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Original Paper

Expression of Syndecan-1 in Human B Cell Chronic Lymphocytic Leukaemia

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Syndecan-1 is considered an important transmembrane proteoglycan in cell–microenvironment interactions, but its exact function in normal or in transformed B cells is still unknown. In this study, RNA was isolated from peripheral cells of chronic lymphocytic leukaemia (B-CLL) and 'normal', non-leukaemic patients, as controls. Reverse PCR showed no or very low syndecan-1 mRNA expression in controls, while in 11/13 B-CLL the circulating leukaemic cells expressed syndecan-1. Similar results were obtained for interleukin-1β (IL-1β) and interleukin-6 (IL-6). Furthermore, syndecan-1 protein was detected in the majority of circulating B-CLL cells by flow cytometry and immunocytochemistry using anti-syndecan-1 MAb. Control cells were practically negative. Further study is required to understand the biological significance of syndecan-1 on B-CLL cells. © **1997 Elsevier Science Ltd.**

Key words: B-CLL, syndecan-1, proteoglycan, IL-1β, IL-6
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INTRODUCTION

ALTHOUGH CANCER is considered to be a genetic disease, and the continuous accumulation of malignant cells is dictated mainly by autocrine factors, the progression of a given tumour is also highly dependent on cell–microenvironmental interactions. Syndecans are a gene family of four transmembrane heparan sulphate (HS) proteoglycans, that mediate a variety of these interactions by binding—via their sugar chains—diverse components of the cellular microenvironment. Syndecan-1 can influence cell shape, cell–cell contact, cell–matrix interactions and can participate in signal transduction, as receptor or coreceptor for collagen I-III, fibronectin as well as for bFGF, aFGF, GM-CSF, IL-3, γIFN) [1–3]. It is known that each syndecan family member is expressed selectively in a cell-, tissue- and development-specific manner [4, 5]. Syndecan-1 is expressed in distant stages of differentiation, of B cells, including pre-B cells (B cells maturing in the bone marrow) and immunoglobulin-producing plasma cells [6, 7]. The function of syndecan-1 in normal B cells is still not understood and there are hardly any data on its expression in lymphoproliferative diseases, except myelomas. If tumour progression is strongly dependent on the relationship between tumour cells and their microenvironment, and syndecan can influence this relationship, it would be interesting to determine how syndecans are expressed in malignant forms of B-cells. Here, we studied syndecan-1 expression in the circulating cells of chronic lymphocytic leukaemia (CLL) patients together with the expression of IL-1β and IL-6 which can interact with proteoglycans and seem to be present in CLL [8, 9].

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MATERIALS AND METHODS

Cells

10–15 ml peripheral blood from 6 'normal' (non-leukaemic or healthy) individuals and from 15 leukaemic (13 B-CLL, 1 T-CLL, 1 CML) patients were placed on Ficoll-Paque (Pharmacia, Sweden) gradient and mononuclear cells were isolated from the interphase. The morphology of the cells was verified on cytospin slides stained with routine haematoxylin and eosin.

Preparation of RNA

RNA was prepared from 2×10^8 cells of each case using the RNeasy Total RNA kit (Quiagen, Germany). The yield and quality of the preparation were determined by $OD_{260/280}$.

Reverse PCR

0.5 μg RNA was used as template in each case. The reaction mixture consisted of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs (50 μM each) (Boehringer-Mannheim, Germany), 5 mM DTT, 10 U RNasin, 0.25 μg random primer (Promega, U.S.A.), 200 U MMLV reverse transcriptase (Gibco-BRL, U.S.A.) in 20 μl total volume. The reaction was carried out at 42°C for 40 min and followed by 5 min denaturation at 95°C. 3 μl of the first strand synthesis product (corresponding to 0.075 μg original total RNA) was used as a template to amplify each cDNA. The reaction mixture contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM nucleotide mixture, 1.5 U Taq DNA polymerase (Boehringer-Mannheim, Germany), 25 pmol of each primer in 50 μl reaction volume.

