Specific inhibition of FGF-2 signaling with 2-O-sulfated octasaccharides of heparan sulfate

Satoko Ashikari-Hada, Hiroko Habuchi, Noriko Sugaya, Takashi Kobayashi, and Koji Kimata

Introduction

Heparan sulfate (HS) proteoglycans interact with a variety of heparin-binding growth factors (HB-GFs), morphogens, and their receptors via HS chains. These interactions regulate the activity, gradient formation, and stability of these ligand molecules. HS is a polysaccharide of repeating disaccharide units with glucuronyl N-acetylglucosamine as a back bone and undergoes several modification reactions in the Golgi apparatus catalyzed by the following enzymes: N-deacetylation, N-sulfotransferases. C5 epimerase, HS 2-O-sulfotransferase (HS2ST), HS 6-O-sulfotransferases (HS6ST), and HS 3-O-sulfotransferases (Pellegrini et al. 2000; Pye et al. 1998; Delehedde et al. 2000). These modifications yield HS with a variety of structures. Since the interaction of HS with HB-GFs is thought to be dependent upon the patterns of sulfate residues and hexuronic acid isomers besides upon their negative charge (Gallagher 2001; Nakato and Kimata 2002; Allen and Rapraeger 2003; Ashikari-Hada et al. 2004; Powell et al. 2004; Kreuger et al. 2006), such a structural diversity of HS may give rise to differences in the response of cells to various HB-GFs. Thus, HS is thought to have important regulatory functions in a variety of developmental, morphogenetic, physiological, and pathogenic processes. 

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we transfected the human FGFR-1 gene into CHO-K1 cells but the expressions of Fgfr-1, Fgfr-3 were examined the species and the expression levels of the FGFRs using this cell line in order to test our hypothesis. For this, we et al. 2003), we initially established an experimental system of interactions between HS and FGF-2 (Richard et al. 1995; Lundin et al. 2000). It has been reported that the minimal lengths of heparin required for FGF-2-dependent activation are octasaccharide, decasaccharide, or dodecasaccharide (Ornitz et al. 1992; Guimond et al. 1993; Ishihara et al. 1993; Tyrrell et al. 1993; Ostrovsky et al. 2002) and that both the 2-O-sulfate groups of IdoUA and the 6-O-sulfate groups of N-sulfated GlcN are required for the FGF-2 mitogenic activity (Guimond et al. 1993; Ishihara et al. 1994; Pye et al. 1998). It is interesting to note that when cell surfaces are deficient in HS or when the surface HSs exhibit a low degree of O-sulfation, exogenous heparin promotes FGF-2 activity, probably due to the substituted role of cell surface HS (Rapraeger et al. 1991; Yayon et al. 1991). Conversely, when cell surface HSs are substantially sulfated, exogenous heparin inhibits FGF-2 activity. The latter response of exogenous heparin appears to be caused by a competition effect with cell surface HSs (Ishihara 1994; Lundin et al. 2000). Such opposite effects of exogenous heparin on FGF-2 activity reflect the fact that the FGF-2/FGFR/HS ternary complex formation is necessary for FGF-2 signaling.

In this study, we postulated that certain HS oligosaccharides that can bind to FGF-2, but that cannot form a signaling ternary complex, may inhibit FGF-2 activity, whether endogenous HS is present on the cell surface or not. In order to test this hypothesis, we prepared an octasaccharide library consisting of well-defined sulfated octasaccharides (Ashikari-Hada et al. 2004). The library comprised 2-O-sulfated and 6-O-sulfated octasaccharides generated by in vitro reactions with heparan sulfate 2-O-sulfotransferase (HS2ST) or 6-O-sulfotransferase-1 (HS6ST-1) from the octasaccharide, octa-N2,6sulfated and N-sulfated heparin (CDSNS heparin). We also established an experimental system by transfecting CHO-K1 cells with the human FGFR-1 gene and by cloning the stable mutants (CHO-K1/FR1 cells). We then screened the library for octasaccharides that could specifically inhibit the FGF-2-induced phosphorylation of FGFR-1 and ERK1/2. We found that the inhibitory effect of octasaccharide on the FGF-2 activity was dependent on the number of 2-O-sulfate groups within a molecule but not dependent on the number of 6-O-sulfate groups. In contrast, both the 2-O-sulfate and the 6-O-sulfate groups were necessary for the inhibitory effect of octasaccharides on FGF-4 activity. Our results suggest that the activity of particular heparin-binding factors may be inhibited by octasaccharides with distinctive structures, which can bind FGF and/or FGFR but cannot form a functional signaling complex.

**Results**

**Establishment of an experimental system for CHO-K1 cells**

Since CHO-K1 cells have often been used to investigate the interactions between HS and FGF-2 (Richard et al. 1995; Lundin et al. 2003), we initially established an experimental system using this cell line in order to test our hypothesis. For this, we examined the species and the expression levels of the FGFRs in CHO-K1 cells by RT-PCR. CHO-K1 cells expressed Fgfr-3 but the expressions of Fgfr-1, -2, and -4 were below the level of detection (Figure 1A, left panel). Since FGFR-1 is one of the major tyrosine kinase receptors for FGF-2 (Ornitz et al. 1996), we transfected the human FGFR-1 gene into CHO-K1 cells and cloned the transfectants (CHO-K1/FR1 cells). The CHO-K1/FR1 cells thus obtained stably expressed human FGFR-1 in addition to Fgfr-3 (Figure 1A, right panel).

