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B lymphocytes express and lose syndecan at specific stages of differentiation

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Lymphopoietic cells require interactions with bone marrow stroma for normal maturation and show changes in adhesion to matrix during their differentiation. Syndecan, a heparan sulfate-rich integral membrane proteoglycan, functions as a matrix receptor by binding cells to interstitial collagens, fibronectin, and thrombospondin. Therefore, we asked whether syndecan was present on the surface of lymphopoietic cells. In bone marrow, we find syndecan only on precursor B cells. Expression changes with pre-B cell maturation in the marrow and with B-lymphocyte differentiation to plasma cells in interstitial matrices. Syndecan on B cell precursors is more heterogeneous and slightly larger than on plasma cells. Syndecan 1) is lost immediately before maturation and release of B lymphocytes into the circulation, 2) is absent on circulating and peripheral B lymphocytes, and 3) is reexpressed upon their differentiation into immobilized plasma cells. Thus, syndecan is expressed only when and where B lymphocytes associate with extracellular matrix. These results indicate that B cells differentiating in vivo alter their matrix receptor expression and suggest a role for syndecan in B cell stage-specific adhesion.

Introduction

In adult mammals, hematopoiesis occurs in the bone marrow where stromal cells and extracellular matrix provide microenvironments permissive for precursor cell proliferation and differentiation (Trentin, 1970; Bentley, 1981; Dexter *et al.,* 1984; Campbell *et al.,* 1985). This matrix is comprised predominantly of collagens, fibronectin, laminin, and proteoglycans (Bentley and Foidart, 1980; Gallagher *et al.,* 1983; Zuckerman and Wicha, 1983), and interruption of normal matrix synthesis changes hematopoiesis drastically (Spooncer *et al.,* 1983; Zuckerman *et al.,* 1985).

Lymphocytes undergo a number of adhesive changes during their development; e.g., during the early stages of B cell development, cells adhere to the stromal matrix, but following immunoglobulin gene rearrangement, a portion of the now mature B cells leave the bone marrow, enter the peripheral circulation, and home to specific secondary lymphoid organs. Homing requires the expression of cell adhesion molecules to mediate lymphocyte binding to the high endothelial cell venules of these organs. The B cells can circulate out of the lymphoid organ and, when they differentiate into plasma cells, become immobilized to interstitial tissues.

One possible mechanism for these adhesive changes is altered expression by the differentiating B cell of the cell surface receptors that mediate adhesion. Previous studies using lymphoid cell lines indicate that B cells blocked at distinct differentiation stages differ in their adhesion to fibronectin (Bernardi *et al.,* 1987; Liao *et al.,* 1987). Lymphoid cell lines blocked prior to immunoglobulin gene rearrangement bind to the arg-gly-asp-containing domain of fibronectin. Cell lines blocked following light chain gene rearrangement bind to the carboxy-terminal heparin binding fragment of fibronectin. Mature, circulating B lymphocytes, however, fail to bind to fibronectin, presumably due to the loss of the adhesion receptor(s) (Bernardi *et al.,* 1987). A similar loss during differentiation of adhesion to fibronectin has been described on murine erythroleukemia cells (Patel and Lodish, 1986).

In the present study, we have examined the expression of syndecan on bone marrow-derived hemotopoetic precursors. Syndecan, an integral membrane proteoglycan whose core protein has been recently cloned (Saunders *et al.,* 1989), behaves as a matrix receptor. It contains an extracellular domain that binds with a high affinity to interstitial collagens (types I, III, and V) (Koda *et al.,* 1985), fibronectin (Saunders and Bernfield,

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1988) and thrombospondin (Sun *et al.,* 1989). When cross-linked at the cell surface, its cytoplasmic domain associates with the actin cytoskeleton (Rapraeger *et al.,* 1986). A protease-susceptible site is located adjacent to the cell membrane and cleavage at this site releases cells from their association with the extracellular matrix (Jalkanen *et al.,* 1987). In mature mouse tissues, syndecan is found predominantly on the surfaces of epithelial cells (Hayashi *et al.,* 1987), whereas its expression is induced in embryonic mesenchyme (Thesleff *et al.,* 1988; Vainio *et al.,* 1989). In this report we show that, within the bone marrow, syndecan is detected solely on cells of the B lymphocyte lineage. Syndecan expression changes during pre-B cell maturation and differentiation: it is lost when B cells leave the bone marrow, is absent on circulating and peripheral B cells, but is re-expressed upon B cell differentiation into plasma cells, where it is a slightly smaller molecular form. This correlation between syndecan expression and adhesion of B cells to matrix suggests that syndecan plays a role in mediating B cell stage-specific adhesion.

