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Authors

Sanderson, RD

Sneed, TB

Young, LA

et al.

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ADHESION OF B LYMPHOID (MPC-11) CELLS TO TYPE I COLLAGEN IS MEDIATED BY THE INTEGRAL MEMBRANE PROTEOGLYCAN, SYNDECAN[1]

RALPH D. SANDERSON,[2*†] THOMAS B. SNEED,[*] LANNE A. YOUNG,[*] GARY L. SULLIVAN,[*] AND ARTHUR D. LANDER[‡]

From the Departments of [*]Pathology and [†]Anatomy and the Center for Musculoskeletal Research, University of Arkansas for Medical Sciences, Little Rock, AR 72205; and [‡]Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

Differentiating B lymphocytes undergo changes in cell-cell and cell-matrix adhesion that control their movement through a series of distinct microenvironments. The integral membrane proteoglycan, syndecan, is a candidate for mediating B lymphocyte-matrix interactions because it is expressed on B lymphocytes only at times when they associate with matrix, and because syndecan is known to behave as a matrix receptor on simple epithelia. However, syndecan from B lymphocytes is significantly smaller in molecular mass than syndecan from simple epithelia (85 vs 160 kDa) suggesting that syndecan may have distinct functions on these two cell types. Our study was undertaken to determine if syndecan mediates adhesion of B lineage cells to extracellular matrix. The murine myeloma cell line MPC-11 was used because syndecan is the only major heparan sulfate proteoglycan detected on these cells and because they express a form of syndecan almost identical to that found on normal B lymphocytes. Cell binding assays demonstrate that syndecan binds MPC-11 cells to type I collagen. Binding is inhibited by heparin, by pretreatment of cells with heparitinase or by growth of cells before the assay in chlorate, an inhibitor of sulfation. Solid phase assays show that syndecan purified from MPC-11 cells binds to type I collagen but not type IV collagen, laminin, or fibronectin. The interaction of MPC-11-derived syndecan with type I collagen is of relatively high affinity (K_d app = 143 nM) as measured by affinity coelectrophoresis. However, the 160-kDa form of syndecan isolated from epithelial cells has a greater than fourfold higher affinity for type I collagen (K_d app = 31 nM) than does the MPC-11 syndecan, suggesting that different molecular forms of syndecan have distinct ligand binding properties. These results demonstrate that syndecan can mediate B lymphocyte interactions with matrix and suggest that changes in syndecan expression during B cell differentiation are a mechanism for controlling B cell localization within specific microenvironments.

Differentiation of B cell precursors into plasma cells is a multistep process requiring localization of these cells within a series of distinct microenvironments (1). These microenvironments are not always in close proximity to each other, thus necessitating release of B cells from one location, migration, recognition, and subsequent attachment of cells at another location, a process requiring exquisite coordination of both cell-cell and cell-matrix interactions. B lymphocyte cell-cell interactions have been extensively examined and are mediated by at least several structural families of adhesion receptors including integrins (2), Hermes/CD44 (3), Ig superfamily-related molecules (4) and selectins (5). However, much less is known about the receptors mediating B lymphocyte cell-matrix interactions, with the exception of various integrins that can mediate binding of B lymphocytes to fibronectin (2).

Cell surface heparan sulfate proteoglycans interact with extracellular matrix molecules (6) and are likely mediators of hematopoietic cell adhesion to matrix (7). A direct role for heparan sulfate in binding B lineage cells to collagen has been proposed (8), and it has been hypothesized that hematopoietic cell precursors progressively lose their surface heparan sulfate as they differentiate, thus becoming less adhesive before release into the circulation (9, 10). However, a direct role for heparan sulfate proteoglycans in binding hematopoietic cells to matrix has not been demonstrated.

Syndecan is an integral membrane proteoglycan expressed by B lymphocytes at distinct stages of their differentiation. Syndecan is expressed on pre-B cells within the marrow, lost as cells mature before release from the marrow, absent on circulating and peripheral B cells, and reexpressed on plasma cells within interstitial matrices (11). Thus, during murine B lymphocyte differentiation, syndecan is expressed only when and where B cells associate with matrix, making it a likely candidate for mediating B cell interactions with matrix.

Syndecan was initially isolated from NMuMG[3] epithelial cells and on these simple epithelial cells syndecan behaves as a matrix receptor. It 1) anchors these cells to interstitial collagens (types I,III, and V) (12), fibronectin (13) and thrombospondin (14); 2) interacts with the actin

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[2] Address correspondence and reprint requests to Dr. Ralph D. Sanderson, Department of Pathology, Slot 517, University of Arkansas for Medical Sciences, 4301 West Markham St., Little Rock, AR 72205.

[3] Abbreviations used in this paper: NMuMG, normal murine mammary gland; ABCase, chondroitin sulfate ABC lyase.

cytoskeleton via its cytoplasmic domain (15); 3) is linked to the cell surface via a hydrophobic domain within the core protein (16); and 4) can be rapidly released from the cell surface when cells detach from matrix and round up (17). Syndecan also binds basic fibroblast growth factor (18, 19) and thus could play a role in basic fibroblast growth factor-mediated cell signaling (20).

In adult murine tissues, syndecan is located predominantly on epithelial cells (21), although its expression during development is less restricted and at times includes mesenchymal cells (22–25). Syndecan molecular structure varies with tissue type due to differences in the type, number, and size of its glycosaminoglycan chains (26, 27). For instance, NMuMG epithelial cells express syndecan with a modal molecular mass of 160 kDa but plasma cells express syndecan of 85 kDa. This difference in mass is due primarily to differences in glycosaminoglycan content. However, the functional significance of these differences in syndecan structure remains obscure.

Our study was undertaken to determine if syndecan functions as a matrix receptor on B lymphocytes. The MPC-11 myeloma cell line was used in this study because syndecan is the only major heparan sulfate proteoglycan detected on these cells and because they express a molecular form of syndecan nearly identical in structure to syndecan isolated from normal murine B lymphocytes. We find that syndecan binds MPC-11 cells to type I collagen. Binding to collagen is mediated by heparan sulfate chains on syndecan because pretreatment of collagen with heparin or removal of heparan sulfate from the cell surface before incubation of cells with collagen inhibits cell binding. Furthermore, growth of cells in the presence of chlorate, an inhibitor of sulfation, also inhibits cell binding to collagen, suggesting that proper sulfation of syndecan heparan sulfate chains is required for the interaction of syndecan with collagen. In addition, solid phase assays demonstrate that syndecan purified from MPC-11 cells binds to type I collagen, but does not bind to type IV collagen, laminin, or fibronectin. Analysis by affinity coelectrophoresis indicates that the interaction of MPC-11-derived syndecan with type I collagen is of relatively high affinity (K_d app = 143 nM), but differs from that of NMuMG-derived syndecan that has an even higher affinity for type I collagen (K_d app = 31 nM). We conclude that syndecan mediates adhesion of B lymphocytes to type I collagen and propose that regulation of syndecan expression during B lymphocyte differentiation may, in part, be responsible for the control of B lymphocyte localization within specific microenvironments.

MATERIALS AND METHODS

Cell Culture

MPC-11 mouse myeloma cells (28) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. NMuMG epithelial cells (passages 13–22) were maintained in bicarbonate-buffered DMEM as described previously (29). For radiolabeling, cells were placed in fresh complete media containing 50 μCi/ml $H_2{}^{35}SO_4$ (ICN Biomedicals Inc., Costa Mesa, CA) for 24 h and for double labeling experiments, cells were placed in media containing 2.5 mM glucose with 10 μCi/ml [3H]glucosamine hydrochloride (Dupont, Wilmington, DE) and 50 μCi/ml $H_2{}^{35}SO_4$. For [3H]leucine labeling, cells were placed in leucine-free RPMI 1640 (ICN) containing 10 μCi/ml [3H]leucine (Du Pont, Wilmington, DE) for 4 h. Media containing reduced amounts of sulfate were not used in any experiments to insure that aberrant sulfation did not occur.

