Regulation of FGF-1 mitogenic activity by heparan sulfate oligosaccharides is dependent on specific structural features: differential requirements for the modulation of FGF-1 and FGF-2

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The interaction of heparan sulfate (HS) and the closely related molecule heparin with FGF-1 is a requirement for enabling the growth factor to activate its cell surface tyrosine kinase receptor. However, little is known about the regulatory role of naturally occurring cell surface HS in FGF-1 activation. We have addressed this issue by utilizing a library of HS oligosaccharides, which are defined in both length and sulfate content. Mitogenic activation assays using these oligosaccharides showed that HS contained both FGF-1 activatory and inhibitory sugar sequences. Further analysis of these oligosaccharides showed a clear correlation between FGF-1 promoting activity and their 6-O-sulfate content. The results, in particular with the dodecasaccharide sequences, suggested that specific positioning of 6-O-sulfate groups may be required for the promotion of FGF-1 mitogenic activity. This may also be true for 2-O-sulfate groups though the evidence was not as conclusive. Differential activation of FGF-1 and FGF-2 was also observed and found to be mediated by both oligosaccharide length and sulfation pattern, with different specific O-sulfate positioning being implicated for the promotion of different growth factors. These results suggest that variation and tight control of the fine structure of HS may allow cells to not only control their positive/negative responses to individual FGFs but also to change specificity towards promotion of different members of the FGF family.

Key words: heparan sulfate/oligosaccharides/FGF-1/FGF-2/ activity

Introduction

Acidic fibroblast growth factor FGF-1 is a member of a large family of structurally related polypeptides (Burgess and Maciag, 1989; Tanaka et al., 1992; Miyamoto et al., 1993; Burgess and Winkles, 1996; Yamakita et al., 1996), that are known to stimulate a number of cell processes such as proliferation, migration, differentiation and survival (Burgess and Winkles, 1996). FGF-1 is one of the more widely studied members along with basic fibroblast growth factor (FGF-2) and is known to bind to two classes of receptor. A family of four high affinity tyrosine kinase receptors (FGFRs), FGFR-1 (Lee et al., 1989), FGFR-2 (Dionne et al., 1990), FGFR-3 (Keegan et al., 1991), and FGFR-4 (Partanen et al., 1991), are the signaling receptors for the FGFs while the second class of receptor is comprised of low affinity cell surface HS proteoglycans (HSPGs) (Moscatelli, 1987). The interaction of growth factor/HSPG complexes with FGFRs results in receptor dimerization and subsequent tyrosine autophosphorylation of the receptor (Friesel et al., 1989; Bellot et al., 1991), which is followed by the phosphorylation of other proteins (Burgess et al., 1990a; Zhan et al., 1993; Vainikka et al., 1994; Wang et al., 1994; Goh et al., 1996). Interestingly, FGF-1 is the only member of the growth factor family identified to date, which binds with a high affinity to all four FGFRs and their major splice variants (Chellaiah et al., 1994). Both this and the wide tissue distribution of FGFRs probably make FGF-1 the broadest specificity growth factor yet characterized. FGF-1 binds with a low affinity to the HS chains of HSPGs (Brown and Parish, 1994; Brown et al., 1995a; Rahmoun et al., 1998) K₄ between 0.4 and 8.6 μM and the structurally related molecule heparin with a K₄ of approximately 90 nM (Lee and Lander, 1991). Heparin and various structural analogues have been shown to potentiate the mitogenic (Thornton et al., 1983; Damon et al., 1989; Mueller et al., 1989; Burgess et al., 1990b; Kaplow et al., 1990; Belford et al., 1992, 1993; Brown et al., 1995b; Wiedlocha et al., 1995), chemotactic (Terranova et al., 1985), neurotrophic (Unsickel et al., 1987; Damon et al., 1989; Belford et al., 1992, 1993; Renaud et al., 1996), cell survival promoting (Tamm et al., 1991), and angiogenic activities (Lobb et al., 1985; Watanabe et al., 1990; Passaniti et al., 1992) of FGF-1. These properties of heparin are believed to reflect the significant enhancement of FGF-1 activity by cell surface HS chains (Tanahashi et al., 1995).

