Expression of thrombospondin receptor (CD36) in B-cell chronic lymphocytic leukemia as an indicator of tumor cell dissemination

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ABSTRACT

Background and Objective. The expression of CD36 antigen has not been conclusively associated with human B-lymphocytes although CD36 was recently detected in a human B-cell angiotropic lymphoma where it might be involved in lymphoblast-endothelial cell adhesion. We investigated the expression of CD36 in B-cell chronic lymphocytic leukemia (CLL) by multiparameter flow cytometry; results were correlated with clinical features.

Design and Methods. CD36 expression was evaluated on peripheral blood and bone marrow samples from 24 patients affected by CD5+ B-CLL. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation, were labeled with fluorochrome-conjugated monoclonal antibodies under standard experimental conditions and were analyzed by flow cytometry. CD36 expression was quantified both in terms of frequency of CD19+CD36+ cells and of mean fluorescence intensity (MFI-R) of CD36+ cell populations. The intensity of CD36 expression was arbitrarily classified as weak (MFI-R ranging from 3 to 6; score 0), moderate (MFI-R ranging from 6 to 9; score 1), intermediate (MFI-R ranging from 9 to 11; score 2) or strong (MFI-R ranging from 11 to 17; score 3).

Results. CD36 could be detected on 3% (range 2-5) of normal CD19+ B-lymphocytes and on 45% (range 30-75) of neoplastic CD19+ B-cells. When CLL patients were stratified according to CD36 staining intensity, higher hemoglobin levels (Hb) were recorded in patients assigned to score 0 (Hb = 14.3 g/dL; range 13.9-15.1) compared to patients scoring 1-2 (Hb = 11.2; range 10.3-12.2) or 3 (Hb = 9.8; range 9.6-11.6; p=0.0053). Similarly, higher platelet counts (Ptu) were found in patients scoring 0 (Ptu = 282*10^3/µL; range 244-319) compared to patients with intermediate (MFI-R ranging from 9 to 11; score 2) or strong (MFI-R ranging from 11 to 17; score 3).

Interpretation and Conclusions. The present report provides the first evidence of CD36 expression on CD19+ B-cells from CLL; the correlations with clinical parameters strongly support the view that CD36 might favor tumor cell spreading. Whether high CD36 expression levels on CLL CD19+ B-cells identify an aggressive disease subset remains to be further confirmed in larger series of patients.

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Key words: CD36, chronic lymphocytic leukemia, flow cytometry, tumor spreading

CD36 is a membrane-bound glycoprotein receptor for thrombospondin (TSP), identical to platelet GPIV; CD36 is expressed on a variety of hematopoietic cell types and cultured cell lines and mediates several TSP-dependent reactions, namely inflammation, chemotaxis, atherosclerosis, cell proliferation and tumor metastasis.1,2 Among hematopoietic cells, monocytes, megakaryocytes and erythroid precursors express CD36; in particular, CD36 regulates the adhesion of erythroid progenitors to marrow matrix fibroblasts and its expression declines during erythroid differentiation, while blasts from megakaryocytic leukemias have been shown to up-regulate CD36 following culture in the presence of lymphocyte conditioned medium and interleukin-3.3 Interestingly, the expression of CD36 is restricted to vessels in tonsils and lymph nodes and has been detected by immunohistochemistry in scattered cells of thymic medulla and cortex.4 Furthermore, CD36 mediates adhesion of human erythrocytes infected by P. falciparum to cap-
illary endothelial cells, thus contributing to morbidity and mortality of human malaria.1
An association of CD36 with tyrosine kinases of the src gene family has been described in platelets and endothelial cells, thus extending the role of CD36 to that of a signal transduction molecule;2 interestingly, an intracellular localization of CD36 has been demonstrated in human uninduced monocyctic cell lines and in platelets, where CD36 is stored in α-granules and is rapidly transported to the plasma membrane upon activation.3
The expression of CD36 has not been conclusively associated with human B lymphocytes although CD36 was recently detected in a human B-cell angiotropic lymphoma, where it might be involved in lymphoblast-endothelial cell adhesion.4 In contrast, primary murine B cells and cell lines have shown to express CD36.5 In the present investigation, B-cells from chronic lymphocytic leukemia (CLL) at diagnosis were analyzed by multiparameter flow cytometry for CD36 expression. Given the potential role of CD36 in mediating adhesion to endothelial cells and in promoting tumor cell spreading, the results were correlated with clinical features, namely peripheral blood lymphocytosis, platelet count and hemoglobin level, presence of splenomegaly and/or extranodal involvement, pattern of bone marrow infiltration and staging according to Rai.6–8

