Journal of Cellular Biochemistry 64:605–617 (1997)

Production of Sulfated Proteoglycans by Human Breast Cancer Cell Lines: Binding to Fibroblast Growth Factor-2

Maryse Delehedde,[1] Elisabeth Deudon,[2] Benoni Boilly,[1] and Hubert Hondermarck[1]*

[1]Unité de ''Dynamique des Cellules Embryonnaires et Cancéreuses,'' Centre de Biologie Cellulaire, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France
[2]Laboratoire de Biologie Cellulaire INSERM U402, Faculté de Médecine Saint-Antoine, Paris, France

Abstract The cellular distribution and nature of proteoglycans synthesised by human breast cancer cells in culture were studied. Proteoglycans were labelled with [^{35}S] sulfate, purified, and characterised after ion-exchange chromatography followed by gel-filtration chromatography and treatment with glycosaminoglycan degrading enzymes. Proteoglycans were isolated from the culture medium and from cell layers of the hormono-dependent well-differentiated MCF-7 cell line, the hormono-independent poorly-differentiated MDA-MB-231 and the HBL-100 cell line which is derived from non malignant breast epithelium. HBL-100 and MDA-MB-231 cells produced larger amounts of proteoglycans which had a lower degree of sulfation than MCF-7 cells. Gel-filtration chromatography on Sepharose CL-6B indicated that HBL-100 and MDA-MB-231 cells accumulated cell surface heparan sulfate proteoglycans (HSPG), with a high apparent molecular weight (K_{av} 0.1). In contrast, the MCF-7 cell monolayers synthesised small sulfated macromolecules (K_{av} 0.4) which possessed mostly chondroitin sulfate chains. Moreover, considerable differences in the nature of the sulfated proteoglycans released into the culture medium of these breast epithelial cell lines were observed. MCF-7 cells released into the culture medium HSPG as the main proteoglycan component while MDA-MB-231 and HBL-100 cells released mainly chondroitin sulfate proteoglycans. In these three cell lines, medium-released sulfated macromolecules have a higher hydrodynamic size than cell-associated ones. Proteoglycans purified by ion-exchange chromatography were tested for their ability to bind ^{125}I FGF-2. We demonstrated that HBL-100 and MDA-MB-231 cells bind more FGF-2 to their heparan sulfate proteoglycans than MCF-7 cells. Taken together, these results suggest that differences in proteoglycan synthesis of human breast epithelial cells could be responsible for differences in their proliferative and/or invasive properties. J. Cell. Biochem. 64:605–617.

Key words: breast cancer; proteoglycans; heparan sulfate; chondroitin sulfate; sulfation; fibroblast growth factor-2

Proteoglycans (PG) are complex sulfated macromolecules composed of linear polysaccharide chains of glycosaminoglycans (GAG) such as heparan sulfate and chondroitin/dermatan sulfate covalently attached to a core protein [Kjellen and Lindahl, 1991]. These GAG chains contain sulfate groups at various positions, giving them a high density of negative charges, and allowing them to interact with extracellular matrix molecules. Heparan sulfate glycosaminoglycans have been shown to interact with various growth factors such as fibroblast growth factor (FGF) or hepatocyte growth factor (HGF) [reviewed in Fernig and Gallagher, 1994]. Thus, qualitative or quantitative changes in PG may have profound consequences on cell proliferation and differentiation [Iozzo, 1988; Timar and Kovalszky, 1995]. In the developing mammary gland, sulfated proteoglycans can interact with extracellular matrix components including collagen I and IV, laminin, and fibronectin in order to constitute a functional matrix. Thereby they participate in the morphogenesis and differentiation of mammary epithelial cells [Parry et al., 1985; Lee et al., 1985; Pe-

Abbreviations used: FGF-2, fibroblast growth factor-2; GAG, glycosaminoglycan; PG, proteoglycan; HSPG, heparan sulfate proteoglycan; EDTA, ethylenediamine tetraacetic acid; CS, chondroitin sulfate; HA, hyaluronic acid.

Contract grant sponsors: Ligue Nationale Contre le Cancer, Comité du Nord, Ministère de l'Education Nationale, Ministère de la Recherche et de l'Enseignement Supérieur.

*Correspondence to: Hubert Hondermarck, Unité de Dynamique des Cellules Embryonnaires et Cancéreuses, Centre de Biologie Cellulaire, Bâtiment SN3(3), Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France. email: Hubert.Hondermarck@univ-Lille1.fr

Received 6 June 1996; Accepted 17 October 1996

tersen et al., 1992; Beck et al., 1993]. Furthermore, transfection of S115 malignant mammary carcinoma cells with syndecan-1 (a prototype of heparan sulfate proteoglycan) restores their epithelial morphology and reduces their tumorigenicity in nude mice [Leppa et al., 1992]. In human breast cancer, some studies have shown variations in the content and composition of glycosaminoglycans in tissue biopsies [Takeuchi et al., 1976; Alini and Losa, 1991; Losa and Alini, 1993] and in cell culture [Chadransekaran et al., 1979; Channe-Gowda et al., 1986; Emerman et al., 1988]. Invasive mammary tissues generally show a higher content in chondroitin sulfate chains compared to normal or benign breast tissues [Takeuchi et al., 1976; Olsen et al., 1988; Alini and Losa, 1991]; concerning the heparan sulfate component, Mangakis et al. [1990] showed an increase in more invasive tumors while Alini and Losa [1991] described a reduced content of heparan sulfate per cell volume in neoplastic breast tissues.

In this study, we have investigated the synthesis of sulfated PG in HBL-100 cell line which is derived from non malignant breast epithelium and in two breast cancer cell lines: the well-differentiated epithelioid MCF-7 and the poorly-differentiated, invasive MDA-MB-231 cells [Sommers et al., 1994]. We first compared the cell-associated and medium-released proteoglycans and found significant differences in the nature and cellular distribution of PG synthesised by these three cell lines. Thereafter, we purified the sulfated proteoglycans by ion-exchange chromatography and examined their capacity to bind FGF-2. Our results show that HBL-100 and MDA-MB-231 cells present a higher capacity for binding of ^{125}I FGF-2 to their heparan sulfate proteoglycans than MCF-7 cells.

MATERIALS AND METHODS

Materials

Earle's minimum essential medium (MEM), fetal calf serum, L-glutamine, Hepes buffer, nonessential amino acids, penicillin, streptomycin, gentamycin, and trypsin/EDTA solutions were obtained from Eurobio (Les Ulis, France). DEAE-Sephacel, Sepharose CL-6B were from Pharmacia LKB Biotechnology (Upsala, Sweden). FGF-2 was from Amgen (USA). Cationic membranes (Zeta-Probe, Thousand Oaks, CA) were from Biorad. Guanidinium chloride, Triton X-100, CHAPS, bovine serum albumin, chloramine T, heparin, chondroitin sulfate, or hyaluronic acid were purchased from Sigma (St. Louis, MO). Heparitinase (EC 4.2.2) and chondroitinase ABC (EC 4.2.2.4) and $Na_2{}^{35}SO_4$ were from ICN (Orsay, France).

Cell Culture

Human breast epithelial cell lines MDA-MB-231, MCF-7, and HBL-100 were cultured in improved modified Eagle's medium (MEM) containing 10% heat-inactivated fetal calf serum (FCS), 20 mM Hepes, 2 g/l sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamycin sulfate, and 0.6 µg/ml of insulin. Cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C.

Labelling and Isolation of Proteoglycans

Sulfated proteoglycans were purified as shown in Figure 1. Exponentially growing breast cancer cells were incubated with 25 µCi/ml $Na_2{}^{35}SO_4$ for 24 h in complete MEM medium. A parallel sample seeded at the same density was used to determine the cell number and protein content. At the end of the labelling period, the culture medium was removed, and the underlying cell layer rinsed twice with PBS. The first rinse being pooled with the medium which was centrifugated and stored at −20°C until use. The proteoglycans that were subsequently isolated from the supernatant were denoted medium proteoglycans. Monolayers were then scrapped into buffer containing 4 M guanidinium chloride, 50 mM sodium acetate, protease inhibitors (10 mM EDTA, 5 mM benzamidine, 0.1 mM phenylmethylsulfonylfluoride), and 0.3% Triton X-100 pH 5.8.