Primers for PCR amplification

syndecan[1]—extracellular part with GAG binding region
sense: 5′ ATG TCG ACG GAG GTG GAG 3′
antisense: 5′ GCT GAG CCT GCA GCT GGC 3′
size: 230 bp

syndecan[2]—extracellular + transmembrane regions
sense: 5′ AGC TGA CCT TCA CAC TCC 3′
antisense: 5′ TCG GCT CCT CCA AGG AGT 3′ 360 bp

IL-1β
sense: 5′ ATG GCA GAA GTA CCT GAG CTC GC 3′
antisense: 5′ ACA CAA ATT GCA TGG TGA AGT CAG TT 3′
803 bp

IL-6
sense: 5′ TTG TAC TCA TCT GCA CAG CTC TGG C 3′
antisense: 5′ CGG TAC ATC CTC GAC GGC ATC TCA G 3′
271 bp

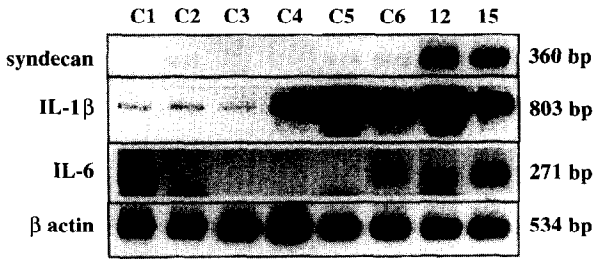

Figure 1. Expression of syndecan-1, IL-1β, IL-6, β-actin genes (RT-PCR analysis) in control (C1-6) and B-CLL samples (cases 12 and 15). Equal amounts of the same cDNA were used as a template for amplification of each message.

β-actin
sense: 5′ GTG GGG CGC CCC AGG CAC CA 3′
antisense: 5′ CTC CTT AAT GTC ACG CAC GAT TTC 3′
538 bp

We designed syndecan primers according to the cDNA sequence of human syndecan-1 [10]. The IL-6 primers and the sequences of IL-1β and β-actin were kindly provided by Professor András Falus and Professor Giuliano Ramadori, respectively.

The cycling parameters after denaturation (95°C 5 min) were 94°C 1 min—62°C (60°C for IL-6) 1 min—75°C 1 min, 30 cycles with a final extension at 75°C for 10 min. Products were run on 1.5% agarose gel in the presence of 1 μg/ml EBr. Gels were visualised under UV light (Eagle Eye Still Video System, Stratagen, U.S.A.).

Flow cytometry

For syndecan-1 staining, the fixed (80% methanol for 30 min) and washed cells isolated from interphase were incubated in 3% BSA (bovine serum albumin) for 30 min and

Table 1. Clinical data and RT-PCR results

	Sex	Age (years)	CD5	IgM	dg	Therapy	WBC	Rai(*)	syn1/syn2	IL1β	IL6
1	m	75	+	+	1990	steroid	20 900	B; III/I	+/+	+	+
2	f	76	+	+	1988	—	68 200	B; 0/0	−/−	−	−
3	f	74	+	−	1993	CT/st	210 000	T; III/IV	+/+	+	+
4	f	59	+	−	1985	CT	23 900	B; I/IV	−/−	+	+
5	m	71	+	+	1987	CT	138 000	B; III	+/+	+	+
6	m	54	+	+	1985	CT	47 800	B; 0/IV	+/+	+	+
7	m	56	−	−				CML	+/−	−	+
8	m	79	+	+	1994	—	235 000	B; 0/0	+/+	+	+
9	f	64	+	+	1984	—	90 300	B; 0/0	+/−	+	+
10	f	62	+	+	1992	—	96 000	B; 0/0	+/+	−	−
11	m	65	+	+	1995	—	59 600	B; I/I	+/+	+	+
12	f	72	+	+	1990	—	19 300	B; II/II	+/+	+	+
13	m	81	+	+	1992	—	20 000	B; 0/0	+/+	+	+
14	m	54	+	+	1992	CT	18 300	B; I/IV	+/+	+	+
15	m	65	+	+	1989	CT	68 000	B; II/IV	+/+	+	+
C1	f	49						hypertension	nd/−	+	+
C2	m	29						depression	nd/+	+	−
C3	f	27						normal	nd/−	+	−
C4	m	25						normal	nd/−	+	−
C5	m	45						hypertension	nd/+	+	−
C6	f	70						oesophagitis	nd/+	+	+

dg, year of diagnosis; st, steroid; WBC, white blood cells/μl; Rai (*), at diagnosis/present; C, control; syn1/syn2, syndecan 1 and 2 primer pairs; nd, not determined; CT, chemotherapy.

