Expression of Syndecan Regulates Human Myeloma Plasma Cell Adhesion to Type I Collagen

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The syndecans comprise a family of integral membrane proteoglycans that regulate cell behaviors by binding to extracellular matrix and binding growth factors. In mouse blood cells, syndecan expression is restricted to cells of the B-cell lineage where it is expressed by pre-B cells and plasma cells, but is absent from circulating B cells. In the present study, we examined the expression, structure, and function of syndecan on human myeloma cell lines and myeloma patient bone marrow cells. On myeloma cells, syndecan is a small (modal relative molecular mass [Mr] ~ 120 Kd) heparan sulfate proteoglycan localized at the cell surface. Syndecan was detected by immunodot blotting on 7 of 10 human myeloma cell lines and by reverse transcriptase polymerase chain reaction on 10 of 14 patient samples. Cell binding assays show that myeloma cells expressing syndecan bind to type I collagen via heparan sulfate chains, while those cell lines not expressing syndecan do not bind to collagen. Furthermore, the cell lines expressing syndecan were negative for CD19 and CD45 staining, indicating that syndecan expression is restricted to tumors having a well-differentiated phenotype. We conclude that syndecan acts as a matrix receptor on human myeloma cells but is not expressed by all tumors, suggesting that syndecan may participate in regulating myeloma cell adhesion to the bone marrow stromal matrix.

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mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. For radiolabeling, $5 \times 10^5$ cells/mL were placed in fresh complete media containing $50 \mu$Ci/mL Na$^{35}$SO$_4$ (ICN Biomedicals Inc, Costa Mesa, CA) for 24 hours.

The myeloma cell lines ark, ram, ard-I, mer, and col were established from peripheral blood mononuclear cells (col) or from Histopaque-1077 (Sigma, St Louis, MO) separated BM aspirates obtained from myeloma patients. All cell lines express the same monoclonal Ig genes as the original myeloma cells. ARH77 and U266 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD), U266 from Dr K. Nickson, RPMI 8226 (8226) from Dr W. Dalton (University of Arizona) and clone-2-positive and clone-2-negative cells from Dr Thomas M. Grogan (University of Arizona). None of the cell lines used require the addition of exogenous IL-6 for growth, with the exception of ard-I cells.

**Isolation of syndecan and analysis by Sepharose CL-4B chromatography.** Cells were washed three times in ice-cold phosphate-buffered saline (PBS), followed by extraction for 1 hour on ice in 10 mmol/L Tris pH 7.4, containing 1% Triton X-100, 0.15 mol/L NaCl, 5 mmol/L N-ethylmaleimide, 5 mmol/L benzamidine, 5 µg/mL pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride. Extracts were centrifuged for 15 minutes at 16,000g; supernatants brought to 6 mol/L urea, 50 mmol/L sodium acetate pH 4.5, boiled, and centrifuged again at 16,000g for 15 minutes. DEAE Sepharose beads (100 µL of beads/10^7 cell equivalents; Pharmacia, Piscataway, NJ) were added to the supernatants, and the mixture placed on a rocker for 1 hour at room temperature. DEAE beads were pelleted by gentle centrifugation (240g for 2 minutes) placed in a clean 0.5 mL microcentrifuge tube, washed four times with PBS by centrifugation, and eluted with PBS containing a final NaCl concentration of 1 mol/L. The DEAE eluates were brought to a final concentration of 0.15 mol/L NaCl by dilution with 10 mmol/L Tris pH 7.4 buffer and chondroitin sulfate chains degraded by the addition of 0.05 U/mL of ABCase (Seikagaku, Rockville, MD) and incubation for 45 minutes at 37°C, followed immediately by addition of another 0.05 U/mL of ABCase and incubation for an additional 45 minutes. Samples were boiled for 10 minutes to destroy remaining enzyme, cooled on ice, and concentrated on DEAE beads. The bound heparan sulfate proteoglycans were eluted from beads by boiling in 4 mol/L GdnHCl containing 50 mmol/L sodium acetate, pH 5.8 and 0.1% Triton X-100 (buffer A). 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 A. Fractions were collected and assayed by liquid scintillation counting.