Ion-Exchange Chromatography

For characterization of the PG charge density, cell extracts, and incorporation media were diluted respectively 40- and 2-fold and applied to a DEAE-Sephacel column equilibrated in 0.1 M NaCl Tris-HCl buffer. After loading samples, columns were washed with equilibration buffer until no further radioactivity eluted. This ion-exchange chromatography step separated PG from hyaluronic acid and glycoproteins. Elution was then performed with 60 ml of a continuous linear gradient of 0.1-1.5 M NaCl in 50 mM Tris-HCl pH 7.4 containing 0.5% CHAPS. Frac-

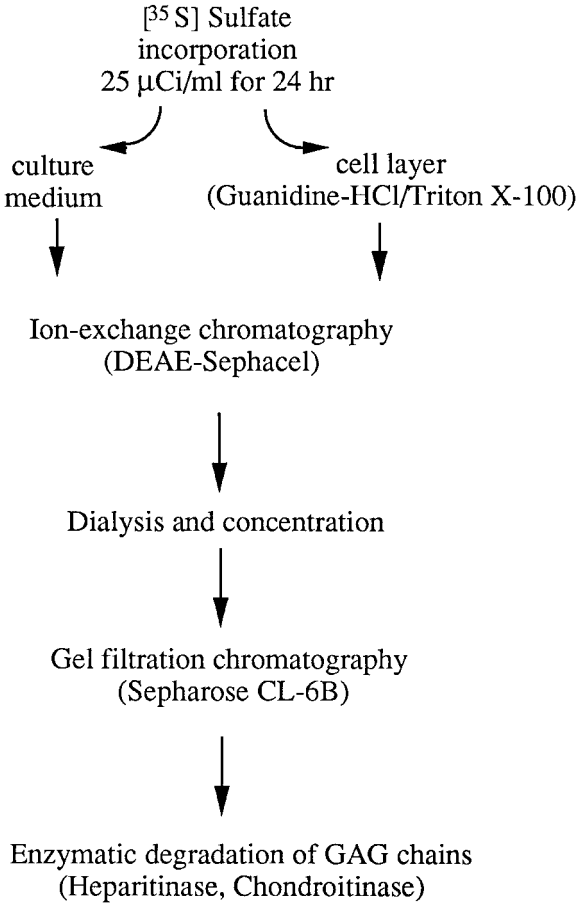

Fig. 1. Schematic survey of the method. Breast cancer cells were incubated in medium containing 25 µCi/ml of [^{35}S] sulfate for 24 h. Labelled proteoglycans were purified by ion-exchange chromatography. A linear NaCl gradient eluted macromolecules according to their charge density. Samples were then dialysed, lyophilized and finally exposed to specific enzyme treatments to determine their GAG composition. The PG size was determined by gel-filtration chromatography (Sepharose CL-6B).

tions were collected and aliquots (50 µl) taken from measurement of radioactivity.

Characterization of GAG Chains

Pooled fractions from the DEAE-Sephacel columns were extensively dialysed against digestion buffer containing 50 mM Hepes, 100 mM NaCl, 1 mM $CaCl_2$, 50 mM hexanoic acid, CHAPS 0.1% (pH 7). Thereafter samples were incubated for 60 mn at 37°C with heparitinase or chondroitinase ABC in digestion buffer containing 1 mg/ml bovine serum albumin. The digested and control material was then chromatographed on a Sepharose CL-6B column (0.9 × 60 cm) in 4 M guanidine-HCl, 50 mM Tris-HCl, 0.3% Triton X-100, pH 8, eluted at a flow rate of 7 ml/h and 1 ml fractions were collected. Aliquots were removed and the radioactivity determined with an LKB liquid scintillation counter.

^{125}I FGF-2/Proteoglycan Interaction Assay

To study the binding of FGF-2 to DEAE-purified proteoglycans, samples volumes corresponding to 500,000 cells were dialyzed into buffer containing 10 mM Tris-HCl and loaded onto cationic membranes as described by Rapraeger and Yeoman [1989]. Briefly, filters were prehydrated in TBS (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl) for 30 mn on a shaker at room temperature and placed into a Bio-Dot apparatus (Millipore) before washing by drawing 0.5 ml of TBS through each well under vacuum. Samples (up to 0.3 ml) in TUT (10 mM Tris-HCl, pH 8.0, 8 M urea, 0.1% Triton X-100) were then applied to the wells and pulled through by vacuum. Each well was then washed with 2 × 0.5 ml TUT. Filters were removed and washed several times in 50 ml of TBS to eliminate urea (20 mn, at room temperature). Filters were then incubated in 3 × 5 ml hybridization buffer (20 mM Hepes, 0.15 M NaCl, 1% bovine serum albumin, pH 7.4) for 30 mn at room temperature, with gentle shaking. They were then incubated in hybridization buffer containing 0.4 ng/ml of ^{125}I FGF-2 for 2 h at room temperature with or without 1 µg/ml unlabelled FGF-2, on a shaking platform. FGF-2 was iodinated using the chloramine-T method as previously described [Hondermarck et al., 1990]. The specific activity averaged 80,000 cpm/ng on the day of iodination. After the hybridization period, each filter was removed, washed several times with hybridization, buffer, and dried after immersing in 96% ethanol. Filters were then subjected to autoradiography for 12–24 h (X-OMAT film Kodak). In some cases, heparin, chondroitin sulfate or hyaluronic acid (0 to 100 µg/ml) were added to the hybrization buffer containing ^{125}I FGF-2.

RESULTS

^{35}S Incorporation Into Proteoglycans

The amount of cell-associated and medium-released proteoglycans was determined from the [^{35}S] incorporated radioactivity eluted with a NaCl gradient from DEAE-Sephacel ion-exchange columns. Experiments were always performed on cells in the exponential growth

phase. After 24 h of metabolic labelling, we observed that the total ^{35}sulfate incorporated into proteoglycans of HBL-100 and MDA-MB-231 cells was about two-fold higher than into MCF-7 cells (Table I). Moreover, for each cell line, the amount of proteoglycans in the medium was the same as the amount in cell extract.

Cell-Associated Proteoglycans

^{35}Sulfated proteoglycans of MDA-MB-231 cells. MDA-MB-231 cell extract material resolved into two peaks with elution molarities of 0.40 and 0.55 M NaCl when chromatographed on DEAE-Sephacel. These two fractions were then subjected to further analysis, respectively peak 1 and peak 2 as shown in Figure 2A. The molecular size of the isolated material was analysed by Sepharose CL-6B gel chromatography in dissociative conditions. The peak 1 material eluted as two peaks with K_{av} of 0.1 and 0.7, representing respectively 40 and 60% of the sulfated material. The peak 2 material consisted of intermediate sized macromolecules eluting with a K_{av} of 0.35. Consistent chromatogram profiles were obtained for several extracts. Samples were then treated with polysaccharide lyases as described in the Materials and Methods, and breakdown products were analysed by chromatography on Sepharose CL-6B. Heparitinase resulted in near complete degradation of sulfated material in the peak with K_{av} 0.1 which hence contained HS-like chains. The peak in the Vt position corresponded to heparitinase digestion products. The second broad peak with a K_{av} of 0.7 represented small sulfated products sensitive to heparitinase. Chondroitinase treatment modified only slightly the elution profile. In fact, peak 1 contained 80% of HS-like chains and 20% of CS-like chains. Peak 2 material was subjected to the same treatments as peak 1. This material was partially sensitive to heparitinase (30%) while approximately 70% was digested by chondroitinase ABC and eluted at the Vt position. The specific degradation of peak 1 and peak 2 material allowed us to determine precisely the total amount of PG; the MDA-MB-231 cell layer therefore contained a mixture of HSPG (56%) and CSPG (44%) as reported in Table II.