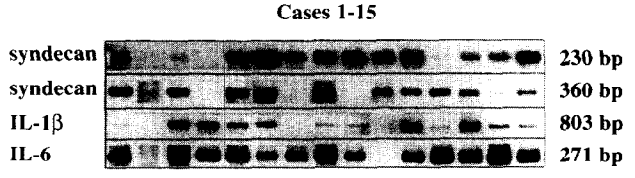

Figure 2. Expression of syndecan-1, IL-1β and IL-6 genes (RT-PCR analysis) in 15 human leukaemic patients (CLL: cases 1–6, 8–15; case 7: CML).

for 60 min with anti-human syndecan-1 antibody (Serotec MCA 681, U.K. 10 μl/10^6 cells). The bound primary antibody was detected by anti-mouse IgG-FITC antibody (Amersham, U.K. diluted 1:60 in phosphate-buffered saline). For double immunostaining, the Ficoll-separated unfixed peripherial blood mononuclear (PBMN) cells were labelled by syndecan-l antibody (see above). Secondary anti-mouse IgG1 FITC or RPE conjugated antibodies were used to detect syndecan-1 labelling (1:25 FITC, 1:200 RPE Southern Biotechnology Associates, U.S.A.). The free binding sites of anti-mouse antibody were saturated with mouse serum. Next, the cells were incubated by one of the direct labelled monoclonal anti-human CD19 (RPE, Dako, Denmark) or CD3 (FITC, Becton Dickinson, U.S.A.) antibodies in 1% BSA. For double-staining experiments, we used several negative controls and the HT58 human NHL (B) cell line [11] as a positive control for syndecan-1 expression. The immunophenotype of the CLL cases was characterised with anti-human IgM and anti-human CD5 (Dako, Denmark) MAbs.

After washings, the cells were measured for fluorescence at 530/30 and 575/42 nm in FACStar Plus flow cytometer (Becton Dickinson, U.S.A.) and the analysis was performed with Winlist Software (Verity Software House, U.S.A.).

Immuncytochemistry

Cytospin slides were fixed in methanol for 5 min and anti-human syndecan-1 MAb (see above) in 1:50 dilution and Vectastain anti-mouse IgG-ABC kit (Vector, U.S.A.) were used to detect syndecan-1 protein. After the last step—ABC incubation—biotinylated tyramin (0.056 μM) diluted in 0.03% H_2O_2 was added and the slides were incubated for 10 min and a second round of ABC reaction was carried out to amplify the sensitivity of the reaction [12]. Diaminobenzidine tetrahydrochloride was used as a chromogen.

RESULTS

In controls (non-leukaemic patients), the peripheral mononuclear cells (not only B cells) showed either no or very low syndecan mRNA expression (360 bp product), similarly

