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Original Article

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 (Le^X) in FGF-2-mediated proliferation of embryonic stem (ES) cells. We have found that the addition of sulfated Le^X 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 Le^X. Other fucosylated motifs, basic Le^X trisaccharide and sialylated Le^X, 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 Le^X 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 Le^X oligosaccharides. Our results show that (A) O-linked Le^X 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-2. 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; Le^X, Lewis X.

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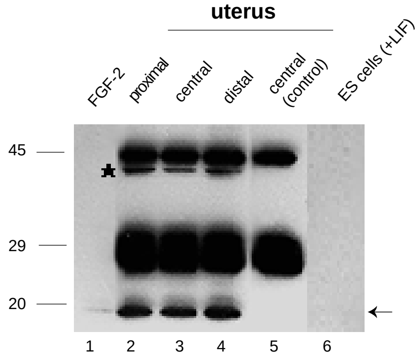

Fig. 1. **Expression of FGF-2 in mouse uterus**. *Human recombinant FGF-2 served as a positive control (lane 1). All parts of pregnant mouse uterus, proximal (lane 2), central (lane 3), and distal (lane 4) contain secreted, low molecular mass isoform of FGF-2 (arrow). Additional bands present in uterine 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.*

octasaccharide (Ornitz *et al.,* 1992) as well as large 2-O-desulfated and 6-O-desulfated heparin (Guimond *et al.,* 1993) do not activate FGF-2. Recently, however, Ornitz *et al.* (1995) revealed that even nonsulfated trisaccharides, corresponding to structures found within heparin/heparan sulfate, activate FGF-2 at concentrations comparable to those of heparin. The proteoglycan activity is believed to stem from the interaction between acidic, and thus negatively charged oligosaccharide chain, and basic, positively charged, peptide growth factor. According to this concept, the saccharides incorporated into proteoglycans in the majority of somatic cells are negatively charged due to their highly sulfated nature (Ruoslahti and Yamaguchi, 1991; Yanagishita, 1993). In mouse preimplantation embryos, however, the majority of proteoglycan/embryoglycan carbohydrate ectodomains is nonsulfated and thus neutral (Kimber *et al.,* 1993), although some sulfated forms are still also present (Smith *et al.,* 1997). In this context, we have recently proposed a new concept that these embryoglycan domains, including their shedded ectodomains, could also act as FGF-2 co-receptors contributing to regulation of mesoderm induction (Dvorak *et al.,* 1997). We have shown that FGF-2 low-affinity binding function is displayed by an embryoglycan-specific epitope recognized by TEC-1 antibody that corresponds to Le^X trisaccharide Gal(β1$\rightarrow$4)-[Fuc(α1$\rightarrow$3)]GlcNAc or its isoforms (Dvorak *et al.,* 1998). Besides this, some other functions of Le^X structures have been suggested. They include adhesion of embryonal carcinoma (EC) cells and mechanism of cell recognition based on carbohydrate-carbohydrate interaction (Kojima *et al.,* 1994; Boubelik *et al.,* 1996). Interestingly, Le^X is strongly expressed in peri-implantation embryos and in undifferentiated EC and ES cells (Ozawa *et al.,* 1985; Kimber *et al.,* 1993) but almost disappears later during development and differentiation (Brown *et al.,* 1993), indicating that its functions are limited to only a short time window. In addition to basic Le^X trisaccharide, Rosenman *et al.* (1989) detected sialylated Le^X, NeuNAc(α2$\rightarrow$3)-Gal(β1$\rightarrow$4)-[(Fucα1$\rightarrow$3)]GlcNAc, in undifferentiated EC cells. Among the other cell types, sialylated Le^X 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 Le^X 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 ectodomains. 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 isoforms –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

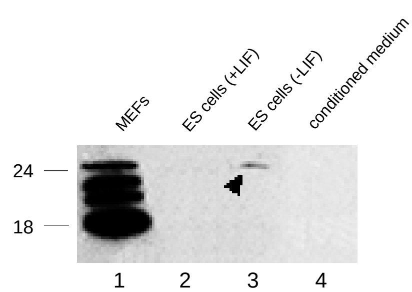

Fig. 2. Expression of FGF-2 in ES cells. *Mouse embryonic fibroblasts (MEFs) that express four known isoforms of FGF-2 were used as a positive control (lane 1). Undifferentiated ES cells do not synthesize any FGF-2 (lane 2), however, differentiated cells express high molecular mass isoform of FGF-2 (arrowhead) 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 Le^X isoforms in proliferation experiments (Table 1).