Isolation, Purification, and Analysis of Syndecan

Trypsin-released syndecan. Previous studies have shown that the extracellular domain of syndecan is released from the cell surface by mild trypsin treatment (17), presumably at the site of a dibasic sequence located adjacent to the extracellular face of the transmembrane domain (16). MPC-11 cells were harvested by centrifugation and washed three times in PBS containing 0.5 mM disodium EDTA (buffer A). NMuMG cell monolayers were washed in buffer A, released from the culture dish by scraping and washed three more times in buffer A. Cells were resuspended in buffer A and syndecan was released from the cell surface by the addition of 20 μg/ml trypsin (GIBCO, Grand Island, NY; catalog no. 610–5200) for 10 min on ice. Soybean trypsin inhibitor (Calbiochem, La Jolla, CA) was added to a concentration of 100 μg/ml and cells pelleted by centrifugation at 735 × *g* for 3 min. Supernatants containing the released syndecan were then brought to 6 M urea, 50 mM sodium acetate, pH 4.5, 1 mM PMSF, and boiled for 10 min. Tubes were cooled to room temperature and DEAE Sepharose beads (100 μl of beads/10^7 cell equivalents; Pharmacia Fine Chemicals, Piscataway, NJ) were added and the mixture placed on a rocker for 1 h at room temperature. DEAE beads were pelleted by gentle centrifugation (240 × *g* for 2 min) placed in a clean 0.5-ml microcentrifuge tube and washed four times with PBS. The beads were then eluted with PBS containing a final NaCl concentration of 1 M. Elution of beads was accomplished by placing a small hole in the bottom of the 0.5-ml microcentrifuge tube with an 18-gauge needle, adding 50 μl of PBS-1 M NaCl to the beads and then placing the 0.5 ml tube within a 1.5-ml microcentrifuge tube. Eluate was collected in the 1.5-ml tube by centrifugation of the assembled tubes at maximum speed (16,000 × *g*) in an Eppendorf microcentrifuge (Brinkmann, Westbury, NY) for 10 s. The beads remain in the 0.5-ml tube. Three sequential elutions were performed in this manner to insure all of the DEAE-bound material was released. Eluates were then diluted with 10 mM Tris, pH 7.4, to a final concentration of 0.15 M NaCl and incubated with mAb 281–2 that was covalently bound to Sepharose CL-4B beads (281–2 beads). 281–2 is an mAb that binds with high affinity to the core protein of syndecan (30). After incubation overnight at 4°C with gentle rocking, beads were washed extensively in PBS then distilled water and bound syndecan was eluted from beads by boiling in SDS-PAGE sample buffer.

Detergent-extracted syndecan. After washing three times in ice cold PBS, cells were extracted for 1 h on ice in 10 mM Tris, pH 7.4, containing 1% Triton X-100, 0.15 M NaCl, 5 mM *N*-ethylmaleimide, 5 mM benzamidine, 5 μg/ml pepstatin A, and 1 mM PMSF (buffer B). Extracts were centrifuged for 15 min at 16,000 × *g*, supernatants brought to 6 M urea, 50 mM sodium acetate, pH 4.5, boiled and syndecan purified as described above except 0.1% Triton X-100 was included in all buffers.

SDS-PAGE and Western blotting. SDS-PAGE, Western blotting, and removal of syndecan glycosaminoglycan chains with *Flavobacterium heparinum* heparitin sulfate lyase (heparitinase, Seikagaku, Rockville, MD) or chondroitin sulfate ABC lyase (ABCase, Seikagaku) were performed as previously described (11), except SDS-PAGE gradients were 3.5 to 15%.

Sepharose CL-4B chromatography. For analysis of MPC-11 proteoglycans by Sepharose CL-4B chromatography, $^{35}SO_4$-labeled MPC-11 cells were extracted with buffer B, extracts were brought to 6 M urea, 50 mM sodium acetate, pH 6.0, boiled, and incubated with DEAE beads as described above except elution from the beads was with 2 M NaCl. The eluate was then brought to a final concentration of 0.15 M NaCl by dilution with 10 mM Tris, pH 7.4, buffer and chondroitin sulfate chains degraded by the addition of 0.05 U/ml of ABCase (Seikagaku) and incubation for 45 min at 37°C, followed immediately by addition of another 0.05 U/ml of ABCase and incubation for an additional 45 min. Samples were boiled for 10 min to destroy remaining enzyme, cooled on ice, and 281–2 beads added. After incubation on a rocker at 4°C overnight, 281–2 beads were harvested, washed, and bound syndecan eluted by boiling beads in 4 M guanididne hydrochloride containing 0.1% Triton X-100 and 50 mM sodium acetate, pH 5.8 (buffer C). Proteoglycans in the 281–2 unbound fraction were concentrated on DEAE beads and eluted with buffer C. The samples (50 μl) were applied to Sepharose CL-4B (Pharmacia) columns (0.5 × 50 cm) and eluted at a flow rate of 6 ml/h in buffer C. Fractions were collected and assayed by liquid scintillation counting.

For analysis of syndecan molecular size after growth of cells in chlorate, trypsin released [3H]glucosamine and $^{35}SO_4$-labeled syndecan was purified as described above. The syndecan bound to 281.2 beads was eluted by boiling in buffer C and applied to a Sepharose CL-4B column as described above.

Sepharose CL-6B chromatography. For analysis of syndecan heparan sulfate chain size, purified syndecan was treated with

ABCase to remove chondroitin sulfate chains followed by incubation with 0.1 N sodium hydroxide in 1 M potassium borohydride for 24 h at 37°C to release heparan sulfate chains from the core protein. Samples were neutralized with glacial acetic acid, desalted by passing over prepacked Excellulose G-5 columns (Pierce Chemical Co., St. Louis, MO), and applied to a Sepharose CL-6B (Pharmacia) column (0.5 × 50 cm) and eluted in buffer containing 1% SDS, 0.15 M NaCl, 50 mM sodium acetate, pH 5.0, and 0.02% sodium azide. Glycosaminoglycan chain size was determined by comparison with a molecular size calibration curve generated with chondroitin sulfate standards (31).

DEAE chromatography. The polyanionic properties of syndecan derived from cells grown in the presence of various concentrations of chlorate were analyzed by DEAE chromatography. $^{35}SO_4$-labeled trypsin released syndecan was purified and eluted from 281.2 beads by boiling in buffer containing 10 mM Tris, pH 7.4, and 0.05 M NaCl (buffer D). Samples were applied to a 0.4 ml DEAE column equilibrated in buffer D and eluted with a 0.05 to 1.0 M NaCl gradient. As an internal standard, [3H]glucosamine-labeled syndecan purified from NMuMG cells was added to each sample just prior to application of the sample to the column.

Solid Phase Binding of Syndecan to Matrix Ligands

Binding of syndecan to matrix ligands was analyzed using a previously described solid phase assay (32). The matrix proteins type I collagen (rat tail), type IV collagen (mouse Engelbreth-Holm-Swarm), laminin (Engelbreth-Holm-Swarm), and fibronectin (human plasma) all purchased from Collaborative Research (Bedford, MA), were blotted on to nitrocellulose filters (0.45-μm pore, Schleicher & Schuell, Kenne, NH), using a MilliBlot-D dot blotting apparatus (Millipore, Bedford, MA). After removal from the apparatus, filters were incubated for 1 h at room temperature with PBS containing 10 mg/ml BSA (fraction V, catalog no. A-4503; Sigma Chemical Co., St. Louis, MO). Filters were then washed three times with PBS and placed on a rocker overnight at 4°C in PBS containing 7500 cpm/ml of either $^{35}SO_4$-labeled intact syndecan or $^{35}SO_4$-labeled heparan sulfate from intact syndecan. Filters were washed extensively with PBS, dried, and the dots punched out and counted in a liquid scintillation counter.

