



L-selectin expression is low on CD34⁺ cells from patients with chronic myeloid leukemia and interferon- α up-regulates this expression

GREGORIO ANGEL MARTÍN-HENAO,* REGINA QUIROGA,* ANNA SUREDA,^o JUAN RAMÓN GONZÁLEZ,# VÍCTOR MORENO,# JUAN GARCÍA*

*Department of Cryobiology and Cell Therapy, Cancer Research Institute; ^oHospital de la Santa Creu i Sant Pau;

#Institut Catalán de Oncología, Barcelona, Spain

ABSTRACT

Background and Objectives. Altered adhesive interaction between bone marrow (BM) stroma and progenitors in chronic myeloid leukemia (CML) may be in part caused by abnormal expression of cell adhesion molecules (CAMs) on malignant progenitor cells. Treatment of CML with interferon- α (IFN- α) re-establishes normal hemopoiesis in some patients in part by restoring normal adhesive interactions between CML progenitors and BM microenvironment, which may in turn be mediated by correcting CAM expression on progenitors.

Design and Methods. We investigated the expression of CAMs (L-selectin, β_2 -integrin, LFA-3, ICAM-1, ICAM-3, NCAM) on purified BM CD34⁺ cells from CML patients (n= 34) and healthy adults (n= 15) by flow cytometry. Modulation of L-selectin expression on CD34⁺ cells from CML after *in vitro* treatment with IFN- α was also investigated.

Results. The mean percentage of CD34⁺ cells expressing L-selectin was significantly lower in CML patients (25.4 \pm 12.8%) than in normal controls (68.7 \pm 8.3%, n=15). CD34⁺/HLA-DR^{-/low} and CD34⁺/CD38^{-/low} co-expressing L-selectin were also significantly lower in untreated CML (27.4 \pm 21.5% and 39.8 \pm 26.7%, respectively, n=8) than in controls (61 \pm 17% and 83.7 \pm 10%, respectively, n=7). *In vitro* treatment with IFN- α of purified CD34⁺ BM cells from untreated CML patients (n=8) induced a significant, dose and time-dependent increase in the L-selectin expression as indicated by FACS analysis.

Interpretation and Conclusions. We hypothesize that this L-selectin deficiency reflects a cell surface adhesion defect of progenitors from CML that is partially restored by *in vitro* IFN- α treatment. These data may help to explain the adhesive abnormalities of CML progenitors to the BM microenvironment and the *in vitro* restoration of adhesion capacity after IFN- α treatment.

©2000, Ferrata Storti Foundation

Key words: chronic myeloid leukemia, CD34⁺ cells, adhesion molecules, L-selectin, interferon- α

Chronic myeloid leukemia (CML) is a clonal malignancy of the hematopoietic stem cell¹ characterized by the Philadelphia chromosome (Ph1).² This disease has an initial chronic phase characterized by an expanded myeloid clonal hematopoiesis with increased, unregulated malignant cell proliferation and a premature release of progenitor cells into the peripheral blood.³ Several studies have demonstrated that Ph1⁺ progenitors adhere poorly to bone marrow (BM) stroma feeders compared to normal progenitor cells.^{4,5} This diminished capacity of adhesion seems to be partially due to an abnormal expression and/or function of cell adhesion molecules (CAM) on the malignant cell surface. An abnormal expression of LFA-3, β_1 -integrin and L-selectin CAMs on CML progenitors has been previously reported by other groups.⁶⁻⁸ Adhesion receptors retain stem and progenitor cells within the BM microenvironment during normal steady-state hemopoiesis.⁹⁻¹⁵ Moreover, interactions between hematopoietic progenitors and progenitor-stromal components via adhesion molecule-ligand pairs have also been implicated in the regulation of progenitor cell proliferation and differentiation.^{9,16,17} Therefore, diminished adhesion capacity in CML may contribute to the premature release of progenitors into the peripheral blood and their malignant cell growth advantage.^{18, 19}

Interferon- α (IFN- α) treatment induces a significant reduction of the Ph1⁺ cells restoring residual normal hematopoiesis in some CML patients.²⁰⁻²² The mechanisms by which IFN- α suppresses malignant hematopoiesis remain poorly understood. *In vitro* studies have demonstrated that CML progenitors can adhere to normal stromal layers after the treatment of the stroma with IFN- α .²³ It has been also demonstrated that IFN- α acts directly on CML progenitors by restoring deficient expression of LFA-3⁸ and modulating the function of β_1 -integrin receptor.⁷ These data suggest that IFN- α may enhance the expression of other critical adhesion molecules that are deficient on CML progenitors, such as L-selectin.⁶

We investigated the co-expression of CAMs on CD34⁺ BM cells from CML patients. L-selectin (selectin family), intracellular CAM-1 and 3 (ICAM-1 and ICAM-3), neural CAM (NCAM), leukocyte function antigen-3 (LFA-3) (CAM immunoglobulin family) and β_2 -integrin chain receptor (integrin family) were analyzed. Here we show that, compared to normal controls, CD34⁺ cells of CML patients have a low co-expression of L-selectin and that *in vitro*

Correspondence: G.A. Martín-Henao, MD, Department of Cryobiology and Cell Therapy, Cancer Research Institute, Hospital Duran i Reynals, Av. Castelldefels, Km 2.7, L'Hospitalet de Llobregat, 08907 Barcelona, Spain. Phone: international +34-93-3363015/2633817 - Fax: international +34-93-2607776 - E-mail: gmartinh@iro.es

treatment of CD34⁺ purified cells with IFN- α 2b is able to modulate this deficient expression.