**FGF-2-induced tyrosine phosphorylation of FGFR-1 and ERK1/2**

Most of the previous reports on FGF-induced signaling are concerned with the effects on cell proliferation and/or mitogenic activity, which at least need the incubation for several hours to be investigated (18 h; Guimond et al. 1993; 46 h; Pye et al. 1998; 4 h; Lundin et al. 2000; 4 days; Googder et al. 2008). We previously observed that the binding of FGF-2 to HS prevented FGF-2 from proteolytic degradation (Habuchi et al. 1992). The long-term assays for the effects on cell proliferation and/or mitogenic activity as described above might include such secondary effects in addition to those on the signaling. Therefore, in this study, FGF-induced signaling was determined by the phosphorylation of FGFR-1 and ERK1/2, which can be performed within a short period. We used an antibody against phospho(Tyr766)-FGFR-1 to detect phosphorylated FGFR-1 by Western blotting. The same membrane was stripped and reprobed with an anti-ERK1/2 (ERK) antibody. (C) Phospho-FGFR-1 (closed circles) and phospho-ERK1/2 (open triangles) were determined using Multi-Gauge image analysis software. Nonspecific phosphorylation was determined in the absence of FGF-2. The maximal levels of phosphorylation were set to 1. The experiments were independently repeated three times. The data show mean values ± SD.
Fig. 2. FGF-2-bound, but not unbound, modified heparins inhibited FGF-2-induced phosphorylation. (A and C) CHO-K1/FR1 cells were stimulated with 2 ng/mL FGF-2 in the absence or presence of FGF-2-bound and unbound modified heparins for 5 min at 37°C. Samples were subjected to gel electrophoresis, with subsequent transfer to PVDF membranes. Phospho-FGFR-1 and phospho-ERK1/2 were stained with specific antibodies. (B) Phospho-FGFR-1 (closed circles) and phospho-ERK1/2 (open triangles) were determined using Multi-Gauge image analysis software. Nonspecific phosphorylation was determined in the absence of FGF-2. The experiments were independently repeated three times. The mean values ± SD represent relative phosphorylations, when the values without heparin were set to 1.

Effect of modified heparins on FGF-2-induced signaling

In the case of HS-deficient CHO cells (CHO-677) overexpressing FGFR-1, it has previously been reported that heparin stimulates FGF-2 signaling in the concentration range 10–1 μg/mL (Lundin et al. 2000). In CHO-K1/FR1 cells, high concentrations of heparin (100 ng/mL) decreased the FGF-2-induced phosphorylation of FGFR-1 and ERK1/2, although at lower concentrations (1–10 ng/mL) the effect was not significant (Figure 2A and B). Heparin alone did not affect basal levels of phosphorylation of FGFR-1 or ERK1/2 (data not shown). In order to clarify which sulfate groups are important for the inhibitory effect on FGF-2 signaling, we investigated the effects of the following modified heparins on FGF-2-induced FGFR-1 and ERK1/2 phosphorylations in CHO-K1/FR1 cells: completely desulfated N-sulfated heparin (CDSNS heparin), 2-O-desulfated heparin (2ODS heparin), and 6-O-desulfated heparin (6ODS heparin). The major disaccharide components of heparin, CDSNS heparin, 2ODS heparin, and 6ODS heparin were HexUA(2SO₄)GlcNSO₃(6SO₄), HexUA-GlcNSO₃, HexUA-GlcNSO₃(6SO₄), and HexUA(2SO₄)-GlcNSO₃, respectively (Habuchi et al. 2000; Ashikari-Hada et al. 2004). Interestingly, 6ODS heparin impeded these FGF-2-induced phosphorylations, whereas CDSNS heparin and 2ODS heparin did not (Figure 2C). Considering our previous observation that FGF-2 specifically binds to HS containing an IdoUA(2SO₄)-GlcNSO₃ unit (Ashikari-Hada et al. 2004), these results clearly suggest that only the modified heparin that had the capacity to bind FGF-2 inhibited the FGF-2-inducible signaling.

Effect of an octasaccharide library on FGF-2-induced signaling

The results above further suggest that the FGF-2-binding structure in exogeneous HS may compete with endogenous HS proteoglycan for binding to FGF-2 so as to interrupt FGF-2-induced signaling. We investigated whether HS oligosaccharides that have the capacity to bind FGF-2 could inhibit FGF-2 signaling. We first prepared an octasaccharide library as described below. The oligosaccharide, ΔHexUA-GlcNSO₃-(HexUA-GlcNSO₃)₃, was prepared from CDSNS-heparin as described in Material and methods and designated as Octa-N₂₄₀₆₀. Hereafter, this designation is used. Octa-N₂₄₀₆₀ was sulfated by HS2ST to yield Octa-N₂₄₁₆₀, Octa-N₂₄₂₆₀, and Octa-N₂₄₃₆₀, which had one, two, and three 2-O-sulfate groups, respectively, compared to Octa-N₂₄₀₆₀. Octa-N₂₄₂₆₀ was sulfated by HS6ST₁ to yield N₂₄₂₆₁ which had three 6-O-sulfate groups compared to Octa-N₂₄₂₆₀. Octa-N₂₄₃₆₃ was prepared from heparin and contained three HexUA(2SO₄)GlcNSO₃(6SO₄) disaccharide units within the octasaccharide. We demonstrated previously that, among these octasaccharides, FGF-2 bound Octa-N₂₄₂₆₀, Octa-N₂₄₃₆₀, Octa-N₂₄₂₆₁, and Octa-N₂₄₃₆₁, whereas FGF-4 only bound to Octa-N₂₄₂₆₃ (Ashikari-Hada et al. 2004). We probed with an anti-phospho-ERK1/2 antibody as described in Material and methods. Western blots (Figure 1B and C) demonstrated that FGF-2 dramatically increased the phosphorylation of FGFR-1 and ERK1/2, although at lower concentrations (1–10 ng/mL) the effect was not significant (Figure 2A and B). Heparin alone did not affect basal levels of phosphorylation of FGFR-1 or ERK1/2 (data not shown). In order to clarify which sulfate groups are important for the inhibitory effect on FGF-2 signaling, we investigated the effects of the following modified heparins on FGF-2-induced FGFR-1 and ERK1/2 phosphorylations in CHO-K1/FR1 cells: completely desulfated N-sulfated heparin (CDSNS heparin), 2-O-desulfated heparin (2ODS heparin), and 6-O-desulfated heparin (6ODS heparin). The major disaccharide components of heparin, CDSNS heparin, 2ODS heparin, and 6ODS heparin were HexUA(2SO₄)GlcNSO₃(6SO₄), HexUA-GlcNSO₃, HexUA-GlcNSO₃(6SO₄), and HexUA(2SO₄)-GlcNSO₃, respectively (Habuchi et al. 2000; Ashikari-Hada et al. 2004). Interestingly, 6ODS heparin impeded these FGF-2-induced phosphorylations, whereas CDSNS heparin and 2ODS heparin did not (Figure 2C). Considering our previous observation that FGF-2 specifically binds to HS containing an IdoUA(2SO₄)-GlcNSO₃ unit (Ashikari-Hada et al. 2004), these results clearly suggest that only the modified heparin that had the capacity to bind FGF-2 inhibited the FGF-2-inducible signaling.
2-O-Sulfated octasaccharides inhibit FGF-2 signaling

