



## CD27 in B-cell chronic lymphocytic leukemia. Cellular expression, serum release and correlation with other soluble molecules belonging to nerve growth factor receptors (NGFr) superfamily

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### Abstract

**Background and Objective.** CD27, a transmembrane homodimer belonging to the nerve growth factor (NGF) receptor superfamily, is typically expressed on leukemic CD5<sup>+</sup> cells in B-cell chronic lymphocytic leukemia (CLL) and found in soluble form in the serum of CLL patients. Therefore, we investigated clinico-biological implications of increased serum levels of sCD27 in an unselected series of B-CLL patients.

**Design and Methods.** Serum CD27 (sCD27) levels were determined at the time of diagnosis in 82 previously untreated B-CLL patients using a sandwich enzyme-linked immunoassay (ELISA). Results were correlated with either clinico-hematological or biological features. Finally, quantitative flow cytometric analyses of membrane CD27 (mCD27) expression were carried out on peripheral blood (PB) cells of 22 B-CLL patients and 5 healthy controls, respectively.

**Results.** CD27 was found to be expressed on the surface of both resting normal and leukemic B cells. sCD27 levels were significantly higher in B-CLL patients (median value 2150 U/mL) than in healthy controls (median value 220 U/mL) ( $p < 0.0001$ ). There was a close relationship between sCD27 and soluble TNF- $\alpha$ , another molecule belonging to the NGF receptor superfamily. Changes in sCD27 level correlated with clinical stage,  $\beta_2$  microglobulin and LDH.

**Interpretation and Conclusions.** These findings indicate that sCD27 is a reliable marker of tumor mass in B-CLL. Its potential prognostic value should be tested in prospective studies.

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Key words: chronic lymphocytic leukemia, CD27, TNF- $\alpha$ , APO-1/Fas

CD27 is a transmembrane disulfide-linked homodimer belonging to nerve growth factor receptors (NGFr) superfamily, a group of homologous molecules involved in the processes of lymphocyte differentiation and selection that include

the two tumor necrosis factor (TNF) receptors, the B-cell antigen CD40, the lymphocyte activation antigen CD30, and APO-1/Fas (CD95) a molecule which controls lymphocyte survival.<sup>1</sup> Expression of CD27 has been considered a peculiar finding of leukemic CD5<sup>+</sup> B-CLL cells; as a matter of fact normal follicular CD5<sup>+</sup> mantle zone cells, which represent the non-neoplastic counterpart of B-CLL, are CD27 negative.<sup>2-4</sup> The expression on B-CLL leukemic cells is generally accompanied by the release in the serum of an appreciable amount of sCD27 which correlates with clinico-hematological parameters representative of tumor burden.<sup>3</sup> To further extend these observations, we studied 82 previously untreated CD5<sup>+</sup> B-cell CLL in whom serum levels of sCD27 were measured using a commercial sandwich enzyme-linked immunosorbent (ELISA) assay. We demonstrate that sCD27 is a reliable disease marker in B-cell CLL, thus confirming previous reported results.<sup>3</sup> In addition, results of the present study provide information lacking in the literature, which concern the relationship between increased levels of serum sCD27 and some serological markers of common use in B-CLL such as  $\beta_2$ -microglobulin ( $\beta_2$ M), LDH and soluble CD23 (sCD23). Finally, we explored whether changes of sCD27 serum levels matched those of TNF- $\alpha$  and sAPO-1/Fas, two molecules belonging, like sCD27, to the NGFr superfamily.

