Expression of Adhesion Molecules on Leukemic B Cells from Chronic Lymphocytic Leukemia Patients with Predominantly Splenic Manifestations

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Abstract

Background: The highly tissue-specific trafficking of normal and malignant lymphocytes to particular organs is mediated by adhesion molecules, or "homing receptors." Among our patients with B cell chronic lymphocytic leukemia 15% demonstrate predominantly splenic manifestations and are classified as stage II(S).

Objective: To investigate whether expression of cell surface adhesion molecules can distinguish stage II(S) patients from stage 0 or stage 0 and I CLL patients.

Methods: Expression of adhesion molecules belonging to different families was studied in CD19-positive cells isolated from the blood of 42 patients by dual color flow cytometry. The families included: immunoglobulin superfamily (CD54, CD58), integrin family (β1, β2 and β3 chains, CD11a, CD11c CD49d), selectin family (L-selectin), and lymphocyte homing receptor family (CD44).

Results: The average percentage of leukemic cells expressing CD11c in the 23 patients with stage II(S) was 25.7 compared with 13.2% in the 14 patients with stage 0 disease (P = 0.047). The average percentage of leukemic cells expressing CD44 in patients with stage II(S) was 90.5 compared with 77.2% in patients with stage 0 (P = 0.007) and 80% in patients with stages 0 and I together (n = 19, P = 0.008). Other adhesion molecules tested did not show a statistically significance difference in expression between the different disease stages.

Conclusions: The higher expression of CD44 and CD11c in cells of CLL patients with predominantly splenic manifestations may account for the tendency of their lymphocytes to home to the spleen.

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Chronic lymphocytic leukemia is a neoplastic disease characterized by the accumulation of small mature-appearing lymphocytes in the blood, marrow and lymphoid tissues [1]. The staging system initially described by Rai et al. [2] in 1975 stratified patients according to the clinical estimate of the tumor load and was later confirmed to be the single most important predictor of prognosis [3–5]. The Rai system defines stage II CLL as splenomegaly or hepatomegaly with or without lymphadenopathy. Approximately half of all CLL patients present with mild to moderate splenomegaly [6]. In some CLL patients the clinical course is predominated by splenomegaly. In 1977, Binet et al. [7] distinguished a "pure splenic form" (stage II, isolated splenomegaly) that accounted for 4–6% of all cases of CLL and was characterized by a favorable prognosis, with a slow and uneventful course even when not treated [8]. Several years later Baccarani and co-workers [9] reported that patients with Rai stage II CLL were not a homogeneous group: those presenting with isolated splenomegaly (15% of stage II patients and 3% of all CLL patients) had a long survival (median >10 years), similar to that of patients with stage 0. Thereafter, Singh [10] identified a stage II(S) of the disease, characterized by splenomegaly and complete absence of lymphadenopathy, as a distinct entity with a unique clinical course. In 13 of the patients, who accounted for 10% of the total CLL series, the splenomegaly progressed with time, and when it became marked the clinical course was predominated by hypersplenism yet the survival was similar to that in stage 0.

The tissue-specific trafficking of normal and malignant lymphocytes to particular organs is mediated by adhesion molecules, or "homing receptors" [11,12]. These molecules constitute a structurally diverse group of cell membrane receptors belonging to distinct families, namely, the immunoglobulin (CD54, CD58), integrin (β1, β2, β3 chains, CD11a, CD11c, CD49d), selectin (L-selectin), and lymphocyte homing receptor (CD44) families. It has been postulated [12] that the regulation and function of adhesion molecule expression might explain the dissemination pattern, localization, and biological behavior of lymphoid malignancies. The studies conducted to date in the B-chronic lymphoproliferative disorders have been extensive and were designed to determine whether the expression of adhesion molecules is unique to the different disorders and whether it can help in their differential diagnosis [13–18]. In none of the studies was a large group of patients with predominantly splenic manifestation evaluated. The purpose of the present study was to evaluate the correlation between the expression of adhesion molecules on CLL cells and the development of a "pure splenic form" – stage II(S) – of CLL.

Materials and Methods

Materials

All antibodies used were purchased from commercial sources [Table 1]. Fluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson FACSCalibur, Mountain View, CA, USA).

