JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 499–503

Received September 27, 2007
Accepted January 14, 2008
DOI 10.1211/jpp.60.4.0013
ISSN 0022-3573

Induction of hepatocyte growth factor by fucoidan and fucoidan-derived oligosaccharides

Kazuhiro Fukuta and Toshikazu Nakamura

Abstract

Fucoidan, which is extracted from brown seaweed, is a complex sulphated polysaccharide that is mostly composed of L-fucose and sulphated ester groups. The structural and anionic characteristics of fucoidan are similar to those of heparin. Heparin stimulates production of hepatocyte growth factor (HGF), which has key roles in tissue regeneration. We have shown that fucoidan and fucoidan-derived oligosaccharides have similar ability to stimulate production of HGF as heparin and heparin-derived oligosaccharides. This induction of HGF by heparin or fucoidan and their oligosaccharide derivates occurs primarily at the level of translation, probably via the same mechanism. Fucoidan may thus be useful to protect tissues and organs from various injuries and diseases, via mechanisms involving HGF.

Division of Molecular Regenerative Medicine, Department of Biochemistry and Molecular Biology, Osaka University Graduate School of Medicine, 2-2-B7 Yamadaoka, Suita, Osaka 565-0871, Japan

Kazuhiro Fukuta, Toshikazu Nakamura

Kringle Pharma Joint Research Division for Regenerative Drug Discovery, Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Toshikazu Nakamura

Correspondence: Toshikazu Nakamura, Division of Molecular Regenerative Medicine, Department of Biochemistry and Molecular Biology, Osaka University Graduate School of Medicine, 2-2-B7 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: nakamura@onbich.med.osaka-u.ac.jp

Acknowledgements and funding: We thank Mr Mamoru Shibata, President of Shibata Pharmaceutical Co. (12-20 Ohe 3, Otsu 520-2141, Japan), for supplying low-molecular-weight fucoidan (Power Fucoidan). We also thank Dr James L. McDonald for editing the manuscript. This work was supported by grants from the Ministry of Education, Science, Technology, Sports and Culture of Japan (nos. 18013031 and 18390087) to T. Nakamura, and the 21st Century Center of Excellence programme to T. Nakamura.

Introduction

Fucoidan is a complex sulphated polysaccharide derived from brown seaweed, the jelly coat of sea urchin eggs and the body wall of sea cucumbers (Berteau & Mulloy 2003). Fucoidan is mostly composed of L-fucose and sulphated ester groups, and its structural and anionic characteristics resemble those of heparin. Fucoidan possesses biological activities similar to those of heparin, including anti-coagulant (Colliec et al 1991), anti-inflammatory (Shimaoka et al 1996; Tissot & Daniel 2003), anti-tumour (Itoh et al 1993) and anti-viral activities (Baba et al 1998).

Heparin and heparin-derived oligosaccharides induce production of hepatocyte growth factor (HGF) (Matsumoto et al 1993; Sakiyama et al 2007). HGF, originally identified and cloned as a potent mitogen for mature hepatocytes (Nakamura et al 1984; 1989; Miyazawa et al 1989), exerts multiple biological activities on a variety of cells through activation of the Met receptor tyrosine kinase, and plays critical roles in development and tissue regeneration (Matsumoto & Nakamura 1997; Birchmeier & Gherardi 1998).

Administration of recombinant HGF to experimental animals has remarkable therapeutic effects on various injuries and diseases (Matsumoto & Nakamura 2001; Funakoshi & Nakamura 2003; Mizuno & Nakamura 2007). Heparin or heparin-derived oligosaccharides may facilitate tissue regeneration by inducing production of HGF. Given the similarities between fucoidan and heparin, this study compared the HGF-inducing activities of fucoidan and fucoidan-derived oligosaccharides with those of heparin and heparin-derived oligosaccharides.

Materials and Methods

Materials

Fucoidan from brown seaweed, *Fucus vesiculosus*, was obtained from Sigma-Aldrich (St Louis, MO, USA). A low-molecular-weight (LMW) fucoidan solution (commercially available as 'Power Fucoidan') was a generous gift from Shibata Pharmaceutical Co. (Otsu, Japan). The LMW fucoidan was prepared by abalone glycosidase digestion of high-molecular-weight fucoidan isolated from the brown seaweed Limu Moui (*Cladosiphon novae-caledoniae kylin*) from the Kingdom of Tonga (Ye et al 2005). Porcine mucosal heparin was obtained

from Scientific Protein Laboratories, Inc. (Waunakee, WI, USA). The heparin disaccharide △UA-GlcNS was purchased from Seikagaku Co. (Tokyo, Japan). (△UA denotes a $\triangle^{4,5}$ unsaturated hexuronic acid; GlcNS indicates N-sulfated glucosamine.) Actinomycin D, cycloheximide and interleukin (IL)-1α were purchased from Calbiochem (San Diego, CA, USA), Nacalai Tesque (Kyoto, Japan) and R&D Systems (Minneapolis, MN, USA), respectively.