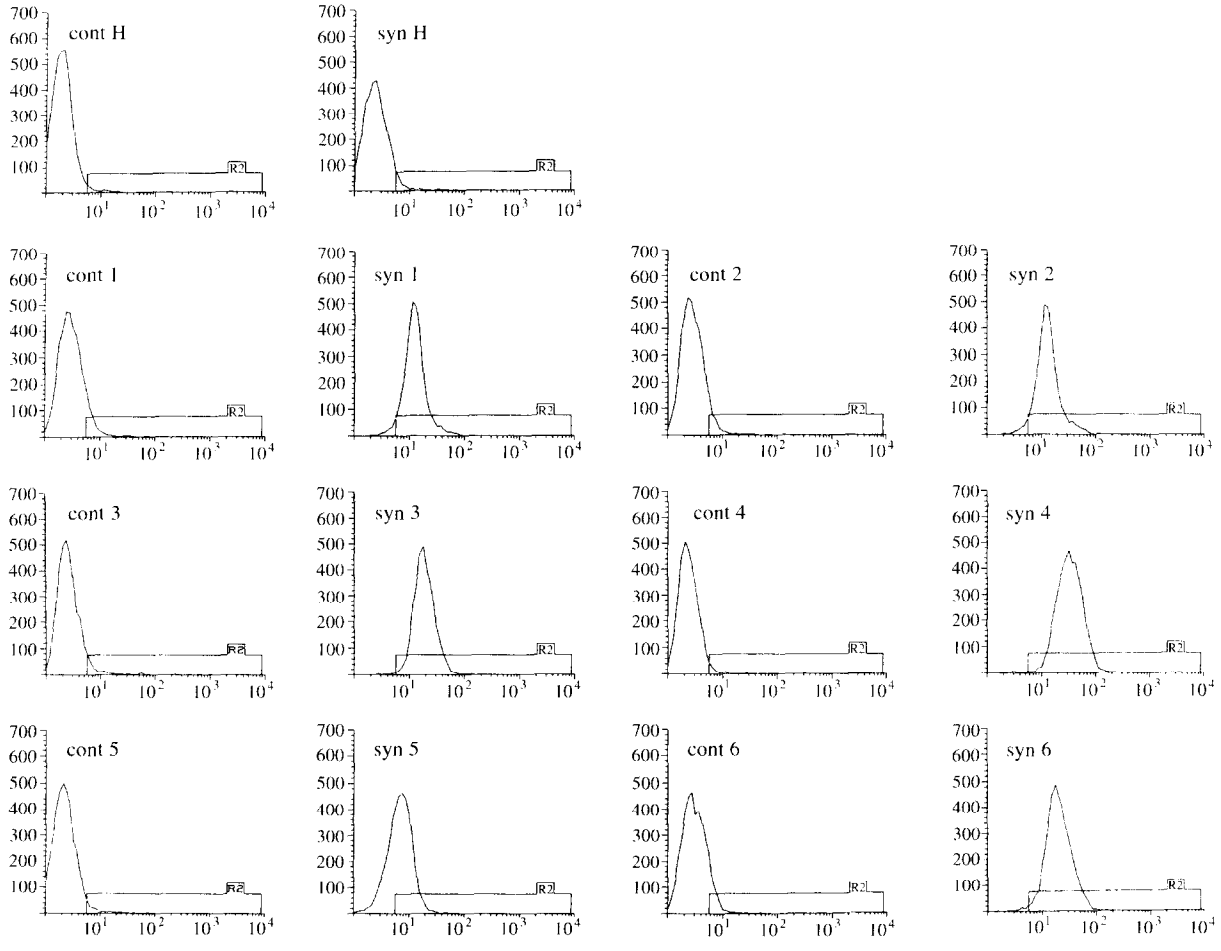

Figure 3. Analysis of syndecan-1 protein expression by flow cytometry: cont (negative controls), syn (syndecan), H (healthy patient), 1–6 (B-CLL patients). The percentages of positive cells: contH: 1.47%, synH: 2.66%; cont1–6: 1.34–3.93%, syn1,2,3,4,6: 88.84–98.34%, syn5: 58.56%.