Patients

The diagnosis of CLL was based on the following criteria: persistent, unexplained lymphocytosis of > 5,000/μL; mainly small, mature-appearing lymphocytes that expressed dim surface immunoglobu-

CLL = chronic lymphocytic leukemia

Table 1. Monoclonal antibodies

Specificities	Cluster of differentiation	Clone	Source
	CD19-PE	HD37	Dako, Copenhagen, Denmark
LFA-1	CD11a	MHM24	Dako
α_L	CD11c	KB90	Dako
Integrin β_2	CD18	CLB-LFA-1/1	Caltage, Burlingame, CA, USA
Integrin β_1	CD29	2A4	Caltage
LHR	CD44	DF1485	Dako
VLA-4	CD49d	44H6	SeroTec, Ltd. Oxford, UK
ICAM-1	CD54	6.5B5	Dako
Integrin β_3	CD61	Y2/51	Dako
L-selectin	CD62L	DREG-56	Caltage
Irrelevant IgG1		Dako-601	Dako

lins, and CD19, CD23 as well as CD5 [19]. Patients with cells expressing strong surface immunoglobulins were excluded, as were patients with other disorders such as prolymphocytic leukemia, hairy cell leukemia, splenic lymphoma with villous lymphocytes, and non-Hodgkin's lymphoma in the leukemic phase.

Patients were chosen based on their clinical characteristics. Clinical stages 0 and I were defined according to the Rai staging system [2]. Stage II(S) patients were those with a clinical course dominated by splenomegaly and only minimal peripheral lymphadenopathy (<2 cm). Splenic size was measured in all patients by palpation (edge length below the costal margin in the mid-axillary line) and by ultrasonography where splenic index was also calculated (spleen length x width x thickness).

The clinical characteristics of the patients at the time of sampling are shown in Table 2. Follow-up time was defined from the date of diagnosis to the date of sample collection.

Adhesion molecule studies by immunofluorescence analysis

Peripheral blood cells from 42 patients with CLL were collected after informed consent was obtained. The 42 patients included 23 with stage II(S), 14 with stage 0 and 5 with stage I. The cells from patients at different stages of the disease were examined simultaneously.

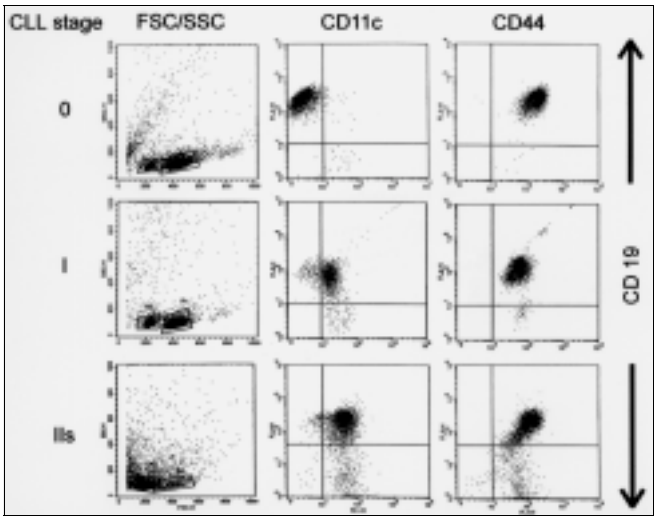

Figure 1. The two right columns show a representative two-color immunofluorescence analysis of CD11c, CD44 and CD19 co-expression on B-CLL cells from three patients: stage 0 CLL, stage I CLL and stage II(S) CLL. In the left column we can see the different size groups in these patients: large lymphocytes in the right square and small lymphocytes in the left square.

Mononuclear cells were isolated from heparinized peripheral blood samples by Ficoll-Hypaque density gradient centrifugation and frozen at -70°C until assayed. This step was based on the reported observation that there is no difference in fluorescence intensity between freshly isolated and frozen cells, even for molecules shed from the cell surface [14]. In several patients whose samples from fresh isolated cells were compared with frozen cells, the results were similar. The cells were thawed by standard procedure. Only samples with good viability (>97%) were included. Adhesion molecule expression was studied by using direct immunofluorescence. In brief, 10^6 cells were double-labeled with phycoerythrin-conjugated murine anti-human CD19 monoclonal antibody together with one of the anti-adhesion monoclonal antibodies conjugated with fluorescein isothiocyanate [Table 1]. In this way, the evaluation was restricted to the CD19-positive (CD19+) cells. The gate did not exclude CD19+ normal B cells but almost all the CD19+ cells were found to have the typical CLL phenotype (>90% were CD19/CD5 positive).