Separation of fucoidan- and heparin-derived oligosaccharides by gel filtration

LMW fucoidan (10 mg) was applied to a Bio-Gel P10 column (1 × 48 cm) (BioRad, Hercules, CA, USA) equilibrated with 0.2 M NH_4HCO_3 and eluted with the same solution at room temperature at a flow rate of 6 ml h^{-1}. Fractions (0.5 mL) were collected.

Fucoidan (10 mg) from *F. vesiculosus* was depolymerized by mild acid hydrolysis in 1 mL 10 mM HCl. The solution was incubated at 60°C for 6 h, after which the reaction was stopped by neutralizing the solution with 0.1 mL 1 M Tris-HCl buffer (pH 7.5) on ice. The products of the acid hydrolysis were separated on a Bio-Gel P10 column. LMW fucoidan and the size-fractionated oligosaccharides were quantified using the phenol-H_2SO_4 method (Dubois et al 1956); absorbance was measured at 490 nm; fucoidan from *F. vesiculosus* was used as the standard.

Porcine mucosal heparin (10 mg) was digested with heparinase as described previously (Sakiyama et al 2007). The digest was separated on a Bio-Gel P10 column in the same manner as fucoidan. Fractions were monitored by measuring absorbance at 232 nm. The size-fractionated heparin oligosaccharides were quantified using the carbazole method (Bitter & Muir 1962) with heparin as the standard.

Measurement of HGF-inducing activity

The HGF-inducing activity of fucoidan, heparin and the corresponding oligosaccharides was determined using MRC-9 human embryonic lung fibroblasts (CCL-212, obtained from the American Type Culture Collection) as described previously (Sakiyama et al 2007). MRC-9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were seeded on 96-well plates at a density of 15000 cells per well and cultured for 24 h. After replacing the medium with fresh medium supplemented with 1% FCS, the test solutions were added and the cells cultured for 24 h. The amount of HGF in the medium was then determined by ELISA using rabbit polyclonal antibody raised against human HGF, as described previously (Matsumoto et al 1992b).

To assess the effects of actinomycin D and cycloheximide on HGF-inducing activity, MRC-9 cells were preincubated in DMEM supplemented with actinomycin D or cycloheximide (both 5 μg mL^{-1}) and 1% FCS. After 30 min' preincubation, the medium was replaced with fresh medium containing fucoidan (1 μg mL^{-1}), heparin (1 μg mL^{-1}), fucoidan- or heparin-derived oligosaccharides (both 50 μg mL^{-1}), or IL-1α (10 ng mL^{-1}) in the presence of actinomycin D or cycloheximide (both 5 μg mL^{-1}), and 1% FCS. The cells were cultured for 12 h and the amount of HGF in the medium was determined by ELISA.

Statistical analysis

Results are expressed as mean ± s.d. of four experiments. Statistical analysis was performed using SPSS software (Chicago, IL, USA). Differences between treatments and controls were evaluated using one-way analysis of variance, followed by Dunnett's test for pairwise comparison. In all cases, $P < 0.05$ was considered significant.

Results and Discussion

First, the HGF-inducing activity of fucoidan isolated from *F. vesiculosus* was measured in cultured MRC-9 human embryonic lung fibroblasts (Figure 1). Neither heparin nor fucoidan affected proliferation of the cells. Induction of HGF was evaluated by measuring the concentration of HGF in the conditioned medium. Fucoidan stimulated HGF production in a dose-dependent manner. Activity was maximum at fucoidan concentrations of 0.5 μg mL^{-1} and above. HGF production in response to fucoidan was comparable to that induced by heparin. When heparin and fucoidan were added to the cells as a mixture (0.05 or 0.1 μg mL^{-1} of each), the concentration of HGF in the medium was higher than with the addition of either compound alone. However, addition of the two together did not increase the maximum production of HGF.