The HS binding site on FGF-1 has been identified by x-ray crystallography, with the major residues involved being N18, K112, K113, K118, and R122 (Zhu et al., 1996; DiGabriele et al., 1998). The primary FGFR binding domain of FGF-1 has been less well studied; however, two FGFR binding sites (high and low affinity) have previously been identified on FGF-2 (Springer et al., 1994). Recent crystallographic studies have shown the FGF-2 primary high
affinity FGFR binding site to be conserved in FGF-1 (Y15, R35, N92, Y94, L133, and L135) with the exception of a Leu substitution for Met at position 135 (Blaber et al., 1996). The second FGF-2 low affinity site in FGF-1 was also located and found to be composed of a seven-residue loop from positions K101 to H106 and the adjacent W107 (Blaber et al., 1996). This loop is longer than the corresponding region in FGF-2 (Springer et al., 1994), and it is the variation in the low affinity site which is believed to determine FGFR subtype specificity (Seddon et al., 1995). HS chains are highly complex molecules with very heterogeneous structure. This heterogeneity is due to a series of structural modifications, which occur after the initial synthesis of the repeating -GlcUAβ1,4GlcNAc- polymer precursor. The subsequent modification pathway proceeds in a stepwise manner, beginning with the N-deacetylation and N-sulfation of some regions of the chains (Lindahl, 1989). These sulfated domains (S-domains) are then further transformed by epimerization of GlcUAβ1,4- to IdUAα1,4-, followed by 2-O-sulfation of selected IdoUA residues. Further alterations include 6-O-sulfation of GlcNAc/GlcNSO3 (Gallagher et al., 1986; Lindahl and Kjellen, 1987) also the more infrequent 3-O-sulfation of GlcNSO3 (Marcum et al., 1986; Pejler et al., 1987; Edge and Spirio, 1990) and 2-O-sulfation of GlcUA (Lindahl et al., 1994). S-domains are separated by regions of low or zero sulfation, in which the main disaccharide repeat is unmodified GlcUAβ1,4GlcNAc (Turnbull and Gallagher, 1991; Lyon et al., 1994; Pye and Kumar, 1995). Several studies have shown that HS chain structure differs extensively depending on the cell type (Turnbull and Gallagher, 1990; Kato et al., 1994; Lyon et al., 1994; Sanderson et al., 1994; Pye and Kumar, 1995), or the tissue it is extracted from (Maccarana et al., 1996). Changes are also seen in malignant transformation (Winterbourne and Mora, 1981; Pejler and David, 1987; Jayson et al., 1998), development (David et al., 1992; Brickman et al., 1998a), aging (Feyzi et al., 1998), and injury (Challacombe and Elam, 1995).

Various groups have identified sulfated oligosaccharide sequences (S-domains) within heparin and HS that interact strongly with FGF-1. These oligosaccharides were found to be enriched in IdUA2S(or1,4GlcNSO3(6S) disaccharides, with their affinity for FGF-1 increasing with 2-O- and 6-O-sulfate content (Mach et al., 1993; Ishihara, 1994; Fromm et al., 1997; DiGabriele et al., 1998). Indeed, an x-ray crystallographic study of FGF-1 and heparin showed a direct role for 2-O- and 6-O-sulfates in the FGF-1 interaction (DiGabriele et al., 1998). This is in contrast to HS interaction with FGF-2, in which there is no role for 6-O-sulfation in the interaction with the primary HS binding site (Faham et al., 1996). The minimum length of oligosaccharide binding to FGF-1 with relatively high affinity and promoting its mitogenic activity has been reported as being an octa- or decasaccharide (Mach et al., 1993; Ishihara, 1994; Fromm et al., 1997). Chemically desulfated heparins have also been utilized, in order to show the importance of 2-O- and 6-O-sulfate groups in the promotion of FGF-2 and FGF-1 mitogenic activity (Guimond et al., 1993; Ishihara et al., 1995, 1997). Intracellular signaling by FGFs is believed to be initiated by FGF dimerization and a number of models have been proposed, by which HS/FGF/FGFR interact to form a signaling complex. These models include the oligomerization of FGF-1 by HS, thereby presenting FGF-1 dimers/oligomers to FGFRs (Mach et al., 1993; Spivak-Kroizman et al., 1994).