**Immunodot blots.** Fractions from the Sepharose CL-4B column were diluted to a final concentration of 0.15 mol/L guanidine HCl with 10 mmol/L Tris, pH 7.4 buffer containing 0.1% Triton X-100. For analysis of syndecan expression on cell lines, syndecan was extracted with detergent and brought to 6 mol/L urea, 50 mmol/L sodium acetate pH 4.5 as described above. Samples were loaded on to Genetran (Plasco Inc, Woburn, MA), a cationic nylon membrane using an immunodot apparatus (Milliblot D; Millipore, Bedford, MA). Membranes were removed from the apparatus and remaining binding sites were blocked for 1 hour with a solution containing 3% Carnation instant nonfat dry milk (Carnation, Los Angeles, CA), 0.5% bovine serum albumin (BSA), 10 mmol/L Tris, pH 8.0, 0.15 mol/L NaCl, and 0.3% Tween-20 (solution B). Blots were probed with immune serum overnight at 4°C followed by washing for 60 minutes with five changes of 10 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl, 0.3% Tween-20, 1% bovine serum (solution C), and incubation with 5 µg/mL biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) in solution C for 1 hour at room temperature. Membranes were washed for 60 minutes with five changes of PBS followed by incubation for 30 minutes at room temperature with Vector ABC reagent coupled

**MATERIALS AND METHODS**

**Cell culture.** Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 0.05 mol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. For radiolabeling, $5 \times 10^5$ cells/mL were placed in fresh complete media containing 50 µCi/mL Na$^{35}$SO$_4$ (ICN Biomedicals Inc, Costa Mesa, CA) for 24 hours.
The sample was digested with detergent, and the extracts incubated with DEAE beads at pH 4.5. Bound proteoglycan was eluted with 1 mol/L NaCl, dialyzed against PBS, and a portion of the sample was digested with heparitinase. After separation by SDS-PAGE and transfer to a cationic nylon filter, the intact or heparitinase digested syndecan was detected using an antisera prepared against a 7-amino acid synthetic peptide corresponding to the highly conserved COOH terminus of syndecan core protein.

Syndecan analysis by flow cytometry. Cells were prepared by incubation with a 1:200 dilution of anti-syndecan (anti-extracellular domain antiserum) or, as a control, a 1:200 dilution of normal rabbit serum. Sorted cells contained only myeloma cells as determined morphologically, immunocytochemically (concordant, monoclonal clg content), and by differences in the patterns of cytokine gene expression (IL-6, IL-1β, tumor necrosis factor-α [TNF-α]) between the purified cells and those of the simultaneously sorted monocytes and other nonmyeloma hematopoietic cells.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using a microadaptation of the guanidinium thiocyanate/cesium chloride procedure. Briefly, cell pellets containing 1 to 10 × 10⁶ purified myeloma and control cells were resuspended in 100 μL of 4 mol/L guanidinium thiocyanate, vortexed for 1 to 2 minutes, layered over a 100-μL cushion of 5.7 mol/L cesium chloride in a polycarbonate tube, and centrifuged at 80,000 rpm for 2 hours at 20°C in a refrigerated tabletop ultracentrifuge (TL-100, Beckman, Fullerton, CA). The RNA pellet was reuspended in 50 μL DEPC-treated water, precipitated with ethanol, dried, and resuspended in 10 μL cDNA synthesizing solution. First-strand cDNA was synthesized using Moloney murine leukemia virus (MMV) GIBCO BRL, Grand Island, NY) reverse transcriptase and oligo dT (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 42°C. After inactivation of the reverse transcriptase at 95°C for 5 minutes, 20 μL of the reaction mixture was added to the PCR amplification mixture and subjected to 35 cycles of amplification by PCR. The amplification primers used were: 5'-TCTGACAACCTTCTCC-
Wells with 10 mM heparin before addition of cells abolishes cell binding to type I collagen (b through g). After incubation of cells within wells for 30 minutes, the plate was gently centrifuged. Unbound cells form a pellet in the center of the well, at the cell surface. Cells do not bind BSA (a) but do bind to collagen (b). Incubation of collagen coated wells with 10 μM heparin before addition of cells abolishes cell binding to collagen (c). Removal of heparan sulfate from the surface of cells with heparitinase, before their introduction to wells, also inhibits cell binding to collagen (d), but removal of chondroitin sulfate has no detectable effect (e). Cells grown for 24 hours before the assay in media containing 20 mM/L sodium chloride, a competitive inhibitor of proteoglycan sulfation, fail to bind to collagen (f). Control cells grown in media containing 20 mM/L sodium chloride and 10 mM/L sodium sulfate bind collagen (g).