**Design and Methods**

**Patients and samples**

Twenty-four consecutive patients [median age 64 years (range 55-70)] referred to our Institution from April 1997 and affected by CD5- B-cell chronic lymphocytic leukemia (B-CLL) were evaluated at diagnosis, except for 4 patients who, at the time of inclusion in the present study, were in disease remission after conventional chemotherapy.9 Patients were clinically evaluated to assess nodal involvement, pattern of bone marrow infiltration and staging according to Rai.8,9

**Immunologic markers**

Mononuclear cells (MNCs) were isolated from heparinized peripheral blood (PB) and/or bone marrow (BM) as follows: samples were diluted 1:2 in RPMI 1640 complete culture medium, layered onto Ficoll-Hypaque gradient (density=1,077 g/L, Uppsala, Sweden) and centrifuged at 1700 rpm for 30 minutes at 20°C. Cells at the interface were harvested, washed in RPMI 1640 at 1500 rpm for 6 minutes and suspended at a final concentration of 1×10⁶/mL.

In 15 out of 24 CLL patients, BM samples were available and could be used for flow cytometry analysis.

Cytophilic immunoglobulins were shed by precubation at 37°C for 30 minutes.10 For surface membrane labeling, PB or BM MNCs were incubated for 30 minutes at 4°C with pre-titrated dilutions of the following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or peridinin chlorophyll protein (Per-CP)- conjugated monoclonal antibodies (mAb): CD5 (L17F12 clone, IgG₂₃), CD3 (SK7 clone, IgG₁), CD19 (4G7 clone, IgG₁), CD20 (L27 clone, IgG₁), CD22 (S-HCL-1 clone, IgG₂₀), CD23 (EBVCS-5 clone, IgG₁), CD30 (L243 clone, IgG₁), CD4 (SK3 clone, IgG₂₃), CD8 (B73.1 clone, IgG₁), CD16 (B73.1 clone, IgG₁), CD11c (S-HCL-3 clone, IgG₂₀), CD25 (2A3 clone, IgG₁); all from Becton Dickinson (BD), CA), CD138 (B-B4 clone, IgG₁; Valter Occhiena, Turin, Italy), anti-κ/anti-λ immunoglobulin light chain (IgG₁; BIB D, Bari, Italy) or fluorochrome-conjugated isotype-matched irrelevant mAb to establish background fluorescence.10 After labeling, cells were thoroughly washed with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and were kept on ice until flow cytometry analysis.

**Detection of CD36 antigen**

Aliquots of PE- and/or PerCP-surface labeled normal and neoplastic MNCs were incubated for 30 minutes at 4°C with pre-titrated dilutions of uncon-

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<th>Table 1. Characteristics of CLL patients (n = 24).</th>
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WBC = white blood cells; Ly = lymphocytes; Hb = hemoglobin; Plt = platelets; Spl = splenomegaly; Extramedial = extramedial disease. Data were expressed as median and interquartile range. All patients were analyzed at diagnosis, except for 4 patients who, at the time of inclusion in the present study, were in disease remission (CR) after conventional chemotherapy.
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jugated anti-CD36 mAb (SMO clone, IgM, BIO D) or with irrelevant isotype-matched mAb as control; after washings in PBS supplemented with EDTA 0.1 mM, cells were incubated for 30 min at 4°C with saturating amounts of FITC-conjugated goat-anti-mouse mAb (Ortho Diagnostics Syst., Raritan, NJ, USA). After incubation, cells were washed in PBS-1%BSA and were kept on ice until flow cytometry analysis. Platelet-rich plasma (PRP) obtained after centrifugation of normal PB samples at 800 rpm for 10 minutes was used as the positive control for CD36 staining.

