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Ovarian Carcinoma Cells Synthesize Both Chondroitin Sulfate and Heparan Sulfate Cell Surface Proteoglycans That Mediate Cell Adhesion to Interstitial Matrix

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Abstract Metastatic ovarian carcinoma metastasizes by intra-peritoneal, non-hematogenous dissemination. The adhesion of the ovarian carcinoma cells to extracellular matrix components, such as types I and III collagen and cellular fibronectin, is essential for intra-peritoneal dissemination. The purpose of this study was to determine whether cell surface proteoglycans (a class of matrix receptors) are produced by ovarian carcinoma cells, and whether these proteoglycans have a role in the adhesion of ovarian carcinoma cells to types I and III collagen and fibronectin. Proteoglycans were metabolically labeled for biochemical studies. Both phosphatidylinositol-anchored and integral membrane-type cell surface proteoglycans were found to be present on the SK-OV-3 and NIH:OVCAR-3 cell lines. Three proteoglycan populations of differing hydrodynamic size were detected in both SK-OV-3 and NIH:OVCAR-3 cells. Digestions with heparitinase and chondroitinase ABC showed that cell surface proteoglycans of SK-OV-3 cells had higher proportion of chondroitin sulfate proteoglycans (75:25 of chondroitin sulfate:heparan sulfate ratio), while NIH:OVCAR-3 cells had higher proportion of heparan sulfate proteoglycans (10:90 of chondroitin sulfate:heparan sulfate ratio). RT-PCR indicated the synthesis of a unique assortment of syndecans, glypicans, and CD44 by the two cell lines. In adhesion assays performed on matrix-coated titer plates both cell lines adhered to types I and III collagen and cellular fibronectin, and cell adhesion was inhibited by preincubation of the matrix with heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, or chondroitin glycosaminoglycans. Treatment of the cells with heparitinase, chondroitinase ABC, or methylumbelliferyl xyloside also interfered with adhesion confirming the role of both heparan sulfate and chondroitin sulfate cell surface proteoglycans as matrix receptors on ovarian carcinoma cells. J. Cell. Biochem. 83: 259–270, 2001.

Key words: glycosaminoglycan; syndecan; glypican; CD44; collagen; fibronectin

Ovarian carcinomas, a major health threat to women, originate from the coelomic epithelium of the ovary [Auersperg et al., 1998]. The mechanism of dissemination of ovarian carcinoma is unique, because it occurs mostly via intraperitoneal adhesion to the mesothelium [Cannistra, 1993], and subsequent penetration of the sub-mesothelial matrix. Invasion of the sub-mesothelial matrix is influenced by cell surface matrix receptors that mediate the adhesion of carcinoma cells to the components of the mesothelial matrix. The most abundant proteins of the mesothelial matrix are type I and III collagens, fibronectin [Harvey and Amlot, 1983; Stylianou et al., 1990]. Adhesion of the ovarian carcinoma cells to type I collagen and fibronectin has been shown to be mediated by β1 integrins [Moser et al., 1996; Buczek-Thomas et al., 1998; Strobel and Cannistra, 1999]. However, blocking the integrin function of ovarian carcinoma cells did not completely prevent binding to type I collagen or fibronectin [Fishman et al., 1998] indicating that other, hitherto undescribed, functional matrix receptors are present on ovarian carcinoma cells. Matrix receptors that mediate adhesion of ovarian carcinoma cells to type III collagen have not been described.

Abbreviations used: BSA, bovine serum albumin; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; RT-PCR, reverse transcriptase-polymerase chain reaction.

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Cell surface proteoglycans constitute a group of matrix receptors, but the role of these receptors in ovarian carcinoma cells has not been investigated. Proteoglycans are composed of a core protein and glycosaminoglycan chains covalently attached to the core protein [Silbert et al., 1997]. Syndecans and glypicans are the most ubiquitous cell surface proteoglycans. The syndecan family of cell surface proteoglycans has transmembrane core proteins that carry chondroitin and/or heparan sulfate glycosaminoglycans [Bernfield et al., 1999]. The glypican family of cell surface proteoglycans has phosphatidylinositol-anchored core proteins that carry heparan sulfate glycosaminoglycans [David, 1993]. The binding of cell surface proteoglycans to matrix molecules such as collagens, fibronectin, thrombospondin, and vitronectin [Bernfield et al., 1992] happens via the heparan sulfate chains of the proteoglycans. Glycosaminoglycan-binding sequences have been described from collagens and fibronectin [Cardin and Weintraub, 1989], and direct binding of heparan sulfate and heparin to fibrillar collagens have been demonstrated [San Antonio et al., 1994; Sweeney et al., 1998]. Adhesion of mammary epithelial cells, myeloma cells, and melanoma cells via syndecans to types I and III collagen [Koda et al., 1985; Sanderson et al., 1992] and fibronectin [Saunders and Bernfield, 1988] have been described. Glypicans have been implicated in cell adhesion to fibronectin [Drake et al., 1992]. Expression of syndecans and glypicans has not been studied in ovarian carcinoma cells. CD44 splice variants may be synthesized as proteoglycans [Jackson et al., 1995]; therefore, CD44 may be able to carry out cell-matrix receptor activity via its glycosaminoglycan chains (binding to collagens and fibronectin), in addition to binding to hyaluronan via the protein core. A non-proteoglycan form of CD44 has been described as hyaluronan receptor on primary and cultured ovarian carcinoma cells [Cannistra et al., 1995; Schroeder et al., 1999].

Our goal was to determine whether cell surface proteoglycans are synthesized by ovarian carcinoma cells, and whether these proteoglycans contribute to cell-matrix adhesion. We used two ovarian carcinoma cell lines, SK-OV-3 and NIH:OVCAR-3, because they are widely used models of ovarian carcinoma biology and biochemistry, and both were derived from ascites fluid of carcinoma patients; thus, they likely represent the adhesive properties of carcinoma cells that participate in peritoneal dissemination of ovarian carcinoma.

MATERIALS AND METHODS

Materials

Type I collagen, heparin (from porcine intestinal mucosa), chondroitin sulfate A (from bovine trachea), chondroitin sulfate C (from shark cartilage), dermatan sulfate (from porcine intestinal mucosa), heparan sulfate (from bovine kidney), Sepharose CL-4B, Sepharose CL-6B, DPCC-treated trypsin (type XI), soybean trypsin inhibitor, methylumbelliferyl β-D-xyloside, and phosphatidylinositol-specific phospholipase were from Sigma. Type III collagen was from Chemicon. Cellular fibronectin (derived from human fibroblast cultures) was from Calbiochem. $H_2[^{35}S]O_4$ (5 mCi/ml) was from New England Nuclear. DEAE-Sephacel was from Pharmacia. Chondroitin (desulfated chondroitin sulfate A), heparitinase (containing mostly heparitinase I from Flavobacterium), and chondroitinase ABC were from Seikagaku America.