We considered positive the samples that showed fluorescence intensity $>10^1$ on the fluorescence channel or an intensity that was higher than that of the negative control if the latter was higher. Ten thousand events were recorded for each sample and the appropriate isotype-matched negative controls were used. Two populations of lymphocytes were observed on FSC/SSC graph [Figure 1, left column]. R1 large cells had an FCS value about double that of R2, which consisted of small cells.

Table 2. Clinical and laboratory characteristics (mean values ± standard deviation) of patients with CLL stages 0-I and II(S), at the time of adhesion molecule determination

	Stages 0+I (n=19)	Stage II(S) (n=23)	Statistical significance ($P < 0.05$)
Age (yrs)	72.4 ± 7.9	71.0 ± 5.9	Not significant
Hb (g/dl)	13.8 ± 1.3	12.3 ± 1.4	0.001
WBC count (cells/μl)	32,900 ± 23,300	77,600 ± 77,000	0.002 (of log WBC)
Lymphocytes (%)	77% ± 12	90.5% ± 5.0	<0.001
Thrombocyte count (cells/ μl)	217,400 ± 52,500	110,000 ± 32,800	<0.001
β2-microglobulin (mg/L)	1.67 ± 0.8	3.29 ± 1.7	0.012
Soluble CD23 (U/ml)	1,100 ± 1,090	3,040 ± 1,480	0.007
Follow-up time (months)	91 ± 90	109 ± 69	Not significant

Measurement of β2-microglobulin

β2-microglobulin was measured by standard method in an IMx system (Microparticle Enzyme Immunoassay System, Abbott Laboratories, IL, USA). The normal serum levels range from 0.7 to 3.4 mg/L

Measurement of soluble CD23

Serum samples were stored at -70^{O}C and serum was thawed at room temperature before use. The level of soluble CD23 was measured using a commercially available enzyme-linked immunosorbent assay kit (Endogen Inc., Woburn, MA, USA) in accordance with the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with the SPSS statistical software program (Chicago, USA). Either *t*-test or χ^2 analysis (Pearson's correlation or Fisher's exact test) was used for statistical comparison of the clinical characteristics and adhesion molecule levels among the different CLL groups. A *P* value <0.05 was considered statistically significant.

Table 3. Expression of the different adhesion molecules (average percentage of CD19+ cells ± standard deviation) and of small and large lymphocytes in CLL patients with stage 0, stage 0 and I, and those with stage II(S) disease

	Stage 0	Stage 0-I	Stage II(S)	P value
Small cells	16.8 ± 11.8*	21.7 ± 18	30.5 ± 21.1*	0.016
Large cells	59.9 ± 11.4*	52.2 ± 23.0	40.0 ± 28.6*	0.006
CD19	82.7±13.7*	83.5±12.6**	91.3±5.6*,**	0.042* 0.02**
CD11a	13.8 ± 15.0	17.2 ± 19.1	23.05 ± 19.8	NS
CD11c	13.2 ± 12.0*	18.8 ± 23.8	25.7 ± 24.5*	0.047
CD18	3.2 ± 3.1	4.3 ± 4.3	5.1 ± 3.5	NS
CD29	8.85 ± 9.0	11.5 ± 12.0	14.0 ± 20.7	NS
CD44	77.2 ± 15.0*	80.05 ± 14.3**	90.5 ± 7.7*,**	0.007* 0.008**
CD49d	7.8 ± 10.1	10.2 ± 12.4	12.7 ± 18.6	NS
CD54	3.25 ± 1.9	3.1 ± 2.2**	4.7 ± 3.3**	0.06**
CD61	3.8 ± 2.4	4.3 ± 3.7	7.8 ± 14.2	NS
CD62L	6.9 ± 9.5	7.4 ± 9.7	11.8 ± 12.4	NS

