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Functional and clinical relevance of CD44 variant isoform expression on B-cell chronic lymphocytic leukemia cells

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Abstract

Background and Objective. **Recent studies have shown that expression of adhesion molecules of the Ig superfamily, of integrins and of selectins allows definition of high vs low risk B-cell chronic lymphocytic leukemia (B-CLL). The proteoglycan CD44 is an adhesion molecule that may be expressed as a *standard* form of 85-95 KD or as several *variant* isoforms. The presence of certain CD44 variant (v) isoforms on neoplastic cells indicates poor prognosis in epithelial and lymphoid malignancies, as it is associated with tumor progression and metastasis.**

Design and Methods. **The expression of CD44 v3, 4, 5, 6, 7, 9 and 10 was analyzed in cells from 85 B-CLL patients. Indirect immunofluorescence and flow cytometry were used to identify CD44v. Functional studies were performed by analysis of adhesion to hyaluronate (HA), one CD44 ligand, and HA-induced Ca^{2+} influx. A variety of statistical methods were used to define phenotypic and functional differences between the various clones, to calculate survival curves, and for multivariate analyses.**

Results. In 17/85 B-CLL (20%), one or more CD44v were detectable by indirect immunofluorescence, whereas in 68/85 cases (80%) this technique yielded negative results. However, moAb "mixes" against CD44v and patching of surface molecules on B-CLL cells have shown that all B-CLL clones express CD44v. This has been confirmed by Western blot in a number of cases. Thus, two groups of patients whose cells bear CD44v at high or low density, are distinguished. Functions of the two clonotypes were investigated, namely their adhesion to a CD44 ligand and hyaluronate (HA), and effect on HA-induced Ca^{2+} influx. Cells expressing high density CD44v adhere to HA-coated substrates more efficiently than cells with low density CD44v. In all clones, HA-signaling via CD44 yields Ca^{2+} influx. This indicates that CD44 mediates activatory signals following interaction with the ligand.

Interpretation and Conclusions. **The clinical relevance of these findings has been ascertained. The 17/85 cases whose cells bore high density CD44v had significantly worse prognostic features than those of patients with low density CD44v, namely more advanced disease stage, LDT < 12 months and therapy requirement. Moreover, the median survival in the former group of patients was < 5 years as opposed to > 12 years in the latter. Therefore, analysis of CD44v expression provides indications of biological and clinical relevance also in low grade lymphoproliferative disorders.**

Key words: adhesion molecules, B-CLL, CD44 standard, CD44 variant isoforms, prognostic factors

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The CD44 proteoglycan is a multistructural and multifunctional adhesion molecule that recognizes hyaluronic acid (HA) and other extracellular matrix (ECM) components, such as collagens, fibronectin and laminin, and is capable of mediating activatory signals via cytoskeletal rearrangements.[1-5] The CD44 surface receptor is expressed on a variety of hematopoietic and non-hematopoietic normal and malignant cells in which it plays a role both in cell function regulation and in the pathophysiology of neoplastic disorders.[6,7]

CD44 is found on lymphocytes of B and T lineage, with the exception of sessile cells such as cortical thymocytes and germinal center B cells.[8] This indicates that all trafficking lymphocytes bear CD44, also termed the *lymphocyte homing receptor*.[9] Interestingly, co-capping experiments have shown that, on both T and B cells, CD44 is physically associated with the antigen receptor complex, and it has been suggested that it may deliver co-activatory signals following antigen recognition (Dianzani U., personal communication, and ref. #10).

In addition to the *standard* form of 85-95 KD (CD44st), several *variant* isoforms (CD44v) are generated by pre-mRNA alternative splicing of at least 20 different exons.[11-13] These variants differ from the standard form in that they contain additional peptide domains inserted into the membrane-proximal extracellular portion of the molecule, and therefore

display a higher molecular weight.[14] The CD44st and CD44v repertoire is further enriched by N- and O-glycosylation and by glycosaminoglycanation (by heparan sulfate and chondroitin sulfate).[7,15]

CD44v isoforms are not detected on resting B and T lymphocytes. However, CD44v6 and v9 are transiently expressed on activated T cells.[13,16] Their presence is necessary for HA adhesion, and these isoforms possess signaling and effector function activation capabilities.[17-19] In addition, HA-mediated signaling appears to be required for variant-dependent adhesion to HA.[19] This indicates that CD44v isoforms play a functional role in T lymphocytes.

Splice variants carrying sequences encoded by exon v6 are selectively detected in metastatic animal cancer cell lines, and they confer metastatic behavior to non-metastatic tumor cells.[20,21] In some cancers, expression of CD44v5 and v6 is associated with disease progression and with metastatic potential.[22-27] In certain malignancies, such as melanoma, tumor progression is accompanied by up-regulation of v5 and down-regulation of v10.[28] Overexpression of CD44v6 has also been described in high grade, but not in low grade non-Hodgkin's lymphomas.[16,29,30] Although the significance of CD44v in lymphoproliferative disorders as well as in other malignancies has been challenged recently,[31] it is generally agreed that they may help define neoplastic clones in more or less aggressive disease settings.

