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Stimulation of Hepatocyte Growth Factor Production by Heparin-derived Oligosaccharides

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We previously reported that heparin post-transcriptionally stimulates the production of hepatocyte growth factor (HGF). In this study, we addressed the size-dependency of heparin fragments on the HGF-inducing activity aiming to obtain fragments without antiblood coagulant activity. Heparin fragments, produced by digestion with heparinase, were size-fractionated and tested for HGF-inducing activity in cultured human fibroblasts. The HGF-inducing activity deceased with the reduction in oligosaccharide size. Decasaccharides exerted an activity comparable with undigested heparin, while smaller oligosaccharides showed lesser activities. The anticoagulant activity of heparin fragments also decreased with size and anticoagulant activity of decasaccharides was <13% that of undigested heparin. Further fractionation of decasaccharides by anion-exchange chromatography revealed that most of the decasaccharides had HGF-inducing activity and the extent of sulfation was roughly related to the activity. The lack of *N*-sulfation in heparin markedly reduced HGF-inducing activity, whereas 2-*O*-desulfation or 6-*O*-desulation had a lesser influence. Moreover, an *N*-sulfated disaccharide showed significant HGF-inducing activity, suggesting the involvement of *N*-sulfation in HGF-inducing activity. Because of the much reduced anticoagulant activity, potential applications of heparin-derived oligosaccharides such as decasaccharides is considerable as a therapeutic agent for many diseases.

Key words: glycosaminoglycan, heparan sulfate, heparin, HGF, oligosaccharide.

Abbreviations: HGF, hepatocyte growth factor; HS, heparan sulfate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; UA, hexuronic acid; ΔUA, $\Delta^{4,5}$ unsaturated hexuronic acid; GlcN, *N*-unsubstituted glucosamine; GlcNS, *N*-sulfated glucosamine; GlcNAc, *N*-acetylglucosamine; 2S, 2-*O*-sulfate; 6S, 6-*O*-sulfate.

Heparin is a polysaccharide comprised of repeating disaccharide units consisting of hexuronic acid and glucosamine residues, in which the hexuronic acid is present either as D-glucuronic acid or its C-5 epimer, L-iduronic acid (Fig. 1A). The glucosamine residues are either *N*-acetylated or *N*-sulfated or unsubstituted at the C-2 position. The disaccharide unit can also be *O*-sulfated, primarily at the C-2 position of an L-iduronic acid residue and the C-6 position of a D-glucosamine residue. Sulfation does not occur at every potential sulfation site, thereby conferring structural complexity and heterogeneity to the heparin molecule (*1*).

Heparin is known to possess antiblood coagulation activity (*2*) and the use of heparin as an anticoagulant agent is well established in clinical practice. In addition, heparin experimentally shows a wide range of biological activities, including lipolytic activity (*3*), inhibition of complement activation (*4*), regulation of angiogenesis (*5*, *6*), antiviral activity (*7*) and anti-inflammatory activity through modulating the functions of immune cells such as T cells and neutrophils (*8*), although most of these activities may be exerted by heparan sulfate (HS) *in vivo*. Heparin also regulates the activities or functions of a variety of proteins *in vitro*, including enzymes, growth factors, extracellular matrix proteins and cell surface proteins, through interaction with them (*9*).

Apart from the afore-mentioned functions, we previously reported that heparin has a unique biological function as an inducer of hepatocyte growth factor (HGF) (*10*). HGF, originally identified and cloned as a potent mitogen for mature hepatocytes (*11–13*), exerts multiple biological activities, including mitogenic, motogenic and morphogenic activities on a variety of cells, and thus plays roles in development, regeneration and malignant transformations (reviewed in *14*, *15*). Because heparin is released from mast cells that play important roles in tissue repair, heparin may physiologically play roles in tissue repair as an HGF inducer. The detailed mechanism(s) of how heparin stimulates HGF production remains unknown, but heparin stimulates the