Affinity Coelectrophoresis

Binding of syndecan to type I collagen was also analyzed in solution using the method of affinity coelectrophoresis. Briefly, agarose gels (1% w/v) were cast in which nine distinct lanes were present containing type I collagen (rat tail, Collaborative Research) at concentrations from 1–750 nM (assuming a M_r for collagen of 300,000). Dilute samples of $^{35}SO_4$-labeled syndecan were electrophoresed through these lanes and the mobility shifts incurred in each lane were used to follow binding (33, 34). Electrophoresis buffers and conditions were as described by Lee and Lander (33). Collagen-containing lanes were formed by diluting a collagen stock (4.05 mg/ml) into 20 mM acetic acid to concentrations 10 times those desired, and then adding nine volumes of premelted, 37°C agarose (1.11% w/v) in electrophoresis buffer containing 0.55% CHAPS and supplemented with 2.22 mM NaOH. Immediately on addition of agarose, samples were mixed and pipeted into precast lanes and allowed to gel at room temperature. Samples of purified trypsin-released syndecan (100 μl) from $^{35}SO_4$-labeled cells were then loaded into gels at final concentrations of 360,000 cpm/ml (MPC-11 syndecan) and 500,000 cpm/ml (NMuMG syndecan). After running and drying gels, syndecan migration was measured autoradiographically using a Phoshorimager (Molecular Dynamics). Average distances migrated in each collagen-containing lane were determined densitometrically using the Phosphorimager.

Cell Binding Assays

MPC-11 cell binding to type I collagen was assayed by the method of Koda et al. (12). Polyvinyl 96-well U-bottom plates (Dynatech, Chantilly, VA) were incubated with type I collagen (rat tail, 1 mg/ml; Collaborative Research) or BSA (fraction V, 1 mg/ml, Sigma) overnight at 4°C. Wells were washed with PBS and remaining protein binding sites within the wells were saturated by incubation with 10 mg/ml BSA for 30 min. Plates were then washed three times with PBS before addition of cells. MPC-11 cell cultures were established at a concentration of 2×10^5 cells/ml 24 h before performing cell binding assays. Cells were harvested by centrifugation at 4°C, washed with ice cold PBS, and resuspended in cold PBS at a concentration of 4×10^5 cells/ml. Cells were added to microtiter wells (200 μl/well), incubated for 30 min at room temperature, and the plate was centrifuged at 120 × *g* for 10 min in a Beckman TJ-6 centrifuge (Beckman Instruments Inc., Fullerton, CA). After centrifugation, 50 μl of 4% gluteraldehyde in PBS were added to each well and the plate placed at 4°C overnight. After removal of the buffer, cells were stained with 4% Trypan blue in PBS. In this assay, if cells do not bind to the protein-coated wells a clearly visible pellet is formed in the bottom of the well after centrifugation. If cells bind to wells, they remain as a uniform coating over the well surface.

To determine the effects of potential inhibitors of cell binding, wells were preincubated with heparin, cells were pretreated with heparitinase or ABCase or cells were grown in the presence of chlorate before the assay. The effect of heparin was tested by incubation of 10 μM heparin in PBS (based on an M_r for heparin of 10,000; porcine intestinal heparin, precipitated three times with ethanol; Sigma, catalog no. H-3393) with collagen coated wells for 30 min (13). Before the addition of cells, heparin was removed and the wells washed three times with PBS. For removal of glycosaminoglycan chains, washed cells were treated for 30 min at 37°C with either 33 μIU/ml of heparitinase or 0.05 U/ml of ABCase. Before addition of cells to wells, cells were washed in ice cold PBS and enzyme was again added in the same concentrations given above. To determine the effects of chlorate on MPC-11 cell binding to collagen, cells were grown in culture media containing the described amount of sodium chlorate (Mallinckrodt, Paris, KY) for 24 h, washed in ice cold PBS, and assayed as described above. In some experiments, as a control for chlorate specificity, sodium sulfate was included in culture media in addition to chlorate.

For quantitative cell binding assays, cells were prepared and assayed exactly as described above for the qualitative assay except [^{3}H]leucine-labeled cells were used and, after incubation of cells in the wells, plates were not centrifuged, but placed on a belly dancer shaker (Stovall Life Science, Inc., Greensboro, NC) on setting 4 for 1 min and unbound cells removed by two washes with PBS. Wells were then cut from the plate and subjected to liquid scintillation counting to determine the amount of ^{3}H remaining on bound cells. Percentage of bound cells was determined by the formula: percent bound = [(cpm from bound cells)/(input cell associated cpm-spontaneously released cpm)] × 100.

RESULTS

Syndecan on MPC-11 cells is similar to syndecan on normal B lymphocytes in molecular size, glycosaminoglycan composition, and cellular localization. Syndecan from normal murine plasma cells is a heparan sulfate proteoglycan of modal size 85 kDa with a core protein of 62 kDa (11). To determine if the MPC-11 cell line expresses syndecan with molecular properties similar to normal cells, $^{35}SO_4$-labeled cell surface syndecan was purified and subjected to SDS-PAGE (Fig. 1*A*). The autoradiograph reveals that MPC-11-derived syndecan is a relatively small proteoglycan similar in molecular mass to syndecan from normal plasma cells. Western blotting of MPC-11 syndecan purified from detergent extracts and treated with enzymes to remove glycosaminoglycan chains reveals a core protein of 62 kDa (Fig. 1*B*), identical in apparent size to the core protein of normal plasma cell syndecan. In addition, both MPC-11 and normal plasma cell syndecan contain heparan sulfate glycosaminoglycan. However, unlike normal plasma cells, a small population of the MPC-11 syndecan molecules apparently contain small amounts of chondroitin sulfate because heparitinase treatment alone does not reduce all of the syndecan molecules to the 62-kDa core protein size (Fig. 1*B*).

Immunocytochemistry has demonstrated that syndecan is localized on the cell surface of normal plasma cells (21). To assess the location of syndecan on MPC-11 cells, $^{35}SO_4$-labeled MPC-11 cells were subjected to mild trypsinization, a procedure that quantitatively releases syndecan from the surface of cells (17, 35). After trypsinization, cells were extracted with Triton X-100 to solubilize remaining syndecan and both trypsin released and Triton extracted material was incubated with 281.2

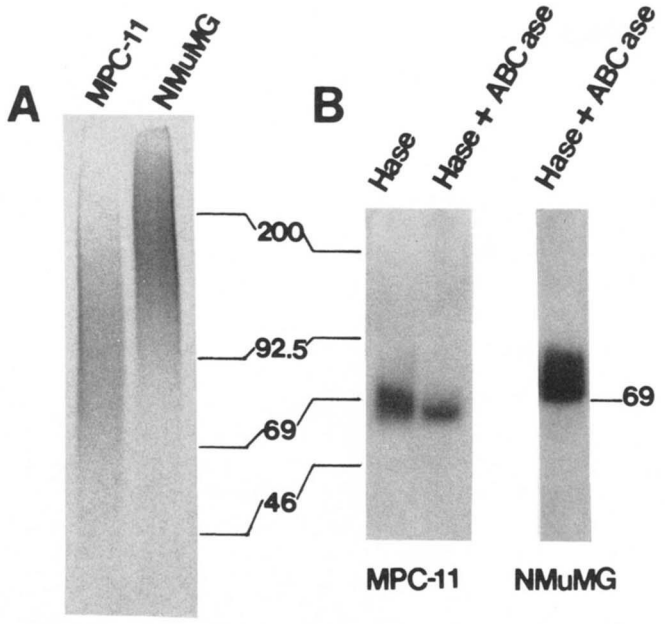

Figure 1. PAGE analysis of syndecan from MPC-11 cells. *A*, The trypsin released $^{35}SO_4$-labeled syndecan was purified by DEAE and 281–2 affinity chromatography, run on SDS-PAGE and visualized by autoradiography as described in *Materials and Methods*. 281–2 is a mAb that binds with high affinity to the core protein of syndecan (30). For comparison, syndecan from NMuMG epithelial cells is included. *B*, To determine the size of the core protein of syndecan, MPC-11, and NMuMG cells were extracted with detergent, and the syndecan purified as above. Glycosaminoglycan chains were removed by treatment with heparitinase (*Hase*) or heparitinase and chondroitinase ABC (*Hase + ABCase*). Samples were then run on SDS-PAGE, blotted on to a cationic nylon membrane (Genetrans, Plasco, Inc., Woburn, MA) and bound syndecan was detected with ^{125}I-labeled 281–2 antibody.

