O-linked carbohydrates are required for FGF-2-mediated proliferation of mouse embryonic cells

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ABSTRACT During development, fibroblast growth factors (FGFs) serve highly specific functions that are mediated through high-affinity transmembrane receptors and modulated by membrane-bound proteoglycans. Proteoglycans, in an embryonic environment called embryoglycans, contain numerous carbohydrate ectodomains, the structure of which undergoes rearrangement. Since they can be lost from the cell surface, they are sometimes found in extracellular space where they may also serve some regulatory function. Here we address the potential roles of three naturally occurring isoforms of Lewis X (LeX) in FGF-2-mediated proliferation of embryonic stem (ES) cells. We have found that the addition of sulfated LeX to ES cells at a concentration of 17 nM promotes FGF-2 mitogenic activity while a 10-fold higher concentration leads to a reduction of FGF-2-mediated proliferation. Notably, this dose-dependent modulation operated only for sulfated LeX. Other fucosylated motifs, basic LeX trisaccharide and sialylated LeX, also affected ES cell proliferation but the mechanism cannot be clearly correlated with the presence or absence of FGF-2. The suppression of biosynthesis of O-linked carbohydrates including LeX reduced basal proliferation of ES cells and interfered with the mitogenic effect of FGF-2. However, in inhibitor-treated cells, the stimulatory activity of FGF-2 can be reestablished to its original level by exogenous LeX oligosaccharides. Our results show that (A) O-linked LeX oligosaccharides can regulate mitogenic activity of FGF-2 in embryonic cells, (B) and this ability varies with subtle modifications in their structure. Importantly, our data represent the first insight into the mechanism of how growth factor activities might be modulated by shedded embryoglycan ectodomains.

KEY WORDS: embryonic stem cells, FGF-2, Lewis X, mouse, proliferation

Introduction

FGF-2 belongs to a growing family of signaling molecules believed to induce proliferation and differentiation of various cell types (Basilico and Moscatelli, 1992; Dvorak et al., 1998) through high-affinity transmembrane receptors (Givol and Yayon, 1992). Besides cognate fibroblast growth factor receptors (FGFRs), FGFs also interact with nonsignaling co-receptors, proteoglycans, that modulate their biological activity (Venkataraman et al., 1996). These FGF co-factors are largely described as heparin-like molecules occurring on the cell surface and in the extracellular matrix (Klagsbrun and Baird, 1991; Rapraeger, 1995), since the reduction of mitogenic activity of FGF-2 has been shown in chlorate-treated cells (Guimond et al., 1993) and/or mutant CHO cells unable to synthesize heparan sulfate proteoglycans (Yayon et al., 1991). As an important regulatory mechanism, carbohydrate fragments may be released from the core proteoglycan in a manner dependent on the enzymatic activity of milieu (Kato et al., 1998), and thus associate with peptide growth factors before their interaction with transmembrane receptors (Ornitz et al., 1992). The mechanism by which carbohydrate fragments may be secreted into the luminal fluid has been shown for mouse uterine epithelial cells and lactosaminoglycan-bearing glycoproteins (Dutt and Carson, 1990). The size and composition of proteoglycan ectodomains that directly interact with signaling peptides has been shown to alter dramatically biological activities of FGF. Previously, it was reported that heparin sequences shorter than

Abbreviations used in this paper: benzyl-αGalNAc, benzyl N-acetyl-α-D-galactosaminide; ES, embryonic stem; EC, embryonal carcinoma; FGF-2, fibroblast growth factor-2; LeX, Lewis X.
and 6-O-desulfated heparin (Guimond et al., 1993) do not activate LeX, NeuNAc(α2→3)Gal(β1→4)-[Fucα1→3]GlcNAc, in undifferentiated EC cells. Among the other cell types, sialylated LeX isoform is synthesized in cancer cells as a ligand for cell adhesion molecules (Kannagi, 1997) and its expression is associated with tumor progression (Dabelsteen, 1996). Similarly, sulfated LeX has been shown to be a dominant structural motif in mucin that is produced by human colon carcinoma cells (Capon et al., 1997).

In this report we demonstrate that, depending on their structure and concentration, free carbohydrate sequences regulate FGF-2-stimulated proliferation of ES cells in vitro in concert with membrane-bound embryoglycan ectodomain. We extrapolate this finding in vivo, hypothesizing that the same mechanisms are being employed to regulate FGF-2 signaling during the earliest stages of embryonic development.