Cell Culture

SK-OV-3 and NIH:OVCAR-3 cells were purchased from American Type Culture Collection (ATCC) and maintained according to instructions supplied by ATCC.

Radiolabeling of Proteoglycans

Metabolic labeling was accomplished by adding 50 μCi/ml of carrier-free $H_2[^{35}S]O_4$ to nearly confluent cultures which were then incubated for 24 h. The conditioned medium was then removed, the cell layer was rinsed three times with ice-cold PBS, and cell surface proteoglycans were isolated as described below.

Isolation of Cell Surface Proteoglycans

The radiolabeled cell layer was rinsed with ice-cold PBS/0.02% EDTA, the cells were scraped into ice-cold PBS/EDTA, and washed in the same buffer. Total cells surface proteo[^{35}S]glycans were released by trypsinization of the cells with 20 μg/ml DPCC-treated trypsin for 10 min on ice [Rapraeger and Bernfield, 1985]. Phosphatidylinositol-anchored cell surface proteo[^{35}S]glycans were released by treatment of the scraped and rinsed cell with 0.4 U/ml of phosphatidylinositol-specific phospholipase (PI-PLC) in the presence of serum-free medium

TABLE I. Oligonucleotide Sequences for RT-PCR

Protein	Forward primer	Reverse primer	Amplicon size (bp)
GAPDA	5′-cgtattgggcgcctggtcaccag-3′	5′-ggaaggccatgccagtgagcttc-3′	663
Syndecan-1	5′-cctgaagatcaagatggctctgg-3′	5′-ctgtttggtgggcttctggtagg-3′	824
Syndecan-2	5′-cgcgtggatcctgctcaccttg-3′	5′-ttagtaggtgccttctgataagc-3′	581
Syndecan-4	5′-aggcggagtcgccgagtcgatcc-3′	5′-cgcgtagaactcattggtggccc-3′	552
CD44	5′-cctggatcaccgacagcacagaca-3′	5′-ttctgacgactccttgttcacca-3′	382*
Glypican-1	5′-cgccgagtggaggaacctcctgg-3′	5′-ctgggggcagctggcagccgagg-3′	747
Glypican-3	5′-tcccttgaagaacttgtgaatgg-3′	5′-gaatgaacgttcccgaggttgtg-3′	775
Glypican-5	5′-tccggtcgttggaagaactctcg-3′	5′-tcagtcgccactgcacatccagc-3′	718

*Amplicon size for CD44s splice variant.

for 30 min at 37°C [Yanagishita, 1992]. The digestion mixtures were centrifuged in a microfuge for 5 min, and the supernatants containing the released cell surface proteo[^{35}S]glycans were processed further. The cell surface proteo[^{35}S]glycans were partially purified by ion-exchange chromatography on DEAE-Sephacel column as previously described [Kokenyesi and Silbert, 1995], with the modification that the proteo[^{35}S]glycans were eluted with 4 M guanidine HCl.

Glycosamino[^{35}S]glycans were prepared by alkali elimination from the partially purified proteo[^{35}S]glycans. The eluate from the DEAE column was dialyzed against distilled water for overnight, NaOH was added (to a final concentration of 0.5 M) to the dialyzed proteo[^{35}S]-glycans and the alkali elimination was allowed to proceed for 16 h at room temperature.

Molecular Sieve Column Chromatography

For separation of proteoglycans with different hydrodynamic sizes the partially purified cell surface proteo[^{35}S]glycans were chromatographed on a Sepharose CL-4B column (1×47 cm) equilibrated and eluted with 0.05 M Tris/HCl buffer, pH 7.4, containing 4 M guanidine HCl. For hydrodynamic size estimation of glycosaminoglycans and degradation products the alkali-released glycosamino[^{35}S]glycans were chromatographed on a Sepharose CL-6B column (1×47 cm) eluted with 0.5 M ammonium bicarbonate. An aliquot of the fractions were counted in a scintillation counter, and fractions containing glycosamino[^{35}S]glycans were pooled and lyophilized. Void volume and total volume were determined by the elution positions of Blue Dextran and [^{3}H]glucosamine, respectively. The hydrodynamic size of glycosaminoglycans was estimated based on the calibration curve previously published by Wasteson [1971].

Enzymatic Degradations of Isolated Glycosaminoglycans

The lyophilized glycosamino[^{35}S]glycans were dissolved in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and 0.1% Triton X-100. Treatments with chondroitin ABC lyase were done using 0.005 U of the enzyme for 30 min at 37°C, as previously described [Kokenyesi and Silbert, 1995]. Treatments with heparitinase were done with 0.05 U of enzyme for 30 min at 42°C for treatment of glycosaminoglycan preparations.

RT-PCR

mRNA was isolated from the cultured cell using the Micro Poly(A)Pure kit (Ambion); first strand cDNA was synthesized using oligo-d(T) priming with the SuperscriptII Preamplification System (Gibco BRL). PCR reactions were carried out using Vent DNA polymerase (New England Biolabs) on 2% of the first strand reaction as template with the oligonucleotide primers shown in Table I. The same fist strand reaction mixture was used for all PCR reactions. The amplified DNA was analyzed on a 2% ethidium bromide-agarose gels.

Coating of Culture Plates With Matrix Molecules

96-well, flat-bottom PVC titer plates were coated with the matrix molecules using the following methods. Cellular fibronectin (50 μl of a 50 μg/ml solution in PBS) was dried onto the wells overnight at room temperature, and the wells were rinsed twice with PBS. Type I collagen (50 μl of a 1 mg/ml neutralized solution) was incubated in the wells overnight at 4°C as previously described [Sanderson et al., 1992], the collagen solution was removed and the wells were washed twice with PBS. Type III collagen was adsorbed onto wells pre-coated

with nitrocellulose. The pre-coating was done by adding 50 μl of nitrocellulose solution (0.3 cm^2 nitrocellulose membrane/ml in methanol) to the well and drying the well overnight. The type III collagen (50 μl of a 0.66 mg/ml neutralized solution) was incubated in the pre-coated well for 30 min at room temperature, and the wells were rinsed twice with PBS.

All substrate-coated wells were incubated with 10 mg/ml BSA in PBS for 30 min at room temperature to block uncoated PVC surfaces. The wells were rinsed twice with PBS before addition of cells. In some experiments the BSA-blocked wells were treated for 30 min with 100 μg/ml concentration of heparin or other competitive inhibitor (heparan sulfate, chondroitin sulfate A, dermatan sulfate, chondroitin sulfate C, chondroitin), then the wells were rinsed twice with PBS before adding the cells to the wells.