Results

Preparation of heparinase III generated HS oligosaccharides

Heparinase III derived dodeca-, deca-, and octasaccharides were those used previously (Pye et al., 1998) and were obtained using a combination of gel filtration and SAX-HPLC (Figure 1). Samples were designated by both size (i.e., dp12 = dodecasaccharide) and alphabetically, by their order of elution from SAX-HPLC (i.e., dp12A, dp12B, etc.) The pooled fractions in each size class are indicated in Figure 1 by horizontal bars. Analysis of these pooled fractions by polyacrylamide electrophoresis showed them to be heterogeneous (results not shown).

Effect of dodeca-, deca-, and octasaccharides on FGF-1 and FGF-2 mitogenic activities

The effects the oligosaccharide fractions had on the mitogenic activities of FGF-1 and FGF-2 were assessed using the F32 cell assay system as described in Materials and methods. The level of zero activity was defined as being the level of 3H-thymidine incorporated with FGF-1 alone or FGF-2 alone. Octasaccharide activation profiles against both FGF-1 and FGF-2 are shown in Figure 2. The results showed that only the most sulfated octasaccharide fraction dp8D activated FGF-1, the other three fractions dp8A, dp8B, and dp8C were nonactivating, and to a certain extent displayed an inhibitory effect on basal FGF-1 activity (Figure 2A). A striking difference was seen in the FGF-2 activation assay in which none of the octasaccharide fractions were able to promote activity (Figure 2B). It is possible that differences in sulfation pattern are involved in the differential activation of FGF-1 and FGF-2 by the octasaccharides. However, this seems unlikely given that no FGF-2 activation was seen with the wide range of octasaccharides structures tested. It therefore seems more plausible that for the octasaccharides at least, the differential activity is principally the result of FGF-1 being able to utilize an 8-sugar sequence, albeit a highly sulfated one. In order to validate the
length of the octasaccharides, all the fractions tested were analyzed by analytical size exclusion HPLC. The elution profiles shown in Figure 3 clearly indicate that all the octasaccharide fractions are of the expected size and are free from cross-contamination by larger oligosaccharides.

Decasaccharide (dp10) FGF-1 activation profiles (Figure 4A) were much the same as those seen previously with FGF-2 (Pye et al., 1998) with one nonactivating/inhibitory oligosaccharide fraction dp10A and three FGF-1 activating fractions dp10B, dp10C, and dp10D with biological activity increasing with the SAX elution time (i.e., sulfate content). Figure 4B clearly shows that no significant differential FGF-1/FGF-2 promoting activity was observed with these decasaccharide fractions.

Analysis of the dodecasaccharides (dp12) clearly shows the dp12E oligosaccharide fraction to be the most active FGF-1 stimulator, with the other active oligosaccharide fractions dp12C, dp12D, dp12F, and dp12G being significantly weaker promoters of FGF-1 activity (Figure 5A). Two nonactivating dodecasaccharide fractions dp12A and dp12B were also identified which again positively inhibited basal FGF-1 activity. Closer examination of the modulatory activities of the dodecasaccharides (Figure 5B) shows them to have differential FGF promoting capacities. The results show that oligosaccharide fractions dp12F and dp12G, which have almost identical FGF-2 promoting activity, have dramatically different activity against FGF-1. Also, the most active FGF-1 promoter, dp12E, had substantially higher levels of FGF-1 stimulatory activity than dp12F.
and dp12G, but these same fractions had remarkably similar FGF-2 promoting activities (Figure 5B). Another major difference in activity of the dodecasaccharides was seen with fraction dp12D, which activated FGF-1 with levels comparable to dp12F. This is significant since fraction dp12D was unable to activate FGF-2 whilst fraction dp12F was the most active promoter of FGF-2 (Figure 5B). Since these oligosaccharides are of uniform length, the results suggest that the sulfation pattern of an HS oligosaccharide can determine both FGF-1 promoting activity and differential activation of FGF-1 and FGF-2. Finally, no promotion of FGF-1 or FGF-2 mitogenic activity was observed with heparinase III-resistant hexa- and tetrasaccharide fractions (results not shown).

