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Enterocytic Differentiation Correlates with Changes in the Fine Structure and Sulfation of Perlecan in HT29 Human Colon Carcinoma Cells

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Undifferentiated HT29 and differentiated HT29G- human colon carcinoma cells have been used to study the changes in proteoglycan production and structure associated with enterocytic cell differentiation. Differentiated cells incorporate twice as much sulfate than undifferentiated cells when labeled with [^{35}S]sulfate. Both cell lines produce a heparan sulfate proteoglycan which was purified by ion-exchange. The heparan sulfate proteoglycan from differentiated HT29G- cells is larger and more homogeneous in size than that produced by undifferentiated HT29 cells. No differences in the core protein structure were observed. The detailed structural analysis of the heparan sulfate chains revealed that the structure of these chains follows the standard rules for these glycosaminoglycans with N-sulfated domains and N-acetylated domains. The main finding was that differentiated HT29G- cells have a degree of higher sulfation than HT29 cells. These differences were found to affect primarily 6-O-sulfated positions.

Cell-cell and cell-substrate interactions are one of the main mechanisms involved in the control of tissue development and remodeling. These interactions take place between specific proteins involved in cell adhesion and communication. One of these molecules is the proteoglycans, macromolecules composed of a glycoprotein core linked to long sulfated glycosaminoglycan chains. Changes in proteoglycan composition and structure have been correlated with development and differentiation in several cell systems (1). Among the several glycosaminoglycans, the heparan sulfate (HS) chains have the ability to bind many proteins as extracellular matrix components and growth factors, and it is thought that HS chains with the appropiate composition are required for the formation of correct basement membrane structures and the specific binding to soluble growth factors (2).

Colon carcinoma is a good model to study whether there is a relationship between heparan sulfate proteoglycan structure and cell differentiation due to the existence of cell lines which show a different degree of differentiation. Thus, the human colon carcinoma cell line HT29 grown in the standard conditions in the presence of glucose consists of >95% undifferentiated cells, whereas when cultured in the absence of sugar (HT29G-), it displays typical enterocytic differentiation with apical brush border, tight junctions and presence of intestinal hydrolases (3,4). There are several evidences that indicate that changes in protein glycosylation occur during the process of cell differentiation in the HT29 cell line under lack of glucose as, for example, the accumulation of several glycosylation precursors that correlates with the inability of HT29 cells to differentiate (5,6).

The main surface HS proteoglycan produced by human colon carcinoma cells (7,8) has been identified as perlecan (9-11). Perlecan is a basement membrane heparan sulfate proteoglycan consisting in a 467 kDa core protein composed of several domains (10,11). Perlecan is responsible for cell/cell interactions and binding to growth factors as FGF-2 (12,13).

This paper presents evidence that changes in glycosaminoglycan sulfation and structure are associated to the process of cell differentiation in the HT29 human colon carcinoma model.

MATERIALS AND METHODS

Materials

Poly-Prep columns were from BioRad. Sepharose CL2B and Sephadex G-15 were from Pharmacia. Chondroitinase ABC was from Seikagaku Kogyo. Heparitinase was from ICN Biochemicals. Reagents for cell culture were from Gibco. Radiochemicals were from Amersham.

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All the other reagents were analytical grade from Sigma or Boehringer Mannheim.

Cell Culture and Metabolic Labeling

HT29 human colon carcinoma cells were grown in a humidified atmosphere at 37°C with 5% CO_2 in DMEM supplemented with 10% heat inactivated fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. HT29G- cells are a cell line obtained after culturing HT29 cells in the absence of glucose (3) and were grown in the same conditions but in a medium devoid of glucose supplemented with 10% dialyzed fetal calf serum. Both cell lines were kindly provided by Dr. J. C. Murat (Université Paul Sabatier, Toulouse, France).

All the experiments were carried out in cultures maintained in confluence for 20 days, when cells have achieved a complete degree of differentiation (4). Cultures were grown in DMEM with 1% serum for 16 hours. Cells were then incubated for 24 hours in 1% serum with 50 mCi/ml of carrier free [^{35}S]sulfate in low sulfate (0.2 mM) medium, or with 25 mCi/ml L-[^{35}S]methionine in methionine-free medium or with 50 mCi/ml D-[6-^{3}H]glucosamine. The medium was then removed, the cell layer rinsed with cold PBS, a cocktail of protease inhibitors were added (10 mM EDTA, 5 mM benzamidine, 5 mM N-ethylmaleimide, 1 mg/ml pepstatin A, 0.5 mg/ml leupeptin and 1 mM PMSF) and the medium was made up to 0.5 g/ml GndHCl to avoid aggregation of proteoglycans. Cell extracts were prepared in a buffer containing 2% Triton X-100, 0.5 M sodium acetate pH 5.8, 4 M GndHCl and protease inhibitors.