Design and Methods

Patients

BM samples were obtained from 41 Ph¹⁺ CML patients, 26 males and 15 females, with a median age of 44 years (range; 20-80). All patients were in chronic phase when studied. Twenty-two patients were studied at diagnosis before receiving any treatment (untreated patients), 15 had been treated with IFN- α 2b (Schering-Plough S.A., Madrid, Spain) for a variable period of time (between 3 and 86 months, median 12) and 4 with hydroxyurea alone. The median time between diagnosis and study was 38 (8-99) and 47 months (1-63) in CML patients treated with IFN- α and hydroxyurea, respectively. Eight of 15 CML patients treated with IFN- α showed some degree of cytogenetic response. The percentage of Ph¹ negative metaphases in the responding patients ranged from 10% to 100% (median 30%). The control group comprised BM samples from 16 healthy adult subjects, with a median age of 46 years (range; 39-52) undergoing BM harvest for allogeneic transplantation.

Samples were obtained in accordance with the Institutional Human Research Committee guidelines.

CD34⁺ cell purification

CD34⁺ cells were isolated by positive selection by magnetic activated cell sorting (mini-MACS, Miltenyi Biotec, Gladbach, Germany) as reported elsewhere.²⁴ Briefly, mononuclear cells (MNC) were washed twice in Ca²⁺/Mg²⁺/red phenol free Hank's saline solution (HBS) and human serum albumin (HSA) 1%, pH=7.5, after separation by Ficoll-Hypaque density gradient ($d=1.077$) (Pharmacia, Sweden). Cells were diluted to a concentration of 3×10^8 MNC/mL in degassed HBS with HSA 1% and ACD-A 6% (selection buffer) (Baxter, Barcelona, Spain), pH=7.5. Cells were then filtered through a nylon mesh (30 μ m diameter) to remove clumps and cellular aggregates and incubated for 15 min at 4° C on a rotator at 4 rpm with a human IgG to block the Fc receptor and an anti-CD34 mouse monoclonal antibody (MoAb) (QBEND-10) modified with an hapten. Cells were washed once with selection buffer and incubated for 20 min at 4° C, 4 rpm, with an anti-hapten mouse MoAb conjugated to colloidal superparamagnetic microbeads (Miltenyi Biotec) and filtered through a nylon mesh. The high gradient magnetic columns (MS columns, mini-MACS, Miltenyi Biotec) were filled with 1 mL of cold selection buffer before use. Labeled cells were applied and the unbound cells were washed out with 0.5 mL \times 4 of selection buffer. Positive cells were eluted from the column out side of the magnet with 1 mL of selection buffer at high rate. A second column was used to improve enrichment. All manipulations were performed in a laminar flow hood. Cell viability was evaluated by the trypan blue dye exclusion and/or propidium iodide tests.

Flow cytometry

Samples were analyzed with an Epics-XL-MCL flow cytometer (Coulter Electronic, Inc., Hialeah, FL, USA)

equipped with a 488 nm excitation Argon laser. MNC and CD34⁺ enriched cells were incubated with AB inactive human serum 1% (v/v) for 10 min at 4° C. Afterwards, cells were washed with HBS with 0.2% of sodium-azide (Merck, Germany) and 2% of bovine serum albumin (Sigma Chemical Co, Sant Louis, USA) (HBS-BSA), centrifuged and diluted in a final volume of 50 mL after tube decantation. Cells were incubated with MoAbs for 30 min to 4° C in the dark at the concentration indicated by previous titration. Samples were then washed twice in HBS-BSA and incubated with 7-aminoactinomycin-D (7-AAD, Molecular Probes, Eugene, Oregon, USA) at 5 μ g/mL for 5 min at room temperature. Cells were acquired by setting an amorphous gate of forward side (FS) and 7-AAD, to exclude 7-AAD⁺ dead cells. A second gate was established on FS and side scatter (SS) to include cells with low SS and low to high FS. FS, SS (linear amplification) and fluorescence signals (logarithmic amplification) were determined for each cell (minimum of 10,000 CD34 purified events per sample) and stored in list mode data files. The following direct phycoerythrin (PE) labeled MoAbs were used in a single color: HPCA-2 (CD34, 8G12, IgG1), Leu8 (L-selectin, CD62L, SK11, IgG2a), Leu54 (ICAM-1, CD54, LB-2, IgG2b), β_2 integrin (CD18, L130, IgG1), N-CAM (Leu19, CD56, My31, IgG1), (Becton Dickinson, San José, FL, USA); ICAM-3 (CD50, HP2/19, IgG2a), L-selectin (CD62L, Dreg56, IgG1), LFA-3 (CD58, AICD58, IgG2a) (Coulter Immunology, Hialeah, FL, USA). Double staining with FITC-HPCA-2 and PE-Leu8 was performed on 6 samples before (MNC) and after the CD34⁺ cell selection including also 7-AAD, to reveal whether L-selectin expression pattern on CD34⁺ cells could be affected by the enrichment procedure. Triple color immunofluorescence analysis was performed to determine the L-selectin co-expression by CD34⁺/HLA-DR^{-low} and CD34⁺/CD38^{-low} primitive progenitor cell subsets. For these studies, purified CD34⁺ cells were simultaneously stained with MoAb to PerCP-anti-CD34 (HPCA-2-PerCP, Becton Dickinson), PE-anti-HLA-DR (L246, IgG2a, Becton Dickinson) or PE-anti-CD38 (T16, IgG1, Immunotech, SA) and FITC-anti-Leu8 (Becton Dickinson). Negative controls with isotype non-relevant MoAb (mouse IgG1, IgG2a, Becton Dickinson) were done in all experiments. Data from 20,000 to 30,000 CD34⁺ events were collected on triple color studies. Gates defining stained cells were set to include 1% of cells stained with the isotypic control. Data analysis was performed with the Epics-XL-MCL v. 1.5 workstation software (Coulter Electronic).