CGGCTC-3' and 5'-CCACTTCTGGCAGGACTACA-3' (expected product size = 210 bp). Temperature cycling was 1 minute at 95°C, 2 minutes at 55°C, followed by 3 minutes at 72°C. Aliquots of 10 μL of the amplification product were analyzed by electrophoresis on 2% agarose gel containing 0.5 μg/mL ethidium bromide.

RESULTS

Biochemical characterization of syndecan on the human myeloma cell line, ark. The newly established cell line designated ark was metabolically labeled with H235SO4 for 24 hours and extracted with detergent to release membrane-bound proteoglycans. Extracts were treated with 6 mol/L urea and 50 mM/L sodium acetate, pH 4.5, and bound to DEAE. Bound material was eluted with 1 mol/L NaCl and diluted with 10 mM/L Tris pH 7.4 to a final concentration of 0.15 mol/L NaCl. The sample was then treated with chondroitinase ABC to digest chondroitin sulfate chains and again bound to DEAE to concentrate the sample. After elution with buffer containing 4 mol/L guanidine hydrochloride, the sample was passed over a Sepharose CL-4B column. High and low molecular weight peaks were detected (Fig 1). Fractions from the column were then diluted to lower the salt concentration, bound to filters using a dot blot manifold, and probed with a 1:500 dilution of antisera prepared against the purified extracellular core protein domain of murine syndecan.35 Dots positive for syndecan correspond with the high molecular weight peak. The low molecular weight peak, which is negative for syndecan, likely represents heparan sulfate glycosaminoglycan not bound to a core protein and/or fragments of chondroitin sulfate glycosaminoglycan generated during the enzyme digestion.

To further assess the molecular mass and glycosaminoglycan composition of syndecan, ark cells were extracted and proteoglycan partially purified using DEAE as described above. Material eluted from DEAE was digested with heparitin sulfate lyase and both intact and digested samples were then subjected to SDS-PAGE and Western blotting. Syndecan was detected with an antisera prepared against a 7-amino acid synthetic peptide corresponding to the predicted COOH terminus present in both mouse and human syndecan. Syndecan migrates as a broad smear that is reduced to a band of approximately 77 Kd after the removal of heparan sulfate (Fig 2). Treatment of samples with both heparan sulfate lyase and chondroitin ABC lyase also showed a band of 77 Kd, suggesting that syndecan on these cells contains little if any chondroitin sulfate (not shown).

To determine if syndecan is localized at the surface of human myeloma cells, we stained ark cells and cells obtained by BM aspiration from a patient whose marrow contained approximately 80% myeloma cells. Analysis by flow cytometry indicates the presence of syndecan on the surface of a high percentage of cells from both the cell line and the patient sample (Fig 3).

Together, these data demonstrate that human myeloma cells express a form of syndecan that is predominantly heparan sulfate, relatively small in molecular mass and present at the cell surface.

Binding of human myeloma cells to type I collagen. Binding of myeloma cells to type I collagen was assessed using a solid-phase assay. In this assay, wells of microtiter plates are coated with collagen and cells are added. After a 30-minute incubation, the plates are gently centrifuged. Bound cells remain as a uniform coating of the well surface, and unbound cells form a pellet in the bottom of the well. Results show that ark cells bind to type I collagen, but this binding can be inhibited by pretreatment of wells with heparin or by removal of heparan sulfate from cell surfaces before their addition to wells (Fig 4, b through g). Removal of chondroitin sulfate does not inhibit binding to collagen (Fig 4e). Furthermore, cells grown in chlorate, an inhibitor of proteoglycan sulfation, do not bind to collagen (Fig 4f). This inhibition of binding of cells grown in chlorate is apparently specific for changes in sulfation of cell surface molecules because inclusion of sodium sulfate along with chlorate abolishes chlorates inhibitory effect (Fig 4g). Thus, binding of ark myeloma cells to type I collagen is mediated by heparan sulfate.