Fig. 3. FGF-2-bound, but not unbound, octasaccharides inhibited FGF-2-induced phosphorylation. Effect of the addition of an octasaccharide library on the FGF-2-induced phosphorylation of FGFR-1 (first row in panels) and ERK1/2 (second row in panels) in CHO-K1/FR1, CHO-K1 (third row in panels), and CHO-677 (fourth row in panel) cell lines. CHO-K1/FR1, CHO-K1, and CHO-677 cells were stimulated with 2 ng/mL FGF-2 in the absence or presence of various octasaccharides of indicated concentrations (nmol as HexUA/mL) for 5 min at 37°C in duplicate. Samples were subjected to gel electrophoresis, with subsequent transfer to PVDF membranes. Phospho-FGFR-1 and phospho-ERK1/2 were stained with the respective specific antibodies. Similar results were obtained in two other different experiments.

then examined the effects of these octasaccharides on the phosphorylation of FGFR-1 and ERK1/2 in CHO-K1/FR1 cells, which were stimulated with 2 ng/mL FGF-2 for 5 min at 37°C (Figure 3, first and second rows of panels). Octa-N42160, Octa-N42260, and Octa-N42360 inhibited FGF-2-induced FGFR-1 phosphorylation in a concentration-dependent manner. At a concentration of 5 nmol as HexUA/mL, all of these octasaccharides had decreased the FGFR-1 phosphorylation activity to 17% or less. At a concentration of 2 nmol as HexUA/mL, Octa-N42160, Octa-N42260, and Octa-N42360 significantly decreased the FGFR-1 phosphorylation activity to 22%, 64%, 67%, and 34%, respectively. FGF-2-induced phosphorylations of ERK1/2 were also decreased by the addition of the octasaccharides in a concentration-dependent manner. At a concentration of 5 nmol as HexUA/mL, Octa-N42160, Octa-N42260, and Octa-N42360 decreased the ERK1/2 phosphorylation activity to 5%, 46%, 27%, and 21%, respectively, when the phosphorylation without the addition of the octasaccharides was set as 100%. Interestingly, Octa-N42063 failed to inhibit both FGFR-1 and ERK1/2 phosphorylations. These results indicate that the octasaccharides possessing some 2-O-sulfate groups and with the capacity to bind FGF-2 can inhibit FGF-2-induced FGFR-1/ERK phosphorylation, whereas those lacking the binding capacity cannot.

In order to avoid the possibility that FGF-2-binding octasaccharides simply delayed or sustained the duration of the FGF-2-induced signaling, the time courses of the effects of the octasaccharides on the phosphorylation of FGFR-1 and ERK1/2 were determined. FGFR-1 and ERK1/2 phosphorylations were decreased by the presence of 2 nmol as HexUA/mL Octa-N42363 at every time point examined (Figure 4). Both phosphorylations in the presence and the absence of Octa-N42363 reached maximum levels 10 min after FGF-2 was added, and the phosphorylation of FGFR-1 and ERK1/2 in the presence of Octa-N42363 was decreased to 57% and 65%, respectively, compared to that in the absence of Octa-N42363. In the presence of 1 µg/mL heparin, which is approximately equivalent to 2 nmol as HexUA/mL octasaccharide, the FGF-2-induced phosphorylation of FGFR-1 and ERK1/2 was reduced to 25% and 17%, respectively, at 10 min (data not shown). These results indicated that inhibition of FGF-2 signaling with FGF-2-binding octasaccharides and heparin continued at least up to 30 min after induction and was not due to a delay in phosphorylation.

Fig. 4. The time course of the effects of Octa-N42363 on FGF-2-induced phosphorylation. CHO-K1/FR1 cells were stimulated with 2 ng/mL FGF-2 in the absence (open circles) or presence (closed circles) of 2 nmol as HexUA/mL Octa-N42363 at 37°C in duplicate. Cell lysates from CHO-K1/FR1 cells stimulated for the indicated times were analyzed by immunoblotting with specific antibodies for the indicated proteins. The experiment was carried out independently three times. The data are representative of the three experiments. Phospho-FGFR-1 (B) and phospho-ERK1/2 (C) were determined using Multi-Gauge image analysis software. Nonspecific phosphorylation was determined in the absence of FGF-2.
CHO-K1 cells, which only express FGFR-3, were stimulated with 2 ng/mL FGF-2 in the absence or presence of various octasaccharides for 5 min at 37°C (Figure 3, third row of panels). The FGF-2-induced phosphorylation of ERK1/2 was decreased by FGF-2-binding octasaccharides such as Octa-N21260, Octa-N2260, Octa-N2260, and Octa-N2260 but not by FGF-2 unbound octasaccharides such as Octa-N2260: this indicated that FGF-2-bound octasaccharides inhibited the FGF-2/FGFR-3/ERK signaling pathway as well as the FGF-2/FGFR-1/ERK signaling pathway.

A previous study (Lundin et al. 2003) has demonstrated that in the HS-deficient CHO-677 cell line, FGF-2 alone had a very limited but significant stimulatory effect on ERK1/2 kinase activation and that the addition of exogenous heparin increased FGF-2-induced ERK1/2 phosphorylation. In the present study, the addition of exogenous 1 μg/mL heparin to a culture of CHO-677 cells increased FGF-2-induced ERK1/2 phosphorylation (data not shown), which is consistent with the previous observation. In contrast, interestingly, the addition of Octa-N21260 to the culture decreased FGF-2-induced ERK1/2 phosphorylation significantly (Figure 3, bottom row of panel). The lack of cell surface HS chains changed the effect of heparin on FGF-2 activity to a stimulatory one; however, this effect was not observed with Octa-N21260. The FGF-2-binding octasaccharide did not behave similarly to heparin in the HS-deficient CHO-677 cell line. These results suggest that FGF-2-binding octasaccharides but not a polysaccharide inhibited FGF-2 signaling irrespective of the presence of cell surface HS chains.

