Matrix-associated Heparan Sulfate Proteoglycan: Core Protein–specific Monoclonal Antibodies Decorate The Pericellular Matrix of Connective Tissue Cells and the Stromal Side of Basement Membranes

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Abstract. Cultured human lung fibroblasts produce a large, nonhydrophobic heparan sulfate proteoglycan that accumulates in the extracellular matrix of the monolayer (Heremans, A., J. J. Cassiman, H. Van den Berghe, and G. David. 1988. J. Biol. Chem. 263: 4731–4739). A panel of four monoclonal antibodies, specific for four distinct epitopes on the 400-kD core protein of this extracellular matrix heparan sulfate proteoglycan, detects similar proteoglycans in human epithelial cell cultures. Immunohistochemistry of human tissues with the monoclonal antibodies reveals that these proteoglycans are concentrated at cell–matrix interfaces. Immunogold labeling of ultracryosections of human skin indicates that the proteoglycan epitopes are nonhomogeneously distributed over the width of the basement membrane. Immunohistochemical investigations and amino acid sequence analysis indicate that the proteoglycan from the fibroblast matrix shares several structural features with the large, low density heparan sulfate proteoglycan isolated from the Engelbreth-Holm-Swarm sarcoma. Thus, both epithelial cell sheets and individual mesenchymal cells accumulate a large heparan sulfate proteoglycan(s) at the interface with the interstitial matrix, where the proteoglycan may adopt a specific topological orientation with respect to this matrix.

Heparan sulfate proteoglycans (HSPGs) occur as integral components of basement membranes in probably all mammalian tissues and in basement membrane–producing tumors (Anderson and Fambrough, 1983; Edge and Spiro, 1987; Hassell et al., 1980; Heathcote and Orkin, 1984; Kanwar et al., 1984; Kato et al., 1988; Oohira et al., 1982; Robinson and Gospodarowicz, 1984; Tyree et al., 1984). Their functions have not yet been well established, but the HSPGs are known to interact with several other basement membrane constituents (Fujiwara et al., 1984; Gallagher et al., 1986; Paulsson et al., 1986). These interactions probably contribute to the general architecture of the basement membrane and may play a role in tissue morphogenesis and growth control (David and Bernfield, 1982). Basement membrane HSPGs may also influence the specific properties of certain matrices since they determine the permeability of the glomerular basement membrane (Kanwar et al., 1980) and have been implicated in the anchorage of asymmetric acetylcholinesterase to the extracellular matrix (ECM) of the neuromuscular junction (Anderson and Fambrough, 1983; Brandan et al., 1985). Finally, by activating protease inhibitors, HSPGs may also modulate serine protease activity as documented for the Reichert's membrane (Pejler et al., 1987) and for the HSPGs of mouse mammary epithelial cells (Pejler and David, 1987). Perhaps the latter property of the HSPGs contributes to the metabolic stability of the basement membranes.

Immunohistochemical and cytochemical data have suggested that the basement membrane HSPGs occur specifically in basement membranes (Couchman, 1987; Hassell et al., 1980). Basement membrane HSPGs from different sources appear antigenically related to each other (Wewer et al., 1985) but are unrelated to the cell-associated HSPGs (Dziadek et al., 1985; Jalkanen et al., 1988; Keller and Furthmayr, 1986; Stow and Farquhar, 1987). Yet, despite antigenic cross-reactivity, structural variations have been described for HSPGs isolated from different basement membranes. Most tissues and basement membrane–producing cells synthesize a large, low density HSPG (LDPG) with a 400-kD core protein (Anderson and Fambrough, 1983; Fenger et al., 1984; Hassell et al., 1985; Tyree et al., 1984), but small, high density HSPGs have also been reported (Edge and Spiro,
the different forms represent precursor-product relationships is still a matter of discussion, but there is a possibility that basement membranes may contain a family of proteoglycans (Couchman, 1987). Some investigations suggest that the different forms represent precursor-product relationships (Hassell et al., 1985; Klein et al., 1988).

HSPGs are also found in the ECM of several cultured epithelial and mesenchymal cells (David and Bernfield, 1981; Eldridge et al., 1986; Hedman et al., 1979; Johansson et al., 1985; Nevo et al., 1984; Norling et al., 1981; Oohira et al., 1983; Robinson and Gospodarowicz, 1984; Singer et al., 1987; Wewer et al., 1985; Woods et al., 1985). In previously reported investigations, we have isolated and identified the major HSPG from the ECM of cultured human lung fibroblasts. In the present work, we provide evidence that this matrix HSPG is related to the large HSPGs that occur in basement membranes and we propose that, in basement membranes, the HSPGs might be vectorially inserted in the matrix.

