Differential Expression of Cell Surface Heparan Sulfate Proteoglycans in Human Mammary Epithelial Cells and Lung Fibroblasts*

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Treating the liposome-intercalatable heparan sulfate proteoglycans from human lung fibroblasts and mammary epithelial cells with heparitinase and chondroitinase ABC revealed different core protein patterns in the two cell types. Lung fibroblasts expressed heparan sulfate proteoglycans with core proteins of ~35, 48, 90 (fibroglycan), 64 (glypican), and 125 kDa and traces of a hybrid proteoglycan which carried both heparan sulfate and chondroitin sulfate chains. The mammary epithelial cells, in contrast, expressed large amounts of a hybrid proteoglycan and heparan sulfate proteoglycans with core proteins of ~35 and 64 kDa, but the fibroglycan and 125-kDa cores were not detectable in these cells. Phosphatidylinositol-specific phospholipase C; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PGs, proteoglycans; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; C, core protein; PI, phosphatidylinositol; PLC, phospholipase C; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

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The abbreviations used are: PGs, proteoglycans; HS, heparan sulfate; CS, chondroitin sulfate; PI, phosphatidylinositol; PLC, phospholipase C; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

To further investigate this possibility we have characterized the hydrophobic cell surface PGs from human mammary epithelial cells and attempted to relate these to the cell-surface PGs from human mammary epithelial cells with heparitinase and chondroitinase ABC revealed different core protein patterns in the two cell types. Lung fibroblasts expressed heparan sulfate proteoglycans with core proteins of ~35, 48, 90 (fibroglycan), 64 (glypican), and 125 kDa and traces of a hybrid proteoglycan which carried both heparan sulfate and chondroitin sulfate chains. The mammary epithelial cells, in contrast, expressed large amounts of a hybrid proteoglycan and heparan sulfate proteoglycans with core proteins of ~35 and 64 kDa, but the fibroglycan and 125-kDa cores were not detectable in these cells. Phosphatidylinositol-specific phospholipase C; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PGs, proteoglycans; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; C, core protein; PI, phosphatidylinositol; PLC, phospholipase C; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s).

To further investigate this possibility we have characterized the hydrophobic cell surface PGs from human mammary epithelial cells and attempted to relate these to the cell-surface PGs from human mammary epithelial cells and mouse mammary epithelial cells. It appeared that the human mammary epithelial cells and mouse mammary epithelial cells two major core proteins were identified, i.e., that of syndecan and the 38-kDa core protein of a distinct HSPG (David and Van den Berghe, 1989). Human fibroblasts, in contrast, expressed at least four major structurally distinct HSPGs (Lories et al., 1989) and two CSPGs (David et al., 1989). Two of these fibroblast HSPGs, fibroglycan (Marynen et al., 1989) and glypican (David et al., 1990) have been cloned and shown to be distinct from syndecan. These data imply that different cell types or different situations may engage different proteoglycans.

Materials and Methods

Cell Culture—The human fetal lung fibroblasts, and the human (HBL100) and mouse (NMuMG) mammary gland epithelial cell lines...
were cultured on plastic substrata in a (1:1) mixture of Ham's F-12 and Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. For metabolic labeling of the proteoglycans confluent monolayers were incubated with 5 μCi/ml carrier-free H135SO4, during 24-48 h (Lories et al., 1986).

**Isolation and Analysis of the Hydophobic Cell Surface Proteoglycans**—Only confluent cultures were used to avoid variations in proteoglycan expression due to different growth states of the cells. The cultures were rinsed with phosphate-buffered saline and extracted with ice-cold Triton X-100 buffer. Triton X-100 buffer contained 0.5% (v/v) Triton X-100, 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and several protease inhibitors as described previously (Lories et al., 1986). The extracts were cleared by centrifugation (10,000 x g, 60 min) and concentrated by adsorption on DEAE-Sepharose Fast Flow. The PG fractions were isolated from the DEAE eluates by ion-exchange chromatography on Mono Q. For experiments which included autoradiography the isolated PGs were adsorbed on DEAE NaCl, and several protease inhibitors as described previously (Lories et al., 1987). Further fractionations by SDS-polyacrylamide gel electrophoresis were as described before (Lories et al., 1989).

**Gel Electrophoresis and Western Blotting**—Electrophoresis was performed in SDS-polyacrylamide gradient (4-16% T; 2.5% or 10% C) gels. Running conditions, sample preparation, and autoradiography were as described previously (Lories et al., 1988). For immunostaining, the electrophoresed samples were immunoblotted (0.5 A, 3-4 h) to Hybond-N membranes. Immunoblotting using the appropriate antibodies was as described before (Lories et al., 1989). Apparent molecular weights were determined by comparison to prestained molecular weight markers (GIBCO BRL, Ghent, Belgium).

