Research Paper

Identification of core active disaccharides in heparin for HGF-inducing activity

Takashi Kato[1,2], Ryoichi Sakiyama[3], Kiyomasa Oka[4], Toshikazu Nakamura[4]

[1]Department of Pathology, Faculty of Medicine, Kinki University, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka, [2]Department of Clinical Engineering, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, [3]Research and Development, Neurogen Inc., 7-7-20 Saito-asagi, Ibaraki, Osaka 567-0085, [4]Division for Regenerative Drug Discovery, Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received: 14-04-2014 **Revised: 30-05-2014** **Accepted: 15-09-2014**

ABSTRACT

Objectives: To ascertain the positions of sulfated groups for HGF-inducing activity using differently sulfated heparin disaccharides and to investigate whether the heparin disaccharide elevates HGF levels in plasma *in vivo* and exerts protective effects on acute liver injury. **Materials and Methods:** The heparin disaccharides ΔUA-GlcNS, ΔUA (2S)-GlcN, ΔUA-GlcNAc (6S), ΔUA-GlcNS (6S), ΔUA (2S)-GlcNS, ΔUA (2S)-GlcNAc (6S), ΔUA-GlcNAc and ΔUA (2S)-GlcNS (6S) were added to MRC-9 fibroblasts and HGF concentrations in culture media were determined by enzyme-linked immunosorbent assay. Furthermore, ΔUA-GlcNS (100 μg/head) was injected into C57BL/6 mice and plasma levels of HGF measured at 12 h. After acute hepatitis was induced by CCl_4 (15 mg/kg) in mice, liver specimens were stained with hematoxylin and eosin (H and E). Levels of aspartate aminotransferase and alanine aminotransferase were measured at 24 h. **Results:** Among the disaccharides investigated, ΔUA-GlcNS, ΔUA-GlcNAc (6S) and ΔUA-GlcNS (6S) stimulated HGF production in MRC-9 fibroblasts. However, none of the 2-*O*-sulfated disaccharides [ΔUA (2S)-GlcNS, ΔUA (2S)-GlcNAc (6S) and ΔUA (2S)-GlcNS (6S)] showed any activity despite the presence of *N*-sulfated and/or 6-*O*-sulfated disaccharides. Thus, 2-*O*-sulfation of hexuronic acid has an inhibitory effect. Moreover, ΔUA-GlcNS administration increased plasma levels of HGF in normal mice and prevented CCl_4-induced liver injury in mice. **Conclusions:** *N*-sulfation and/or 6-*O*-sulfation of glucosamine with nonsulfated hexuronic acid provides a structural basis for the HGF-inducing activity of disaccharides. ΔUA-GlcNS increases plasma levels of HGF and protects against CCl_4-induced acute liver injury.

Key words: Disaccharide, heparin, HGF, sulfation

INTRODUCTION

Heparin is a complex mixture of polysaccharides that are composed of repeating disaccharide units of glucosamine and uronic acids (glucuronic acid or iduronic acid), which are modified heterogeneously by carboxylate groups and *N*- or *O*-linked sulfate groups.[1] One of the well-known functions of heparin is its inhibition of blood clotting.[2] Additionally, heparin modulates a wide range of biological functions. Heparin inhibits inflammation, viral activity, complement activation and metastatic spread of tumor cells, and regulates angiogenesis[3] by the interactions of some proteins: Antithrombin III, fibroblast growth factor, hepatocyte growth factor (HGF), fibronectin, annexin, phospholipase-A2 and interleukin-8.[4,5] A unique pentasaccharide of heparin

Access this article online	
Quick Response Code:	Website: www.jpharmacol.com
	DOI: 10.4103/0976-500X.155483

Address for correspondence:
Toshikazu Nakamura, Research and Development, Neurogen Inc., 7-7-20 Saito-asagi, Ibaraki, Osaka 567 - 0085, Japan.
E-mail: info.neurogen@gmail.com

(the synthetic analog fondaparinux) is necessary for binding to antithrombin. Fondaparinux induces a conformational change and accelerates its interaction with the active site of factor Xa to inhibit activity.[6] Fondaparinux is too short to bridge antithrombin to thrombin. It catalyzes only the inhibition of factor Xa and exhibits better bioavailability, longer half-life and lower contamination risk.[7] Thus, structure–function studies are important for more efficacious clinical applications of heparin.

