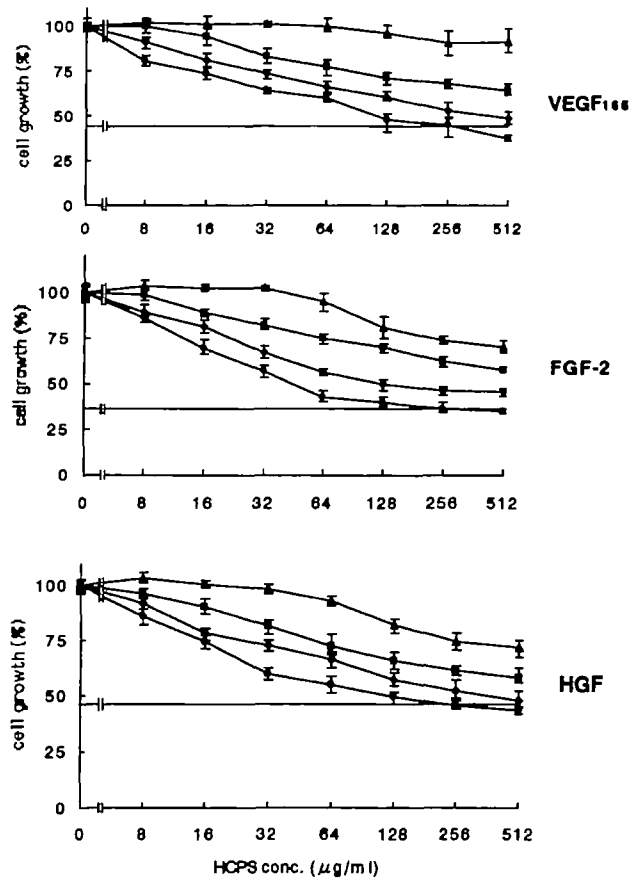

Fig 3. **Inhibition of heparin-binding growth factor–induced coronary endothelial cell proliferation by ppHCPSs with various molecular weights.** Human coronary endothelial cells were grown in the presence of the indicated concentrations of HCMS (▲), LMW-ppHCPS (■), MMW-ppHCPS (◆), or HMW-ppHCPS (●) The growth of cells incubated with either $VEGF_{165}$, FGF-2, or HGF in the absence of any heparinoids was defined as a 100% growth, and the growth data were calculated as percentages. The straight line in each panel represents the level of cell growth obtained in the absence of both heparinoid and growth factor. The results represent mean ± SD of triplicate determinations

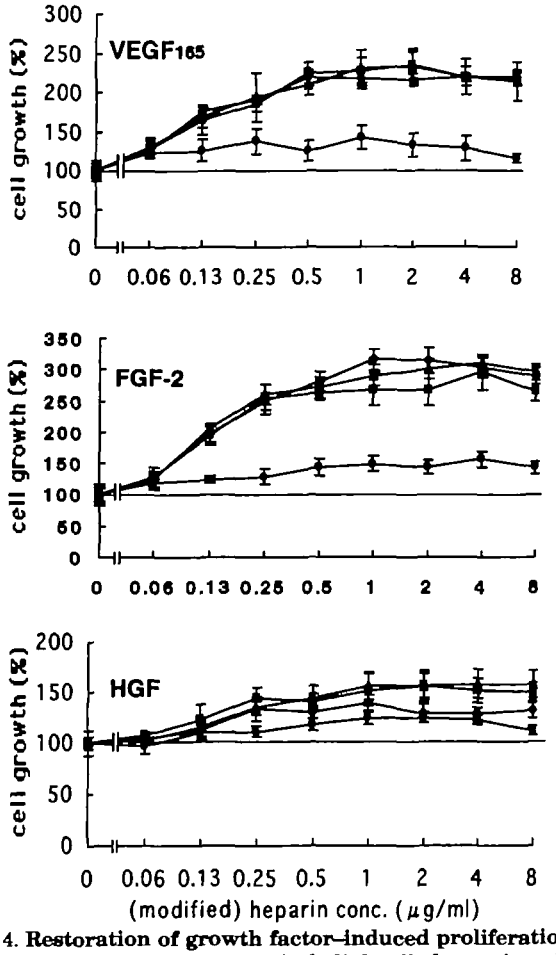

Fig. 4. **Restoration of growth factor–induced proliferation of chlorate-treated coronary endothelial cells by various heparinoids.** Human coronary endothelial cells were treated with chlorate in the presence of the indicated concentrations of either hpHCPS (●), native heparin (■), IO_4-heparin (▲), or IO_4-LMW-heparin (◆) The growth of cells in the presence of either $VEGF_{165}$, FGF-2, or HGF, but in the absence of any heparinoids was defined as a 100% growth, and the growth data were calculated as percentages. The straight line in each panel represents the 100% growth. The results represent mean ± SD of triplicate determinations.