Results

Secreted form of FGF-2 is synthesized in pregnant mouse uterus where it may stimulate FGFRs that are expressed in embryonic cells

In view of specific signaling activity of FGF-2 to embryonic ectoderm, we used western blot analysis to determine the presence of secreted low-molecular mass FGF-2 isoform in mouse uterus during the process of implantation. We show that the 18 kDa isoform that is known to be secreted (Mignatti et al., 1992; Florkiewicz et al., 1995), is present in all proximal (the most frequent implantation area), central and distal parts of uterus. (Fig. 1).

To investigate possible effects of endogenous FGF-2 on early embryonic cells, we examined the expression of FGF-2 in undifferentiated and differentiated ES cells. Morphologically undifferentiated ES cells cultured without feeder layer but in medium supplemented with LIF do not express any FGF-2. However, 24 kDa isoform of FGF-2 that is known to be targeted into the nucleus (Florkiewicz et al., 1991) was detected in ES cells that were cultured for five days without both feeder layer and LIF. Embryonic fibroblasts that express four known FGF-2 isomers –18, 22, 5, 23 and 24 kDa were used as positive control (Fig. 2). To confirm these results we precipitated heparin-binding growth factors contained in ES cell-conditioned media harvested from

Fig. 1. Expression of FGF-2 in mouse uterus. Human recombinant FGF-2 served as a positive control (lane 1). All parts of pregnant mouse uterus, proximal (lane 2), central (lane 3), and distal (lane 4) contain secreted, low molecular mass isoform of FGF-2 (arrow). Additional bands present in uterine proteins (asterisk) of approximate molecular mass of 42 kDa appear to be FGF-2 oligomer. Negative controls are presented in lane 5 (western blot where primary antibody was omitted) and in lane 6 (undifferentiated ES cells which resemble ectodermal cells of approximately 6 to 8 day old embryo). Seven µg of total protein was loaded onto lanes 2 to 6. Molecular masses are given in kDa.

7 served as a positive control (lane 1). Undifferentiated ES cells do not synthesize any FGF-2 (lane 2), however, differentiated cells express high molecular mass isoform of FGF-2 (arrowhead) that is supposed to be targeted into the nucleus (lane 3). This is confirmed by the absence of any isoform of FGF-2 in medium conditioned by ES cells differentiating for 5 days in culture (lane 4). Molecular masses are given in kDa.

Fig. 2. Expression of FGF-2 in ES cells. Mouse embryonic fibroblasts (MEFs) that express four known isoforms of FGF-2 were used as a positive control (lane 1). Undifferentiated ES cells do not synthesize any FGF-2 (lane 2); however, differentiated cells express high molecular mass isoform of FGF-2 (arrowhead) that is supposed to be targeted into the nucleus (lane 3). This is confirmed by the absence of any isoform of FGF-2 in medium conditioned by ES cells differentiating for 5 days in culture (lane 4). Molecular masses are given in kDa.
both undifferentiated and 5 days-differentiated cells using heparin-agarose. Precipitates were then analyzed for FGF-2 by western blotting. As expected, we did not detect any FGF-2.

Given the fact that FGFR-1 and -2 are predominant receptors capable of binding FGF-2, we examined whether or not both of them are present in ES cells. The mRNAs of both cognate receptors for the FGF-2 were expressed in undifferentiated (data not shown) as well as in differentiated ES cells (Fig. 3).

Together, the expression of significant amounts of FGF-2 in the uterus indicates that besides endogenous FGF-2 produced by differentiated ES cells themselves, exogenous ligand may also participate in regulating their behavior via FGFR-1 and FGFR-2. These data allow us to consider our experimental model involving ES cells and exogenous FGF-2 as resembling the situation in vivo during and several days after implantation.

**Soluble embryoglycan ectodomains regulate FGF-2-mediated proliferation in a concentration-dependent manner**

Inner cell mass-derived cells, the in vivo counterpart of ES cells, express large amounts of embryoglycans, the ectodomains of which can be released by glycosidases during growth and differentiation. To investigate the involvement of free embryoglycan ectodomains in the regulation of FGF-2 activity in ES cells, we assayed two different molar concentrations of synthetic LeX isoforms in proliferation experiments (Table 1).