* Comparison between stage II(S) and stage 0 patients
** Comparison between stage II(S) and stages 0 and I patients

Results

We previously found that CLL stage II(S) patients comprise 15% of our total CLL population [20]. Clinical and laboratory characteristics of 23 CLL stage II(S) patients were compared with those of 19 CLL patients with stages 0-I disease [Table 2]. We found that the II(S) group was associated with many poor prognostic factors. The mean hemoglobin level in patients with stage II(S) disease was significantly lower than the level in stage 0+I disease (12.3 and 13.8 g/dl respectively, *P* = 0.001). Patients with stage II(S) disease had a higher white blood cell count, with a mean count of 77,600 cells/μl vs. 32,900 cells/μl in patients with stage 0 and I disease (*P* of log WBC = 0.002). The percent of lymphocytes in the total WBC count was also different between the two groups, with patients in stage II(S) showing a mean of 90.5% compared to 77% in those in stages 0 and I (*P* < 0.001). Patients in stage II(S) showed lower platelet counts (mean 110,000 cells/μl) than patients with stages 0 and I disease (mean 217,400 cells/μl, *P* < 0.001). Patients in stage II(S) showed higher levels of serum β2-microglobulin (mean value 3.29 mg/L in 16 evaluated II(S) patients compared to 1.67 mg/L in 9 patients with stages 0 and I, *P* = 0.012) and serum sCD23 (mean value 3,040 U/ml in 8 patients with stage II(S) disease compared to 1,100 U/ml in 9 patients with stages 0 and I, *P* = 0.007).

The mean percentage of leukemic cells expressing the different adhesion molecules and of lymphocytes of different size (small vs. large) in CLL patients with stage 0, stage 0 and I, and stage II(S) disease are shown in Table 3, and a representative immunofluorescence analysis in Figure 1. The expression of CD19, CD11c and CD44 was significantly higher in the CLL cells from patients with a predominantly splenic manifestation. On average, CD19 was expressed on 91.3% of lymphocytes from patients with stage II(S) compared to 82.7% of those in stage 0 (*P* = 0.042) and 83.5% of those in stages 0 and I disease (*P* = 0.02); CD11c was expressed on 25.7% of cells from patients with stage II(S) compared to only 13.2% of cells from patients with stage 0 disease (*P* = 0.047); CD44 was expressed on 90.5% of cells from patients with stage II(S) compared to only 77.2% of cells from patients with stage 0 (*P* = 0.007) and 80.05% of patients with stage 0 and I disease (*P* = 0.008). None of the other adhesion molecules examined showed a statistically significant difference in percent of expression between the different disease stages.

When only patients with greater than 20% expression of the adhesion molecule in the CD19+ cells were considered positive, 56% of stage II(S) patients demonstrated positive CD11a expression compared with 14% of the stage 0 patients (*P* = 0.016) and 21% of the stages 0 and I patients (*P* = 0.029).

There was a significant difference in the percentage of small versus large lymphocytes between patients with stage 0 and stage II(S) disease, with a higher percentage of small lymphocytes in the latter (16.8% vs. 30.5% respectively, *P* = 0.016). On average, large cells comprised 40% of lymphocytes in patients with stage II(S) disease compared to 60% in patients with stage 0 disease (*P* = 0.006). A direct correlation was found between the percentage of lymphocytes in the blood count and the amount of small lymphocytes. An increase in the percentage of lymphocytes was accompanied by an increase in small-sized lymphocytes (*P* = 0.026) and a concomitant decrease in large lymphocytes (*P* = 0.035). The number of large lymphocytes increased over time, concomitant with a decrease in small lymphocytes. The expression of CD11c was higher in the small lymphocytes than in the large lymphocytes (32.5% and 20.4% respectively, *P* = 0.003). The expression of CD11a was higher in the large lymphocytes than in the small ones (30.5% and 17% respectively, *P* = 0.02).