B-cell chronic lymphocytic leukemia (B-CLL) is regarded as a low grade lymphoproliferative disorder. However, a high degree of phenotypic heterogeneity has been observed among B-CLL clones.[32,33] On clinical grounds, the course of the disease is also quite variable, with cases that remain stable for long periods of time and others that are rapidly progressive.[34] Most often this is not paralleled by significant phenotypic differences. We and others have shown that expression of adhesion molecules of the immunoglobulin superfamily, of integrins and of selectins, allows definition of high vs low risk B-CLL patients.[33,35-39] As for CD44st expression, two groups of B-CLL clones can be distinguished that bear the receptor at low ($CD44^{low}$) or at high ($CD44^{high}$) density. The two groups of patients have a significantly different survival.[40] We have also shown that the levels of serum CD44st, but not those of CD44v5 and v6, provide useful information for tumor bulk assessment, for detection of high risk cases and for monitoring disease progression.[41]

In this study we analyzed the expression of CD44v isoforms on B-CLL cells. All the leukemic clones express CD44v, which are detected at high density in 20% of the cases and at low density in the remaining 80%. We also ascertained the functional relevance of CD44v expression for substrate adhesion of, and signaling to B-CLL cells. Finally, we showed that the density of CD44v expression allows significant clinical correlations between B-CLL phenotype, disease stage and survival.

Materials and Methods

Patients

Eighty-five patients were enrolled in this study after informed consent. The mean age of the patients was 57.6 years, and the male to female ratio was 50:35. Diagnosis of B-CLL according to generally accepted criteria[42] was confirmed by immunophenotypic analysis of IgH and L chains, CD5, CD19 and HLA-DR expression. The pattern of bone marrow infiltration[43] was evaluated in bone marrow biopsies from 71/85 patients. Biopsy was not performed in 14 patients due to advanced age, cardiovascular risk factors, or refusal of informed consent. The clinical evaluation included assessment of peripheral blood lymphocytosis, liver, spleen and lymph node enlargement, lymphocyte doubling time (LDT),[44] and staging according to Binet *et al.*[45] At the time of study, 62 patients were in Binet stage A, 12 were in stage B and 11 were in stage C of disease. For an evaluation of survival and for the construction of survival curves, none of the above patients was censored because of disease-unrelated death. Not all of the patients were studied at diagnosis. Therefore our study is not prospective.

Surface phenotype of B-CLL cells and expression of CD44st and of CD44v isoforms

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Freshly isolated cells or cells stored in liquid nitrogen were used in all of the studies without appreciable differences.

In addition to mouse moAb to IgH and L chains, CD5, CD19 and HLA-DR, that allowed evaluation of the bulk of B-CLL cells, moAb to CD3 and CD16 were used to enumerate residual T cells and NK cells. All of the above moAb were from Becton Dickinson (San José, CA, USA). We also used moAb to CD44st (Immunotech, Luminy-Marseille, France) and to the variant isoforms v3 (R&D Systems, Abingdon, UK), v5, v6, v7, v10 (Bender MedSystems, Vienna, Austria), v4 and v9 (a kind gift of C.R. Mackay, the Basel Institute for Immunology, Switzerland). In some experiments, anti-CD44v moAb were used as the primary reagent in the following combinations or *mixes*: v3+v4+v5, v5+v6+v7, v7+v9+v10. The secondary reagents used in the indirect immunofluorescence assays were FITC-conjugated goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Birmingham, AL, USA). Cells were subsequently analyzed by flow cytometry (FACScalibur, Becton Dickinson). Controls were provided by use of unrelated primary moAb of the same isotype, followed by the secondary reagent, and by use of the latter reagent alone.

In 10 selected patients the phenotype of leukemic cells was re-assessed, within 6 months after the first analysis.

Modulation of B-CLL cell surface molecules

To determine the redistribution of cross-linked sur-

face molecules, cells were incubated with the primary moAb for 30 min on ice. The secondary labeled reagent was then added, and cells were incubated at 37°C for 4 hours. Analyses of surface molecule redistribution were performed by both fluorescence microscopy and flow cytometry. In addition to moAb to CD44st and variant isoforms (used one by one or in *mixes*, see preceding section), we used moAb to IgM (Becton Dickinson) as a control for the modulation experiments.

To assess whether or not the CD44 ligand HA could induce a redistribution of CD44 molecules similar to that obtained by moAb cross-linking, B-CLL cells were incubated overnight at 37°C on plastic surfaces coated with human umbilical cord HA (Calbiochem, San Diego, CA, USA) at 1 mg/mL. Cells were subsequently harvested by vigorous pipetting, stained with moAb to CD44st followed by the secondary labeled reagent, and analyzed by flow cytometry and fluorescence microscopy.

Analysis of CD44 by immunoprecipitation and immunoblotting

Immunoprecipitation/blotting experiments were performed on peripheral blood mononuclear cells from three patients whose cells expressed CD44v detectable by indirect immunofluorescence, and from three patients whose cells were negative by this assay. In all of the cases, HLA-DR$^+$ cells exceeded 90%.

Ten$\times 10^6$ cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer containing 1% Triton X 100 and 0.15M NaCl, for 1 hour in the cold. Cell lysates were centrifuged for 1 hour at 10,000 rpm and supernatants were analyzed. Cell supernatants were precleared using protein A-Sepharose (Pharmacia, Uppsala, Sweden), and immunoprecipitated with anti-CD44st moAb (clone J 173, Immunotech). As a control moAb for immunoprecipitation we used an anti-CD4 moAb (Serotech, Oxford, England). An additional control was provided by the A-549 lung adenocarcinoma cell line which expresses the standard form of CD44, but no variant isoforms (see ref. #41). After several washings, immunoprecipitates were eluted by boiling in non-reducing running buffer, and separated in 7.5% SDS-PAGE. Proteins were blotted onto nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). Membranes were saturated overnight with non-fat dry milk (Merck, Darmstad, Germany) in PBS, and subsequently incubated with 10 µg anti-CD44st moAb in PBS, supplemented with 1% non-fat dry milk, for 1 hour under continuous stirring. After exhaustive rinsing with PBS containing 0.5% Tween 20, membranes were incubated with affinity-purified, peroxidase-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark). Protein bands were revealed using the ECL system (Amersham). Molecular weight markers were from Biorad (Richmond, VA, USA).