Disaccharide composition and structure activity relationships

Analysis of the disaccharide composition of the dodeca-, deca-, and octasaccharide fractions (Table I) showed them to be rich in the 2-O-sulfated hexuronic acid containing disaccharides ∆HexUA(2S)α1,4GlcNSO3 and ∆HexUA(2S)α1,4GlcNSO3(6S), in which ∆HexUA(2S) would most probably correspond to IdoUA(2S) in the intact oligosaccharides (Turnbull and Gallagher, 1991; Turnbull et al., 1992) and not GlcUA(2S), which has been shown previously to be a rare component of HS (Maccarana et al., 1996). Comparison of the disaccharide composition of the octasaccharide fractions (Table I) and SAX elution time (Figure 1) showed that the only clear correlation was with increasing 6-O-sulfate content, with the FGF-1 stimulating octasaccharide fraction dp8D having the highest total 6-O-sulfate content. No clear correlation was seen for 2-O- and N-sulfation levels with either their SAX elution position or biological activity (Figure 6A). Indeed, the non-activating octasaccharide fraction dp8A had similar 2- and N-sulfation levels to that of the FGF-1 activating octasaccharide dp8D. The increase in 6-O-sulfation from dp8A to dp8D was mainly a result of increases in the content of the ∆HexUA(2S)α1,4GlcNSO3(6S) and ∆HexUA(2S)α1,4GlcNSO3(6S) disaccharides (Table I). These results indicate a distinct requirement for the 6-O-sulfation of HS octasaccharides in order for them to stimulate FGF-1 biological activity. However, the lower levels of 2-O-sulfation in the dp8B and in particular dp8C fractions, may also contribute to their inability to stimulate FGF-1 (Figure 6A).

The requirement for 6-O-sulfation in HS mediated FGF-1 activation is clearly demonstrated with the decasaccharide fractions with compositional and activation data showing a

Table I. Disaccharide composition of HS oligosaccharides. Size defined heparinase III–resistant dodeca-, deca- and octasaccharides (dp12, dp10, and dp8) were previously fractionated by SAX-HPLC and pooled as shown in Figure 1; these were then depolymerized using a combination of heparinases and the resulting disaccharides resolved by SAX-HPLC (see Materials and methods)

| Disaccharide structure | dp8A | dp8B | dp8C | dp8D | dp10A | dp10B | dp10C | dp10D | dp12A | dp12B | dp12C | dp12D | dp12E | dp12F | dp12G |
|------------------------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| HexUA-GlcNAc | 22.0 | 23.4 | 13.5 | 16.0 | 16.3 | 15.9 | 10.5 | 7.7 | 19.4 | 5.1 | 16.1 | 15.8 | 11.1 | 7.6 | 7.7 |
| HexUA-GlcNAc(6S) | 2.0 | 12.1 | 19.1 | 10.3 | 8.3 | 9.3 | 11.9 | 15.4 | 2.6 | 8.8 | 4.3 | 6.5 | 6.3 | 7.9 | 12.1 |
| HexUA-GlcNSO3 | 22.6 | 18.9 | 33.4 | 14.3 | 21.2 | 16.8 | 17.9 | 12.6 | 15.8 | 23.0 | 12.1 | 14.2 | 16.4 | 12.1 | 9.9 |
| HexUA-GlcNSO3(6S) | 0.8 | 5.1 | 5.5 | 13.0 | 4.2 | 7.5 | 6.6 | 10.5 | 4.9 | 3.9 | 2.4 | 5.5 | 4.8 | 6.9 | 10.2 |
| HexUA(2S)-GlcNSO3 | 45.7 | 24.1 | 6.3 | 11.5 | 40.7 | 37.9 | 29.9 | 14.9 | 48.9 | 40.7 | 53.4 | 49.5 | 45.9 | 43.5 | 39.2 |
| HexUA(2S)-GlcNSO3(6S) | 6.7 | 14.8 | 17.8 | 33.5 | 7.2 | 12.4 | 20.9 | 36.8 | 7.6 | 7.5 | 6.3 | 8.5 | 13.1 | 21.3 | 20.9 |
| HexUA(2S)-GlcNAc | nd | 1.4 | 3.4 | 1.4 | 2.1 | nd | 2.3 | 2.0 | 0.8 | 11.0 | 5.5 | nd | 2.4 | 0.7 | nd |