Discussion

CLL patients with predominant splenic manifestation (stage II(S) patients) comprise an interesting subgroup that has been little investigated until recently. Compared to patients with stages 0 and I

WBC = white blood cells
sCD23 = soluble CD23

CLL, patients with stage II(S) had a significantly lower hemoglobin level, higher WBC count, lower platelet count in addition to higher levels of serum β2-microglobulin and serum soluble CD23, both reliable prognostic parameters associated with aggressive disease and poor outcome [21,22].

In patients with CLL and splenomegaly, thrombocytopenia can be expected to occur at a high frequency because of splenic factors and not bone marrow insufficiency. Redistribution of total body platelet mass (splenic pooling), reduced platelet life span and an increase in the plasma volume are all contributing factors to the thrombocytopenia, with the most important one presumably being splenic pooling of platelets. Although patients with CLL stage II(S) manifest many poor prognostic factors, they are reported to have an indolent clinical course with a mean survival closer to that of stage 0 disease [9,10,20] and thus it is important to distinguish them from patients with high risk disease stages III-IV in whom the anemia and thrombocytopenia are mainly the result of bone marrow failure.

In the present study, we sought to understand why lymphocytes of patients with stage II(S) CLL accumulate mainly in the spleen and usually spare the peripheral lymph nodes. Since the cell surface expression of adhesion molecules may determine the patterns of lymphocyte dissemination and infiltration, we compared the expression of nine adhesion molecules from different families between patients with stage II(S) and patients with stages 0-I CLL. We found that when splenomegaly is the prominent feature, there is significantly higher CD44 and CD11c expression on the surface of the leukemic cells and an increase in percentage of small lymphocytes [Table 3].

Binet et al. [23] reported that an increase in the ratio of large unstained cells, as determined by Hemalog D, to total lymphocytes is a statistically significant criterion of poor prognosis in CLL and that it is characteristic of patients with advanced disease. In the present study, we found that as follow-up time increased the percentage of large lymphocytes increased too, whereas that of small lymphocytes decreased, but as the percentage of total lymphocytes increased there were significantly more small lymphocytes. Stage II(S) CLL patients had a mean of 30.5% small cells compared with only 16.8% in stage 0 patients ($P = 0.016$).

B-CLL has been reported to be the most heterogeneous of the B-chronic lymphoproliferative disorders with respect to adhesion molecule expression [13]. In general, the disease is characterized by low CD11a and CD54, high L-selectin and CD44, and variable CD11c levels. Angelopoulou and colleagues [13] studied the adhesion molecule phenotype in 32 patients with B-CLL, including 7 with splenomegaly as the sole clinical finding. The latter subgroup had high CD11c, CD11a, and CD18 expression on the surface of the leukemic cells (defined as expression of the adhesion molecule in >25% of the CD19+ cells and mean fluorescence intensity over the 10^1 fluorescence channel compared with the negative control), with negative findings for L-selectin in all of them. CD44 expression was uniformly strongly expressed in all cases. De Rossi et al. [14] found that a lack of β_1, β_2 and β_3 chain expression was associated with a significantly lower incidence of splenomegaly and the most favorable prognosis. CD44 was detected on B-CLL cells from 73 of these 74 CLL patients. A significantly increased incidence of diffuse bone marrow infiltration was found in the group with high intensity CD44 expression. Molica et al. [24] evaluated CD11c expression in 69 patients with CLL. They found that 77% had more than 30% CD11c-positive cells but that the intensity of expression was low. However, they did not find a correlation between CD11c expression and Binet's clinical stages. CD44 is a cell surface glycoprotein that is the major receptor for hyaluronate, the principal glycosaminoglycan of the extracellular matrix. It is involved in lymphopoiesis, lymphocyte activation, and lymphocyte migration and homing [11]. CD44 expression is an unfavorable prognosticator in high grade B cell lymphomas. De Rossi et al. [25] reported that B-CLL cells express surface CD44 at variable density, and that this phenotypic feature allows for the distinction of two groups of patients with significantly different survival rates.

In summary, cells from CLL patients with predominantly splenic manifestation and only minimal peripheral lymphadenopathy [stage II(S)] demonstrate higher expression of CD44 and CD11c than cells of patients with stage 0 disease. The different size of the lymphocytes and the different expression of CD44 and CD11c may account for the tendency of the lymphocytes in CLL stage II(S) to target the spleen.

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