



## Clinico-biological implications of increased serum levels of interleukin-8 in B-cell chronic lymphocytic leukemia

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### ABSTRACT

**Background and Objective.** Constitutive cellular expression and serum release of biologically active interleukin-8 (IL-8) has been reported in B-cell chronic lymphocytic leukemia (CLL). Given the autocrine role played by IL-8 in the process of cell accumulation characteristic of this disease we tried to investigate clinico-biological implications of increased serum levels of this cytokine in an unselected series of B-cell CLL patients.

**Design and Methods.** Serum levels of IL-8 were determined at the time of diagnosis in 58 previously untreated B-CLL patients using an immunoenzyme assay. Results were correlated with main clinico-hematologic features as well as with the risk of disease progression. Finally, we looked for associations between IL-8 and molecules directly involved in apoptosis, such as intracellular bcl-2 and soluble APO-1/Fas.

**Results.** Increased serum levels of IL-8 were found in 15 out of 58 (25.8%) B-cell CLL patients. Serum levels of IL-8 did not reflect clinico-biological features representative of tumor mass such as clinical stage, histopathologic pattern of bone marrow (BM) involvement,  $\beta_2$ -microglobulin, sCD23 and sCD27 titers. Interestingly, circulating levels of IL-8 paralleled those of intracellular bcl-2 ( $r = 0.522$ ;  $p = 0.01$ ), thus confirming that the antiapoptotic effect of IL-8 can be exerted through a bcl-2 dependent pathway. Levels of IL-8 did not match those of soluble Apo-1/Fas ( $r = -0.013$ ;  $p = 0.943$ ). Finally, stage A patients with levels of IL-8 above the median value (i.e. 4.5 pg/mL) were more likely to progress to a more advanced clinical stage than those with levels below the median value ( $p < 0.05$ ).

**Interpretation and Conclusions.** IL-8 is an interesting marker in B-cell CLL, closely involved in the pathogenesis of disease. Furthermore, it is useful for predicting the pace of disease progression in early clinical stages.

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Key words: IL-8, B-CLL, apoptosis, disease progression

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Over the last few years several cytokines have been shown to play a regulatory role in the mechanisms leading to the growth of B-cell chronic lymphocytic leukemia (CLL) cells.<sup>1-3</sup> Interleukin-8 (IL-8), a multifactorial chemokine constitutively expressed by B-CLL cells and released in the serum, contributes via an autocrine pathway to the cell accumulation characteristic of this disease.<sup>4</sup> In order to extend these preliminary observations, we studied 58 CD5<sup>+</sup> B-cell CLL patients in whom serum levels of IL-8 were measured at the time of diagnosis using a commercial immunoenzyme assay (ESIA). Results were correlated with either biological or clinical parameters of disease activity in B-CLL. In addition, we explored whether changes of circulating IL-8 concentrations matched intracellular amounts of bcl-2 oncoprotein and soluble levels of Apo-1/Fas, thus providing a contribution to understand mechanisms underlying the antiapoptotic effect of IL-8 in B-cell CLL. Finally, IL-8 level was investigated as an indicator of disease progression in stage A CLL patients whose outcome is not accurately predicted by currently used clinico-hematologic features.

### Design and Methods

#### Patient characteristics

Fifty-eight patients diagnosed as having CLL at our institution form the basis of this study. Their mean age was 64 years (SD 6.4) and the male to female ratio 50 to 38. CLL was diagnosed according to generally accepted criteria which included peripheral blood (PB) lymphocytosis  $> 5 \times 10^9/L$  and bone marrow (BM) lymphocytosis  $> 30\%$ .<sup>5</sup>

The patients' disease stage was classified according to the Binet staging system<sup>6</sup> and distributed as follows: Stage A, 36 patients; Stage B, 14; Stage C, 8. BM biopsy available in 47 (81%) patients, made it possible to identify a non-diffuse pattern of BM involvement in 38 patients and a diffuse pattern in 9.<sup>7</sup>