Cell adhesion assays

Cell adhesion to HA-coated surfaces was evaluated using the technique described by Miyake *et al.*,[3] with modifications.[19] Cells were labeled with ^{51}Cr (Amersham) and added (2×10^5 cells/well) to 96-well plates (Costar, Cambridge, MA, USA) precoated with 1 mg/mL HA. Control wells as well as non-specific binding sites of HA-coated wells were blocked with 10% fetal calf serum (FCS) in PBS.[19] Plates were centrifuged at 600 rpm for 5 min, incubated at 37°C for 5 min, and unbound cells were removed by 5 washing cycles in pre-warmed medium. Bound cells were lysed with 1N HCl and ^{51}Cr was measured in a gamma counter. Percentages of bound cells were determined as: percent bound=[(cpm from bound cells)/(input cell associated cpm – spontaneously released cpm)] $\times$ 100.

Measurement of intracellular Ca^{2+} mobilization

Five $\times 10^6$ B-CLL cell aliquots (10^6/mL) were incubated with 5 mM Fura 2-AM (Sigma, St. Louis, MO, USA) for 30 min at room temperature in a standard loading buffer,[46] washed and resuspended in a 0.7 mL volume of the same buffer, and dispensed in a quartz cuvette alternatively illuminated by 340 and 380 nm light using a Hitachi 2000 fluorescence spectrophotometer. The intracellular Ca^{2+} concentration was calculated according to Tsien and Poenie.[47] The Fura 2/Ca^{2+} signal was calibrated by adding 125 µM digitonin followed by 2.5 mM EGTA (Sigma). To evaluate the effect of the CD44 ligand, HA was added at 100 µg/mL final dilution. A control to this experiment was provided by treatment of the cells with anti-CD44 moAb (clone J-173, Immunotech) prior to incubation with HA (see ref. #19). The total intracellular Ca^{2+} was determined by addition of 0.5 µM ionomycin (Sigma).

Statistical analyses

Each single clinical and phenotypic parameter was matched with all of the others using the statistical technique of cross tabs. We used the Pearson χ^2 test (SPSS/PC) to define the significance of each cross and analyzed the percentages in each tab. When the probability value was less than 0.05, the hypothesis that the two examined variables were independent was rejected. The Student t-test was used to evaluate statistical differences in the mean values of percentage HA adhesion. Survival curves were calculated according to the Kaplan-Meier method and the statistical difference was evaluated using the log-rank test. For a multivariate analysis we used the proportional Cox's hazard regression model (see Biomedical Package BMPD, 1981). The model is in keeping with the partial likelihood theory. Estimates of the regression coefficients are provided together with the standard measures of the significance of covariates (prognostic factors). The set of variables analyzed in the Cox's model was: bone marrow histologic pattern (non-diffuse vs. diffuse); Binet stage (A vs. B+C);

splenomegaly (no vs. ≥ 3 cm below the costal margin); LDT (≥ 1 yr vs. < 1 yr); therapy requirement (no vs. yes); lymphocytosis (≤ 60,000 vs > 60,000/μL); CD44v expression (high vs. low).

Results

Detection of CD44v on B-CLL cells by indirect immunofluorescence

Expression of CD44v3, v4, v5, v6, v7, v9 and v10 on B-CLL cells from 85 patients was analyzed. One or more isoforms in various combinations were readily detectable in 17/85 cases (20%) and this phenotype, reassessed within six months, remained stable in a group of 10 patients selected from among both positive and negative cases. Flow cytometric profiles of one negative and one positive representative case are shown in Figure 1. In Table 1 we report percentages of CD44v$^+$ cells, of residual CD3$^+$ T cells and of CD19$^+$-HLA-DR$^+$ cells, providing a rough indication of the proportion of leukemic B cells in the 17 cases whose cells expressed CD44v isoforms detectable by indirect immunofluorescence. The data show that variable numbers of CD44v isoforms, from 1 to 6, are detected in each case. CD44v4 is most frequently expressed (14/17 cases), whereas CD44v9 was not found in any case. It should be noted that moAb against CD44st reacted with nearly 100% of the cells in cases that expressed CD44v, as well as in cases that were CD44v-negative by indirect immunofluorescence (not shown).

All B-CLL cell clones express CD44v isoforms

The finding that in 20% of the B-CLL cases the malignant cells bear CD44v at levels detectable by indirect immunofluorescence raises the possibility that, in the remaining negative cases, cells might express the variant isoforms at a very low density, which is not be detectable by this technique. In order to prove this hypothesis, we set up two types of experiments. B-CLL cells that were negative for each individual moAb were stained with mixes of moAb to v3+v4+v5, v5+v6+v7, and v7+v9+v10. Data in Table 2 show that in some cases this approach was successful in detecting CD44v. In many cases, however, the results were negative (see e.g. patients 1, 3, 6 etc. in Table 2). Therefore, in a second set of experiments, modulation of surface molecules was performed as described in the *Materials and Methods* section. The rationale behind these experiments is that, due to cytoskeletal defects, B-CLL cells, at variance to normal B cells, remain arrested at the *patching* phase of surface molecule modulation.[48] *Patching* of surface membrane molecules should increase the fluorescence signal, thus allowing detection of molecules expressed at very low density that would be otherwise undetectable. The comparison of fluorescence micrographs with flow cytometric profiles of modulated B-CLL cells shows that patching of cross-linked surface molecules is indeed paralleled by a definite increase in the fluorescence signal (Figure 2), and data in Table 2 demonstrate that CD44v is expressed on all the B-CLL clones tested. Additional modulation experiments in the remaining 80% of the B-CLL cases that were negative