Cell Adhesion Assays

Cells were grown in T-25 flasks and used one day or three days after plating of SKOV-3 or NIH:OVCAR-3 cell, respectively. The cell layer was rinsed with ice-cold PBS, and the cells were scraped into PBS on ice. The cells were counted in a hemacytometer, and the cell suspension was diluted to obtain a cell suspension with 200,000 cells/ml. Fifty microliter of this cell suspension was added to each coated well, and incubated at room temperature for 30 min. The cell suspension was then removed from the well, the wells were rinsed twice with PBS, the cells remaining in the well were fixed with 5% glutaraldehyde for 30 min, and the cells were stained with cresyl violet (type I collagen and fibronectin) or with hematoxylin (type III collagen). The stained cells in the entire well were counted using a light microscope. Each adhesion assay was carried out in quadruplicate.

Xyloside Treatment of Cells

In some experiments the cells were incubated for 12 h in regular medium in the presence of 0.5 mM methylumbelliferyl β-D-xyloside to inhibit glycosaminoglycan biosynthesis. The controls were treated with equal volume of vehicle (dimethyl sulfoxide). At the end of the treatment the cells were processed as described for the regular adhesion assay.

Enzymatic Degradation of Cell Surface Proteoglycans on Intact Cells

The enzyme treatments followed in general the protocol described previously [Saunders and Bernfield, 1988]. The cells were scraped and counted as in a regular cell adhesion assay. The cell suspension was treated for 30 min at 37°C with heparitinase or chondroitinase ABC at 0.1 U/ml final concentration. The cells were chilled on ice before adding them to the matrix-coated wells. The controls for these adhesion assays were cells that were incubated at 37°C for 30 min, but not treated with enzyme.

RESULTS

Synthesis of Cell Surface Proteoglycans by Ovarian Carcinoma Cells

The carcinoma cells were incubated with [^{35}S]sulfate in order to metabolically label pro\teoglycans. The radiolabel incorporates into the glycosaminoglycan side chains of proteoglycans during the sulfation steps of their biosynthesis. The radiolabeled cell surface proteo[^{35}S]glycans of the SK-OV-3 and the NIH:OVCAR-3 cells were removed from the cells either by mild trypsin digestion or by treatment with PI-PLC. Digestion with trypsin removes both integral membrane proteoglycans and phosphatidylinositol-anchored proteoglycans, while treatment with PI-PLC removes only phosphatidylinositol-anchored proteolycans. The phosphatidylinositol-anchored proteo[^{35}S]glycans constituted ~5 and 1% of the total cell surface proteo[^{35}S]glycans from the SK-OV-3 and the NIH:OVCAR-3 cells, respectively.

Analysis of Proteoglycan Size

Trypsin-removable and PI-PLC-removable cell surface proteo[^{35}S]glycans were first partially purified by ion-exchange chromatography on DEAE-Sephacel, and then were chromatographed on a Sepharose CL-4B column to determine the size distribution. The trypsin-removable (total) proteo[^{35}S]glycans from the SK-OV-3 and the NIH:OVCAR-3 cells contained at least two proteoglycan populations with different hydrodynamic size (Fig. 1A). The largest proteo[^{35}S]glycans from both cells eluted in the void volume of the column. The smaller proteo[^{35}S]glycans of the SK-OV-3 cells were resolved into three overlapping peaks with

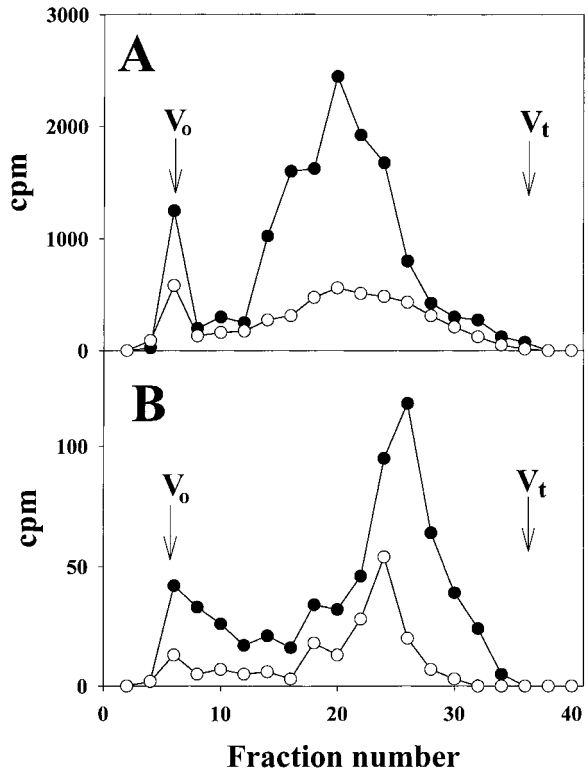

Fig. 1. Molecular sieve chromatography of cell surface proteo[^{35}S]glycans from ovarian carcinoma cells. Cell surface proteo[^{35}S]glycans were harvested either by trypsin treatment **(panel A)** or PI-PLC treatment **(panel B)** of the cells as described in the ''Materials and Methods''. SK-OV-3 cells (●); NIH:OVCAR-3 cells (○). The partially purified proteo[^{35}S]glycans were chromatographed on a Sepharose CL-4B molecular sieve column (1 × 47 cm) eluted with 0.05 M Tris/HCl buffer, pH 7.4, containing 4 M guanidine HCl. Vo, void volume; Vt, total volume.

a Kav of 0.35 and 0.45, and 0.55, respectively. The smaller proteo[^{35}S]glycans from the NIH:OVCAR-3 cells eluted as a broad peak with a Kav of 0.6. The PI-PLC-removable proteo[^{35}S]-glycans also contained several populations (Fig. 1B). The largest proteo[^{35}S]glycans from both cell lines eluted in the void volume. The smaller proteo[^{35}S]glycans of the SK-OV-3 cells eluted as a single broad peak at a Kav of 0.65 with a shoulder peak at Kav of 0.40. The smaller proteo[^{35}S]glycans of the NIH:OVCAR-3 cells eluted as a single peak at a Kav of 0.55 with a shoulder peak at a Kav of 0.4.

Analysis of Glycosaminoglycan Composition

An aliquot of the DEAE-purified trypsin-removable (total) and PI-PLC-removable proteo[^{35}S]glycans was degraded in alkali to liberate the glycosamino[^{35}S]glycan chains for further analysis on Sepharose CL-6B column. The proportion of heparan sulfate and chondroitin sulfate in the glycosamino[^{35}S]glycans was determined by enzymatic degradation of heparan sulfate with heparitinase, and chondroitin sulfate with chondroitinase ABC; the degradation products were detected by chromatography of the digestion mixture on a Sepharose CL-6B column. The degradation products (mostly disaccharides) from chondroitinase ABC treatment chromatographed at Kav of 0.9. However, the degradation products from heparitinase treatments chromatographed at variable Kav values. Heparitinase preferentially degrades the low-sulfated domains of heparan sulfate chain, therefore, the sulfate-labeled degradation products of heparitinase represent the heparitinase-resistant, highly-sulfated heparan sulfate domains. The length of these domains can vary between proteoglycan pools (phosphatidylinositol-anchored vs. integral membrane type), and cell lines.