^{35}Sulfated proteoglycans of MCF-7 cells. As shown in Figure 2B, the MCF-7 cell extract material eluted as a broad peak with an elution molarity of 0.62 M NaCl after ion-exchange chromatography. All fractions were pooled together and analysed by gel-filtration chromatography (Sepharose CL-6B). MCF-7 cell-associated sulfated macromolecules appeared to be smaller than those in MDA-MB-231 cells and eluted in a peak with a K_{av} of 0.42. Heparitinase treatment partially degraded them (38%) and revealed a CS containing component of small apparent molecular size (K_{av} 0.42). This material was sensitive to chondroitinase treatment (62%) and degradation products eluted at the Vt position. Sulfated products insensitive to chondroitinase treatment eluted at K_{av} 0.3 and correspond to the minor HS containing component of the MCF-7 cell extract. In conclusion, we observed that well-differentiated MCF-7 cells synthesise in their cell layer mostly chondroitin sulfate proteoglycans which are characterised by their smaller apparent size.

^{35}Sulfated proteoglycans of HBL-100 cells. Figure 2C shows that the proteoglycans of HBL-100 cells eluted as a broad peak with an elution molarity of about 0.45 M NaCl. CL-6B chromatography shows that these proteoglycans posses a mean Kav value of 0.45. HSPG constitute the major part of these proteoglycans since treatment by heparitinase degraded 78% of this material. Chondroitinase degraded only 22% of sulfated products and insensitive material (corresponding to HSPG) eluted at Kav 0.45. These results show that HBL-100 cells produce about the same amount of sulfated proteoglycans than MDA-MB-231. Moreover, proportion of HSPG is higher for the cell layer of HBL-100 than for the two other cell lines.

TABLE I. [^{35}S] Sulfate Incorporation Into Breast Cancer Cell Proteoglycans*

	MCF-7 ^{35}S cpm/10^6 cells	MDA-MB-231 ^{35}S cpm/10^6 cells	HBL-100 ^{35}S cpm/10^6 cells
Cell layer	11409 ± 2898	22615 ± 4029	29004 ± 4409
Culture medium	14020 ± 863	21548 ± 2858	25336 ± 2696
Total	25429 ± 3761	44163 ± 6887	54340 ± 7015

*Total incorporation was determined after purification of sulfated proteoglycans on DEAE-Sephacel ion-exchange columns. Results are expressed as cpm/10^6 cells.

Culture Medium Released Proteoglycans

^{35}Sulfated proteoglycans of MDA-MB-231 cells. Culture medium proteoglycans from MDA-MB-231 cells eluted in two peaks, respectively at 0.5 M and 0.65 M NaCl on ion-exchange chromatography (Fig. 3A). Analysis of the pooled peaks by gel-filtration chromatography shows that the sulfated proteoglycans possess a high apparent molecular weight, eluting at a K_{av} value of 0.16. Treatment of this material with heparitinase resulted in partial depolymerisation of these macromolecules (34%). The undepolymerised material still eluted at a K_{av} of 0.16 and contained CS-like chains as shown by its sensitivity to chondroitinase. In fact. 66% of the ion-exchange purified PG was sensitive to chondroitinase. In contrast to cell-associated PG, no additional peak of small free GAG chains was observed for medium-released PG. In conclusion, poorly-differentiated MBA-MB-231 cells released into their culture medium PG with a high molecular size which contain chondroitin sulfate chains as their major GAG component (Table II).

^{35}Sulfated proteoglycans of MCF-7. Medium-released PG from MCF-7 cells eluted in a sharp peak with a elution molarity of 0.5 M NaCl on ion-exchange chromatography (Fig. 3B). Heparitinase treatment depolymerised 68% of the sulfated material. The peak at K_{av} 0.05 disappeared totally and digestion products were observed at the Vt position. Heparitinase insensitive material eluted with a K_{av} of 0.35 and are hence of the CSPG type (32%). Chondroitinase ABC treatment confirmed these results; the peak with K_{av} 0.05 was insensitive (HSPG type) to chondroitinase treatment and only 32% of the material was depolymerised. These results suggest that this sulfated material was composed of 68% HSPG and 42% CSPG. In contrast to MDA-MB-231 cells, MCF-7 cells produce in the culture medium essentially HSPG with a higher apparent hydrodynamic size.

^{35}Sulfated proteoglycans of HBL-100 cells. Sulfated proteoglycans from culture medium of HBL-100 cells eluted from DEAE columns in large two peaks (numbered 1 and 2) at elution molarities of about 0.5 and 0.65 M NaCl (Fig. 3C). These two peaks were then separately analysed. Peak 1 eluted at K_{av} 0.1 from a CL-6B column and was mainly degraded by heparitinase whereas peak 2 exhibits the same K_{av} but mainly degraded by chondroitinase. These results show that HBL-100 cells released about the same amount of proteoglycans than MDA-MB-231 cells and that the proportion of HSPG is lower in the culture medium of HBL-100 and MDA-MB-231 cells than in MCF-7 cells.

^{125}I FGF-2 Interaction With Proteoglycans

In order to examine if breast cancer cell proteoglycans could interact with FGF-2, we loaded DEAE-purified PG on a cationic nylon membranes and incubated the filters in a buffer containing ^{125}I FGF-2. Our results showed that the three cell lines produced cell layer and medium proteoglycans which are able to bind specifically ^{125}I FGF-2 (Fig. 4). Treatment with heparitinase abolished ^{125}I FGF-2 binding. The FGF-2 binding was deplaced with 10 µg/ml heparin thus confirming that HSPG are required for binding. Inversely, other GAG tested (chondroitin sulfate or hyaluronic acid) have no effect on FGF-2 binding whatever concentrations used (data not shown). Moreover, we observed that the ^{125}I FGF-2 binding capacity was greater for medium-released HSPG than for cell-associated HSPG for all cell lines. Interestingly, MDA-MB-231 and HBL-100 cell-associated HSPG exhibited a higher capacity to bind FGF-2 than MCF-7 cell-associated HSPG.

DISCUSSION

In this study, we examined the distribution and nature of proteoglycans synthesised by human breast cancer cell lines in culture. We determined the amount of HSPG and CSPG in the cell layer or in the culture medium of well-differentiated epithelioid MCF-7 cells, poorly-differentiated invasive MDA-MB-231 cells, and transformed but non malignant HBL-100 cells. We observed that MDA-MB-231 and HBL-100 cells incorporated two- to three-fold higher amounts of [^{35}S] sulfate into GAG than MCF-7 cells and demonstrated that MDA-MB-231 and HBL-100 cells accumulated specifically heparan sulfate proteoglycans (HSPG) in their cell layer. Accumulation of HSPG in the cell layer has been reported for metastatic melanoma cells [Caux et al., 1992; Moczar et al., 1993] and invasive lung cells [Timar et al., 1987] and seems to be associated with the metastatic phenotype of cells [Mangakanis et al., 1990; Lapis et al., 1992]. Timar et al. [1995] demonstrated that melanoma cells with a high HSPG content are more likely to form metastases than cancer-