Short-term cultures

The culture medium was composed of RPMI-1640 (GibcoBRL, Life Technologies Ltd, Paisley, Scotland) with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Purified BM CD34⁺ cells from 8 untreated CML patients as well as the IFN- α growth-sensitive Daudi B lymphoblastoid cell line (obtained from ATCC) were incubated *in vitro* with culture medium containing different concentrations of IFN- α 2b (100, 1,000, 5,000, 10,000 U/mL) (kindly supplied by Schering Plough, Madrid,

Spain). Cells without IFN- α 2b added to the medium were used as controls. Cells were plated in 96-well round-bottomed plates ($1-2 \times 10^5$ cells per well, $0.5-1 \times 10^6$ cells/mL) and incubated at 37°C with 5% CO₂ for different times (24 h, 48 h and 72 h). Assays were run in triplicate. After washing and blocking with AB human serum, the cells were stained with PE-anti-Leu8 MoAb or with isotypic control including 7-AAD in all cases as previously described, then analyzed by flow cytometry.

Statistical analysis

Data obtained by flow cytometry analysis are presented as the percentage of positive events. Quantification of adhesion molecule expression was evaluated as the ratio of mean fluorescence intensity between the test and the negative control histogram (logarithmic scale) (MFI). Results of experimental points obtained from multiple experiments are reported as arithmetic mean and standard deviation (s.d.). Normality of the parameters was evaluated by the Kolmorov-Smirnov test. The Mann-Whitney U-test was used to compare the expression of the adhesion molecules. A *p* value lower than 0.05 was considered statistically significant.

General linear-mixed models were used to estimate the effect of IFN- α 2b on the expression of L-selectin (percentage of positive events and MFI). These models allowed analysis of the overall effect, at any time and any dose. The dose-effect relationship of IFN- α 2b was also modeled, overall and in relation to the incubation time. For these latter analyses, the logarithm of the dose was used to improve homoscedasticity. To ease interpretation of coefficients, decimal logarithms were used, thus an increase in one unit of log-dose corresponds to multiplying the dose by 10. Time effect measured differences at 48 and 72 hours of incubation with respect to 24 hours. All models included the subject as a random effect. The test for a random effect for IFN- α 2b was not statistically significant. Estimation of coefficients and standard errors to compute 95% confidence intervals were based on restricted maximum likelihood. Comparisons of models were based on likelihood ratio tests derived from model fits using maximum likelihood fit. All analyses were done with S-PLUS functions using the library NLME.²⁵

Results

Cell surface expression of the adhesion molecules

The percentage of CD34⁺ cells after the selection procedure and after gating the cell population to exclude 7-AAD⁺ events and to include low SS and variable FS was always superior to 95% (Figure 1).

Co-expression of CAMs on purified CD34⁺ BM cells is shown in Table 1. The percentage of CD34⁺ cells expressing L-selectin was lower in CML than in controls ($25.4 \pm 12.8\%$ Vs $68.7 \pm 8.3\%$, $p < 0.005$) (Figure 2). L-selectin reactivity was measured before and after the CD34⁺ selection procedure in 5 CML samples (2 untreated and 3 treated with IFN- α) and 1 control, to reveal whether this molecule could be affected by the enrichment procedure. No differences were

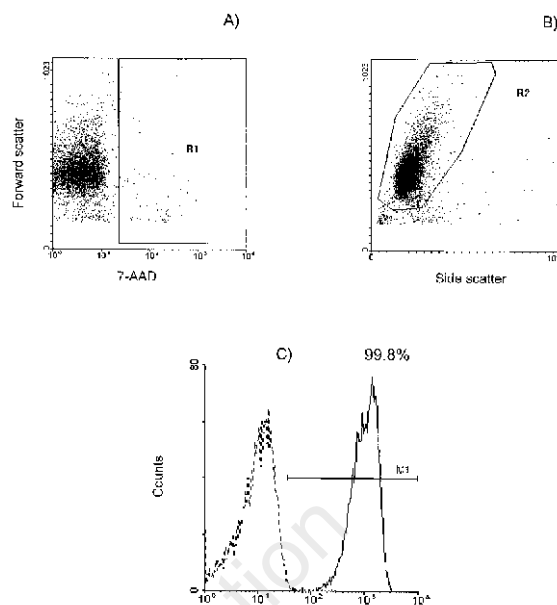


Figure 1. FACS analysis of a representative case of BM from an untreated CML patient after CD34⁺ cell purification with the MACS system. **A)** A region was drawn on 7 aminoactinomycin-D (7AAD) vs FS to exclude 7AAD⁺ dead cells (R1). **B)** A second gate of SS Vs FS was drawn to include events with low SS and low to high FS (R2). **C)** The expression of CD34 was analyzed in the first histogram of FL2 (log scale). The positive cells are cells stained above 1% of the negative control (dashed line).