#### Immunophenotype analyses

Fresh PB cells were used for immunologic analyses. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation and cells were stained

using both direct and indirect techniques. The different immunomarkers used included CD3, CD5, CD22,  $\kappa$  and  $\lambda$  chain immunoglobulins (Ortho Raritan, NY, USA); CD19, CD20, CD23, CD11c (Becton Dickinson, San José, CA, USA); FMC7 (Immunotech, Marseille, France). A marker was considered positive when it was expressed in over 30% of cells analyzed. The stained cells were analyzed on an ABSOLUTE flow cytometer (ORTHO Diagnostic System). In 20 patients we assessed levels of bcl-2 protein using a quantitative indirect immunofluorescence assay (QIFI kit, DAKO, Copenhagen, Denmark). In order to detect cytoplasmic bcl-2 in individual cells, the cells were fixed and permeabilized by means of a commercially available kit (Fix and Perm, Permeabilization Kit, CALTAG, Burlingame, CA, USA). Indirect staining was performed by incubating permeabilized cells with anti-bcl-2 MoAb (124 Clone: IgG1,  $\kappa$  isotype, DAKO, Copenhagen, Denmark) as previously reported. The bcl-2 antigen density was assessed using the QIFI kit assay and was expressed as antibody binding capacity (ABC) molecules/cell.<sup>8</sup>

#### Determination of serum levels of IL-8 and other assays

All serum samples were taken at diagnosis and stored at  $-70^{\circ}\text{C}$ . Quantitative determination of serum levels of IL-8 was carried out by means of a solid phase Enzyme Amplified Sensitivity Immunoassay (IL-8 EASIA™, Medgenix Diagnostics, Fleurus, Belgium). The detection limit was 0.7 pg/mL. The inter-assay coefficient of variance (CV) ranged between 3% and 4.3% and the intra-assay CV between 3.5% and 4.9%. Sera previously analyzed for IL-8 were selected for determination of a tumor necrosis factor (TNF) (TNF EASIA™ kit, Medgenix, Bio Source, Belgium); sCD23 (CELL FREE CD23 test kit; T cell Diagnostic Inc, Cambridge, MA, USA), sCD27 (CD27 ELISA kit, CLB, Amsterdam, The Netherlands);  $\beta_2$  microglobulin and LDH. In a subset of 20 patients in whom quantitative determinations of intracellular bcl-2 were obtained, we measured serum levels of sAPO-1/Fas by using a commercial ELISA assay (Bender, Med System, Vienna, Austria).

#### Statistical analyses

Results were analyzed with the statistical program GraphPAD Software 2.00 (GraphPAD Software Inc, San Diego, CA, USA). Non-parametric tests (Mann-Whitney test, Kruskal-Wallis test) were used to evaluate differences between medians. Calculation of Pearson's coefficient was used for comparing two continuous variables. Disease progression curves were plotted according to the method of Kaplan-Meier and compared with the log-rank test.

#### Results

The serum levels of IL-8 in B-cell CLL patients were not significantly different (4.52 pg/mL; range, 0.54-268.5) than those in an age-matched control popu-

lation (4.41 pg/mL; range, 2.4-12.17;  $p = 0.819$ ). Increased levels of circulating IL-8 (i.e., higher than mean value  $\pm 2$  SD, 11.9 pg/mL) were found in 15 out of 58 (25.8%) patients. However, when we sought to evaluate the clinical implications of such an increase, it was found that serum concentrations of IL-8 did not reflect features representative of tumor mass such as clinical stage (Stage A, 3.67 pg/mL, range, 0.85-268.5; Stage B, 4.76 pg/mL, range, 0.54-59.14; Stage C, 11.20 pg/mL, range, 2.1-19.7;  $p = 0.543$  for Kruskal-Wallis analysis of variance), BM histology (non-diffuse, 4.06 pg/mL, range, 0.54-268; diffuse, 4.18 pg/mL, range, 1.7-59.14;  $p = 0.871$ , Mann-Whitney test),  $\beta_2$  microglobulin ( $r = 0.336$ ,  $p = 0.06$ ), sCD23 ( $r = 0.07$ ,  $p = 0.559$ ), sCD27 ( $r = 0.267$ ,  $p = 0.161$ ). Interestingly, levels of IL-8 correlated with those of  $\alpha$ -TNF ( $r = 0.408$ ,  $p = 0.02$ ) a molecule belonging to the nerve growth factor receptor (NGFr) superfamily.