To rule out the possibility that synthetic LeX oligosaccharides themselves influence cell proliferation, ES cells were exposed to each oligosaccharide in the absence of FGF-2. In such unstimulated ES cells, 10 ng/ml of LeX (19 nM) and its sulfated form (17 nM) increase cell proliferation by 5.1 and 1.6%, while sialylated LeX (12 nM) decreases proliferation by 6.7%. Essentially the same results were obtained using 10-fold higher concentration of LeX oligosaccharides. As seen in Table 1, 100 ng/ml of basic isoform of LeX (190 nM) increases cell proliferation by 6.7%, sulfated LeX (170 nM) does not influence cell growth, and sialylated LeX (120 nM) inhibits proliferation of ES cells by 4.2%.

The next series of experiments was to establish to what extent free embryoglycan ectodomains can modulate the effect of FGF-2 on ES cell proliferation (Table 1). In this particular set of experiments, FGF-2 alone stimulated the proliferation of ES cells by 9.9%. Combining FGF-2 with either basic or sialylated LeX in a concentration of 10 ng/ml, which represents a molar excess of 32-fold for basic and 20-fold for sialylated LeX over FGF-2, did not dramatically alter the effect of FGF-2. Specifically, FGF-2 combined with basic LeX stimulated the proliferation by 10.3%, and FGF-2 plus sialylated LeX elevated the proliferation of ES cells by 13.5%, as compared to basal growth of ES cells. Thus, the effect of basic LeX or sialylated LeX on FGF-2-mediated proliferation is either none or only very minor. In contrast, when 10 ng/ml of sulfated LeX is added to the ES cell culture in the presence of FGF-2, the increase of proliferation reaches 21.9%. In other words, sulfated LeX elevates the potency of FGF-2 to stimulate proliferation of ES cells in culture by the factor of 2. Strikingly, free embryoglycan ectodomains in 10-fold higher concentration elicit thoroughly different effects on FGF-2-mediated proliferation, compared to those observed when using the concentration of 10 ng/ml. First, basic and sulfated forms of LeX inhibit the proliferation of ES cells by 6.1% and 3.8%, respectively. In other words, they not only fully prevent the stimulatory effect of FGF-2, but even reduce the proliferative activity to below than in untreated cultures. In contrast, the effect of sialylated LeX at a concentration of 100 ng/ml, is to maintain the proliferative effect of FGF-2 at the level of 7.7%; this is reminiscent to its effect observed at a 10-fold lower concentration.

Altogether, sulfated LeX (A) at a low concentration increases the stimulatory effect of FGF-2. (B) dramatically suppresses this effect at high concentrations, and (C) does not alter the proliferation of ES cells when not combined with FGF-2. Those characteristics point to sulfated LeX as the only specific regulator of the

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**Table 1**

<table>
<thead>
<tr>
<th>Concentration of LeX</th>
<th>The change in proliferation (cell number/well) expressed as the percentage related to untreated control</th>
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<tbody>
<tr>
<td>ng/ml</td>
<td>nM</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>LeX</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>190</td>
</tr>
<tr>
<td>sulfated LeX</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>170</td>
</tr>
<tr>
<td>sialylated LeX</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>120</td>
</tr>
</tbody>
</table>

The values represent the level of cell growth obtained in the presence of FGF-2, exogenous LeX derivatives, and combination of both as compared to basal cell growth of control ES cells (0).

The changes in proliferation of ES cells were highly significant (**; P<0.01), significant (*; P<0.05), or nonsignificant as compared to control cell growth. Data for each group were received as the mean of 30 wells from one of the two independent experiments showing very similar pattern of cellular response to various treatments.
action of FGF-2 from our panel. Basic LeX itself stimulates basal proliferation of ES cells and at high molar excess inhibits the effect of FGF-2. This might suggest an ability of basic LeX to interact somehow with FGF-2 but also with heparin-binding factors other than FGF-2. Finally, while sialylated LeX inhibits the proliferation of ES cells in the absence of FGF-2, its effect on FGF-2-induced proliferation is only minor. Therefore, it seems very unlikely that sialylated LeX regulates the mitogenic activity of FGF-2 towards ES cells under those conditions.