Chromatographic Procedures

(A) Ion exchange chromatography in DEAE cellulose. The dialyzed sample was applied to a Poly-Prep column (3 ml, Bio Rad) equilibrated with a buffer containing 6 M urea, 50 mM Tris HCl pH 7.2, 0.2% Triton X-100, 0.2 M NaCl. Elution was carried out with a linear salt gradient up to 1.5 M NaCl at 5 ml/hour flux rate. The eluate was collected in 1 ml fractions and the radioactivity determined by scintillation counting. The proteoglycan-containing fraction was recovered, dialyzed, concentrated with Centriprep (Amicon) or by precipitation with ethanol/ammonium acetate and used for further analysis.

(B) Gel chromatography on Sepharose CL2B. Purified proteoglycans radioactively labeled with [^{35}S]sulfate were applied to a Sepharose CL2B column (1 × 100 cm) equilibrated with 4 M GndHCl, 50 mM Na_2SO_4, 0.2% Triton X-100, pH 7.0 Fractions of 1.3 ml were collected at a rate of 15 ml/h and analyzed by liquid scintillation counting.

(C) Gel chromatography on Sephadex G-100. Isolated GAGs before and after deamination at pH 1.5 (see below) were applied to a Sephadex G-100 column (1× 100 cm) equilibrated with 1M NaCl. Fractions of 1.25 ml were collected at a rate of 5 ml/h and analyzed by liquid scintillation counting.

(D) Gel chromatography on Sephadex G-15. The HS degradation products obtained after deamination were separated on a column of Sephadex G-15 (1 × 200 cm) equilibrated with 0.2M NH_4HCO_3. Fractions of 2 ml were collected at a rate of 6.5 ml/h and analyzed by liquid scintillation counting.

Enzymatic Digestions and Isolation of HS Chains

Enzymatic digestions were performed at 37°C for 16 hours with chondroitinase ABC (50 mU/ml in 33 mM sodium acetate, 33 mM Tris-HCl pH 8.0) or heparitinase (10 mU/ml in 10 mM calcium acetate, 100 mM sodium acetate, pH 7.0). The incubations were terminated by boiling the samples for 5 min.

Heparan sulfate chains were isolated by alkaline hydrolysis by treating the samples with 0.5M NaOH for 16 h at 4°C.

Compositional Analysis of the Glycosaminoglycan Chains

The heparan sulfate chains were degraded at N-sulfated glucosamine units by treatment with nitrous acid at pH 1.5 (14) and the resulting di-, tetra- and oligosaccharides were reduced with $NaBH_4$. The fragments were separated by gel chromatography on a column of Sephadex G-15. The disaccharide fractions, containing hexuronosyl-anhydromannitol disaccharides with or without sulfate groups in various positions, were analyzed further by anion-exchange HPLC on a Partisil-10 SAX column (Whatman) as described (15).

Tetrasaccharides eluted from the Sephadex G-15 column were analyzed by high voltage paper electrophoresis (40 V/cm) on Whatman 3MM paper in 1.6 M formic acid (pH 1.7). Paper strips were cut into 1 cm segments that were eluted with water, and the aqueous extract was analyzed by scintillation counting.

RESULTS

Isolation and Characterization of Proteoglycans from Undifferentiated HT29 and Differentiated HT29G- Colon Carcinoma Cells

Cells were simultaneously labeled with [^{35}S]sulfate and [^{3}H]glucosamine as described in Materials and Methods. The conditioned medium and the cell extracts were dialyzed, concentrated and the radioactivity in both fractions determined. Differentiated cells incorporate twice as much sulfate than undifferentiated cells, which is mainly incorporated into proteoglycans. The samples were subjected to ion exchange chromatography in DEAE cellulose (Poly Prep columns) and eluted in denaturing conditions in the presence of 6M urea. The results for the conditioned media are shown in Fig. 1. Cell extracts gave similar results. The peak in the gradient (peak II) eluted at 0.28M NaCl, concording with the results obtained by Simmon-Assman et al. (16) and contained the proteoglycans (see below). Peak II was free of mucins, which can be also sulfated, as analyzed by western blot using antibodies against these molecules (two different antibodies against native and deglycosylated mucins were used (kindly given by Dr. F. X. Real, I. M. I. M., Barcelona, Spain) (not shown)). Both cell lines presented a similar profile, although the ^{3}H/^{35}S ratio in peak II was higher in HT29 cells (29 *vs* 19 for media, 26 *vs* 14 for cell extracts), indicating a higher sulfation degree in differentiated HT29G- cells.