One important function of heparin and heparin-derived oligosaccharides is the increased production of HGF.[8,9] HGF exerts multiple biological activities (e.g., mitogenic, motogenic, morphogenic) on various cells. The multiple functions of HGF have important roles in embryogenesis and organ regeneration.[10] When heparin and low-molecular heparin have been injected into rats with hepatic injury, plasma levels of HGF have become elevated and liver regeneration has been enhanced.[11,12] Thus, heparin might have clinical potential as an HGF-inducer, but its anticoagulant activity could elicit side-effects. Heparin-derived oligosaccharides retain HGF-inducing activity while having low anticoagulant activity.[9] Therefore, heparin-derived oligosaccharides might be useful as HGF inducers.

In the course of our studies, we have found that the extent of sulfation in heparin is approximately related to HGF-inducing activity and that some disaccharides of a specific structure (ΔUA-GlcNS) have such activity.[9] However, the details of the structure–function relationship have not been determined. Therefore, we assessed the positions of sulfate groups involved in HGF-inducing activity using heparin disaccharides of defined structure (sulfated to different extents) under cell culture. Furthermore, we investigated whether specific heparin disaccharides elevate HGF levels in plasma *in vivo* and exert protective effects on acute injury to the liver.

MATERIALS AND METHODS

Reagents

Porcine mucosal heparin was obtained from Scientific Protein Laboratories (Waunakee, WI, USA). Heparin disaccharides ΔUA (2S)-GlcNS (6S), ΔUA-GlcNS (6S), ΔUA (2S)-GlcNS, ΔUA-GlcNAc (6S) and ΔUA-GlcNAc were purchased from Seikagaku (Tokyo, Japan) and ΔUA (2S)-GlcN and ΔUA (2S)-GlcNAc (6S) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). In these disaccharides, ΔUA denotes a $\Delta^{[4,5]}$ unsaturated hexuronic acid; GlcN refers to *N*-unsubstituted glucosamine; GlcNS denotes *N*-sulfated glucosamine; GlcNAc refers to *N*-acetylated glucosamine; and 2S and 6S represent the 2-*O*- and 6-*O*-sulfate groups, respectively. ΔUA-GlcNS was purchased from Seikagaku and Iduron (Manchester, UK).

Analyses of HGF induction in cell culture

HGF-inducing activity was determined using MRC-9 human embryonic lung fibroblasts, as described previously.[9] For the culture of MRC-9 cells at 4°C, cells were replaced with the fresh cold medium supplemented with 1% fetal calf serum. HGF levels were measured by enzyme-linked immunosorbent assay.[13]

Animal experiments

Animal experiments were carried out according to the *Guideline for Experimental Animal Care* issued by the Prime Minister's Office of Japan and approved by the Committee on Animal Experimentation of Osaka University (Osaka, Japan). Male-specific pathogen-free C57BL/6 mice (8–9 weeks; SLC, Hamamatsu, Japan) were used. To analyze the effect of heparin disaccharides on plasma levels of HGF, 100 μg/head of ΔUA-GlcNS (Iduron) was injected via the intraperitoneal route and plasma was collected at 12 and 24 h. The effect of ΔUA-GlcNS on liver injury was examined using mice with acute hepatitis. To induce acute hepatitis, carbon tetrachloride (CCl_4) in olive oil was administered orally at 15 mg/kg body weight. In the pretreatment group, 100 μg/head of ΔUA-GlcNS was administered via the intraperitoneal route 1 h before CCl_4 injection. Plasma levels of HGF were analyzed 12 and 24 h after CCl_4 injection. After 24 h, mice were killed under anesthesia and subjected to histologic examinations and serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed.

Histologic examination and measurement of serum levels of aminotransferases

Liver specimens were fixed with 10% buffered formalin, embedded with paraffin and stained with hematoxylin and eosin (H and E). Serum levels of AST and ALT were measured using a Transaminase CII Test kit (Wako Pure Chemicals, Osaka, Japan).

