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EXPRESSION AND FUNCTION OF L-SELECTIN MOLECULES (LECAM-1) IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT

Background. **The notion that adhesion molecules play a crucial role in lymphoma/leukemia dissemination is widely accepted. Individual cases of B-cell chronic lymphocytic leukemia (B-CLL) show well-defined variables in the extent and pattern of peripheral blood and nodal involvement. The L-selectin adhesion molecule (TQ1/Leu-8, LAM series and LECAM-1) initiates the attachment of lymphocytes to the high endothelial venules (HEVs), and as a consequence the entrance of lymphocytes from the blood into the peripheral lymph node (*recirculation* which may be operative in lymphoma/leukemia dissemination as well).**

Materials and Methods. **The constitutional expression of L-selectin molecules (LECAM-1) on peripheral blood mononuclear cells (PBMCs) from B-CLL (16 cases) was examined and correlated with receptor function in an HEV-binding assay and in a ligand immobilization test.**

Results and Conclusions. **A correlation was found between constitutional expression and function of the L-selectins, namely the higher the number of cells expressing L-selectin molecules at a measurable level on the cell surface, the greater the number of cells showing attachment in the tests. It is suggested that many aspects of the biological and clinical heterogeneity of B-CLL will be explained by revealing the exact adhesion profile and function in different subtypes of the disease.**

Key words: B-CLL, L-selectins, carbohydrate immobilization, HEV-binding assay

Selectins are lectin molecules belonging to the family of $Ca2^{+}$-dependent animal lectins. L-selectins (Mel-14 antigen in rodents, TQ1/Leu-8, LAM-1, LECAM-1 in humans)[1] bind to their ligand molecules (glycosylation-dependent cell adhesion molecules: GlyCAMs) at the glycocalyx of endothelial cells[2] and initiate a series of dynamic interactions (the adhesion cascade) between different adhesion molecules.[3] Lymphocytes then are able to leave the blood stream and enter the secondary lymphoid organs via the high endothelial venules (HEVs).

The notion that the receptors of normal recirculation play a crucial role in the dissemination of some lymphomas/leukemias is well substantiated.[4] Cells from chronic lymphocytic leukemia (B-CLL cells) express L-selectins similar to normal lymphocytes;[5] however, the L-selectin expression (constitutional expression: % of positive cells) is variable in different series.[5-7] Spertini et al. correlated constitutional expression and density of L-selectins (LAM-1) and their functional activity in a limited num-

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ber (four) of cases.[5]

Different methods are available for measuring L-selectin activity in experimental settings, such as the HEV-binding assay and the application of ligand-analogue molecules (phosphomannan core polysaccharide: PPME, sulfated fucose polymer: fucoidin) as fluorescein-derivatized probes, or as immobilized ligands.[5, 8, 9]

In this study the constitutional expression of L-selectins on peripheral blood mononuclear cells (PBMCs) from B-CLL was compared with their functional activity as measured by the HEV-binding assay and a ligand-analogue immobilization test.

Materials and methods

Patients

Sixteen patients with B-CLL at Rai stage 0-IV (median age 64 years, range 49-78; male-female ratio 10/6) were included in the study. The defining criterion for diagnosis was the presence of a persistent lymphocytosis $>10\times10^9$/L in all cases;[10] bone marrow cytology/histology and lymph node biopsy (in cases of lymphadenomegaly) were utilized as additional diagnostic procedures.

Cell separation

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by standard Ficoll-gradient centrifugation (Pharmacia, Uppsala, Sweden). The standard cell suspension medium was RPMI-1640 (Sigma Chemical Co., St. Louis, MO, USA).

Cell viability was tested by trypan-blue exclusion, and suspensions with a viability ratio over 90% were used in the experiments.

Phenotyping

Immunocytochemical studies were performed either on freshly prepared or on cryopreserved cells.