**Materials and Methods**

**Materials**

 Unless stated otherwise in the text, the origin of the chemicals, enzymes, and culture media was as indicated in previous reports (Heremans et al., 1988; Lories et al., 1989). Cultures of amniotic epithelial cells, fetal human lung fibroblasts, and normal adult human skin fibroblasts were established in our laboratory (Lories et al., 1986). Human mammary epithelial cells (HBL 100) were provided by the EG&G Mason Research Institute (Worcester, MA) through the courtesy of Dr. Jensen. These cultures were grown in Dulbecco's modified Eagle's minimal medium containing 10% (vol/vol) FCS.

**Enzyme Treatments, Electrophoresis, and Western Blotting**

Samples of resuspended matrix, media, or cell extracts were incubated for 4 h at 37°C either with or without 1 mM of heparitinase in 100 mM NaCl, 1 mM CaCl₂, 50 mM Hepes, pH 7, in the presence of protease inhibitors (50 mM 6-aminohexanoic acid, 5 mM benzamidine, 3 µM pepstatin A, 5 mM N-ethylmaleimide, 1 µg/ml leupeptin, 1 mM PMSF) and 100 µg/ml BSA. The enzyme-treated and the untreated matrix fractions were pelleted by centrifugation at 10,000 × g for 10 min at 4°C, resuspended in 10 mM Tris-Cl, 1 mM EDTA, 2% SDS, pH 6.8, and boiled for 3 min. Electrophoresis was performed in SDS-gel agarose slab gels (4% NuSieve agarose; FMC Bioproducts, Rockland, ME) in 90 mM Tris, 90 mM borate, 2 mM EDTA, 0.1% SDS, pH 8 for 3 h at 50 V as described before (Heremans et al., 1988).

**Immunoperoxidase Reaction on Cells and Tissues for Light Microscopy**

Cultured cells were analyzed as described before (David et al., 1989). Human tissue samples were frozen in isopentane that was precooled in liquid nitrogen. After preincubation in PBS containing 1% casein (15 min), cell cultures and 6-µm cryosections of tissues were reacted, successively, with the first antibody preparation (1 h), with peroxidase-linked rabbit anti-mouse IgG (DAKOPATTS) or with 50 µl of rabbit antiserum against the LDPG of the murine Engelbreth-Holm-Swarm (EHS) sarcoma (anti-EHS LDPG antiserum) and characterized as described before (Paulsson et al., 1987). Rabbit anti-human type IV collagen was kindly provided by Dr. Charles Lapierre (University of Liège, Liège, Belgium).

**Antibodies**

Four mAbs (F64-3H8, F65-4B1, F65-7H9, and F65-9C9) were raised against HSPG purified from the ECM of cultured human lung fibroblasts (anti-ECM HSPG mAbs) and were shown to recognize four different epitopes on the core protein of this matrix HSPG (Heremans et al., 1988). These four mAbs do not cross react with cell surface HSPGs isolated from the same cultures (Heremans et al., 1988). mAb F58-7F9, which reacts with the 48- and 90-kD core proteins of these cell surface-associated HSPGs, was raised and characterized as described previously (Lories et al., 1989). Rabbit antiserum was raised against the LDPG from the murine Engelbreth-Holm-Swarm (EHS) sarcoma (anti-EHS LDPG antiserum) and characterized as described before (Paulsson et al., 1987).

**Processing of Culture Media, Cell Layers, and ECMS of Cultured Cells**

Culture media of confluent monolayers were decanted. The cell layers were briefly rinsed with PBS before three extractions with 10 ml Triton X-100 buffer (0.5% [vol/vol] Triton X-100, 10 mM Tris-Cl, 50 mM 6-aminohexanoic acid, 10 mM EDTA, 5 mM N-ethylmaleimide, 5 mM benzamidine, 1 mM PMSF, 3 µM pepstatin A, pH 8) for 10 min at 4°C. The media and the detergent extracts of the cell layers were cleared by centrifugation and concentrated as described before (Lories et al., 1987). The ECM remaining on the dial after detergent extraction was scraped in 10 ml PBS and pooled with the pellet of the cell extract. The combined pellets were resuspended in 1 ml PBS containing 50 mM 6-aminohexanoic acid, 5 mM benzamidine, 5 mM N-ethylmaleimide, 1 mM PMSF, and 3 µM pepstatin A.
immunogold Labeling for Electron Microscopy