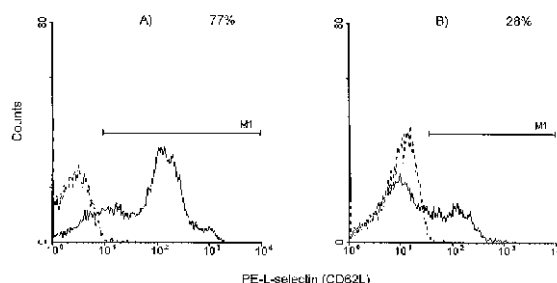


Figure 2. Expression of L-selectin on purified BM CD34⁺ cells by single color FACS analysis. The analysis was performed as described in Figure 1. The percentages of positive events represent the cells stained above 1% of the negative control (dashed line). **A)** Normal control. **B)** Untreated CML.

shown in L-selectin before and after the CD34⁺ enrichment technique ($30.4 \pm 27\%$ vs $35 \pm 27\%$, respectively, $p = ns$). L-selectin expression on CD34⁺ purified cells was measured with another MoAb (Dreg56) in some cases and was not different from that measured with the antibody used in this study (Leu8) (data not shown).

A subpopulation of CD34⁺/NCAM⁺ cells, not seen in the BM of healthy adults was detected in 14 of 34 CML patients, representing more than 5% of the

Table 1. Expression of cell adhesion molecules on purified bone marrow CD34⁺ cells from controls and chronic myeloid leukemia patients.

	n	L-selectin		β_2 -integrin		ICAM-1		ICAM-3		N-CAM		LFA-3	
		%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
Controls	15	68.7 [†] (8.3)	21.1 [†] (12.2)	48.9 (15)	6 (2.7)	31 (16)	2 (0.8)	93.3 (4.5)	104 (61)	1 [†] (1.5)	0.9 (0.0)	97 (2)	19 (10)
CML													
Diagnosis	15	30.2 ^{1§} (12)	2.7 [†] (1.6)	52.9 (6.9)	3.6 (0.5)	33.7 (23.4)	2.3 (1.5)	98.1 [†] (0.9)	144 (63)	19.4 ^{1#} (8)	1.7 [†] (0.4)	92.2 (12)	18 (1.4)
IFN- α	15	22.3 [†] (11.9)	2.5 [†] (1.6)	49 (35)	4.3 (1.9)	22.1 (17.2)	1.6 (0.6)	96.8 [†] (4.3)	128 (53)	9.2 ^{1#} (8.9)	1.3 [†] (0.3)	92.3 (11)	18.7 (6.9)
Hydroxyurea	4	11.1 ^{1§} (4.8)	1.9 [†] (0.6)	64 (37)	6.4 (1.3)	26 (16)	1.9 (0.5)	98 [†] (1.7)	144 (4)	10.6 ^{1#} (7.3)	1.1 [†] (0.0)	98.3 (1.9)	17.9 (3.9)

The expression of adhesion molecules is presented as percentage of positive events and MFI (ratio between mean fluorescence intensity of test and negative control histograms). Results are expressed as mean value and standard deviation. $p < 0.05$: comparison between [†]normal controls and CML patients; [§]hydroxyurea vs CML at diagnosis and IFN- α treated patients; [#]CML at diagnosis vs IFN- α and hydroxyurea treated patients.

Table 2. Co-expression of L-selectin molecule on CD34⁺ purified cells (HLA-DR or CD38) from untreated CML patients and control group.

	HLA-DR ^{-low}	HLA-DR ⁺	CD38 ^{-low}	CD38 ⁺
Untreated CML (n = 8)				
Mean (SD)	27.4 (21.5)	24.8 (16.7)	39.8 (26.7)	21.2 (5.4)
Median (range)	20 (7-70)	23.5 (8-58)	38 (7-74)	23 (15-27)
Normal controls (n = 7)				
Mean (SD)	61 (17)	71 (7)	83.7 (10)	67.1 (6.9)
Median (range)	60.5 (34-94)	72 (63-81)	83.7 (70-96)	65 (59-79)
p^*	0.03	0.001	0.009	0.002

Percentage of L-selectin co-expression on CD34⁺/HLA-DR or CD38 fractions as determined by triple color immunofluorescence analysis. The CML patients and control group studied were 4 of the 15 cases and 7 of the 15 cases contributing to the data in table 1, respectively.

*Mann-Whitney U-test.

CD34⁺ cells. Co-expression of ICAM-3 was higher in CD34⁺ cells from CML patients than in those from controls. No differences were shown with regards to the expression of LFA-3, β_2 -integrin or ICAM-1.

The L-selectin expression rate was lower in hydroxyurea than in IFN- α treated and untreated CML patients (Table 1). A higher co-expression of NCAM was found in untreated than in IFN- α or hydroxyurea treated patients. No differences were found with respect to the others molecules analyzed.

Eight of fifteen CML patients achieved some degree of cytogenetic response after *in vivo* treatment with IFN- α . No differences were found between percentages of adhesion molecule expression on CD34⁺ cells between responding and non-responding patients. Expression of CAMs in IFN- α responding patients was independent of the degree of cytogenetic response.

Expression of L-selectin on CD34⁺/CD38 and CD34⁺/HLA-DR subpopulations

Purified CD34⁺ BM cells from 7 controls and 8 untreated CML patients were stained with MoAb to Leu8, CD34 and CD38 or HLA-DR and analyzed by three-color flow cytometry. After gating in FS/SS to exclude debris, two regions were drawn on dot-plots of FL2 vs FL3: CD34⁺/HLA-DR^{-low} or CD34⁺/CD38^{-low} and CD34⁺/HLA-DR⁺ or CD34⁺/CD38⁺ (regions of positive events were allowed to include 1% of the negative controls). Each fraction was evaluated for the expression of L-selectin in histograms of FL1 (Table 2). The mean percentages of CD34⁺/HLA-DR^{-low} cells were 1.8 \pm 1.1% in controls and 1.5 \pm 0.9% in CML patients. CD34⁺/CD38^{-low} cells were expressed in 3.4 \pm 3.4% and 2.2 \pm 1.2% in controls and CML patients, respectively. The cells expressing L-selectin in all CD34⁺ cell fractions (HLA-DR^{-low}, HLA-DR⁺, CD38^{-low} and CD38⁺) from CML patients were significantly fewer than those from controls.