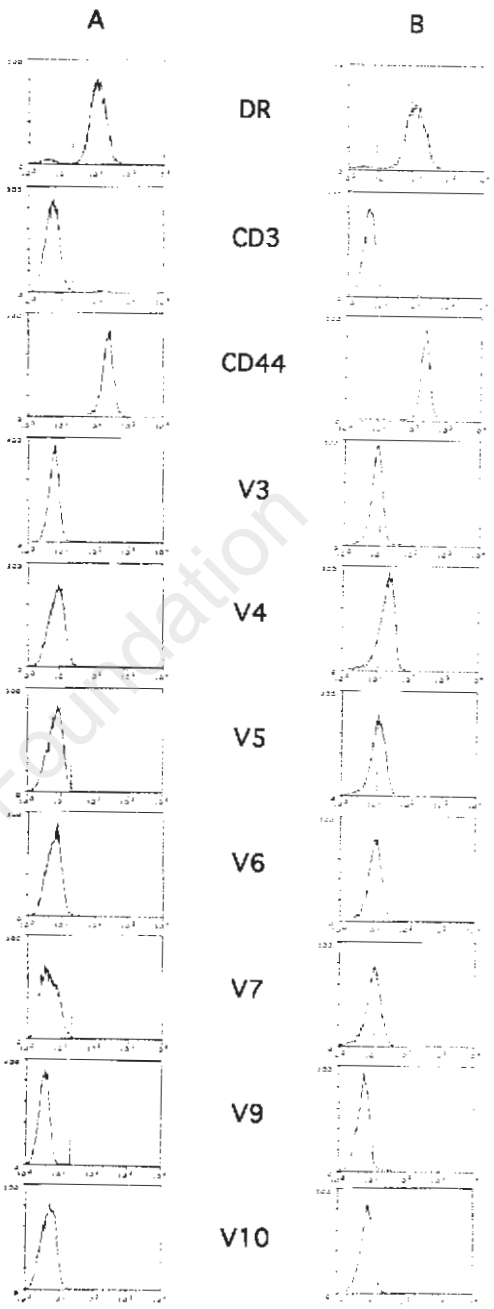

Figure 1. Flow cytometric analysis of CD44v expression on B-CLL cells from two representative cases. A) Cells are negative for all of the variant isoforms analyzed. B) Several variant isoforms are detected in variable percentages of B-CLL cells. CD44st is expressed on all of the cells. HLA-DR and CD3 provide a rough estimate of B-CLL cells and of residual T cells, respectively.

Table 1. Expression of CD44v on B-CLL cells from 17 (out of 85) patients.*

Pt.	HLA-DR°	CD3	CD44	v3	v4	v5	v6	v7	v9	v10
1	97	1	99	30#	87	61	51	48	3	16
2	88	7	100	12	54	25	23	11	0	2
3	82	2	97	3	2	22	1	29	1	1
4	72	20	100	12	29	5	3	5	1	2
5	94	8	100	18	12	3	1	1	1	1
6	99	2	98	19	43	31	33	30	4	10
7	79	5	99	6	38	17	35	25	0	6
8	93	5	100	2	21	2	4	0	1	3
9	96	4	99	1	33	10	5	5	0	0
10	95	5	100	1	26	2	2	2	1	0
11	86	10	100	4	28	14	19	11	1	2
12	97	2	100	33	52	40	30	24	1	4
13	98	0	100	3	17	14	25	10	0	5
14	62	7	93	2	21	4	10	7	0	1
15	90	11	100	3	49	4	5	21	1	1
16	77	8	100	99	12	2	1	2	1	0
17	93	5	100	33	88	74	75	57	3	24

**Percentages of positive cells determined by indirect immunofluorescence and flow cytometric analyses. °Percentages of CD19⁺ cells were virtually superimposable to those provided by HLA-DR determination. #Underlined numbers indicate significant percentages of CD44v⁺ cells. Percentages lower than 15% were considered negative.*

Table 2. Effect of moAb "mixes" and of their modulation on the detection of CD44v isoforms.

Pt.*	v3+v4+v5	MOD.°	v5+v6+v7	MOD.	v7+v9+v10	MOD.
1	1#	81#	0	72	3	68
2	47	98	20	99	9	96
3	0	49	0	49	0	10
4	22	73	10	58	13	47
5	21	75	19	77	20	67
6	3	50	2	49	2	48
7	7	77	2	81	1	67
8	7	77	6	77	5	66
9	10	78	6	77	5	62
10	2	69	2	64	1	54
11	2	62	1	48	1	44
12	16	88	13	87	8	83
13	1	95	4	91	2	82
14	0	42	0	51	0	12
15	0	48	0	38	0	25
16	3	78	2	72	1	52

**In all of these patients, staining with single moAb to CD44v had yielded negative results. °Mod = Modulation performed at 37°C after staining with primary and secondary antibodies (see Materials and Methods). #Percentages of positive cells, before and after modulation, as determined by indirect immunofluorescence and flow cytometric analysis.*

in the straight indirect immunofluorescence assay yielded similar results (not shown).

We, therefore, demonstrated that two groups of B-CLL cell clones exist. In the first, that accounts for 20% of the cases, CD44v isoforms are expressed at high density on B-CLL cells and are readily detectable by indirect immunofluorescence. In the second group, CD44v isoforms are undetectable because they are expressed on the cell surface at a very low density. They can, however, be demonstrated using moAb *mixes* or by modulating the surface molecules into *patches*.