**Enzyme Treatments**—Before electrophoresis the PG samples were digested with 0.5 million units of heparitinase, or with 25 million units of chondroitinase ABC, or with both enzymes, in 50 mM NaCl, 1 mM CaCl2, 0.1% Triton X-100, 50 mM 6-amino-hexanoic acid, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml pepstatin A, and 20 μg/ml leupeptin. Both enzymes were obtained from Seikagaku Kogyo (Tokyo, Japan). The treatment with phosphatidylinositol-specific phospholipase C (Boehringer Mannheim, BRD) was as reported before (David et al., 1990).

**Isolation and Sequencing of Human Syndecan cDNA**—Sequences coding for human syndecan were amplified from cDNA obtained in the first strand synthesis reaction during the construction of a human lung fibroblast cDNA library (David et al., 1990). The reaction mixture contained 2 units of Taq polymerase, 1 ng of cDNA, and 140 pmol of the sense and antisense primers. These were 42-mers identical, respectively, to the murine syndecan cDNA sequence (from residue 240 to 281) and to the complement of this sequence (from residue 1131 to 1172), as reported by Saunders et al. (1990). After 40 thermal cycles (1 min of denaturation at 94°C, 1 min of annealing at 55°C, 1 min of extension at 68°C), the amplification products were analyzed in 1% agarose gels and detected by ethidium bromide staining. The ~900-base pair amplification product was electrophoretically separated, ethanol precipitated, phosphorylated, and ligated in the Smal restriction site of pGEM-3Z (Promega Corporation, Madison, WI). The termini of five independent clones and, after subcloning, both strands of the PsiI fragments of three of these clones were sequenced by the dideoxy chain termination method (Sanger et al., 1977), using sequencing primers (Promega Biotechnology, Uppsala, Sweden), T7 and SP6 primers, and both dGTP and C7-deaza dGTP.

**Construction of the Expression Plasmids and Identification of the Recombinant Protein**—For expression as a recombinant β-galactosidase-syndecan fusion protein, one of the fully sequenced syndecan cDNA clones obtained by polymerase chain reaction (HUSYN-2) was linearized with EcoRI and blunt-link with Klenow enzyme. The insert and flanking sequences were liberated from the vector by cleavage with SalI and ligated into a Smal and SalI-restricted pE3 expression vector (Genofit, Geneva, Switzerland). In this construct (HUSYN-PEx3) the human syndecan cDNA sequence is inserted, in frame, into β-galactosidase-coding sequences through the intermediate of a short open reading frame derived from the multiple cloning site of the pGEM-3Z vector. Transformed POP 2136 cells were selected at 28°C on ampicillin plates. Exponentially growing cultures were initiated from single colonies and induced by shifting the incubation temperature from 28 to 42°C. After treatment with lysozyme, the cells were solubilized in hot SDS and the extracts analyzed by polyacrylamide gel electrophoresis. Western blotting and immunostaining with the anti-HSPG monoclonal antibodies 281-2, 2E9, and 1C7, and with a monoclonal anti-β-galactosidase antibody (Promega Corporation) were as reported before (Lories et al., 1988).

**Isolation and Analysis of RNA**—The isolation of polyA RNA from confluent human lung fibroblasts and from confluent human mammary epithelial cells was as described before (Marynen et al., 1988). Total RNA samples from cells at different stages of confluence were prepared by the acid guanidinium thiocyanate phenol-chloroform extraction protocol (Chomczynski and Sacchi, 1987). For Northern analysis, aliquots of 3 μg of polyA+ RNA or 10 μg of total RNA/lane were separated in formaldehyde-containing 1.2% agarose gels. The RNA was transferred to Nytran membranes and cross-linked to the membrane by exposure to UV light. The blots were prehybridized (4 h at 42°C) in 50% formamide, 0.1% SDS, 5× SSPE, 5× Denhardt’s, 100 μg/ml denatured salmon sperm DNA, and 100 μg/ml heparin. Hybridization (16 h at 42°C) was in the same buffer containing 4.106 cpn °P-oligolabeled cDNA/ml. The filters were washed in 2× SSPE, 0.1% SDS at room temperature (2 × 15 min) and at 42°C (1 × 30 min), and in 0.1× SSPE, 0.1 SDS at 55°C (1 × 20”) and 65°C (1 × 25”). Methylene blue-stained RNA markers (GIBCO BRL) and 18 S and 28 S ribosomal RNA were used to determine the molecular size of the hybridizing RNA species.