Total cell surface glycosamino[^{35}S]glycans of the SK-OV-3 cells chromatographed as a single peak at a Kav of 0.45 with a shoulder at the approximate Kav of 0.55 (Fig. 2A). These Kav values correspond to the approximate hydrodynamic sizes of 25 and 17 kDa. Chondroitinase ABC treatment of the glycosamino[^{35}S]glycans resulted in a decrease in the magnitude of the original peak and in a formation of a new peak at Kav of 0.9 (fractions 28–34) corresponding to the degradation products. Treatment with heparitinase resulted in a shift in the position of the original peak and in the formation of a new peak (as a shoulder) at a Kav of 0.7 (fractions 24–30) corresponding to degradation products. The relative amounts of the degradation products indicate that the glycosamino[^{35}S]glycans of the total cell surface proteoglycans of the SK-OV-3 cells are composed of ~75% chondroitin sulfate and 25% heparan sulfate. Glycosamino[^{35}S]glycans of the NIH:OVCAR-3 cells chromatographed as a single peak at a Kav of 0.5 that corresponds to an approximate hydrodynamic size of 20 kDa (Fig. 2B). The relative amounts of the degradation products from heparitinase and chondroitinase ABC treatments indicate that the glycosamino[^{35}S]glycans of the total cell surface proteoglycans of the NIH:OVCAR-3 cells are composed of ~10% chondroitin sulfate and 90% heparan sulfate.

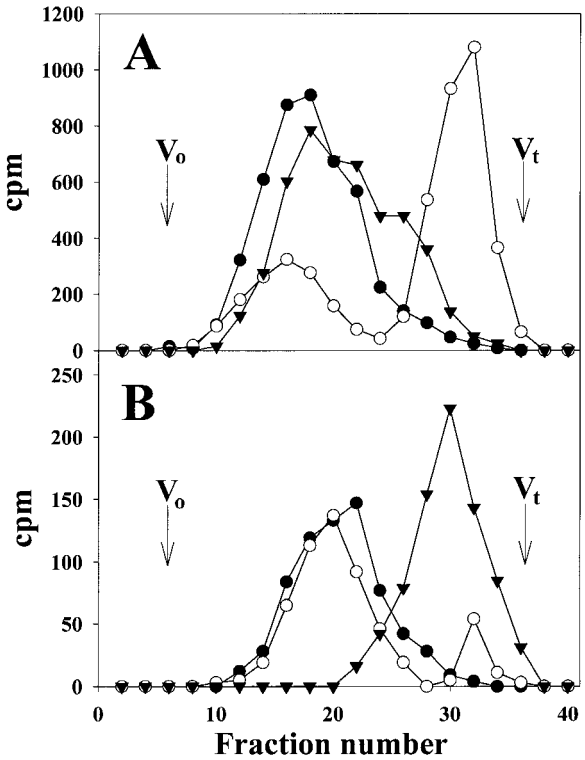

Fig. 2. Molecular sieve chromatography of glycosamino[^{35}S]-glycans derived from total cell surface proteoglycans of ovarian carcinoma cells. Glycosamino[^{35}S]glycans were liberated from trypsin-removable cell surface proteoglycans by alkali elimination as described in the ''Materials and Methods''. **Panel A**, SK-OV-3 cells; **Panel B**, NIH:OVCAR-3 cells. Untreated (●); chondroitinase ABC-treated (○); heparitinase-treated (▼). The samples were chromatographed on a Sepharose CL-6B molecular sieve column (1 × 47 cm) eluted with 0.5 M ammonium bicarbonate. Vo, void volume; Vt, total volume.

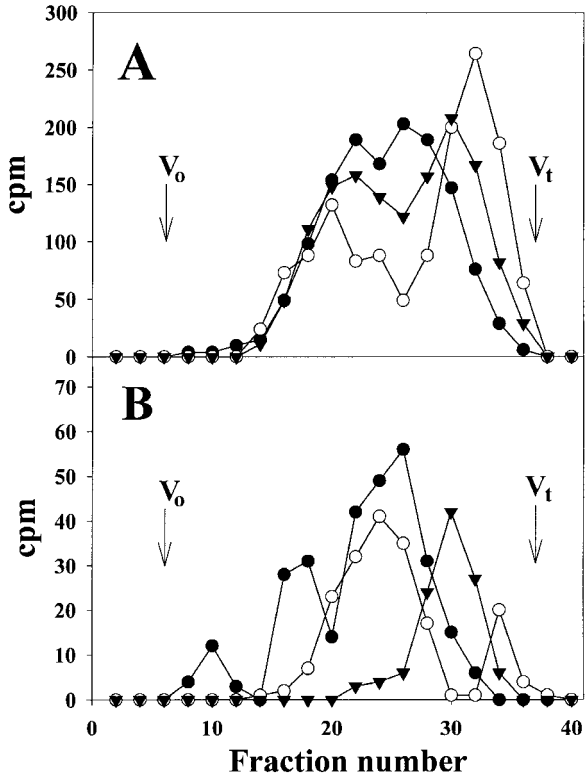

Fig. 3. Molecular sieve chromatography of glycosamino[^{35}S]-glycans derived from PI-PLC-removable cell surface proteoglycans of ovarian carcinoma cells. Glycosamino[^{35}S]glycans were liberated from PI-PLC-removable cell surface proteoglycans by alkali elimination as described in the ''Materials and Methods''. **Panel A**, SK-OV-3 cells; **Panel B**, NIH:OVCAR-3 cells. Untreated (●); chondroitinase ABC-treated (○); heparitinase-treated (▼). The samples were chromatographed on a Sepharose CL-6B molecular sieve column (1 × 47 cm) eluted with 0.5 M ammonium bicarbonate. Vo, void volume; Vt, total volume.

The PI-PLC-removable glycosamino[^{35}S]glycans of the SK-OV-3 cells chromatographed as two overlapping peaks at a Kav of 0.6 and 0.7 corresponding to 12 and 8 kDa hydrodynamic size, respectively (Fig. 3A). The relative amounts of the degradation products indicate that the PI-PLC-removable glycosamino[^{35}S]-glycans of the SK-OV-3 cells are composed of ~65% chondroitin sulfate and 35% heparan sulfate. The PI-PLC-removable glycosamino[^{35}S]glycans of the NIH:OVCAR-3 cells chromatographed as three peaks at a Kav of 0.1, 0.4, and 0.6 corresponding to 60, 30, and 12 kDa hydrodynamic size, respectively (Fig. 3B). The relative amounts of the degradation products indicate that the PI-PLC-removable glycosamino[^{35}S]glycans of the NIH:OVCAR-3 cells are composed of ~15% chondroitin sulfate and 85% heparan sulfate.