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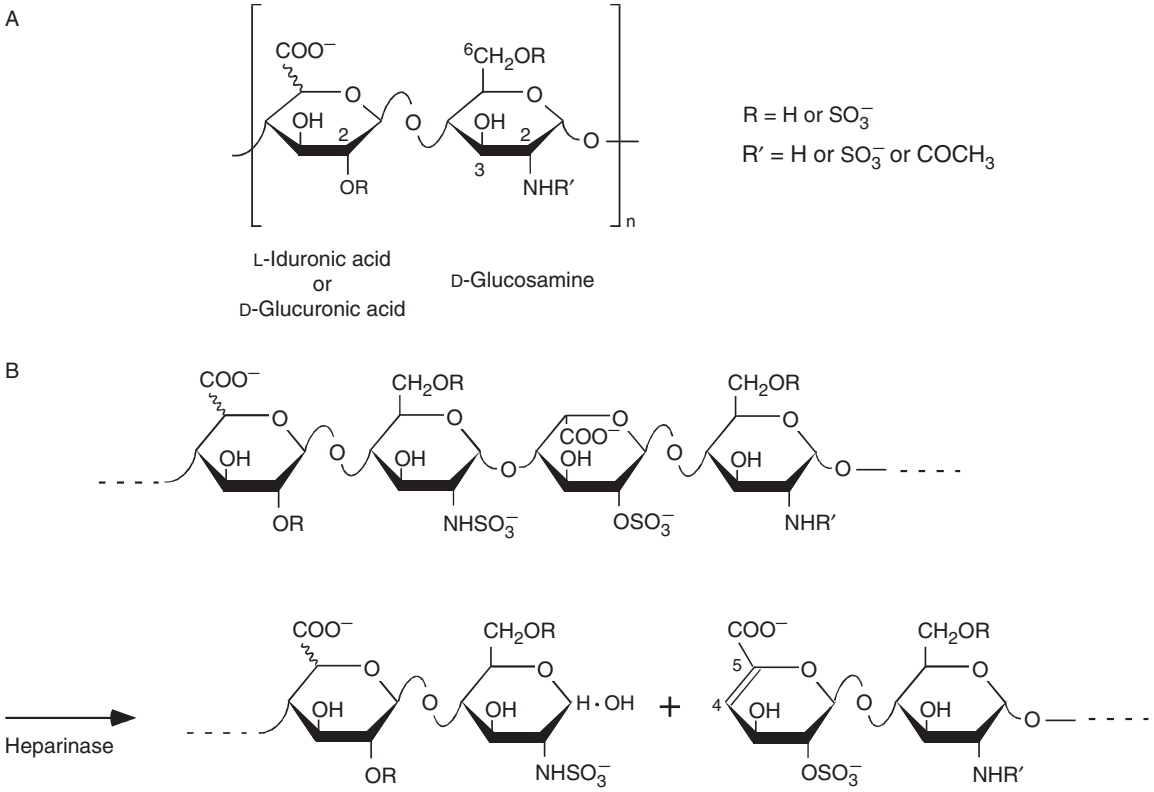

Fig. 1. **Structure of the disaccharide unit that comprises heparin (A) and the cleavage of heparin by heparinase (B).** Three major sulfation sites (the C-2 position of the L-iduronic acid residue, and the C-2 and C-6 positions of the D-glucosamine residue) are located in the disaccharide unit. The C-3 position of the D-glucosamine residue can also be sulfated but the frequency of this is low. Heparinase cleaves the glycosidic bond between an *N*-sulfo-6-*O*-sulfo (or OH) glucosamine residue and a 2-*O*-sulfo iduronic acid residue, forming a $\Delta^{4,5}$ double bond in the uronic acid at the non-reducing end of the product.

biosynthesis of HGF by affecting post-transcriptional process(es) (*10*).

The function of heparin as an HGF inducer might have clinical potential, however, the anticoagulant activity of heparin will be a side effect for this purpose. If the removal of anticoagulant activity from heparin while maintaining its activity for stimulating HGF production could be achieved, the use of heparin as an HGF inducer seems to be practical. Most of the functional properties of heparin are regulated by specific structural motifs within the heparin molecule. The depolymerization of heparin to a low molecular size often causes a reduction in its individual activities according to the extent of removal of the relevant structural motifs. It is also known that the anticoagulant activity of heparin is reduced by depolymerization (*16*, *17*).

Based on this background, we speculated that some fragments derived from heparin might retain HGF-inducing activity but might have a reduced anticoagulant activity. To obtain such fragments, we digested heparin with heparinase and assessed the activity of the resulting fragments. In the present study, we report on decasacharide fragments that have activity for stimulating HGF production with low anticoagulant activity. In addition, information on the structural characteristics of heparin molecules involved in HGF-inducing activity is provided.