Next, we investigated the effect of the size of the fucoidan- and heparin-derived oligosaccharides on induction of HGF production. For this comparison, commercially available LMW fucoidan (Ye et al 2005), which was prepared by enzymatic digestion of high-molecular-weight fucoidan from *C. novae-caledoniae kylin*, was fractionated on a Bio-Gel P10 column, yielding fucoidan-derived oligosaccharides.

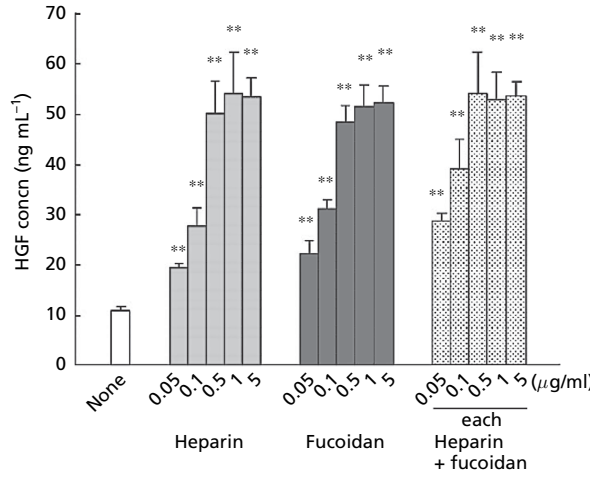

Figure 1 Production of hepatocyte growth factor (HGF) in MRC-9 cells stimulated by fucoidan and heparin. Cells were cultured with test samples for 24 h and the HGF concentration in the medium measured. Data represent mean ± s.d. of four experiments. $^{**}P < 0.01$ vs control.

The elution profile of LMW fucoidan was shifted to lower molecular weights (Figure 2B) compared with that of high-molecular-weight fucoidan (Figure 2A). The fractions were divided into four groups, designated F-I, F-II, F-III and F-IV. Heparin was digested with heparinase and the digest separated using a Bio-Gel P10 column (Figure 2C). The di-, tri-, tetra-, hexa-, octa- and deca-saccharide fractions of the heparin digest were designated hp2, hp4, hp6, hp8 and hp10, respectively. F-IV of LMW fucoidan was the same size as hp6, and no peaks appeared after F-IV, indicating that there were no oligosaccharides smaller than hexasaccharides in LMW fucoidan. This may have been because the enzyme used to produce LMW fucoidan was unable to produce fragments smaller than hexasaccharides. To obtain smaller oligosaccharides, fucoidan from *F. vesiculosus* was chemically depolymerized by mild acid hydrolysis. When the reaction products were separated using a Bio-Gel P10 column, a peak (designated F-V) corresponding to disaccharides appeared (Figure 2D).