Effect of an octasaccharide library on binding FGF-2 to CHO-K1/FR1 cells

FGF-2-binding octasaccharides, which have some 2-O-sulfate residues, inhibited FGF-2/FGFR-1/ERK signaling. In order to confirm our hypothesis, we further examined if FGF-2-binding octasaccharides could decrease the binding of FGF-2 to HS on the cell surface. Digoxigenin-labeled FGF-2 with octasaccharides was prepared as described in Material and methods and added to cultured CHO-K1/FR1 cells. The binding of digoxigenin-labeled FGF-2 to the cell surface was greatly reduced by the addition of Octa-N21260 in a concentration-dependent manner (Figure 5A). The degree of inhibition induced by Octa-N21260 was comparable to that of heparin. With 20 nmol as HexUA/mL Octa-N21260 or Octa-N2260, the binding of digoxigenin-labeled FGF-2 to the cell surfaces was reduced to 42% and 23%, respectively. In contrast, N21260 had no significant effect on FGF-2 binding to the cell surface (Figure 5B). The results indicate that the binding of FGF-2 to the CHO-K1/FR1 cell surface is specifically inhibited by FGF-2-binding octasaccharides. Treatment of cells with a mixture of heparitinase I, heparitinase II, and heparinase decreased the binding of digoxigenin-labeled FGF-2 to 42% (Figure 5C), but the extent of the reduction was less than that by the addition of FGF-2-binding octasaccharides. Treatment with chondroitinase-ABC had no significant effect (Figure 5C). These results suggest that FGF-2-binding octasaccharides inhibit not only the binding of FGF-2 to heparan sulfate on the cell surface but also the binding of FGF-2 to cell surface molecules other than heparan sulfate and chondroitin sulfate chains of cell surface proteoglycans.

Fig. 5. Binding of FGF-2 to CHO-K1/FR1 cells was inhibited by the FGF-2-bound, but not unbound, octasaccharides. CHO-K1/FR1 were seeded at 5 × 10⁵ cells per well in 96-well plates. Digoxigenin-conjugated FGF-2 (50 ng/mL) with various concentrations of heparin (A, closed circles), Octa-N21260 (A, open circles), or 20 nmol as HexUA/mL octasaccharide (B) were added to each well for 30 min at 4°C. After unbound digoxigenin-conjugated FGF-2 was removed, the cell contents were fixed and blocked. Alkaline phosphatase-conjugated Fab fragments of the anti-digoxigenin antibody were then added. The enzyme activity of alkaline phosphatase in each well was measured by using a microplate reader. (C) Before the addition of digoxigenin-conjugated FGF-2, cells were digested with HSase mixture and/or chondroitinase ABC at 37°C for 15 min. Control wells were left undigested. Nonspecific binding was determined in the absence of digoxigenin-conjugated FGF-2. Each variable was tested in triplicate. The columns show mean values ± SD. The experiments were independently repeated twice, and statistical analyses were performed using Student’s t-test. *P < 0.05 with respect to digoxigenin-labeled FGF-2 alone.
were obtained in two different experiments.

In a previous study, we demonstrated that Octa-N 42363 was the only member of the octasaccharide library to which FGF-4 binds (Ashikari-Hada et al. 2004). Therefore, we asked whether only the FGF-4-binding octasaccharide, Octa-N 42363, could inhibit FGF-4-induced signaling. CHO-K1/FR1 cells were stimulated with 2 ng/mL FGF-4 in the absence or presence of octasaccharides for 5 min at 37°C. Immunoblotting of the respective cell lysates revealed that the FGF-4-induced phosphorylation of FGFR-1 and ERK1/2 was decreased to the baseline level by the addition of 5 nmol as HexUA/mL Octa-N 42363 (Figure 6). In contrast, Octa-N 42260 and Octa-N 42063 had no effect on FGF-4-induced phosphorylation. As expected from the experiment with FGF-2, the FGF-4 activity was specifically inhibited by FGF-4-binding octasaccharides. The data suggest that the specific HS-oligosaccharides bound to a particular HB-GF can inhibit the HB-GF activity.

Effect of an octasaccharide library on FGF-4-induced FGFR-1 phosphorylation

In a previous study, we demonstrated that Octa-N 42363 was the only member of the octasaccharide library to which FGF-4 binds (Ashikari-Hada et al. 2004). Therefore, we asked whether only the FGF-4-binding octasaccharide, Octa-N 42363, could inhibit FGF-4-induced signaling. CHO-K1/FR1 cells were stimulated with 2 ng/mL FGF-4 in the absence or presence of octasaccharides for 5 min at 37°C. Immunoblotting of the respective cell lysates revealed that the FGF-4-induced phosphorylation of FGFR-1 and ERK1/2 was decreased to the baseline level by the addition of 5 nmol as HexUA/mL Octa-N 42363 (Figure 6). In contrast, Octa-N 42260 and Octa-N 42063 had no effect on FGF-4-induced phosphorylation. As expected from the experiment with FGF-2, the FGF-4 activity was specifically inhibited by FGF-4-binding octasaccharides. The data suggest that the specific HS-oligosaccharides bound to a particular HB-GF can inhibit the HB-GF activity.

Effects of an octasaccharide library on FGF-2-induced ERK-1 phosphorylation in mouse embryonic fibroblast

We further examined using mouse embryonic fibroblast (MEF) whether it is general in cells other than CHO-K1 cells for octasaccharides possessing 2-O-sulfate residues to inhibit the FGF-2-induced ERK phosphorylation, regardless of whether endogenous HS was present on the cell surface. MEF expresses FGFR2 and FGFR4 other than FGFR1 and FGFR3. In MEF, the addition of FGF-2 increased the phosphorylation of ERK1/2 in a dose-dependent manner and the level of pERK reached almost a plateau at 2 ng/mL (Figure 7A).

The addition of 2 nmol HexA/mL heparin reduced markedly FGF2-induced pERK (Figure 7A). We next examined effects of oligosaccharides on the FGF-2-induced phosphorylation. The addition of Octa-N 42260 inhibited the phosphorylation up to the level of no addition of FGF-2 at the concentrations of both 1 and 5 nmol as HexUA/mL. However, Octa-N 42063 was not significant inhibitory even at 5 nmol/mL (Figure 7B). These results were consistent with those obtained using CHO-K1 cells and CHO-K1/FR1 cells. To examine the effects on the FGF-2-induced signaling in HS-deleted MEF, MEF was treated with a mixture of heparitinases as described in Material and methods. The response to FGF-2 in these cells was markedly decreased. FGF-2-induced phosphorylation in the treated cells was rescued by the addition of 1 μg/mL heparin (Figure 7C). As predicted from the results shown in Figure 3, Octa-N 42363 even at the high concentration of 5 nmol as HexUA/mL could not rescue the phosphorylation and rather slightly inhibited the phosphorylation. Interestingly, as also predicted, 5 nmol as HexUA/mL Octa-N 42260 slightly inhibited the phosphorylation, but the same concentration of Octa-N 42063 did not give any effect on the phosphorylation (Figure 7C). These results further support that at least octasaccharides possessing 2-O-sulfate residues but not 6-O-sulfate residues alone inhibit FGF-2-dependent signaling in MEF irrespective of whether HS is present or absent on the cell surfaces.