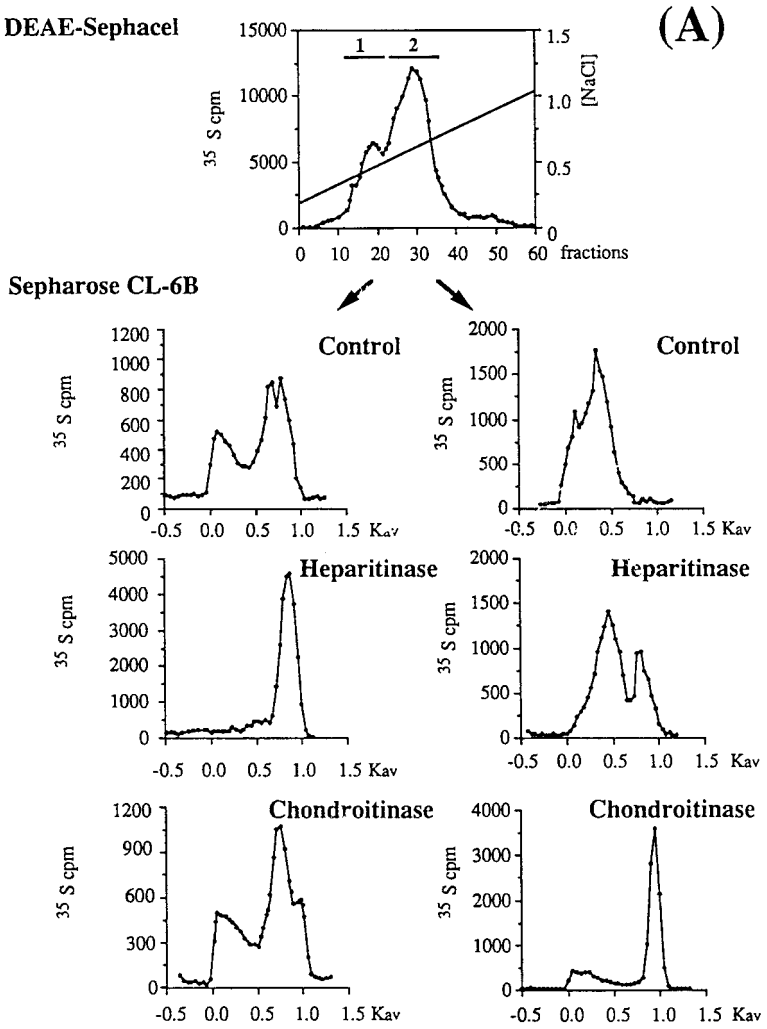

Fig. 2. Cell-associated sulfated proteoglycans. After [^{35}S] sulfate incorporation, cell-associated PG were extracted from MDA-MB-231 (**A**), MCF-7 (**B**), and HBL-100 (**C**) cell layer. Using 4 M guanidine-HCl containing protease inhibitors as described in the Materials and Methods section. They were purified by ion-exchange chromatography (DEAE-Sephacel) with a linear NaCl gradient (0.15–1.5 M). Eluted material was then subjected to Sepharose CL-6B gel chromatography. Resulting chromatograms of untreated material, heparitinase, and chondroitinase ABC treated material are shown. Elution was realised with 4 M guanidine-HCl, 50 mM Tris-HCl, 0.3 Triton X-100 (pH 8).

ous cells stimulated to produce a greater amount of CSPG. Furthermore, Esko et al. [1988] have shown that HSPG are required for tumorigenesis in vivo; they demonstrated that mutant Chinese Hamster Ovary cells (CHO) which are deficient in HSPG synthesis are unable to induce tumors in nude mice in contrast to wild type CHO cells.

Cell-associated HSPG synthesised by MDA-MB-231 and HBL-100 cells were also characterised by a low degree of sulfation. Undersulfation of HSPG has been reported for transformed and cancerous cells [Fransson and Sjöberg, 1981; David and Van den Berghe, 1983; Robinson et al., 1984; Pejler and David, 1987; Romaris et al., 1994; Piedagniel et al., 1994]. The undersulfation of HSPG in cancer cells could modify interactions with other molecules of the extracellular matrix. Hepatoma cells produce undersulfated HSPG, with strongly reduced binding to fibronectin compared to normal counterpart HSPG [Robinson et al., 1984]. Furthermore, HSPG synthesised in the presence of sodium chlorate (a competitive inhibitor of GAG sulfation) lost their capacity to bind fibronectin [Keller et al., 1989] or laminin [Brauer et al., 1990]. Oncogenic transformation might also affect the level of sulfation of the glycosaminoglycans chains [Piedagnel et al., 1994; Romaris et al., 1994]. In mouse mammary glands, HSPG

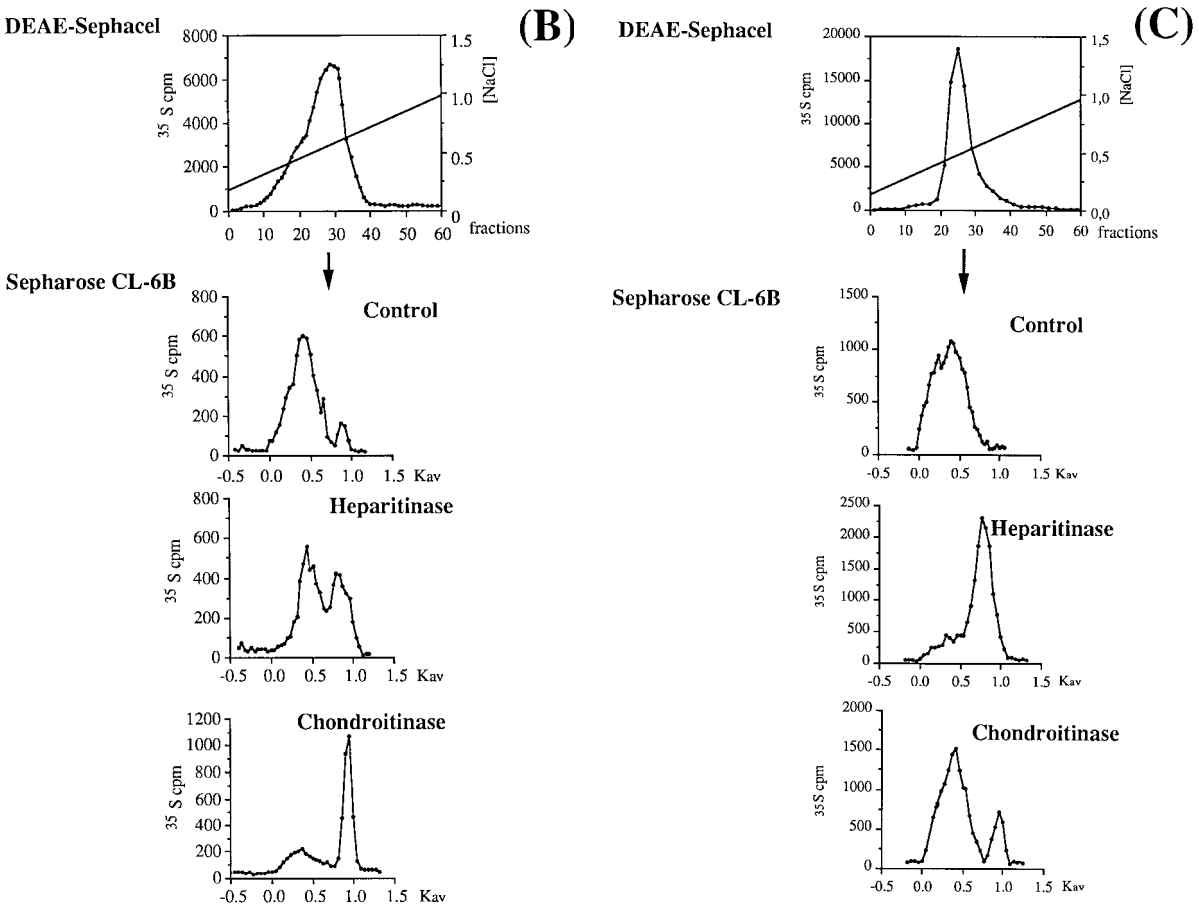

Figure 2. (Continued.)