The proteoglycan in peak II was characterized as a heparan sulfate by deamination with nitrous acid at pH 1.5 of the glycosaminoglycan chains (Fig. 2) and heparitinase digestion (Fig. 3), confirming that both cell lines contained mainly a heparan sulfate proteoglycan (HSPG) that was degraded by these treatments. Chondroitinase ABC did not have any effect (not shown).

The HSPG isolated after ion exchange chromatography was dialyzed and submitted to gel chromatography on Sepharose CL2B in dissociative conditions in the presence of 4M GndHCl. In both cell lines a single peak

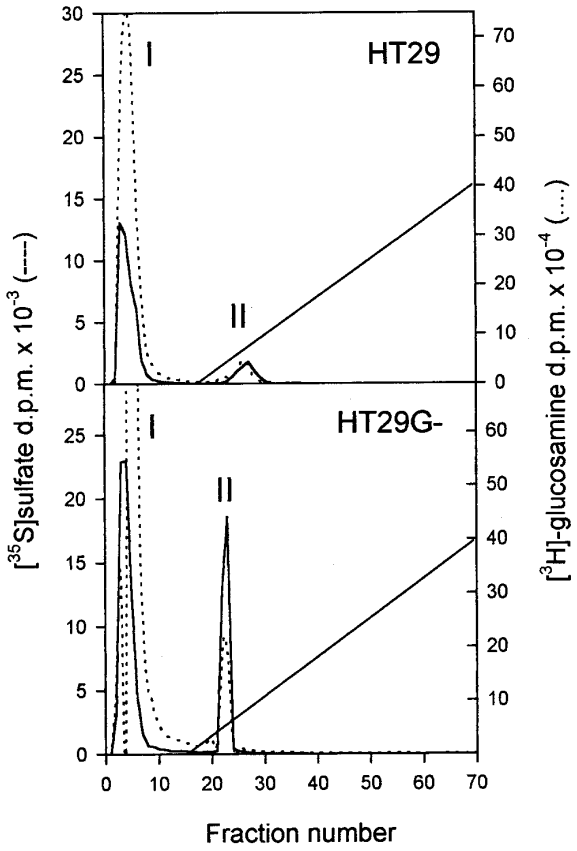

FIG. 1. Ion exchange chromatography on DEAE- cellulose of the extracellular medium from HT29 and HT29G- colon carcinoma cell lines. The media from [^{35}S]-sulfate labeled cells (-----) and from [^{3}H]-glucosamine labeled cells (– – – –) was recollected and dialyzed. The samples were applied to a Poly Prep column as described in Materials and Methods.

was observed, although with a higher molecular mass in differentiated cells (Kav 0.53 in HT29G- cells *vs* 0.57 in HT29 cells). The HSPG from undifferentiated HT29 cells was also more heterogeneous in size, as is clearly evidenced when samples were subjected to gel electrophoresis (Fig. 3).

The protein core of the HSPG was visualized after heparitinase digestion of the peak II isolated from [^{35}S]-methionine labeled cells. Differentiated and undifferentiated cells gave an analogous pattern consisting in a double band of approximately 250 and 315 kDa (Fig. 4).

Structural Analysis of the Heparan Sulfate Chains from Colon Carcinoma Cells

The HS chains were isolated after alkaline hydrolysis of the purified [^{3}H]glucosamine-labeled HSPG (Materials and Methods). The distribution and amount of N-sulfated GlcN units along the HS chains was analyzed by deamination at pH 1.5 followed by reduction with $NaBH_4$. Under these conditions the chains are cleaved at the sites of N-sulfated GlcN units whereas N-acetylated units remain intact. Consecutive N-sulfated GlcN residues ("contiguous" N-sulfated sequences) will be recovered in disaccharides, whereas alternating N-sulfated and N-acetylated GlcN residues ("alternating" sequences) will give rise to tetrasaccharides. Finally, solitary N-sulfate groups ("spaced" sequences) will yield oligosaccharides of at least hexasaccharide size.