EXPERIMENTAL PROCEDURES

Reagents—Porcine mucosal heparin was obtained from Scientific Protein Laboratories, Inc. (Waunakee, WI, USA). Heparin disaccharides ΔUA(2S)-GlcNS(6S), ΔUA-GlcNS, ΔUA-GlcNAc, were purchased from Seikagaku Co. (Tokyo, Japan), where ΔUA denotes a $\Delta^{4,5}$ unsaturated hexuronic acid, GlcN an *N*-unsubstituted glucosamine, GlcNS an *N*-sulfated glucosamine, GlcNAc an *N*-acetylglucosamine and 2S and 6S 2-*O*- and 6-*O*-sulfate groups, respectively. HS and completely desulfated *N*-acetylated HS were also obtained from Seikagaku Co., chemically *N*-desulfated, *N*-acetylated heparin and chemically 2-*O*-desulfated or 6-*O*-desulfated heparin were obtained from Neoparin (Alameda, CA, USA). Fragmin (low-molecular-weight heparin) was obtained from Kissei Pharmaceutical Co. (Matsumoto, Japan). The basic peptide $(arg\text{-}gly)_{19}arg$ was obtained from Medical and Biological Laboratories Co. (Nagoya, Japan).

Heparinase Treatment and Size Fractionation—Porcine mucosal heparin (50 mg) was digested with 0.25 U of heparinase (heparinase I, heparin lyase I, from *Flavobacterium heparinum*, EC 4.2.2.7, Seikagaku Co. No.100700) in 1 ml of 50 mM acetate-NaOH buffer, pH 7.0, containing 3 mM $Ca(OAc)_2$. The mixture was incubated at 37°C for 10 h and then heated at 100°C for

2 min to terminate the reaction. The digest was size-fractionated by gel filtration high performance liquid chromatography (HPLC) on a column (1.6×60 cm) of Superdex 30 pg (Amersham Biosciences, Piscataway, NJ, USA). Elution was performed with 0.2 M NH_4HCO_3 at a flow rate of 0.4 ml/min and the eluate was monitored by absorption at 232 nm, caused by the $\Delta^{4,5}$ site of the hexuronic acid at the non-reducing end (Fig. 1B). Fractions that contained oligosaccharides were pooled and lyophilized.

Anion-exchange Chromatography—The fractions obtained after size fractionation were re-dissolved in H_2O and further separated by anion-exchange HPLC using a MiniQ^{TM}4.5/50 column (Amersham Biosciences). Elution was performed at a flow rate of 0.5 ml/min with a linear gradient of 0–1.0 M NaCl in H_2O, and the absorption was monitored at 232 nm. Each peak was collected and tested for the activity to stimulate HGF production.

Quantitation of Oligosaccharides—The amount of oligosaccharides obtained after heparinase digestion was determined on the basis of uronic acid by analysis by the carbazole method (*18*) using heparin as a standard.

Cell Culture and Measurement of HGF in Conditioned Medium—MRC-9 cells (human embryonic lung fibroblasts, CCL212) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). MRC-9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were seeded on 48-well plates at a density of 5×10^4 cells/cm^2, and cultured for 24 h. After replacing the medium with fresh medium supplemented with 1% fetal calf serum, the test samples were added, the cells were cultured for 24 h, and the amount of HGF in the medium was then determined by an enzyme-linked immunosorvent assay (ELISA) as described (*19*).

Assay of Antiblood Coagulant Activity—Antiblood coagulant factor Xa and antifactor IIa activities were measured using the chromogenic substrate method (*20*, *21*), with minor modifications. Briefly, for the measurement of antifactor Xa activity, 22.5 μl of test sample was mixed with 2.5 μl of antithrombin III solution (2.5 mU), and the mixture was incubated at 37°C for 3 min. Then, 12.5 μl of factor Xa solution (7.1 ncat S-2222/ml) was added to the mixture followed by incubation for 30 sec at 37°C. The reaction was started by adding 25 μl of a solution of chromogenic substrate S-2222 (Daiichi Pure Chemicals Co., Tokyo, Japan) (0.75 mg/ml), stopped after 3 min by adding 37.5 μl of 50% acetic acid, and the absorbance at 405 nm was measured. An international standard low molecular weight heparin (National Institute of Health Sciences, Tokyo, Japan) was used as a standard. Measurement of anti-factor IIa activity was performed similarly, except that solutions of factor IIa (0.4 U/ml) and S-2238 (0.625 mg/ml) were added instead of factor Xa and S-2222, respectively. The reaction was stopped by the addition of 37.5 μl of 0.01 M citric acid.