Statistical analyses

We used the Student's *t*-test to determine statistical significance. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Previously, we disclosed that the HGF-inducing activities of heparin are dependent on its molecular size.[9] Heparin fragments smaller than decasaccharides showed low activity, and disaccharides did not stimulate HGF production, but *N*-sulfated disaccharides retained HGF-inducing activity. The disaccharide fraction is composed of a mixture of various disaccharides and contains various sulfated disaccharides. Some types of disaccharides sulfated at specific positions could have HGF-inducing activity. We assessed the positions of sulfate groups required for HGF-inducing activity using various heparin disaccharides of defined structure (sulfated to different extents)

[Figure 1a]. Disaccharides were added to cultures of MRC-9 cells and the amounts of HGF in the culture medium were measured [Figure 1b]. A disaccharide containing no sulfate group (ΔUA-GlcNAc) did not increase HGF production even at 50 μg/mL. This finding suggested that the basal disaccharide composed of hexuronic acid and glucosamine residue did not have HGF-inducing activity. Conversely, consistent with other reports,[9] ΔUA-GlcNS (mono-(*N*-) sulfated) showed significant activity at >5 μg/mL, and the level of HGF production at 50 μg/mL ΔUA-GlcNS was the same as the maximum level in the case of unfractionated heparin. Thus, *N*-sulfation in the glucosamine residue was found to confer HGF-inducing activity to the basal disaccharide. In addition to ΔUA-GlcNS, ΔUA-GlcNAc (6S) (mono-(6-*O*-) sulfated) also showed significant activity. Because ΔUA (2S)-GlcN (mono-(2-*O*-) sulfated) had no activity, an essential component for HGF-inducing activity was limited to the *N*-sulfation and/or 6-*O*-sulfation of glucosamine residue in the disaccharide. The disaccharide disulfated at the *N*- and 6-*O*- positions, i.e. ΔUA-GlcNS (6S), showed almost the same activity as that of monosulfated ΔUA-GlcNS and ΔUA-GlcNAc (6S), indicating that the effect of *N*-sulfation and 6-*O*-sulfation is not additive. None of the 2-*O*-sulfated disaccharides [ΔUA (2S)-GlcNS, ΔUA (2S)-GlcNAc (6S) and ΔUA (2S)-GlcNS (6S)] showed any activity despite the presence of *N*-sulfated and/or 6-*O*-sulfated glucosamine residues, indicating that the 2-*O*-sulfation of hexuronic acid has an inhibitory effect on HGF production. The major structure in the disaccharide fraction is a trisulfated form, ΔUA (2S)-GlcNS (6S), which does not have HGF-inducing activity. Therefore, a fraction of disaccharides did not stimulate HGF production, but HGF-inducing disaccharides might exist. We summarized that the HGF-inducing activity of heparin disaccharides is exerted by the *N*-sulfation and/or 6-*O*-sulfation of the glucosamine residue in the disaccharide unit, whereas the 2-*O*-sulfation of the residue of hexuronic acid has an inhibitory effect [Figure 2].

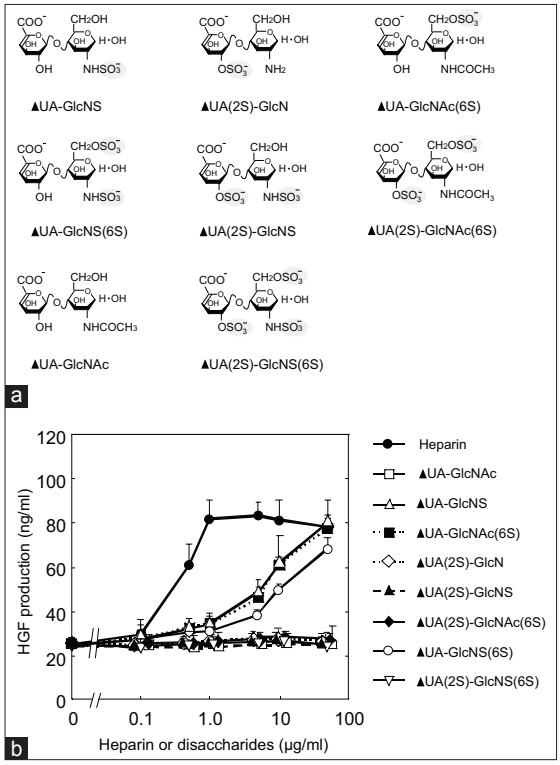

Figure 1: Hepatocyte growth factor (HGF)-inducing activity of disaccharides composed of hexuronic acid and glucosamine sulfated at different positions. (a) Heparin-derived disaccharides used for the assay. (b) Dose-dependent activity of heparin or heparin-derived disaccharides for stimulating HGF production. MRC-9 cells were cultured at 37°C for 24 h in the presence of disaccharides and the HGF concentration in the medium was measured by enzyme-linked immunosorbent assay. Each value is the mean ± SD (n = 4 in each group)