That B-CLL clones express CD44v was further confirmed by immunoprecipitation/blotting experiments on B-CLL cell lysates belonging to the two groups described above. In both CD44v⁺ and CD44v⁻ cells defined by indirect immunofluorescence, moAb to CD44st detect the 85 KD hematopoietic form, together with several thicker bands of higher molecular weight (Figure 3). At least five bands of molecular weight comprised between 94 KD and 224 KD are demonstrated in both types of B-CLL cells. Increased glycosylation, glycanation and insertion of peptide domains (i.e. variant isoforms) may account for the high molecular weight forms of CD44. No high molecular weight bands were detected in A-549 cell lysates (not shown, but see Figure 3 in our ref. #41).

HA induces patching of surface CD44

To determine whether or not the CD44 natural ligand, HA, induced the same CD44 redistribution pattern observed in modulation experiments using moAb to CD44, B-CLL cells were incubated on HA-coated plastic surfaces and subsequently stained with anti-CD44st moAb. An increased fluorescence signal was observed following incubation on HA-coated surfaces in comparison with that from control B-CLL cells which were incubated on uncoated surfaces (Figure 4A). The occurrence of patching was confirmed by fluorescence microscopy observation (not shown).

As a control for these experiments and for those described in the preceding section, B-CLL cells were stained for surface IgM and subsequently modulated. Patching, shown by an increased fluorescence signal was again observed (Figure 4B). This indicates that, due to an intrinsic cytoskeletal defect of B-CLL cells, all surface molecules undergo incomplete redistribution following cross-linking and modulation.

HA adhesion of B-CLL cells expressing CD44v at high or low density

HA adhesion was tested in 26 B-CLL cases. In 12 of these, CD44v isoforms were readily detectable by indirect immunofluorescence (high density CD44v⁺) whereas, in the remaining 14, cells were negative by this assay (low density CD44v⁺). Percent adhesion was widely variable in the two groups (range 15% to 44%, and 3% to 25%, respectively). However, when the mean values in the two groups were compared (35.8±21.0 and 13.6±6.7, respectively), a signifi-

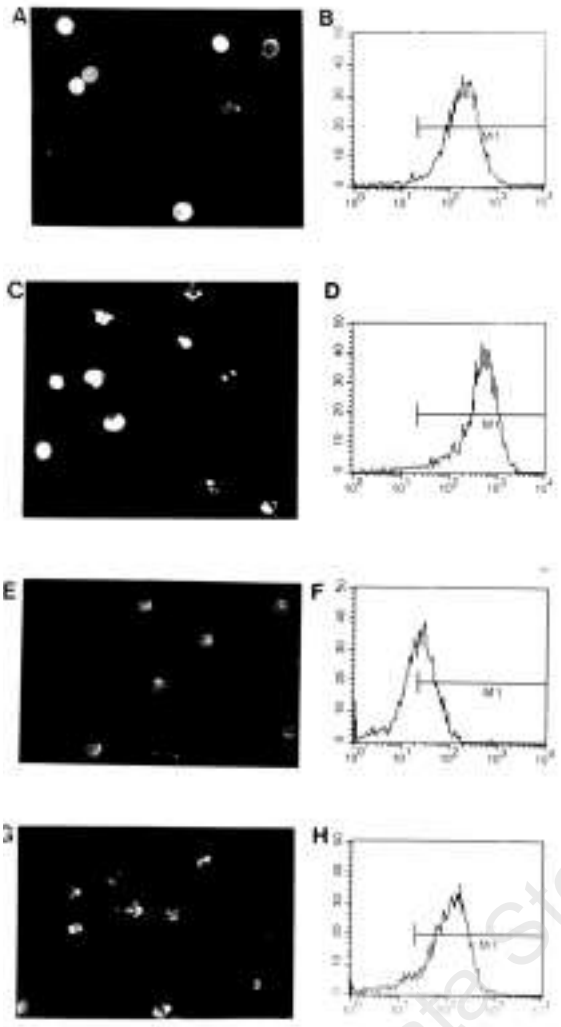

Figure 2. Fluorescence microscopic and flow cytometric analysis of B-CLL cells stained by indirect immunofluorescence for CD44st (A,B) and CD44v6 (E,F). The effect of modulation is shown in C, D for CD44st and in G, H for CD44v6. In C and G it is evident that modulation induces a redistribution of surface molecules in the form of patches. A comparison between B and D, and F and H demonstrates that patching of surface molecules results in a distinct increase of the fluorescence signal measured by flow cytometry. A, C, E, G, ×250.

cantly higher HA adhesion was found for B-CLL cells that expressed CD44v isoforms at high density vs B-CLL cells that were negative by indirect immunofluorescence ($p<0.006$).

HA-mediated signaling to B-CLL cells

Redistribution of surface CD44 molecules induced by HA suggests that the ligand-receptor interaction could deliver activatory signals to B-CLL cells. To test this hypothesis, Ca^{2+} influx was measured following

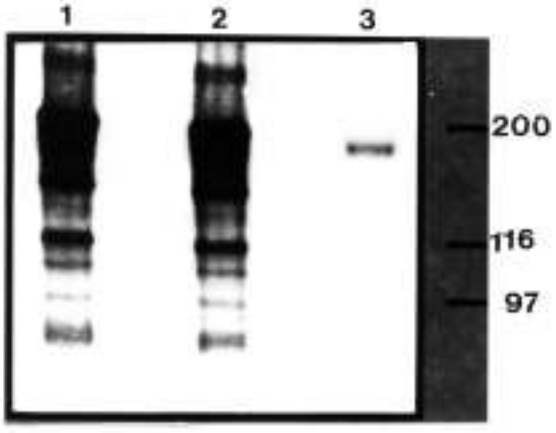

Figure 3. Immunoprecipitation/blotting of CD44 from two B-CLL cell lysates (lanes 1 and 2). Arrowhead indicates the band of 85 KD that corresponds to the hematopoietic form of CD44. Several bands of molecular weight comprised between 94 and 224 KD are also evident. Anti-CD44st moAb have been used for both precipitation and blotting. In lane 3 a control experiment using moAb to CD4 is shown. The band of approximately 150 KD is that of the mouse moAb used.