20-μm cryosections of tissue were fixed with 2% acrolein (Polysciences, Inc., Warrington, PA) in 0.1 M phosphate buffer, pH 7.4, for 15 min on ice. The cytochemical reaction procedure was analogous to that used in light microscopy. After the immunohistochemical reaction and a supplementary fixation in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, the cells or tissues were dehydrated, embedded in epon, and sectioned with an ultramicrotome (Ultratome 2128 IV; LKB Instruments, Inc., Bromma, Sweden). Alternatively, to avoid possible antibody penetration problems, the immunogold labeling was performed on ultracryosections. For that purpose, mouse and human skin and human umbilical cord were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer for 20 min at 4°C and then treated with 0.1 M glycine in PBS. Freezing, sectioning, and treatment of the ultracryosections were essentially as described by others (Geuze et al., 1981; Tokuyasu and Singer, 1976). The primary antibody solutions contained 0.1% Tween 20 and 0.1% Triton X-100. The anti-ECM HSPG mAbs were used at a concentration of 0.1 mg/ml. The dilution of the anti-EHS LDPG and of the anti-type IV collagen antibodies was 1:200 and 1:100, respectively. The auroprobes (GAM-G5 and GAR-G5; Janssen Pharmaceutica, Beerse, Belgium) were diluted 1:10 in PBS containing 1% gelatin, 0.1% Tween, and 0.1% Triton X-100 and were applied for 1 h at room temperature. In some experiments, the detergents were omitted from the buffer solution. Fixation was a critical step. Other tested fixatives or longer fixation times sometimes led to better tissue preservation but markedly reduced the intensity of the labeling obtained with the mAbs.

Amino Acid Sequence Analysis

Fibroblast matrix HSPG was purified by cesium chloride density ultracentrifugation, ion exchange chromatography on mono Q, and gel filtration on Sepharose CL4B as described previously (Heremans et al., 1988). Purified matrix HSPG was reduced with 10 mM DTT in 4 M guanidine hydrochloride, 50 mM Tris-Cl, 50 mM octylglucoside, pH 8, for 4 h at room temperature, and alkylated with 40 mM iodoacetamide overnight at room temperature. The sample was then precipitated with 3 vol of 95% ethanol, resuspended in 0.1% SDS, 44 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, 166 mM bis Tris, pH 7.35, and incubated for 1 h at 37°C with 0.25 μg of S. aureus V8 protease. After digestion, the sample was boiled in 2% SDS and subjected to SDS-PAGE (gradient 6–26% total acrylamide and 2.5% cross-linker) using the Jovin system 3328.IV (Moos et al., 1988). The fractionated digest was electrotransferred to a polyvinylidene difluoride sheet (Immobilon TM; Millipore Continental Water Systems, Bedford, MA) in a phosphate buffer (10 mM NaH2PO4, 20% [vol/vol] methanol) 68.5% for 6 h at 0.5 A. After electrotransfer, the sample was stained for 10 min at room temperature with 1% (w/vol) Coomassie brilliant blue R-250 in 50% (vol/vol) methanol and destained in 10% glacial acetic acid, 50% (vol/vol) methanol. One major protein band at 13.5 kD was excised, cut into small pieces (2 x 4 mm), and placed on top of a polybrene-conditioned glass fiber filter in the cartridge block of a protein sequencer (477A; Applied Biosystems, Inc., Foster City, CA). Phenylthiohydantoin derivatives of amino acids produced by the sequencer were identified on-line using an analyzer (1220A; Applied Biosystems, Inc.).

Results

Immunodetection of Matrix HSPG in Cultured Cell Lines

The panel of four mAbs reacting with four distinct epitopes in the core protein of the major matrix HSPG of lung fibroblasts was used to analyze other cultured human cell lines: i.e., mammary epithelial cells, keratinocytes, and amnion cells. When Western blots of the matrix fractions of the cell layers were reacted with a mixture of the anti-ECM HSPG mAbs, one single heparitinase-sensitive protein was detected in each cell line examined (Fig. 1a). Staining with each mAb separately yielded similar results, indicating that all four core epitopes were expressed by all cells tested (not shown). The fibroblast core protein comigrated with the A chain of laminin (not shown), suggesting an apparent relative molecular mass of ~400,000. The fibroblast core protein and all other cross reacting heparitinase-resistant cores had similar sizes. This similarity of the sizes of the heparitinase-digested cores contrasted with the variation of the apparent relative molecular masses of the intact HSPGs in the different monolayers (Fig. 1a). This suggests tissue variation in the length or the number of the glycosaminoglycan chains. The detergent extracts of the cells contained little cross-reactive material (not shown). Variable amounts of immunoreactive proteoglycan were secreted in the culture media of all analyzed cell types (Fig. 1b). After heparitinase digestion, the culture media contained some immunoreactive 400-kD core protein but also smaller proteins that carried the matrix HSPG epitopes. Interestingly, these proteins were of similar mobility within the different cell lines. A further confirmation of the similarity of the HSPGs that were detected in the matrix of epithelial cells (mammary gland cells and amniotic epithelial cells) and mesenchymal cells (lung fibroblasts) was achieved by V8 protease digestion of heparitinase-treated matrix preparations. After SDS-PAGE and electrotransfer of the digests, staining with the mixture of the four mAbs yielded very similar patterns of immunoreactive V8 peptides in the different cell lines (Fig. 2). Similar peptide patterns in different cell types were also obtained when mAbs were used individually (not shown). Thus, several cultured epithelial cells seem to express the fibroblast matrix HSPG. Moreover, the epitopes defined by the mAb panel appear unique and specific for this type of proteoglycan since only heparitinase-sensitive molecules were detected.