Results

Syndecan is expressed by precursor B cells

Antibodies against lineage-specific cell surface markers and the syndecan core protein were used to identify the cells in the bone marrow that express syndecan. Analyses with the fluorescence-activated cell sorter (FACS) reveal that 93% of the cells that stain for syndecan also express the B-cell lineage specific antigen B220 (Figure 1a). A small proportion of the syndecan-positive cells (7%) do not stain for B220. None of the syndecan-positive cells stain for Thy-1 (Figure 1b).

Patterns of light scatter were used to establish the size and nuclear-cytoplasmic ratio of the bone marrow cells that express syndecan. Of the syndecan-positive cells, 84% show small forward and wide-angle light scatter, signals that characterize immature or pre-B cells (Herzenberg *et al.,* 1987) (Figure 1, c and d); the remainder appear to have characteristics of large B cell precursors (not shown). The cells which show large forward and wide-angle light scatter include granulocyte and megakaryocyte/monocyte precursors and the very abundant large number of erythroid precursors; none of these express syndecan (Figure 1d, broken line). Antibodies identifying megakaryocytes/monocytes (Mac-1) and granulocytes (RB6-8C5, anti-Gr-1) confirm this observation (data not shown).

Syndecan expression changes with B cell differentiation

The developmental stages of the B cells expressing syndecan were defined by analyzing for stage-specific surface antigens (Figure 2). Three distinct populations of B220-positive cells are seen in bone marrow: 1) pre-B cells (B220^{dull}, IgM$^-$), 2) immature B cells (B220^{dull}, IgM$^+$), and 3) mature B cells (B220^{bright}, IgM$^+$) (Figure 2a). Syndecan is expressed on a substantial proportion of pre-B (46%) and immature B cells (40%), but on a negligible proportion of mature B cells (Figure 2, b and c). Pre-B and immature B cells reside within the marrow, but leave the marrow upon maturation. Thus, syndecan is expressed on B-lymphocyte precursors but is lost from their surfaces before their release as mature cells.

Following their entry into the peripheral circulation, the mature B cells home to specific secondary lymphoid organs (node, spleen, Peyer's patches). To determine if these peripheral B cells express syndecan, we analyzed cells from blood and spleen (Figure 2, d and e). B lymphocytes in these locations do not express syndecan as detectable by FACS.

Following antigen stimulation, some mature B cells migrate out of the secondary lymphoid compartments and localize in microenvironments that are rich in interstitial matrix materials where they differentiate into antibody-producing plasma cells. Immunostaining reveals that syndecan is on the surface of plasma cells in the lamina propria of the duodenum, in the medulla of lymph nodes and in the spleen, but is not detected on B lymphocytes or other nonepithelial cell types within these organs (Figure 2f and Hayashi *et al.,* 1987).

Syndecan from B cells and plasma cells has distinct molecular characteristics

Immunoisolation and immunoblotting with antibody 281-2 was used to confirm that the antigen detected on cell surfaces was syndecan. Hematopoietic cells from the marrow show a smear characteristic of a heterogeneous proteoglycan of modal size ~92 kDa on Western blots (Figure 3). Pretreatment with chondroitin sulfate ABC lyase to remove chondroitin sulfate chains results in no detectable change in mobility (data not shown), but pretreatment with heparitinase to remove heparan sulfate chains greatly reduces the heterogeneity and yields a broad core protein band at ~76 kDa. Mesenteric lymph nodes of both control and lipopolysaccharide-stimulated animals show a proteoglycan smear that is less heterogeneous than that seen in syndecan isolated from bone marrow. Lymph node syndecan