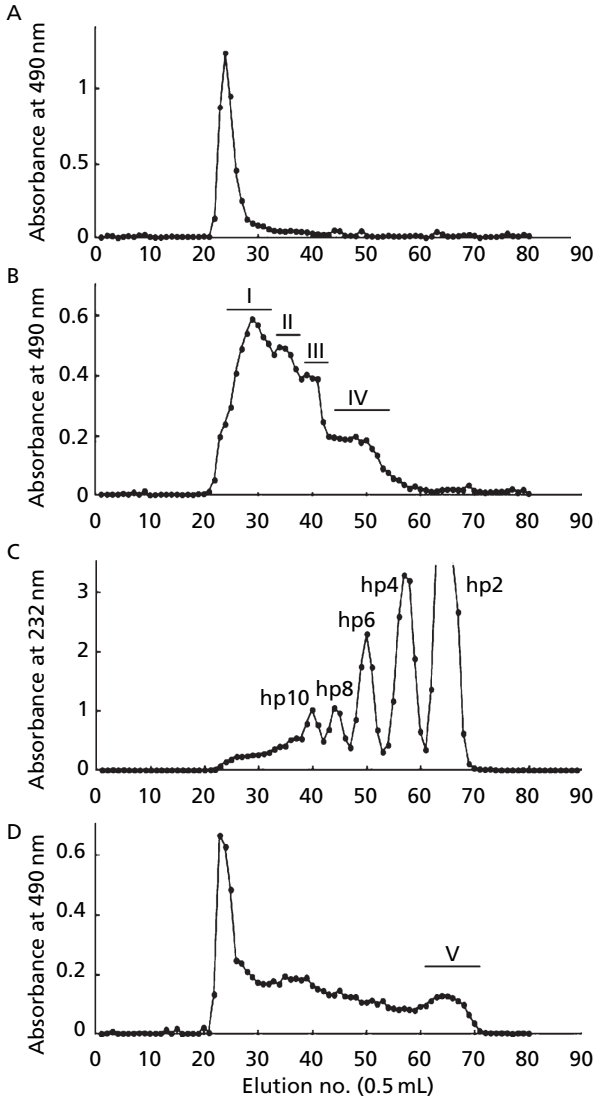

Figure 2 Elution profiles of fucoidan from *Fucus vesiculosus* (A), LMW fucoidan (B), heparin fragments (C) and acid-hydrolysed fucoidan (D) on a Bio-Gel P10 column. Fucoidan or fucoidan-derived oligosaccharides in the fractions were quantified using the phenol–H_2SO_4 method. Fractions of heparin-derived oligosaccharides were monitored at 232 nm.

The HGF-inducing activity of each fraction was measured in MRC-9 cells (Figure 3). Of the heparin-derived oligosaccharides, both hp6 and hp4 stimulated production of HGF; the activity of hp6 was weaker than that of undigested heparin, and the activity of hp4 was less than that of hp6. The heparin disaccharide △UA-GlcNS also stimulated production of HGF. Similarly, fucoidan-derived oligosaccharide fractions F-I to F-V stimulated HGF production, although the activity of fraction F-V, which corresponds to the disaccharide hp2, was significantly reduced compared with undigested fucoidan. The weak activity of fraction F-V might be due to desulphation during acid hydrolysis. Overall, stimulation of HGF production was comparable between heparin- and fucoidan-derived oligosaccharides.

The detailed mechanism by which heparin stimulates HGF production remains to be clarified. However, it is known that heparin stimulates HGF production without any change in HGF mRNA levels. Pulse-labeling with [^{35}S]methionine has shown that stimulation of HGF production is due to a net increase in HGF protein synthesis (Matsumoto et al 1993). Thus, heparin stimulates HGF production at the level of translation.

To determine whether the HGF-inducing activity of fucoidan also occurs at the level of translation rather than transcription, we tested the effects of actinomycin D (a transcription inhibitor) and cycloheximide (a protein synthesis inhibitor) on HGF-inducing activity (Figure 4). IL-1α (10 ng mL^{-1}), which is known to stimulate HGF production by regulating transcription of the HGF gene (Matsumoto et al 1992a; Tamura et al 1993), enhanced HGF production two-fold in the absence of actinomycin D, whereas it had no effect in the presence of actinomycin D. The production of HGF stimulated by heparin, fucoidan and the related oligosaccharides was only partly reduced by actinomycin D, suggesting that transcriptional regulation plays only a minor role in the HGF-inducing activities of these oligosaccharides.

Cycloheximide largely prevented induction of HGF production in response to heparin, fucoidan and the corresponding oligosaccharides, suggesting that stimulation of HGF production occurs primarily at the level of translation. Furthermore, co-treatment with heparin and fucoidan did not have an additive effect to increase the maximum level of HGF production, indicating that the two compounds are likely to stimulate production of HGF via the same mechanism. Identification of the mechanism of HGF induction may guide synthesis of oligosaccharides that possess greater HGF-inducing activity.

HGF plays critical roles in tissue regeneration, and HGF has been shown to have therapeutic effects in various disease models (Matsumoto & Nakamura 2001; Funakoshi & Nakamura 2003; Mizuno & Nakamura 2007). Ingestion of fucoidan may protect tissues or organs from injury and disease by stimulating production of HGF. Currently, fucoidan and LMW fucoidan are attracting attention because of their remarkable ability to maintain health, although the

