CELLULAR EXPRESSION AND SERUM CIRCULATING LEVELS OF CD23 IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA. IMPLICATIONS FOR PROGNOSIS

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ABSTRACT

Background. CD23 is a functionally relevant molecule in B-cell chronic lymphocytic leukemia (CLL) which mediates growth and differentiation signals in B-cells. An intriguing feature of CD23 is its ability to be cleaved from the cell surface and released into the serum.

Materials and Methods. Serum levels of soluble CD23 (sCD23) were determined with a sandwich enzyme immunoassay at the time of diagnosis in 90 previously untreated CLL patients, in order to evaluate whether they reflected disease activity and tumor load. Results were correlated with those dealing with CD23 expression on leukemic cells to verify whether the cellular counterpart determines serum levels.

Results. CD23 was detected on peripheral blood mononuclear cells (PBMC) from 78 out of 90 (86.6%) B-CLL patients, without correlation with clinical stage. Circulating levels of sCD23 in the serum of patients with CLL were highly elevated in comparison to 15 normal controls (p < 0.0005); this increase reflected tumor mass as defined by either clinical stage (p < 0.0005) or bone marrow (BM) histology (p < 0.0005). Neither percentage nor absolute number of CD23+ cells correlated with circulating levels. Interestingly, life expectancy was significantly shorter in patients with higher serum levels of sCD23 (p < 0.0005). When integrated into the Binet clinical staging system, sCD23 led to isolation of two subgroups with different prognosis among intermediate-risk patients. Furthermore, longitudinal studies support the idea that sCD23 can be utilized as an indicator of disease progression.

Conclusions. sCD23 is a highly sensitive and suitable marker with prognostic potential in B-CLL.

Clinical stage, bone marrow (BM) histology, peripheral blood (PB) lymphocyte count, lymphocyte doubling time (LDT), number of prolymphocytes in PB and cytogenetic abnormalities are considered reliable prognostic factors in chronic lymphocytic leukemia (CLL).[1-9] More recently, prognostic assessment of patients with CLL has begun moving from an era in which the emphasis was focused on clinical parameters to a new one in which serological data are emerging as potentially important.[10] Given the regulatory role of the CD23 molecule in B-cell proliferation, clinical studies which have focused on soluble CD23 (sCD23) as a disease marker with prognostic relevance are of special interest.[11-16] Moreover, longitudinal analyses support the idea that serum levels of sCD23 can be used as an indicator of disease progression.[17]

Keeping these concepts in mind, we analyzed the prognostic impact on survival of serum levels of sCD23 in an unselected series of 90 previously untreated B-cell CLL patients. Interestingly, sCD23 could be integrated into the Binet clinical staging system, thus allowing two prognostic subgroups with different prognosis to be identified among stage B patients who usually exhibit similar survival to the one of entire series.

Materials and Methods

Patient characteristics

Ninety patients diagnosed as having CLL in two different hematological institutions form the basis of this study. The mean age of patients was 66 years (SD 8.2), and the male to female ratio was 58 to 32. B-CLL was diagnosed according to generally accepted criteria that included peripheral blood lymphocytosis greater than 5×10^9/L and BM lymphocytosis greater than 30%.[18] B-cell phenotype was demonstrated in all patients by means of immunological markers: HLA-DR (anti MHC-class II), OKT3-CD3 (T lymphocytes), OK CLL-CD5 (T lymphocytes, B lymphocyte subset, B-CLL cells), OK BCALLA-CD10 (lymphoid progenitor cells, C-ALL, granulocytes) (Ortho, Raritan NJ, CA), Leu 16-CD20 (B lymphocytes, malignant B cells), Leu 20-CD23 (activated B-cells, B-CLL) (Becton-Dickinson, San José, CA, USA), IOM 11c-CD11c (monocytes, granulocytes, NK-cells, hairy cell leukemia-strong, B-cell subset-weak), IOL 54-CD54 (endothelial cells, many cell subtypes upon activation) (Immunotech, Marseille, France), κ and λ light chains (SIg) (Ortho, Raritan, NJ, USA).

Flow cytometric measurements using single color immunofluorescence were carried out by means of a CYTORON (Ortho Diagnostic System, Raritan, NJ, CA) or a FACS-SCAN cytofluorograph (Becton-Dickinson, San José, CA, USA). On the basis of the most common marker profile in CLL (i.e. CD5⁺, CD23⁺, and weak expression of SmIg), 78 out of 90 (86.6%) cases met the criteria for typical CLL. The remaining patients who lacked one or more of the immunological criteria were called atypical B-CLL.[19] Phycoerythrinated (HLA-DR; Ortho Diagnostic System, Raritan, NJ, CA) and fluorescinated (Leu 20-CD23, Becton-Dickinson, San José, CA) monoclonal antibodies (MoAb) were used for two-color direct immunofluorescence.