Data for the dodeca- and decasaccharides was obtained from Pye et al. (1998). nd, not detected.
positive correlation between increasing 6-O-sulfate content and maximum FGF-1 activation. In this case, however, no significant differences were observed in 2-O-sulfate and N-sulfate levels (Figure 6B). Increases were seen with all 6-O-sulfate containing disaccharides, i.e., $\Delta$HexUA$\alpha_1,4$GlcNAc(6S), $\Delta$HexUA$\alpha_1,4$GlcNSO$_3$(6S) and $\Delta$HexUA(2S)$\alpha_1,4$GlcNSO$_3$(6S) (Table I), with the latter trisulfated disaccharide being the main contributor towards the overall 6-O-sulfate increase.

Comparisons of the disaccharide analysis and the activity data for the dodecasaccharides did not display the clear relationship between 6-O-sulfate content and biological activity seen with the decasaccharide fractions. For example, the most sulfated dodecasaccharide fraction dp12F and dp12G had lower FGF-1 activatory ability than dp12E, even though they had a considerably higher 6-O-sulfate content (Figure 6C). Moreover, the dp12D fraction, which has approximately half the 6-O-sulfate content of dp12F, had similar FGF-1 stimulatory activity. However, perhaps the most significant observation on structure/activity relationships was seen when comparing the composition of the most powerful FGF-1 activating dodecasaccharide fraction dp12E, and non-activating dp12B fraction. Only small variations in total 6-O, 2-O, and N-sulfate content were observed in these preparations despite the differences in biological activity. Analysis of the dp12E fraction by size exclusion chromatography showed that the deviation from the correlation between 6-O-sulfate content and biological activity, seen with the decasaccharide fractions, was not a result of cross-contamination with shorter oligosaccharides (Figure 7). Relating the relevant levels of dodecasaccharide FGF-1 stimulatory activity to the content of individual disaccharide components proved difficult with no clear correlation being observed. Differences in composition and activity between FGF-1 and
FGF-2 were also difficult to ascertain with no particular disaccharide contributing significantly to differential activity. Thus qualitative and quantitative characteristics of the dodecasaccharides determine their activation properties; this applies to both FGF-1 and FGF-2 as discussed below.

Discussion

In this study, we have created a library of heparinase III–generated HS oligosaccharides, which vary in both their length and sulfation pattern. When co-introduced with either FGF-1 or FGF-2 into an HS dependent mitogenic assay, these oligosaccharide fractions showed both activatory and inhibitory actions, which were dependent on their size and sulfation pattern. The smallest oligosaccharide fraction found to be capable of activating FGF-1 was an octasaccharide. Certain HS oligosaccharide fractions were also seen to have differential FGF modulatory activities, which are believed to be a function of both oligosaccharide chain length and sulfation pattern.