MALDI-TOF/MS Analysis—Matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF/M S) analysis was performed using an Ultraflex (Bruker Daltonics, Bremen, Germany) reflectron time-of-flight instrument in the linear mode. Heparin fragments were dissolved in H_2O to give a 2 mg/ml solution. One microlitre of the solution was added to 1 μl of matrix solution (12 mg/ml caffeic acid in 30% acetonitrile) containing a 10-fold molar excess of basic peptide $(arg\text{-}gly)_{19}arg$ (calculated mass of the $[M+H]^+$ ion = 4226.8). The basic peptide was added to specifically chelate heparin oligosaccharides (*22*, *23*). Samples were spotted on the AnchorChip^{TM} target (Bruker Daltonics), and mass spectra were then acquired. The observed mass spectra were $[M+H]^+$ ions of a 1: 1 complex of the basic peptide and an oligosaccharide. The mass of the oligosaccharide was determined by subtracting the *m/z* value of the $[M+H]^+$ ion of the basic peptide from that of the 1:1 complex. All spectra were calibrated externally using Protein Calibration Standard I (Bruker Daltonics) under identical instrumental parameters.

RESULTS

Relationship Between Molecular Size of Heparin Fragments and HGF-inducing Activity—We first analysed the relationship between the molecular size of the heparin fragments and their activity for stimulating HGF production. Porcine mucosal heparin was partially digested with heparinase and the digest was size-fractionated by gel filtration (Fig. 2A). Fractions containing oligosaccharides were individually collected and each fraction was designated hp2, hp4, hp6, hp8, hp10 and hp12, respectively, from the last fraction. The elution position of hp2 corresponded to that of an authentic sample of heparin disaccharide ΔUA(2S)-GlcNS(6S). When the individual fractions were analysed by MALDI-TOF/MS as non-covalent complexes with the basic peptide $(arg\text{-}gly)_{19}arg$ (*22*, *23*), the average masses of hp2, hp4, hp6, hp8, hp10 and hp12 coincided with the estimated molecular sizes of di-, tetra-, hexa-, octa-, deca- and dodeca-oligosaccharides, respectively (data not shown).

To determine the activity to stimulate HGF production, fractions were desalted and added to cultures of MRC-9 human embryonic lung fibroblasts and the amount of HGF in the culture medium was measured (Fig. 2B). Undigested heparin stimulated HGF production in a dose-dependent manner and the maximum activity was found at concentrations >1 μg/ml. The stimulatory effect of hp12 was almost the same as that of undigested heparin. The stimulatory effect of hp10 was slightly lower than that of undigested heparin at concentrations <5 μg/ml, however, the maximal stimulatory effect at concentrations >5 μg/ml was almost the same as that of undigested heparin. In the case of oligosaccharides smaller than hp10, the stimulatory effects decreased with the reduction in molecular size. At concentrations of 10 μg/ml, hp8 and hp6 showed 83.6% and 64.3%, respectively, of the stimulatory activity of undigested heparin. A weak stimulatory effect was seen for hp4 at a concentration of 10 μg/ml, whereas hp2 did not show any stimulatory effect at any of the concentrations tested (~10 μg/ml).

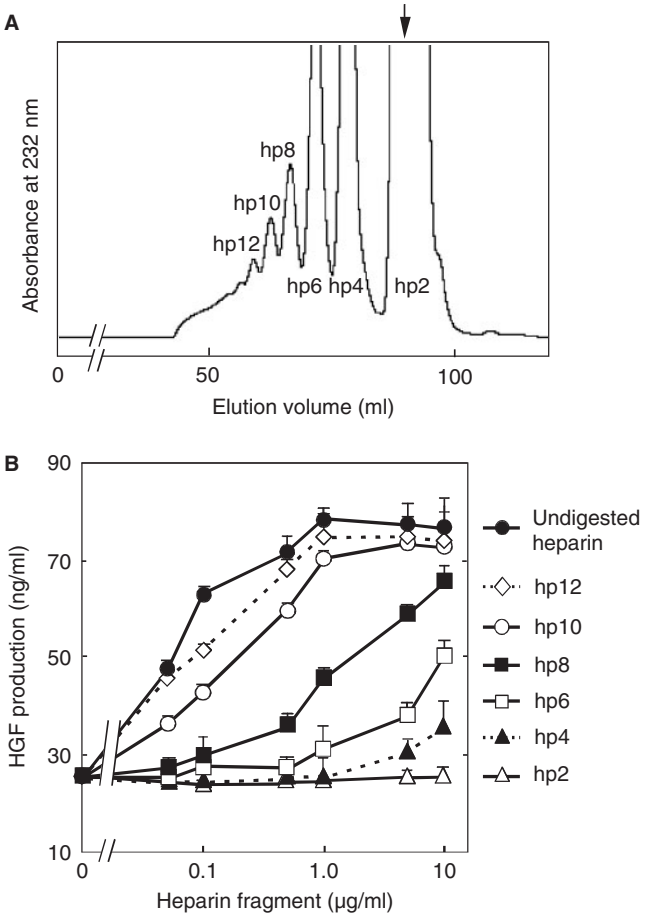

Fig. 2. **Size-fractionation of heparin-derived oligosaccharides and their HGF-inducing activity.** (A) Elution profile of heparin-derived oligosaccharides in gel-filtration HPLC. Fractions were collected and are designated as hp2, hp4, hp6, hp8, hp10 and hp12, respectively, from the smallest size. The position of elution of an authentic disaccharide is indicated by an arrow. (B) HGF-inducing activity in size-fractionated oligosaccharide pools. Each fraction was lyophilized, re-dissolved in distilled water, and its activity for stimulating HGF production was assayed using cultured MRC-9 cells. Cells were cultured for 24 h and the HGF concentration in the medium was measured by ELISA.