**RESULTS**

**Distinctive Cell Surface Proteoglycan Patterns**—The hydophobic cell surface PGs synthesized by the human mammary epithelial cells were purified from detergent extracts of the cells by ion-exchange chromatography and liposome incorporation and were radiiodinated to analyze their protein cores. During gel electrophoresis these °P-labeled hydophobic proteoglycans run as a high molecular mass (>200 kDa) smear (Fig. 1, lane 1). After treatment with heparitinase, two major core proteins of ~35 and 64 kDa and a residual smear (200-90 kDa) were obtained (Fig. 1, lane 2). This smear was converted into an 88-kDa protein band when the PG fraction was digested with both chondroitinase ABC and heparitinase (Fig. 1, lane 3). Besides this 88-kDa core the combined digestion yielded two additional proteins of 115 and 130 kDa. These were derived from chondroitin sulfate proteoglycan as they were also observed after chondroitinase digestion only (Fig. 1, lane 4). Reduction of the disulfide bonds did not change the electrophoretic behavior of the °I-bands (results not shown). Thus, cultured human mammary epithelial cells synthesized multiple types of cell surface PG, expressing core proteins of ~35 and 64 kDa which carried only HS chains, core proteins of 115 and 130 kDa which carried only CS chains, and core proteins of ~40 and 65 kDa which carried both HS and CS chains.

**Fig. 1. Electrophoresis of unfraccionated °P-labeled hydophobic proteoglycans.** The liposome-intercalatable heparan sulfate proteoglycans isolated from HBL-100 cells (E) and from fetal human lung fibroblasts (F) were iodinated, reduced, and analyzed by gel electrophoresis in SDS-polyacrylamide (4-16%) gradient gels without enzyme digestion (lanes 1 and 5), after heparitinase (Hase) digestion (lanes 2 and 6), after combined heparitinase and chondroitin ABC digestion (lanes 3 and 7), and after chondroitinase ABC digestion (lanes 4 and 8).
chains, and an 88-kDa core protein which carried both HS and CS glycosaminoglycan chains. Similar analyses on radiiodinated cell surface heparan sulfate proteoglycans from human lung fibroblasts (Fig. 1, lanes 5–8) yielded several core protein bands as reported before (Lories et al., 1987, 1989). These included prominent bands of ~125 and 48 kDa, not seen in the detergent extracts of the epithelial cells. These results were suggestive for differential expression of proteoglycan in epithelial and fibroblastic cells.

**Immunological Relationships between Human Epithelial and Fibroblast Proteoglycans**—A panel of mAbs raised against the cell surface HSPGs of human lung fibroblasts (Lories et al., 1989) was used to investigate possible relationships between the PGs from fibroblastic and epithelial cells. From initial immunodot blot assays it appeared that the core protein epitopes recognized by mAb 2E9 and by mAb S1 (anti-glypican) were detectable in the detergent extracts of the human epithelial cells, but that the epitopes of mAb 6G12 (anti-fibroglycan) and mAb 1C7 were absent (not shown).

The antibodies were then used to further immunopurify the radioiodinated epithelial and fibroblastic PGs prior to electrophoresis and autoradiography. The epithelial PGs recognized by mAb 2E9 migrated as a smear >88 kDa after heparitinase digestion (Fig. 2, lane 5) and as an ~88-kDa band after combined heparitinase and chondroitinase ABC digestion (Fig. 2, lane 6). In contrast, the 125-kDa PGs of fibroblasts which bound to mAb 2E9 yielded both a 125-kDa core protein and a smear >82 kDa after heparitinase digestion (Fig. 2, lane 3). This smear was converted into a sharp band after combined heparitinase and chondroitinase digestion (Fig. 2, lane 4), indicating (more clearly than in experiments with whole proteoglycan extracts as shown in Fig. 1) that fibroblasts also synthesized a HS/CS hybrid PG. Although both hybrids shared the mAb 2E9 epitope, the core protein of the fibroblast hybrid PG appeared to be somewhat smaller in size than the core protein of the epithelial hybrid PG (82 versus 88 kDa). Interestingly, mAb 1C7, which did not react with extracts from epithelial cells, bound only the "125 K"-HSPG from fibroblast PG extracts (Fig. 2, lanes 1 and 2) suggesting that the 125 kDa and the ~82-kDa cores contained structurally distinct peptides.

The mAb S1-reactive proteoglycans from epithelial cells and fibroblasts, finally, resembled each other very closely. They migrated as 64-kDa core proteins after heparitinase digestion (Fig. 2, lanes 7–20). Proteinase K digestion (Fig. 2, lane 7) nearly completely abolished the ability of the mAb S1-reactive epithelial proteoglycans to intercalate into liposomes, causing them to elute in the included volume of a Sepharose CL4B column (Fig. 3A). Control preparations of S1-reactive proteoglycans which had not been treated with PI-PLC, coeluted with the liposomes, mostly in the excluded volume of the column (Fig. 3A). The epithelial proteoglycans which had not been retained on the S1 column, in contrast, coeluted with the liposomes, whether treated with PI-PLC or not (Fig. 3B).