Heparinase III is believed to produce resistant HS oligosaccharides with the general structure ΔHexUAα1,4-(GlcNSO3(±6S)α1,4IdoUA(±2S))α1,4GlcNR with R being Ac or SO3 (Linhardt et al., 1990). Recently, however, it has been suggested that the enzyme works more extensively to produce fragments enriched in IdoUA(2S)-containing disaccharides (Desai et al., 1993; Yamada et al., 1995). This information along with our disaccharide compositional analysis suggests that the oligosaccharide fractions used in this study contain a backbone of IdoUA(2S)α1,4GlcNSO3(6S) disaccharides. A correlation was initially observed between total sulfate content (as judged by SAX elution times) and FGF-1 promoting activity with the dodeca-, deca-, and octasaccharide fractions tested, and in particular with the deca- and octasaccharide fractions. Disaccharide analysis generally showed this to be a result of increasing 6-O-sulfate level, with the main contributor being the disaccharide IdoUA(2S)α1,4GlcNSO3(6S).

Although some notable variations were seen with 2-O- and N-sulfate levels of the octasaccharide fractions they could not be clearly related to changes in FGF-1 stimulatory activity. The data in this study therefore implicate 6-O-sulfation in playing an important role in the modulation of FGF-1 activity. These results are supported by others who showed that chemically 6-O-desulfated heparins were unable to promote FGF-1 or FGF-2 activity (Guimond et al., 1993; Ishihara, 1994; Ishihara et al., 1995). Little, however, is known about the sulfation patterns of naturally occurring cell surface HS and their abilities to affect FGF-1 activities. The exact role that 6-O-sulfate groups play in the final assembly of the FGF-1/HS/FGFR signaling complex is also unclear, although a role for 6-O-sulfate groups in the interaction with FGF-1 has been suggested (Ishihara, 1994; DiGabriele et al., 1998). In the case of FGF-2, it has been proposed that HS oligosaccharides acts as a bridge between FGF-2 and the FGFR (Guimond et al., 1993; Rusnati et al.,
The presence of nonactivating/inhibitory oligosaccharides was an interesting finding. Additionally, since each oligosaccharide fraction is comprised of a mixture of structures, the different levels of FGF-1 promoting activities seen are likely to be due to the relative amounts of activating and inhibitory oligosaccharides present in each fraction. As suggested previously for FGF-2 (Pye et al., 1998) the direct correlation between the 6-O-sulfate content of an HS-decasaccharide fraction and its biological activity implies that the occurrence of 6-O-sulfate groups in specific positions is necessary for the promotion of FGF-1 mitogenic activity. The increased frequency of occurrence of 6-O-sulfate(s) at precise position(s) is presumably reflected in the increased activity of the more 6-O-sulfated decasaccharides. However, it could be argued that the relationship between 6-O-sulfate content of HS oligosaccharides and FGF-1 biological activity is purely a result of a nonspecific requirement for a high negative charge density. Indeed, it has previously been shown using chemically desulfated heparins, that substantially higher levels of 6-O-sulfation were required for FGF-1 promoting activity than with FGF-2 (Ishihara et al., 1995). In the present study, however, the case for specific sulfate positioning was strengthened by the data obtained from the analysis of the dodecasaccharide fractions, in which it was clearly seen that only relatively low levels of 6-O-sulfation were needed for FGF-1 stimulatory activity. Also, and perhaps even more supportive, is the fact that small differences in 6-O-sulfate content of the dodecasaccharide fractions resulted in considerable diversity in FGF-1 mitogenic promoting activities. Although no specific positional requirement for 2-O-sulfation could be seen with the decosa- and decasaccharide fractions, the presence of obligatory 2-O-sulfate groups cannot be ruled out, on account of a significant divergence in the total 2-O-sulfate levels of the individual octasaccharide fractions.