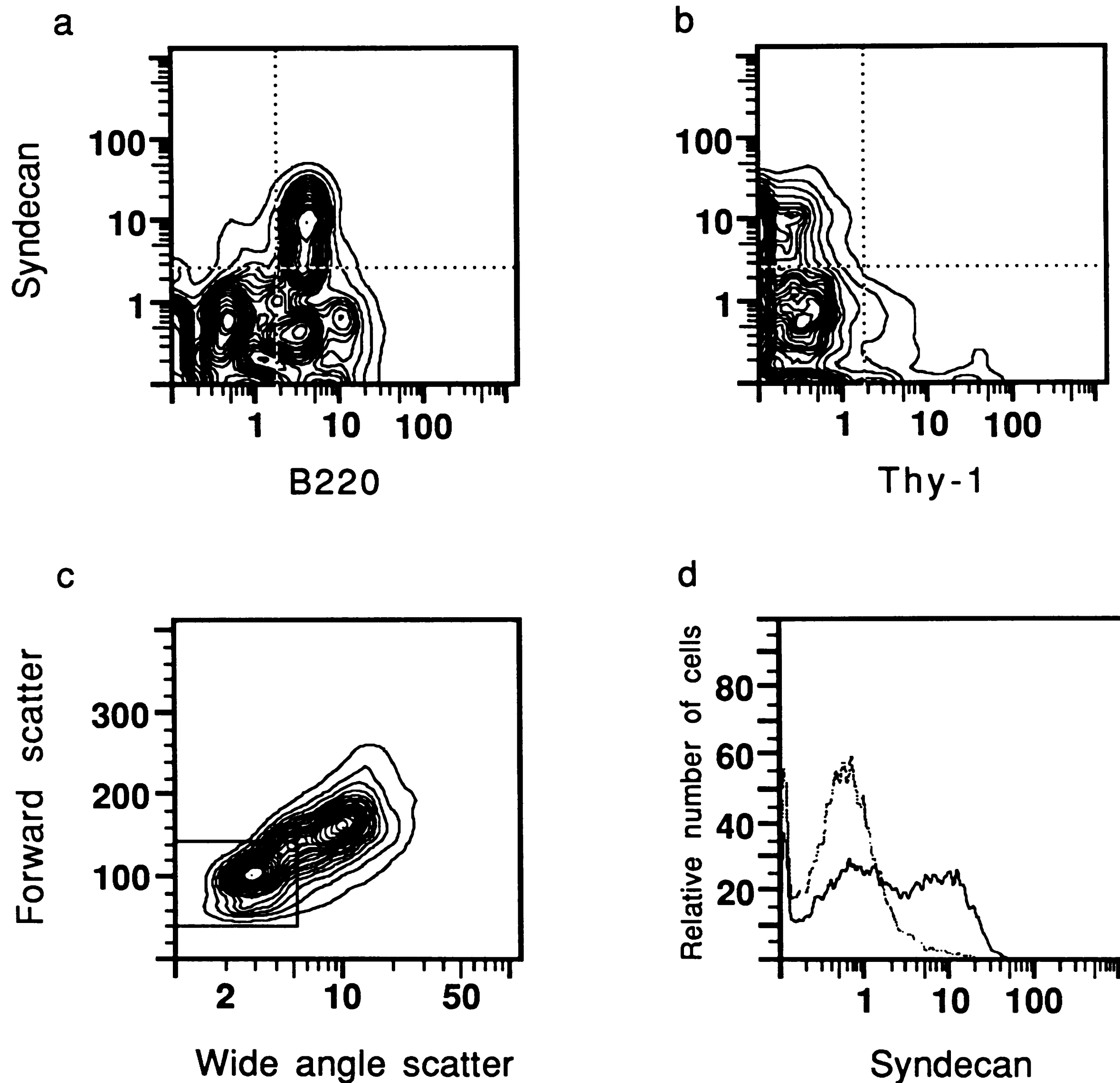