Expression of Cell Surface Proteoglycan Core Protein mRNA

RT-PCR analysis was performed with the intention to determine whether multiple cell surface proteoglycans are produced by the ovarian carcinoma cells. The analysis is not quantitative; however, it reflects the relative proportion of the proteoglycan mRNAs within a single cell line. RT-PCR on mRNA derived from SK-OV-3 cells resulted in strong signal for syndecan-1, -2, and -4, CD44 and glypican-1 (Fig. 4). No expression of glypican-3 and -5 were detected using the regular 30 cycles of amplification. Increasing the number of amplification

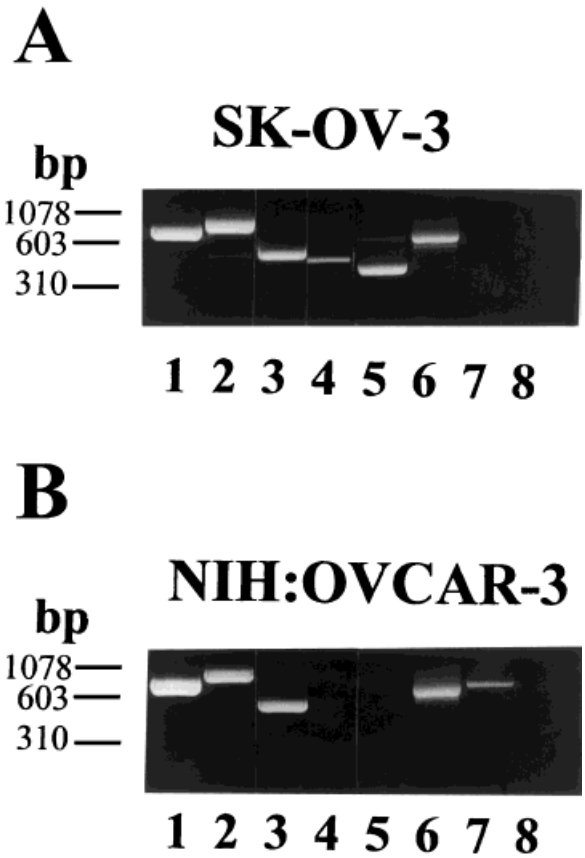

Fig. 4. RT-PCR analysis of cell surface proteoglycan core protein expression in ovarian carcinoma cells. **Panel A**: SK-OV-3 cells; **Panel B**: NIH:OVCAR-3 cells. **Lane 1**, glyceraldehyde-3-phosphate dehydrogenase; **lane 2**, syndecan-1; **lane 3**, syndecan-2; **lane 4**, syndecan-4; **lane 5**, CD44; **lane 6**, glypican-1; **lane 7**, glypican-3; **lane 8**, glypican-5. The analysis was carried out on reverse transcribed mRNA from the cell lines. The primer sequences were as indicated in Table I. For each cell lines the same first strand reaction was used for the PCR. The DNA size markers are indicated at the left.

cycles to 40 resulted in weak signal for glypican-3, but still no signal was detected for glypican-5. RT-PCR on mRNA derived from NIH:OVCAR-3 cells resulted in strong signal for syndecan-1, and -2, glypican-1, and glypican-3, while no signal was obtained for syndecan-4, CD44, and glypican-5 (Fig. 4). Increasing the number of amplification cycles to 40 resulted in a moderate signal for syndecan-4 and CD44, but still no signal was detected for glypican-5.

The sizes of all amplicons were similar to the expected size (Table I). The size of the major CD44 amplicon was 370 bp (as calculated from the agarose gel; Fig. 4, lane 5), while a minor amplicon was seen at 700 bp. Because a forward primer from exon 5 and a reverse primer from exon 19 was used the data show that the major CD44 form is the CD44s (standard form) containing exons 1–5, 15–17, and 19. The minor amplicon was not analyzed further.

Cell Adhesion to Type I and III Collagens and Fibronectin Via Cell Surface Proteoglycans

Types I and III collagen and cellular fibronectin are abundant components of the interstitial matrix of the peritoneum. Because metastasizing ovarian carcinoma cells adhere to the peritoneal matrix, and because cell surface proteoglycans can mediate cell-matrix adhesion we studied the involvement of cell surface proteoglycans in the cell-matrix adhesion of ovarian carcinoma cell lines. The glycosaminoglycan portion of the cell surface proteoglycans is the mediator of the adhesion event, so we utilized glycosaminoglycan-specific inhibitors in an attempt to disrupt cell-matrix adhesion. In one set of experiments the matrix was incubated with various glycosaminoglycans before cells were added to the assay. The blocking effect of glycosaminoglycans is derived from the binding of the soluble glycosaminoglycans to the specific binding sites on the collagen or fibronectin molecules. The bound glycosaminoglycans reduce the number of potential binding sites for cell surface proteoglycans. This competitive inhibition of the interaction of matrix with cell surface proteoglycans is expected to result in decreased cell adhesion mediated by cell surface.

Both the SK-OV-3 and the NIH:OVCAR-3 cells adhered to type I and III collagens and cellular fibronectin (Fig. 5). For both cell lines the largest number of cells adhered to types I and III collagen, while adhesion to cellular fibronectin was less efficient. Pre-incubation of adhesion substrates with heparin caused significant decrease in the number of adhering cells (35–60% inhibition at $P < 0.001$, or $P < 0.05$ for NIH-OVCAR-3 cells on fibronectin) indicating that cell surface proteoglycans may mediate the cell adhesion of ovarian carcinoma cells. Pre-incubation of the adhesion substrates with heparan sulfate, chondroitin sulfate A, dermatan sulfate, chondroitin sulfate C, or chondroitin caused a decrease in the number of adhering cells providing further evidence for the involvement of cell surface proteoglycans in cell adhesion (Table II). For both cell lines chondroitin sulfate A blocked best the adhesion to type I collagen, while chondroitin sulfate C