This study has also yielded novel information on differential activation of FGFs by HS oligosaccharides. It was plainly seen with the octasaccharide fractions, that chain length could play a role in determining specificity in differential FGF-1/FGF-2 activation. A role for sulfation pattern changes in the contrasting ability of HS to affect FGF-1 and FGF-2 activities was also implicated, by the dodecasaccharide fractions capacities to differentially modulate either FGF-1 or FGF-2 action. Indeed, it has been shown that neural cells are able to switch from FGF-2 to FGF-1 mediated potentiating activity at a crucial stage in neuronal development (Nurcombe et al., 1993). This has recently been shown to be a result of controlled variation in HS chain structure (Brickman et al., 1998a). Changes in HS sulfation patterns have been associated with a variety of biological processes and disease states, with the most notable changes occurring in the 6-O-sulfate content of HS (Winterbourne and Mora, 1981; Pelger and David, 1987; Pelger et al., 1987; David et al., 1992; Kato et al., 1994; Brickman et al., 1998a,b; Feyzi et al., 1998; Ghiselli et al., 1998; Jayson et al., 1998; Safaiyan et al., 1998; Salmivirta et al., 1998), with the main contributor to these variations being GlcNSO(6S) containing disaccharides. This indicates a possible regulatory role for the HS biosynthetic pathway, and in particular with the sulfotransferase enzyme controlling 6-O-sulfation within heparinase III resistant domains. One possible explanation therefore for the differential FGF activating characteristics of the HS oligosaccharides used in this study, may again lie in the precise positioning of specific O-sulfate groups. For example, 6-O-sulfate groups are required for the interaction of FGF-1 with HS, but not for the interaction with FGF-2 (Ishihara, 1994; DiGabriele et al., 1998). Other positional differences may also influence different FGF activatory capacities, for instance it is believed that 6-O-sulfate groups play a role in HS interactions with FGFRs (Kan et al., 1993; Pantoliano et al., 1994). However, it is not known if a specific 6-O-sulfate position is conserved for this purpose within HS oligosaccharides on the formation of different FGF ternary complexes.

Changes in the expression of different FGFR variants have been implicated in the ability of cells to modulate their activities to various members of the FGF family. Our results show that variation in the fine structure of domains within HS chains may also allow cells to control their positive and negative responses to individual FGFs and in addition to change their specificity towards promotion of different growth factors. This study also shows promise for the pharmaceutical development of bio-active oligosaccharide analogues that can discriminate between individual members of the FGF family.

Materials and methods

Materials

Cell culture media and horse serum were obtained from Gibco BRL (Paisley, U.K.). Human recombinant FGF-1 and FGF-2 were supplied by R and D Systems (Abingdon, Oxon, UK). Porcine mucosal HS was obtained from Organon (Oss, The Netherlands). Grampian Enzymes (Orkney, UK) supplied Heparinases I, II, and III. Sephadex G-25 was obtained from Amersham Pharmacia Biotech (St. Albans, Herts, UK). NEN Life Science Products (Hounslow, UK) supplied [methyl-³H] thymidine. Bio-Gel P-6 and P-2 were obtained from Bio-Rad (Hemel Hempstead, Herts, UK). ProPac PA1 analytical columns were purchased from Dionex (Camberley, Surrey, UK). TSK3000PW, TSK3000SW, and TSK2000SW columns were purchased from Phenomenex (Macclesfield, Cheshire, UK). All other reagents were supplied by BDH-Merk LTD (Lutterworth, Leicester, UK).

Preparation of HS oligosaccharides

The HS oligosaccharide fractions utilized in this study were the same samples used previously (Pye et al., 1998) and were produced as follows. Porcine mucosal HS (200 mg) was exhaustively digested by heparinase III 50 mIU in 100 mM sodium acetate, 0.5 mM calcium acetate buffer pH 7.0 for 24 h at 37°C. The progress of the enzyme was monitored by absorbance at 232 nm, and further additions of enzyme were made until digestion was complete. The digestion products were then size separated using a Bio-Gel P-6 column (170 cm x 1.5 cm). Samples were eluted at a flow rate of 6 ml/h in 0.5 M ammonium bicarbonate and 1 ml fractions collected. Peaks were detected by measuring the absorbance of fractions at 232 nm, and further additions of enzyme were made in order to ensure complete digestion. Size fractionated samples were then further purified by strong anion-exchange (SAX) HPLC, as described previously for dodeca- and decasaccharides (Pye et al., 1998). These gradient conditions were varied for the octasaccharide mixture, with saccharides being eluted using a gradient of 0.1 M NaOH.
0.04–1.3 M linear NaCl gradient in Milli Q water pH 3.0 over 1 h at a flow rate of 1 ml/min. Fractions (1 ml) were collected and the elution profile monitored by absorption at 232 nm. Oligosaccharide-containing fractions were then pooled as indicated in Figure 1. Oligosaccharides were next freeze-dried and desalted by application to a Sephadex G-25 column eluted with water. Finally, samples were quantified by drying to constant weight and by measuring their absorbance at 232 nm.