Immunocytochemistry of human epithelial (Fig. 3a) and fibroblast (Fig. 3b) monolayers using the anti-ECM HSPG mAb panel assigned the epitopes to the reticular network of fibrillar material surrounding the cultured cells. In fibroblasts, this matrix staining pattern was clearly distinct from the cell surface staining obtained with an antibody directed against an integral membrane HSPG (Fig. 3c). There was no difference in staining pattern among the four anti-ECM HSPG mAbs available, and no additional staining was obtained after permeabilization of the cells with acetone (data not shown). Heparitinase digestion of the cultures before the incubation with the antibodies did not affect the staining intensity or pattern (not shown). Control experiments in which the mAbs were omitted were negative (not shown).

Immunolocalization of Matrix HSPG in Human Tissues

To locate the matrix HSPG in vivo, several human tissues were screened with the anti-ECM HSPG mAbs. In light microscopy, the most prominent reaction of the mAbs occurred at the level of the basement membranes (Fig. 4). In human skin sections (Fig. 4a), the dermo–epidermal junction and the basement membranes limiting the epidermal adnexae (hair follicles, sebaceous glands, and sweat glands) were heavily stained. The keratinocytes were negative. In the underlying dermis and at the light microscopic level, staining around individual fibroblasts was not a prominent feature. Blood vessels and smooth muscle cells, however, were surrounded by a strongly reactive belt. Intense staining of the
Figure 1. Production of matrix HSPG by cultured cells. Suspensions of scraped ECM material (a) and concentrated culture media (b) of keratinocytes (lanes 1 and 2), lung fibroblasts (lanes 3 and 4), amnion cells (lanes 5 and 6), and mammary epithelial cells (lanes 7 and 8) were incubated at 37°C for 4 h with (+) or without (−) 1 mIU of heparitinase. After incubation, the samples were made 2% in SDS, subjected to electrophoresis in 4% NuSieve agarose gels, and electrotransferred onto Z probe. The blots were stained with a mixture of 20 μg/ml of each of the four anti-ECM HSPG mAbs (4B1, 7H9, 9C9, and 3H8). The arrow indicates the position of the sample wells. Similar results were also obtained with skin fibroblasts (not shown).

Figure 2. Peptide mapping of the immunoreactive HSPGs from cultured epithelial and fibroblastic cells. The heparitinase-digested scraped ECM of amniotic fluid cells (lanes 1 and 2), mammary epithelial cells (lanes 3 and 4), and lung fibroblasts (lanes 5 and 6) were incubated with (+) or without (−) S. aureus V8 protease, subjected to SDS-PAGE, and electrotransferred onto Z probe. The blot was stained with the four anti-ECM HSPG mAbs as in Fig. 1. Assays with single antibody probes also detected similar peptides in different cells. mAb 4B1 reacted only with 100–200-kD peptides, whereas mAbs 3H8 and 7H9 also stained bands of lower relative molecular mass. mAb 9C9, in contrast, produced only very weak signals on blots of the protease digests (not shown).

Topological Arrangement of the Matrix HSPG

The localization of the epitopes within pericellular matrix structures, either the matrix surrounding connective tissue cells or the basement membrane underlying epithelial cells, was confirmed and specified by electron microscopical studies.

Immunogold labeling of 20-μm skin sections with the anti-ECM HSPG mAbs resulted in the scattered deposition of grains on irregular fibrillar material in the immediate vicinity of the dermal fibroblast cell surface (Fig. 6 a). A clear space always separated the fibroblast plasma membrane from the gold deposits (Fig. 6 b). Similarly, immunogold labeling of ultracytosections of umbilical cord revealed the deposition of gold on plaque-like material in the immediate vicinity of the cells in the Wharton's jelly (Fig. 6 c).