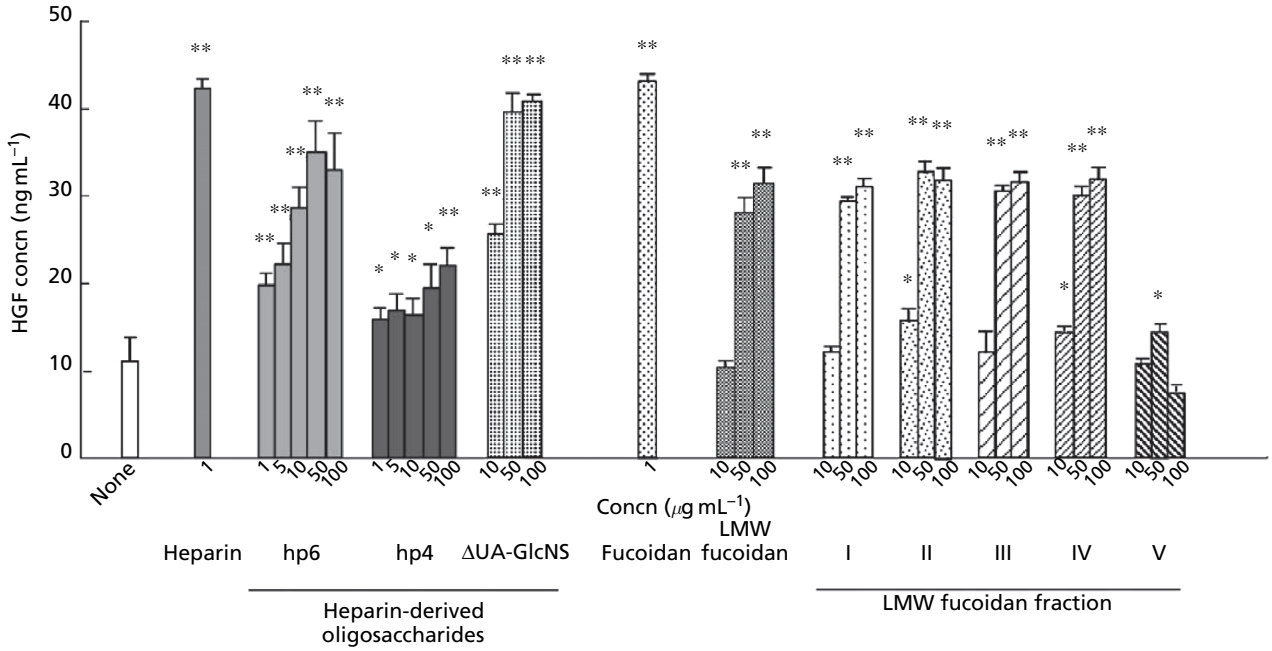

Figure 3 Production of hepatocyte growth factor (HGF) in MRC-9 cells stimulated by fucoidan- and heparin-derived oligosaccharides. Cells were cultured with test samples for 24 h and the HGF concentration in the medium measured. δUA-GlcNS is a heparin disaccharide. Data represent mean ± s.d. of four experiments. *$P < 0.05$; **$P < 0.01$ vs control.

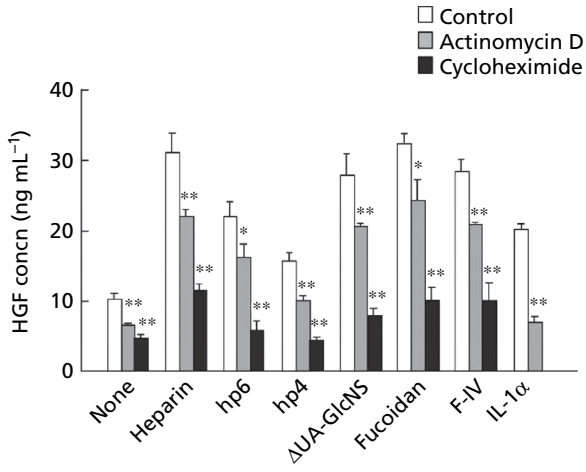

Figure 4 Effects of actinomycin D (5 μg mL^{-1}) and cycloheximide (5 μg mL^{-1}) on the production of hepatocyte growth factor (HGF) stimulated by fucoidan (1 μg mL^{-1}), heparin (1 μg mL^{-1}) and the corresponding oligosaccharides (F-IV and hp4/6, respectively, both 50 μg mL^{-1}) in MRC-9 cells. The cells were cultured with test samples for 12 h and the amount of HGF in the medium measured. Interleukin (IL)-1α (10 ng mL^{-1}) stimulates production of HGF by regulating transcription of the HGF gene. △UA-GlcNS is a heparin disaccharide. Data represent mean ± s.d. of four experiments. *$P < 0.05$; **$P < 0.01$ compared with relevant control.

mechanism of this effect is not fully understood. The ability of fucoidan and LMW fucoidan to stimulate production of HGF suggests that these compounds may be useful as natural medicines.