**F32 cell mitogenesis assay**

BaF3 cells transfected with FGFR1 (designated F32 cells; Ornitz et al., 1992) were routinely maintained in RPMI-1640 medium, 10% horse serum supplemented with interleukin-3-conditioned medium (prepared from WEHI 3b cells) at 37°C, 5% CO₂. For the assay system F32 cells were plated into 96-well plates at a density of 50,000 cells/well in 100μl RPMI-1640 medium supplemented with 10% horse serum and the appropriate growth factor + oligosaccharide test samples. Cells were incubated for 46 h before addition of [3H]thymidine (0.3 μCi/well) for a further 2 h. Incorporation of [3H]thymidine was stopped by harvesting cells on a Filtermate-196 cell harvester. Plates were allowed to air dry, before addition of 25μl of Microscint O to each well and incorporated radioactivity counted on a top count system (Packard, Pangbourne, Berks, UK).

**Strong anion-exchange HPLC of disaccharides**

The HS oligosaccharide fractions were depolymerized to disaccharides by complete digestion with a mixture of heparinases I, II, and III followed by Bio-Gel P-2 chromatography as described previously (Turnbull et al., 1992). The resulting disaccharides were then resolved by SAX-HPLC on a ProPac PA1 analytical column (25 x 0.48 cm, Dionex). The column was first equilibrated with Milli Q water (adjusted to pH 4.0 with HCl), samples were then injected and the disaccharides resolved using a two stage NaCl gradient (0–0.12 M over 90 min followed by 0.12–1.0 M over 45 min) in Milli Q water pH 4.0. The elution positions of specific disaccharides, detected by absorbance at 232 nm, were established by comparison with authentic standards.

**Analytical size exclusion HPLC**

The oligosaccharide fractions obtained by SAX-HPLC of the sized oligosaccharide mixtures were applied (25–50 μg in 20μl of Milli Q water) to three TSK columns connected in series (TSK3000PW 30 x 0.75 cm, TSK3000SW 60 x 0.75 cm and TSK2000SW 60 x 0.75 cm). The columns were eluted with 0.5 M NaCl at a flow rate of 0.6 ml/min, and the elution profiles monitored by absorbance at 232 nm, 0.005 AUFS. Retention times were determined for each oligosaccharide fraction and the void and total volumes measured using dextran blue and potassium dichromate.

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**Abbreviations**

FGF-1, acidic fibroblast growth factor; FGF-2, basic fibroblast growth factor; FGF-R, fibroblast growth factor receptors; dp, degree of polymerization (i.e., disaccharide = dp2); HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; SAX, strong anion exchange; GlcNAc, N-acetylglucosamine; GlcNAc(6S), N-acetylglucosamine 6-sulfate; GlcNSO₃, N-sulfated glucosamine; GlcNSO₃(6S), N-sulfated glucosamine 6-sulfate; GlcUA, glucuronic acid; GlcUA(2S), glucuronic acid 2-sulfate; IdoUA, iduronic acid; IdoUA(2S), iduronic acid 2-sulfate; HexUA, unsaturated hexuronic acid residue; HexUA(2S), unsaturated hexuronic acid 2-sulfate.

**References**


Fibroblast growth factor (FGF)  

**FGF activities of HS oligosaccharides**