These fractions were separated by gel chromatography on Sephadex G-15 and the composition of the disaccharide fraction ("contiguous" sequences) was determined by ion exchange HPLC. As summarized in Table 1, these disaccharides contained largely 2-O-sulfated IdoA (2-OSO_3)-aMan_R units and IdoA (2-OSO_3)-aMan_R(6-OSO_3) units, according to the idea that these regions are the most extensively modified. From the same data, the extent of polymer modification was calculated to estimate the ratio of O-sulfation in contiguous sequences and the results are described in Table 2. It can be seen that these contiguous sequences in the HS chains are heavily sulfated, with more than one O-sulfated group per disaccharide. Around 2/3 of the

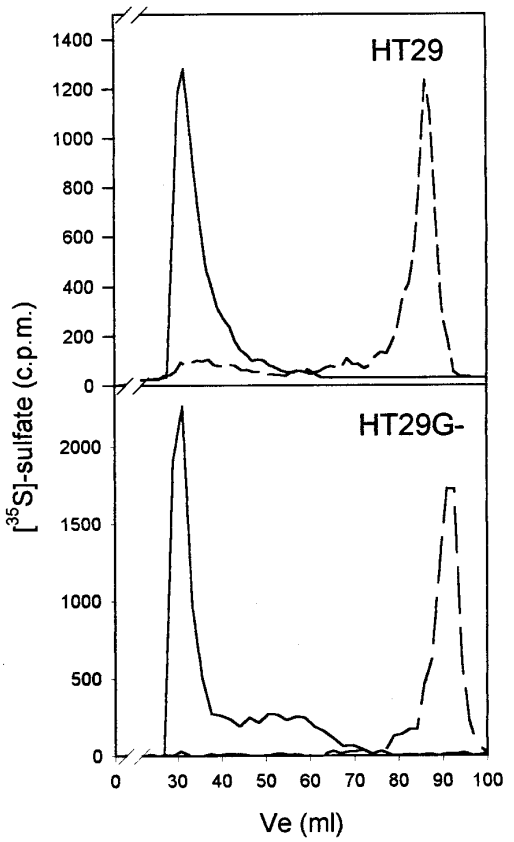

FIG. 2. Gel chromatography on Sephadex G-100 of the HSPG from HT29 and HT29 G- colon carcinoma cells before and after deamination at pH 1.5. 10000 cpm of isolated GAGs from the extracellular medium of HT29 and HT29G- cells were subjected to gel chromatography on Sephadex G-100 before (----) and after (– – –) deamination at pH 1.5 as described in Materials and Methods.

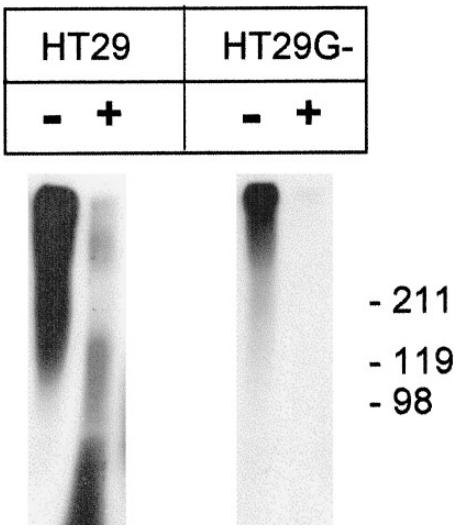

FIG. 3. Electrophoretic analysis of the proteoglycan isolated from [^{35}S]sulfate labeled colon carcinoma cells. Peak II from [^{35}S]sulfate labeled HT29 and HT29G- cells was digested without (−) and with (+) heparitinase, subjected to SDS-PAGE in a 3-15% gradient gel and visualized by fluorography. Molecular weight markers are shown on the right.

sulfated groups are modified in the C-2 position, whereas the rest are sulfated in the C-6. We found a moderate increase in O-sulfation in differentiated HT29G- cells (116 O-sulfated groups/100 disaccharides) *versus* the undifferentiated HT29 cells (111 O-sulfated groups/100 disaccharides). In both cases, IdoA was mainly found in the sulfated disaccharides (91-93 %) with respect to GlcA.

Separation of tetrasaccharides by high voltage electrophoresis at pH 1.7 ("alternating" N-sulfated sequences) showed the presence of nonsulfated, mono-O-sulfated, di-O-sulfated and tri-O-sulfated species, with again a higher degree of total O-sulfation in differentiated HT29G- cells (Table 3). A similar study was undertaken with the oligosaccharide fraction from the Sephadex G-15 column ("spaced" sequences), which was analyzed by ion exchange HPLC. These sequences are composed mainly of non-sulfated disaccharides, although the percentage is again lower in differentiated HT29G- cells (82 % *vs* 90% in HT29 cells). The sulfated disaccharides were of the IdoA (2-OSO_3)-aMan_R(6-OSO_3) type (not shown).