**Glypican Proteoglycans**—To test whether, like glypicans in fibroblasts (David et al., 1990), some epithelial proteoglycans might be membrane-anchored through a glycosyl phosphatidylinositol moiety, the epithelial proteoglycans were treated with PI-specific phospholipase C and tested for their residual hydrophobic properties (Fig. 3). PI-PLC nearly completely abolished the ability of the mAb S1-reactive epithelial proteoglycans to intercalate into liposomes, causing them to elute in the included volume of a Sepharose CL4B column (Fig. 3A). Control preparations of S1-reactive proteoglycans which had not been treated with PI-PLC, coeluted with the liposomes, mostly in the excluded volume of the column (Fig. 3A). The epithelial proteoglycans which had not been retained on the S1 column, in contrast, coeluted with the liposomes, whether treated with PI-PLC or not (Fig. 3B).

**Relationships between the Human Hybrid Proteoglycans and Syndecan**—To test whether the human hybrid PGs might be related to syndecan (Saunders et al., 1989), Western blots of partially purified cell surface PGs from mouse mammary epithelial cells were assayed with mAb 3G10, which reacts with ~4-uronate generated by heparitinase and therefore stains the heparitinase-generated core of any HSPG (Lories et al., 1989); with mAb 2E9, the anti-human HSPG mAb, and with mAb 281-2, an anti-mouse syndecan mAb (Jalkanen et al., 1985). mAb 3G10 revealed multiple "HS stub"-carrying core proteins, among which an ~84-kDa core protein which ran as a smear after heparitinase digestion (Fig. 4, lane 2) but as a sharp band after combined heparitinase and chondroitinase digestion (Fig. 4, lane 3). The two anti-core antibodies mAb 281-2 and mAb 2E9 reacted only with the ~84-kDa core protein of the hybrid proteoglycan (Fig. 4, lanes 7 and 9), indicating that syndecan and the human hybrid proteoglycans shared the mAb 2E9 epitope.

The presence of a syndecan homologue in human fibroblasts was confirmed by amplifying the corresponding cDNA from human lung fibroblasts using the polymerase chain

![FIG. 2. Electrophoresis of immunopurified 125I-labeled hydrophobic proteoglycans.](image-url)

| Designation | Core size | Glycan side chains | Expression | Cross-reacting antibodies |
|-------------|-----------|--------------------|------------|--------------------------|
| Fibroglycan  | 35        | HS                 | NA         | 6G12                     |
| Glypican     | 48        | HS                 | F          | S1                       |
| Syndecan     | 64        | HS, CS             | F, E       | 2E9                      |
|              | 82        | HS                 | F, E, M    | 2E9, 1C7                 |

*Approximate apparent sizes (in kDa) of the major cell surface proteoglycan cores in SDS-polyacrylamide gel electrophoresis.

Expression detected, by antibody or cDNA probes, in human lung fibroblasts (F), in human mammary epithelial cells (E), and in mouse mammary epithelial cells (M). The latter was tested for syndecan only. NA, non-applicable.
The hydrophobic proteoglycans were isolated from the phobic proteoglycans. Proteoglycan fractions eluted in the included volume of the column if and fell through fractions after treatment with enzyme buffer only (○). Association with the liposomes was tested by fractionating the proteoglycan-liposome mixture over Sepharose-CL4B in 4 M guanidinium chloride buffer. All proteoglycan fractions eluted in the included volume of the column if detergent was added to the samples and elution buffer (not shown).

Cell Surface Heparan Sulfate Proteoglycans

Fig. 3. Gel filtration of PI-PLC-treated 125I-labeled hydrophobic proteoglycans. The 125I-labeled proteoglycans from HBL-100 cells were fractionated by immunomicrotography on mAb S1. The hydrophobic proteoglycans were isolated from the S1 eluate (A) and fall through fractions (B) by liposome incorporation, and retested for their ability to intercalate into phospholipid vesicles after treatment with phosphatidylinositol-specific phospholipase C (○), or after treatment with enzyme buffer only (○). Association with the liposomes was tested by fractionating the proteoglycan-liposome mixture over Sepharose-CL4B in 4 M guanidinium chloride buffer. All proteoglycan fractions eluted in the included volume of the column if detergent was added to the samples and elution buffer (not shown).

Fig. 4. Cross-reactivity of murine syndecan mAb 2E9. The cell-associated proteoglycans of NMuMG cells were left untreated (lanes 1 and 5) or were digested with heparitinase only (lanes 2 and 6), with both heparitinase and chondroitinase ABC (lanes 3, 7, and 9), or with chondroitinase ABC only (lanes 4 and 8) before electrophoresis and transfer to nylon membranes. The Western blots were stained with mAb S1 before electrotransferred to nylon membranes. The Western blots were stained with mAb 2E9 (lanes 1–4), mAb 2E9 (lanes 5–8), and mAb 281-2 (lane 9).

reaction technique and primers derived from the murine syndecan cDNA sequence (Fig. 5). The amplified human fibroblast cDNA sequences coded for a protein which was 75% homologous to the mouse syndecan core protein (Saunders et al., 1989) and identical, except for one amino acid, to the protein sequence predicted by human syndecan cDNA clones isolated from a HBL-100 library with the aid of the murine syndecan probe (Mali et al., 1990).