**Inhibitor of synthesis of O-linked oligosaccharides reduces the expression of LeX ectodomain in ES cells**

We next attempted to inhibit the biosynthesis of glycoconjugates in ES cells specifically in order to study the effect of the depletion of embryoglycan carbohydrate side chains. Therefore, we first tested the usefulness of several inhibitors of the synthesis of O- and/or N-linked carbohydrates by determining their effects on the expression of LeX determinants (Table 2). Briefly, ES cells were grown in the presence of the particular inhibitor for 72 h and then immunocytochemically probed with the antibody against TEC-1 epitope that corresponds to LeX trisaccharide Gal(β1→4)[Fuc(α1→3)]GlcNAc. Generally, no observable downregulation of LeX determinants resulted from the inhibition of synthesis of N-linked oligosaccharides. In contrast, 72 h exposure to the inhibitor of synthesis of O-linked carbohydrates, 2 mM benzyl N-acetyl-α-D-galactosaminide (benzyl-αGalNAc), caused complete loss of LeX determinants in about 37% of cell colonies and the great reduction of the expression of LeX in the remaining ones (Fig. 4). Notably, prolongation of treatment with 2 mM benzyl-αGalNAc up to five days does not result in significantly stronger suppression of LeX synthesis. For both periods of the treatment, the expression of LeX is completely restored at 24 h after removal of the inhibitor.

Thus, treating the cells with 2 mM benzyl-αGalNAc for 72 h causes enough inhibition of LeX synthesis without any observable changes in cell-substrate attachment, compactness of colonies and cell viability.

**Inhibition of O-linked oligosaccharide synthesis suppresses FGF-2-mediated proliferation and is restored by exogenous LeX**

To investigate the specific role of O-linked oligosaccharides in FGF-2-mediated proliferation further we designed the following experiment. First, intact ES cells were stimulated only with 10 ng/ml FGF-2. In this particular experiment, FGF-2 treatment increased basal cell proliferation by average 9.1%, as measured after 72 h. Second, ES cells were grown only with 2 mM benzyl-αGalNAc that reduced basal cell proliferation by 19.7%, while not affecting cell adhesion and viability. However, when benzyl-αGalNAc-treated cells were exposed simultaneously to 10 ng/ml

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**TABLE 2**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Point of inhibition</th>
<th>Molar concentration</th>
<th>Percentage of LeX positive colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzyl-αGalNAc</td>
<td>N-acetyl-α-D-galactosaminyl transferase (inhibitor of O-linked glycans)</td>
<td>1 mM</td>
<td>87*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM **</td>
<td>63*</td>
</tr>
<tr>
<td>castanospermine</td>
<td>glucosidases (inhibitor of N-linked glycans)</td>
<td>0,5 mM</td>
<td>100</td>
</tr>
<tr>
<td>swainsonine</td>
<td>mannosidase II. (inhibitor of N-linked glycans)</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>deoxymanojirimycin</td>
<td>mannosidase I. (inhibitor of N-linked glycans)</td>
<td>5 mM</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM</td>
<td>100</td>
</tr>
</tbody>
</table>

* Only low percentage of cells among these colonies shows some positive reaction with anti-TEC-1 antibody, however, the intensity of staining is much lower than in control cells.

** 2 mM concentration of benzyl-αGalNAc has been used in all experiments since it exhibits a significant reduction of the expression of LeX determinants, but still does not alter cell-substrate adhesion and cell viability.
FGF-2, the proliferation was significantly increased, that means, it stayed almost unaffected compared to the basal growth of untreated cells. In other words, FGF-2 alone is able to rescue the proliferation phenotype imposed by the inhibition of synthesis of O-linked oligosaccharides.

To evaluate the differences in biological activities of three naturally occurring isoforms of Le^X_ oligosaccharide, basic Le^X_ trisaccharide, sialylated Le^X_, and sulfated Le^X_, ES cells were exposed simultaneously to 2 mM inhibitor, 10 ng/ml FGF-2, and the particular synthetic Le^X_. The addition of 100 ng/ml of any Le^X_ isomer does not influence cell proliferation that remains near to the FGF-2-mediated growth of benzyl-αGalNAc-treated cells (data not shown). However, ES cells supplemented that interact, FGF-2, and 10-fold higher concentration, 1 μg/ml, of basic Le^X_ trisaccharide increase proliferation by 29.5% as compared to those cells treated only with benzyl-αGalNAc. Similar increase, 31.6%, was obtained with sialylated Le^X_. Analysis of the effect of sulfated isomer of Le^X_ shows stimulation by 24.1% above control benzyl-αGalNAc-treated cells (Fig. 5).