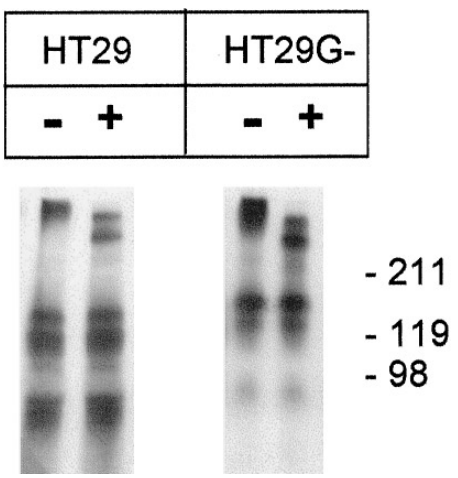

FIG. 4. Electrophoretic analysis of the proteoglycan isolated from [^{35}S]methionine labeled colon carcinoma cells. Peak II from [^{35}S]-methionine labeled HT29 and HT29G- cells was digested without (−) and with (+) heparitinase, subjected to SDS-PAGE in a 3-15% gradient gel and visualized by fluorography. Molecular weight markers are shown on the right.

TABLE 1

Disaccharides Derived from "Contiguous" N-Sulfated Sequences in HS Chains of the HSPG from Colon Carcinoma Cells

Disaccharides	HT29	HT29G−
HexA-aMan_R	17.5	21.2
GlcA(2-OSO_3)-aMan_R	2.8	3.1
GlcA-aMan_R(6-OSO_3)	3.3	4.2
IdoA-aMan_R(6-OSO_3)	1.3	1.8
IdoA(2-OSO_3)-aMan_R	46.0	31.3
IdoA(2-OSO_3)-aMan_R(6-OSO_3)	29.1	38.4

Note. The disaccharides obtained after deaminative cleavage at pH 1.5 (see Materials and Methods) were separated by anion exchange HPLC. Values are given as mole % of total disaccharides and were calculated from peak areas.

DISCUSSION

It is now widely recognized that the precise disaccharide composition of the heparan sulfate chains in proteoglycans define the active sites for the binding of other proteins, as growth factors, and that this interac-

TABLE 2

Substituent Patterns within "Contiguous" N-Sulfated Sequences of HS Chains from Colon Carcinoma Cells

	HT29 (%)	HT29G− (%)
O-sulfate/disac.	111	116
2-O-sulfate/disac.	78	72
6-O-sulfate/disac.	34	44
2-O-sulfate/O-sulfate	0.69	0.62
6-O-sulfate/O-sulfate	0.30	0.38
2-O-sulfate/6-O-sulfate	2.3	1.6
IdoA/disac.-O-sulfate[a]	92.6	91.0
GlcA/disac.-O-sulfate[a]	7.4	9.0

Note. The percentages were calculated from the compositional analysis shown in Table 1 and are expressed as mol % in relation to different types of disaccharide units.

[a] Proportion of IdoA and GlcA in O-sulfated disaccharides.

TABLE 3

Substituent Pattern of Tetrasaccharides Recovered after Deaminative Cleavage at pH 1.5 of HS Chains from Colon Carcinoma Cells ("Altered Sequences")

Tetrasaccharide	HT29	HT29G−
	% of total tetrasaccharides	
Nonsulfated	47	44
Mono-O-sulfated	44	38
Di-O-sulfated	6.3	13.6
Tri-O-sulfated	2.3	4.7
	sulfate groups/50 tetrasacch. (100 dis.)	
Total O-sulfate	31.7	39.6

Note. The tetrasaccharide fraction obtained after chromatography on Sephadex G-15 was subjected to high voltage electrophoresis at pH 1.7 (see Materials and Methods). Percentages of disaccharides were calculated from peak areas.

tion is important for the activity of the protein (17). Variations on the heparan sulfate composition can be thus a new point of regulation of the biological activity of growth factors and for the assembly of the extracellular matrix. In this context, our study was undertaken to show whether there could be changes in the fine structure of the disaccharide composition of the heparan sulfate chains associated to cell differentiation.