### Materials and Methods

#### Patient characteristics

Eighty-two patients diagnosed as having CLL in two different hematological institutions form the basis of this study. The mean age was 65 years (SD, 6.1) and the male to female ratio was 51 to 31. B-CLL was diagnosed according to generally accepted criteria that included peripheral blood lymphocytosis greater than  $5 \times 10^9/L$  and bone marrow (BM) lymphocytosis greater than 30%.<sup>5</sup> According to the Binet clinical staging system,<sup>6</sup> 50 patients were in stage A, 20 in B and 12 in stage C. BM biopsy was performed in 75 patients. A non-diffuse pattern of BM infiltration could be recognized in 48 and a diffuse one in 27.<sup>7</sup>

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### Immunophenotype analyses

A complete immunological profile which included CD3, CD5, CD19, CD20, CD23, CD11c (Becton Dickinson, Mountain View, CA, USA), FMC7 (Immunotech, Marseille, France),  $\kappa$  and  $\lambda$  chains (Ortho Diagnostic, Raritan, NJ, USA) was obtained in each patient at the time of CLL diagnosis. Fresh peripheral blood (PB) samples were used for immunological analyses. Mononuclear cells were separated by Fycoll-Hypaque density gradient centrifugation and cells were stained using both direct and indirect techniques. A marker was considered positive when it was expressed in over than 30% of the cells analyzed. The stained cells were analyzed on an ABSOLUTE flow cytometry (Ortho Diagnostic System, Raritan, NJ, USA). Controls were provided by cells incubated with an adequately titrated mouse Ig of the isotype identical to that of the MoAb to be tested. In all cases the predominant cell population shared B-cell markers (i.e., CD19, CD20) and CD5 antigen. B-cells were monoclonal with regard to expression of either  $\kappa$  or  $\lambda$  light chain (LC) surface immunoglobulins. Immunological studies made it possible to diagnose immunologically typical CLL (i.e., CD5<sup>+</sup>, CD23<sup>+</sup> and weak expression of LC immunoglobulins) in 72 out of 82 (87.8%) cases while 10 patients lacked CD23 expression.<sup>8</sup>

Twenty-two B-CLL patients and 5 normal controls were analyzed for CD27 (PharMingen, San Diego, CA, USA) cellular expression. In all experiments a double staining immunofluorescence was used (i.e., CD27-FITC/CD19-PE or CD27FITC/CD3-PE). A quantitative immunofluorescence indirect assay (QIFI kit, DAKO, Copenhagen, Denmark) was employed for assessing the amount of CD27 in either B-CLL or normal cells (B and T lymphocytes).

### Determination of soluble CD27 and other assays

All serum samples analyzed were taken at diagnosis of CLL and stored at  $-70^{\circ}$ . Due to the well-known effect of renal failure on sCD27 levels, all patients included had serum creatinine levels higher than 100  $\mu\text{mol/L}$ . Quantitative determinations of sCD27 levels were carried out by means of a Compact soluble CD27 enzyme-linked immunosorbent assay (ELISA) kit (CLB, Amsterdam, The Netherlands). The detection limit was 2 U/mL.

Sera previously analyzed for sCD27 were selected for determination of TNF- $\alpha$ , sAPO1/Fas, sCD23,  $\beta_2\text{M}$  and lactate dehydrogenase (LDH). As for TNF- $\alpha$  measurements we used a solid phase enzyme amplified sensitivity immunoassay (ESIATM Kit, MEDGENIX TNF- $\alpha$ , BioSource, Belgium) based on an oligoclonal system of blend of MoAbs directed against distinct epitopes of TNF- $\alpha$ . The detection limit of test was estimated to be 3 pg/mL. The inter-assay coefficient of variance (CV) ranged between 8% and 9.9% and intra-assay CV between 3.7% and 5.2%.

Serum levels of sAPO-1/Fas were measured in 39

out of 82 (47.5%) patients by means of a commercial ELISA assay (Bender, MedSystems, Vienna, Austria). The lower limit of detection of the assay was 0.2 U/mL. Mean value of healthy controls was  $3.2 \pm 5.2$  U/mL; values of sAPO-1/Fas corresponding to the mean of normal donors  $\pm 2$  SD (13.8 U/mL) were considered elevated. Finally, sCD23,  $\beta_2\text{M}$  and LDH were determined according to previously reported methods.<sup>10,11</sup>

### Statistical analyses

Results were analyzed with the statistical program GraphPAD Software V 2.00 (GraphPAD Software Inc, San Diego, CA, USA). Non parametric tests (Mann-Whitney test, Kruskal-Wallis test) were used to evaluate differences in the median among various groups. Calculation of Pearson's correlation coefficient was performed when comparing two continuous variables.