Short-term culture with IFN- α

Analysis of the effect of IFN- α on the cell surface expression of L-selectin was first performed using the Daudi cell line. This cell line constitutively expresses low but detectable levels of L-selectin, with a mean-expression rate of 15%. This cell line has been reported to increase L-selectin expression in response to *in vitro* treatment with IFN- α ²⁹ and was used in this study as the positive control for the culture conditions. Overall, *in vitro* treatment of the Daudi cell line with IFN- α increased the expression of L-selectin to 44.7%. (95% confidence interval [CI]=39.6,49.7). This induced expression was dose-dependent with an additional 11.7% ([CI]= 9.5,13.9) increment of the L-selectin expression rate every time IFN- α 2b dosage was increased 1 log. The effect was also time-dependent with additional 20% ([CI]= 10.5,29.4) and 14.5% ([CI]= 7.1,21.8) increments at 48 h and 72 h with respect to the 24 h culture.

The incubation of CD34⁺ enriched cells from

untreated CML patients (n=8) with IFN- α 2b significantly increased the percentage and fluorescence intensity of L-selectin expression at any point of the culture (Table 3). Overall, L-selectin expression rate increased 8.3% with IFN- α compared to cells cultured without IFN- α . This increase was dose-dependent (with an additional 2.41% increment every time IFN- α 2b dosage added to the culture was increased 1 log) and time-dependent (with additional 2.84% and 14.11% increment at 48 h and 72 h vs 24 h of culture, respectively). In contrast, no differences were shown in L-selectin expression rate on CD34⁺ BM cells from normal controls (n= 2) between cultured cells without or with IFN- α (overall expression and MFI of 74.8% [CI] = 62.91, 86.73 vs 75.0% [CI] = 59.1, 90.9, $p = 0.9$, and 15.05 [CI] = 7.22, 22.88 vs 15.66 [CI] = 7.19, 24.12, $p = 0.6$, respectively).

Discussion

In this report we show that L-selectin adhesion receptor is abnormally co-expressed on CD34⁺ BM-cells from CML patients at a mean of ten fold lower fluorescence intensity than normal controls. In addition, the mean percentage of CD34⁺ cells expressing L-selectin from CML patients treated with hydroxyurea was lower than that from IFN- α treated or untreated patients. Since CML is a stem cell disorder, we further studied the L-selectin expression pattern in untreated CML patients on a more primitive progenitor cell subset as defined by the expression of CD34⁺ and the lack co-expression of HLA-DR or CD38 antigens. Triple-color flow cytometry analysis demonstrated that CD34⁺/CD38^{-/low} and CD34⁺/CD38⁺ also co-expressed significantly lower percentages of L-selectin than control cells. This finding is in agreement with results from Kawaiishi *et al.*⁵ who have recently reported a lower expression rate of L-selectin on CD34⁺/CD38⁺ cells from untreated CML patients. Our data also show that the CD34⁺/CD38^{-/low} subpopulation has a significantly lower mean percentage of L-selectin expression than normal controls. With regards to HLA-DR antigen expression, both CD34⁺ cell subpopulations, HLA-DR^{-/low} and HLA-DR⁺, also co-expressed lower percentages of L-selectin than controls. CD34⁺/HLA-DR^{-/low}, but not CD34⁺/CD38^{-/low} fractions, have been reported to be enriched in benign progenitors in CML patients.²⁶⁻²⁸ Thus, one could speculate that this primitive CD34⁺/CD38^{-/low} cell subset might be predominantly malignant CML progenitors expressing lower L-selectin. However, a similar expression rate of L-selectin in CML and controls could be expected in the CD34⁺/HLA-DR^{-/low} fraction. One possible interpretation for these observations is that the CD34⁺/HLA-DR^{-/low} cell fraction expressing lower L-selectin might be expanded in CML. Another explanation could be the variability of the proportion of normal progenitors in these fractions.

We next investigated the effect of IFN- α *in vitro* treatment of purified CD34⁺ cells from untreated CML patients on the abnormally co-expressed L-selectin molecule. Our data show that L-selectin expression was significantly up-regulated on CML CD34⁺ cells upon *in vitro* culture with IFN- α . Approximately, 10-20% of the BM CD34⁺ cells were found to

Table 3. Effect of *in vitro* treatment with interferon- α 2b (IFN) on the expression of L-selectin on bone marrow CD34⁺ purified cells from CML patients.