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

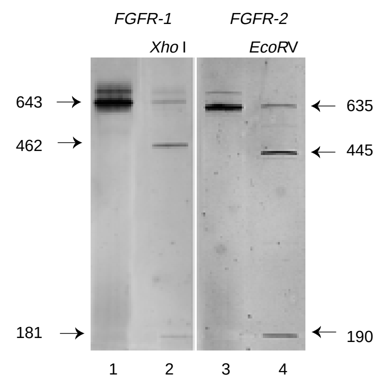

Fig. 3. Expression of *FGFR-1* and *-2* mRNAs in ES cells. *RT PCR analysis showed that ES cells differentiated for 5 days express both, tyrosine kinase domain of FGFR-1 (lane 1; 643bp) and tyrosine kinase domain of FGFR-2 (lane 2; 635bp). Two bands of predicted size (462 and 181bp) were detected after digestion of FGFR-1 transcript with Xho I (lane 2) and two bands of predicted size (445 and 190bp) were generated by digestion of FGFR-2 transcript with EcoR V (lane 4).*

TABLE 1

MITOGENIC RESPONSES OF ES CELLS EXPRESSING NATIVE Le^X TO FGF-2, EXOGENOUS Le^X, AND THE COMBINATION OF BOTH

	Concentration of Le^X		The change in proliferation (cell number/well) expressed as the percentage related to untreated control	
			FGF-2	
	ng/ml	nM	-	10 ng/ml (0.6nM)
Control	–		**0**	+9,9
Le^X	10	19	+5,1*	+10,3*
	100	190	+6,7*	-6,1*
sulfated Le^X	10	17	+1,6*	+21,9**
	100	170	+0,3*	-3,8*
sialylated Le^X	10	12	+6,7*	+13,5*
	100	120	-4,2*	+7,7*

The values represent the level of cell growth obtained in the presence of FGF-2, exogenous Le^X derivates, 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.

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 Le^X in a concentration of 10 ng/ml, which represents a molar excess of 32-fold for basic and 20-fold for sialylated Le^X over FGF-2, did not dramatically alter the effect of FGF-2. Specifically, FGF-2 combined with basic Le^X stimulated the proliferation by 10.3%, and FGF-2 plus sialylated Le^X elevated the proliferation of ES cells by 13.5%, as compared to basal growth of ES cells. Thus, the effect of basic Le^X or sialylated Le^X on FGF-2-mediated proliferation is either none or only very minor. In contrast, when 10 ng/ml of sulfated Le^X is added to the ES cell culture in the presence of FGF-2, the increase of proliferation reaches 21.9%. In other words, sulfated Le^X 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 Le^X 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 Le^X 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 Le^X (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 Le^X as the only specific regulator of the

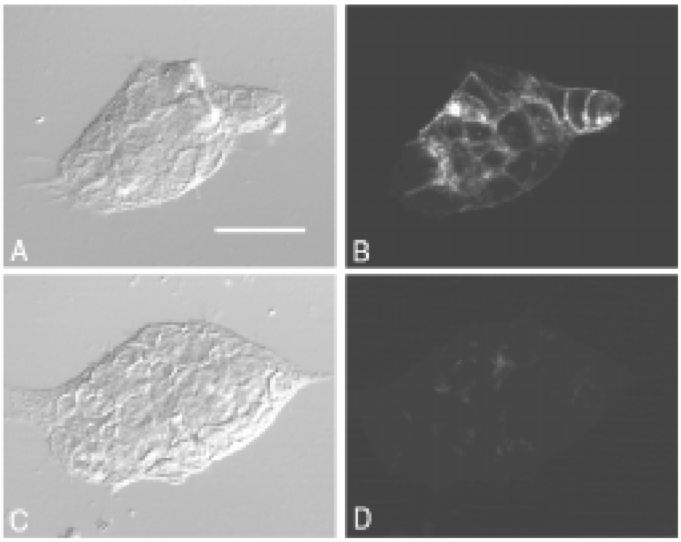

Fig. 4 Inhibition of biosynthesis of O-linked oligosaccharides in ES cells. *The morphology of ES cells in cultures grown for 72 h in control DMEM* **(A)** *and in the presence of 2 mM benzyl-αGalNAc* **(C)** *was similar, indicating that the inhibitor was not toxic. However, exposure of cells to the inhibitor resulted in complete loss or great downregulation of Le^X determinants* **(D)** *as compared to untreated control cultures* **(B)**. *Analysis was performed using the Fluoview confocal laser scanning microscope system equipped with Nomarski DIC (Olympus). Immunofluorescence images were acquired using the same confocal settings. Bar, 100 mm.*

action of FGF-2 from our panel. Basic Le^X 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 Le^X to interact somehow with FGF-2 but also with heparin-binding factors other than FGF-2. Finally, while sialylated Le^X 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 Le^X regulates the mitogenic activity of FGF-2 towards ES cells under those conditions.