Immunogold labeling of ultracytosections of human skin also confirmed the localization of the ECM HSPG epitopes in the basement membrane (Fig. 7). Using a mixture of all four mAbs, the gold deposits were located in both the lamina rara and the lamina densa, but they were more abundant at the side of the lamina densa facing the underlying dermis (Fig. 7 c). The ECM HSPG epitopes were also present in small clusters of amorphous material and associated with fibrils in the interstitial areas just beneath the lamina densa. Omission of the detergent from the incubation mixtures did not markedly influence the distribution or abundance of the grain deposits (Fig. 7 d). When the sections were incubated with individual mAbs, a differential staining pattern was observed (Fig. 8). While the epitope recognized by mAb 4B1 (Fig. 8, a and c) appeared to be more distributed throughout the basement membrane, mAb 3H8 recognized a determinant that was concentrated at the interstitial side of the basement membranes by the anti-ECM HSPG mAbs was confirmed in several other tissues. In trachea, the epithelial-mesenchymal interface and the basement membrane surrounding blood vessels were demarcated (Fig. 4 b). In liver, the mAbs delineated the sinusoids and the portal vessels but also stained the portal fibroblasts (Fig. 4 c). In kidney, the antigen was detected primarily at the boundary of the tubuli, in the glomerular capsule, and in capillaries (Fig. 4 d). In placenta, reactive material was present at the basal surface of the trophoblast and around blood vessels (Fig. 4 e). The matrix HSPG was detected in umbilical cord (Fig. 4 f), in association with endothelial cells and the surface of smooth muscle cells, and also around mesenchymal cells of the Wharton's jelly. This staining of the basement membranes in human skin (Fig. 5, a–c) and of the Wharton's jelly (Fig. 5, d–f) could be completely inhibited when the sections were incubated with the anti-ECM HSPG mAbs in the presence of purified fibroblast matrix HSPG (Fig. 5, compare b with c and e with f).
Figure 3. Assembly of the HSPG in the pericellular matrix of cultured cells. Indirect immunoperoxidase staining of a normal human lung fibroblast cell culture (a) and an epithelial (HBL 100) cell culture (b) using mAb 9C9 stains the fibrillar network between the cells. In contrast, staining of a normal human fibroblast cell culture with mAb F58-7F9, an antibody that reacts with a cell surface-associated HSPG, lightly stains the contours of the cells (c). Bars, 100 μm.

lamina densa (Fig. 8, b and d). In contrast, staining of the human skin basement membrane cryosections with a polyclonal antiserum directed against collagen type IV showed a grain distribution throughout the whole thickness of the basement membrane (Fig. 7 b). Taken together, these results suggest that the core proteins penetrate both layers of the basement membrane but that a specific domain of the molecules is facing or might even be inserted in the underlying connective tissue.

Relationship of the Matrix HSPG to the LDPG from the EHS Tumor

Because of this occurrence in basement membranes and because a similar proteoglycan is expressed by epithelial cells, we investigated the cross-reactivity of the fibroblast matrix HSPG with antibodies raised against the LDPG of the basement membrane-producing EHS tumor. Western blots of human fibroblast ECM that were stained with the anti–EHS LDPG antiserum showed heparitinase-sensitive proteins with similar relative molecular masses as those recognized by the anti–ECM HSPG mAb panel (Fig. 9a). Both the antiserum and the panel stained the ~400-kD core protein and some smaller degradation products present in the sample. The anti–EHS LDPG antiserum also recognized two major core protein fragments that were detected by the mAb panel in protease V8 digests of the matrix proteoglycans (Fig. 9b).

To exclude the possibility that the anti–EHS LDPG antiserum and the anti–ECM HSPG mAbs would recognize two antigenically different, but structurally similar proteoglycans of the fibroblast ECM, 125I-labeled fibroblast matrix HSPG was immunopurified using the mAbs (see Materials and Methods) and tested for binding to protein A–Sepharose beads substituted with either rabbit anti–mouse IgG or with the rabbit anti–EHS LDPG antiserum. Nearly 84% of the immunopurified fibroblast matrix HSPG was bound by the rabbit anti–EHS LDPG–protein A beads, whereas the control rabbit anti–mouse IgG–protein A beads, as a test for possible mAb–HSPG complexes, bound only 29% of the 125I label. Separate immunopurification experiments, in which the anti–ECM HSPG mAbs were used individually and consecutively, established that all ECM HSPG epitopes were carried by a single type of 125I–fibroblast matrix proteoglycan (not shown). Moreover, the fibroblast matrix HSPG was able to completely block the labeling of the basement membranes of human tissues by the anti–EHS LDPG antiserum, suggesting that the HSPG from the fibroblast matrix contained (at least) the same cross-reactive epitopes as the HSPG present in human basement membranes (Fig. 10, compare b with c). Interestingly, at the ultrastructural level, the incubation of human skin sections with the anti–EHS LDPG antiserum yielded a similar staining pattern as obtained with the mAb panel, labeling predominantly the stromal side of the lamina densa (Fig. 11a). In mouse skin basement membranes, in contrast, this antiserum showed strong and uniform reactivity throughout the whole basement membrane (Fig. 11, compare a with b).