Given the autocrine role played by IL-8 in the process of cell accumulation, we looked for correlations between IL-8 and some molecules involved in apoptosis, such as bcl-2 and Apo-1/Fas. To this purpose intracellular levels of bcl-2 oncoprotein were evaluated in flow cytometry and assessed as antibody binding capacity (ABC) molecules/cell.<sup>8</sup> Levels of bcl-2 paralleled those of circulating IL-8 ( $r = 0.522$ ;  $p = 0.01$ ; Figure 1), thus confirming that the anti-apoptotic role of IL-8 is exerted through a bcl-2 dependent pathway. No correlation could be demonstrated between circulating levels of IL-8 and those of soluble Apo-1/Fas ( $r = -0.013$ ;  $p = 0.943$ ).

Finally, to assess the prognostic value of serum levels of IL-8, an analysis of risk of disease progression was performed in 32 stage A patients. In this analysis a cut-off of 4.5 pg/mL was chosen because it was the median value of our series of patients. Clinical characteristics of the two groups of patients stratified

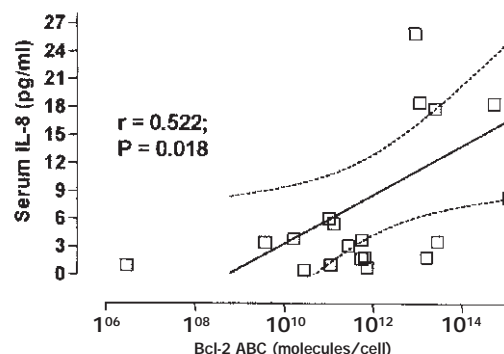


Figure 1. Correlation between serum levels of interleukin 8 and intracellular amount of bcl-2 oncoprotein.

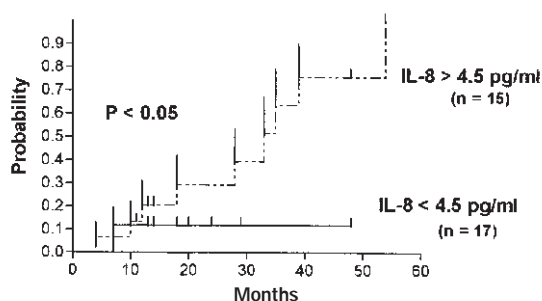
**Table 1. Clinico-hematologic characteristics of stage A patients stratified on the basis of serum levels of IL-8.**

Characteristics	IL-8 <4.5 pg/mL n=17	IL-8 ≥4.5 pg/mL n=15	p
Rai stage (0/I-II)	10/7	7/8	0.734
BM histology (nD/D)	12/1	13/1	0.496
PB lymphocytosis (10 <sup>9</sup> /L)	15.6 (2.9-120)	14 (5.6-82.3)	0.482
LDT (>12 mo./<12 mo.)	16/1	13/2	0.909
β <sub>2</sub> M (mg/mL)	2.28 (1.2-2.9)	2.3 (1.3-3.5)	0.257
sCD23 (U/mL)	1245 (24-5,475)	847 (72-9,209)	0.819

according to circulating levels of IL-8 are depicted in Table 1. As shown, they were alike with respect to main prognostic features such as Rai substages, BM histology, absolute PB lymphocytosis, LDT, β<sub>2</sub> microglobulin and sCD23 levels. However, median time of disease progression was significantly shorter (33 months) in patients with levels of IL-8 higher than 4.5 pg/mL than in patients with IL-8 levels lower than 4.5 pg/mL (median not reached at 50 months) (p < 0.05; relative risk 0.272, 95% confidence interval, 0.08 to 1.04) (Figure 2).