Thus, cells having suppressed biosynthesis of O-linked oligosaccharides and significantly reduced cell growth are still able to respond to FGF-2. It is likely that O-linked oligosaccharides that survive the treatment by inhibitor may interact with FGF-2. No further enhancement of FGF-2-mediated proliferation is observed when these cells are supplemented with Le^X_ oligosaccharides at concentration 100 ng/ml. However, Le^X_ oligosaccharides in 10-fold higher concentration generate a proliferative response to FGF-2 similar to that found in intact cells exposed to FGF-2 only. We, therefore, postulate that from all possible O-linked carbohydrates the addition of any of Le^X_ isomer is enough to rescue the mitogenic potential of FGF-2.

Discussion

In the present study, we first confirm the existence of secreted isoform of FGF-2 within the tissues of mouse uterus (Wordinger et al., 1994; Yoshida, 1996), the absence of FGF-2 (Taniguchi et al., 1998), the expression of FGFs (Mummery et al., 1993) in ES cells, a strong mitogenic effect of exogenous FGF-2 on ES cells (Dvorak et al., 1998), and the expression of Le^X_ epitopes on ES cells (Kimber et al., 1993) that may be reversibly suppressed by inhibitor of O-glycosylation (Kuan et al., 1989; Nagata et al., 1994). Then, we provide evidence that O-linked Le^X_ structures regulate FGF-2-mediated proliferation of ES cells, and that this regulation varies with both the structure and molar concentration of exogenous Le^X_ oligosaccharides. The possibility that other member of FGF family, FGF-4, also play a role in our experimental system is unlikely because even FGF-4 mRNA and FGF-4 protein are expressed in preimplantation mouse embryos (Rappolee et al., 1994), FGF-4 does not appear to act as an autocrine modulator for cultured ES cells (Wilder et al., 1997).

The biological response of cells to FGFs requires high affinity proteoglycan co-receptors (Yayon et al., 1991). This requirement includes contacting of monomers and stabilization of growth factor oligomers (Waksman and Herr, 1998), and probably also the induction of a conformational changes of FGFs that enhance receptor binding and activation (Yayon et al., 1991). In crystal complexes between FGF-2 and synthetic oligosaccharides three binding sites on one molecule of FGF were found (Lam et al., 1998). Theoretically, all or some of these binding sites can be occupied by the small carbohydrate sequences and this may result either in promotion of oligomerization of peptide growth factors (up to the certain molar ratio that physically allows crosslink between one carbohydrate and two FGF molecules) or in inhibition of oligomerization (in a large excess of oligosaccharides when all binding sites on all FGFs are saturated). Only the first situation leads to enhanced dimerization and activation of FGF-cognate receptors and the positive cellular response (Ornitz et al., 1992, 1995). In this context, we have previously shown that synthetic Le^X_ ectodomain binds and oligomersize FGF-2 and elicits the cellular response in a concentration-dependent manner (Dvorak et al., 1998). However, this might not be true for all cell types. For example, in Swiss 3T3 fibroblasts and F32 lymphoid cells treated with chlorate excess of heparin competes for high-affinity binding of FGF-2 and receptor transphosphorylation but does not alter mitogenesis (Krufta et al., 1996).

Regarding this seemingly controversial effect of concentration, we have assayed 10 and 100 ng/ml of synthetic Le^X_ oligosaccharides in FGF-2-mediated proliferation of ES cells that carry their own native embryoglycan ectodomains. The structure of very abundant ectodomain Le^X_ supports the existence of three variants, Le^X_ trisaccharide, sulfated Le^X_, and sialylated Le^X_. Although sulfated and sialylated Le^X_ determinants have been reported as prominent among the membrane glycoconjugates of transformed cells (Dabelsteen, 1996; Capon et al., 1997; Kannagi, 1997), neutral Le^X_ trisaccharide might still be dominant in particular embryonic cells (Kimber et al., 1993). It is apparent that the
structural modification that most significantly favors the interaction with positively charged peptide growth factor comes from sulfate residue. Realizing this, we expected that sulfated LeX would have the most significant influence on the mitogenic activity of FGF-2. Here, we first show that synthetic LeX derivatives themselves affect the proliferation of ES cells. This effect is dependent on oligosaccharide structure rather than on its concentration. Specifically, while LeX has low stimulatory effect, sulfated LeX does not modify the proliferation, and sialylated LeX even inhibits ES cell proliferation. Thus interestingly, LeX trisaccharide and sialyl LeX exhibit antagonistic effects on the proliferation of ES cells, regardless of FGF-2 signals. The unexpected activity of sialyl LeX might be of special interest, since sialyl LeX has been reported to be the most abundant O-linked saccharide in several types of cancer cells (Kannagi, 1997; Ravindranath et al., 1998).