Discussion

In this study, we demonstrated that 2-O-sulfated octasaccharides, Octa-N 42260, Octa-N 42360, and Octa-N 42063, which were prepared by specific sulfation with HS2ST, when added to cultured CHO-K1/FR1 cells, inhibited FGF-2-induced FGFR-1 and ERK1/2 phosphorylation. In contrast, when a 6-O-sulfated octasaccharide, Octa-N 42063, which was prepared by specific sulfation with HS6ST-1, was added, phosphorylations were not inhibited (Figure 3). Such different effects observed between 2-O-sulfated octasaccharides and 6-O-sulfated octasaccharides could also be seen in the culture of MEFs (Figure 7). We previously demonstrated that Octa-N 42360, Octa-N 42260, and Octa-N 42063, but not Octa-N 42260, were able to bind FGF-2 (Ashikari-Hada et al. 2004). These results suggest that octasaccharides with HB-GF binding capacities were competent.
to inhibit HB-GF activities. Indeed, FGF-4-induced phosphorylation was inhibited by FGF-4-bound octasaccharides, such as Octa-N_{2,6,6}, but not by FGF-4-unbound octasaccharides, such as Octa-N_{2,6,6} and Octa-N_{2,6,6} (Figure 6). Therefore, it is possible that, in general, oligosaccharides containing certain HB-GF-bound structures are able to inhibit such HB-GF activities, but not other HB-GF activities, and this may be common to various types of cells.

A previous in vitro study (Robinson et al. 2005) has demonstrated that in the presence of heparin octasaccharide, 2:2:1 complexes are formed between FGF-1, FGFR-2c, and the octasaccharide; the octasaccharide was the shortest chain length that bound FGFR-2c, dimerized FGF-1, and promoted a strong mitogenic response to FGF-1 through FGFR-2c. This study also demonstrated that heparin hexasaccharide and various selectively desulfated heparin decasaccharides failed to bind FGFR-2c and could only interact with one FGF-1 monomerically and, thus, these saccharides only formed 1:1:1 ternary complexes with FGF-1 and FGFR-2c, although their recent study (Goedger et al. 2008) suggested that these complexes somehow showed the mitogenic activity by the mechanism distinct from the one that induces a rapid and efficient formation of the asymmetric 2:2:1 complex. A previous in vitro study (Jastrebova et al. 2006) has demonstrated that the ternary complex formation between FGF-2, FGFR-1c or -3c, and octa- or decasaccharides requires more than four O-sulfate groups (over-all number of both 2-O- and 6-O-sulfate residues). These results suggest that Octa-N_{2,6,6}, Octa-N_{2,6,6}, and Octa-N_{2,6,6} are unable to form the ternary complexes to induce the FGF-2 activity since they have no O-sulfate groups and less than four 2-O-sulfate groups. It should be noted here that we have demonstrated that digoxigenin-labeled FGF-2 binding to the surface of these cells was decreased by the addition of these 2-O-sulfated octasaccharides (Figure 5B). Considering these results together, we speculate that Octa-N_{2,6,6}, Octa-N_{2,6,6}, and Octa-N_{2,6,6} could compete with cell surface HS chains for binding FGF-2, and thus impede the formation of the FGF-2/HS/FGFR ternary complex and the FGF-2/HS binary complex on cell surfaces, which are necessary for FGF-2 signaling.

Most cells have HS proteoglycans on their cell surface. The addition of heparin and heparin-derived oligosaccharides to cultured cells inhibited FGF-2 activities at higher concentrations (Ishihara 1994; Ishihara et al. 1994; Lundin et al. 2000). However, when cells lack HS synthesis (Ornitz et al. 1992; Pye et al. 1998), when cells are genetically only capable of synthesizing low-sulfated HS (Lundin et al. 2000, 2003), or when cells encounter sulfate- or sulfate precursor-depleted environments (Guimond et al. 1993; Ishihara 1994), the situation is different—heparin promotes FGF-2 signaling. Heparin-derived oligosaccharides also induce activation (Ornitz et al. 1992; Guimond et al. 1993; Ishihara et al. 1994; Pye et al. 1998; Lundin et al. 2000), which fulfill the structural requirements for the ternary complex formation necessary for the FGF-2 activation, although the minimum length of such biologically active oligosaccharides varies from study to study—octasaccharides (Ornitz et al. 1992), decasaccharides (Ishihara et al. 1994; Pye et al. 1998; Lundin et al. 2000), or decasaccharides (Guimond et al. 1993). Considering the above speculation, it is likely that heparin and such heparin-derived oligosaccharides play a stimulatory role in FGF-2 signaling, as a substitute for deficient HS proteoglycans on cell surfaces, but that 2-O-sulfated octasaccharides of Octa-N_{2,6,6}, Octa-N_{2,6,6}, and Octa-N_{2,6,6} do not. In this study, we noticed that CHO-677 cells lacking the synthesis of HS chains still exhibited FGF-2-induced ERK1/2 phosphorylation, although the level is low compared to the wild-type cells, and this phosphorylation in CHO-677 cells was inhibited by the addition of Octa-N_{2,6,6} (Figure 3). Chondroitin sulfate/dermatan sulfate hybrid chains from embryonic pig brain exhibit binding to various FGFs, including FGF-2 (Steer et al. 2004), and chondroitin sulfate/dermatan sulfate-containing proteoglycans participate in the metastatic melanoma cell FGF-2-induced mitogenic response (Nikitovic et al. 2008). It is likely, therefore, that the observed FGF-2-induced ERK1/2 phosphorylation in CHO-677 cells might be due to chondroitin/dermatan sulfate-containing proteoglycans on the cell surface. However, in the present study, we also observed that the binding of digoxigenin-conjugated FGF-2 to CHO-K1/FR1 cells was not affected by chondroitinase ABC digestion (Figure 5C). Further, Lundin et al. (2003) demonstrated that, as well as CHO-677 cells, CHO-745 cells devoid of the xylosyltransferase activity required for the initiation of HS and chondroitin sulfate chain syntheses exhibit FGF-2-induced ERK1/2 phosphorylation. They also demonstrated that tyrosine 463 in the juxtamembrane domain of FGF-1 was phosphorylated in the absence of heparin, whereas heparin was required for tyrosine 766 to be phosphorylated. Therefore, CHO cells may have a signal transducing system via tyrosine 463 in response to FGF-2 treatment alone which is independent of HS and/or the chondroitin/dermatan sulfate chains of cell surface proteoglycans and is inhibited by Octa-N_{2,6,6}, possibly by depriving such a unique signal system of FGF-2. Here it should be noted that Octa-N_{2,6,6} may not form the signaling 2:2:1 complex with FGF-2 and FGFRs because this octasaccharide contains three units of HexUA(2SO4)-GlcNS-O3(6SO4) disaccharide component and is not fully sulfated, compared to heparin octasaccharide, Octa-N_{2,6,6}, that is composed of four units of HexUA(2SO4)-GlcNS-O6SO4 and has been demonstrated to be able to form the signaling 2:2:1 complex with FGF-2 and FGFR1c (Robinson et al. 2005; Goedger et al. 2008). Therefore, it is likely that, different from heparin and heparin octasaccharide, the binding of Octa-N_{2,6,6} to FGF-2 by forming the 1:1 complex reduces the concentration of FGF-2 available to a unique signal system described above.