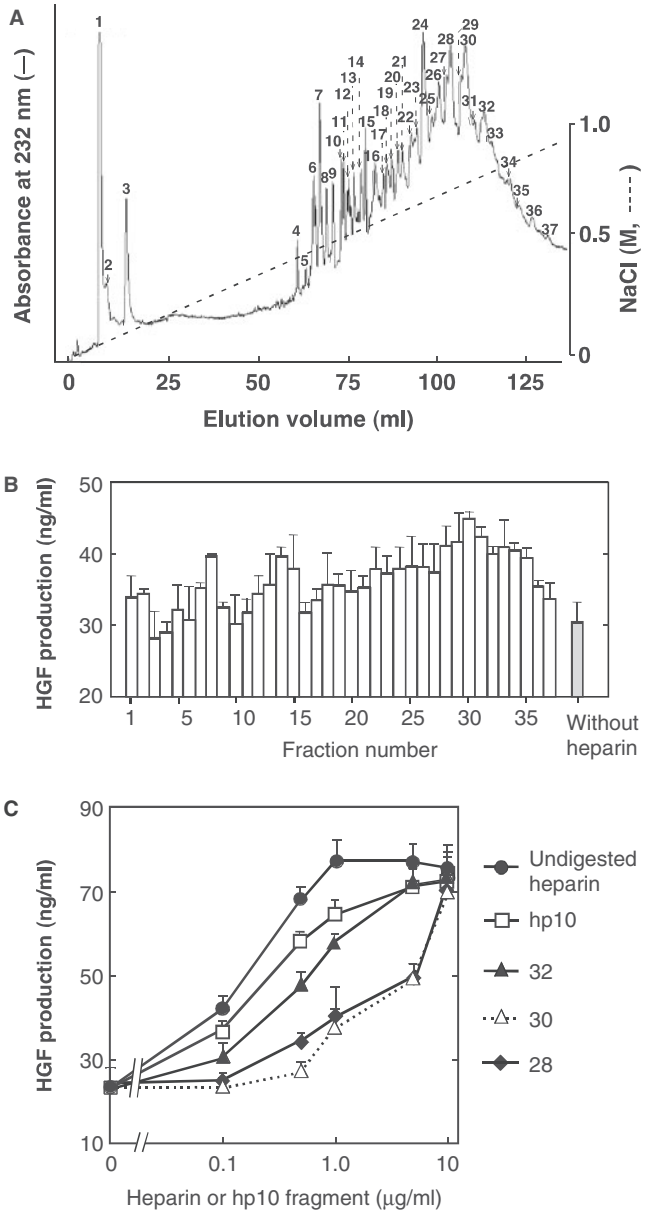

Fig. 3. **Fractionation of decasaccharides by anion-exchange chromatography and their HGF-inducing activity.** (A) Elution profile of the anion-exchange HPLC of decasaccharide fraction. The hp10 fraction obtained by gel-filtration was separated on an anion-exchange column. (B) HGF-inducing activity of each peak. An aliquot of each fraction was directly added to the culture medium of MRC-9 cells at 5% in volume. (C) Dose-dependent stimulation of HGF production in MRC-9 cells by selected fractions obtained by anion-exchange HPLC. Fractions 28, 30, 32 were desalted, re-dissolved in distilled water, and their activity to stimulate HGF production was determined using MRC-9 cells.

Separation of hp10 Fraction by Anion-Exchange Chromatography—Because hp10 showed the maximal HGF-inducing activity comparable with undigested heparin, the hp10 fraction was further separated by anion-exchange HPLC (Fig. 3A). The hp10 fraction was separated into a series of peaks. When the activities of individual fractions were roughly analysed by adding the fraction solution directly to the culture medium of MRC-9 cells so that the fraction solution was 5/100 of the medium, most fractions contained HGF-inducing activity to varying extents (Fig. 3B). Even so, the HGF-inducing activities of fractions around No. 30, that were eluted at high salt concentrations, were relatively higher compared with the other fractions. To analyse the HGF-inducing activities of fractions around No. 30 more precisely, selected fractions (No. *28, 30, 32*) were desalted and their concentration-dependent stimulatory effect on HGF production was analysed (Fig. 3C). Among these fractions, fraction 32 showed the highest activity and stimulated HGF production to a 3.2-fold higher level than the control level at a concentration of 10 μg/ml, similar to the activity of the hp10 fraction mixture.