Consistently, supernatants of lysed POP2136 cells which had been transfected with pEX3 plasmids containing a human fibroblast syndecan cDNA insert and induced by heat shock contained the mAb 2E9 epitope. Western blotting indicated that the 2E9 epitope occurred in an ~180-kDa protein (Fig. 6, lane 1) which was also traced by an anti-β-galactosidase antibody (lanes 1 and 2), with mAb 2E9 (lanes 3 and 4), and with mAb 1C7 (lanes 5 and 6).

Northern Blot Analysis—To study the expression of the proteoglycans. The hydrophobic proteoglycans were isolated from the phobic proteoglycans. Proteoglycan fractions eluted in the included volume of the column if and fell through fractions after treatment with enzyme buffer only (○). Association with the liposomes was tested by fractionating the proteoglycan-liposome mixture over Sepharose-CL4B in 4 M guanidinium chloride buffer. All proteoglycan fractions eluted in the included volume of the column if detergent was added to the samples and elution buffer (not shown).

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were hybridized with 32P-oligolabeled 48K3, a fibroglycan-specific cDNA (Marynen et al., 1989), with 64K3, a glypican-specific cDNA (David et al., 1990), and with HUSYN-2, one of the human syndecan-specific cDNAs, and submitted to autoradiography (Fig. 7). Mammary epithelial cells did not contain a mRNA which hybridized with the 48K3 probe, whereas in human lung fibroblasts a major ~2.3 kb and a minor ~3.5-kb mRNA were recognized (Fig. 7, lanes 1 and 2). The 64K3 probe detected a major 3.7-kb mRNA in both human epithelial cells and fibroblasts (Fig. 7, lanes 3 and 4). Epithelial poly(A)+ RNA also contained a second, less abundant 64K3-labeled RNA of 1.9 kb. This band was also observed in poly(A)+ RNA from fibroblasts after prolonged exposure (not shown). The human syndecan probe revealed two mRNA bands of 2.5 and 3.3 kb in both cell types (Fig. 7, lanes 5 and 6), but the syndecan message appeared to be relatively less abundant in lung fibroblasts than in the epithelial cells. In fibroblasts there was a clear difference in the relative abundance of the syndecan message depending on the growth state of the cells. Fibroblasts from confluent and post-confluent cultures expressed higher levels of syndecan message than cells from exponentially growing cultures (Fig. 8). Although not necessarily reflecting syndecan, immunodot blot assays suggested that the increase in syndecan message was paralleled by enhanced expression of the 2E9 epitope (not shown). Under similar circumstances no such differences were seen with the “48K” (Fig. 8) and the “64K” probes (not shown). Growing HBL-100 cells, in contrast, yielded strong signals for both syndecan and glypican, but the 48K message remained undetectable (not shown).

Finally, Northern analysis of total RNA samples from a panel of cultured human cells revealed large individual differences in the message levels for these three proteoglycans, extending the findings of proteoglycan heterogeneity and variability, as observed in human fibroblasts and mammary epithelial cells, to other cell lines (Fig. 9).

**DISCUSSION**

In the present study we have isolated and characterized the cell surface HS PGs of human mammary epithelial cells and related the human epithelial PGs to previously described human fibroblast and mouse epithelial PGs by using a panel of anti-fibroblast HS PG mAbs and PG-specific cDNA probes. The results are indicative of differential expression of the cell surface proteoglycans (Table I).

**Cell Surface Proteoglycans with Restricted Distributions**

The major cell surface HS PG of human lung fibroblasts has a core protein with apparent M, of 48,000 (see Fig. 1). From cDNA sequencing it appears to have the characteristics of an integral membrane protein featuring a membrane spanning hydrophobic domain, a cytoplasmic domain at the carboxyl terminus, and an NH2-terminal extracellular domain with the attachment sites for the heparan sulfate side chains (Marynen et al., 1989). This proteoglycan reacts with mAbs 6G12 and 10H4 (Lories et al., 1989), and has been named “fibroglycan” (David, 1990).

Detergent extracts of human mammary epithelial cells showed no reactivity with the 48K-specific mAbs 6G12 or mAb 10H4 and contained no core proteins of 48 kDa, suggesting that these cells did not synthesize a HS PG related to fibroglycan. This was confirmed by Northern blot analysis of both proliferating and stationary human mammary epithelial cells, which also ruled out that in these epithelial cells the core protein might be expressed in an alternative glycoprotein form that would behave differently from the proteoglycans during the purification procedure. Of all human cells analyzed in the panel, skin and lung fibroblasts, neuroblastoma, and retinoblastoma cells had the highest levels of fibroglycan mRNA (Fig. 9). The fibroglycan message was also detectable in glioma, sarcoma, teratoma, vascular endothelial, and HEP 3B cells, but it was virtually absent from the other cell lines, suggesting that high levels of this proteoglycan may be characteristic for cells of mesenchymal and neuroectodermal origin. Syndecan and glypican also showed variable levels of expression, but their messages were detectable in most of the cell lines tested. With the exception of HEP 3B cells, all cell

**FIG. 7. Northern blot analysis of human fibroblast and mammary epithelial mRNA.** Poly(A)+ RNA from confluent cultured human lung fibroblasts (F) and from the HBL-100 epithelial cell line (E) was size fractionated in denaturing formaldehyde agarose gels, blotted to nylon membranes, and analyzed by hybridization to the fibroglycan probe 48K3 (FIB), the glypican probe 64K3 (GLY), and the syndecan probe HUSYN-2 (SYN).