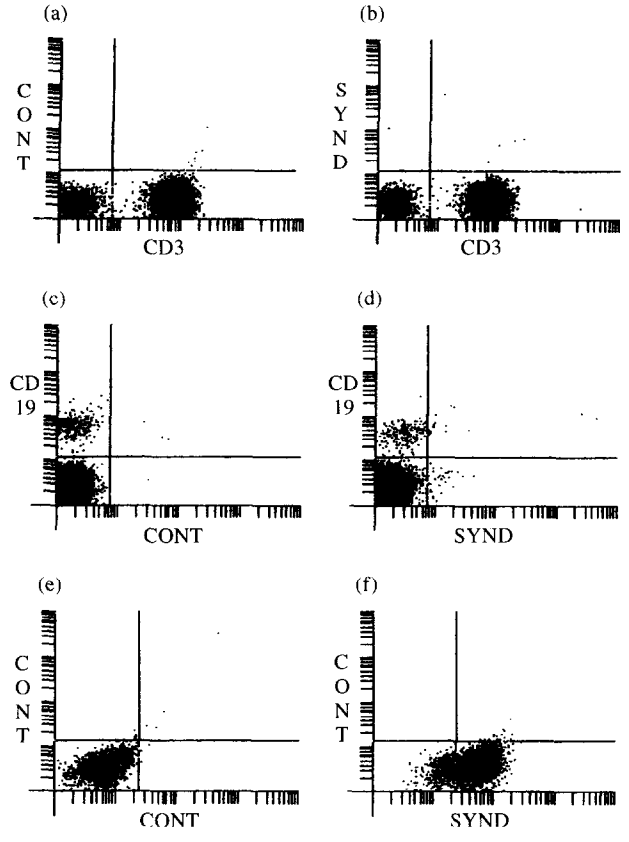

Figure 4. Co-expression of syndecan with lymphocyte markers in normal peripheral blood mononuclear cells (a–d) and expression of syndecan on HT58 lymphoma cells (e–f). Cont, control (no primary antibody); synd, syndecan-1.

to IL-6 (Table 1). IL-1β message was expressed in all samples but at different intensities (Figure 1). Most (12/14) CLL cases (case 7 was a CML) showed syndecan mRNA expression. In case 9, only syndecan-1 was expressed, while case 4 was negative for both syndecans (it was positive for both ILs). The results for case 2, which was also negative for both syndecans, are probably inaccurate due to technical failure since there was a lack of signals for any primers. IL-6 mRNA was absent in two, IL-1β mRNA in three samples (Figure 2).

Since the presence of mRNA does not necessarily indicate protein production [13, 14], 6 CLL cases (positive for mRNA by RT-PCR) were further analysed. Approximately 88–98% of circulating B-CLL cells reacted positively with anti-syndecan-1 MAb in all cases except one, where the positivity was 58.5%. All controls remained below 5% (Figure 3). A similar reaction pattern was observed using the same MAb and tyramine amplified immuncytochemistry on cytospin slides. In positive cells, the syndecan-1 protein was located on the cell surface.

To verify syndecan-1 expression on normal PBMN cells, they were double stained with anti-syndecan as well as anti-CD3 or anti-CD19 antibodies. No or hardly any positive cells were identified by flow cytometry, while HT58 B-NHL cells showed the expected positivity (Figure 4).

DISCUSSION

The low level of syndecan-1 mRNA and the absence of syndecan-1 protein in control mononuclear cells are in agreement with previously published data showing that syndecan-1 is not expressed in circulating murine B cells, only in those in tissue environment [6] and that it is expressed in human pre-B and plasma cell lines, but not in activated B cells (using RT–PCR) [15]. Here, we provide the first evidence that peripheral B-CLL cells express both mRNA and protein of syndecan-1. Also, our data support the fact that there is no syndecan-1 expression on human peripheral lymphoid cells. What is the biological significance of syndecan-1 in circulating B-CLL cells? One explanation would be the promotion of a more efficient adherence and cooperation with growth factors to stimulate accumulation of leukaemic cells in distant sites. However, this appealing idea is just the opposite of what has been observed for non-haematopoietic (epithelial) malignancies where syndecan-1 expression decreased or disappeared during malignant transformation, both *in vivo* and *in vitro* [16–18]. This difference between epithelial and haematopoietic cells was also indicated in another study, when syndecan transcription was detected in human myeloma cell lines by RT–PCR and also by immunodot blotting [19], similarly to the normal counterparts, plasma cells.

Direct evidence that syndecan-1 plays a role in homotypic adhesion comes from experiments where human B lymphoid cells (ARH-77) and human lymphoblastoid (Raji) cells were transfected with cDNA of murine syndecan-1 and the transfectants formed large multicellular aggregates in suspension cultures. The aggregation was inhibited by the addition of heparin and heparin-like glycosaminoglycans and by the removal of HS from the cell surface [20]. The transfected Raji cells, showing strong syndecan-1 expression, could bind to and spread on thrombospondin and fibronectin [21]. These observations support the idea that syndecan-1 can function as a receptor or co-receptor for different factors including growth factors (e.g. which are ligands for the HS chains of the proteoglycans like bFGF) [22], or others (e.g. which can promote homotypic/heterotypic or matrix adhesions) [2, 3, 23].