Flow cytometry
All samples were run through a FACScan flow cytometer (BD), equipped with an argon laser emitting at 488nm. Forward (FSC) and side scatter (SSC) were collected as linear signals and all fluorescent emissions were collected on a four-decade logarithmic scale. FITC, PE and PerCP signals were measured at 530 nm, 575 nm and 670 nm, respectively, and spectral overlap was minimized by electronic compensation with Calibrite Beads (BD) before each determination series. A minimum of 10,000 events was acquired in list mode using CellQuest® software (BD). The percentage of CD19+ leukemic cells co-expressing a given antigen (Ag) was calculated according to the formula:

\[
\% \text{Ag}^+ = \frac{\text{Nr of CD19}^+\text{Ag}^+}{\text{Nr of CD19}^+\text{Ag}^- + \text{Nr of CD19}^+\text{Ag}^+} \times 100
\]

The staining intensity was calculated as the mean fluorescence intensity ratio (MFI of test histogram : MFI of control histogram), as previously reported.14

Immunofluorescence analysis
MNCs were smeared on glass slides by cytospin centrifugation and were fixed for 5 minutes at room temperature with absolute methanol and for 2 minutes at -20°C with acetone. After rehydration in PBS, cells were incubated for 30 minutes at 37°C with a proper dilution of unconjugated anti-CD36 mAb. After extensive washing in PBS, cells were incubated for 30 minutes at room temperature with a 1:20 dilution of FITC-conjugated goat-anti-mouse mAb (Ortho Diagnostics Syst.). The slides were then washed in PBS, mounted in PBS/glycerol and observed under an epifluorescence microscope (Axophot; Zeiss, Jena, Germany), using a x63 objective. Cells were photographed using a 400 ASA black and white film (Tmax; Eastman Kodak Co., Rochester, NY, USA).

Statistical methods
To test the approximation of population distribution to normality, y (g) statistics (for symmetry and kurtosis testing) as well as probability plots were used.16 Data were asymmetrically distributed and were presented as median and interquartile range (25th and 75th percentile). Consequently, statistical analyses were performed using the Mann Whitney U test or the Kruskal-Wallis test for unpaired determinations and the χ²-test, as appropriate. The correlations between series of data were calculated using Spearman rank analysis. Multivariate regression models were formed to examine the relation between the dependent variable (BM histology) and potential predictor variables, including percentage of CD36-expressing B-cells, CD36 staining intensity, PB lymphocytosis, Hb level and Plt count. The criterion for statistical significance was defined as p<0.05.

Results
Expression of CD36 on normal peripheral blood cells
CD36 could be detected on 3% (range 2-5) of normal CD19+ B-lymphocytes and on a subset (<2%) of CD4+ as well as CD8+ peripheral T-lymphocytes; 4% (range 2-5) of natural killer (NK) cells, defined by the co-expression of CD16 and CD56 and by the lack of CD3 antigen, were dimly stained by the anti-CD36 mAb. In accordance with previously published reports,1 more than 90% of circulating CD14+ monocytes expressed detectable amounts of CD36 (MFI-R = 5, range 3.2-6.5); conversely, no reactivity above background fluorescence could be evidenced on circulating CD16+ granulocytes. As expected, normal platelets were uniformly and intensely stained by the anti-CD36 mAb (MFI = 10, range 7.7-11.5).

Expression of CD36 on CLL B-cells
CD36 antigen could be readily detected on 45% (range 30-75) of neoplastic B-cells from patients with CLL at diagnosis (Figure 1). These findings were fur-
the majority of CLL (62%) expressed CD36 on <40% of CD19+ B-cells; the remaining CLL cases expressed CD36 on intermediate-to-high percentages (40-80%) of B lymphocytes. No differences were observed when comparing either the frequency of CD36-coexpressing B-cells or CD36 staining intensity in PB and BM samples from individual patients (data not shown).

Since the entire CD19+ CD36+ B-cell population appeared to be shifted by the CD36 staining in bimodal cytograms, the intensity of CD36 expression was arbitrarily classified as weak (MFI-R ranging from 3 to 6; score 0), moderate (MFI-R ranging from 6 to 9; score 1), intermediate (MFI-R ranging from 9 to 11; score 2) or strong (MFI-R ranging from 11 to 17; score 3).