All patients were staged according to the Binet clinical staging system[2] (stage A, 41; stage B, 21; stage C, 18), and the pattern of BM involvement was evaluated in 82 patients. Biopsy was not performed in the remaining 8 patients, mainly due to refusal of informed consent. Although

four histological patterns were recognized (i.e. nodular, interstitial, mixed and diffuse),[3] for the purposes of the current study interstitial, nodular and mixed patterns were considered together and formed the non-diffuse group. Therefore 49 patients could be classified as having a non-diffuse and 33 a diffuse histological pattern of BM involvement.

LDT was retrospectively evaluated in 78 patients, all having observation times greater than 1 year, according to previously described methods.[7]

Quantitative determination of soluble CD23 levels

Quantitative determinations of sCD23 were carried out by means of an enzyme immunoassay kit (CELL FREE CD23 test kit, T cell Diagnostic Inc, Cambridge, MA). All serum samples were taken at diagnosis of CLL and stored at –70°. In brief, standard or diluted samples were added to the polystyrene microliter wells precoated with murine monoclonal antibody to human CD23.

A second horseradish peroxidase-conjugated murine MoAb to human CD23 was then used to bind a second epitope on the molecule captured by the first antibody. After removing unreacted components by washing, a chromogen solution was added to the wells, forming a colored end product that was proportional to the amount of CD23 present in the sample.

The reaction was terminated by the addition of a stop solution and the absorbance was measured at 490 nm. Serum samples were diluted as far as necessary to yield CD23 values which fell within the standard curve range. Finally, the mean value of absorbance from duplicate samples was plotted on a standard curve and converted in units per milliliter (U/mL).

The detection limit of the test was 15 U/mL. sCD23 displayed an interassay coefficient of variance (CV) between 5.1% and 9.9% and an intra-assay CV which varied between 2.8% and 9.4%.

Treatment and follow-up data

As a rule patients in stage C or symptomatic B were treated at diagnosis. In the remaining cases indications for treatment were progressive disease with clear B-symptoms (fever, night sweats,

a weight loss not due to other causes), clinical stage progression (from A to B or C), a rapid LDT (i.e. shorter than 6 months). An alkylating agent, usually chlorambucil (CLB) associated with low doses of corticosteroids, was the drug chosen. As far as survival is concerned, median duration of follow-up was 35 months (range 8-66 months). At the time of the present study 28 patients (31.1%) had died. Actuarial median survival was 60 months.

Statistical analyses

The Student's t-test and analysis of variance were used to evaluate differences in the mean among various groups. When dealing with discrete variables, statistical analyses were carried out by means of the chi-square test. The *r* correlation coefficients between parameters were computed in least-squares regression equations. Survival curves were plotted according to the method of Kaplan and Meier[20] and compared by using the log-rank test.[21]

Results

Cellular expression of CD23 and correlation with its soluble form

CD23 was detected on PBMC from 78 out of 90 (86.6%) B-cell CLL patients. In both normal controls and CLL patients, CD23 expression was associated with HLA-DR positivity. When investigated by two-color fluorescence flow cytometry, the mean percentage of CD23+ cells was 2.7±2.7% in 15 normal controls and 67.5±28.3% in CLL patients (p < 0.0005). Mean fluorescence intensity (MFI) of CD23+ on B-CLL cells was also

significantly higher than that of normal controls (MFI 94.6±17.4 versus 50.7±3.1; p < 0.001) (Figure 1). Finally, the percentage of CD23+ cells among PBMC did not show any change as a function of the Binet clinical stages (stage A 66.3±26.3%; stage B 71±25.9%; stage C 65.4±32.9%; p = not significant in the analysis of variance). Circulating levels of sCD23 in the serum of patients with B-CLL were highly elevated in comparison to 15 normal controls (2774.7 ±2480.1 U/mL versus 108±64 U/mL; p< 0.0005): this increase reflected tumor mass as defined by the Binet clinical stages. The same applied when patients were analyzed according to either BM histology or LDT (Table 1).

To test whether CD23 expression on leukemic B-CLL cells determined its serum levels, we tried to correlate both these parameters. No correlation was found between the absolute number of PB CD23+ cells and circulating levels of sCD23 (r = 0.01). When the total amount of membrane CD23 expression was calculated as the product of the percentage of CD23+ cells and the MFI of the CD23 antigen, again no correlation with sCD23 levels could be detected (r = 0.172). On the other hand, patients who displayed membrane CD23 (mCD23) positivity (i.e. more than 30% CD23+ cells) had serum levels of sCD23 that were not significantly different from those with mCD23 negativity (2779.3± 2405 U/mL vs. 3392.2±3064.9 U/mL; p = NS).