The present study has demonstrated that Octa-N_{2,6,6} was unable to inhibit FGF-2-induced phosphorylation and FGF-2 binding to cells. In addition, 2ODS heparin was also unable to inhibit FGF-2-induced phosphorylation. Our previous study also indicated that Octa-N_{2,6,6} is unable both to bind FGF-2 on affinity columns and to release FGF-2 from FGF-2/HS complexes coated on plastic wells (Ashikari-Hada et al. 2004). These observations are consistent with the results of previous studies by others. For example, 2-O-sulfate-deficient oligosaccharides, even decasaccharides, have no effect on FGF-2-induced proliferation, regardless of the status of cell surface HS (Ishihara et al. 1994), and that 2ODS heparin does not inhibit FGF-2-induced FGFR-1 activation by heparin (Lundin et al. 2000). Further, although Jenthin et al. (2002) reported that IdoUA-GlcNSO_{6SO4}-IdoUA-aManQ has some affinity to FGF-2, Jastrebova et al. (2006) have demonstrated that the affinity is definitely weaker than that of GlcUA-GlcNS-IdoUA(2SO4)-GlcNSO_{6SO4}-GlcUA-GlcNSO_{6SO4}-GlcUA-aManQ to FGF-2. Furthermore, in fibroblasts derived from HS2ST-null mice, FGF-2

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promoted ERK1/2 phosphorylation; however, the interaction of FGF-2 with HS derived from HS2ST-null mice was weaker than that with HS derived from wild-type mice (Merry et al. 2001). Our previous study (Ashikari-Hada et al. 2004) indicated that the dissociation constant value of the FGF-2/2ODS heparin interaction was 15-fold greater than that of the FGF-2/heparin interaction and that desulfation of the 2-O-sulfated groups of heparin caused the high dissociation rate. Taken together with these results, 6-O-sulfate-rich and 2-O-sulfate-devoid oligosaccharides or polysaccharides, although they have an affinity to FGF-2 in some cases as described above, do not play a significant role in competitively inhibiting the interaction of FGF-2 with cell surface HS. A 6-O-sulfate group could not compensate for the loss of a 2-O-sulfate group in the inhibition of FGF-2 activity.

The wild-type CHO-K1 cells do not express FGFR-1. However, FGF-2 treatment alone induced a strong ERK1/2 phosphorylation (Figure 3). FGF-2 probably enhances FGFR-3-mediated signal transduction since CHO-K1 cells express Fgf-3 mRNA (Figure 1). 2-O-Sulfated octasaccharides, Octa-N42160, Octa-N42260, and Octa-N42360, inhibited not only FGF-2-induced FGFR-1 phosphorylation in CHO-K1/FRI cells but also FGF-2-induced ERK1/2 phosphorylation in CHO-K1 cells (Figure 3). These results suggest that those oligosaccharides preclude both FGFR-1- and FGFR-3-mediated signaling pathways. Jastrebova et al. (2006) have demonstrated that FGF-2 requires decasaccharides with a 6-O-sulfated group for complex formation with FGFR-1c, FGFR-2c, FGFR-3c, or FGFR-4. These results suggest that 2-O-sulfated octasaccharides act as competitive inhibitors of the interactions between FGF-2 and HS but do not inhibit interactions between any of the above-mentioned FGFRs and HS.

FGF-2, together with the hepatocyte growth factor (HGF) and vascular endothelial growth factor-A (VEGF), is a major angiogenesis factor. A line of study on tumor cells has revealed an increased production of FGF-2 by tumor cells themselves (Ribatti et al. 2007), and that FGF-2 produced by tumor cells exerts autocrine and paracrine functions in angiogenesis. Octa-N42160, Octa-N42260, and Octa-N42360 might inhibit such angiogenesis. Since Octa-N42160 and Octa-N42260 bind not only FGF-2 but also HGF (Ashikari-Hada et al. 2004), they might inhibit both FGF-2- and HGF-mediated signaling. In contrast, since Octa-N42260 and Octa-N42360 do not bind VEGF165 (Ashikari-Hada et al. 2004), these octasaccharides should not inhibit VEGF165 signaling. However, since FGF-2 and VEGF have been found to exert a synergistic effect in angiogenesis models (Presta et al. 2005; Ribatti et al. 2007) and HGF has also been found to induce a significant increase in VEGF production (Dong et al. 2001), 2-O-sulfated octasaccharides might indirectly regulate VEGF activation, and thus be effective as anti-angiogenesis reagents.

**Expression of the FGFR-1 protein**

For the production of stable transfectants of FGFR-1, a 3.4-kb cDNA encoding the IIic splice variant of FGFR-1 was inserted into the pcDNA3 expression vector at the EcoRV and NotI sites. The full open reading frame of FGFR-1, EcoRV-NotI fragment obtained from a full-length cDNA clone (accession number BC018128, purchased from Open Biosystems, Huntsville, AL), was ligated into pcDNA3. The orientation of the cDNA insert was confirmed by DNA sequencing. The pcDNA3/FGFR-1 construct was then transfected into CHO-K1 cells using the Transfast mammalian transfection system (Promega, Southampton, UK) following the manufacturer’s recommendations. Putative transfectants were selected by antibiotic resistance in the cell medium containing 500 μg/mL G418. After 4 weeks in culture in the presence of G418, the surviving clones were tested for the presence of FGFR-1 mRNA and FGFR-1 protein expression.