In parallel experiments, FGF-2 in combination with two concentrations of exogenous LeX oligosaccharides produced very diverse biological responses in ES cells. Basic LeX as well as its sulfated form promote the mitogenic activity of FGF-2 in low concentration of 10 ng/ml and inhibit the effect of FGF-2 in high concentration of 100 ng/ml. This suggests that dimerization of FGF-2 in an extracellular space may occur with high efficiency only up to a certain molar ratio of free oligosaccharide ectodomains to FGF that favors optimal crosslinks and simultaneously does not compete with membrane LeX determinants. However, these results must be interpreted cautiously because they were done using one particular ES cell line having its own characteristics. Previously, we showed that mitogenic response of C3H-derived ES cells to FGF-2 is blocked already at low concentration of 10 ng/ml of extracellular basic LeX (Dvorak et al., 1998). It raises the question of whether individual ES cell lines differ in their carbohydrate moiety or whether the ratio of membrane-bound and free oligosaccharide ectodomains play the same role in vivo.

Krusius et al. (1986) and Smalheiser et al. (1998) reported that carbohydrate sequences bearing LeX epitope are mostly O-linked to mannos. Thus, we used benzyl-αGalNAc, a competitive inhibitor of N-acetyl-α-D-galactosaminy transferase which is crucial in the biosynthesis of the majority of O-linked oligosaccharides (Kuan et al., 1989), to gain insight into the mechanism of how growth factor activity may be modulated by this particular embryoglycan ectodomain. We have shown that the expression of LeX epitopes was strongly inhibited after 72 h of treatment with benzyl-αGalNAc. The remaining activity may be due either to the extremely high rate of synthesis of LeX in embryonic cells that cannot be completely blocked by inhibitor without side effects, or to the possibility that not all LeX structures are O-linked to mannos. Regarding the first possibility, we have used 2 mM benzyl-αGalNAc that strongly decreases the expression of LeX determinants, while basal cellular features remain unaffected. Those involve cell-substratum interactions that was shown to be mediated, at least partially, by LeX structures (Boubelik et al., 1996; Sarkar et al., 1997) and cell viability. The second possible reason is very unlikely because no inhibitor that is known to be N-glycan specific affects the expression of LeX.

Notably, the level of proliferation was significantly lower in benzyl-αGalNAc-treated ES cells. This might be partly due to (A) the inhibition of cell-cell interactions which we are not able to define and to (B) the generally reduced acceptance of signals from the culture medium. Moreover, the reestablished proliferative capacity (up to the basal level) of benzyl-αGalNAc-treated cells upon the addition of exogenous FGF-2 suggests that the remaining O-linked oligosaccharides interact with FGF-2, and/or that FGF-2 mitogenic activity on ES cells is not completely O-linked glycan-dependent.

In another set of experiments, we employed synthetic analogs of LeX oligosaccharide in FGF-2-mediated proliferation assay using benzyl-αGalNAc-treated cells to demonstrate that these chemically defined embryoglycan ectodomains may be essential (or somehow limiting) for FGF-2 signaling. In the experiments with benzyl-αGalNAc-treated cells, we revealed that extracellular basic LeX as well as sialylated LeX are more potent in the restoration of FGF-2-mediated proliferation than sulfated LeX. However, it should be noted that these data were obtained with the high concentration of 1 µg (as mentioned above, 100 ng/ml had no effect on FGF-2-mediated proliferation in benzyl-αGalNAc-treated cells), which might not be optimal, specifically for sulfated LeX isoform. Moreover, no data are available on relative amounts of the particular forms of carbohydrate ectodomains before and after the treatment by the inhibitor. A general requirement for high concentrations of exogenous LeX may reflect the action of inhibitor toward the processing of all types of O-linked carbohydrates, from which the particular LeX normally represents only a small part and thus must be added at a relatively high concentration to rescue the mitogenic effect of FGF-2.

In conclusion, combining our data enables us to propose that ES cells exert strict requirements for both structure and molar concentration of free embryoglycan ectodomains in FGF-2-dependent proliferation. Our results may represent a starting point for designing specific modifiers of FGF-2 mitogenic activity in various cell types.