Activities of fractions 28 and 30 were lower than that of fraction 32 at concentrations under 5 μg/ml. Thus, oligosaccharides eluted at high salt concentrations showed relatively high activity. Although we expected the presence of fractions showing higher activity than unfractionated hp10, none were found. Combinations of active decasaccharides with specific structures might have an additive effect on HGF production and, thus, an hp10 mixture might have the highest activity.

Oligosaccharides eluted at higher salt concentrations are generally considered to be more highly sulfated. Therefore, a higher degree of sulfation of oligosaccharides appeared to be preferable. To address the involvement of the extent of sulfation in HGF-inducing activity, selected fractions from the anion-exchange HPLC were analysed by MALDI-TOF/MS (Supplementary Data, Fig. S1, Table S1). As expected, the decasaccharides in fraction 32 were sulfated to a greater extent than those in fractions 24, 28 and 30. Thus, a higher degree of sulfation in heparin-derived oligosaccharides appeared to confer a higher activity for enhancing HGF production, at least to a certain extent. However, the highest level of sulfation may not be associated with the highest activity for enhancing HGF production.

Antiblood Coagulant Activities of Heparin Fragments—The anti-blood coagulant activity of heparin fragments decreases depending on their size (*16*, *17*). To examine the size dependency of heparin fragments on anticoagulant activity in comparison with that on HGF-inducing activity, we measured the anticoagulation factor Xa and antifactor IIa activities of the size-fractionated heparin fragments (Table 1). The anticoagulant activities of size-fractionated heparin fragments were significantly lower than undigested heparin and low molecular weight heparin, Fragmin, which is clinically used as an anticoagulant agent. The anticoagulant activity of hp10, which exhibited HGF-inducing activity comparable with that of undigested heparin, was only 12.2% and 1.3% that of undigested heparin on the basis of antifactor Xa activity and antifactor IIa activity, respectively. Heparin fragments smaller than octasaccharides, i.e. hp6, hp4, hp2 fractions, showed much lesser anticoagulant activities than hp8 and hp10, and their activities were marginal or undetectable.

Influences of Sulfated Positions on the HGF-inducing Activity—To characterize the structural characteristics involved in HGF-inducing activity, we analysed the activities of chemically desulfated heparin and HS (Fig. 4A). HS stimulated HGF production with a lesser potential than heparin as we previously reported (*10*). However, completely desulfated, *N*-acetylated HS showed no activity, suggesting that a skeletal structure comprised of repeating disaccharide units of hexuronic acid and glucosamine residues has no activity. Removal of individual sulfated groups showed different influences on the activity. In the case of *N*-desulfated, *N*-acetylated heparin, the activity was largely reduced compared with undigested heparin. The reduction in activity was also observed in the case of 2-*O*-desulfated and 6-*O*-desulfated heparins, however, the extent of the reduction was less than that of *N*-desulfated, *N*-acetylated heparin.

Table 1. **Anticoagulant activity of heparin and size-fractionated heparin fragments.**

	Antifactor Xa activity		Antifactor IIa activity	
	unit/mg	(%)	unit/mg	(%)
Heparin	203.9 ± 24.2	(100.0)	184.1 ± 24.1	(100.0)
Fragmin	152.0 ± 7.7	(74.5)	58.2 ± 0.6	(31.6)
hp10	24.9 ± 0.9	(12.2)	2.4 ± 1.2	(1.3)
hp8	26.0 ± 0.5	(12.8)	1.0 ± 0.5	(0.5)
hp6	4.0 ± 0.2	(2.0)	–	(–)
hp4	1.8 ± 0.1	(0.9)	–	(–)
hp2	0.6 ± 0.3	(0.3)	–	(–)

Antifactor Xa and antifactor IIa activities of heparin, Fragmin and size-fractionated heparin fragments were assayed using synthetic peptides S-2222 and S-2238 as chromogenic substrates. Activities were calculated against a standard curve for an international standard low-molecular-weight heparin. –, undetectable.

Thus, the *N*-sulfation of glucosamine residues appears to play a substantial role in stimulating HGF production, although 2-*O*-sulfation and 6-*O*-sulfation also appeared to contribute to the activity to a certain extent.