There is only limited information on the regulatory mechanisms of syndecan expression in lymphoid cells [24–26]. In murine B lymphoid cells, syndecan-1 expression is regulated by IL-6 via post-transcriptional mechanisms [27], but data on the expression and production of IL-6 and other suggested regulatory cytokines in human CLLs are conflicting. In one study, B-CLL cells produced IL-1β, TNFα, TGFβ and IL-6, similarly to the cytokine pattern of normal resting B lymphocytes [28]. Others have shown no significant release of IL-6 in B-cell leukaemia or lymphomas, including B-CLL [29]. Similarly, no RNA message for IL-6 or IL-1 and only low levels of TNFα mRNA could be detected in cultured mononuclear cells from patients with B-CLL. *In situ* hybridisation revealed no IL-6 mRNA, but TNFα and IL-1β mRNA were detected in a minority of mononuclear cells [30]. In our study, 12/14 circulating leukaemic cells of CLL patients expressed IL-6 mRNA, compared with 2/6 normal controls, and this difference was not seen for IL-1β. Due to the fact that almost all cases were positive, an estimation of the relationship between the expression of syndecan-1 and clinical, biochemical data (Table 1) had no relevance. We had only one 'true' syndecan-1 negative B-CLL case, and this was also IgM negative. To understand this finding and to find any correlation between clinical data and the syndecan expression would require more syndecan-1 negative B-CLL cases.

Considering all possibilities, we believe that this first evidence of the presence of syndecan mRNA and protein on

circulating B-CLL cells is an important result and could be one of the starting observations clarifying the biological role of transmembrane HS proteoglycans in lymphomas and leukaemia.