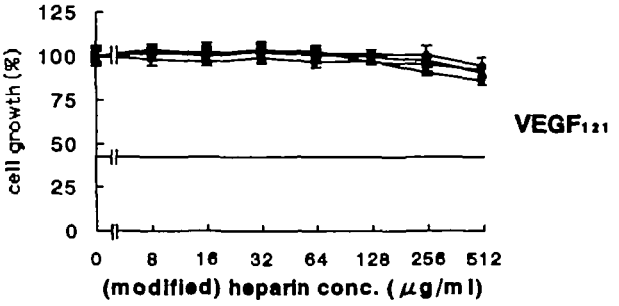

Fig. 5. **Absence of inhibition of $VEGF_{121}$-induced coronary endothelial cell proliferation by various heparinoids.** Human coronary endothelial cells were grown in the presence of hpHCPS (●), native heparin (■), IO_4-heparin (▲), or IO_4-LMW-heparin (◆) The growth of cells incubated with 15 ng/ml $VEGF_{121}$ in the absence of any heparinoid was defined as a 100% growth, and the growth data were calculated as percentages. The straight line represents the level of cell growth obtained in the absence of both heparinoid and $VEGF_{121}$ The results represent mean ± SD of triplicate determinations

bated in sulfate-free medium containing 15 mM sodium chlorate and either 6 ng/ml $VEGF_{165}$, 3 ng/ml FGF-2, or 20 ng/ml HGF did not grow well (data not shown), whereas the addition of native heparin, IO_4-heparin or IO_4-LMW-heparin restored growth factor–induced cell growth in a concentration-dependent manner (Fig. 4). Interestingly, hpHCPS that exhibited a strong ability to interact with these growth factors showed almost no ability to restore growth factor–induced cell growth. Similarly, the additions of HMW-ppHCPS and MMW-ppHCPS did not restore growth factor–induced cell growth (data not shown).

No Inhibitory Activity of HCPSs on $VEGF_{121}$-Induced Endothelial Cell Growth—The 121–amino acid isoform of VEGF ($VEGF_{121}$) differs from larger VEGF isoforms ($VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) in that it is the only VEGF type that does not bind to heparin and HS (*16*, *17*). In fact, with our ELISA assay (Table II) no $VEGF_{121}$ bound to heparin-beads was detected, even in the absence of HCPSs, native heparin and heparin-derivatives (data not shown). However, the addition of 15 ng/ml $VEGF_{121}$ stimulated endothelial cell growth (Fig. 5). In contrast to the inhibitory effect of HCPSs on heparin-binding growth factor ($VEGF_{165}$, FGF-2, or HGF)–induced endothelial cell growth, hpHCPS, native heparin, and heparin-derivatives did not inhibit $VEGF_{121}$-induced endothelial cell growth up to concentrations of 512 μg/ml (Fig. 5). Furthermore, HMW-ppHCPS, MMW-ppHCPS, LMW-ppHCPS, and HCMS also showed no ability to inhibit $VEGF_{121}$-induced endothelial cell growth (data not shown). These results suggest that the inhibitory ability of HCPSs on heparin-binding growth factor–induced endothelial cell growth might be due to extremely strong interactions of the HCPSs with the heparin-binding growth factors, and that high concentrations of HCPSs (up to 512 μg/ml) do not cause direct cell damage.

DISCUSSION

We previously reported that hpHCPS is efficiently adsorbed to the surface of polystyrene dishes and that this hpHCPS-coating provides an excellent adhesive substratum for various cultured cells (*10*). In addition, cell growth can be selectively controlled by hpHCPS-coating and the preservation of heparin-binding growth factors on the hpHCPS-coated surface (*10*). On the other hand, hpHCPS molecule in solution contains a high density of heparin chains towards solution that can be ascribed to the following: (i) the IO_4-LMW-heparin chains are attached to all repeating units along a polystyrene backbone and (ii) this hydrophobic polystyrene might be coiled and buried inside the molecule to form a hydrophobic core that is hidden from the water. The hydrophilic carbohydrate chains tend to be oriented to the outside of the polymer, resulting in a higher concentration of carbohydrates on the polymer surface. It is known that the ability of a cell surface receptor to recognize its target is greatly enhanced by an increase in the density of carbohydrate chains in an interacting molecule (*18*, *19*). Similarly, enhanced biological activities due to the carbohydrate-clustering effect and immobilization of carbohydrate-clustered proteoglycans has been reported and ascribed to the presence of multiple glycosaminoglycan chains in one core pro-

tein (*20–22*). The purpose of the present study was to examine the carbohydrate-clustering effect of HCPSs in culture medium solution and to determine its ability to inhibit the activities of various heparin-binding growth factors to endothelial cells. The main conclusions from this study are that HCPSs in culture medium solution have enhanced abilities to interact with heparin-binding growth factors and to inhibit the growth factor–induced endothelial cell growth in a molecular weight–dependent manner.