TABLE I

MPC-11 cell syndecan is localized predominantly at cell surface[a]

	Syndecan (cpm ± SD)	Percent of Total Syndecan
Trypsin released	81,929 ± 5,062	97.9
Residual	1,728 ± 449	2.1
Total	83,657 ± 4,640	100.0

[a] ^{35}Sulfate-labeled syndecan from 2×10^7 cells was released by mild trypsin treatment. Remaining intact cells (more than 98% viable as determined by Trypan Blue exclusion) were extracted with Triton X-100 to release remaining syndecan. Further extraction of the Triton X-100 insoluble pellet with Gdn HCl released no detectable syndecan (not shown). The syndecan present in each fraction was isolated with 281.2 beads and quantitated by scintillation counting. Values represent mean of triplicate samples ± SD.

beads. More than 97% of the total syndecan present on MPC-11 cells is released by trypsin and <3% remains and is released by detergent extraction of the trypsinized cells (Table I), indicating that syndecan is localized predominantly on the surface of these cells.

Thus, the molecular size, glycosaminoglycan composition, and cellular localization of syndecan on MPC-11 cells is almost identical to that of normal plasma cells.

Syndecan on MPC-11 (B lymphoid) cells differs from syndecan on NMuMG (epithelial) cells in molecular size and glycosaminoglycan composition. Because the structure and function of syndecan from NMuMG cells has been well characterized (27), we compared the molecular composition of syndecan from MPC-11 cells to that of NMuMG cells. Intact MPC-11 syndecan is substantially smaller in molecular mass than NMuMG syndecan (Fig. 1*A*). Comparison of the residual core proteins after degradation of the intact molecule with heparitinase and chondroitinase ABC indicates that the MPC-11 core protein is slightly smaller than the NMuMG core protein (apparent mass 62 vs 69 kDa, respectively; Fig. 1*B*) (26). Analysis of heparan sulfate chain size demonstrates that MPC-11 heparan sulfate chains are considerably smaller than NMuMG heparan sulfate chains (approximately 17 vs 36 kDa, respectively; Fig. 2). In addition, although MPC-11 syndecan contains very little chondroitin sulfate (Fig. 1*B*), previous studies have shown that NMuMG syndecan contains an average of two chondroitin sulfate chains of 17 kDa (26). Therefore, the molecular composition of MPC-11 syndecan is distinct from that of NMuMG syndecan due predominantly to differences in glycosaminoglycan chain size and amount.

Syndecan is the only major heparan sulfate proteoglycan detected on MPC-11 cells. Before examining the role of syndecan in MPC-11 cell adhesion, it was necessary to determine if other heparan sulfate proteoglycans were present on these cells that could participate in heparan sulfate-mediated interactions with ligands. $^{35}SO_4$-labeled detergent extracts of MPC-11 cells were subjected to DEAE chromatography in the presence of 6 M urea at pH 6.0 and bound material was eluted with 2 M NaCl. Analysis of DEAE bound and unbound fractions by cetylpyridinium chloride-TCA precipitation indicated that more than 92% of the heparan sulfate proteoglycan present in the Triton extracts binds to DEAE under these conditions (data not shown). After elution, the DEAE bound fraction was treated with chondroitinase ABC to degrade chondroitin sulfate chains, and 281.2 positive and 281.2 negative fractions were obtained and subjected to Sepharose CL-4B chromatography (Fig. 3). Syndecan eluted as a single peak with a K_{av} of 0.57. Only a small

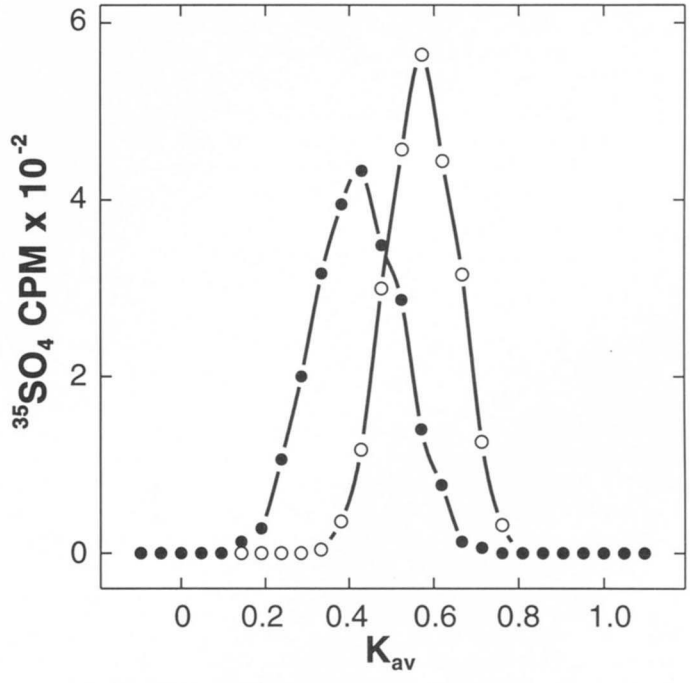

Figure 2. Sepharose CL-6B chromatography of syndecan heparan sulfate chains from MPC-11 (○) and NMuMG (●) cells. Chondroitin sulfate was removed from purified syndecan by treatment with ABCase and the remaining intact heparan sulfate chains released from the core protein with alkaline borohydride and chromatographed on Sepharose CL-6B. The resulting elution positions of the heparan sulfate chains were compared to a molecular size calibration curve generated with chondroitin sulfate standards (31). Heparan sulfate chains from MPC-11 and NMuMG syndecan have an estimated molecular mass of 17 and 36 kDa, respectively.

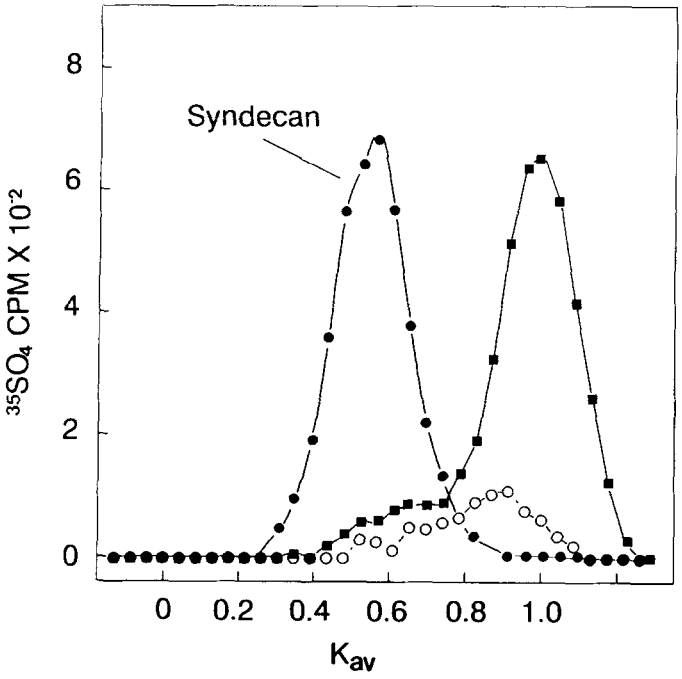

Figure 3. Sepharose CL-4B chromatography of $^{35}SO_4$-labeled extracts from MPC-11 cells. MPC-11 cells were extracted with buffer containing Triton-X 100 and the extracted material subjected to DEAE chromatography. Material eluted from DEAE was desalted and treated with ABCase to degrade chondroitin sulfate chains. The sample was then incubated with 281-2 beads and the bound (●) and unbound (○) fractions chromatographed on Sepharose CL-4B in 4 M guanidine hydrochloride, pH 5.8, containing 0.1% Triton X-100. In addition, the Triton X-100 insoluble material was further extracted with 4 M guanidine hydrochloride, purified by DEAE chromatography, treated with ABCase, and chromatographed as above (■).