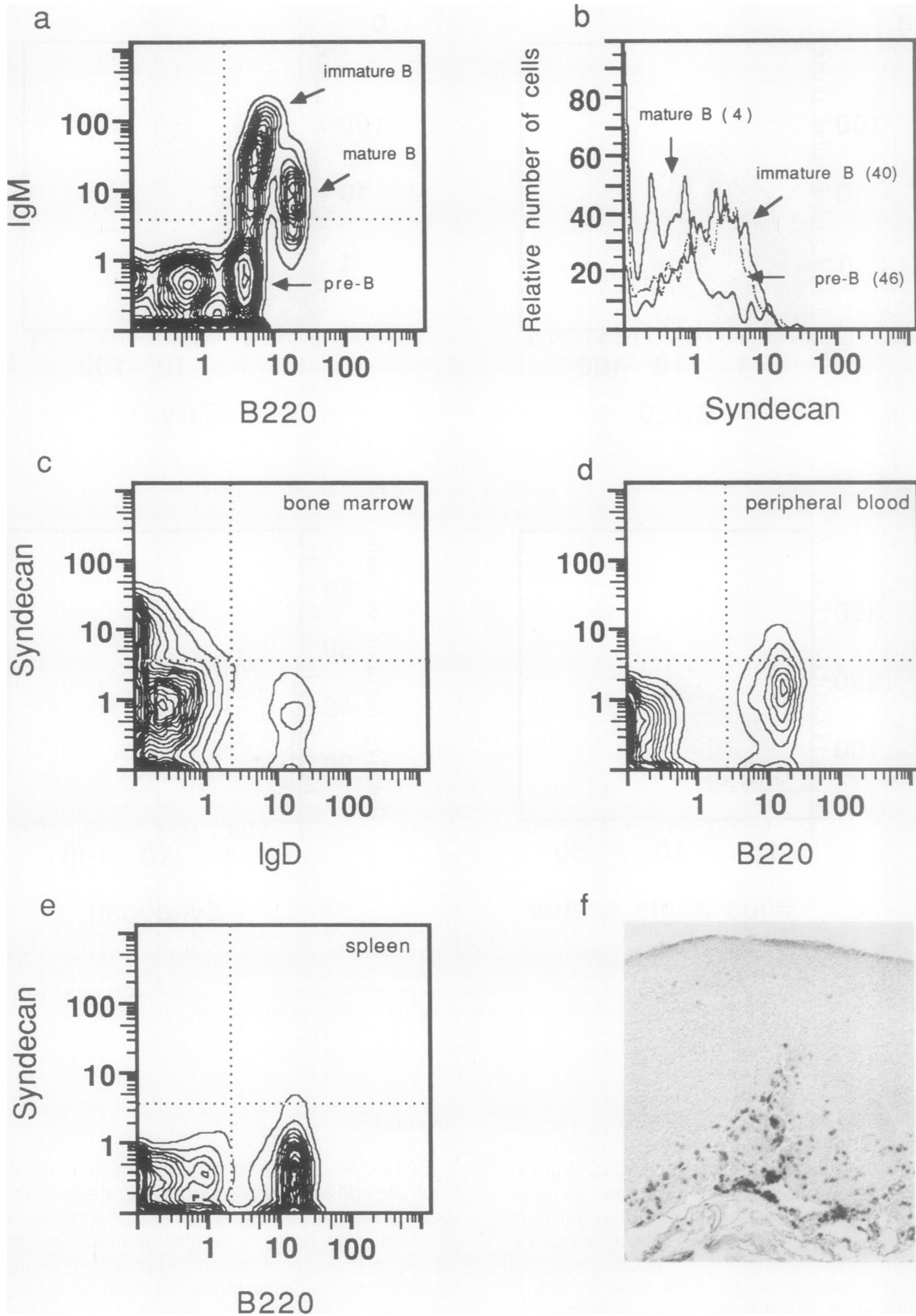