**Expression level of FGFR messenger RNA in CHOK1 and CHOK1/FRI**

Total cellular RNA was prepared using the RNeasy Mini Kit (QIAGEN, Germany). Aliquots of total cellular RNA (1.0 μg) were subjected to first-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and the cDNA was diluted five times with water. One microliter of the diluted cDNA was used for each PCR reaction. The PCR primer set used for the hFGFR-1 gene was: forward, 5'-AACCTGATTTAGCCAC-3' and reverse, 5'-CAGGGTCGCTGCGCAATC-3'. The size of this reaction product was 297 bp. The PCR primer sets used for the mFGFR-1~4 genes were described previously (Sugaya et al. 2008). After 30 cycles of amplification according to the manufacturer’s specifications (Promega, France), PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

**Preparation of octasaccharide library**

Octa-N42160, an oligosaccharide composed of HexUA-GlcNSO3 and Octa-N42260, an oligosaccharide composed of HexUA(2SO4)-GlcNSO3(6SO4), were prepared from CDSNS-heparin and heparin, respectively, as described previously (Ashikari-Hada et al. 2004). The structure of Octa-N42160 was HexUA-GlcNSO3-(HexUA-GlcNSO3)3. On the other hand, 3 mol of HexUA(2SO4)-GlcNSO3(6SO4), 0.5 mol of HexUA-GlcNSO3(6SO4), and 0.5 mol of other disaccharide components were released from 1 mol of Octa-N42260. Therefore, the structure of Octa-N42260 was thus a mixture containing three units of HexUA(2SO4)-GlcNSO3(6SO4) per molecule. 2-O-Sulfated derivatives of and 6-O-sulfated derivatives of Octa-N42260 were prepared as follows. For 2-O-sulfation of Octa-N42260, the reaction mixture contained, in a final volume of 500 μL, 10 μmol of acetic anhydride, pH 5.5, 37.5 μg of protease chloride, 5 nmol as HexUA of Octa-N42260, 40 nmol of PAPS, and soluble HS2ST, man FGF-2 for analysis of phosphorylation was purchased from R&D Systems (Minneapolis, MN). Recombinant human FGF-2 for labeling of digoxigenin was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Recombinant human FGF-4 was purchased from R&D Systems. CHO-677 cells which are deficient in HS biosynthesis were kindly gifted by Dr Jeffrey Esko.

**Material and methods**

Completely desulfated, N-sulfated heparin (CDSNS heparin), heparitinase I (Flavobacterium heparinum, EC 4.2.2.8), heparitinase II (F. heparinum, EC 4.2.2.7), 2-O-desulfated heparin (2ODS heparin), and 6-O-desulfated heparin (6ODS heparin) were obtained from Seikagaku Corp. (Tokyo, Japan). Recombinant hu-
For 6-O-sulfation, the reaction mixture (500 μL) contained 25 μmol of imidazole-HCl, pH 6.8, 37.5 μg of proteamine chloride, 5 nmol as HexUA of Octa-N2,4,6,9,0, for 5-O-sulfated derivatives of Octa-N2,6,0, and 6-O-sulfated derivatives of Octa-N2,6,0. The sulfated products were precipitated with 3 volumes of ethanol containing 1.3% potassium acetate and 0.5 mM EDTA and then separated with Mono Q chromatography, which was performed with a linear gradient from 0.2 to 0.8 M NaCl in 50 mM glycine–HCl, pH 3.0. Both 2-O-sulfated derivatives of Octa-N2,4,6,9,0, and 6-O-sulfated derivatives of Octa-N2,6,0 were separated into three peaks: Octa-N2,4,6,9,0, Octa-N2,4,6,9,0, and Octa-N2,4,6,9,0 for 2-O-sulfated derivatives of Octa-N2,6,0 and Octa-N2,6,0, Octa-N2,6,0, and Octa-N2,6,0 for 6-O-sulfated derivatives of Octa-N2,6,0, Octa-N2,6,0, Octa-N2,6,0, and Octa-N2,6,0 contained one, two, and three units, respectively, of the HexUA(2SO4)–GlcNSO3 component and Octa-N2,6,0, Octa-N2,6,0, and Octa-N2,6,0 have one, two, and three units, respectively, of the HexUA-GlcNSO3(6SO4) component.

Cloning and expression of soluble forms of HS2ST and HS6ST-1

The eDNAs of soluble forms of HS2ST and HS6ST-1 (sHS2ST and sHS6ST-1, respectively) were constructed by removing the sequences corresponding to 27 amino acids comprising the N-terminal transmembrane domain, respectively. The gene for truncated HS2ST was obtained by a PCR reaction using a 5′ primer GACACCGCTGGAACCCAGATCC with a HindIII site (underlined) and a pFLAG-CMV-2 reverse 3′ primer AACCCGGGATCCTCTAGATGCGACT with a pFLAG-CMV-2 template plasmid containing the HS2ST gene. The gene for truncated HS6ST-1 was obtained by a PCR reaction using a 5′ primer GACACGGCTCAGGACTGCTGAT with a HindIII site (underlined) and a pFLAG-CMV-2 reverse 3′ primer AACCCGGGATCCTCTAGATGCGACT with a pFLAG-CMV-2 template plasmid containing the HS6ST-1 gene. These PCR products were cleaved by HindIII/KpnI and cloned into a HindIII/KpnI-digested pFLAG-CMV-3 vector that was constructed by inserting the preprotrypsin secretion signal sequence and a FLAG peptide sequence at N-terminal. pFLAG-CMV-3 template plasmids containing the genes for sHS2ST or sHS6ST-1 served as a PCR template using the 5′ primer ACCCTTAGAATGTCTGCACTTCTGA with a XbaI site (underlined) and the 3′ primer AACCCGGGATCCTCTAGATGCGACT. The PCR products were digested and cloned into the XbaI and KpnI restriction sites of a pFastBac1 vector. The reading frame and coding region were confirmed by sequencing. pFastBac vectors containing the sHS2ST or sHS6ST-1 gene were transformed into DH10Bac Escherichia coli cells to allow site-specific transposition into bacmid BMON14272 (BAC-TOBAC Baculovirus Expression System, Life Technologies, Inc., Gaithersburg, MD). High molecular weight recombinant bacmids were isolated and transfected into Sf21 cells to produce virus stocks that were amplified as described by the manufacturer. For protein production, 1.7 x 10^8 High Five cells were infected with the recombinant baculoviruses. After 3 days, each culture medium was collected and cells were removed by low speed centrifugation. The harvested medium was mixed with Tris-HCl, pH 7.2 (final concentration of 10 mM), Trion X-100 (0.1%), NaCl (0.2 M), glycerol (20%), MgCl₂ (10 mM), and CaCl₂ (2 mM). Recombinant each HSST was purified with the heparin affinity column and anti-FLAG M2 antibody-conjugated affinity column. All the purification steps were carried out at 4°C.