**FIG. 8. Northern blot analysis of exponentially growing cells.** Human lung fibroblasts were plated at one-tenth of their confluent density. The RNA was extracted after 1, 2, 3, 4, 7, and 9 days of culture. Ten µg aliquots of total RNA from each of the extracts were fractionated in agarose gels and hybridized to the EcoRI-PstI (bases 1-1389) generated fragment of the 48K3 probe (FIB) and to the HUSYN-2 probe (SYN). Only the major (2.3 kb) fibroglycan message is shown.

**FIG. 9. Northern blot analysis of a panel of cultured human cells.** Ten µg aliquots of total RNA from human H5 883 glioma (I), skin fibroblast (2), HBL-100 breast (3), lung fibroblast (4), HT-1080 sarcoma (5), TR-14 neuroblastoma (6), HEP 3B hepatoma (7), MOLT-4 acute lymphoblastic leukemia (8), MG-63 osteosarcoma (9), A-431 epidermoid carcinoma (10), Y79 retinoblastoma (11), Tera-2 embryonal carcinoma (12), U-937 histiocytic lymphoma (13), umbilical vein endothelial (14), MCF-7 breast adenocarcinoma (15), A-375 malignant melanoma (16), IM-9 myeloma (17), CaCo-2 colon adenocarcinoma (18), and HEP G2 hepatocarcinoma (19) cell cultures were fractionated in agarose gels, blotted, and hybridized to the fibroglycan (FIB), syndecan (SYN), and glypican (GLY) probes.
lines with relatively high levels of fibroglycan mRNA contained relatively low levels of syndecan mRNA, suggesting that these two proteoglycans may have opposite patterns of expression. Further experiments are needed to confirm this and to assess whether additional proteoglycan forms with a restricted expression might exist, since a 125-kDa core and the 1C7 epitope were also not detected in the mammary epithelial cells (Fig. 1).

Glypican Expression in Epithelial Cells—The cell surface HS PG from human fibroblasts which is characterized by the 64-kDa core protein and the S1 epitope is associated with the cell surface through a glycosyl phosphatidylinositol anchor and has therefore been named glypican (David et al., 1990). Glypican cDNA clones isolated from fibroblasts predict a protein with a short stretch of hydrophobic amino acids at its carboxyl terminus and apparently no cytoplasmic domain (David, 1990). They also indicate that glypican is molecularly distinct from fibroglycan (Marynen et al., 1989) and from syndecan (Saunders et al., 1989; Mali et al., 1990). Apparently, human mammary epithelial cells also express glypican. They synthesize a 64-kDa protein which carries HS chains (Fig. 1, lane 2) and the S1 epitope (Fig. 2, lane 7). Moreover, enzyme susceptibility tests confirmed the presence of the phospholipid anchor in the epithelial forms (Fig. 3). The expression of glypican in both cell types was confirmed by Northern blot analysis, revealing a major ~3.7-kb mRNA in both epithelial and fibroblast poly(A)+ RNA preparations, and perhaps a minor 1.9-kb mRNA in the epithelial preparations (Fig. 7). This smaller mRNA could also be detected in the fibroblast preparation after prolonged exposure of the filter but was not detected with a 64K3 probe from which the 5'-untranslated sequences had been removed. Although probably nonspecific, the origin of this minor signal remains to be further unraveled and its occurrence implies that the possibility of variant forms of glypican should be considered, especially in epithelial cells. Partial or even complete resistance to phospholipase C is not uncommon among proteins with known glycosyl phosphatidylinositol anchors, probably due to additional modification of the lipid moiety, but the 5–15% of residual hydrophobic glypican following the enzyme treatment could be representative of such variant forms. A cell surface HS PG with a core protein of about 64 kDa and with a phospholipid membrane anchor has also been isolated from Schwann cells (Carey and Stahl, 1990), and may represent the equivalent of glypican. The screening of the cell panel indeed indicates that glypican is widely distributed.