Figure 1. **Expression of syndecan on bone marrow cells.** (a) Staining of cells with antibodies to syndecan and B220, a B-cell lineage marker, and (b) with antibodies to syndecan and Thy-1. (c) Scatter profile of bone marrow cells showing 2 distinct cell populations. The box in c and the solid line in d identify cells of low forward and wide-angle scatter (pre-B and immature B cells). Cells outside the box in c have high forward and wide-angle scatter and do not stain for syndecan (d, broken line).

has a modal size of 85 kDa which yields a narrow core protein band (62 kDa) following heparitinase treatment. Syndecan from lipopolysaccharide-treated mice is twofold more abundant (as determined by scanning densitometry) on a per node basis, presumably reflecting an increased number of plasma cells. Syndecans isolated from several B cell and myeloma cell lines have structural characteristics similar to those described here for marrow and plasma cells (data not shown).

Discussion

Lymphocyte differentiation and migration involve multiple adhesive interactions mediated by members of several structural families of adhesion receptors. The present results demonstrate that changes in the expression of syndecan, an integral membrane proteoglycan and matrix receptor, correlate in time and location with changes in adhesion that occur during B-lymphocyte differ-


a
immature B
mature B
pre-B
IgM
B220
b
mature B (4)
immature B (40)
pre-B (46)
Relative number of cells
Syndecan
c
bone marrow
Syndecan
IgD
d
peripheral blood
B220
e
spleen
Syndecan
B220
f

entiation and migration. Thus, the syndecan group of proteoglycans may also mediate these interactions.

Of bone marrow hematopoietic cells, syndecan is expressed only on B lineage cells

Bone marrow contains various classes of hematopoietic stem cells and precursors that can be distinguished by lineage-specific cell surface antigens and by size. Over 90% of the bone marrow cells that express syndecan have characteristics of B-cell precursors and express B220, the cell surface antigen present on precursor and mature B cells but not on other hematopoietic cells (Coffman and Weissman, 1981). In contrast, T lymphocytes, granulocyte, megakaryocyte/monocyte, and erythroid precursors fail to express syndecan, suggesting that adhesion of these cells to matrix is mediated by other receptors. The small proportion of syndecan-positive cells that do not express B220 may represent B cell precursors that have not yet acquired the B220 antigen, or syndecan may be present on a small number of non-B lineage cells.

This highly selective expression of syndecan among hematopoietic cells contrasts with the less discrete expression of syndecan at other tissue sites. As assessed by 281-2 staining, syndecan is expressed on various simple and stratified epithelia of the mature mouse (Hayashi *et al.*, 1987) and on a variety of condensing mesenchymal cells in embryos (Thesleff *et al.*, 1988; Vainio *et al.*, 1989). Thus, restriction of expression on hematopoietic cells to cells of the B-lineage is unique to this tissue.

Syndecan expression changes with B cell differentiation

Pre-B cells arise in the marrow from undifferentiated stem cells and give rise to immature and mature B cells. Upon maturation, the cells release from the marrow, enter the circulation, and migrate into organized lymphoid tissues where they may be stimulated to become antibody-producing plasma cells. Syndecan is expressed on ~40% of pre-B and immature B cells, but is lost when B cells mature and enter the peripheral circulation. The subset of B lineage syndecan-negative cells

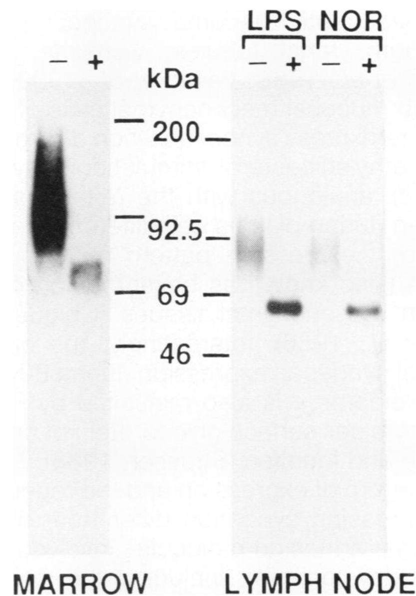


Figure 3. **Western blot of immunoisolated syndecan from bone marrow and mesenteric lymph nodes before (−) and after (+) heparitinase treatment to remove heparan sulfate chains.** Mesenteric lymph nodes were removed from control mice (NOR) or 3 d after injection of lipopolysaccharide.

in the marrow could represent cells that have not yet or have previously expressed syndecan. Alternatively, syndecan may not be expressed at all by certain subsets of B cells.

Syndecan is re-expressed during the differentiation of B-lymphocytes into plasma cells. Staining of plasma cells for syndecan is readily apparent in the lamina propria of the duodenum and is intense in lymph nodes where it is restricted to the medullary cords. Therefore, syndecan is present during B-lymphocyte development in the marrow, lost upon release into the circulation, absent during homing to peripheral lymphoid organs and re-expressed on antibody-producing cells.