Variable	L-selectin (%)		MFI	
	Coefficient	CI95%	Coefficient	CI95%
Model 1				
Intercept	35.72	(26.35,45.09)	2.66	(1.91,3.40)
IFN				
No	0		0	
Yes	8.37	(6.09,10.65)	0.68	(0.50,0.86)
Random patient (variance)	249.50		1.43	
Model 2				
Intercept	30.54	(19.96,41.12)	2.45	(1.65,3.25)
Doses IFN (log ₁₀)	2.41	(1.76,3.06)	0.19	(0.13,0.25)
Time				
24 h	0		0	
48 h	2.84	(0.37,5.31)	0.31	(0.11,0.51)
72 h	14.11	(10.50,17.72)	0.47	(0.18,0.76)
Random patient (variance)	249.42		1.45	

Increased expression rate and mean fluorescence intensity (MFI) of L-selectin on purified CD34⁺ bone marrow cells from untreated CML patients at diagnosis (n=8) after *in vitro* treatment with interferon- α 2b (IFN- α 2b). The patients investigated were 2 and 1 of the cases contributing to the data in Tables 2 and 3, respectively, plus 5 further cases who were studied only for the effect of the *in vitro* treatment with IFN- α on the expression of L-selectin. Purified CD34⁺ cells incubated with or without IFN- α 2b at different doses and times, were labeled with antibodies to the L-selectin (leu8) or with isotypic control, including 7-AAD. The expression of L-selectin was measured using FACS analysis. MFI represents the ratio between the MFI of the test and the isotypic control histograms (log scale). Statistical analysis was done using general linear-mixed models. Model 1 estimates the effect of *in vitro* treatment with IFN- α 2b for all times and doses. Model 2 estimates the effect of different doses and time. The coefficient, confidence interval 95% (CI95%) and the variance interpatients (random patient) are shown. IFN- α 2b increases the overall expression rate of L-selectin on CD34⁺ purified cells by 8.37% and the MFI by 0.68. The effect was dose-dependent, with an additional 2.41% of increase in the expression rate every time the dose of IFN- α 2b added to the culture was increased 1 log. The effect was also time-dependent, with an additional increase of 2.8% and 14% with respect to 24 h of culture after 48 h and 72 h of incubation.

increase L-selectin expression rate in response to IFN- α treatment. This effect was dose- and time-dependent. Similarly, IFN- α was also found to induce L-selectin expression on the human B-lymphoid Daudi cell line in a dose and time-dependent manner, which is in agreement with results published by Evans *et al.*²⁹ concerning this cell line. Of note, L-selectin enhancement on the cell surface of the Daudi cell line was much more marked than that observed on CML CD34⁺ cells with a rate increased to nearly 50% over the control cells without IFN- α . Therefore, the proportion of CD34⁺ BM cells from CML responding to IFN- α appears to be relatively restricted and is similar to that reported for tissue-derived human B-cells.²⁹ In addition, no effect was shown on CD34⁺ cells treated with IFN- α *in vitro* from normal controls. These data may suggest that the effect of IFN- α *in vit-*

ro is specific to CD34⁺ cells from patients with CML.

IFN- α treatment in CML can result in the selective suppression of malignant hemopoietic progenitor cells and restoration of normal hematopoiesis.²⁰⁻²² The IFN- α action mechanism in CML is unknown, but recent *in vitro* studies have demonstrated that IFN- α increases the adhesion of CML progenitors to stroma. Such an effect has been shown via an action of IFN- α on stroma cells²³ and on CML progenitors correcting the deficient expression and/or function of some CAMs.^{7,8} Bathia *et al.*⁷ demonstrated that IFN- α partially restored the adhesion of CML progenitor cells to BM stroma by correcting β_1 -integrin receptor functions. Kawaishi *et al.*⁶ reported an inverse correlation between L-selectin expression rate in the CD34⁺⁺/CD38⁻ cells of CML patients and the percentage of Ph⁺ve cells in patients treated with IFN- α . They speculate that this cell subpopulation could be restored to normal in these patients or that IFN- α may directly induce L-selectin expression on CML CD34⁺ cells. The data presented in this study suggest that IFN- α acts on CML progenitors to up-regulate L-selectin surface expression and thus, may potentially use this adhesion receptor to increase adhesive interactions between CML progenitors and stroma. Nevertheless, this *in vitro* effect of IFN- α is partial and other mechanism(s) that explain the beneficial effect of this drug in CML patients are possible.³⁰

The L-selectin molecule belongs to a new family of selectin receptor adhesion molecules expressed on neutrophils, monocytes, T, B and natural killer cells, in addition to hematopoietic progenitor cells. L-selectin is involved in the regulation of leukocyte traffic, and in both initial attachment and rolling of lymphocytes and neutrophils to endothelium.¹³ Although the function of L-selectin on normal and CML hematopoietic progenitors remains unknown, accumulated evidence suggests that it plays a role in progenitor adhesion.^{13,31,32} Malignant progenitor cells from CML have defective adhesion to BM stroma and this has been reported to be partially mediated by dysfunction of β_1 -integrins, since CML progenitor cells co-express similar levels of this molecule to the levels expressed by normal progenitor cells.⁷ It has also been demonstrated that signaling through L-selectin serves as an activation/priming step on leukocyte adhesion and facilitates subsequent firm cell-cell adhesion through β_1 -integrin during an inflammatory response.³³ Based on these data, we speculate that the decreased L-selectin expression on CD34⁺ cells from CML patients may make initial cell attachment to stroma defective and thus, these progenitors might not be capable of generating signals to activate pathways that firmly attach progenitors to the BM microenvironment. Up-regulation of L-selectin expression in CML progenitors by IFN- α treatment could subsequently result in an enhanced affinity of the normally co-expressed β_1 -integrin receptors.

A subset of CD34⁺ CML cells (6-26%) expressed significant amounts of NCAM on their surface. By contrast, this adhesion receptor was co-expressed by less than 1% of purified CD34⁺ cell from normal individuals. These data are in agreement with those previously reported by Lanza *et al.*³⁴ who also observed a NCAM subpopulation within the CD34⁺ cells from 20% of CML patients.