We have used the HT29 colon carcinoma cell line for several reasons. First, is a good model for studying cell differentiation: we have used the parental HT29 cell line which is mainly undifferentiated, and the HT29G- cell line, which grows in the absence of glucose and shows a stable differentiated phenotype with all the characteristics of a enterocytic cell (presence of brush border, hydrolase activity and tight junctions) (4). Secondly, these cell lines have been widely used to study several aspects referred to epithelial cell differentiation and intestinal development (18,19). Furthermore, the main proteoglycan found in the extracellular medium of these cells has been identified as perlecan (9,11), a proteoglycan which has been described as responsible for cell/cell interactions and binding to growth factors (12,13).

Differences were found between the purified HSPG from undifferentiated and differentiated cells. First, the ratio $^{3}H/^{35}S$ in the purified HSPG (peak II) indicated a higher sulfation degree in differentiated HT29G- cells. Second, differentiated HT29G- cells synthesized a larger proteoglycan (Kav 0.53) than the undifferentiated HT29 cells (Kav 0.57). Third, the HSPG from differentiated cells is more homogeneous in size, indicating a more uniform GAG chain length. A increase in GAG size upon differentiation has been also described in Caco-2 cells (20). We did not detect proteoglycans of the chondroitin sulfate type, although other reports have described the presence of chondroitin sulfate in HT29G- cells but not in HT29 cells (16).

Heparan sulfate, along with heparin, are the GAGs with the greatest structural variability. Due to the incompleteness of the modification reactions during elongation of the HS chains, these GAGs present a wide spectrum of sulfation patterns. The detailed composition of the HS chains is determined after deamination at pH 1.5, which cleaves the HS chains at the sites of N-sulfated units. This technique allows the separation of N-sulfated blocks (contiguous sequences), which are highly sulfated and epimerized, from blocks were N-sulfated units are alterned with N-acetylated disaccharides (alterned sequences) and from poorly modified N-acetylated domains (spaced sequences). The disaccharide composition can be then determined by HPLC ion exchange chromatography.

When analyzing the results, it becomes clear that cell differentiation correlates with a higher degree of HS sulfation. Disaccharides from the N-contiguous sequences were isolated and analyzed by HPLC and an increase in O-sulfated disaccharides in differentiated cells is observed in these regions (116 in HT29G- *vs* 111% in HT29 cells), as well as in the alterned sequences isolated as tetrasaccharides after deamination at pH 1.5 (39.6% in HT29G- *vs* 31.7% in HT29 cells). The oligosaccharide fraction which corresponds to spaced sequences presents a very low degree of modification, although in this case the presence of sulfated disaccharides is also higher in differentiated HT29G- cells. This difference in sulfation is mainly due to an increase in 6-O-sulfation (Table 1 and 2). A high proportion of disaccharides containing IdoA(2-SO_3) are observed in these sequences, in accordance with the general model where O-sulfation is found mainly in N-sulfated sequences (17, 21-23).

As it has been stated above, small changes in the sulfation pattern of the HS chains could have important functional consequences. Thus, Nurcombe et al. (24), have described that a single HSPG binds FGF in neural precursor cells, but that the FGF binding specificity resides in the different GAG side chains of the HSPG. The current model for FGF-2 mitogenic action involves the formation of a ternary complex involving the growth factor, the FGF-2 receptor and the HSPG, requiring the presence of 6-O-sulfate groups (25-27). Other experiments suggest that receptor signaling by other members of the FGF family, FGF-1 and FGF-4, require factor-specific saccharide structures (25), as well as HGF (28). Furthermore, structural studies suggest that different members of the FGF family may use different contacts for the binding to heparin (29).

Finally, a low degree of sulfation of the HS chains can be a quite general characteristic of transformed/undifferentiated cells as compared to their differentiated counterpart. Thus, besides the present study,

other authors have found a lower degree of sulfation in hepatoma (30), in SV40-transformed 3T3 cell line (31), in E1A-immortalized rat fetal intestinal cells (32), in transitional carcinoma cells (33) and in transformed mouse mammary epithelial cells (34, 35). In the cases where a rigorous study was performed, mainly differences in O-sulfation were found. In SV40-transformed mouse embryo fibroblasts, a decrease in 6-O-sulfated glucosamine residues occurs in regions of the chain containing few sulfate groups (36), similarly to the HT29 model described here. That means that certain sequences of charged groups will be present in HS chains from differentiated cells and only rarely in transformed cells, thus possibly contributing to the abnormal behaviour of cancer cells.

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