The acquisition of syndecan, then its loss and subsequent reappearance during B lymphocyte development is not seen during the development of a variety of epithelia. Embryonic mesenchymal cells express syndecan initially when they condense during their interaction with an epithelium,

Figure 2. **Cell surface syndecan expression is lost as B cells mature but reappears upon terminal differentiation.** (a) Contour plot showing 3 populations of B-lineage cells in the bone marrow, as defined by staining for B220 and surface IgM. (b) Histogram showing the relative number of syndecan-positive cells from each B-lineage cell population in a as determined by 3-color FACS analysis. Syndecan-positive cells as a percentage of each population are indicated in parentheses. (c) Staining of bone marrow cells with antibodies to syndecan and IgD. (d) Peripheral blood cells and (e) spleen cells analyzed for syndecan and B220. (f) Expression of syndecan on plasma cells in mesenteric lymph nodes. Sections of mesenteric lymph nodes from lipopolysaccharide-stimulated mice were stained with monoclonal 281-2. The stained cells are plasma cells, as verified by electron microscopy (Hayashi *et al.*, 1987).

but subsequently become syndecan-negative (e.g., tooth, renal tubules, vibrissae, vagina) (Thesleff *et al.,* 1988; Vainio *et al.,* 1989). However, sub-mucosal mesenchymal cells of the vagina will re-express syndecan when differentiating under diethylstilbesterol stimulation (Hayashi *et al.,* 1988), analogous with the re-expression of syndecan during plasma cell differentiation. This "on-off-on" expression pattern mimics that of tenascin (also known as hexabrachion or cytotactin) in mesenchymal tissues (Chiquet-Ehrismann *et al.,* 1986). Interestingly, the on-off-on pattern of syndecan expression during B-lymphocyte development is also mimicked by PB76, a lymphocyte cell surface glycoprotein of unknown structure and function (Strasser, 1988).

The pattern of expression and the repertoire of cells expressing syndecan differ from those of some other adhesion molecules involved in lymphocyte development, including the integrin fibronectin receptor (Bernardi *et al.,* 1987; Cardarelli and Pierschbacher, 1987), lymphocyte homing receptors (Gallatin *et al.,* 1986), and lymphoid function associated antigen adhesion molecules (Springer *et al.,* 1987). Interestingly, like syndecan, the Hermes/CD44 antigen, an adhesion receptor on B-lymphocytes, can bind to interstitial matrix molecules (Carter and Wayner, 1988), can exist as a proteoglycan (Jalkanen *et al.,* 1988) and is widely distributed among hematopoietic, epithelial, and mesenchymal cells (Stoolman, 1989).

Syndecan structure changes with B cell differentiation

Syndecan exists in tissues in different molecular forms. Simple epithelia contain a larger size syndecan than stratified epithelia, due to differences in number and size of glycosaminoglycan chains, but the core protein size is the same, migrating on PAGE at 69 kDa (Sanderson and Bernfield, 1988; Saunders *et al.,* 1989). The structure of syndecan also changes with B cell differentiation. Differences in size and/or abundance of heparan sulfate chains on syndecan may affect its affinity for various ligands (Klebe and Mock, 1982). Syndecan from the marrow is exceedingly heterogeneous, possibly reflecting the heterogeneity of the pre-B and immature B cells. This syndecan is a heparan sulfate proteoglycan that contains no apparent chondroitin sulfate. The GAG-free core protein migrates as a broad band at 76 kDa presumably due to glycosylation by other *N*- or *O*-linked sugars. Syndecan from plasma cells in lymph nodes is also a heparan sulfate proteoglycan, but is substantially less heterogeneous and smaller, due to fewer or smaller sized heparan sulfate chains. Its GAG-free core protein migrates as a narrow band at 62 kDa, presumably due to less extensive non-GAG glycosylation.

Syndecan function during B cell differentiation

Syndecan on mammary epithelial cells acts as a matrix receptor: it binds cells via its heparan sulfate chains to components of the interstitial matrix and associates with the actin cytoskeleton when cross-linked at the cell surface. We now find that it is expressed as a heterogeneous heparan sulfate proteoglycan on pre-B and immature B lymphocytes, lost when the cells are released into the periphery, and re-expressed as a smaller, more discrete heparan sulfate proteoglycan on plasma cells. These findings are consistent with evidence that heparan sulfate anchors B-lineage (myeloma) cells to type I collagen (Stamatoglou and Keller, 1983) and the suggestion that heparan sulfate is lost as hematopoietic cells mature and become less adhesive (Chiarugi and Vannucchi, 1976).