Analysis of FGFR-1 and ERK1/2 phosphorylation

Chinese hamster ovary (CHO-K1) cells in Dulbecco’s modified Eagle’s medium (DMEM) were seeded at 1.9 x 10^5 cells per well in 12-well plates and serum starved overnight. MEF were seeded at 1 x 10^5 cells per well in a 24-well plate and starved of serum overnight as described previously (Sugaya et al. 2008). The cells were then stimulated with FGF-2 or FGF-4 in the presence or absence of glycosaminoglycan for 5 min at 37°C. The cells were lysed in the cell lysis buffer containing 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Triton X-100, 1.5 mM EDTA, 1 mM Na3PO4, 25 mM NaF, 1 mM Na3VO4, and 1 tablet of protease inhibitor mixture per 10 mL (Complete Mini, Roche Applied Science). Lysates were stirred gently and clarified by centrifugation (10,000 rpm for 30 min). Protein concentrations were determined using a QuickPro BCA assay kit (Sigma, St. Louis, MO), and 30 μg of protein from each sample was used for analysis. For immunoblotting, cell lysates were subjected to 8.0% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were incubated with 10% skim milk containing phosphate-buffered saline (PBS) and probed with the primary antibody diluted in 1% skim milk containing PBS. FGFR-1 phosphorylation and ERK1/2 phosphorylation were detected with a polyclonal antibody specific to phosphorylated Tyr766) FGFR-1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal anti-phosphorylated (Thr202/Tyr204) ERK1/2 (1:1000; Cell Signaling Technology, Danvers, MA). The reaction was visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Western Lightning Plus; Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer’s instructions. Band density was determined using Image Gauge or Multi Gauge image analysis software (Fuji Film, Tokyo, Japan). The membranes were then stripped by incubating in the stripping buffer (Restore™ Western blot stripping buffer; Pierce, Rockford, IL) at room temperature for 15 min and reprobed with total ERK1/2 antibodies to quantify the ERK1/2 loaded onto each lane. Total ERK1/2 was detected by a polyclonal anti-ERK1/2 antibody (1:1000; Cell Signaling Technology). The experiments were independently repeated three times for statistical analyses. The representative Western blots were demonstrated in the figures.

Digestion of cell surface/ECM HS

Mouse embryonic fibroblasts were digested with 10 μU of heparinase I, 5 μU of heparinase II, and 10 μU of heparinase at 37°C before the addition of FGF-2. After 15 min, the enzymes and the digested HS were removed by washing twice with DMEM containing 0.1% BSA and the cells were then stimulated as described above.

Analysis of binding FGF-2 to CHO-K1/FR1 cells

CHO-K1/FR1 in DMEM were seeded at 5 x 10^4 cells per well in 96-well plates and serum starved overnight. Cells in some wells were digested with four millunits of heparinase I, two
milliunits of heparitinase II, and four milliunits of heparinase (HSase mixture), and/or 10 milliunits of chondroitinase ABC (C-ABC) at 37°C for 15 min. The contents of control wells were left undigested. The wells were washed three times with PBS, and then 50 μL aliquots of PBS containing 50 ng/mL digoxigenin-conjugated FGF-2, 10 mg/mL BSA, and 0.9 mM CaCl2 with or without heparin or octasaccharides were added to each well. After 30 min at 4°C, unbound digoxigenin-conjugated FGF-2 was removed by three washes with PBS, and the cells were then fixed in neutral buffered formaldehyde (4% final concentration) for 30 min at 4°C. The wells were washed three times with PBS and then blocked with PBS containing 10 mg/mL BSA with gentle shaking. After 1 h at 4°C, the wells were washed, and then alkaline phosphatase-conjugated Fab fragments of the anti-digoxigenin antibody (1:1000 dilution) were added. After 1 h at room temperature, unbound Fab fragments were removed by three washes with PBS containing 0.05% (v/v) Tween 20 (PBST), and the alkaline phosphatase substrate (1 mg/mL p-nitrophenyl phosphate in 1 M diethanolamine, pH 9.8) was added to each well. The enzyme activity in each well was measured by a color reaction using a microplate reader. The experiments were independently repeated twice, and statistical analyses were performed using Student's t test. The criterion for statistical significance was P < 0.05.

Preparation of digoxigenin-conjugated FGF-2
Digoxigenin-conjugated FGF-2 was prepared by the method reported previously (Ashikari et al. 1995). Briefly, 10 μg of FGF-2 in the 0.2 M phosphate buffer, pH 8.5, were added into N-acetylated heparan sulfate and then mixed with 8.75 nmol of digoxigenin-3-O-methylcarbonyl-s-aminoacaproyl-N-hydroxysuccinimide ester (Roche, Mannheim, Germany) in ethanol, followed by the incubation for 2 h at room temperature. The FGF-2 solution was applied to 0.3 mL of heparin-Sepharose gel equilibrated with PBST containing 1 mg/mL BSA. Heparin-Sepharose gels were washed with 3 mL of PBST containing 1 mg/mL BSA, Digoxigenin-conjugated FGF-2 was then eluted with 1 mL of 2 M NaCl in PBST containing 1 mg/mL BSA.

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Conflict of interest statement
None declared.

Abbreviations
CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GlcNAc, N-sulfoglucosamine; GlcUA, L-glucuronic acid; HB-GF, heparin-binding growth factor; HexUA, hexuronic acid; HS, heparan sulfate; HS6ST, heparan sulfate 6-O-sulfotransferase; IdoUA, L-iduronic acid; MEF, mouse embryonic fibroblast; p-ERK, phosphorylated ERK; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References
Ishihara M. 1994. Structural requirements in heparin for binding and activation of FGF-1 and FGF-4 are different from that for FGF-2. Glycobiology. 4:817–824.