## Results

### mCD27 expression on normal and B-CLL leukemic cells

Membrane CD27 (mCD27) antigen was constitutively expressed by leukemic cells from all 22 CD5<sup>+</sup> B-CLL patients tested. In all instances a moderate to strong amount of mCD27 was detected; mean value expressed as ABC accounted for  $98.2 \pm 26.8 \times 10^3$  molecules/cell (range,  $70-138 \times 10^3$  molecules/cell). A representative example of coexpression of CD19 and CD27 on either normal or leukemic B-CLL cells is presented in Figure 1. To have a basis for quantitative comparison of mCD27 antigen density, results obtained on either normal or malignant B cells were compared with those observed on normal peripheral blood (PB) T cells. As shown, the amount of CD27 membrane-bound, expressed as mean value of ABC molecules/cell, was similar on B (normal and B-CLL) and on T cells (Table 1).

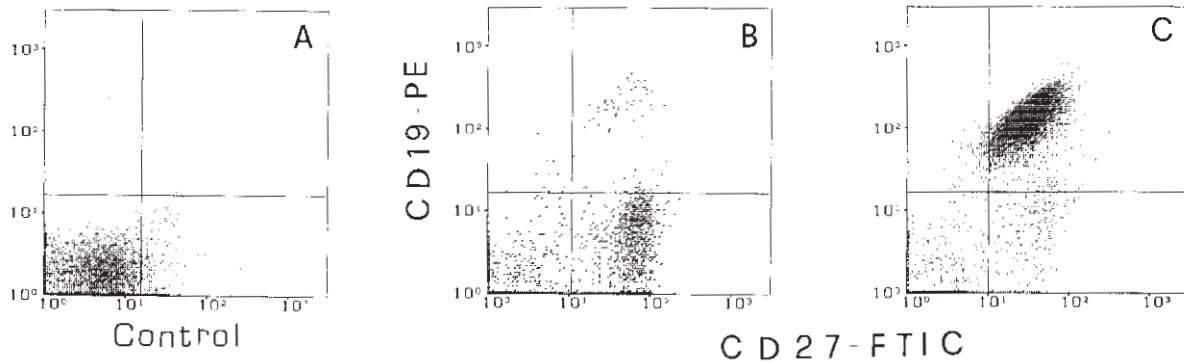
### Serum levels of soluble CD27 in patients with B-CLL

In B-cell CLL patients serum levels of sCD27 were significantly higher (2150 U/mL; range 169-17640) in comparison to those of an age-matched healthy control population (220 U/mL; range, 146-366; p

**Table 1.** mCD27 expression on normal and B-CLL peripheral blood cells.

Cell samples	N. pts.	Positive cases	ABC ( $10^3$ ) molecules/cell
B-CLL	22	22/22	$98.2 \pm 26.8$
Normal T-cells	5	5/5	$81.5 \pm 3.2$
Normal B-cells	5	5/5	$94.7 \pm 13.6$

ABC: antibody binding capacity.



**Figure 1. Coexpression of CD19 and CD27 on normal peripheral blood lymphocytes (B) and B-CLL leukemic cells (C). Isotype controls are also provided (A).**

< 0.0001). Very high levels of sCD27 (i.e., > 5000 U/mL; range, 5,115-17,640) were found in 14 out of 82 (17%) patients. Furthermore, in 77 out of 82 (93.9%) patients sCD27 was above the mean  $\pm$  3SD (477.3 U/mL) of the control group.