Binding of syndecan to matrix could sequester B cell precursors in the marrow. Loss of syndecan, which is rapidly shed and not replaced when adherent cells are suspended (Jalkanen *et al.,* 1987), could be a mechanism for release of mature B cells from the marrow. Lack of syndecan expression could allow peripheral B-lymphocytes to circulate in and out of lymphoid tissues and migrate into inflammatory lesions. Upon differentiation into plasma cells, syndecan is re-expressed, possibly enabling these cells to bind to the interstitium, as in lymph node medullae or lamina propria.

Heparan sulfate from bone marrow binds hematopoietic growth factors (Gordon *et al.,* 1987; Roberts *et al.,* 1988). Syndecan on B-lymphocyte precursors could compete for these growth factors and/or be a reservoir (Saksela *et al.,* 1988) for those involved in promoting B-cell development, such as interleukin-7 (Lee *et al.,* 1989).

In summary, we have found that the expression of syndecan, a matrix receptor, correlates with the developmental stage, location, and adhesion of cells of the B-cell lineage. Thus, differentiating B-lymphocytes regulate their expression of syndecan. We hypothesize that this regulation controls B-lymphocyte adhesion to matrix: 1) in the marrow, syndecan anchors maturing B cells to stromal matrix; 2) in lymphoid organs, syndecan anchors plasma cells to the interstitial matrix; and 3) during the interval between these two stages, syndecan is not expressed, allowing the cells to be released from the marrow, to circulate within the peripheral compartments and to migrate to their sites of terminal differentiation.

Materials and Methods

Harvesting of bone marrow cells

Bone marrow cells were obtained from the femur of 12-wk-old BALB/c or C.B-17 mice. Cells were flushed from the femur with media containing 10 mM Hepes, 5% newborn calf serum, and RPMI-1640 containing no phenol red. Cells were centrifuged and resuspended in media. Cell aggregates were removed by filtering through a 30 μm mesh and red blood cells were lysed in 140 mM ammonium chloride, 20 mM Tris pH 7.5. Cells were collected by centrifugation, resuspended in media, and incubated with antibody.

Fluorescence-activated cell sorting

Cells were analyzed by use of a modified, dual laser fluorescence-activated cell sorter (FACS II; Becton-Dickinson, Mountain View, CA) interfaced with a VAX 11/78 computer (Parks *et al.,* 1984). Data are presented as 5% probability contour maps (Moore and Kautz, 1986). Dead cells were excluded by propidium iodide staining (Loken and Stall, 1982). Monoclonal antibodies used in experiments were directly conjugated to either biotin or purified fluorochromes (Hardy, 1986). Antibody conjugated to biotin was revealed with Texas Red/avidin. Antibodies used included anti-syndecan, 281-2/biotin (Jalkanen *et al.,* 1985); anti-B220, RA3-6B2/FITC (Coffman and Weissman, 1981; Coffman, 1982); anti-Thy-1/FITC (Letbetter and Herzenberg, 1979); anti-IgM, 331.12/allophycocyanin (Kincade *et al.,* 1981); anti-IgD, AMS 9.1/FITC (Stall and Loken, 1984); anti-Gr-1/FITC (Spangrude *et al.,* 1988); and anti-Mac-1/allophycocyanin (Springer *et al.,* 1979).