Finally, two amino acid sequences obtained from the analysis of protease V8 fragments of the core protein of the human fibroblast matrix HSPG showed a striking similarity to the amino acid sequence of the mouse EHS basement membrane LDPG that has been deduced from the analysis of corresponding cDNA clones (Noonan et al., 1988) (Fig. 12). One amino acid sequence was identical (except for two unknowns) to a sequence of the EHS LDPG that shows homology to N-CAM and to α1-β glycoprotein (cDNA clone BPG7, amino acids 293–313). The other sequence was homologous (except for two mismatches—Gln vs. Arg and His
Figure 4. Distribution of the matrix HSPG in human tissues. Staining of human skin sections (a) with mAb 9C9 reveals the presence of high concentrations of HSPG in the basement membranes of endothelia and epidermis. Staining of human trachea (b), liver (c), kidney (d), placenta (e), and umbilical cord (f) with mAb 9C9 displays the HSPG predominantly at the level of the basement membrane but also reveals stain around portal fibroblasts (c) and around cells from the Wharton's jelly (f). SM, smooth muscle; WH, Wharton's jelly. Bars, 100 μm.

Figure 5. Specificity of the immunostaining of basement membranes and fibroblast pericellular matrices. Indirect immunoperoxidase staining of human skin (a, c, and e) and human umbilical cord (b, d, and f) sections that were pretreated without first antibody (a and b) or with a mixture of the four anti-ECM HSPG mAbs (c-f) either in the absence (c and d) or the presence (e and f) of competing HSPG isolated from the matrix of cultured human fibroblasts. The staining of the basement membranes of human epidermis (EP), smooth muscle cells (SM), and the matrix around cells of the Wharton's jelly (WH) was completely blocked by the fibroblast proteoglycan. Bars, 100 μm.
Figure 6. Immunogold labeling of the pericellular matrix of stromal cells with the anti-ECM HSPG mAbs. In a and b, a cryosection of human skin is labeled with mAb 9C9. This reveals the HSPG in amorphous material in immediate proximity to a dermal fibroblast (FB). No gold is seen on the cell membrane (M) itself or on the more distant collagen fibers (CF).

In c, an ultrathin cryosection of umbilical cord is stained with a mixture of the mAbs, revealing the HSPG in plaque-like material in the vicinity of a stromal cell (SC) from the Wharton's jelly. The arrows point to areas where the cell boundaries are more distinct. Bars, 1 μm.

Discussion

Our previous studies on cultured human lung fibroblasts have provided evidence for several different forms of membrane-associated HSPGs (Lories et al., 1987; De Boeck et al., 1987; Lories et al., 1989) but for only a single matrix-associated HSPG (Heremans et al., 1988). This ECM component is a major proteoglycan of cultured fibroblasts, representing nearly 40% of the [35S]HSPG in the monolayer (Lories et al., 1986). The data reported here provide biochemical and morphological indications that this matrix HSPG is a ubiquitous component of epithelial and mesenchymal cell–matrix interfaces: i.e., that it is identical to or related to the “basement membrane proteoglycan(s).” Immunogold microscopy using mAbs suggests that this pericellular matrix HSPG might be vectorially inserted in basement membranes: i.e., that these proteoglycans may adopt a specific orientation with respect to the cell–matrix interface.

Epithelial and mesenchymal cells in culture seem to produce this large matrix proteoglycan as shown by immunochromatic and immunomicroscopical analysis using four mAbs specific for four different epitopes in the core protein of the fibroblast matrix HSPG (Figs. 1 and 3). The relative molecular mass of the fibroblast core protein is estimated at ~400kDa since it comigrated with the A chain of laminin in SDS-electrophoresis and was even somewhat more retarded than this marker protein after complete reduction of its disulfide bonds. This relative molecular mass estimate represents a correction with respect to previously reported values of 300,000–360,000 (Heremans et al., 1988) where this marker had not been included. The similarity of the ~400-kDa core proteins within the different cultured cell types could be confirmed by V8 protease peptide mapping (Fig. 2). In this context, the similarity of the sizes of the smaller cross-reactive core proteins that were detected in the culture media of the different cell lines and that could be degradation products of the large 400-kDa core proteins (Fig. 1 b) may also be the reflection of a similar core protein structure in the different cell lines.