amount of sulfate-labeled material is present in the 281.2 negative fraction, most of which eluted in a peak at K_{av} 0.92. Treatment of this fraction with alkaline borohydride does not alter its elution position on 4B chromatography suggesting that it is composed of free heparan sulfate not bound to a core protein (not shown). The remaining sulfated material eluting between K_{av} 0.5 and 0.8 is less than 5% of the total detergent extractable proteoglycan. Therefore, more than 95% of the detergent extractable heparan sulfate proteoglycan that binds to DEAE is syndecan. To determine if any heparan sulfate proteoglycans remained insoluble after Triton X-100 extraction, the Triton insoluble pellet was extracted with Triton buffer containing 4 M guanidine hydrochloride. After dilution of the extract, concentration of proteoglycans with DEAE beads and enzymatic degradation of chondroitin sulfate chains, a single major peak close to the V_t of the 4B column is detected, likely representing free glycosaminoglycan fragments of degraded chondroitin sulfate. Minor peaks, similar to those found in the samples of syndecan negative detergent extracts, elute between K_{av} 0.5 and 0.8. These results demonstrate that syndecan is the only major heparan sulfate proteoglycan detected after extraction of MPC-11 cells. Furthermore, incubation of $^{35}SO_4$-labeled cells with 20 mM heparin fails to release detectable $^{35}SO_4$-labeled material, indicating that these cells do not have sulfated molecules that associate with their cell surfaces via heparan sulfate chains (data not shown).

Syndecan from MPC-11 cells binds to type I collagen. To evaluate syndecan binding to matrix ligands, dot blots were prepared by aspirating matrix molecules onto nitrocellulose filters then incubating these filters with purified $^{35}SO_4$-labeled syndecan (Fig. 4). Syndecan from MPC-11 cells binds to type I collagen in a concentration-dependent fashion, but does not bind to type IV collagen, laminin, or fibronectin in this assay. Binding to type I collagen is completely inhibited by treatment of syndecan with heparitinase before the assay. Interestingly, syndecan heparan sulfate chains, free of core protein, also bind poorly to collagen suggesting that intact syndecan is required for high affinity binding of syndecan to type I collagen.

In the same experiment, syndecan from NMuMG cells apparently bound better to type I collagen than did syndecan from MPC-11 cells, suggesting that different molecular forms of syndecan have different affinities for collagen. To confirm this, the technique of affinity coelectrophoresis was used to measure the affinity of NMuMG and MPC-11 syndecan for type I collagen. In this technique, purified radiolabeled proteoglycan is electrophoresed through an agarose gel that contains a known concentration of protein ligand (33). Because at neutral pH the electrophoretic mobility of most proteins is much lower than those of proteoglycans, the interaction of proteoglycan and protein retards the proteoglycan mobility. An apparent affinity constant can then be arrived at by plotting the shift in proteoglycan mobility, R, vs the protein concentration (34). As measured by this technique, NMuMG syndecan has a greater than fourfold higher affinity for type I collagen than does MPC-11

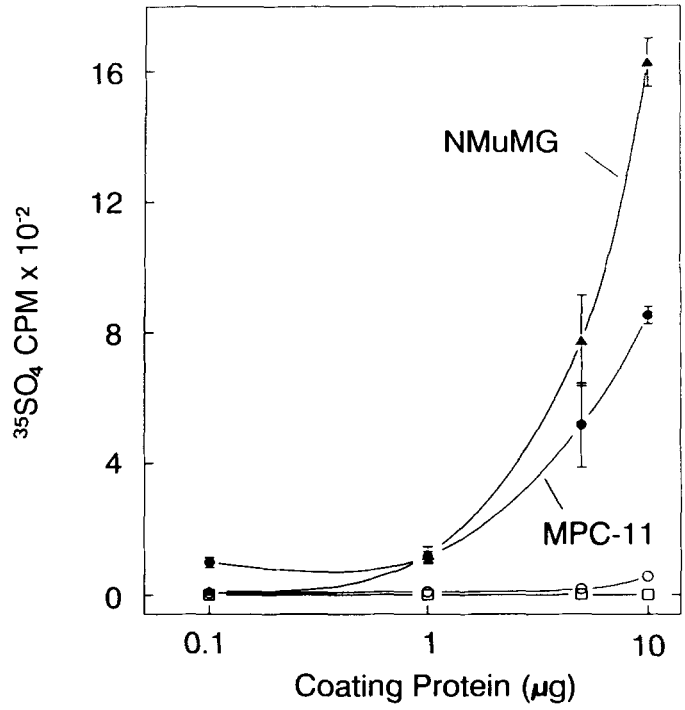

Figure 4. Purified syndecan binds to type I collagen in a solid phase dot assay. Trypsin-released syndecan from $^{35}SO_4$-labeled MPC-11 cells was purified by DEAE and 281-2 affinity chromatography and incubated with type I collagen immobilized on nitrocellulose. Intact syndecan binds to type I collagen (●), but syndecan glycosaminoglycan chains generated by alkaline borohydride treatment of intact syndecan bind poorly (○). For comparison, NMuMG syndecan binding to type I collagen is also shown (▲). No cpm above background were detected for intact MPC-11 syndecan binding to type IV collagen, laminin, or fibronectin or for heparitinase-treated syndecan binding to type I collagen; for clarity, these are all represented by the same symbol (□). Bound syndecan was quantitated by liquid scintillation counting.

syndecan (Fig. 5; apparent K_d of 31 and 143 nM, respectively).

The K_d of 31 nM for NMuMG-derived syndecan binding to collagen that we report is different from the value of 0.77 to 2.8 nM previously reported by Koda et al. (12). In our experiments, syndecan concentration was held constant and collagen concentration was varied, while in Koda's experiments, collagen concentration was held constant and syndecan concentration was varied. Only if syndecan and collagen interact by simple one-to-one binding would Koda's and our procedures be expected to give identical values. In fact, it is likely that collagen and syndecan are multivalent in their interaction with each other. For example, the difference between the value of 31 nM reported here and the 0.77 to 2.8 nM reported by Koda might be an indication that each syndecan molecule has 10 to 40 potential sites for collagen binding. This is certainly possible given the fact that each syndecan molecule has multiple glycosaminoglycan chains and that each chain likely has multiple sulfated sites capable of binding to collagen.

Syndecan binds MPC-11 cells to type I collagen. A qualitative cell binding assay was used to determine if syndecan mediates the binding of MPC-11 cells to type I collagen. In this assay, cells are introduced into microtiter wells coated with type I collagen (12). After a 30-min incubation, plates are gently centrifuged causing unbound cells to form a distinct visible pellet, while bound cells do not pellet and remain as a uniform coating over the surface of the well. This assay demonstrates that MPC-11 cells bind to collagen but not to control wells coated with BSA (Fig. 6, *A* and *B*). Binding to collagen is significantly reduced by pretreatment of collagen-coated wells with 10 μM heparin (Fig. 6*C*), or degradation of heparan sulfate chains on cell surfaces before their introduction into wells (Fig. 6*D*). Removal of chondroitin sulfate chains from cell surfaces only slightly inhibited cell adhesion to type I collagen (Fig. 6*E*).