Conclusions

Fucoidan and fucoidan-derived oligosaccharides have been shown to stimulate production of HGF, with activities comparable to those of heparin and heparin-derived oligosaccharides. Both heparin and fucoidan stimulated HGF production primarily at the level of translation, probably via the same mechanism.

References

Baba, M., Snoeck, R., Pauwels, R., de Clercq, E. (1998) Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. *Antimicrob. Agents Chemother*. **32**: 1742–1745

Berteau, O., Mulloy, B. (2003) Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* **13**: 29R–40R

Birchmeier, C., Gherardi, E. (1998) Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends Cell Biol*. **8**: 404–410

Bitter, T., Muir, H. M. (1962) A modified uronic acid carbazole reaction. *Anal. Biochem*. **4**: 330–334

Colliec, S., Fischer, A. M., Tapon-Bretaudiere, J., Boisson, C., Durand, P., Jozefonvicz, J. (1991) Anticoagulant properties of a fucoïdan fraction. *Thromb. Res*. **64**: 143–154

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem*. **28**: 350–356

Funakoshi, H., Nakamura, T. (2003) Hepatocyte growth factor: from diagnosis to clinical applications. *Clin. Chim. Acta* **327**: 1–23

Itoh, H., Noda, H., Amano, H., Zhuaug, C., Mizuno, T., Ito, H. (1993) Antitumor activity and immunological properties of marine

algal polysaccharides, especially fucoidan, prepared from Sargassum thunbergii of Phaeophyceae. *Anticancer Res*. **13**: 2045–2052

Matsumoto, K., Nakamura, T. (1997) Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochem. Biophys. Res. Commun*. **239**: 639–644

Matsumoto, K., Nakamura, T. (2001) Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. *Kidney Int*. **59**: 2023–2038

Matsumoto, K., Okazaki, H., Nakamura, T. (1992a) Up-regulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. *Biochem. Biophys. Res. Commun*. **188**: 235–243

Matsumoto, K., Tajima, H., Hamanoue, M., Kohno, S., Kinoshita, T., Nakamura, T. (1992b) Identification and characterization of "injurin," an inducer of expression of the gene for hepatocyte growth factor. *Proc. Natl Acad. Sci. U S A*. **89**: 3800–3804

Matsumoto, K., Tajima, H., Okazaki, H., Nakamura, T. (1993) Heparin as an inducer of hepatocyte growth factor. *J. Biochem. (Tokyo)* **114**: 820–826

Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N. Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Gohda, E., Daikuhara, Y., Kitamura, N. (1989) Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. *Biochem. Biophys. Res. Commun*. **163**: 967–973

Mizuno, S., Nakamura, T. (2007) Hepatocyte growth factor: a regenerative drug for acute hepatitis and liver cirrhosis. *Regen. Med*. **2**: 161–170

Nakamura, T., Nawa, K., Ichihara, A. (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun*. **122**: 1450–1459

Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A. Tashiro, K., Shimizu, S. (1989) Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342**: 440–443

Sakiyama, R., Fukuta, K., Matsumoto, K., Furukawa, M., Takahashi, Y., Nakamura, T. (2007) Stimulation of hepatocyte growth factor production by heparin-derived oligosaccharides. *J. Biochem. (Tokyo)* **141**: 653–660

Shimaoka, M., Ikeda, M., Iida, T., Taenaka, N., Yoshiya, I., Honda, T. (1996) Fucoidin, a potent inhibitor of leukocyte rolling, prevents neutrophil influx into phorbol-ester-induced inflammatory sites in rabbit lungs. *Am. J. Respir. Crit. Care Med*. **153**: 307–311

Tamura, M., Arakaki, N., Tsubouchi, H., Takada, H., Daikuhara, Y. (1993) Enhancement of human hepatocyte growth factor production by interleukin-1α and -1β and tumor necrosis factor-α by fibroblasts in culture. *J. Biol. Chem*. **268**: 8140–8145

Tissot, B., Daniel, R. (2003) Biological properties of sulfated fucans: the potent inhibiting activity of algal fucoidan against the human complement system. *Glycobiology* **13**: 29G–30G

Ye, J., Li, Y., Teruya, K., Katakura, Y., Ichikawa, A., Eto, H., Hosoi, M. Nishimoto, S., Shirahata, S. (2005) Enzyme-digested fucoidan extracts derived from seaweed *Mozuku* of *Cladosiphon novae-caledoniae kylin* inhibit invasion and angiogenesis of tumor cells. *Cytotechnology* **47**: 117–126