TABLE II. Distribution of Sulfated Proteoglycans in the Cell Layer and Medium of Human Breast Cancer Epithelial Cells*

		MCF-7 ^{35}S cpm/10^6 cells	MDA-MB-231 ^{35}S cpm/10^6 cells	HBL-100 ^{35}S cpm/10^6 cells
Cell layer	HSPG	38%	56%	78%
		4335 ± 1101	12664 ± 2256	22624 ± 2779
	CSPG	62%	44%	22%
		7074 ± 1797	9951 ± 1179	6380 ± 1630
Culture Medium	HSPG	68%	34%	45%
		9534 ± 534	7326 ± 972	11410 ± 1213
	CSPG	32%	66%	55%
		4486 ± 279	14222 ± 1886	13926 ± 1483

*[^{35}S] Sulfate labelled PG are purified and digested with heparitinase or chondroitinase ABC as described in the Materials and Methods section. Results are expressed as cpm/10^6 cells and are the average of independent triplicate experiments.

synthesised by transformed epithelial cells have a reduced charge density compared to corresponding normal NMuMg cells [David and Van den Berghe, 1983]. These undersulfated HSPG prevent cell matrix interactions and formation of extracellular matrix [David and Bernfield, 1982].

The other sulfated macromolecules which were sensitive to heparitinase in MDA-MB-231 cell extracts, eluted at a Kav of 0.7. The small sized products correspond to 30% of the total sulfated material synthesised by MDA-MB-231 cells and interestingly they were not found in MCF-7 and HBL-100 cells. These molecules are

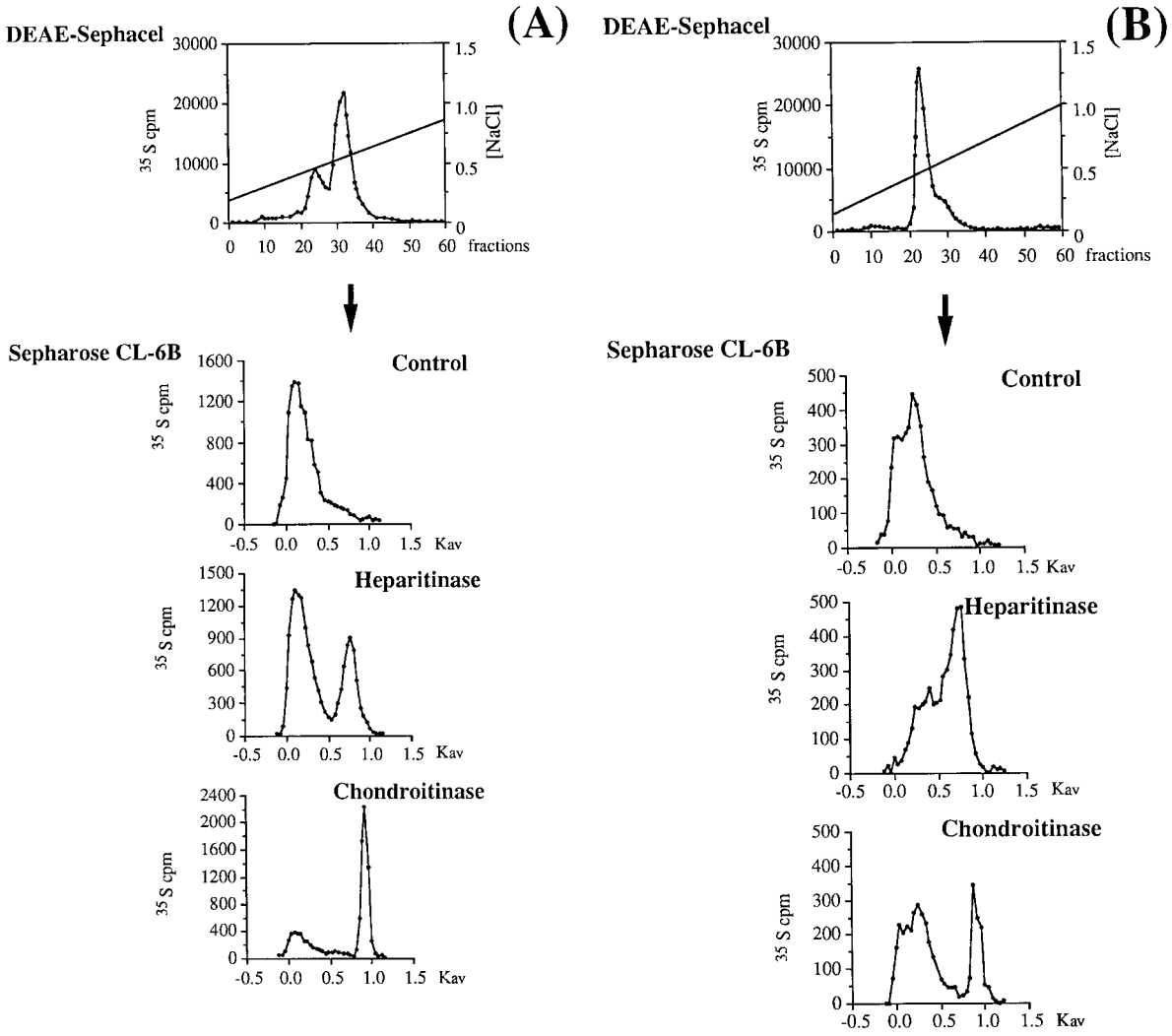

Fig. 3. Culture medium-released proteoglycans. Medium-released proteoglycans from MDA-MB-231 (**A**), MCF-7 (**B**), and HBL-100 cells (**C**) were purified by ion-exchange chromatography (DEAE Sephacel). Radiolabelled PG were then analysed by gel-filtration chromatography (Sepharose CL-6B) as described in the Materials and Methods section.

presumably composed of free heparan sulfate chains, as already described for normal and cancerous cells in culture [Rapraeger and Bernfield, 1985; Piepkorn et al., 1991] and might correspond to degradation products of cell-associated PG [Hovingh et al., 1993; Schmidtchen and Fransson, 1994].

Compared to MDA-MB-231 and HBL-100 cells, MCF-7 cells produced less cell-associated HSPG. In fact, these cells released mostly sulfated HSPG into their culture medium. These HSPG were characterised by a higher apparent molecular size. In contrast, HBL-100 and MDA-MB-231 cells released mostly CS proteoglycans into their culture medium. Channe-Gowda et al. [1986] already reported that CSPG are the main PG component found into the culture medium of MDA-MB-231 and HBL-100 cells. Elevated concentrations of CSPG were also described in breast tumor biopsies [Takeuchi et al., 1976; Alini and Losa, 1991] and in other cancerous tissues [reviewed in Iozzo, 1988; Timar and Kovalszky, 1995]. The presence of an increased proportion of CSPG to HSPG in culture medium in cells with an increased metastatic potential suggest that modification in the PG content could be related to pathogenic and metastatic processes.

One important property of HSPG is their ability to interact with growth factors as FGF-2 or Hepatocyte Growth Factor (HGF) [reviewed in Fernig and Gallagher, 1994]. Specific sequences of HS mediating binding to FGF-2 were described in different models [Turnbull et al.,