Myeloma cells that do not express syndecan do not bind type I collagen. Expression of syndecan on 10 human myeloma cell lines was examined on dot blots (Fig 5A). Mouse MPC-11 myeloma cells were used as a positive control because they are known to bind type I collagen via syndecan.30 Human lines included both recently established lines (ark, ram, ard-1, mer, col, clone-2-pos, clone-2-neg) and older lines (8226, ARH-77, U266). Syndecan was not expressed by three
SYNDECAN REGULATES MYELOMA CELL ADHESION

Fig 5. Syndecan expression correlates with myeloma cell adhesion to type I collagen. The level of syndecan expression by myeloma cell lines was determined by dot blots of cell extracts using the antibody to the cytoplasmic domain of syndecan (A) (lanes 1 through 5: 1 x 10⁷, 5 x 10⁶, 2.5 x 10⁶, 1.25 x 10⁶, and 6.25 x 10⁵ cell equivalents per dot, respectively). All dots are from the same experiment. Murine MPC-11 cells were used as a positive control because they are known to express syndecan and because syndecan mediates their adhesion to type I collagen. In (B), the ability of these same lines to bind to type I collagen was assessed using the same cell-binding assay as in Fig 4. Cells with relatively high amounts of syndecan (ark, clone-2-pos, clone-2-neg) bind well to collagen, while those with lower amounts (8226, ard-1, U266) appear to have small pellets formed near the center of the well, suggesting they bind less well than those cells with higher amounts of syndecan. Those cells with very little or no detectable syndecan (ram, mer, col) do not bind to collagen. Triplicate wells from each cell type are shown and all wells in (B) are from the same experiment.

of the 10 lines examined. Of the seven lines expressing syndecan, the amount of syndecan on a per-cell basis varied.

Cell-binding assays were performed to determine if expression of syndecan correlated with the ability of the cell lines to bind type I collagen (Fig 5B). All lines expressing relatively high levels of syndecan (MPC-11, ark, clone-2-pos, clone-2-neg) bound well in this assay. However, lines with lower amounts of syndecan bound less well to collagen as indicated by the presence of small cell pellets (8226, ard-1, U266). Cells with very low levels of syndecan (ram) and those not expressing detectable amounts of syndecan (mer, col, ARH-77) failed to bind to collagen.

The data in Fig 5 from human myeloma cell lines suggests that syndecan is not always expressed on myeloma tumor cells. To confirm this, highly purified myeloma plasma cells from patient marrow aspirates were analyzed by RT-PCR for syndecan mRNA expression. Results from two separate experiments are shown (Fig 6). A total of 14 patient samples were examined: 10 were positive for syndecan message expression and 4 were negative by this technique.

Myeloma cells that express syndecan do not express CD19 and CD45. Because syndecan expression varies with the stage of B-cell differentiation, the relationship between the expression of syndecan, CD19, and CD45 was examined on the myeloma cell lines (Table I). CD19 is a B-cell antigen lost with differentiation to plasma cells and CD45 is a protein tyrosine phosphatase expressed by B lymphocytes but not by plasma cells. All cell lines expressing syndecan did not express CD19 (n = 4) and the four cell lines with very low or no syndecan expression were CD19-positive (P = .014; Fisher's exact test). Similarly, syndecan expression was restricted to CD45-negative cells with the exception of ard-1 cells, which expressed syndecan and contained 45% CD45-positive cells (without ard-1 cells P = .03, with ard-1 cells P = .07). These results indicate that syndecan is expressed on tumors having a well-differentiated phenotype.
DISCUSSION

In myeloma, tumor cells are usually localized within the BM and axial skeleton and rarely disseminate into the peripheral blood or other lymphoid organs except in the terminal stages of the disease.1 It is likely that this tumor cell adhesion to, and retention within the BM is regulated by the expression of molecules that mediate cell-cell and cell-matrix adhesion. To date, a number of adhesion molecules have been identified on the surface myeloma cells including I-CAM (CD54),98*39 N-CAM (CD56),45.59 CD44,40 and integrins, although the precise functional role of these adhesion molecules on myeloma cells has not been extensively examined. In the present study, we have demonstrated that the integral membrane proteoglycan, syndecan, is expressed on human myeloma plasma cells and that it acts in binding these cells to type I collagen. In addition, we have shown that syndecan is not expressed by all myeloma plasma cell tumors, and that those tumors not expressing syndecan fail to bind to type I collagen. Taken together, these data indicate that syndecan participates in myeloma cell adhesion to the BM extracellular matrix, and that the loss or absence of syndecan expression on myeloma cells may, in part, regulate tumor cell adhesion to the marrow.