1. Elenius K, Jalkanen M. Function of the syndecans—a family of cell surface proteoglycans. *J Cell Sci* 1994, **107**, 2975–2982.
2. Bernfield M, Kokenyes R, Kato M, *et al.* Biology of the syndecans: a family of four transmembrane heparan sulfate proteoglycans. *Ann Rev Cell Biol* 1992, **8**, 365–393.
3. Rapraeger AC. The coordinated regulation of heparan sulfate, syndecans and cell behaviour. *Curr Opin Cell Biol* 1993, **5**, 844–863.
4. Kim CV, Goldberger OA, Gallo RL, Bernfield M. Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue- and development-specific patterns. *Mol Biol Cell* 1994, **5**, 797–805.
5. Salmivirta M, Jalkanen M. Syndecan family of cell surface proteoglycans: developmentally regulated receptors for extracellular effector molecules. *Experientia* 1995, **51**, 863–872.
6. Sanderson RD, Lalor P, Bernfield M. B lymphocytes express and lose syndecan at specific stages of differentiation. *Cell Regul* 1989, **1**, 27–35.
7. Dustin LB, Bullock ED, Hamada Y, Azuma T, Loh DY. Antigen-driven differentiation of naive Ig-transgenic B cells *in vitro*. *J Immunol* 1995, **154**, 4936–4949.
8. diCelle PF, Carbone A, Marchi D, *et al.* Cytokine gene expression in B-cell chronic lymphocytic leukemia: evidence of constituve interleukin-8 (IL-8) mRNA expression and secretion of biologically active IL-8 protein. *Blood* 1994, **84**, 220–228.
9. Caligaris-Cappio F, Gottardi D, Alfarano A, *et al.* The nature of the B lymphocyte in B-chronic lymphocytic leukemia. *Blood Cells* 1993, **19**, 601–613.
10. Mali M, Jaakola P, Arvilommi AM, *et al.* Sequence of human syndecan indicates a novel gene family of integral membrane proteoglycans. *J Biol Chem* 1990, **265**, 6884–6889.
11. Kopper L, Bánkfalvi Á, Mihalik R, *et al. In vitro* line from human (B) non-Hodgkin lymphoma. *Anticancer Res* 1991, **11**, 1645–1648.
12. Merz H, Malisius R, Mannweiler S, *et al.* ImmunoMax: a maximized immunohistochemical method for the retrieval and enhancement of hidden antigens. *Lab Invest* 1995, **73**, 149–156.
13. Yeaman C, Rapraeger AC. Membrane-anchoraged proteoglycans of mouse macrophages: P388D1 cells express syndecan-4-like heparan sulfate proteoglycan and a distinct chondroitin sulfate form. *J Cell Biol* 1993, **122**, 941–950.
14. Miettinen HM, Edwards SN, Jalkanen M. Analysis of transport and targeting of syndecan-1: effect of cytoplasmic tail deletions. *Mol Biol Cell* 1994, **5**, 1325–1339.
15. Engelmann S, Ebeling O, Schwartz-Albiez R. Modulated glycosilation of proteoglycans during differentiation of human B lymphocytes. *BBA* 1995, **1267**, 6–14.
16. Inki P, Stenback F, Talve L, Jalkanen M. Immunhistochemical localization of syndecan in mouse skin tumors induced by UV irradiation. Loss of expression associated with malignant transformation. *Am J Pathol* 1991, **139**, 1333–1340.
17. Inki P, Gomez M, Quintanilla M, Cano A, Jalkanen M. Expression of syndecan in transformed mouse keratinocytes. *Lab Invest* 1992, **62**, 225–233.
18. Kato M, Saunders S, Nguyen H, Bernfield M. Loss of cell surface syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like cells. *Mol Cell Biol* 1995, **6**, 559–576.
19. Ridley RC, Xiao H, Hata H, Woodliff J, Epstein J, Sanderson RD. Expression of syndecan regulates human myeloma plasma cell adhesion to type I collagen. *Blood* 1993, **81**, 767–774.
20. Stanley MJ, Liebersbach BF, Liu W, Anhalt DJ, Sanderson RD. Heparan sulfate mediated cell aggregation: Syndecan-1 and -4 mediate intercellular adhesion following their transfection into human B lymphoid cells. *J Biol Chem* 1995, **270**, 5077–5083.
21. Lebakken CS, Rapraeger AC. Syndecan-1 mediates cell spreading in transfected human lymphoblastoid (Raji) cells. *J Cell Biol* 1996, **132**, 1209–1221.
22. Steinfeld R, Van-Den-Berghe H, David G. Stimulation of fibroblast growth factor receptor-1 occupancy and signaling by cell surface-associated syndecans and glypican. *J Cell Biol* 1996, **133**, 405–416.
23. Elenius K, Salmivirta M, Inki P, Mali M, Jalkanen M. Binding of human syndecan to extracellular matrix proteins. *J Biol Chem* 1990, **265**, 17837–17843.
24. Vihinen T, Auvinen P, Alanen-Kurki L, Jalkanen M. Structural organization and genomic sequence of mouse syndecan-1 gene. *J Biol Chem* 1993, **268**, 17261–17269.
25. Vihinen T, Maatta A, Jaakkola P, Auvinen P, Jalkanen M. Functional characterization of mouse syndecan-1 promoter. *J Biol Chem* 1996, **271**, 12532–12541.
26. Turner CA, Mack DH, Davis MM. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 1994, **77**, 297–306.
27. Sneed TB, Stanley DJ, Yuong LA, Sanderson RD. Interleukin-6 regulates expression of the syndecan-1 proteoglycans on B-lymphoid cells. *Cell Immunol* 1994, **153**, 456–467.
28. Schena M, Gaidano G, Gottardi D, *et al.* Molecular investigation of cytokines produced by normal and malignant lymphocytes. *Leukemia* 1992, **6**, 120–125.
29. Burger R, Wendler J, Antoni K, *et al.* Interleukin-6 production in B-cell neoplasias and Castleman's disease evidence for an additional paracrine loop. *Ann Hematol* 1994, **69**, 25–31.
30. Burke F, Griffin D, Elwood N, *et al.* The effect of cytokines on cultured mononuclear cells from patients with b chronic lymphocytic leukemia. *Hematol Oncol* 1993, **11**, 23–33.

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