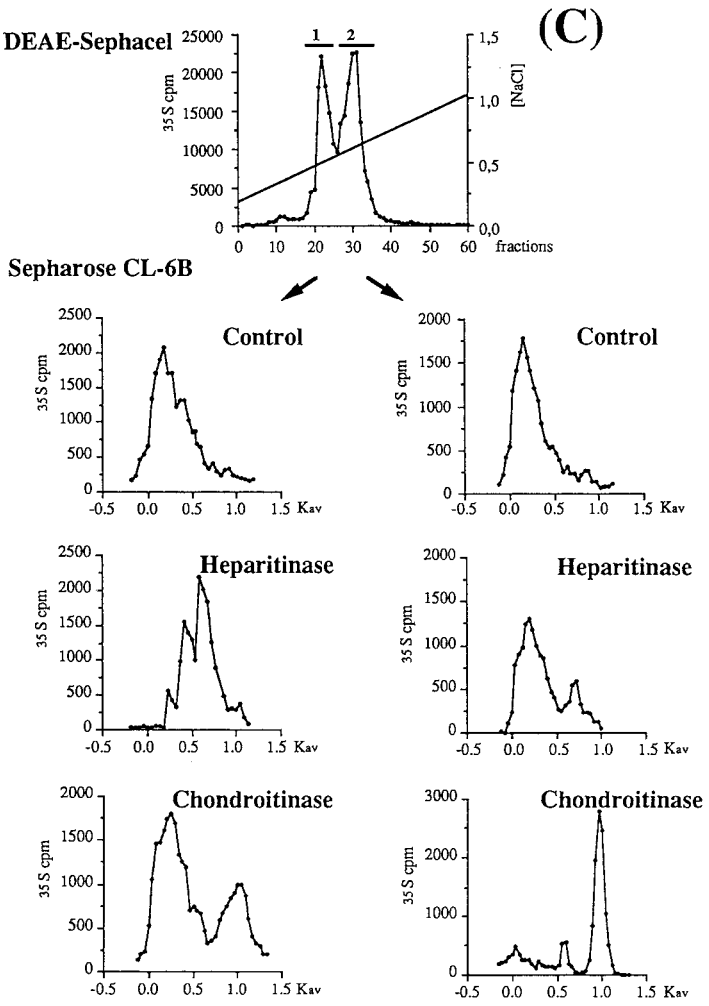

Figure 3. (Continued.)

1992; Habuchi et al., 1992; Ishihara et al., 1993; Maccarana et al., 1993; Tyrrell et al., 1993; Walker et al., 1994]. Several biological advantages of this FGF-2/HSPG interaction are now well-documented. FGF-2 binds to heparin or heparan sulfate chains, and is thereby protected against proteolysis and thermal denaturation [Gospodarowicz and Cheng, 1986; Saksela et al., 1988]. HSPG can also serve as a reserve of growth factor that can support long term responses to FGF-2 [Flaumenhaft et al., 1989, 1990] and are also implicated in its internalisation into cells [Gannoun-Zaki et al., 1991; Roghani and Moscatelli, 1992; Rusnati et al., 1993; Quarto and Amalric, 1994]. Interaction of FGF-2 with heparin or heparan sulfate is reported to be necessary for binding to its tyrosine kinase receptors [Rapraeger et al., 1991; Yayon et al., 1991]. However, a recent study found that heparin or heparan sulfate was not strictly required for binding of FGF-2 to its receptor but increased the affinity of interaction [Roghani et al., 1994; Pantaliano et al., 1994; Steinfeld et al., 1996]. We previously showed that FGF-2 is a strong mitogen for MCF-7 cells but has no effect on MDA-MB-231 and HBL-100 cells [Delehedde et al., 1995]. However, these two last breast cell lines possess high affinity membrane receptors for FGF-2 [Peyrat et al., 1991, 1992; Lucqmani et al., 1992; Jacquemier et al., 1994; Pennault-Llorca et al., 1995; Johnston et al., 1995]. Low affinity sites for FGF-2 are present in higher concentration on MDA-MB-231 membranes than on MCF-7 cells as shown by a radioreceptor assay [Peyrat et al., 1991, 1992]. In the present study, we found that HBL-100 and MDA-MB-231 cells bind higher quantities of FGF2 to their HSPG

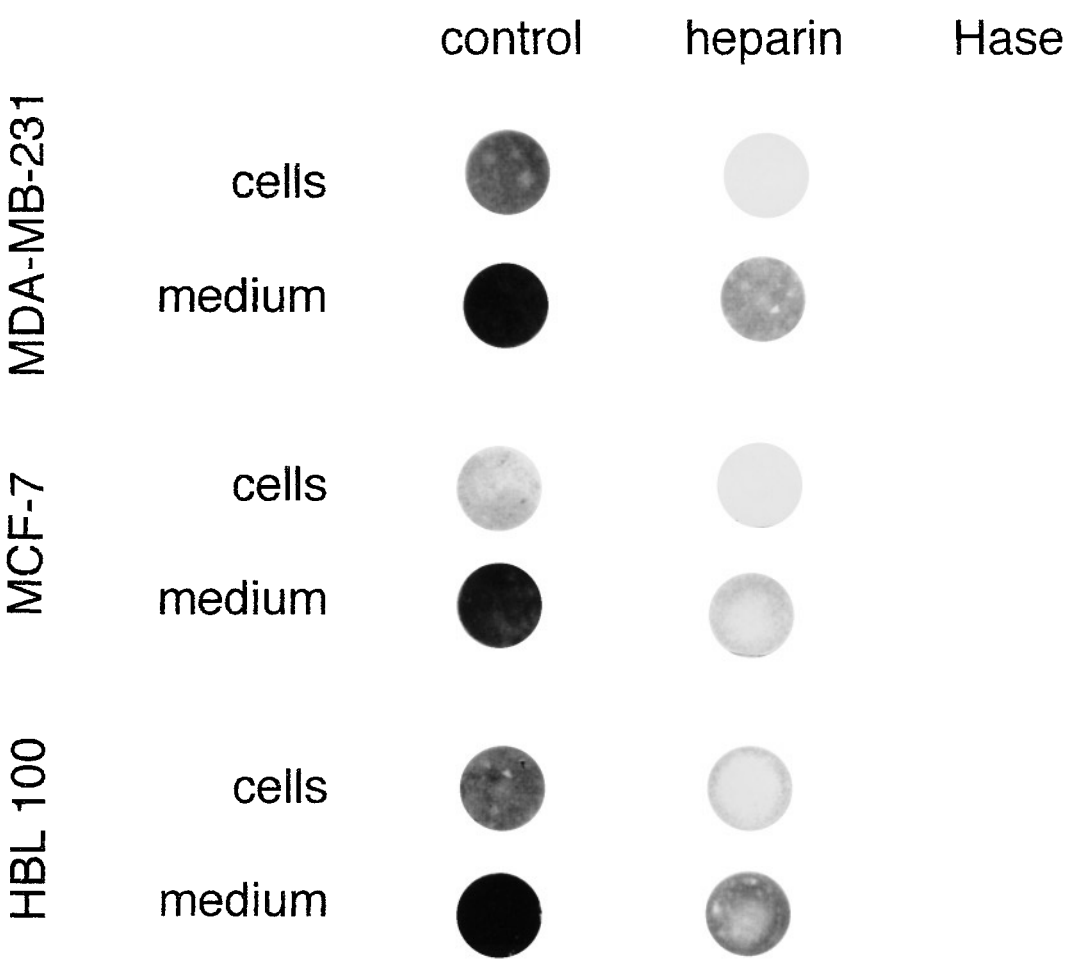

Fig. 4. Binding of ^{125}I FGF-2 to DEAE- purified proteoglycans from human breast cancer cells. Proteoglycans purified by DEAE-Sephacel from MDA-MB-231, MCF-7, and HBL-100 cells (treated or not by heparinase) were loaded onto Zeta-Probe membranes by filtration. The membrane was then incubated in the presence of ^{125}I-FGF-2 with or without heparin (10 µg/ml) for 2 h as described in the Materials and Methods section. The amount of bound FGF-2 was estimated by autoradiography of filters.

than MCF-7 cells. The FGF-2 binding capacity of breast epithelial cell proteoglycans does not appear to depend only on the quantity of HSPG. Indeed, for MDA-MB-231 and HBL-100 cell lines, there is more HSPG in the cell layer than in the culture medium, but medium-derived proteoglycans present a higher capacity to bind FGF-2. The affinity of HSPG for FGF-2 has been shown to depend on both the size and sulfation of HS chains (reviewed in Fernig and Gallagher, 1994), suggesting that the capacity of breast epithelial cells to bind FGF-2 to their HSPG depends both on the HSPG quantity and affinity for this growth factor. We previously showed that HSPG can prevent and inhibit biological activity of FGF-2 [Hondermarck et al., 1992]. Mali et al. [1993] reported that over-expression of syndecan-1 (a prototype of heparan sulfate proteoglycan) totally inhibits FGF-2-induced proliferation in NIH 3T3 cells. This demonstrates that an adequate balance between number of high affinity receptors and low affinity sites (HSPG) is required for obtaining FGF-2 biological activity. The fact that HBL-100 and MDA-MB-231 cells bind higher quantities of FGF-2 on their HSPG than MCF-7 cells strongly suggests that these HSPG present in greater amounts could be responsible for the insensitivity of HBL-100 and MDA-MB-231 cells to this growth factor.