Most of the CD34⁺ BM cells from normal and CML patients co-expressed LFA-3. Upadhyaya *et al.*⁹ have reported a deficient expression of LFA-3 on progenitor cells from untreated CML patients. They used a panning technique and clonogenic assays and recovered only 20% of CML progenitor cells in the adherent-LFA-3-positive fraction. In this work, using a CD34⁺ cell selection technique and a flow cytometry approach, more than 90% of CD34⁺ cells from CML co-expressed this molecule. Our results are in agreement with those reported by Dowding *et al.*²³ who were also unable to detect deficient LFA-3 expression on CD34⁺ cells from CML patients.

ICAM-3, a counter-receptor for LFA1,³⁵ was co-expressed in more than 90% of CD34⁺ cells from CML patients and controls. The slightly higher expression rate of ICAM-3 in CML CD34⁺ cells demonstrated in our study may reflect differences in cell populations between CML patients and normal controls. We have previously shown that CD34⁺/CD19⁺ putative B progenitor cells comprise a very small proportion of CD34⁺ cells in CML patients,³⁶ a cell subset with a lower percentage of ICAM-3 expression (data not shown).

In conclusion, the present study demonstrates that L-selectin is deficiently co-expressed by CD34⁺ cells from CML patients and that expression of this molecule is enhanced after *in vitro* treatment with IFN- α . The role of the partial restoration of L-selectin expression in re-establishing adhesive interactions between CML progenitors and BM stroma deserves further investigations.

Contributions and Acknowledgments

GAMH was the principal investigator, designed the study, conducted the experiments, interpreted the data and wrote the paper. RQ helped to conduct the experiments, and collected and interpreted the data. JRG and VM designed and performed the statistical study. AS was responsible for clinical day-to-day management of the patients and critically reviewed the manuscript; JG critically reviewed the different versions of the manuscript.

The order of the authors tries to take into account the time, work and scientific contribution given by all the authors the first author being the idea promoter and the last the senior member of the research group.

The authors wish to thank the Departments of Hematology of the Hospital Santa Creu i Sant Pau and the Hospital Prínceps d'Espanya for supplying the bone marrow samples.

Funding

This work was partially supported by a grant from the "Ministerio de Educación y Cultura, Dirección General de Enseñanza Superior y Investigación científica, SAF 98-0050."

Schering-Plough kindly supplied the IFN- α 2b and partially supported the work.

Part of this work was awarded the first prize "Modificadores de la Respuesta Biológica en Hemopatías Malignas" from Schering-Plough in the XII National Meeting of the Spanish Society of Hematology and Hemostasis which took place in October 1997 in Barcelona, Spain.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received June 22, 1999; accepted October 29, 1999.

Potential implications for clinical practice

- ◆ Biological characterization of CML progenitors will allow a more profound understanding of the behavior of this disease which will make other therapeutic approaches possible in patients not candidates for allogeneic stem cell transplantation.
- ◆ α -interferon constitutes one of the basic tools in the clinical management of CML patients and its mechanism of action is still unknown; the fact that α -interferon up-regulates L-selectin expression in CD34⁺ cells of CML patients sheds new light on this issue.
- ◆ The up-regulation of L-selectin expression by α -interferon could be evaluated as a predictive test to know whether a patient will achieve a cytogenetic response before starting the treatment.