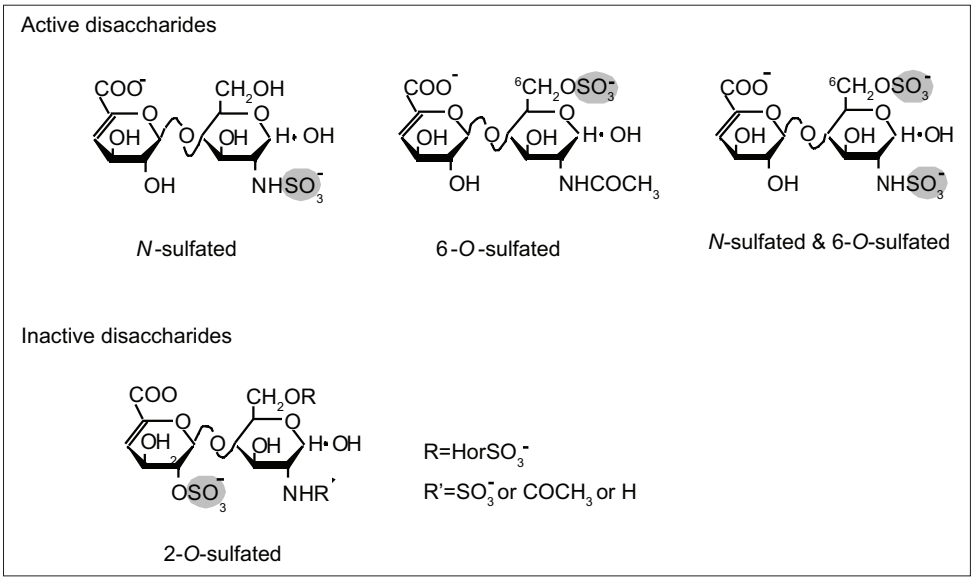

Figure 2: Structural requirement of disaccharides for hepatocyte growth factor (HGF)-inducing activity. Active and inactive disaccharides are summarized. The *N*-sulfation and/or 6-*O*-sulfation of the glucosamine residue provides the disaccharide with HGF-inducing activity. The 2-*O*-sulfated group in the residue of hexuronic acid canceled the effect of *N*-sulfated and/or 6-*O*-sulfated disaccharides

HGF exists on the cell surface by binding to heparan sulfates, and an excess amount of heparin leads to an increase in HGF levels in the culture medium.[14,15] Therefore, we investigated whether the elevation of HGF concentration by heparin disaccharides is due to HGF release from the cell surface through binding to cell surface-bound HGF ("washing effect"). MRC-9 cells were incubated at 37°C for 24 h in the absence of disaccharides. Subsequently, cells were incubated at 4°C for 30 min in the presence of heparin disaccharides [ΔUA-GlcNS (6S), ΔUA-GlcNS and ΔUA-GlcNAc (6S)]. The HGF concentration in the medium was increased slightly, but it had a minor effect [Figure 3]. When cells were incubated with disaccharides at 37°C, the HGF concentration in the medium was elevated much more than that in the former case. These results suggested that the mechanisms of disaccharide-mediated HGF induction include the release of cell surface-bound HGF and increase in HGF production in cells, but the latter is more substantial.