It has been reported that heparin structures enriched in trisulfated disaccharide units (*i.e. N*-sulfate and 6-*O*-sulfate groups in glucosamine residues and a 2-*O*-sulfate group in UA residues) would bind strongly to $VEGF_{165}$, FGF-2, and HGF (*9*). Since only UA residues lacking a sulfate-substituent at their C2 positions are susceptible to oxidation by periodate, the periodate-oxidation of heparin generates a higher ratio of trisulfated disaccharide units (*14*). In fact, trisulfated disaccharide units in native heparin from porcine intestine, IO_4-heparin, and IO_4-LMW-heparin used in this study account for about 69, 86, and 86% of the total disaccharide units, respectively (Table I). Thus, the heparin moieties of HCPSs prepared from IO_4-LMW-heparin are also enriched in trisulfated disaccharide units. Furthermore, HCPSs generate highly negative-charged sites and a high density of interactive saccharide-domains with the heparin-binding growth factors since they contain clustered IO_4-LMW-heparins.

The evaluation of the interaction of HCPSs with heparin-binding growth factors by ELISA showed that binding of $VEGF_{165}$, FGF-2, and HGF to heparin-beads is more strongly inhibited by HCPSs (in a molecular weight–dependent manner) than by native heparin, IO_4-heparin, or IO_4-LMW-heparin (Table II). Especially, hpHCPS and HMM-ppHCPS, which have high molecular weights, showed a 6–18-fold stronger ability to inhibit the binding of growth factors to the heparin-beads when compared to those of HCMS, native heparin, IO_4-heparin, and IO_4-LMW-heparin. These results suggest that the enhanced inhibitory ability of HCPSs might be due to the formations of highly negative-charged sites and high density of interactive saccharide-domains with the growth factors by the clustering of IO_4-LMW-heparin chains in HCPSs.

HCPSs strongly inhibited heparin-binding growth factor–induced endothelial cell proliferation in a molecular weight–dependent manner, whereas HCMS, native heparin, IO_4-heparin, and IO_4-LMW-heparin moderately inhibited these cell proliferations only at rather high concentrations (about 64 μg/ml) (Figs. 2 and 3). It might be possible that HCPSs with high molecular weights can effectively inhibit the specific interaction between the heparin-binding growth factors and cell surface heparan sulfate proteoglycans, which are known to be co-receptors of the heparin-binding growth factors, probably through the extremely strong interaction between HCPSs and heparin-binding growth factors. On the other hand, HCPSs do not inhibit the mitogenic activity of $VEGF_{121}$, being of a non-heparin-binding nature even at a concentration of 512 μg/ml. Thus, HCPSs can strongly inhibit the activity of heparin-binding growth factors without causing direct damage to the endothelial cells.

In previous studies, we showed that $VEGF_{165}$ (*9*) and FGFs (*8*, *11*, *23*) lose significant amounts mitogenic activity upon treatment of the endothelial cells with chlorate. The exogenous addition of native heparin, IO_4-heparin, and IO_4-LMW-heparin partially restored the mitogenic activity of $VEGF_{165}$, FGF-2, and HGF, although hpHCPS also consisting of a cluster of IO_4-LMW-heparin chains showed a very limited ability to restore growth factor–induced cell growth. A summation of effects resulting from the binding of polysaccharides to growth factors and from interactions at the receptor level might explain the restoration of the mitogenic activities of these growth factors by adding native heparin, IO_4-heparin, and IO_4-LMW-heparin. It may be possible that too high density and clustering structure of heparins in hpHCPS molecules does not support the overall interaction of these growth factors with receptors to induce mitogenic activities. Further study is required to better understand the stimulatory effects of heparins on the growth factor activities.

In summary, we demonstrated enhanced abilities of HCPSs to interact with various heparin-binding growth factors and to inhibit activities of certain growth factors. Studies to evaluate the efficacy of HCPS in solution to prevent angiogenesis and restenosis *in vivo* are currently in progress.

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