The cell constituent of each suspension was determined by indirect immunofluorescence analysis with flow cytometry (CYTORON-ABSOLUTE, Ortho Diagnostic Systems Inc., Raritan, NJ, USA); a FACStar PLUS flow cytometer (Becton-Dickinson, Mountain View, CA, USA) was used to detect LECAM-1 expression. The monoclonal antibodies (moabs) applied in this study included: CD14/Leu-M3, CD4/Leu-3a, HLA-DR, CD20/Leu-16, CD5/Leu-1, CD23/Leu-20, CD25, sIgM (Becton-Dickinson), CD3/OKT3, CD8/OKT8 (Ortho), CD21, CD45R, CD45RO/UCHL-1, CD30/Ber-H2 (DAKO) and LECAM-1 (Immunotech).

Cell-suspensions were selected on the basis of immunophenotyping result and only suspensions containing a high percentage of B– ($CD20^+$, or $CD21^+$) and of $CD5^+$ cells were used. The proportion of contaminating T-cells and monocytes was less than 20% in the study. A further selection of samples was made according to LECAM-1 expression, and only cases considered positive for this marker (>25% of cells reactive with anti-LECAM-1) were counted in the study.

HEV-binding assay

This assay was performed as originally described by Stamper and Woodruff.[8] Rat cervical lymph nodes were used to prepare frozen sections for lymphocyte adhesion.

Preparation of fucoidin and PPME-derivatized polyacrylamide gels and cell adhesion experiments

This procedure was based on methods previously described by Brandley et al.[9]

Briefly, fucoidin was treated by a protease, then dialyzed against running and distilled water. The dialyzate was applied to Sepharose 4B gel filtration and a high molecular weight carbohydrate peak was collected.

PPME preparation was started by mild acid hydrolysis of H. holstii phosphomannan, then saturated with barium hydroxide, and the barium salt of high molecular weight PPME was precipitated by ethanol. After removal of barium by a cation exchange resin, a sodium salt was obtained.

Prior to reduction with sodium borohydride and mild periodate oxidation, PPME and fucoidin were derivatized with hexanediamine. The resulting amino sugars were coupled with a spacer molecule, the N-succinimidyl ester of 6-acrylamidohexanoic acid, and the product was

Table 1. Immunophenotyping of PBMCs from B-CLL patients.

case #	*CD3 (%)*	*CD20/CD21 (%)*	*CD5 (%)*
1	7	89	75
2	10	41	82
3	8	82	92
4	11	75	18
5	16	70	61
6	3	85	89
7	9	81	74
8	0	83	25
9	0	93*	94
10	5	95*	97
11	8	97*	96
12	5	93*	96
13	11	87*	74
14	4	85*	93
15	2	98*	96
16	7	87*	92

copolymerized with acrylamide/ bisacrylamide.

Cell adhesion was performed in conventional ELISA plates with 96 wells; 150 μL of 3.3×10^6 cells/mL in H-DMEM were centrifuged to the gel surfaces (50 g, 5 min, at 4°C).

After 1 hour of incubation at 4°C, the wells were immersed in Hepes buffered saline at 0°C, covered with plastic tape and centrifuged in an inverted position for 10 minutes (50 g for PPME, and 150 g for fucoidin activated gels). Cell adhesion was determined by the lactate dehydrogenase activity of the adherent cells.

Table 2. Immunophenotyping of PBMCs from B-CLL patients.

	mean±SD (%)	*range (%)*
CD14	2.1±1.2	0-4
CD4	4.2±2.4	0-7
CD8	10.6±3.3	6-15
HLA-DR	76.8±14.6	44-90
CD45R	72.5±26.9	8-89
CD45RO	64.8±30.4	10-89
CD23	42.5±27.7	1-85
CD25	11.1±9.6	0-32
CD30	18.3±13.2	5-37
sIgM	35.7±19.6	5-7

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistics

Correlation coefficients (Pearson) and rank correlation coefficients (Kendall and Spearman) were calculated to estimate the association between the variables.

Results

The main cell constituents (T– and B–, as well as $CD5^+$ cells) of each suspension investigated are demonstrated in Table 1.

Additional markers (as a percentage: mean±SD) and the range of positive cells are presented in Table 2. It should be pointed out that not only the number of T-cells (Table 1) but also that of $CD14^+$ positive cells (peripheral blood monocytes) was very small (2.1±1.2%) in the investigated samples.