An intriguing observation of our work is that similar results were obtained with the highly metastatic MDA-MB-231 cells and the transformed but non-tumorigenic HBL-100 cells.

Both cell lines produce a higher proportion of HSPG in their cell layer and a lower proportion of CSPG in the medium compared to MCF-7 cells. In addition, the elution molarity from DEAE as well as the Kav on CL-6B of HSPG and the capacity to bind FGF-2 are also similar in these two cell lines. However, there is a difference between these two cell lines since small size HSPG are present in MDA-MB-231 cells and not in HBL-100 cells. Whether or not differences in the proteoglycan distribution is significant for tumor growth and invasiveness is not yet clearly elucidated. Invasive breast tissues are known to present a higher content in chondroitin sulfate chains (Takeuchi et al., 1976; Olsen et al., 1988; Alini and Losa, 1991). In case of heparan sulfate chains, Mangakis et al. [1990] showed an increase in invasive tumors while Alini and Losa [1991] reported a decrease content of heparan sulfate. Our data indicate that the invasive cell line MDA-MB-231 produces more HSPG than the more differentiated MCF-7 cells, however it appears difficult to draw general conclusions since HBL-100 cells which are known to be transformed but not malignant breast epithelial cells produce even more HSPG compared to MDA-MB-231 cells. Elucidation of the significance of proteoglycans in tumor growth, especially regarding their capacity to bind growth factors such as FGF-2 and examination of the potential of HSPG as prognostic parameter in breast cancer will now require investigations not only on the cellular models but also on tumor biopsies for which histopronostic parameters are determined on a clinical basis.

ACKNOWLEDGMENTS

This work was supported in part by grants from the "Ligue Nationale Contre le Cancer, Comité du Nord" and the "Ministère de l'Education Nationale." M. Delehedde has a fellowship from the "Ministère de la Recherche et de l'Enseignement Supérieur." We are grateful to Dr. C. Brahimi-Horn for reviewing this manuscript. We are thankful to Dr. J.P. Maufroid and Arlette Rousseau for their assistance.

REFERENCES

Alini M, Losa G (1991): Partial characterization of proteoglycans isolated from neoplastic and nonneoplastic human breast tissues. Cancer Res 51:1443–1447.

Beck JC, Lekutis C, Couchman J, Parry G (1993): Stage-specific remodeling of the mammary gland basement membrane during lactogenic development. Biochem Biophys Res Commun 190:616–623.

Brauer PR, Keller KM, Keller JM (1990): Concurrent reduction in the sulfation of heparan sulfate and basement membrane assembly in a cell model system. Development 110:805–813.

Caux F, Timar J, Vigny M, Moczar M (1992): Heparan sulfate synthesised by human melanoma cell variants. Cancer J 5:111–117.

Chandrasekaran EV, Davidson EA (1979): Glycosaminoglycans of normal and malignant cultured human mammary cells. Cancer Res 39:870–880.

Channe-Gowda D, Bhavanandan VP, Davidson EA (1986): Isolation and characterization of proteoglycans secreted by normal and malignant human mammary epithelial cells. J Biol Chem 261:4926–4934.

David G, Bernfield M (1982): Defective basal lamina formation by transformed mammary epithelial cells: a reduced effect of collagen on basal lamina (heparan sulfate-rich) proteoglycan degradation. J Cell Physiol 110:56–62.

David G, Van Den Berghe H (1983): Transformed mouse mammary epithelial cells synthesise undersulfated basement membrane proteoglycan. J Biol Chem 258:7338–7344.

Delehedde M, Boilly B, Hondermarck H (1995): Differential responsiveness of human breast cancer cells to basic fibroblast growth factor. A cell kinetics study. Oncology Res 7:399–405.

Emerman JT, Jones N, Fiedler EE (1988): Glycosaminoglycan accumulation by normal and malignant human mammary epithelial cells in primary culture. Biochem Cell Biol 66:309–318.

Esko JD, Rostand KS, Weinke JL (1988): Tumor formation dependent on proteoglycan biosynthesis. Science 241: 1092–1096.

Fernig DG, Gallagher JT (1994): Fibroblast growth factors and their receptors: an information network controlling tissue growth, morphogenesis and repair. Prog Growth Factor Res 5:353–377.

Flaumenhaft R, Moscatelli D, Saksela O, Rifkin DB (1989): Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth factor for long term stimulation of plasminogen activator production and DNA synthesis. J Cell Physiol 140:75–81.

Flaumenhaft R, Moscatelli D, Rifkin DB (1990): Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor. J Cell Biol 111: 1651–1659.

Fransson LA, Sjöberg I (1981): Copolymeric glycosaminoglycans in transformed cells. J Biol Chem 256:13044–13047.

Gannoun-Zaki L, Pieri I, Badet J, Moenner M, Barritault D (1991): Internalisation of basic fibroblast growth factor by chinese hamster lung fibroblast cells: involvement of several pathways. Exp Cell Res 197:272–279.

Gospodarowicz D, Cheng J (1986): Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 128:475–484.

Habuchi H, Suzuki S, Saito T, Harada T, Yoshida K, Kimata K (1992): Structure of heparan oligosaccharide that binds to basic fibroblast growth factor. Biochem J 285:805–813.

Hondermarck H, Courty J, Ledoux D, Blanckaert V, Barritault D, Boilly B (1990): Evidence of high and low affinity

binding sites for basic fibroblast growth factor in mouse placenta. Biochem Biophys Res Commun 169:272–281.

Hondermarck H, Deudon E, Boilly B (1992): Embryonic brain-derived heparan sulfate inhibits cellular membrane binding and biological activity of basic fibroblast growth factor. Dev Brain Res 68:247–253.

Hovingh P, Piepkorn M, Linker A (1993): Differentially expressed patterns of glycosaminoglycan structure in heparan sulfate proteoglycans and free chains. Eur J Biochem 211:771–779.

Iozzo RV (1988): Proteoglycans and neoplasia. Cancer Metastasis Rev 7:39–50.

Ishihara M, Tyrrell DJ, Stauber GB, Brown S, Cousens LS, Stack RJ (1993): Preparation of affinity-fractionated, heparin-derived oligosaccharides and their effects on selected biological activities mediated by basic fibroblast growth factor. J Biol Chem 268:4675–4683.

Jacquemier J, Adelaide J, Parc P, Penault-Llorca F, Planche J, Delapeyriere O, Birnbaum D (1994): Expression of the FGFR1 gene in human breast-cancer cells. Int J Cancer 59:373–378.

Johnston CL, Cox HC, Gomm JJ, Coombes RC (1995): Fibroblast growth factor receptors (FGFRs) localize in different cellular compartments. A splice variant of FGFR-3 localizes to the nucleus. J Biol Chem 270:30643–30650.

Keller KM, Brauer PR, Keller JM (1989): Modulation of cell surface heparan sulfate structure by growth of cells in the presence of chlorate. Biochemistry 28:8100–8107.

Kjellen L, Lindahl U (1991): Proteoglycans: Structure and interactions. Ann Rev Biochem 60:443–475.

Lapis K, Timar J, Jeney A (1992): Proteoglycans as target in the therapy of metastasis. In Rabes H, Peters PE, Munk K (eds): "Metastasis: Basic Research and Its Clinical Applications." Basel; New York: Karger.