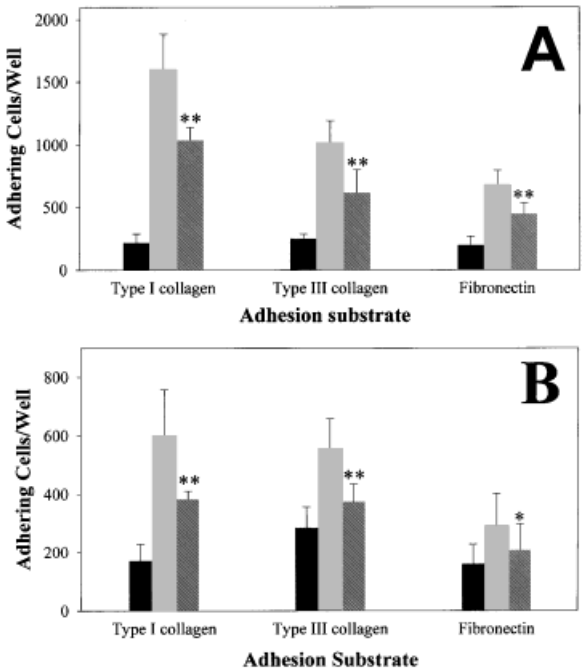

Fig. 5. Cell surface proteoglycan-dependent adhesion of SK-OV-3 cells to extracellular matrix. **Panel A**: SK-OV-3 cells; **Panel B**: NIH:OVCAR-3 cells. Adhesion to BSA control (black bar), extracellular matrix (gray bar), and extracellular matrix molecule coated with heparin (hatched bar) was measured on titer plates as described in the Materials and Methods section. The error bars indicate variation found in three independent quadruplicate measurements on each substrate. Significant inhibition by heparin treatment of the substrate is indicated: *($P < 0.05$); **($P < 0.001$).

was equal or better in blocking the adhesion to type III collagen. These data suggest that there are some specific structural features of glycosaminoglycan chains that are required to interact with types I and III collagen. The data also demonstrate that blocking activity is not necessarily related to the degree of sulfation, because chondroitin (no sulfation) and heparan sulfate (low-degree of sulfation) showed significant inhibition of cell adhesion. In these assays the cells do not adhere to the blocking glycosaminoglycans themselves, because cell adhesion to a glycosaminoglycan-coated BSA surface is not different from adhesion to BSA-coated surface, and there is virtually no cell adhesion to a glycosaminoglycan-coated plastic surface (data not shown).

In another set of experiments specific glycosaminoglycan-degrading enzymes were used to remove the glycosaminoglycan portion of cell surface proteoglycans before the adhesion assay. Thus, carcinoma cells were pre-treated with the glycosaminoglycan-degrading enzymes chondroitin ABC lyase or heparitinase in order to remove chondroitin sulfates or heparan sulfates from the cell surface. This enzymatic degradation of the cell surface proteoglycans resulted in a decreased cell adhesion to the collagens and fibronectin (Table III). These data confirm that cell surface chondroitin sulfate and heparan sulfate proteoglycans of SK-OV-3 and the NIH:OVCAR-3 cells are able to mediate adhesion of these cells to molecules of extracellular matrix. The adhesive interaction may take place via chondroitin sulfate or heparan sulfate chains of the cell surface proteoglycans.

The role of cell surface proteoglycans in cell adhesion was further confirmed by the effect of methylumbelliferyl β-D-xyloside. Treatment of cells prior with methylumbelliferyl β-D-xyloside (an inhibitor of glycosaminoglycan biosynthesis) prior to the adhesion assay significantly reduced cell adhesion (data not shown).

DISCUSSION

Our data from metabolic labeling experiments show that cell surface proteoglycans are indeed synthesized by the ovarian carcinoma

TABLE II. Inhibition of Cell Adhesion by Using Competitive Inhibitors to Saturate Glycosaminoglycan Binding Sites on Matrix Molecules

		Competitive inhibitors					
Cell line	Matrix	Heparin (%)	Heparan sulfate (%)	Chondroitin sulfate A (%)	Dermatan sulfate (%)	Chondroitin sulfate C (%)	Chondroitin (%)
SK-OV-3	Type I collagen	40	38	52	37	32	21
	Type III collagen	49	25	41	31	47	37
	Fibronectin	66	48	57	55	57	65
NIH:OVCAR-3	Type I collagen	48	25	61	32	31	29
	Type III collagen	57	44	39	37	47	37
	Fibronectin	46	29	37	27	39	35

TABLE III. Inhibition of Cell Adhesion by Enzymatic Degradation of Glycosaminoglycans of Cell Surface Proteoglycans

		Glycosaminoglycan-degrading enzymes	
Cell line	Matrix	Heparitinase (%)	Chondroitinase ABC (%)
SK-OV-3	Type I collagen	41	56
	Type III collagen	50	58
	Fibronectin	53	45
NIH: OVCAR-3	Type I collagen	64	62
	Type III collagen	45	32
	Fibronectin	55	30

cells. These proteoglycans are attached to the cell surface either by a transmembrane domain or by a phosphatidylinositol lipid anchor, as shown by the release of proteoglycans by treatment of the cells with trypsin or with PI-PLC, respectively. Analysis of the cells surface proteoglycans showed that proteoglycans of several sizes are present on the cell surface, and that glycosaminoglycans of trypsin-removable or PI-PLC-removable proteoglycans have various sizes. At present it is not possible to determine whether the different proteoglycan populations represent individual gene products, especially because the pool of trypsin-removable proteoglycans may contain some matrix-derived proteoglycans. The glycosaminoglycans of the trypsin-removable proteoglycans from the NIH:OVCAR-3 cells were smaller than glycosaminoglycans of the trypsin-removable proteoglycans from the SK-OV-3 cells. Glycosaminoglycan sizes of the PI-PLC-removable proteoglycans did not differ between the cell lines. Degradation of the glycosaminoglycans with specific enzymes showed that heparan sulfate and chondroitin sulfate chains are components of cell surface proteoglycans. Proteoglycans from the NIH:OVCAR-3 cells had a higher proportion of heparan sulfate, while proteoglycans from the SK-OV-3 cells had higher proportion of chondroitin sulfate. It is unclear whether overall proteoglycan expression or synthesis of specific glycosaminoglycans is the consequence of transformation of ovarian surface epithelium, because proteoglycans of normal ovarian surface epithelium have not been studied.

We have found mRNA of several cell surface proteoglycans expressed in the SK-OV-3 and NIH:OVCAR-3 cell lines. Our data about syndecan and glypican expression constitute novel information. Our data on CD44 expression confirmed findings by other investigators regarding the lack of CD44 on NIH:OVCAR-3 cells, and the presence of CD44 on SK-OV-3 cells [Cannistra et al., 1995]. These data indicate that the ovarian carcinoma cells may be able to use more than one type of cell surface proteoglycans for cell-matrix adhesion. However, we found that both heparin/heparan sulfate and chondroitin/dermatan sulfate are able to inhibit cell-matrix adhesion suggesting that a cell surface proteoglycan carrying both heparan sulfate and chondroitin sulfate chains may mediate cell adhesion. Syndecan-1 is the only cell surface proteoglycans that have been described to have both heparan sulfate and chondroitin sulfate chains. It remains to be determined whether syndecan-1 on ovarian carcinoma cells has both types of glycosaminoglycans.