LECAM-1 expression showed great variability even among the preselected *positive* cases (>25% reactive with anti-LECAM-1):[5] 52±24% (mean±SD), range: 25±89%.

Binding to PPME and fucoidin derivatized gels was 34±19% and 39±16%, respectively (mean±SD).

Constitutional LECAM-1 expression and PPME-binding of cells showed a statistically significant correlation (Figure 1), while no correlation was found between receptor expression and fucoidin binding.

In 9 cases PPME-binding was correlated with the HEV-binding activity (mean±SD: 2.4±1.0) of the cells, and it also tested statistically significant (Figure 2).

Discussion

We previously demonstrated that PBMCs from B-CLL show great variability in their L-selectin function (Csanaky et al., in press).

Simple detection of an adhesion molecule(s) on the cell surface does not necessarily correlate well with the actual adhesive properties of the cell.

In this B-CLL series a correlation between the constitutional expression of L-selectin mole-

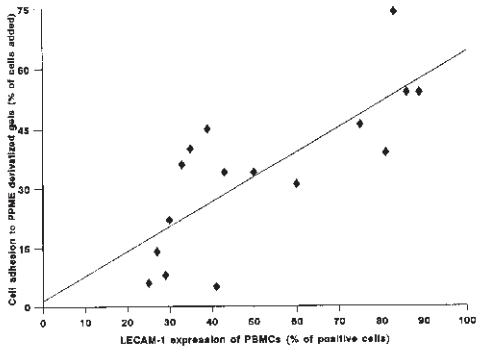

Figure 1. Correlation between LECAM-1 expression and adhesion to PPME-derivatized gels (PPME-binding). In all three different statistical analyses the levels of significance were found to be in the range of $0.001<p<0.004$.

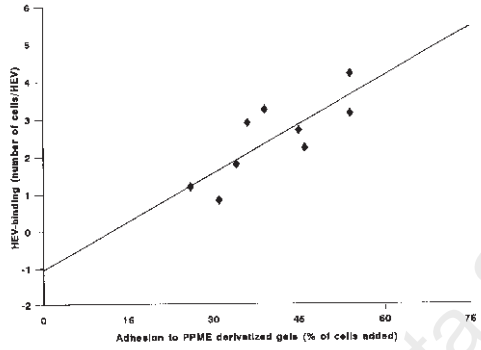

Figure 2. Correlation between PPME- and HEV-binding. The range of the level of significance in the statistical tests: $0.002<p<0.01$.

cules and their function was presented and statistically proven. Failure to find a correlation between L-selectin expression and fucoidin binding can be explained at the light of the existence of additional fucoidin-binding cell surface receptors.[11]

Nevertheless, many questions remain to be answered. Recently some contradictory data have emerged not only in connection with the simple expression and density of L-selectins on B-CLL cells,[5, 6, 12] but with regard to their functional activity as well.[13, 14] Probably even better and more clinically relevant correlations would be achieved if the mean density of the adhesion molecules on clonal proliferations were correlated with their functional activity and dissemination in larger series.

Since the density of L-selectins on the cell surface changes rapidly (up- and down-regulation) and shedding occurs spontaneously,[1] it is very difficult to compare the exact L-selectin expression and density of various lymphocyte subpopulations. Ohgama and Onoé recently suggested a simple method for stabilize the L-selectins by treating cell preparations with sodium azide.[15] The simultaneous application of fluorescent and/or immobilized ligand analogues with flow cytometric detection of LECAM-1 and other differentiation antigens on sodium azide-stabilized cell preparations may give us a better insight into the *in vivo* expression and function of L-selectins.

Besides their interest for lymphoma/leukemia dissemination investigation of L-selectins and other adhesion molecules may have another importance as well. In B-CLL and in some related leukemias therapeutically favored interferon-α (IFN-α)[16-18] increases the expression of certain adhesion molecules and results in enhanced receptor function (Csanaky et al., submitted). The reduction of peripheral leukemic cells following treatment with IFN-α may be attributed to the effect of IFN-α on adhesion molecules.

In the future, many aspects of the biological and clinical heterogeneity of B-CLL will probably be explained on the basis of diverse adhesion molecule expression and function.

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