Prognostic value of increased levels of sCD23

To assess the prognostic value of serum levels of sCD23, a survival analysis was performed after setting the cut-off at the mean value for this soluble molecule. Clear-cut differences in life
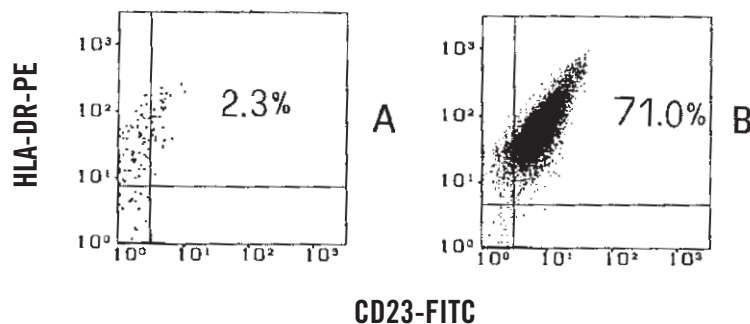

Figure 1. Expression of membrane CD23 among HLA-DR+ PBMC from a healthy donor (A) and a B-CLL patient (B).

Table 1. Relationship between sCD23 and clinico-hematological features.

	N. pts	sCD23(U/mL)	p-value
Clinical stages			
A	41	1726.4±1306.4	
B	31	2828.9±1812.3	0.0001
C	18	5198.3±3790.3	
BM histology			
Non-diffuse	50	1838.3±1419.5	0.0001
Diffuse	34	4373.8±3037.2	
LDT			
> 12 mo	42	1422.0±1006.1	0.0001
12 mo	36	4242.3±3092.4	
PB lymphocytes			
< 50 (10^9/L)	58	2277±912.8	0.0001
50 (10^9/L)	32	4013.4±2801	

LDT: lymphocyte doubling time; PB: peripheral blood; BM: bone marrow.

tion was less than 2700 U/mL and those with sCD23 equal to or more than 2700 U/mL; the 5-year survival rates were 66% (95% CI: 51% to 80%) and 24% (95% CI: 5% to 43%), respectively (p < 0.001; Figure 2).

With the aim of isolating prognostic subgroups within established clinical stages, survival of patients belonging to different Binet clinical stages was analyzed according to the serum levels of sCD23 (i.e. less or more than 2700 U/mL). Life expectancy was better in stage B patients with sCD23 concentrations lower than 2700 U/mL [observed/expected ratio (O/E) 0.48] than in stage B patients with sCD23 higher than 2700 U/mL (O/E ratio 1.85; chi-square = 4.40; p < 0.02). When the same analysis was performed in stage A and C patients, the difference did not reach statistical significance. Indeed the O/E ratios were as follows: stage A-sCD23 < 2700 U/mL, 1.16; sCD23 > 2700 U/mL, 0.58 (chi-square = 0.38, p = NS); stage C-sCD23 < 2700 U/mL, 0.61; sCD23 > 2700 U/mL, 1.20 (chi-square = 1.20, p = NS).

Finally, after integrating sCD23 into the Binet clinical staging system the efficacy of a combined clinico-biological staging was tested. CLL patients could be segregated into four groups: (I) stage A; (II) stage B with sCD23 levels < 2700 U/mL; (III) stage B with sCD23 levels > 2700 U/mL; (IV) stage C. The log-rank test of these survival curves is presented in Table 2. As can be seen, the O/E ratio is always either lower or higher than 1. In other words, all groups designated in this way discriminate with respect to the whole population.

Relationship between sCD23 values and clinical outcome

sCD23 levels were prospectively analyzed on 2 to 4 occasions in 14 stage A patients. In 7 patients considered to be in stable disease on the basis of LDT > 12 months and the absence of clinical stage progression,[22] sCD23 levels examined at intervals ranging from 6 to 38 months were not significantly different from those
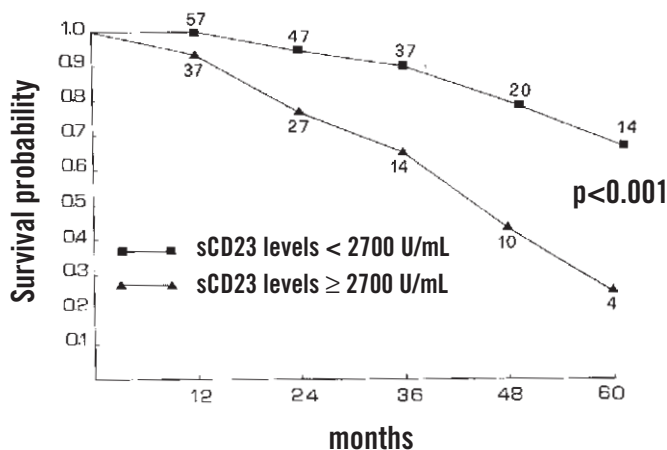