Our data on cell adhesion show that cell surface proteoglycans partly mediate adhesion of ovarian carcinoma cells to type I and type III collagens, and fibronectin. The number of cells binding to each substrate suggests that the ovarian carcinoma cells bind preferentially to type I and type III collagens, while fibronectin is a less preferred substrate. Studies of integrin-based cell-matrix adhesion of ovarian carcinoma cells showed a similar preference for type I collagen vs. fibronectin. Our data is the first to show adhesion of ovarian carcinoma cells to type III collagen. This is an important finding, because type III collagen is an abundant component of the peritoneal matrix, and it is likely that the peritoneal collagen meshwork is composed of heterotypic type I/type III collagen fibrils, as have been shown in other organs [Fleischmajer et al., 1990]. Thus, the ovarian carcinoma cells are likely to have access to, and to utilize type III collagen for adhesion as much

as type I collagen and fibronectin. In addition, the carcinoma cells may use specific receptors (proteoglycan and non-proteoglycan receptors) specific for type III collagen, and, therefore, efforts to inhibit cell adhesion to prevent the spread of ovarian carcinoma must consider effective inhibitors of adhesion to type III collagen. The relative adherence to different extracellular matrix components shows that SK-OV-3 cells adhere most efficiently to type I collagen, significantly reduced adherence is seen on type III collagen and further significant decrease in adherence is seen on fibronectin. NIH:OVCAR-3 cells adhere with comparable efficiency to both collagen types, but significantly reduced adherence was seen on fibronectin. It has been reported that primary ovarian carcinoma cells prefer to bind type I collagen, and show significantly lower binding to fibronectin [Fishman et al., 1998]; our experiments demonstrated that cultured ovarian carcinoma cells retained the cell-matrix adhesion pattern characteristic of primary carcinoma cells. This suggests that cultured cell lines may serve as models for studying cell-matrix adhesion of ovarian carcinoma cells. However, it is unclear whether expression pattern of cell surface proteoglycans correlates with biological characteristics of the tumor. This question might be best answered by studying proteoglycans of primary ovarian carcinoma cells and the primary tumor tissue.

Pre-coating of the adhesion substrates with heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate A, chondroitin sulfate C, or chondroitin significantly inhibited adhesion of ovarian carcinoma cells. Removal of cell surface heparan sulfate or chondroitin sulfate by glycosaminoglycan-degrading enzymes also inhibited cell adhesion confirming the role of cell surface proteoglycans. Pre-treatment of the cells with methylumbelliferyl β-D-xyloside, an inhibitor of glycosaminoglycan biosynthesis, also caused a decrease in cell adhesion.

Our data show that both sulfated and unsulfated glycosaminoglycans are able to block cell adhesion suggesting that both sulfated and unsulfated moieties of the cell surface proteoglycans mediate the interaction with matrix molecules. We have not performed targeted experiments to establish a correlation between a particular heparan sulfate or chondroitin sulfate structure and the ability to bind to types I and III collagens and fibronectin. However, accumulating evidence suggest that specific carbohydrate sequences are present in both heparan sulfate [Lyon and Gallagher, 1998; Perrimon and Bernfield, 2000] and in chondroitin/dermatan sulfate [Maimone and Tollefsen, 1990; Lyon et al., 1998; Buffet et al., 1999] to perform a particular physiological function. A more sophisticated analysis of heparan sulfate and chondroitin sulfate from ovarian carcinoma cell surface is necessary to determine whether the structure or sequence of glycosaminoglycans is different in the transmembrane and phosphatidylinositol-anchored proteoglycans. Such analysis may provide a better understanding of the glycosaminoglycan structures needed for cell-matrix adhesion.

These data demonstrate for the first time that the cultured ovarian carcinoma cell lines can utilize cell surface proteoglycans for cell-matrix adhesion. These findings add to the growing number of adhesion molecules detected on ovarian carcinoma cells. Integrins [Moser et al., 1996; Buczek-Thomas et al., 1998; Strobel and Cannistra, 1999], CD44 as the hyaluronan receptor [Cannistra et al., 1995], and cell surface lectins [Woynarowska et al., 1994; Kiguchi et al., 1998] have been implicated in mediating adhesion of ovarian carcinoma cells to extracellular matrix. The expression of multiple cell adhesion molecules on ovarian carcinoma cells suggests that the carcinoma cells can utilize several molecular mechanisms to adhere to the complex extracellular matrix of the peritoneum. Our data are similar to findings from studies on integrins, CD44, and cell surface lectins in that blocking the function of only one type of adhesion molecule does not result in complete block of cell adhesion. Therapeutic efforts to prevent metastatic spread of ovarian carcinoma will be successful only if they employ a strategy that interferes with multiple adhesion mechanisms.

Proteoglycans on the cell surface may fulfill a variety of functions by interacting with soluble ligands as well, in addition to the insoluble ligands in the extracellular matrix. On non-ovarian cell types the cell surface proteoglycans have been implicated in affecting signaling by acidic fibroblast growth factor [Brickman et al., 1998], basic fibroblast growth factor [Rapraeger et al., 1991], hepatocyte growth factor [Lyon and Gallagher, 1994], platelet-derived growth factor [Feyzi et al., 1997], vascular endothelial growth factor [Neufeld et al., 1999], and heparin-

binding epidermal growth factor [Higashiyama et al., 1993]. It will require futher investigations to find out whether cell surface proteoglycans mediate action of any of the growth factors on normal ovarian epithelium, or on ovarian carcinoma cells.

In summary, our description of cell surface proteoglycans on two cultured ovarian carcinoma cell lines provides new information regarding the molecular mechanisms of cell-matrix adhesion. It will be necessary to investigate the in vivo expression and function of cell surface proteoglycans to determine whether the cell surface proteoglycans contribute to metastatic potential or growth characteristics of ovarian carcinomas.

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REFERENCES

Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. 1998. The biology of ovarian cancer. Semin Oncol 25:281–304.

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ. 1992. Biology of syndecans: a family of transmembrane heparan sulfate proteoglycans. Annu Rev Cell Biol 8:365–393.

Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald MF, Lincecum J, Zako M. 1999. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 68: 729–777.

Brickman YG, Ford MD, Gallagher JT, Nurcombe V, Bartlett PF, Turnbull JE. 1998. Structural modification of fibroblast growth factor-binding heparan sulfate at a determinative stage of neural development. J Biol Chem 273:4350–4359.

Buczek-Thomas JA, Chen N, Hasan T. 1998. Integrin-mediated adhesion and signalling in ovarian cancer cells. Cell Signal 10:55–63.