References

1. Fialkow PJ, Jacobson RJ, Papayannopoulou T. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am J Med* 1977; 63:125-30.
2. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. *Science* 1960; 132:1497.
3. Goldman JM, Shiota F, Th'ng KH, Orchard KH. Circulating granulocytic and erythroid progenitor cells in chronic granulocytic leukaemia. *Br J Haematol* 1980; 46:7-13.
4. Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF. Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. *Nature* 1987; 328:342-4.
5. Verfaillie CM, McCarthy JB, McGlave PB. Mechanisms underlying abnormal trafficking of malignant progenitors in chronic myelogenous leukemia. Decreased adhesion to stroma and fibronectin but increased adhesion to the basement membrane components laminin and collagen type IV. *J Clin Invest* 1992; 90:1232-41.
6. Kawaishi K, Kimura A, Katoh O, et al. Decreased L-selectin expression in CD34-positive cells from patients with chronic myelocytic leukaemia. *Br J Haematol* 1996; 93:367-74.
7. Bhatia R, Wayner EA, McGlave PB, Verfaillie CM. Interferon- α restores normal adhesion of chronic myelogenous leukemia hematopoietic progenitors to bone marrow stroma by correcting impaired β_1 integrin receptor function. *J Clin Invest* 1994; 94:384-91.
8. Upadhyaya G, Guba SC, Sih SA, et al. Interferon- α restores the deficient expression of the cytoadhesion molecule lymphocyte function antigen-3 by chronic myelogenous leukemia progenitor cells. *J Clin Invest* 1991; 88:2131-6.
9. Zannettino AC, Buhning HJ, Niuatta S, Watt SM, Benton MA, Simmons PJ. The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. *Blood* 1998; 92:2613-28.
10. Verfaillie C, Blakolmer K, McGlave P. Purified primitive human hematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated bone marrow stroma. *J Exp Med* 1990; 172:509-12.
11. Simmons PJ, Masinovsky B, Longenecker BM, Berenson R, Torok-Storb B, Gallatin WM. Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. *Blood* 1992; 80:388-95.
12. Teixido J, Hemler ME, Greenberger JS, Anklesaria P. Role of β_1 and β_2 integrins in the adhesion of human CD34^{hi} stem cells to bone marrow stroma. *J Clin Invest* 1992; 90:358-67.
13. Kansas GS. Selectins and their ligands: current concepts and controversies. *Blood* 1996; 88:3259-87.
14. Ghaffari S, Dougherty GJ, Lansdorp PM, Eaves AC, Eaves CJ. Differentiation-associated changes in CD44 isoform expression during normal hematopoiesis and their alteration in chronic myeloid leukemia. *Blood* 1995; 86:2976-85.
15. Healy L, May G, Gale K, Grosveld F, Greaves M, Enver T. The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion. *Proc Natl Acad Sci U S A* 1995; 92:12240-4.
16. Bazil V, Brandt J, Chen S, et al. A monoclonal antibody recognizing CD43 (leukosialin) initiates apoptosis of human hematopoietic progenitor cells but not stem cells. *Blood* 1996; 87:1272-81.
17. Hurley RW, McCarthy JB, Verfaillie CM. Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. *J Clin Invest* 1995; 96:511-9.
18. Gordon MY, Atkinson J, Clarke D, et al. Deficiency of a phosphatidylinositol-anchored cell adhesion molecule influences haemopoietic progenitor binding to marrow stroma in chronic myeloid leukaemia. *Leukemia* 1991; 5:693-8.
19. Lundell BI, McCarthy JB, Kovach NL, Verfaillie CM. Activation-dependent $\alpha_5\beta_1$ integrin-mediated adhesion to fibronectin decreases proliferation of chronic myelogenous leukemia progenitors and K562 cells. *Blood* 1996; 87:2450-8.
20. Allan NC, Richards SM, Shepherd PC. UK Medical Research Council randomised, multicentre trial of interferon- α n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. The UK Medical Research Council's Working Parties for Therapeutic Trials in Adult Leukaemia. *Lancet* 1995; 345:1392-7.
21. Hehlmann R, Heimpel H, Hasford J, et al. Randomized comparison of interferon- α with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. *Blood* 1994; 84:4064-77.
22. Talpaz M, Kantarjian HM, McCredie K, Trujillo JM, Keating MJ, Gutterman JU. Hematologic remission and cytogenetic improvement induced by recombinant human interferon- α in chronic myelogenous leukemia. *N Engl J Med* 1986; 314:1065-9.
23. Dowding C, Guo AP, Osterholz J, Siczkowski M, Goldman J, Gordon M. Interferon- α overrides the deficient adhesion of chronic myeloid leukemia primitive progenitor cells to bone marrow stromal cells. *Blood* 1991; 78:499-505.
24. Martin-Henao GA, Ingles-Estevé J, Cancelas JA, Garcia J. Isolation of CD34⁺ hematopoietic progenitor cells in chronic myeloid leukemia by magnetic activated cell sorting (MACS). *Bone Marrow Transplant* 1996; 18:603-9.
25. Pinheiro JC, Bates DM. Mixed-effect methods and classes for S and S-PLUS. Bell Labs, Lucent Technologies and University of Wisconsin, Madison, 1998.

26. Verfaillie CM, Miller WJ, Boylan K, McGlave PB. Selection of benign primitive hematopoietic progenitors in chronic myelogenous leukemia on the basis of HLA-DR antigen expression. *Blood* 1992; 79:1003-10.
27. Leemhuis T, Leibowitz D, Cox G, et al. Identification of BCR/ABL-negative primitive hematopoietic progenitor cells within chronic myeloid leukemia marrow. *Blood* 1993; 81:801-7.
28. Kirk JA, Reems JA, Roecklein BA, et al. Benign marrow progenitors are enriched in the CD34+/HLA-DR^{lo} population but not in the CD34+/CD38^{lo} population in chronic myeloid leukemia: an analysis using interphase fluorescence in situ hybridization. *Blood* 1995; 86:737-43.
29. Evans SS, Collea RP, Appenheimer MM, Gollnick SO. Interferon- α induces the expression of the L-selectin homing receptor in human B lymphoid cells. *J Cell Biol* 1993; 123:1889-98.
30. Gordon MY, Marley SB, Lewis JL, et al. Treatment with interferon- α preferentially reduces the capacity for amplification of granulocyte-macrophage progenitors (CFU-GM) from patients with chronic myeloid leukemia but spares normal CFU-GM. *J Clin Invest* 1998; 102:710-5.
31. Oxley SM, Sackstein R. Detection of an L-selectin ligand on a hematopoietic progenitor cell line. *Blood* 1994; 84:3299-306.
32. Mohle R, Moore MA, Nachman RL, Rafii S. Transendothelial migration of CD34+ and mature hematopoietic cells: an in vitro study using a human bone marrow endothelial cell line. *Blood* 1997; 89:72-80.
33. Steeber DA, Engel P, Miller AS, Sheetz MP, Tedder TF. Ligation of L-selectin through conserved regions within the lectin domain activates signal transduction pathways and integrin function in human, mouse, and rat leukocytes. *J Immunol* 1997; 159:952-63.
34. Lanza F, Bi S, Castoldi G, Goldman JM. Abnormal expression of N-CAM (CD56) adhesion molecule on myeloid and progenitor cells from chronic myeloid leukemia. *Leukemia* 1993; 7:1570-5.
35. de Fougerolles AR, Springer TA. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J Exp Med* 1992; 175:185-90.
36. Martín-Henao GA, Quiroga R, Sureda A, García J. CD7 expression on CD34+ cells from chronic myeloid leukaemia in chronic phase. *Am J Hematol* 1999; 61:178-86.