The above results are reminiscent of prior investigations on basement membrane proteoglycans in a variety of tissues, where core sizes of 350–400 kDa have been reported, although basement membrane HSPGs with smaller core proteins (ranging from 20 to 180 kDa) have also been described (Edge and Spiro, 1987; Fujiwara et al., 1984; Hassell et al., 1980; Kanwar et al., 1984; Parthasarathy and Spiro, 1984). In our study, all immunoreactive HSPG core proteins that were isolated from the matrices of the different cultures had an apparent relative molecular mass of ~400kDa, but we cannot rule out the possible occurrence of additional, non–cross-reactive HSPG in those matrices. However, the fibroblast matrix core protein appears rather sensitive to proteolytic degradation. It was observed that additional immunoreactive bands may appear with time in stored matrix samples or occasionally be present in the original matrix extracts (see Fig. 9 a). Similar core protein breakdown has been observed during investigations on the EHS LDPG, where the extent of degradation was found to vary with the isolation procedure used (Paulsson et al., 1987).
Like several of the basement membrane HSPGs, the HSPG from the matrix of human fibroblasts shows clear cross-reactivity with an antiserum raised against the LDPG from the mouse EHS tumor. This was demonstrated by Western blotting (Fig. 8) and sequential immunoadsorption experiments which assured that the mAbs and polyclonal antiserum were recognizing the same and not just a similar proteoglycan. Structural similarity was further documented by amino acid sequence analysis of two core protein fragments obtained from the digestion of the fibroblast matrix HSPG with V8 protease (Fig. 12). It is possible that the minor mismatches that were observed only represent species differences. Consistently, the epitopes corresponding to the anti-ECM HSPG mAb panel were detected in the basement membranes of all human tissues examined (Fig. 4), while the staining of the basement membranes, either by the mAbs (Fig. 5) or by the anti-EHS LDPG antiserum (Fig. 10), could be completely inhibited by preincubation of the antibodies with matrix HSPG from fibroblasts. Taken together, these results strongly suggest that the HSPG from fibroblast matrices belongs to the family of the basement membrane HSPGs and extend prior histochemical identifications of anti-EHS LDPG antiserum–cross-reactive matrix fibers in cultured hamster fibroblasts (Singer et al., 1987).

In the above context it seems important to stress that the basement membrane proteoglycan is also accumulating on fibrillar matrix structures that surround fibroblasts in vivo. Both light microscopical (Fig. 5) and ultrastructural investi-
Figure 8. Immunogold labeling of basement membranes with single anti-ECM mAbs. Ultrathin sections of human skin labeled with mAb 4B1 (a and c) or with mAb 3H8 (b and d) in buffer with detergent. EP, epithelium; BM, basement membrane; ST, dermal stroma. Bars, 1 µm.

Figure 9. Antigenic similarity of the human fibroblast matrix HSPG and murine basement membrane HSPG. (a) Suspensions of scraped matrix of human lung fibroblasts were incubated with (+) or without (−) 1 mIU of heparitinase, subjected to NuSieve agarose gel electrophoresis, and electrotransferred onto Z probe. The Western blots were reacted with a mixture of the four anti-ECM HSPG mAbs (lanes 1 and 2) or with the anti-EHS LDPG antiserum (lanes 3 and 4). (b) Human lung fibroblast matrix was digested with S. aureus V8 protease for varying lengths of time, and Western blots of the resulting fragments were analyzed with the mixture of mAbs (lanes 1–3) or with the anti-EHS LDPG antiserum (lanes 4–6).

Gations (Fig. 6), showing the ECM HSPG epitopes in the connective tissue immediately surrounding dermal fibroblasts and around mesenchymal cells in the Wharton's jelly, locate the matrix HSPG at stromal cell–matrix interfaces. These data seem in agreement with observations in the mouse cornea, where plaques of basement membrane–like material that react with anti-EHS LDPG antisera occur in the stroma, in contact with fibroblasts and in association with large collagen fibers (Schittny et al., 1988). The expression of basement membrane proteoglycans by fibroblasts is thus not merely an artifact of tissue culture.

The basement membranes, however, are the pericellular matrix structures where this type of proteoglycan seems to abound (Fig. 4). Interestingly, in human skin basement membranes the epitopes that were recognized by either the anti-ECM HSPG mAb mixture (Fig. 7) or by the anti-mouse EHS LDPG antiserum (Fig. 11 a) seemed located in both the lamina rara and lamina densa, but the gold grains were more concentrated at the basal side of the basement membrane, where they also occurred in association with underlying fibrils in the dermis. This nonhomogeneous staining of the human basement membranes seemed specific for the HSPG probes since, for example, type IV collagen could be detected
throughout the basement membranes. This impression was strengthened by stainings that used single mAb probes, where the 3H8 epitope appeared confined to the stromal side of the basement membrane (Fig. 8). In mouse skin, however, the anti–EHS LDPG antiserum yielded a strong and uniform reaction all across the basement membrane, showing no particular concentration of epitopes at the lamina densa–interstitial matrix interface (Fig. 11 b).

Figure 11. Immunogold labeling of basement membranes with the anti–EHS LDPG antiserum. Human (a) and mouse (b) skin ultra-cryosections labeled with anti–EHS LDPG rabbit antiserum. The control sections using goat anti–rabbit IgG only were negative (not shown). EP, epithelium; BM, basement membrane; ST, dermal stroma. Bars, 1 μm.