Inhibitor of synthesis of O-linked oligosaccharides reduces the expression of Le^X 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 Le^X 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 Le^X trisaccharide Gal(β1→4)-[Fuc(α1→3)]GlcNAc. Generally, no observable downregulation of Le^X 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 Le^X determinants in about 37% of cell colonies and the great reduction of the expression of Le^X 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 Le^X synthesis. For both periods of the treatment, the expression of Le^X 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 Le^X 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 Le^X

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

TABLE 2

INHIBITION OF SYNTHESIS OF O-LINKED OLIGOSACCHARIDES IN ES CELLS

Inhibitor	Point of inhibition	Molar concentration	Percentage of Le^x positive colonies
benzyl-αGalNAc	N-acetyl-α-D-galactosaminyl transferase (inhibitor of O-linked glycans)	1 mM	87*
		2 mM **	63*
castanospermine	glucosidases	0,5 mM	100
	(inhibitor of N-linked glycans)	1 mM	100
swainsonine	mannosidase II.	5 μM	100
	(inhibitor of N-linked glycans)	50 μM	100
deoxymanojirimycin	mannosidase I.	5 mM	100
	(inhibitor of N-linked glycans)	10 mM	100

* 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 Le^x 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 isoform 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 with the inhibitor, 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 isoform 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 isoform is enough to rescue the mitogenic potential of FGF-2.

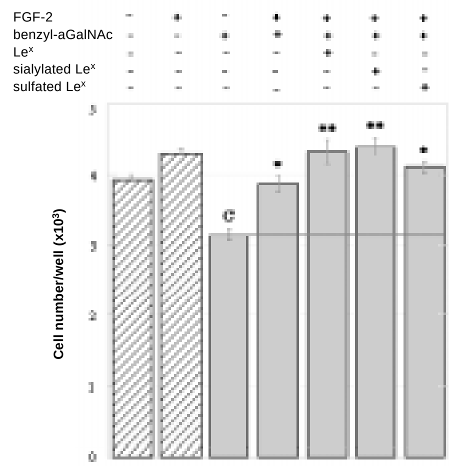

Fig. 5. The effects of benzyl-αGalNAc (2 mM), benzyl-αGalNAc and FGF-2 (0.6 nM), and the combination of FGF-2, benzyl-αGalNAc, and each exogenous oligosaccharide to be tested (1.9 µM Le^X, 1.2 µM sialyl Le^X, 1.7 µM sulfated Le^X) on the proliferation of ES cells. *Cells were cultured for 72 h in medium containing indicated agents and proliferation was measured by WST-1-based colorimetric assay. The changes in proliferation of ES cells were highly significant (**, P<0.01) or significant (*, P<0.05) when compared to control cells which were exposed to the inhibitor of synthesis of O-linked carbohydrates alone (C). For comparison, two striped columns show basal and FGF-2-stimulated growth of intact ES cells. The mean values were derived from 30 wells and from three separate experiments. Standard errors are indicated.*

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 FGFRs (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 low 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 oligomerizes 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 (Krufka *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 Le^X would have the most significant influence on the mitogenic activity of FGF-2. Here, we first show that synthetic Le^X derivatives themselves affect the proliferation of ES cells. This effect is dependent on oligosaccharide structure rather than on its concentration. Specifically, while Le^X has low stimulatory effect, sulfated Le^X does not modify the proliferation, and sialylated Le^X even inhibits ES cell proliferation. Thus interestingly, Le^X trisaccharide and sialyl Le^X exhibit antagonistic effects on the proliferation of ES cells, regardless of FGF-2 signals. The unexpected activity of sialyl Le^X might be of special interest, since sialyl Le^X 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 Le^X oligosaccharides produced very diverse biological responses in ES cells. Basic Le^X 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 Le^X 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 Le^X (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 Le^X epitope are mostly O-linked to mannose. Thus, we used benzyl-αGalNAc, a competitive inhibitor of N-acetyl-α-D-galactosaminyl 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 Le^X 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 Le^X in embryonic cells that cannot be completely blocked by inhibitor without side effects, or to the possibility that not all Le^X structures are O-linked to mannose. Regarding the first possibility, we have used 2 mM benzyl-αGalNAc that strongly decreases the expression of Le^X determinants, while basal cellular features remain unaffected. Those involve cell-substratum interactions that was shown to be mediated, at least partially, by Le^X 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 Le^X.

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 Le^X 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 Le^X as well as sialylated Le^X are more potent in the restoration of FGF-2-mediated proliferation than sulfated Le^X. 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 Le^X 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 Le^X may reflect the action of inhibitor toward the processing of all types of O-linked carbohydrates, from which the particular Le^X 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, 100 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 Le^X isoforms 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; Ca^{2+}- Mg^{2+} -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 *Xho* I (*FGFR-1*) and *EcoR* V (*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 $1x10^3$ 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^X 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 glass coverslips and exposed to the particular inhibitor for 72 h. Then, cells were fixed in ethanol/acetic acid (w/v; 95% EtOH, 1% acetic acid), quenched with 5% bovine serum albumin in PBS and incubated with anti-TEC-1 antibody (a gift from Dr. P. Draber, Institute of Molecular Genetics, Prague, CZ) overnight. After extensive washing with PBS, cells were incubated with anti-mouse IgM/FITC (Sigma), cell nuclei were counterstained with propidium iodide (10 µg/ml; Sigma) and cells were rinsed and mounted in Mowiol (Hoechst) with diazabicyclooctane (DABCO; Aldrich).

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