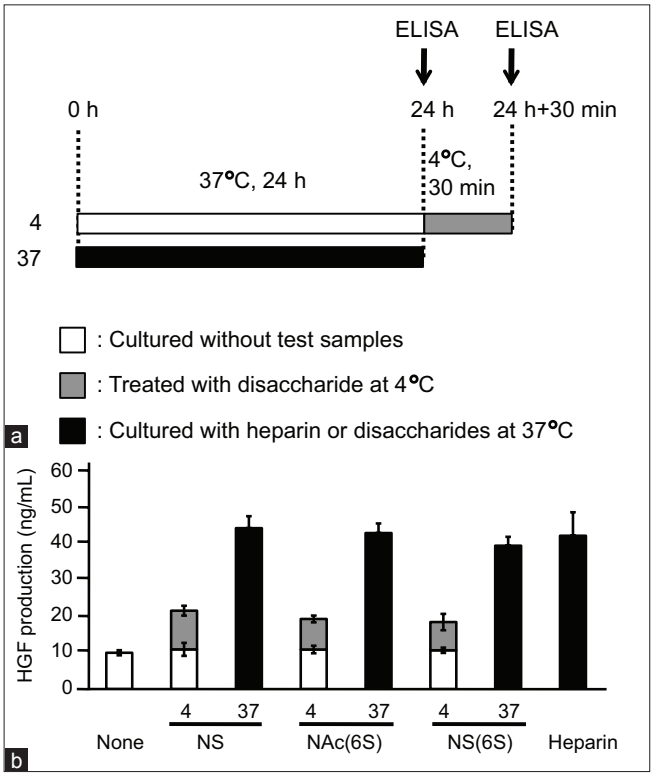

Figure 3: Hepatocyte growth factor (HGF) concentration in the culture medium of MRC-9 cells after incubation with heparin-derived disaccharides at 37°C or after washing cells with disaccharides at 4°C. (a) Experimental design to examine the contribution of HGF release from cells to the increase in HGF levels. MRC-9 cells were incubated with disaccharides at 4°C for 30 min (gray) after preincubation of cells in the absence of disaccharides (white) at 37°C for 24 h compared with incubation in the presence of disaccharides at 37°C for 24 h (black). (b) HGF concentrations in the culture media of MRC-9 cells. NS, NAc(6S) and NS(6S) indicate the disaccharides ΔUA-GlcNS, ΔUA-GlcNAc(6S) and ΔUA-GlcNS(6S), respectively. Disaccharides were used at 50 μg/mL. Heparin (1 μg/mL) was used as a positive control for HGF induction at 37°C for 24 h. Each value is the mean ± SD (n = 4 in each group)

The molecular mechanisms by which the specific disaccharides stimulate HGF production are not known. Some reports have shown a correlation between the sulfation patterns of heparin and its bioactivities. The anti-allergic activity of heparin tetrasaccharide is dependent upon the *N*-sulfation of heparin.[16] Moreover, Ashikari-Hada *et al*. analyzed the specific binding structures for various heparin-binding growth factors using an octasaccharide library.[4] Of interest, HGF was found to bind 2-*O*-sulfate and 6-*O*-sulfate of the heparin octasaccharides. We found the HGF-inducing activity of heparin disaccharides to be assigned to 6-*O*-sulfate, but this activity was suppressed by 2-*O*-sulfate. A possible reason for this discrepancy is that promotion of HGF production by *N*- and/or 6-*O*-sulfated heparin disaccharides is not caused by direct interaction with HGF, but instead is involved with specific molecules having a high affinity to these disaccharides. Then, the resultant complex may induce HGF production, whereas the 2-*O*-sulfated group prevents this association. Thus, identification of the conformational features (e.g., active or inactive) of the specific sulfation in heparin disaccharides (and especially the interaction with molecular partners for HGF induction) is intriguing.

To examine the effects of specific disaccharides on plasma levels of HGF, ΔUA-GlcNS was administered to normal mice. The plasma concentration of HGF was increased 12 h after a single administration of ΔUA-GlcNS, whereas the HGF concentration returned to the basal level after 24 h [Figure 4a]. The influence of ΔUA-GlcNS on the plasma concentration of HGF was also examined in mice with CCl_4-induced liver injury. It is known that the plasma level of HGF increases in response to liver injury as a result of the induction of endogenous HGF production. Consistent with this notion, plasma levels of HGF in CCl_4-treated mice were elevated 12 and 24 h after CCl_4 injection. When CCl_4-treated mice were pretreated with ΔUA-GlcNS, the plasma level of HGF 12 h after CCl_4 treatment was identical to that in mice treated with CCl_4 alone. Thus, the additive effect of ΔUA-GlcNS and CCl_4 on HGF induction was not observed. Furthermore, the plasma level of HGF 24 h after CCl_4 treatment in mice pretreated with ΔUA-GlcNS returned to the basal level, whereas the plasma level of HGF was still high at 24 h in mice treated with CCl_4 alone. Thus, the effect of ΔUA-GlcNS on HGF induction was complex in CCl_4-treated mice compared with that in normal mice. This finding suggested that pretreatment with ΔUA-GlcNS suppressed the liver injury caused by CCl_4 and, therefore, HGF induction due to CCl_4 did not occur. The suppressive effect of ΔUA-GlcNS on liver injury was confirmed by the analyses of the serum levels of ALT and AST as well as histological examination. Serum levels of ALT and AST increased 24 h after CCl_4 treatment, but administration of