Syndecan Expression in Fibroblasts—Both human mammary epithelial cells and fibroblasts produce hybrid HS/CS cell surface proteoglycans with 82–88-kDa core proteins that migrate only as sharp protein bands after both heparitinase and chondroitinase ABC digestion. In fibroblasts, the hybrid PG is a relatively minor component of the total labeled PG fraction, and when heparitinase-digested total PG samples are additionally digested with chondroitinase ABC the change in the migration pattern of the core protein is not easily detected (Fig. 1, compare lane 6 to lane 7). It is only when mAb 2E9-immunopurified samples are doubly digested that the hybrid PG core protein is clearly visualized (Fig. 2, lanes 3 and 4).

These human hybrid proteoglycans share the 2E9 epitope with syndecan (Fig. 4), a hybrid cell surface PG first identified in mouse mammary epithelial cells (Rapprager et al., 1985; David and Ven den Berge, 1985), and seem to represent the human homologue of this molecule. Direct evidence for the presence of syndecan in human lung fibroblasts was obtained by amplifying a cDNA that is homologous to syndecan from lung fibroblast mRNA extracts, and demonstrating the reaction of mAb 2E9 with the encoded recombinant protein (Fig. 6). These data complement the results of Mali et al. (1990) who recently isolated a syndecan-homologous sequence from a HBL-100 cDNA library. Their reported sequence codes for a protein which is identical, except for one amino acid at position 19, to the sequence predicted by the lung fibroblast cDNAs (Fig. 5). The single difference may represent a structural polymorphism, rather than a Taq polymerase error, since it was identified in five independent isolates. The variant codon substitutes a proline for a leucine in the human fibroblast sequence and occurs in a region which has been highly conserved in mouse and man. Incidentally or not, the murine protein also features a proline at this position (Saunders et al., 1989). Then, comparison of the tyrosine contents of the predicted sequences for human fibroglycan (Marynen et al., 1989), syndecan (Mali et al., 1990), and glypican (David et al., 1990) indicates that, at similar specific activities of labeling, the amount of 125I/mole of core protein for fibroglycan will be twice, and for glypican nearly three times as high as for syndecan. Interpretation of the relative intensities of the bands on the autoradiograms in this light underscores that syndecan represents a significant fraction of the cell surface proteoglycans from fibroblasts and the major form in mammary epithelial cells.

Synthesis of syndecan, or at least of a proteoglycan which cross-reacts with mAb 281-2 in mesenchymal cells has been described before. Immunocytochemistry indeed suggests that during tooth (Vaino et al., 1989) and limb (Solursh et al., 1990) morphogenesis, syndecan is transiently detectable in the condensing mesenchyme. In these instances, synthesis appears to be induced by epithelial-mesenchymal tissue interactions. Apparently, confluent in vitro cultured human fetal lung fibroblasts are able to synthesize syndecan in the absence of such tissue interactions. The enhancement of the expression of syndecan in dense fibroblast cultures and in condensing mesenchymal tissues suggests, however, that the expression of this proteoglycan may be modulated, and related to or regulated by the formation of cell-cell contacts. The latter hypothesis was suggested by the observation that the expression of syndecan is higher in human mammary epithelial cells than in mouse mammary cells, and that the expression of syndecan is higher in primary cultures than in cell lines. These findings are reminiscent of the changes in syndecan expression during the ontogeny of B lymphocytes (Saunders et al., 1989) and of the important spatial and quantitative fluctuations in syndecan expression during the steroid-dependent cyclic changes of vaginal epithelial differentiation (Hayashi et al., 1988). The fluctuations in syndecan expression during culture are also consistent with prior findings which had noticed differences in the composition and physical properties of the cell surface proteoglycans in confluent and proliferating fibroblasts (Coster et al., 1986).

Northern blot analysis using the polymerase chain reaction product as a probe revealed the presence of two syndecan mRNAs of 2.5 and 3.3 kb in both human lung fibroblasts and human mammary epithelial cells. The syndecan message was clearly less abundant in fibroblasts than in epithelial cells, but in confluent fibroblasts the signal was quite distinct, indicating that the polymerase chain reaction product did not result from the amplification of an extremely rare fibroblast transcript. The sizes of the two syndecan mRNAs are reminiscent of the 2.4- and 3.5-kb syndecan mRNAs detected in murine cells and tissues with the original murine probe (Saunders et al., 1989) and of the mRNA bands detected in HBL-100 cells by others using cDNA probes isolated from these cells (Mali et al., 1990). The origin of these double mRNA bands is unknown, but they are thought to represent variations in noncoding parts of the messages (Saunders et al., 1989). In this context it is interesting that a cell surface
heparan sulfate proteoglycan was found, in fibroblasts only, which shows partial immunological cross-reaction with syndecan, as it shares the 2E9 epitope, but which is distinguishable from syndecan by the absence of CS chains, by the size of its core protein and by the presence of the 1C7 epitope in its peptide moiety (Lories et al., 1989). Whereas these data imply that the 125-kDa core protein could be a structural variant of the syndecan core protein, it seems unlikely that this variant is encoded by one or the other of these two syndecan messages, as both messages occur in similar relative abundances in the epithelial cells where the 1C7-reactive proteoglycan is not expressed. Otherwise one would have to postulate post-transcriptional or post-translational controls on the expression of this proteoglycan. More knowledge on the primary structure of the 125-kDa core protein should resolve this question. Finally, whereas in human cells the 3.3-kb band appeared to be slightly more intense than the 2.4-kb band (Fig. 7), the mRNA bands in murine cells and tissues created the opposite pattern with the 2.4- and 3.5-kb band occurring in 3:1 proportions (Saunders et al., 1989). Again, the significance of this finding is unknown.