Biochemical analyses of the human myeloma cell line ark suggest that syndecan is the major heparan sulfate proteoglycan on these cells. This is indicated by the elution of a single high molecular weight peak of radiosulfate-labeled material from a Sepharose CL-4B column, and the detection of syndecan in the fractions corresponding to that peak (Fig 1). However, we cannot rule out the possibility that other heparan sulfate proteoglycans are contained within this high molecular weight peak. The syndecan present is a relatively small proteoglycan and contains heparan sulfate glycosaminoglycan chains (Fig 2), and is similar to the molecular form of syndecan found on mouse myeloma cells.30 However, Western blots show that the core protein from human myeloma cells is larger than that from mouse myeloma cells (77 Kd v 62 Kd, respectively; Fig 2 and ref 20). This difference in the apparent core protein molecular size following the removal of glycosaminoglycans is probably not caused by differences in protein structure because the human and mouse form of syndecan present on these cells is similar in predicted core protein size.36,41 Therefore, the size difference seen here is likely caused by differences in N-linked glycosylation or O-linked glycosylation other than glycosaminoglycans.

Our findings indicate that the expression of syndecan regulates myeloma cell adhesion to type I collagen. Solid-phase cell binding assays demonstrate that binding of human myeloma cells to type I collagen is mediated by heparan sulfate chains (Fig 4). Pre-incubation of heparin with collagen-coated wells or removal of heparan sulfate from myeloma cells with heparitinase, before the assay, abolishes cell binding. In addition, for myeloma cell binding to collagen, the heparan sulfate chains must be properly sulfated, because cells grown in media containing sodium chlorate, a competitive inhibitor of sulfation, fail to bind to collagen. Thus, properly sulfated cell surface heparan sulfate is required for binding of myeloma cells to type I collagen. Also, we find a close correlation between syndecan expression and ability of cells from different myeloma tumors to bind to type I collagen (Fig 5). Those cell lines expressing very little or no syndecan fail to bind to collagen. Therefore, the expression of syndecan may, in part, determine the ability of tumor cells to adhere to the BM stromal extracellular matrix.

In mice, syndecan is expressed on pre-B cells, lost before release of mature B cells into the circulation, and re-expressed on plasma cells within interstitial matrices.30 This highly restricted pattern of expression indicates that the timely expression of syndecan is important for normal B lymphocyte differentiation. We find here that myeloma tumors with an immature phenotype similar to circulating B cells (CD19-
and CD45-positive) do not express syndecan, while those tumors with a mature phenotype (CD19- and CD45-negative) express syndecan. This association between syndecan expression and myeloma phenotypic maturity is compatible with the notion that myeloma is disseminated through the circulation of pre-plasma cells and that the expression of adhesion molecules may be a critical step leading to the accumulation of large numbers of plasma cells in the marrow. Syndecan may participate in the homing of tumor cells to the marrow, or following initial cell attachment, syndecan may aid in retaining myeloma cells within the marrow.

In addition, our finding that syndecan is expressed on tumors with a mature phenotype is consistent with the finding that in murine epithelial tumors, syndecan is expressed on moderate- to well-differentiated carcinomas, but is barely detectable at both the protein and mRNA levels in poorly differentiated carcinomas. Thus, the assessment of syndecan expression on myeloma tumors may be of prognostic value as an indicator of disease aggressiveness.

Lastly, syndecan function on myeloma cells may not be limited solely to adhesion. Members of the syndecan proteoglycan family are known to bind to heparin binding growth factors such as basic FGF and participate in basic FGF-mediated cell signaling. Syndecan on myeloma cells may bind to growth factors that can regulate the function and growth of immune cells such as IL-7 and gamma interferon, which have been shown to interact with heparan sulfate. Thus, in addition to mediating myeloma cell adhesion, syndecan may also participate in the cytokine-mediated regulation of myeloma cell growth and differentiation.

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Expression of syndecan regulates human myeloma plasma cell adhesion to type I collagen

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