Figure 2. Actuarial survival curves of CLL patients according to sCD23 serum level. The projected survival at 5 years was 66% (95% CI: 51% to 80%) for patients with sCD23 concentrations < 2700 U/mL and 24% (95% CI: 5% to 43%) for patients with sCD23 concentrations > 2700 U/mL.

	Observed	(Total)	Expected	O/E	5-year survival rate
Stage A	6	(41)	17.754	0.33	75%
Stage B with sCD23 levels < 2700 U/mL	3	(18)	4.241	0.70	55%
Stage B with sCD23 levels 2700 U/mL	7	(13)	3.344	2.03	23%
Stage C	12	(18)	3.027	3.96	0%

Table 2. Log-rank analysis of survival curves in CLL according to a modified version of Binet's staging system in which sCD23 levels are used to subclassify stage B.

O/E = observed/expected ratio; chi-square for heterogeneity (3 d.f.) = 38.43; p < 0.0005; chi-square for trend (1 d.f.) = 27.41; p < 0.0005.

Disease status	N. pts	Median time in months between determinations (range)	sCD23 1st det.	2nd det.	p-value
Stable	7	16 (6-38)	1512.5±944	1554.4±1030	0.93
Progressive	7	18 (17-30)	2930.8±2152.5	6585.1±2948.9	0.021

Table 3. Serial determinations of sCD23 serum levels in 14 B-CLL patients.

(1512.5±944 U/mL vs. 1554.4 ±1030 U/mL; 95% CI: -1192.7 to 1109.1; p = 0.93) (Table 3). Seven patients experienced a change in clinical stage (i.e. from A to B or C) after a median time of 18 months. Interestingly, clinical stage progression paralleled the increase in sCD23 (2930.8±2152.5 U/mL vs 6585.1±2948.9 U/mL; 95% CI: -6660.9 to -647.6; p = 0.021) (Table 3).

Discussion

The expression of CD23 on B-CLL leukemic cells has been strongly correlated with survival; CD23-negative cases showed a significantly shorter survival rate.[23] What emerges from the present study is that the prognostic role of mCD23 expression is far from absolute. Indeed no difference in the expression of mCD23 could be demonstrated between patients in early and advanced clinical stage. As far as sCD23 is concerned, we confirm that serum levels of this molecule reflect either clinical stage or BM histology. In line with the experience of Reinisch et al.,[14] we were unable to demonstrate any correlation between sCD23 levels and the absolute number of CD23+ B-leukemic cells, thus implying a major contribution to sCD23 release and serum accumulation by non-circulating neoplastic cells. This observation is in keeping with the results of other studies which reported only a weak correlation between serum levels of sCD23 and the absolute number of PB lymphocytes bearing the mCD23 antigen.[13-14]

In CLL, LDT provides a stage-independent prognostic parameter, with a short LDT identifying patients with a significantly poorer chance of survival.[24] In this context, our data dealing with the inverse correlation between LDT and sCD23 are of special interest. Moreover, this clinical observation parallels biological *in vitro* results by Fournier et al.,[25] who demonstrated that a selective increase in CD23 type B expression provokes the entry of resting (G0) B-CLL cells into the G1 and S phases of the cell cycle in the absence of other stimuli.

Preliminary data showing that sCD23 levels predict either overall survival or freedom from progression were independently reported, although in abstract form, by two different groups.[15,16] Our results, which confirm and fur-

ther extend these observations, also show that sCD23 can be incorporated into the Binet clinical staging and used to separate stage B patients into two different prognostic subgroups. There is a general reluctance in CLL to treat patients whose disease shows a relatively indolent course.[26] Therefore splitting intermediate-risk patients into two groups (those suitable for a *watch and wait* policy and those requiring early treatment) is mandatory. As shown in the present study, sCD23 is an assay able to identify a subgroup of CLL patients who are expected to have shorter survival and need to initiate treatment early in the disease.

In summary, our results support the clinical usefulness of utilizing biological markers in CLL. sCD23 is a highly sensitive marker with prognostic potential in B-CLL. In addition, its unique properties make it possible to evaluate disease progression of CLL patients through longitudinal determinations of sCD23 levels.

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