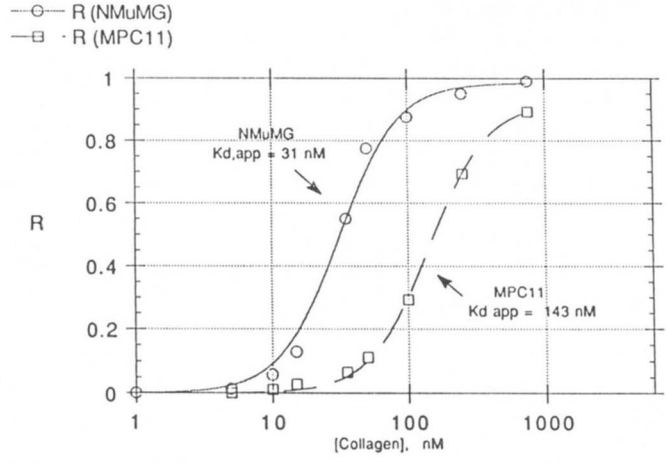

Figure 5. Syndecans from NMuMG and MPC-11 cells have different affinities for type I collagen. Trypsin-released syndecan from $^{35}SO_4$-labeled cells was purified and binding to type I collagen analyzed by affinity coelectrophoresis as described in *Materials and Methods*. Data are presented as plots of the normalized shifts in syndecan mobility, R, vs the collagen concentration. Calculated values of K_d are shown, and were calculated using nonlinear least-squares fits to the equation: $R = R_{max}/[1 + (K_d/[C_{tot}]^n)]$, which derives from the substitution of R for the fraction of syndecan bound into $K_d = [C_{tot}]^n[S]/[C_nS]$, which is the definition of the equilibrium constant under conditions where one component (S, syndecan) has the potential to bind up to "n" ligands (C, collagen) at a time, and where $[S_{tot}] << K_d$ (33, 34). Best fits were obtained with a value of $n = 2$, suggesting that some positive cooperativity may occur in the binding of syndecan to collagen (similar behavior has been observed with the binding of heparin to type I collagen; J. D. San Antonio and A. D. Lander, unpublished observations).

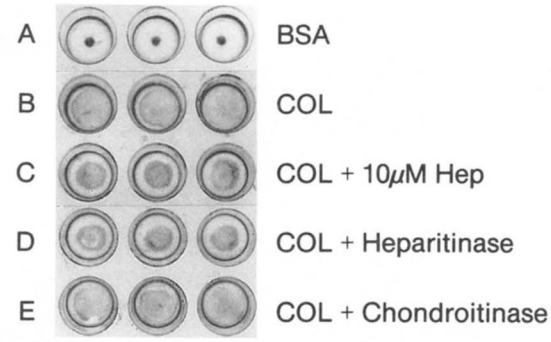

Figure 6. Syndecan binds MPC-11 cells to type I collagen. Microtiter wells were coated with BSA (*A*) or type I collagen (*COL*; *B-E*). After incubation of cells within wells for 30 min, the plate was gently centrifuged. Bound cells form a uniform coating on the well surface, unbound cells form a pellet in the center of the well. Cells bind to type I collagen (*B*) but not BSA (*A*). Incubation of collagen-coated wells with 10 μM heparin before addition of cells abolishes cell binding to collagen (*C*). Treatment of cells with heparitinase before their introduction into wells also inhibits cell binding (*D*), but treatment with chondroitinase ABC has little effect (*E*).

TABLE II

Binding of MPC-11 cells to type I collagen is mediated by syndecan heparan sulfate chains[a]

Coating	Pretreatment	Bound cpm	Percent Cells Bound	Percent Inhibition
BSA	None	1,990 ± 314	7.7 ± 1.3	
Collagen	None	14,923 ± 290	71.3 ± 3.1	
Collagen	Heparin	2,279 ± 255	8.4 ± 0.8	88.2
Collagen	Heparitinase	2,096 ± 205	7.5 ± 0.7	89.5
Collagen	ABCase	15,061 ± 2987	63.0 ± 7.1	11.6

[a] This assay was performed exactly as was the qualitative cell binding assay in Figure 6, except ^3H-labeled cells were used and after incubation of cells in the wells, plates were not centrifuged, but placed on a shaker, washed two times with PBS and bound cpm were determined by liquid scintillation counting. Results are presented as mean values ± SE for quadruplicate wells.

To compliment these qualitative data, we performed quantitative assays to determine the extent of inhibition of cell binding after incubation of collagen with heparin or pretreatment of cells with heparitinase. These assays were performed exactly as the qualitative assay above, except ^3H-labeled cells were used and instead of centrifuging plates, wells were washed, and remaining radioactivity was measured on bound cells. These assays demonstrate that pretreatment of collagen with heparin or pretreatment of cells with heparitinase reduces cell binding by 88.2 and 89.5%, respectively (Table II). Interestingly, pretreatment of cells with chondroitinase ABC results in an 11.6% inhibition of binding, suggesting that chondroitin sulfate may participate in MPC-11 cell adhesion to collagen.

To further analyze syndecan-mediated binding of MPC-11 cells to type I collagen, cell binding assays were performed using cells that had been grown in culture media containing various concentrations of sodium chlorate, a competitive inhibitor of sulfation (36–38). Cells grown without chlorate or with only 1 mM chlorate bind to collagen (Fig. 7, *B* and *C*). However, as chlorate concentration is increased, cell binding to collagen decreases with binding inhibited at a concentration of 10 mM chlorate or higher (Fig. 7, *D-F*). Inclusion of 10 mM sodium sulfate in cultures containing 20 mM chlorate abolishes the negative effect of chlorate on cell binding to collagen

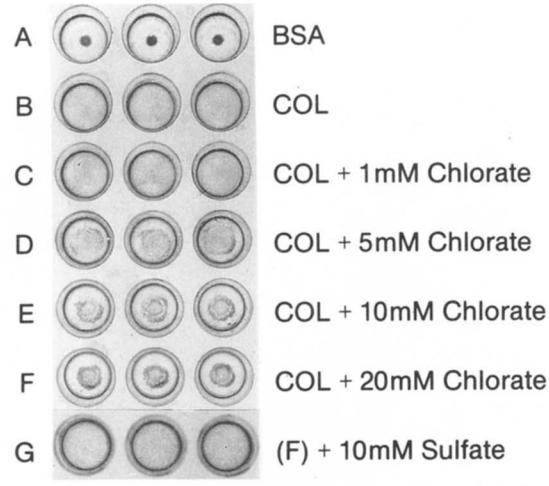

Figure 7. MPC-11 cells grown in the presence of chlorate fail to bind to type I collagen. Cells grown in the absence of chlorate bind type I collagen (*B*) but this binding is decreased as the concentration of chlorate in the growth media is increased (*C-F*). Addition of 10 mM sodium sulfate simultaneously with 20 mM chlorate reverses the inhibitory effect of chlorate on cell binding (*G*). Cells were grown in growth media containing the indicated concentrations of sodium chlorate for 24 h before the assay.

TABLE III

Binding of MPC-11 cells to type I collagen is inhibited by reducing level of syndecan sulfation[a]

mM Chlorate	Bound cpm	Percent Cells Bound	Percent Inhibition
0	21,433 ± 454	69.9 ± 10.9	
1	20,632 ± 1065	73.0 ± 9.3	
5	15,095 ± 1818	49.0 ± 5.3	29.9
10	5,957 ± 527	12.1 ± 0.9	82.7
20	3,769 ± 265	10.7 ± 1.3	84.7
20 + 10 mM Na_2SO_4	15,440 ± 728	56.8 ± 5.3	18.7

[a] Cells grown in the presence of chlorate were prepared and assayed exactly as in the qualitative assay in Figure 7, except ^{3}H-labeled cells were used and after incubation of cells in the wells, plates were not centrifuged, but placed on a shaker, washed two times with PBS and bound cpm were determined by liquid scintillation counting. Results are presented as mean values ± SE for quadruplicate wells.