The majority of CLL (83.3%) expressed CD36 at low-to-intermediate intensity (MFI-R ranging from 3 to 11) and only a minority of CLL (14%) expressed CD36 at intermediate-to-strong intensity (MFI-R ranging from 11 to 17). It was of interest to observe that CLL patients who, at the time of enrollment in the present study, were in disease remission after conventional chemotherapy showed a negligible amount of circulating and BM CD19+CD36+B-lymphocytes; a representative flow cytometric profile from one of these cases is shown in Figure 1.

**Correlation between CD36 expression and clinical and laboratory features**

To ascertain whether CD36 expression level might be associated with a greater propensity to tumor cell spreading, phenotypic features were correlated with laboratory and clinical parameters, namely PB lymphocytosis, Plt count and Hb level, presence of splenomegaly and/or extranodal involvement, pattern of BM infiltration and staging according to Rai.

When CLL patients were stratified according to CD36 staining intensity, higher Hb levels were recorded in patients assigned to score 0 (Hb = 14.3 g/dL; range 13.9-15.1) compared to patients scoring 1-2 (Hb = 11.2; range 10.3-12.2) or 3 (Hb = 9.8; range 9.6-11.6; p=0.0053). Similarly, higher Plt counts were found in patients scoring 0 (Plt = 282×10^3/µL; range 244-319), compared to patients with intermediate (Plt = 175×10^3/µL; range 144-238) and high scores (Plt = 149×10^3/µL; range 103-230; p=0.044). Interestingly, Ly count was significantly higher in patients assigned to score 3-4 (Ly = 23.3×10^3/µL; range 13-30) compared to score 0-2 (Ly = 9.8×10^3/µL; range 8.5-10.8; p=0.045).

CLL expressing CD36 at intermediate-to-strong intensity (MFI-R = 14, range 9-16) were more frequently assigned to Rai stages III-IV than stages I-II (CD36 MFI-R =9, range 6.5-11; p=0.005) or stage 0 (CD36 MFI-R =6, range 4-7.3; p<0.001; Figure 3); similarly, CLL patients expressing CD36 on a relevant percentage of CD19+B-cells (75%, range 28-95) were more frequently assigned to Rai stages III-IV than stages I-II (28% CD19+CD36+B-cells, range 16-44; p=0.015) or stage 0 (10%CD19+CD36+B-cells, range 6-15; p=0.0178; Figure 3).

A well-defined trend, although statistically not significant, towards a brighter CD36 staining intensity was observed in CLL patients with splenomegaly (MFI-R=8; range 6.6-11) compared to those without spleen involvement (MFI-R =6; range 3.4-8.3; p=NS). Similarly, the correlation between presence of extranodal disease and level of CD36 expression failed to achieve statistical significance, although a trend towards brighter fluorescence intensities could be evidenced in patients with more advanced disease (data not shown). Of interest, BM diffuse histology was strongly associated with higher CD36 expression (MFI-R = 17; range 14-18; p=0.0178).

**Figure 2.** Immunofluorescence analysis of CD36 expression in PB samples from two representative B-CLL cases.

**Figure 3.** Correlation between the expression of CD36 antigen on CD19+ B-lymphocytes and clinical stage according to Rai. **° p=0.015 and °° p=0.0178 compared to stages I-II and stage 0, respectively; * p=0.005 and ** p=0.001 compared to stages I-II and stage 0, respectively.**
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8.7; range 4.7-13.9) compared to non-diffuse patterns of BM infiltration (MFI-R = 6.7; range 5.2-9.3; p=0.0019). When the significance and independence of potential predictor variables with respect to BM histology (i.e. percentage of CD19+CD36+ leukemic B-cells, CD36 fluorescence intensity, PB lymphocytosis, Hb level and Plt count) were evaluated in multivariate regression analysis, none of those covariables significantly altered the association between diffuse pattern of BM infiltration and brighter CD36 staining intensities (p=0.033).