Buffet PA, Gamain B, Scheidig C, Baruch D, Smith JD, HernezRivas R, Pouvelle B, Oishi S, Fujii N, Fusai T, Parzy D, Miller LH, Gysin J, Scherf A. 1999. Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. Proc Natl Acad Sci USA 96:12743–12748.

Cannistra SA. 1993. Cancer of the ovary. N Engl J Med 329:1550–1559.

Cannistra SA, DeFranzo B, Niloff J, Ottensmeier C. 1995. Functional heterogeneity of CD44 molecules in ovarian cancer cell lines. Clin Cancer Res 1:333–342.

Cardin AD, Weintraub HJR. 1989. Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis 9:21–32.

David G. 1993. Integral membrane heparan sulfate proteoglycans. FASEB J 7:1023–1030.

Drake SL, Klein DJ, Mickelson DJ, Oegema TR, Furcht LT, McCarthy JB. 1992. Cell surface phosphatidylinositol-anchored heparan sulfate proteoglycans initiates mouse melanoma cell adhesion to fibronectin-derived heparin-binding synthetic peptide. J Cell Biol 117:1331–1341.

Feyzi E, Lustig F, Fager G, Spillman D, Lindahl U, Salmivirta M. 1997. Characterization of heparin heparan sulfate domains binding to the long splice variant of platelet-derived growth factor A chain. J Biol Chem 272:5518–5524.

Fishman DA, Kearns A, Chilukuri K, Bafetti LM, O'Toole EA, Georgacopoulos J, Ravosa MJ, Stack MS. 1998. Metastatic dissemination of human ovarian epithelial carcinoma is promoted by $\alpha 2\beta 1$-integrin-mediated interaction with type I collagen. Invasion Metastasis 18:15–26.

Fleischmajer R, Perlish JS, Burgeson RE, Shaikh-Bahai F, Timpl R. 1990. Type I type III collagen interactions during fibrillogenesis. Ann NY Acad Sci 580:161–175.

Harvey W, Amlot PL. 1983. Collagen production by human mesothelial cells in vitro. J Pathol 139:337–347.

Higashiyama S, Abraham JA, Klagsbrun M. 1993. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. J Cell Biol 12:933–940.

Jackson DG, Bell JI, Dickinson R, Timans J, Shields J, White N. 1995. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. J Cell Biol 128:673–685.

Kiguchi K, Iwamori M, Mochizuki Y, Kishikawa T, Tsukazaki K, Saga M, Amemiya A, Nozawa S. 1998. Selection of ovarian carcinoma cells with high dissemination potential by repeated passage of the cells in vivo into nude mice involvement of Le^x-determinant in the dissemination potential. Jpn Cancer Res 89:923–932.

Koda JE, Rapraeger A, Bernfield M. 1985. Heparan sulfate proteoglycans from mouse mammary epithelial cells. Cell surface proteoglycan as a receptor for interstitial collagens. J Biol Chem 260:8157–8162.

Kokenyesi R, Silbert JE. 1995. Formation of heparan sulfate chondroitin/dermatan sulfate on recombinant domain I of mouse perlecan expressed in Chinese hamster ovary cells. Biochem Biophys Res Commun 211:262–267.

Lyon M, Gallagher JT. 1994. Hepatocyte growth factor/scatter factor: a heparan sulfate-binding pleiotrophic growth factor. Biochem Soc Trans 22:365–370.

Lyon M, Gallagher JT. 1998. Bio-specific sequences domains in heparan sulphate the regulation of cell growth adhesion. Matrix Biol 17:485–493.

Lyon M, Deakin JA, Rahmoune H, Fernig DG, Nakamura T, Gallagher JT. 1998. Hepatocyte growth factor scatter factor binds with high affinity to dermatan sulfate. J Biol Chem 273:271–278.

Maimone MM, Tollefsen DM. 1990. Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. J Biol Chem 265:18263–18271.

Moser T, Pizzo SV, Bafetti LM, Fishman DA, Stack MS. 1996. Evidence for preferential adhesion of ovarian epithelial carcinoma cells to type I collagen mediated by the $\alpha 2\beta 1$ integrin. Int J Cancer 67:695–701.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. 1999. Vascular endothelial growth factor VEGF its receptors. FASEB J 13:9–22.

Perrimon N, Bernfield M. 2000. Specificities of heparan sulphate proteoglycans in developmental processes. Nature 404:725–728.

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

Rapraeger AC, Krufka A, Olwin BB. 1991. Requirement of heparan sulfate for bFGF mediated fibroblast growth myoblast differentiation. Science 252:1705–1708.

San Antonio JD, Lander AD, Karnovsky MJ, Slayter HS. 1994. Mapping the heparin-binding sites on type I collagen monomers and fibrils. J Cell Biol 125:1179–1188.

Sanderson RD, Sneed TB, Young LA, Sullivan GL, Ler AD. 1992. Adhesion of B lymphoid MPC-11 cells to type I collagen is mediated by the integral membrane proteoglycan syndecan. J Immunol 148:3902–3911.

Saunders S, Bernfield M. 1988. Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin behaves as a receptor for interstitial matrix. J Cell Biol 106:423–430.

Schroeder W, Rudlowski C, Biesterfeld S, Knobloch C, Hauptmann S, Rath W. 1999. Expression of CD44v5–10 splicing variants in primary ovarian cancer lymph node metastases. Anticancer Res 19:3901–3906.

Silbert JE, Bernfield M, Kokenyesi R. 1997. Proteoglycans: a special class of glycoproteins. In: Montreuil J, Vliegenthart JFG, Schachter H, editors. Glycoproteins II. Amsterdam: Elsevier. pp 1–31.

Strobel T, Cannistra SA. 1999. β1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. Gynecol Oncol 73:362–367.

Stylianou E, Jenner LA, Davies M, Coles GA, Williams JD. 1990. Isolation culture characterization of human peritoneal mesothelial cells. Kidney Int 37:1563–1570.

Sweeney SM, Guy CA, Fields GB, San Antonio JD. 1998. Defining the domains of type I collagen involved in heparin-binding and endothelial tube formation. Proc Natl Acad Sci USA 95:7275–7280.

Wasteson A. 1971. A method for the determination of the molecular-weight distribution of chondroitin sulphate. J Chromatogr 59:87–97.

Woynarowska B, Skrincosky DM, Haag A, Sharma M, Matta K, Bernacki RJ. 1994. Inhibition of lectin-mediated ovarian tumor cell adhesion by sugar analogs. J Biol Chem 269:22797–22803.

Yanagishita M. 1992. Glycosylphosphatidylinositol-anchored core protein-intercalated heparan sulfate proteoglycans in rat ovarian granulosa cells have distinct secretory endocytotic intracellular degradative pathways. J Biol Chem 267:9505–9511.