To verify the involvement of *N*-sulfation, we assessed the activity of the *N*-sulfated disaccharide (ΔUA-GlcNS)(Fig. 4B). The ΔUA-GlcNS had a lower activity than heparin, but it showed significant activity at concentrations >5 μg/ml and the level of HGF production at 50 μg/ml ΔUA-GlcNS approached the maximum enhancement induced by undigested heparin. On the other hand, a disaccharide containing no sulfate group (ΔUA-GlcNAc) had no activity even at a concentration of 50 μg/ml, indicating that the basal disaccharide composed of hexuronic acid and glucosamine residues has no activity. Thus, *N*-sulfation in the glucosamine residue was confirmed to play roles in the HGF-inducing activity. However, we found that the trisulfated disaccharide [ΔUA(2S)-GlcNS(6S)] does not show any activity despite the presence of *N*-sulfate group, indicating that *N*-sulfation is certainly involved in the HGF-inducing activity, but the activity can be affected by another sulfation.

DISCUSSION

The anticoagulant activity of heparin is potentiated through binding with antithrombin III, a plasma inhibitor of blood coagulation, for which a specific pentasaccharide sequence in a heparin molecule is necessary (*24–26*). The heparin–antithrombin III complex inactivates the proteolytic cascade involved in blood coagulation. The anticoagulant activity of heparin diminishes in a size-dependent manner, because the content of the specific pentasaccharide sequence decreases with size reduction.

In the present study, we clarified that the HGF-inducing activity of heparin is also dependent on the molecular size. The decasaccharide fraction (hp10) obtained after heparinase digestion showed an HGF-inducing activity comparable with undigested heparin, while oligosaccharide fractions smaller than decasaccharides showed a lower activity. Thus, a molecular size of a decasaccharide is necessary to stimulate

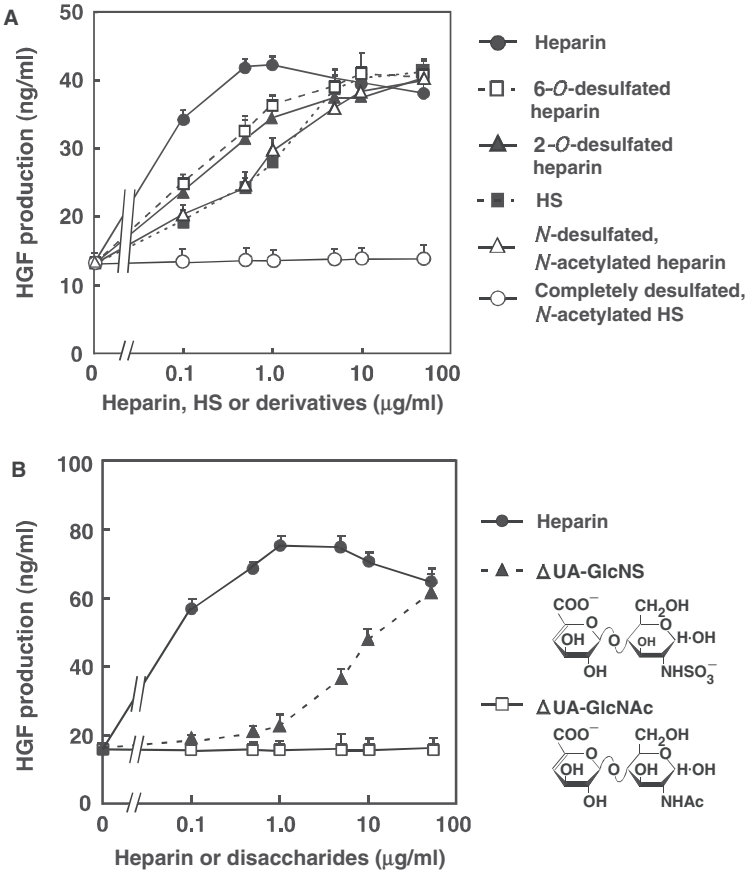

Fig. 4. **Influences of sulfated positions on HGF-inducing activity.** (A) Stimulation of HGF production by heparin, HS and their desulfated derivatives. (B) Stimulation of HGF production by heparin and heparin-derived disaccharides with specific structures. Activities for stimulating HGF production of samples indicated in (A) and (B) were respectively assayed using MRC-9 cells.

HGF production to a level comparable with that of undigested heparin. However, it is noteworthy that some stimulatory activity was retained even in the tetrasaccharide fraction (hp4) and in a specific disaccharide such as ΔUA-GlcNS.