In conclusion, the present data suggest that different cells and a given cell under different circumstances may express different proteoglycans or combinations of proteoglycans at their cell surfaces. It is tempting to speculate that these differences might allow the establishment of different cell-matrix contacts or support responses to different growth factors, or that the different core protein genes and their products might be differentially regulated providing the cells with means to respond differentially to factors that can modulate the abundance of heparan sulfate at the cell surface and thereby affect cell contacts and cell growth.

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REFERENCES

Bernfield, M. (1989) Curr. Opin. Cell Biol. 1, 953–955
Bernfield, M., and Sanderson, R. D. (1990) Phil. Trans. R. Soc. Lond. 327, 171–186
Carey, D. J., and Stahl, R. C. (1990) J. Cell Biol. 111, 2053–2062
Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
Coster, L., Carlstedt, I., Kendall, S., Malmaström, A., Schmidtchen, A., and Fransson, L.-Å. (1986) J. Biol. Chem. 261, 12079–12088
David, G. (1990) J. Cell Biol. 111, 9a
David, G., and Van den Bergh, H. (1985) J. Biol. Chem. 260, 11067–11074
David, G., and Van den Bergh, H. (1989) Eur. J. Biochem. 178, 609–617
David, G., Lories, V., Heremans, A., Van der Schueren, B., Cassiman, J. J., and Van den Bergh, H. (1989) J. Cell Biol. 108, 1165–1175
David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J. J., and Van den Bergh, H. (1990) J. Cell Biol. 111, 3165–3176
Elenius, K., Salmivirta, M., Inki, P., Mali, M., and Jalkanen, M. (1990) J. Biol. Chem. 265, 17837–17843
Gallagher, J. T. (1989) Curr. Opin. Cell Biol. 1, 1201–1218
Hayashi, K., Hayashi, M., Jalkanen, M., Firestone, J. H., Treistad, R. L., and Bernfield, M. (1987) J. Histochem. Cytochem. 35, 1079–1086
Hayashi, K., Hayashi, M., Boutin, E., Cunha, G. R., Bernfield, M., and Treistad, R. L. (1988) Lab. Invest. 58, 68–76
Jalkanen, M., Nguyen, H., Rapraeger, A., Kurn, N., and Bernfield, M. (1986) J. Cell Biol. 101, 976–984
Kiefer, M. C., Stephens, J. C., Crawford, K., Okino, K., and Barr, Ph. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6985–6989
Koda, J. E., Rapraeger, A., and Bernfield, M. (1985) J. Biol. Chem. 260, 8157–8162
Lories, V., David, G., Cassiman, J. J., and Van den Bergh, H. (1996) Eur. J. Biochem. 255, 351–360
Levy, G., De Boeck, H., David, G., Cassiman, J. J., and Van den Bergh, H. (1987) J. Biol. Chem. 262, 854–859, 1987
Lories, V., Cassiman, J. J., Van den Bergh, H., and David, G. (1989) J. Biol. Chem. 264, 7009–7016
Mali, M., Jaakola, P., Arvilommi, A.-M., and Jalkanen, M. (1990) J. Biol. Chem. 265, 6884–6889
Marynen, P., Zhang, J., Cassiman, J. J., Van den Bergh, H., and David, G. (1989) J. Biol. Chem. 264, 7017–7024
Rapraeger, A., Jalkanen, M., Endo, E., Koda, J., and Bernfield, M. (1983) J. Biol. Chem. 260, 11046–11052
Rusnak, E. (1991) Cell Biol. 86, 67–69
Sanderson, R. D., Lalor, P., and Bernfield, M. (1989) Cell Reg. 1, 27–35
Saunders, S., and Bernfield, M. (1988) J. Cell Biol. 106, 423–430
Saunders, S., Jalkanen, M., O’Farrell, S., and Bernfield, M. (1989) J. Cell Biol. 108, 1547–1556
Solursh, M., Reiter, R. S., Jensen, K. L., Kato, M., and Bernfield, M. (1990) Dev. Biol. 140, 83–92
Sun, X., Mosher, D. F., and Rapraeger, A. (1989) J. Biol. Chem. 264, 2885–2890
Theileff, I., Jalkanen, M., Vainio, S., and Bernfield, M. (1988) Dev. Biol. 129, 565–572
Vainio, S., Jalkanen, M., and Theileff, I. (1989) J. Cell Biol. 108, 1945–1954