Discussion

CLL is a clinically and biologically heterogeneous disease, characterized by the clonal proliferation of mature-appearing B-lymphocytes, which represent an antigen-independent pre-follicular state of differentiation.1,2 In early stages of disease, CLL B-lymphocytes can be found primarily in PB and BM; however, disease progression is associated with infiltration of lymphoid and non-lymphoid organs, suggesting that microenvironmental factors might change during the disease course, thus affecting tumor cell adhesion and patterns of dissemination.3,4

Although several investigations have specifically focused on the expression and function of cell-adhesion molecules in chronic B-cell leukemias, few reports have correlated phenotypic features with clinical and/or laboratory parameters.5,6

Recently, receptors for TSP were identified on invasive metastatic cells of squamous and ductal carcinomas;7 the staining intensity, as determined in immunohistochemical sections, predicted the development of metastatic disease within 16 months of initial treatment; interestingly, higher antigen densities were associated with a reduced overall survival, suggesting that the TSP-rich extracellular matrix of those neoplasms might favor tumor cell adhesion and migration.

Human CD36 is a membrane-bound receptor for thrombospondin and can be detected on myelomonocytic cells, platelets, megakaryocytes and endothelial cells.8 Although CD36 has not been apparently associated with human B lymphocytes, the report of a case of CD36-expressing human B-cell angiotropic lymphoma suggests that CD36 might be found at discrete stages of B lymphocyte differentiation.9 In mice, the CD36 gene has been shown to be a target gene through which the Oct-2 transcription factor can affect B-cell differentiation.10 As a membrane receptor on B lymphocytes, CD36 might transduce signals which trigger B-cell differentiation in response to intercellular contact or to as yet unrecognized ligand(s). Given its putative function as a receptor for long-chain fatty acids, CD36 might regulate membrane synthesis and provide energy supply during the differentiation to plasma cells. These hypotheses seem further confirmed by the impairment of the antigen-dependent stage of B-cell development and by the reduced tendency to homotypic cell adhesion in Oct-2−/− mice.11

In the present report, reactivity with the anti-CD36 mAb was found on a minor subset of normal PB B-lymphocytes; conversely, B-cells from CLL unequivocally expressed CD36 antigen. The specificity of these findings was further confirmed by the lack of reactivity with the anti-CD36 mAb in a case of thymic CD1a+ lymphoma and in a case of pre-B acute lymphoblastic leukemia (personal unpublished observation, 1997).

The majority of CLL was assigned to score 0-2, indicating that only a minority of cases expressed CD36 antigen at high density. When CLL patients were stratified according to CD36 fluorescence intensity, higher Hb levels and Plt counts were found in patients assigned to score 0 compared to those assigned to intermediate and high scores; these findings suggest that brighter CD36 fluorescence intensities might be associated with more advanced or aggressive disease as a result of enhanced adhesive interactions of tumor cells with the BM microenvironment. However, the correlation between CD36 score and splenomegaly or presence of extranodal involvement, although intriguing, failed to reach statistical significance. When CLL patients in disease remission were evaluated, a negligible fraction of CD19+CD36+ B-cells was detected (<1%), thus suggesting that the expression of CD36 receptor might be restricted to neoplastic B-lymphocytes.

Recently, patients with diffuse BM infiltration were reported to survive from 2 to 4 years, as opposed to patients with a non-diffuse histology, whose survival ranged between 8 and 10 years.12 In the present investigation, higher intensities of CD36 staining were recorded in CLL patients with diffuse BM histology, an accepted adverse prognostic factor;13 moreover, the significant and independent correlation between CD36 staining intensity and BM diffuse histology in multivariate regression analysis conferred to CD36 antigen expression a predictive value on the propensity to neoplastic cell dissemination.

In conclusion, the present report provides the first conclusive evidence of the expression of CD36 antigen on CD19+ neoplastic B-cells. Further investigations are encouraged to analyze the mechanisms of CD36 gene expression in normal and leukemic B-lymphopoiesis; interestingly, low-grade B-cell non-Hodgkin’s lymphomas in leukemic phase were recently found to express CD36 antigen on relevant percentages of neoplastic B-cells (manuscript in preparation, 1999). The observation of higher CD36 staining intensities in CLL patients with diffuse BM histology and with reduced Hb level and Plt count strongly suggests that CD36 antigen might mediate tumor cell dissemination and that its expression level might identify patients with more aggressive disease.
Contributions and Acknowledgments
SR and CR contributed equally to study design and flow cytometry analyses. SR was responsible for manuscript writing and statistical analyses. PP contributed to flow cytometry experiments. TB performed slide immunofluorescence. AD provided clinical data. LML was responsible for the evaluation of bone marrow trephine biopsies. GL was responsible for the direct supervision of the study and gave final approval.

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