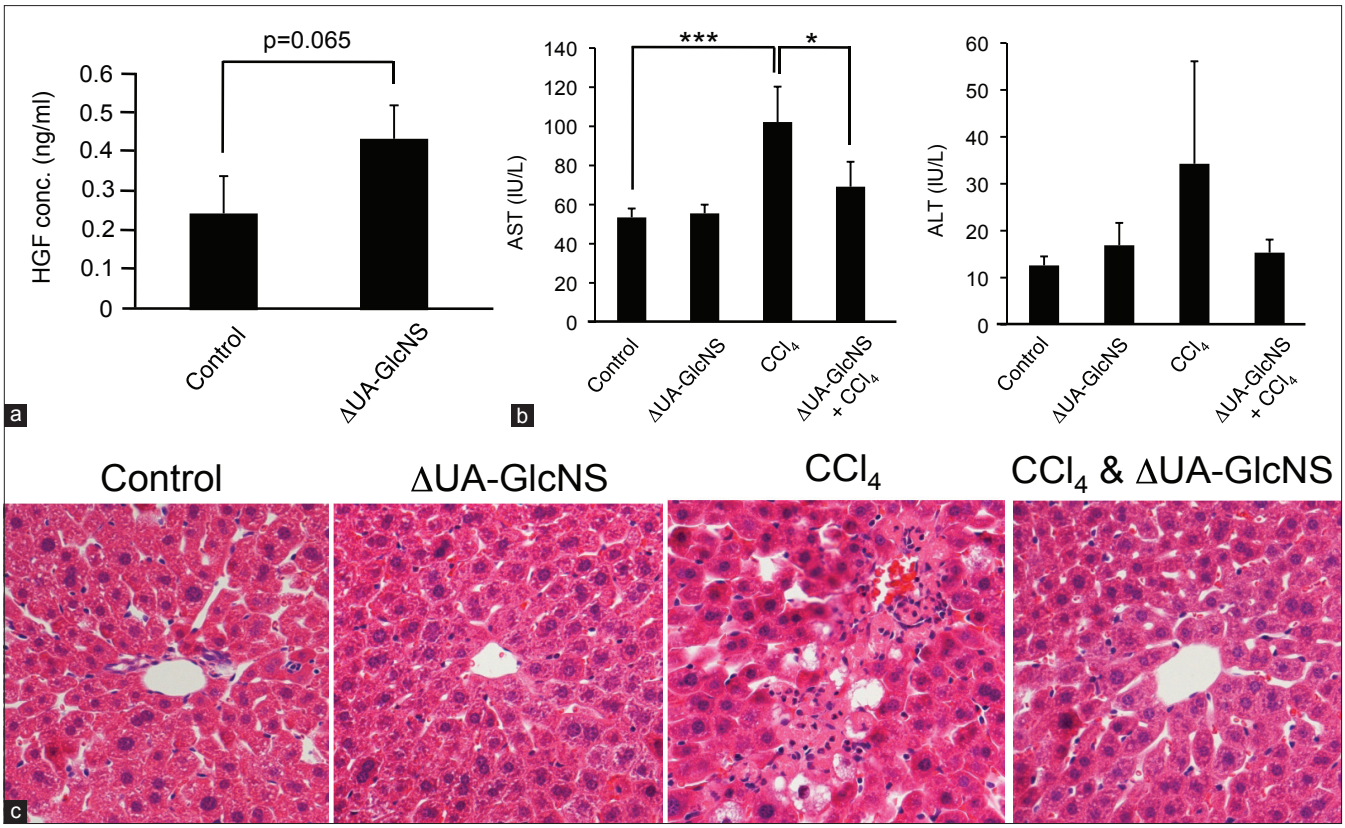

Figure 4: Effects of ΔUA-GlcNS on the plasma concentration of hepatocyte growth factor (HGF) and CCl_4-induced acute liver injury. Mice were given intraperitoneal injection(s) of CCl_4 and/or ΔUA-GlcNS (1 h before CCl_4 injections). (a) Plasma levels of HGF in mice 12 and 24 h after injection(s). Each value is the mean ± SD ($n = 3$). *P*-values represent the results of a two-tailed Student's *t*-test compared with control. (b) Serum levels of alanine aminotransferase and aspartate aminotransferase 24 h after injection(s). Each value is the mean ± SD ($n = 5–7$ in each group). (c) Representative photographs of liver histology (H&E staining) 24 h after injection(s)

ΔUA-GlcNS suppressed the CCl_4-induced increase in levels of ALT and AST [Figure 4b]. Histologic examination of the liver in mice injected with CCl_4 revealed cell swelling and necrotic hepatocytes with pyknosis, especially in centrilobular areas [Figure 4c]. However, these lesions were prevented by ΔUA-GlcNS injection [Figure 4c]. We found that ΔUA-GlcNS injection could prevent CCl_4-induced acute hepatitis.