## Discussion

*In vitro* studies have shown that several cytokines play a role in prolonging survival of B-CLL cells.<sup>1-4</sup> This is the case of IL-4 which may inhibit either spontaneous or induced apoptosis in B-CLL cells.<sup>1,2</sup> The same applies for α-IFN and IL-10.<sup>8,9</sup> As far as IL-8 is concerned, recent data suggest that it is the only



**Figure 2. Risk of disease progression for stage A patients divided on the basis of median value of serum levels of interleukin 8.**

cytokine constitutively expressed by B-CLL cells able to exert a regulatory function on leukemic cells through an autocrine pathway.<sup>3,4</sup> Indeed, *in vitro* experiments carried out by Di Celle *et al.*<sup>4</sup> demonstrated that after treatment with IL-8 an upregulation of mRNA expression of bcl-2 by B-CLL cells occurred. Since the overexpression of bcl-2 is associated with prevention of B-cell death, the upregulation induced by IL-8 highlights the role played by such a cytokine in the process of cell accumulation characteristic of this neoplasia.<sup>11</sup> Our findings, showing an *in vivo* correlation between levels of IL-8 and those of intracellular bcl-2, extend knowledge on the biological properties of B-CLL cells, therefore opening up the prospect of new biological therapies able to influence growth signals mediated by cytokines.

Although a number of independent prognostic factors have been analyzed in B-cell CLL, the clinical course of this disease is often unpredictable.<sup>12,13</sup> In addition to clinical stages, BM histology and LDT increased serum levels of β<sub>2</sub> microglobulin, sCD23, sCD27 and sCD54 have been reported to correlate with an increased risk of disease progression.<sup>13,14</sup> What emerges from results of the present study is that only 26% of the patients had increased levels of IL-8 and there was no correlation between circulating levels of IL-8 and clinico-biological parameters reflecting tumor burden.

Interestingly, we found a close association between levels of IL-8 and those of α-TNF. This finding may further contribute to expand available information on implications of different cytokines in the mechanisms of neoplastic growth and progression. On the other hand, melanoma cells stimulated with α-TNF secrete a biologically active IL-8 protein thus inducing a haptotactic migration of neoplastic cells.<sup>15</sup>

From our observations it can be argued that serum levels of IL-8 are not invariably high in B-cell CLL, levels within the normal limit having been detected in most of patients. Independently of tumor mass, a strong association between the risk of disease progression and IL-8 levels was found. These results corroborate the importance of measuring serum levels of IL-8 at the time of diagnosis as they might provide valuable information in the decision process of initiating chemotherapy. Whether the influence of IL-8 on the pattern of disease progression is exerted through an increase of intracellular levels of bcl-2 is still unclear. A close relationship between high levels of bcl-2 oncoprotein and reduced survival has been reported in two independent studies, however, the impact of bcl-2 on the pattern of disease progression is still unclear and only preliminary data correlating bcl-2/bax ratio with the behavior of disease have been published thus far.<sup>16-19</sup>

In conclusion, IL-8 serum level offers an additional tool in B-cell CLL useful for predicting the pace of disease progression in early clinical stages. Given the lack of correlation between serum levels of IL-8 and

clinico-biological features of disease activity a possible independent prognostic role for IL-8 can be conceived. Whether increased IL-8 concentrations actually reflect the host's immune response to malignant cells or tumor cells is still unclear. Some authors have analyzed IL-8 concentrations after culturing B-CLL cell samples under basal and stimulated conditions.<sup>3</sup> Although IL-8 levels above the test's limit of detection were observed in all instances, significantly increased levels were found in the supernatants of stimulated cell assays. Nonetheless, our observations suggest that IL-8 serum level does not reflect tumor mass in B-cell CLL, therefore the possibility that IL-8 could originate from normal host T- or monocyte cells cannot be ruled out.<sup>20</sup>

### Contributions and Acknowledgments

*SM was the main investigator, designed the study and performed the statistical analyses. He wrote the paper. GV was responsible for the immunoenzyme studies. AD carried out flow cytometric assays. DL and LL were involved in the clinical follow-up of patients. GMG gave final approval to the publication of the study.*

### Disclosures

*Conflict of interest: none.*

*Redundant publications: no substantial overlapping with previous papers.*

### Manuscript processing

*Manuscript received August 26, 1998; accepted November 12, 1998.*

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