(Fig. 7*G*). Quantitative assays confirm these observations and indicate that inclusion of 10 mM chlorate inhibits cell binding by more than 80% of control levels (Table III).

To confirm that loss of cell adhesion is due to a reduction in syndecan sulfate content and not the result of chlorate-induced changes in syndecan abundance or size, we analyzed syndecan from MPC-11 cells after their growth in the presence of chlorate. Quantitative studies using ^{125}I-labeled antibody 281-2 indicate that the amount of syndecan at the cell surface is not significantly altered at a concentration of 10 mM chlorate, and reduced by only 12.1% at a concentration of 20 mM chlorate (Table IV). In addition, syndecan size is not altered dramatically by growth of cells in the presence of chlorate, although syndecan from cells not treated with chlorate elutes at a slightly higher K_{av} on Sepharose CL-4B columns than do chlorate-treated cells (Fig. 8). Although there is little change in the abundance or size of syndecan, the relative amount of sulfate to glucosamine decreases dramatically with increasing concentrations of chlorate, indicating a significant reduction in the level of syndecan sulfation on cells grown in chlorate (Fig. 8). As expected, this reduction in sulfation alters the polyanionic properties of syndecan (Fig. 9). Syndecan isolated from cells grown in the presence of chlorate elutes

TABLE IV

Quantitation of cell surface syndecan on MPC-11 cells grown in presence of chlorate[a]

mM Chlorate	cpm Radioactivity Bound	Percent Inhibition
0	23,486 ± 584	
10	23,431 ± 2110	0.2
20	20,641 ± 505	12.1

[a] Cultures were seeded at 2×10^5 cells/ml in media containing varying concentrations of chlorate. Then 18 h after seeding, cells were harvested by centrifugation, washed, and incubated at 4°C for 30 min with ^{125}I-281-2. Cells were then washed and bound radioactivity was determined by gamma counting. Values represent mean of triplicate samples ± SD, each sample contained 1.5×10^5 cells. Treatment of cells with trypsin to remove syndecan before incubation with ^{125}I-281-2 decreased cell surface bound radioactivity more than 90% (not shown).

from DEAE columns at a lower NaCl concentration than cells grown in the absence of chlorate. Thus, chlorate causes a reduction in syndecan sulfation with a resulting decrease in charge density but does not dramatically effect syndecan size or quantity at the cell surface. Therefore, the changes seen here in MPC-11 adhesion to type I collagen after growth of cells in chlorate are likely due to reduced sulfation of syndecan heparan sulfate.

DISCUSSION

Normal B cell differentiation is dependent on the timely expression of functional receptors that mediate the adhesion of B cells to other cells and to the extracellular matrix. Previous studies indicate that cell surface heparan sulfate proteoglycans play a role in these adhesive interactions (7–10), but the identity and function of the specific proteoglycans involved has remained obscure. Recently, it was discovered that the cell surface heparan sulfate proteoglycan, syndecan, is present on B cells at specific stages during their differentiation. Syndecan is expressed on pre-B and immature B cells in the bone marrow, absent on peripheral B cells in the blood and secondary lymphoid organs and reexpressed on plasma cells (11). Therefore, syndecan is expressed on B cells only when and where they associate with extracellular matrix, leading to the hypothesis that syndecan acts to bind B cells to molecules of the extracellular matrix. We now demonstrate that syndecan mediates adhesion of B lymphocytes to type I collagen. Taken together these data suggest that in early stages of B cell development, syndecan binds cells to interstitial marrow stroma, is lost with cell maturation as a prerequisite for release of cells from marrow and reexpressed on plasma cells to anchor these cells within interstitial matrices. Thus, precise regulation of syndecan expression may be required for normal B cell differentiation and maturation into Ig-secreting plasma cells.

Cell binding assays demonstrate that syndecan mediates binding of MPC-11 cells to type I collagen via heparan sulfate (Fig. 6; Table II). This binding is dependent on syndecan heparan sulfate chains because removal of heparan sulfate or pretreatment of collagen with heparin inhibits binding of cells to collagen. Furthermore, growth of cells in sodium chlorate, an inhibitor of sulfation, inhibits cell binding to collagen in a dose-dependent fashion, indicating that adequate sulfation is required for syndecan-mediated adhesion of cells to collagen (Fig. 7; Table III). This inhibition in cell binding is not due to changes in syndecan amount or size at the cell surface because at a concentration of 10 mM chlorate, cell bind-

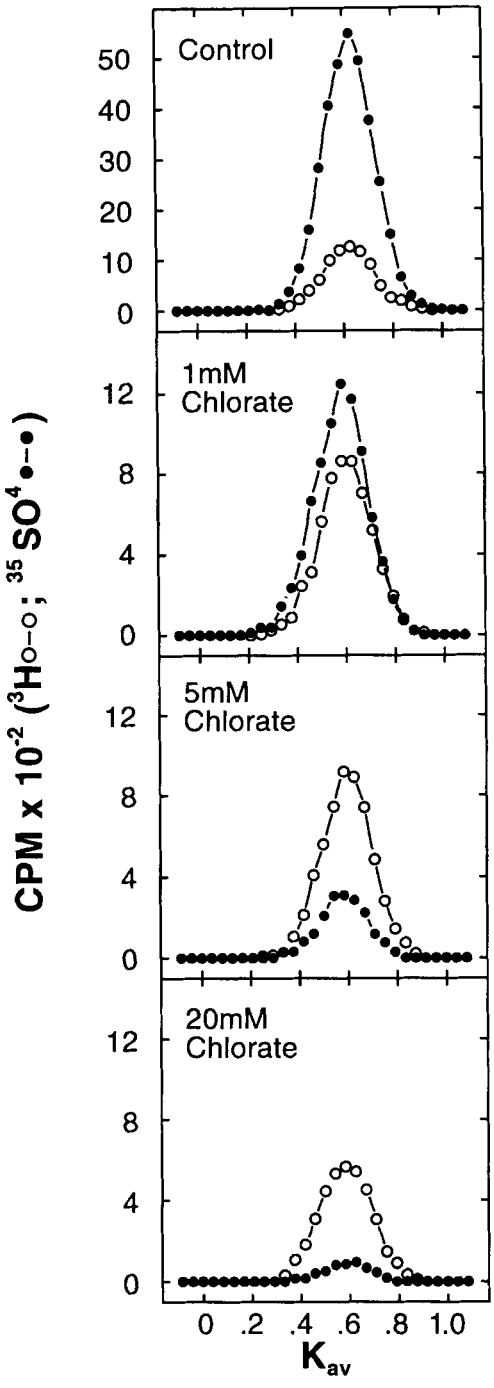

Figure 8. The level of syndecan sulfation is reduced when MPC-11 cells are grown in the presence of chlorate. Syndecan labeled with [^{3}H] glucosamine and $^{35}SO_4$ was purified from cells grown in the presence of the indicated concentrations of chlorate and chromatographed on Sepharose CL-4B. Elution profiles demonstrate a decrease in the level of syndecan sulfation as chlorate concentration is increased. The size of syndecan is not altered as chlorate concentration is increased; however, syndecan from cells grown in the absence of chlorate (control) migrates at a slightly higher K_{av} than does syndecan from chlorate-treated cells.