In the present study, we showed the *N*-sulfation and/or 6-*O*-sulfation of glucosamine with nonsulfated hexuronic acid to be the structural basis for the HGF-inducing activity of heparin disaccharides. Moreover, ΔUA-GlcNS brought about an increase in the plasma levels of HGF and protected against CCl_4-induced acute liver injury in mice. HGF has potent therapeutic effects on various types of disease in distinct tissues; therefore, these specific disaccharides could be valuable for clinical application as HGF inducers.

ACKNOWLEDGMENTS

The authors thank Dr. K. Fukuta for technical advice and Mrs. Y. Yoneda for secretarial assistance.

REFERENCES

1. Casu B. Structure and biological activity of heparin. Adv Carbohydr Chem Biochem 1985;43:51-134.
2. Bourin MC, Lindahl U. Glycosaminoglycans and the regulation of blood coagulation. Biochem J 1993;289:313-30.
3. Ludwig RJ. Therapeutic use of heparin beyond anticoagulation. Curr Drug Discov Technol 2009;6:281-9.
4. Ashikari-Hada S, Habuchi SH, Kariya Y, Itoh N, Reddi AH. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. J Biol Chem 2004;279:12346-54.
5. Yadav VK, Saraswat M, Chhikara N, Singh S, Yadav S. Heparin and heparin binding proteins: Potential relevance to reproductive physiology. Curr Protein Pept Sci 2013;14:61-9.
6. Noti C, Seeberger PH. Chemical approaches to define the structure-activity relationship of heparin-like glycosaminoglycans. Chem Biol 2005;12:731-56.
7. Bauer KA, Hawkins DW, Peters PC, Petitou M, Herbert JM, van Boeckel CA, *et al*. Fondaparinux, a synthetic pentasaccharide: The first in a new class of antithrombotic agents-the selective factor Xa inhibitors. Cardiovasc Drug Rev 2002;20:37-52.
8. Matsumoto K, Tajima H, Okazaki H, Nakamura T. Heparin as an inducer of hepatocyte growth factor. J Biochem 1993;114:820-6.
9. Sakiyama R, Fukuta K, Matsumoto K, Furukawa M, Takahashi Y, Nakamura T. Stimulation of hepatocyte growth factor production by heparin-derived oligosaccharides. J Biochem 2007;141:653-60.
10. Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proc Jpn Acad Ser B Phys Biol Sci 2010;86:588-610.
11. Matsumoto K, Nakamura T. Heparin functions as a hepatotrophic factor

by inducing production of hepatocyte growth factor. Biochem Biophys Res Commun 1996;227:455-61.
12. Abe W, Ikejima K, Lang T, Okumura K, Enomoto N, Kitamura T, *et al*. Low molecular weight heparin prevents hepatic fibrogenesis caused by carbon tetrachloride in the rat. J Hepatol 2007;46:286-94.
13. Matsumoto K, Tajima H, Hamanoue M, Kohno S, Kinoshita T, Nakamura T. Identification and characterization of "injurin," an inducer of expression of the gene for hepatocyte growth factor. Proc Natl Acad Sci U S A 1992;89:3800-4.
14. Naka D, Ishii T, Shimomura T, Hishida T, Hara H. Heparin modulates the receptor-binding and mitogenic activity of hepatocyte growth factor on hepatocytes. Exp Cell Res 1993;209:317-24.
15. Lyon M, Deakin JA, Mizuno K, Nakamura T, Gallagher JT. Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J Biol Chem 1994;269:11216-23.
16. Ahmed T, Smith G. Effect of oral and intravenous heparin tetrasaccharide on allergic airway responses: Critical role of N-sulfation. Pulm Pharmacol Ther 2013;26:180-8.

How to cite this article: Kato T, Sakiyama R, Oka K, Nakamura T. Identification of core active disaccharides in heparin for HGF-inducing activity. J Pharmacol Pharmacother 2015;6:77-82.
Source of Support: Nil, **Conflict of Interest:** None declared.