Materials and Methods

Cell culture and reagents

Embryonic stem cells (C57Bl/6xBALB/c)F1 were established in the Laboratory of Molecular Embryology as described by Hogan et al. (1994). Cells were maintained in DMEM supplemented with 20% fetal calf serum, 100 mM nucleosides, 0.05 mM β-mercaptoethanol, 10 i.u./ml penicillin, 0.1 mg/ml streptomycin and 1000 u./ml leukemia inhibitory factor (LIF) and cultured on mitomycin C-treated mouse embryonic fibroblasts. Human recombinant FGF-2 was obtained from Sigma (St. Louis, MO). Synthetic LeX isomers were purchased from Oxford GlycoSystems (Abingdon, UK).

Immunoblotting

The expression of FGF-2 in mouse uterus was determined by western blotting. Uteri were collected 3.5 days post conception, divided to a proximal, central, and distal parts and homogenized in ice cold lysis buffer (RIPA). The protein amounts were equalized (DC Protein Assay, Bio Rad), samples were mixed with 2x Laemmli sample buffer and subjected to 10% SDS-PAGE. After transfer, the membrane (PVDF; Amersham) was probed with mouse monoclonal anti-FGF-2 antibody (Sigma). Immunodetection was accomplished using anti-mouse IgG/peroxidase (Sigma) and FGF-2 bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham).

For the detection of FGF-2 in undifferentiated and differentiated mouse embryonic cells, ES cells were grown without feeder layer in DMEM with or without LIF for 5 days. The cells were then washed with phosphate buffered saline, pH 7.2 (PBS; Ca²⁺, Mg²⁺-free) and harvested into 1xLaemmli sample buffer. ES cell samples and mouse embryonic
fibroblasts as positive control were resolved by 14% SDS-PAGE and subjected to the same western blot analysis as described above. Simultaneously, conditioned medium from 5 days-differentiated ES cells was examined for the presence of soluble FGF-2. Briefly, conditioned medium was harvested and incubated with heparin-agarose (Sigma) for 12 h. Then, agarose beads with potentially immobilized heparin-binding factors were extensively washed and probed for the presence of FGF-2.

RT-PCR

RNAs isolated from undifferentiated and 5 days-differentiated ES cells that were both depleted of mouse primary fibroblasts were used as templates for RT-PCR amplification of the tyrosine kinase domains of the FGFR-1 and FGFR-2. Primer pairs used for RT-PCR were as follows: for FGFR-1: sense 5‘- TAT AAC CCC AGC CAC AAC - 3‘, antisense 5‘- CAT GAG AGA AGA CAG AGT CC - 3‘; and for FGFR-2 sense 5‘- CCT ATG ACA TTA ACC GTG TCC C - 3‘, antisense 5‘- AAA CAC AGA ATC GTC CCC TG - 3‘. The specificity of amplified sequences of tyrosine kinase domain of FGFR-1 (643bp) and FGFR-2 (635bp) was confirmed by restriction analysis with XhoI (FGFR-1) and EcoRV (FGFR-2). PCR products were resolved on polyacrylamide gels and visualized by silver staining.

Proliferation assays

ES cells were seeded at an initial density of 1 x 10^3 cells/well on 96-well tissue culture plates and cultured for 24 h. Then, cells were washed with PBS and cultured for additional 72 h in DMEM supplemented with various combinations of FGF-2 and Le^4 oligosaccharides. Following the period of culture, the colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells was used. Cell proliferation reagent WST-1 (Boehringer, Mannheim) was added to each well at a final concentration of 10% and plates were incubated for 4 h at 37°C. The absorbance of formazan dye was quantified spectrophotometrically at 450 nm with a reference wavelength of 690 nm. The measured absorbances strongly correlate to the number of viable cells. The data received by spectrophotometry were converted to number of cells/well using a calibration curve calculated from absorbances of formazan dye produced by known cell number/well of 96-well plate.

Inhibition of processing of complex oligosaccharides

To carry out proliferation assay with ES cells that are modified in biosynthesis of glycoconjugates, we have tested several inhibitors of synthesis of O- or N-linked oligosaccharides. Specific activity of each inhibitor was assayed by indirect immunofluorescence for the expression of TEC-1 epitope (Fig. 4 and Table 2). Briefly, ES cells were seeded on slides, coated with gelatin, fixed in ethanol/acetic acid, and stained with propidium iodide (10 µg/ml; Sigma) and cells were rinsed and mounted in Mowiol (Hoechst) with diazabicyclooctane (DABCO; Aldrich).

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