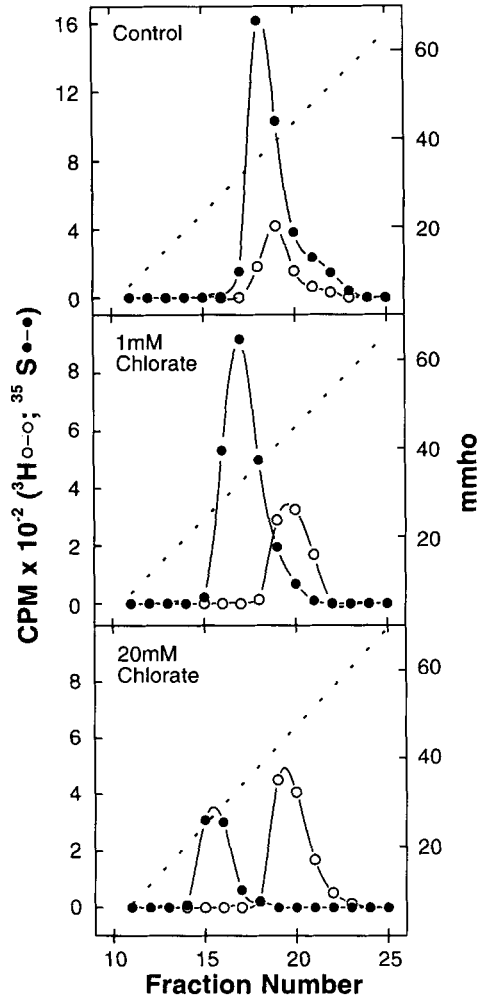

Figure 9. Syndecan charge density is reduced on cells grown in the presence of chlorate. Syndecan was isolated from MPC-11 cells grown in the presence of the indicated concentrations of chlorate, applied to a DEAE column in buffer containing 0.05 M NaCl, and eluted with a 0.05 to 1.0 M NaCl gradient. Syndecan isolated from the chlorate-treated MPC-11 cells (●) elutes at a lower NaCl concentration than does syndecan from untreated (control) cells. As an internal standard, [^{3}H]glucosamine-labeled syndecan purified from NMuMG cells grown in the absence of chlorate was added to each sample just before chromatography (○).

ing is inhibited, but the amount and size of syndecan detected at the cell surface is similar to untreated controls (Table IV; Fig. 8). Also, the dramatic inhibition of cell binding after chlorate treatment is not due to changes in sulfation of cell surface chondroitin sulfate because removal of chondroitin sulfate from cells only slightly inhibits cell binding (Table II). On MPC-11 cells, syndecan is the only major heparan sulfate proteoglycan detected (Fig. 3) and more than 97% of detectable syndecan is at the cell surface (Table I). Thus, the changes seen in cell binding to collagen after removal or alteration of cell surface heparan sulfate can likely be attributed to changes in syndecan heparan sulfate.

As measured by affinity coelectrophoresis, NMuMG syndecan has a more than fourfold higher affinity for type I collagen than does MPC-11 syndecan (Fig. 5). Thus, we have clearly demonstrated that tissue or cell-type specific molecular forms of syndecan can have distinct affinities for collagen. Our analysis of syndecan molecular composition demonstrates distinct differences between NMuMG and MPC-11 syndecan that are likely responsible for their difference in affinity for type I collagen. A typical syndecan molecule from NMuMG cells is composed of a core protein with an apparent size of 69 kDa, two heparan sulfate chains of approximately 36 kDa (Fig. 2) and two chondroitin sulfate chains of 17 kDa for a total molecular mass of approximately 175 kDa (26). Intact syndecan from MPC-11 cells is much smaller than syndecan from NMuMG cells with the typical molecule having two heparan sulfate chains of approximately 17

kDa, and an apparent core protein size of 62 kDa, for a total of 96 kDa, close to the median size of MPC-11 syndecan observed in Figure 1. The molecular basis for the difference in NMuMG and MPC-11 syndecan affinity for collagen is unknown, but previous studies suggest that heparan sulfate affinity for ligands can depend on specific heparan sulfate sequences (39, 40), degree of sulfation (41, 42), chain size (41, 42), or a combination of these. In addition, we cannot rule out the possibility that the higher chondroitin sulfate content of NMuMG syndecan contributes to the differences in NMuMG and MPC-11 affinity for type I collagen, although this is unlikely because removal of chondroitin sulfate from NMuMG syndecan only slightly reduces its binding to type I collagen fibrils (43).

The glycosaminoglycan content of syndecan may also determine syndecan affinity for matrix ligands other than collagen. For instance, in solid phase assays, NMuMG-derived syndecan binds fibronectin (13, 44), but MPC-11-derived syndecan does not (Fig. 4). Similarly, tenascin binds well to syndecan derived from mesenchymal tissues but binds weakly to NMuMG-derived syndecan (44).

The cell type-specific differences in syndecan affinity for matrix ligands seen here may contribute to the different characteristic cell behaviors of simple epithelia and B lymphocytes. Simple epithelia form stable monolayers, bind tightly to matrix and interact with matrix solely at their basal cell surfaces. In contrast, B lymphocytes successively alter their adhesive interactions to facilitate movement to specific tissue locations, remain rounded, and migrate through extracellular matrices. Thus, syndecan with a high affinity for interstitial matrix molecules may be required for maintenance of a rigid epithelial morphology, although syndecan with a lower affinity for matrix may allow an attachment to matrix that is amenable to cell migration. This notion is consistent with the finding that monolayer keratinocytes, which express a 160-kDa form of syndecan, begin expressing a 110-kDa form of syndecan when cells are induced to stratify and migrate upward)[4]. A similar reduction in syndecan size is also seen when NMuMG cells become transformed, lose their monolayer morphology, and pile up in culture (45).

Syndecan may be the major collagen receptor expressed by B cells. Of the other adhesion molecules that are closely regulated during B cell differentiation including integrins (2), Hermes/CD44 (3), Ig superfamily adhesion receptors (4), and selectins (5), apparently only integrins and CD44 interact with extracellular matrix molecules. The integrins primarily bind to fibronectin, although there is some suggestion that the collagen-binding integrin VLA-3 is present in low concentration on some leukocytes (2). However, lymphocytes appear to be mostly negative for VLA-3 (46). CD44 is a receptor for both cell surface and extracellular hyaluronate (47) and can also bind collagen (48, 49). However, antibodies to CD44 do not inhibit cell binding to collagen (48), making it unlikely that CD44 plays a significant role in binding B cells to collagen. Therefore, the association of differentiating B cells with collagen may be predominantly via interaction with syndecan.

[4] **R. D. Sanderson, M. T. Hinkes, and M. Bernfield.** *Submitted for publication.*

Binding of B cells to extracellular matrix appears to be mediated by at least two classes of receptors, syndecans, which bind collagen, and integrins, which bind fibronectin. Interestingly, the pattern of expression of these two types of receptors differs during B cell differentiation in the bone marrow. Syndecan is expressed on pre-B cells but lost as cells mature before their release into the circulation (11). In contrast, two distinct molecular forms of integrins are expressed, the $\alpha_5\beta_1$ form that is present early in differentiation then lost, followed by the $\alpha_4\beta_1$ form that remains on B cells even after they exit the bone marrow and enter the circulation (50–52). These observations imply that adhesion of B cells to collagen and fibronectin is regulated by distinct mechanisms and indicates that the interaction of B cells at specific stages of their differentiation with specific matrix macromolecules may be of functional significance.

Finally, the role of syndecan in B cell differentiation may not be limited solely to adhesion. Heparan sulfate can bind hematopoietic growth factors (53, 54) and the recent demonstration of the direct involvement of heparan sulfate in growth factor-mediated cell signaling (20) raises the possibility that syndecan performs a similar function on B cells. Epithelial syndecan binds basic fibroblast growth factor (18, 19), similarly B cell syndecan may bind hematopoietic growth factors. For instance, the pre-B cell growth factor, IL-7, binds to heparan sulfate and addition of heparan sulfate to culture media inhibits proliferation of IL 7-dependent B and T lymphocyte cell lines (55). Syndecan may compete with the high affinity IL-7 receptor for soluble IL-7 or alternatively participate in IL-7-mediated cell signaling. Thus, it is possible that syndecan plays a multifunctional role in B lymphocyte differentiation by mediating adhesion of cells to matrix and by binding to hematopoietic growth factors.

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