Immunostaining

Mesenteric lymph nodes from lipopolysaccharide-stimulated (L. typhosa 0901; Difco Laboratories, Detroit, MI, 10 μg intraperitoneal injection) 3-mo-old BALB/c mice were fixed in Zamboni's solution containing 2% formaldehyde and 0.18% picric acid in 0.1 phosphate buffer pH 7.4, for 6 h at 4°C. Tissue was dehydrated, embedded in paraffin, sectioned, and deparaffinized in a standard fashion. Sections were incubated with monoclonal 281-2 (3.8 μg/ml) or, as a control, MEL-14, another rat IgG2a anti-mouse monoclonal (Gallatin *et al.,* 1983). Sections were washed six times in PBS and incubated with 5 μg/ml biotinylated rabbit anti-rat IgG (no cross reaction with mouse IgG; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After washing, sections were incubated for 30 min at room temperature with an avidin-horseradish peroxidase reagent prepared from a Vectastain Elite ABC kit. Reaction product was generated with 0.05% diaminobenzidine tetrahydrochloride containing 0.0155% H_2O_2 in PBS. Staining of sections was enhanced with 0.5% $CuSO_4$ in 0.9% NaCl for 5 min. Following mounting with coverslips, photographs were taken in the presence of a blue HOYA 49-mm filter with Tech Pan film on a Zeiss Photomicroscope II.

Syndecan isolation and western blotting

Marrow cells were flushed from 3-mo-old female BALB/c mice and spun over Histopague-1077 (Sigma, St. Louis, MO). The mononuclear cells at the media-Histopaque interface were collected for extraction. Mesenteric lymph nodes were removed from mice 3 d after injection of LPS (10 μg, i.p.) or from control mice. Marrow cells were extracted for 30 min in ice cold 10 mM Tris pH 7.4, containing 1% Triton X-100, 0.5 M KCl, 0.15 M NaCl, 10 mM *N*-ethylmaleimide, 10 mM benzamidine, 0.5 mM EDTA, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonylfluoride. Nodes were extracted in the identical buffer containing 6 M urea. Extracts were diluted with Tris buffered saline (TBS) to a final concentration of 1 M urea, 0.15 M NaCl adjusted to pH 7.4 and incubated with antibody 281-2 covalently bound to Sepharose 4B beads (281-2 beads). After incubation on a rocker overnight at 4°C, beads with bound syndecan were placed on a column, washed with cold TBS containing 0.1% Triton X-100 followed by 20 mM acetic acid pH 2.5 to remove nonspecifically bound molecules. Syndecan was eluted from beads by boiling in PAGE sample buffer (2% SDS [wt/vol], 5% glycerol [vol/vol], 0.025% bromophenol blue, 140 mM Tris, and 60 mM boric acid pH 8.0).

Removal of glycosaminoglycan chains from syndecan was performed on syndecan bound to 281-2 beads. Chondroitin sulfate was removed by treatment with chondroitin sulfate ABC lyase (chondroitinase ABC, ICN Biochemicals, Irvine, CA) in 50 mM Tris pH 7.4, 10 mM sodium acetate, 5 mM calcium acetate, 10 mM *N*-ethyl maleimide, 1 mM phenylmethysulfonyl fluoride, and 5 μg/ml pepstatin A. Digestion with 0.1 U/ml enzyme was carried out for 90 min at 37°C then for another 90 min after addition of an equal amount of fresh enzyme. Heparan sulfate was cleaved by treatment with 1.0 U/ml of *Flavobacterium heparinum* heparitin sulfate lyase (heparitinase, ICN Biochemicals) using the same buffer and incubation periods used above with chondroitinase ABC. Neither enzyme released the syndecan which is bound to the 281-2 beads via the core protein. After digestions, beads were washed extensively with TBS and syndecan was eluted from the beads with SDS sample buffer for analysis by PAGE.

Proteoglycans were fractionated on a 3.8–20% SDS polyacrylamide (7.5% bisacrylamide) gel containing urea and boric acid as described by Koda *et al.* (1985). For Western blotting, gels were transferred by the method of Towbin *et al.* (1979), to Gene-Trans (Plasco, Inc., Woburn, MA), a cationic nylon membrane. To aid in retention of proteins, filter membranes were treated for 15 min in 20 mM phosphate buffer containing 0.025% glutaraldehyde. Membranes were then soaked for 1 h in "blotto" (0.5% BSA, 3% Carnation instant nonfat dry milk, 10 mM Tris pH 8.0, 0.15 M NaCl) followed by 30 min in TBS containing 1% fetal calf serum, and 0.3% Tween 20 (buffer B). Fresh buffer B, containing ^{125}I-labeled mAb 281-2 was added for 4 h at room temperature or overnight at 4°C. Blots containing bound antibody were then washed extensively with buffer B, dried, and exposed to Kodak X-Omat XAR-5 film.

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