Lee EH, Lee WH, Kaetzel CS, Parry G, Bissell MJ (1985): Interaction of mouse mammary epithelial cells with collagen substrata: Regulation of casein gene expression and secretion. Proc Natl Acad Sci USA 82:1419–1423.

Leppa S, Mali M, Miettinen HM, Jalkanen M (1992): Syndecan expression regulates cell morphology and growth of mouse mammary epithelial tumor cells. Proc Natl Acad Sci USA 89:932–936.

Losa GA, Alini M (1993): Sulfated proteoglycans in the extracellular matrix of human breast tissues with infiltrating carcinoma. Int J Cancer 54:552–557.

Luqmani YA, Graham M, Coombes RC (1992): Expression of basic fibroblast growth factor, FGFR-1 and FGFR-2 in normal and malignant human breast, and comparison with other normal tissues. Br J Cancer 66:273–280.

Maccarana M, Casu B, Lindahl U (1993): Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. J Biol Chem 268:23898–23905.

Mali M, Elenius K, Miettinen HM, Jalkanen M (1993): Inhibition of basic fibroblast growth factor-induced growth promotion by overexpression of syndecan-1. J Biol Chem 268:24251–24222.

Mangakis N, Serht B, Mangakis P, Bowe E, Kleemann I (1990): Determining the degree of malignancy of individual cases of mammary carcinoma on the basis of clinical, morphological and biochemical parameters. Bull Cancer 77:235–242.

Moczar M, Caux F, Bailly M, Berthier O, Dore JF (1993) Accumulation of heparan sulfate in the culture of human melanoma cells with different metastatic ability. Clin Exp Metastasis 11:462–471.

Olsen EB, Trier K, Eldov K, Ammitzboll T (1988): Glycosaminoglycans in human breast cancer. Acta Obstet Gyneco Scand 67:539–542.

Pantaliano MW, Horlick RA, Springer BA, Van Dick DE, Tobery T, Wetmore DR, Lear JD, Nahapetian AT, Bradley JD, Sisk WP (1994): Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. Biochemistry 33:10229–10248.

Parry G, Lee EYH, Farson D, Koval M, Bissel MJ (1985): Collagenous substrata regulate the nature and distribution of glycosaminoglycans produced by differentiated cultures of mouse mammary epithelial cells. Exp Cell Res 156:487–499.

Pejler G, David G (1987): Basement membrane heparan sulfate with high affinity for antithrombin synthesised by normal and transformed mouse mammary epithelial cells. Biochem J 248:69–77.

Penault-Llorca F, Bertucci F, Adelaide J, Parc P, Coulier F, Jacquemier J, Birnbaum D, Delapeyriere O (1995): Expression of FGF and FGF receptor genes in human breast cancer. Int J Cancer 61:170–176.

Peterson OW, Ronnov-Jessen L, Howlett AR, Bissel MJ (1992): Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci USA 89:9064–9068.

Peyrat JP, Hondermarck H, Louchez MM, Boilly B (1991): Demonstration of basic fibroblast growth factor binding sites in human breast cancer cell lines. Cancer Commun 3:323–329.

Peyrat JP, Bonneterre J, Hondermarck H, Hecquet B, Adenis A, Louchez MM, Lefebvre J, Boilly B, Demaille A (1992): Basic fibroblast growth factor: mitogenic activity and binding sites in human breast cancer. J Steroid Biochem Biol 43:87–94.

Piedagnel R, Prie D, Cassingena R, Ronco PM, Lelongt B (1994): SV40 Large-T oncogene inhibits transcription of perlecan-related proteoglycans but stimulates hyaluronan synthesis in a temperature-sensitive renal tubule principal cell line. J Biol Chem 269:17469–17476.

Piepkorn M, Hovingh P, Linker A (1991): Proteoglycan and glycosaminoglycan free chain expression in keratinocytes, endothelium and mesenchymal cells. Biochem Biophys Res Commun 179:1281–1288.

Quarto N, Amalric F (1994): Heparan sulfate proteoglycans as transducers of FGF-2 signalling. J Cell Science 107: 3201–3212.

Rapraeger A, Bernfield M (1985): Cell surface proteoglycan of mammary epithelial cells. Protease release of heparan sulfate-rich ectodomain from a putative membrane anchored domain. J Biol Chem 260:4103–4109.

Rapraeger A, Yeoman C (1989): A quantitative solid-phase assay for identifying radiolabeled glycosaminoglycans in crude cell extracts. Anal Biochem 179:361–365.

Robinson J, Viti M, Höök M (1984): Structure and properties of an undersulfated heparan sulfate proteoglycan synthesised by a rat hepatoma cell line. J Cell Biol 98:946–953.

Roghani M, Moscatelli D (1992): Basic fibroblast growth factor is internalized through both receptor-mediated and heparan sulfate-mediated mechanisms. J Biol Chem 267:22156–22162.

Roghani M, Mansukhani A, Dell'Era P, Bellosta P, Basilico C, Moscatelli D (1994): Heparin increases the affinity of basic fibroblast growth factor for its receptor but is not required for binding. J Biol Chem 269:3976–3984.

Romaris M, Villena J, Molist A, Heredia A, Bassols A (1994): Ras transformation alters the composition of extracellular matrix proteoglycans in rat fibroblasts. Biochem Biophys Res Commun 200:925–932.

Rusnati M, Urbinati C, Presta M (1993): Internalisation of basic fibroblast growth factor in cultured endothelial cells: role of the low affinity heparin-like bFGF receptors. J Cell Physiol 154:152–161.

Saksela O, Moscatelli D, Sommer A, Rifkin DB (1988): Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J Cell Biol 107:743–751.

Schmidtchen A, Fransson LA (1994): Analysis of heparan-sulfate chains and oligosaccharides from proliferating and quiescent fibroblasts. A proposed model for endoheparanase activity. Eur J Biochem 223:211–221.

Sommers CL, Byers SW, Thompson EW, Torri JA, Gelmann EP (1994): Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res Treat 31:325–335.

Steinfeld R, Van Den Berghe H, David G (1996): Stimulation of fibroblast growth factor receptor-1 occupancy and signaling by cell surface-associated syndecans and glypican. J Cell Biol 133:405–416.

Takeuchi J, Sobue M, Sato E, Shamoto M, Miura K, Nakagaki S (1976): Variation in glycosaminoglycan components of breast tumors. Cancer Res 36:2133–2139.

Timar J, Moczar E, Timar F, Pal K, Kopper L, Lapis K, Jeney A (1987): Comparative study on Lewis lung tumor cell lines with low and high metastatic capacity: II. Cytochemical and biochemical evidence for differences in glycosaminoglycans. Clin Exp Metastasis 5:79–87.

Timar J, Diczhazi C, Bartha I, Pogany G, Paku S, Raso E, Tovari J, Ladanyi A, Lapis K, Kopper L, Jeney A (1995): Modulation of heparan sulfate/chondroitin sulfate ratio by glycosaminoglycan biosynthesis inhibitors affects liver metastatic potential of tumor cells. Int J Cancer 62:755–761.

Timar J, Kovalszky I (1995): Differential expression of proteoglycans on the cell surface of malignant cells and in the tumor stroma. In Adany R (ed): "Tumor Matrix Biology." Boca Raton, FL: CRC Press.

Turnbull JE, Fernig DG, Ke Y, Wilkinson MC, Gallagher JT (1992): Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. J Biol Chem 267:10337–10341.

Tyrrell DJ, Ishihara M, Rao N, Horne A, Kiefer MC, Stauber GB, Lam LH, Stack RJ (1993): Structure and biological activities of a heparin-derived hexasaccharide with high affinity for basic fibroblast growth factor. J Biol Chem 268:4684–4689.

Walker A, Turnbull JE, Gallagher JT (1994): Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor. J Biol Chem 269:931–935.

Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM (1991): Cell surface, heparin like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64:841–848.