In digested heparin fragments, anticoagulant activity was significantly reduced compared with undigested heparin. The anticoagulant activity of hp10 and hp8 fractions were <13% that of undigested heparin and those of oligosaccharide fractions smaller than hp8 were much more reduced. Although the HGF-inducing activities of hp6 and hp4 were less than those of hp 8 and hp10, the marginal anticoagulant activities of hp6 and hp4 suggest their advantage. Taken together, oligosaccharides not larger than a decasaccharide size could be used as an HGF inducer in potential clinical applications.

We also addressed the structural characteristics of heparin involved in the HGF-inducing activity by mass spectrometric analysis of decasaccharide fragments, and by analysis of the activity of chemically modified heparin, HS and some disaccharides. The repetitive structure of disaccharide units composed of hexuronic acid and glucosamine residues itself does not have HGF-inducing activity, when it is not sulfated. A higher extent of sulfation roughly relates to a higher activity. In particular, the *N*-sulfation of glucosamine residues is a significant contributor to the activity.

Our previous study showed that chondroitin sulfate, dermatan sulfate and keratan sulfate do not stimulate HGF production (*10*). The lack of activity of chondroitin sulfate and dermatan sulfate is likely due to the absence of *N*-sulfated glucosamine residues, since chondroitin sulfate and dermatan sulfate contain galactosamine residues instead of glucosamine residues. On the other hand, the lack of the activity in keratan sulfate is likely due to the difference in the sugar residue paired with glucosamine, since the sugar residue paired with glucosamine is galactose instead of iduronic acid or glucuronic acid in keratan sulfate.

Heparin and HS bind with HGF and the structural requirements for such binding have been characterized (*27–30*). The minimum size of heparin and HS required for HGF binding is a tetrasaccharide, although an octa- to deca-saccharide size is required for maximum binding. Furthermore, the importance of the 2-*O*-sulfation of iduronic acids and the 6-*O*-sulfation of glucosamine residues is suggested, although the contribution of the 2-*O*-sulfation of iduronic acids is controversial (*27–29*). In addition, these studies reported that the contribution by the *N*-sulfation of glucosamine residues is small. The structural characteristics of heparin required for

binding with HGF seem to be somewhat different from that for HGF-inducing activity. Notably, dermatan sulfate can bind to HGF (*31*) but has no HGF-inducing activity (*10*). Moreover, we found that a disaccharide sulfated at the *N*-position shows HGF-inducing activity, even though disaccharides do not bind to HGF. Perhaps, the binding of heparin to HGF does not participate in HGF-inducing activity, although the mechanism for HGF-inducing activity of heparin and heparin-derived oligosaccharides remains to be addressed. We previously showed that heparin stimulates HGF production without any change in HGF mRNA levels (*10*). We also reported that the stimulation of HGF production is due to an enhancement in the net protein synthesis of HGF in a pulse-labelling experiment using [^{35}S]methionine. Thus, heparin stimulates HGF production post-transcriptionally. To address the HGF-inducing mechanism of heparin in more detail, extensive analysis of the structure-activity relationship in HGF-inducing activity of heparin might be helpful. Although we clarified the major role of *N*-sulfation in the present article, the structure-activity relationship seems more complex because a trisulfated disaccharide did not have activity despite the presence of *N*-sulfate group.

In addition to depolymerization, desulfation is another method for reducing the anticoagulant activity of heparin (*32*–*34*). In particular, *N*-desulfation significantly reduces the anticoagulant activity of heparin (*34*). An *N*-desulfated heparin derivative has been reported to retain anti-inflammatory activity, while having low anticoagulant activity, and its application in the treatment of inflammation has been discussed (*35*, *36*). Our results showed that desulfation, particularly *N*-desulfation, largely reduced the HGF-inducing activity of heparin, however, a significant level of HGF-inducing activity still remained as far as all sulfate groups were not eliminated. Therefore, desulfated derivatives might be also considered as alternate candidates as a practical HGF inducer. However, oligosaccharides obtained by the depolymerization of heparin might have an advantage in penetration into tissues because of their reduced molecular size.

In conclusion, we showed that heparin-derived decasaccharide fragments retain an HGF-inducing activity comparable with that of undigested heparin, while anticoagulant activity is largely reduced. Furthermore, oligosaccharides smaller than decasaccharides also showed some HGF-inducing activity while they had marginal or undetectable levels of anticoagulant activity. HGF has therapeutic effects on various disease models (reviewed in *37*, *38*) and a clinical trial of HGF gene therapy is ongoing (*39*). To facilitate tissue regeneration, potential applications of heparin-derived oligosaccharides, e.g. decasaccharides or smaller, that stimulate endogenous HGF production is considerable.

Supplementary data are available at *JB* online.

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