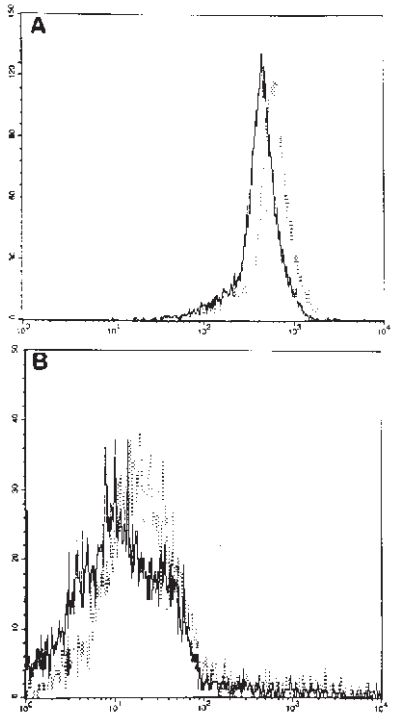

Figure 4. Modulation of CD44 by HA is shown in A. B-CLL cells incubated on plastic (solid line) or on HA-coated surfaces (dotted line) were subsequently stained for CD44st in indirect immunofluorescence and analyzed by flow cytometry. An increased fluorescence signal by HA-incubated cells is due to patching of surface CD44. The controls in B show staining of B-CLL cells for IgM before (solid line) and after modulation (dotted line).

B-CLL cell stimulation with soluble HA, and the results were compared to those obtained using the Ca^{2+} ionophore, ionomycin.

Data in Table 3 demonstrate that, in all cases tested, HA yielded efficient signals for Ca^{2+} influx to B-CLL cells. In 3/11 cases, the signal was even more efficient than that delivered by ionomycin. Two representative cases shown in Figure 5 demonstrate, however, that a large degree of heterogeneity was observed among the B-CLL clones, as for as concerned the time course and the level of the response to HA signaling. Pretreatment of cells with an anti-CD44 moAb directed against the HA-binding domain of CD44 inhibited HA-induced Ca^{2+} mobilization (not shown, see also ref. #19).

Effects of Ca^{2+} mobilization on HA adhesion of B-CLL cells

Our study on CD44v expression in activated T cells demonstrated that cytosolic free Ca^{2+} was required for adhesion to HA, as indicated by the observation that ionomycin increased and EGTA (a Ca^{2+} chelator) inhibited HA adhesion.[19] The effect of ionomycin and EGTA on HA adhesion was, therefore, investigated in 26 B-CLL cases. In the presence of ionomycin, HA adhesion of B-CLL cells was increased (by two- to five-fold) in 5/26 cases only, and in 16/26 cases EGTA significantly decreased HA adhesion (by 20% to 50%). In view of the increased Ca^{2+} influx determined by both HA and ionomycin (see preceding section and Table 2), lack of HA adhesion enhancement by ionomycin in most B-CLL cases is presumably due to cytoskeletal defects preventing focal adhesion.

Clinical relevance of CD44v expression on B-CLL cells

The above phenotypic and functional studies clearly indicate that two groups of patients can be distinguished on the basis of phenotypic and functional characteristics of the malignant cell clones, determined by CD44 expression and by adhesion to HA.

Some clinical features of the patients whose cells did or did not express CD44v at high density are shown in Table 4. Peripheral blood lymphocytosis, LDT, clinical staging, the bone marrow pattern of lymphocyte infiltration, spleen enlargement and therapy requirement were considered since they represent well known prognostic factors for evaluating the clinical course of B-CLL.[43-45] The expression of high density CD44v on B-CLL cells correlated significantly with advanced disease stage (Binet B/C), LDT <12 months and therapy requirement. As for the correlation with disease stage, it should be reiterated that expression of high density CD44v was detected in 9.6%, 25.0% and 72.7% of the patients in Binet stages A, B and C, respectively.

These data are further supported by analyses of the survival curves showing a significantly shorter survival ($p<0.001$) in the group of patients whose B-CLL cells expressed CD44v at high density (Figure 6). It worth repeating that none of the patients included in this evaluation was censored because of disease-unrelated death. Although performed in a limited number of patients, a multivariate analysis showed that LTD < 12 months and lymphocytosis > 60,000/cmm are significant and independent prognostic factors (Table 5). Other variables, including high density CD44v only correlate with the known negative prognostic factors and with disease activity.

Discussion

CD44 is a proteoglycan expressed on the surface membrane of a variety of cell types, including most lymphocytes. This molecule may be present in a standard form of 85-95 KD (CD44st), or it may contain polypeptide inserts, the product of at least twenty exons that may be expressed one by one, or in blocks. This yields variant isoforms (CD44v) of higher molecular weight.[12,14] Variant isoforms are found in certain

Table 3. Effects of HA and ionomycin on Ca^{2+} influx in B-CLL cells.