Figure 10. Cross-reactivity of the anti–EHS LDPG antiserum with fibroblast matrix HSPG. Immunostaining with peroxidase-conjugated swine anti–rabbit antibodies of human skin sections that were pretreated either without first antibody (a) or with anti–EHS LDPG antiserum in the absence (b) and in the presence (c) of competing HSPG isolated from the matrix of cultured human fibroblasts. Bars, 100 μm.
Figure 12. Sequence similarity of the human fibroblast matrix HSPG and the murine EHS LDPG. HSPG isolated from the ECM of cultured human lung fibroblasts was digested with S. aureus V8 protease. The digest was fractionated by SDS-PAGE and electroblotted on an Immobilon membrane. A prominent 13.5-kD band was excised for sequencing. One major and one minor signal, revealing the comigration of two peptides, were obtained. The signals could be read and distinguished unambiguously for 20 cycles, except at two locations (indicated by X in the sequences). Residues 293–313 of the amino acid sequence deduced from cDNA clone BPG7 (a) are aligned with the amino acid sequence from the major signal (b), while the sequence derived from the minor signal (c) is aligned with residues 439–459 of the amino acid sequence deduced from cDNA clone BPG5 (d). Both cDNAs encode different portions of the EHS LDPG (Noonan et al., 1988). The arrows indicate potential V8 protease cleavage sites in the EHS LDPG.

One possible explanation for the findings in human and mouse tissues could be a vectorial insertion (preferential orientation) of the proteoglycan in the basement membrane, whereby a specific part (presumably one end) of the core protein would be in contact with the underlying stroma and the rest of the molecule would penetrate both laminae of the basement membrane. The estimated length (80 nm) of the basement membrane HSPG core protein (Laurie et al., 1988; Paulsson et al., 1987) seems compatible with such a proposal and could account for a scatter of grains over the whole thickness of the basement membrane. In that case, the homogeneous staining of the mouse basement membrane by the anti-EHS LDPG antiserum may be attributable to the recognition of a broad range of epitopes covering the whole length of the core, whereas the human cross-reacting epitopes and the epitopes that are recognized by the mAbs may be restricted to a particular domain of the core, explaining the nonhomogeneous staining of the human basement membranes. This interpretation seems corroborated by the observation that the anti-EHS LDPG antiserum stains only a limited number of the fragments generated by digesting the human core protein with protease V8 (Fig. 9 b). Alternatively, the difference in staining could be due to a species difference in the organization and masking of the HSPG within the different layers of the skin basement membranes. The result with the anti-EHS LDPG serum in the mouse tissue is in agreement with previous ultrastructural studies that have detected the HSPG all across the basement membrane (Grant and Leblond, 1988; Laurie et al., 1982; Schittry et al., 1988). However, an antisera raised against rat glomerular basement membrane HSPG has been found to stain only the lamina rara (Stow et al., 1985). On the assumption that the different antisera were recognizing different parts of equivalent molecules, these findings could also be interpreted in terms of a vectorial insertion of the proteoglycan in the matrix.

To clarify this issue on the organization of the matrix HSPG, additional experiments are needed, including the expression of the mAb panel, the mapping of the mAbs to specific domains of the proteoglycan core proteins, and the enhancement of the sensitivity of the immunolabeling technique on ultracyrosections. This need is emphasized by the available sequence information which predicts the occurrence of structural repeats within the core (Noonan et al., 1988), whereby certain probes may not be informative. So far, the concentration of these HSPGs in plaque-like matrix structures that are bound to collagen fibers in the immediate vicinity of the fibroblast cell surface and at the side of the basement membranes where this membrane abuts the connective tissue implies that these ubiquitous proteoglycans may have a specific function in the anchorage of the pericellular matrices to the interstitial matrix.

We thank Ch. Coomans, G. Degeest, A. Rayé, L. Meekers, and M. Willems for their expert technical assistance. We are very grateful to Dr. P. Marynen and F. Van Leuven for their helpful suggestions, Dr. Ch. Lapière for the gift of antibodies, and Dr. J. Van Damme for the access to protein sequencing facilities.

Indirectly, we owe gratitude to the National Fund for Scientific Research and the Belgian Ministry of Science Policy (Concerted Research Actions) for grants supporting the installation of the protein sequencer. This investigation was supported by grants 3.0330.81 and 3.0066.87 of the Fonds voor Geneeskundig Wetenschappelijk Onderzoek, of Belgium, by the Interuniversity Network for Fundamental Research sponsored by the Belgian Government (1987–1991), by grant HL-31750 from The U. S. Public Health Service awarded to Guido David, and by grants from the Swiss National Science Foundation and the Maurice E. Müller foundation to Mats Paulsson. Guido David is an Onderzoeksleider and Annie Heremans is an Aspirant of the Nationaal Fonds voor Wetenschappelijk Onderzoek of Belgium.

Received for publication 16 May 1989 and in revised form 7 August 1989.

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