Increased levels of sCD27 reflected clinico-hematological features representative of tumor mass such as Binet clinical stage ( $p = 0.009$ ) and absolute PB lymphocytosis ( $r = 0.428$ ;  $p < 0.001$ ) (Table 2). In contrast, only a trend toward a higher sCD27 value could be found in patients with diffuse BM histology ( $p = 0.06$ ). As far as markers claimed to be associated with clinical stages are concerned, we chose to correlate sCD27 with the followings:  $\beta_2$ M, sCD23 and LDH. The first two are suitable for assessing tumor burden, the third is a marker of cell death. Each of these serological markers correlated with sCD27: \* $\beta_2$ M,  $r = 0.508$ ;  $p < 0.001$  (Figure 2); sCD23 = 0.652,  $p = 0.0001$ ; LDH,  $r = 0.446$ ;  $p < 0.001$ .\*

In three patients we had the opportunity to study sCD27 levels longitudinally. A decrease of sCD27 levels (from 1553 U/mL to 400 U/mL) could be observed in a patient considered in partial remission (PR) after 6 monthly courses of intermittent chlorambucil and prednisone. In contrast, the increase of sCD27 (from 577 U/mL to 1360 U/mL) paralleled disease-progression (i.e., change from stage A to C) registered after a 20-month follow-up period. Finally, relatively stable levels of sCD27 (1100 U/mL and 910 U/mL, respectively) were in keeping with a non-progressive disease-status observed over a 12 month-follow-up period in a patient.

#### **sCD27 and other serum markers of NGFr superfamily**

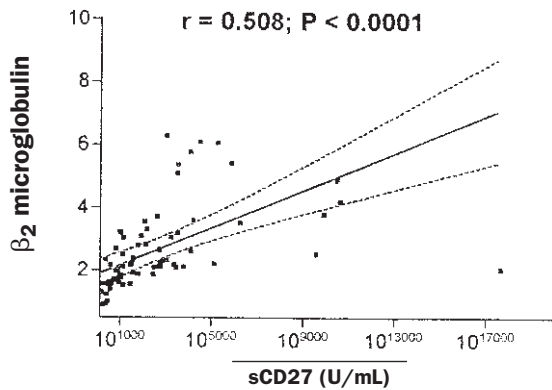
In addition to sCD27 we measured serum levels of soluble TNF- $\alpha$  (sTNF- $\alpha$ ) and soluble Apo-1/Fas (sAPO-1/Fas), two molecules belonging like sCD27 to NGFr superfamily. sTNF- $\alpha$  levels of B-CLL patients

were significantly higher (67 pg/mL; range, 12-863) in comparison to those of healthy controls (11 pg/mL; range, 9-25;  $p < 0.0001$ ). Although such an increase did not parallel neither clinical stages ( $p = 0.136$ ) nor BM histology ( $p = 0.623$ ) (Table 2), a linear correlation with serum levels of sCD27 was found ( $r = 0.525$ ,  $p < 0.001$ ). Serum concentrations of sAPO-1/Fas were available only in 39 patients. Among them, 20 (51.2%) had serum levels of sAPO-1/Fas below the detection limit, 11 (36.6%) showed detectable but not increased levels, while 8 (26.6%) patients had serum levels of sAPO-1/Fas above the mean  $\pm$  2SD (sAPO1/Fas = 13.8 U/mL) of the control group. Serum levels of sAPO1/Fas did not correlate with those of either sCD27 ( $r = -0.105$ ;  $p = \text{NS}$ ) nor sTNF- $\alpha$  ( $r = -0.234$ ;  $p = \text{NS}$ ) therefore suggesting that measuring sAPO-1/Fas levels is of limited clinical value in B-CLL.

**Table 2. Correlation of sCD27 levels and sTNF- $\alpha$  with clinical features.**

Clinical features	N. pts.	sCD27(U/mL), median (range)	sTNF- $\alpha$ (pg/mL) median (range)
<b>Clinical stages</b>			
A	50 (60.9%)	1440 (169-17,640)	52 (14-863)
B	20 (24.3%)	3034 (800- 6,003)	70 (24-297)
C	12 (14.6%)	3522 (788-17,290)	88.5 (18-476)
		* $p = 0.009$	* $p = 0.136$
<b>BM histology</b>			
Non-diffuse	48 (64%)	1203 (220-9570)	67 (15-867)
Diffuse	27 (36%)	2572(169-10161)	70 (15-488)
		$^{\circ}p = 0.06$	$^{\circ}p = 0.623$

\*Kruskal-Wallis test;  $^{\circ}$ Mann-Whitney test.