Pt.	*Basal Ca^{2+}*	*HA*	*Ionomycin*
1	134*	221 (1.6)°	357 (2.6)
2	47	163 (1.8)	652 (13.8)
3	38	175 (4.6)	54 (1.4)
4	55	186 (3.3)	508 (9.2)
5	75	352 (4.7)	155 (2.1)
6	100	310 (3.1)	650 (6.5)
7	110	253 (2.3)	409 (3.7)
8	140	280 (2.0)	300 (2.1)
9	80	250 (3.1)	480 (6.0)
10	150	312 (2.1)	600 (4.0)
11	92	340 (3.7)	322 (3.5)

**Ca^{2+} values are expressed as nM. °Data in parentheses indicate x fold increase.*

Table 4. Clinical features of the B-CLL patients whose cells expressed CD44v at high density.

Clinical features	*CD44v⁺**	*CD44v⁻°*	*Significance (p)*
Advanced Binet stage (B/C)	11/17	12/68	<0.0001
Splenomegaly	11/17	28/68	n.s.#
PB lymphocytosis (>60,000x10^9/mL)	5/17	17/68	n.s.#
Diffuse bone marrow infiltration	4/14@	12/57@	n.s.#
LDT < 12 months	9/16@	17/66@	0.018
Therapy requirement	14/17	37/68	0.035

**CD44v⁺ means that B-CLL cells were positive in an indirect immunofluorescence assay; °CD44v⁻ means that B-CLL cells were positive in an indirect immunofluorescence assay; #n.s.= non significant; @no. of patients available for evaluation.*

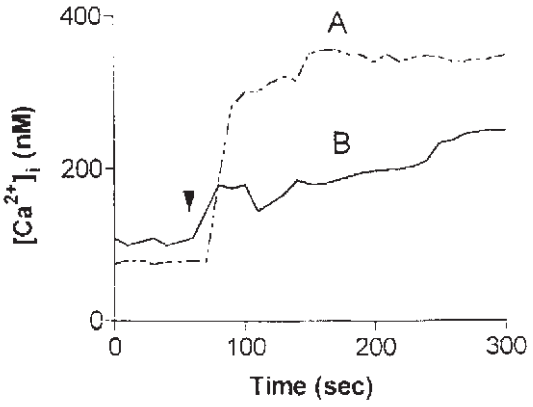

Figure 5. HA-induced cytosolic free Ca^{2+} mobilization in B-CLL cells. Arrow indicates addition of soluble HA. Patterns shown in A and B are representative of a slow and of a fast response observed in two distinct B-CLL clones, cases 5 and 7 reported in Table 3.

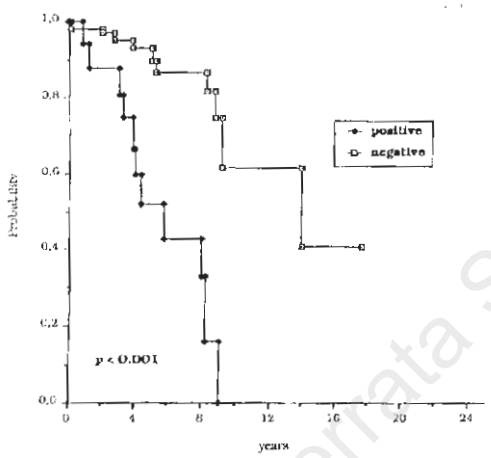

Figure 6. Survival of B-CLL patients according to the expression of CD44v on leukemic cells. Positive refers to the 17 patients whose cells expressed CD44v readily detectable by indirect immunofluorescence (high density CD44v+). Negative indicates the group of 68 patients whose cells were negative using the above technique (low density CD44+). A statistically significant difference exists in the median survival of the two groups of patients.

normal tissues, e.g. epithelia, but are never detected in resting T or B lymphocytes that express CD44st and are unable to bind HA, one natural ligand of CD44.[17] However, upon activation, T cells express v6 and v9 and acquire the ability to bind HA.[19] CD44 does not function solely as an adhesion molecule, but it is also a mediator of activatory signals upon recognition of its ligand(s),[17-19] and CD44v isoforms participate in this activatory pathway.[19]

Interest in the CD44 receptor has increased sharply after the observation that certain CD44v isoforms, particularly v5 and v6, are expressed on malignant

Table 5. Multivariate analysis of the variables entering the regression at significant levels.

	Regression coefficient	*SE*	*Wald*	*Exp (coefficient)*	*p value*
LDT ≤ 12 months	.8021	.2657	9.1110	2.2302	.0025
Lymphocytosis ≥ 60,000x10⁹/L°	.5441	.1923	8.0053	.5803	.0047
BM pattern/diffuse	.1779	.2168	.6738	.8370	.4117
Splenomegaly	.1246	.2076	.3602	1.1327	.5484
Therapy requirement	.0802	.1663	.2325	1.0835	.6297
BC Binet stage	.1369	.3048	.2016	.8721	.6534
$CD44v^{high}$	.0074	.2610	.0008	.9926	.9773

cells and impart a metastatic behavior to them.[20,21] In lymphoproliferative diseases, v5 and v6 have been detected in high grade but not in low grade malignancies, and their expression has been regarded as an additional diagnostic tool and as a prognostic factor.[16,29,30]

B-CLL is generally regarded as a low grade lymphoproliferative disorder because of its smouldering course and generally good response to therapy. However, there are cases that escape this scenario, being rapidly progressive and refractory to conventional treatment. This has prompted the search for novel disease markers and prognostic factors both in B-CLL cells and serum.[41,49-54]

B-CLL cells express CD44st in all cases. This contention is tenable, although a recent immunohistologic study of fixed bone marrow biopsies suggests that CD44-negative cases may exist.[55] Negative cases reported in this study could be due to loss of antigenicity of B-CLL cells expressing CD44st at low density, as our immunofluorescence studies of peripheral blood cells in B-CLL (see refs. #33,40 and the present study) have not detected any negative cases.