**Figure 2. Correlation between serum levels of sCD27 and  $\beta_2$ -microglobulin. Regression analysis for a linear function yields a correlation coefficient of  $r = 0.508$ ;  $p < 0.0001$ .**

## Discussion

Two interesting observations characterize results of the present study, the first one concerns the expression of mCD27 on malignant B-CLL cells the second the presence of significant amount of soluble CD27 levels which can be used as a marker for tumor burden. As far as mCD27 expression is concerned, our results confirm that such a molecule can be detected on either T or normal B PB lymphocytes.<sup>3</sup> Therefore mCD27 becomes the second TNF family member, in addition to the CD30 ligand, that can be expressed on both T and B cells.<sup>12</sup> Furthermore, the amount of mCD27 was assessed using a quantitative indirect assay which makes it possible to evaluate antigen density as ABC molecules/cell.<sup>9,13</sup> In our hands this method failed to demonstrate differences in the density of mCD27 between B-CLL and normal PB B or T cells.

Our findings indicate that in patients with B-CLL high serum levels of sCD27 correlate with a number of unfavorable prognostic features such as high lymphocyte count, advanced disease stage and  $\beta_2$ M, a reliable indicator of tumor burden.<sup>14</sup> As recently shown by Kato and Kipps,<sup>15</sup> sCD27 can be released by purified non-stimulated B-CLL cells. Although the small number of patients considered for the analysis makes results concerning longitudinal sCD27 evaluation of questionable value, sCD27 might represent a promising indicator of disease status because its serum levels vary according to the clinical behavior of the disease.<sup>3</sup>

Another issue we addressed in the present study concerns the correlation between levels of sCD27 and those of other soluble molecules belonging like CD27 to the NGFr superfamily (i.e., TNF- $\alpha$ , APO-1/Fas). Foa *et al.*<sup>16</sup> found raised levels of sTNF- $\alpha$  in the serum of 20 of 24 patients with B-CLL or hairy cell leukemia

(HCL) thus demonstrating that B-CLL cells are a putative source of such a molecule. Our findings showing a correlation between serum levels of sCD27 and those of sTNF- $\alpha$  may further contribute to expand available information on clinical implications of increased serum levels of NGFr superfamily molecules.

Expression of cell-surface protein APO-1/Fas is virtually absent on B-CLL cells, this is in keeping with low amount of sAPO-1/Fas we detected in the serum of B-CLL patients.<sup>17</sup> Nonetheless, about 20% of B-CLL patients had increased levels of sAPO-1/Fas, thus lending support to the idea that increased levels of such a molecule may reflect the T-cell activation status secondary to neoplasia.<sup>18,19</sup> On the other hand, conditions for production of sAPO-1/Fas by neoplastic or accessory cells need to be characterized in order to understand clinico-prognostic implications, if any, of such a finding.<sup>20</sup>

In conclusion, our findings suggest that sCD27 may be a reliable tumor marker in B-CLL. However, its prognostic value<sup>21</sup> should be evaluated in large prospective studies.

## Contributions and Acknowledgments

SM was the main investigator and designed the study; he wrote the paper. GC and AD carried out flow cytometric studies. DL and MD followed up the patients clinically. GV was responsible of immunoenzymatic studies. RM, PM and GMG collaborated in the analysis of data.

Criteria for the order in which authors appear mainly affect the first two and the last two authors, respectively. Indeed, the first two authors (SM and GV) were responsible of the design of study and immunoenzymatic analyses. The last two authors, less directly involved in the study, have had an important role in the analysis of data and in the revision of manuscript. No specific order was followed for authors in between.

## Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

## Manuscript processing

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