So far, expression of CD44v on B-CLL cells has not been extensively investigated. In one study of 14 B-CLL cases,[56] CD44v6, v7 and v10 were found in variable percentages of the malignant cells. The present study of 85 B-CLL cases shows that, in 20% of them, CD44 isoforms v3-v10 are readily detectable on B-CLL cells by indirect immunofluorescence, indicating that they are expressed at high density. All isoforms were found, alone or in combination, on cells from this group of patients, with the exception of v9 (see Table 1).

The finding of 80% CD44v-negative cases, however, does not prove that CD44v are absent, since they could be expressed at a very low density, i.e. below the sensitivity threshold of the immunofluorescence technique. This possibility is raised by our previous observation of activated T cells that transiently express v6 and v9 at a density approximately 10 times lower than that of CD44st.[19] To test this hypothesis, we adopt-

ed two different experimental approaches, i.e. the use of moAb *mixes* to several CD44v, and the modulation of surface CD44v molecules. The first experiment allows detection of different variants expressed in the same or in distinct CD44 molecules, thus increasing fluorescence intensity. The second experiment takes advantage of a peculiar cytoskeletal defect of B-CLL cells which does not allow complete modulation of surface membrane molecules.[48,57-58] In B-CLL cells, patching of these molecules is not followed by capping and internalization. We show that patching yields an increased fluorescence signal that allows detection of CD44v in otherwise negative cells.

These observations clearly delineate the existence of two groups of patients whose cells express CD44v at high (20%) or low (80%) density. In connection with this, it should be noted that repeated testing over a period of several months never showed a *switch* of patients from one group to the other.

That cells from both groups of patients express CD44v was also demonstrated by immunoprecipitation/blotting experiments in which, besides the 85 KD form, several bands of higher molecular weight were detected in all of the B-CLL cases studied. The latter bands could also represent alternatively glycosylated or glycanated forms of CD44.

A second group of experiments was aimed at determining the functional significance of CD44 expression on B-CLL cells. To find out whether or not HA, a CD44 natural ligand, could mimic the effect of moAb to CD44 on the redistribution of membrane molecules, cells were incubated on HA-coated surfaces. Increased fluorescence signal and patching of surface CD44 were observed following HA exposure.

Next, we asked whether HA could mediate signaling to B-CLL cells via CD44. Since Ca^{2+} mobilization provides a good indication that activatory signals are in effect,[19] B-CLL cells were incubated with soluble HA, or with the Ca^{2+} ionophore, ionomycin, as a positive control. In all cases tested, HA produced an increase of cytosolic Ca^{2+} that was abrogated by the Ca^{2+} chelator, EGTA. This indicates that, upon interaction with its ligand, CD44 mediates activatory signals for B-CLL cells.

Cytosolic free Ca^{2+} is necessary for T cell adhesion to HA.[19] Since HA induces Ca^{2+} influx in B-CLL cells, EGTA and ionomycin should reduce or increase HA adhesion, respectively. However, the expected effect was not always observed. In particular, ionomycin enhanced HA adhesion only in approximately 1/5 of the cases. Cytoskeletal defects of B-CLL cells could provide an explanation for this result.

In view of the important role of CD44v isoform expression for T cell adhesion,[13,16,19] we compared HA adhesion of B-CLL cells from the two groups of cases delineated above, i.e. "*high*" and "*low*" density $CD44v^+$ cells. Cells expressing CD44v isoforms at high density adhered to HA significantly more efficiently than low density $CD44v^+$ cells.

All of the above functional studies suggest that B-CLL cells which bear CD44v isoforms at high density may be activated more efficiently than cells with low density isoform expression. In turn, this would mean that the two groups of clones should differ as to their trafficking ability and their activation status. If this were the case, the two groups of patients should present with different clinical features. This was indeed shown by the significant correlation between high density CD44v expression and advanced disease stage (Binet B/C), LDT < 12 months and therapy requirement. Such correlation was further reinforced by analyses of the survival curves in the two groups of cases indicating that of the 85 patients, the 17 whose cells expressed CD44v at high density had a median survival of 5 years, vs a median survival of > 12 years for the 67 patients whose cells expressed CD44v at low density. Although a multivariate statistical analysis points to a significant prognostic independence for lymphocytosis and LDT, but not for high density CD44v expression, it is feasible that the trafficking ability and the activation level of the latter B-CLL clones may account for their proliferative activity, and thus for the reduced LDT, for the advanced clinical stage and for the therapy requirement. It is, therefore, conceivable that high density CD44v expression on B-CLL cells coincides with a number of unfavorable prognostic factors.

Taking into account the results of multivariate analyses we cannot suggest aggressive treatment related to expression of CD44v. So far, repeated testing of the clones over several months has failed to show phenotypic changes in CD44v expression. This reinforces our previous observations[40] on the phenotypic stability of CD44st on B-CLL cells. A long-term (years) follow-up study that we are performing will answer a so far unresolved question, i.e. whether or not high density CD44v expression in previously negative cases occurs and correlates with disease progression.

Contributions and Acknowledgments

GDR and CEG designed the study. DZ, CT and GMC performed all the immunofluorescence and flow cytometric analyses. PM did the Western blot analyses. NA, SF and AV conducted the functional studies (HA adhesion and Ca^{2+} influx). We thank Prof. P. Mancini (Department of Statistics, University La Sapienza, Rome) for all the statistical work herein reported.

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Disclosures

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