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Expression of the mitochondrial bcl-2 oncoprotein and CD 95 on lymphocytes in B chronic lymphocytic leukaemia

Ekspresja bcl-2 mitochondrialnej onkoproteiny i powierzchniowego antygenu CD 95 na B komórkach przewlekłej białaczki limfatycznej

INTRODUCTION

Apoptosis and necrosis are two distinct modes of cell death. Apoptosis, also referred to as "cell suicide" or "programmed cell death", is an active mode of cell death. A complex, multistep mechanism regulates the cell propensity to respond to an environmental or intrinsic signal by apoptosis. The control of cell survival is of central importance in tissues with high cell turnover such as the lymphoid system, and its disruption may be a critical step in tumorigenesis. Genes homologous to bcl-2, play a key role in regulating physiologic cell death (apoptosis). The protein encoded by the bcl-2 (B cell lymphoma/leukemia-2) proto-oncogene has been implicated in the regulation of lymphocyte cell survival and has been shown to interfere with apoptosis. The bcl-2 gene product regulates programmed cell death and a number of experiments suggest that bcl-2 is involved in the selection and maintenance of long-lived memory B cells rescuing them from apoptotic death and leading to their accumulation in the G_0 phase of the cell cycle (6). The bcl-2 proteins enhance the survival of lymphocytes and other cell types but do not promote their proliferation (2). Bcl-2 is not essential for lymphoid development. However, upregulation of bcl-2 appears to be the normal mechanism for positive selection of developing lymphocytes, and its continued expression is critical for survival of mature peripheral B and T cells. In B chronic lymphocytic leukaemia (B-CLL) the leukaemic cells are in the G_0 G_1 phase of the cell cycle and have a very long half-life *in vitro* (4). Freshly isolated (B-CLL) cells express high levels of the bcl-2 protein which may contribute to their extended survival *in vivo*. In contrast to the bcl-2 protein which is able to inhibit apoptic cell death in variety of *in vitro* and *in vivo* experimental systems, the CD 95 (the FAS/APO-1) antigen is able to induce apoptosis.

The CD 95 receptor belongs to the tumor necrosis factor/nerve growth factor receptor superfamily and induces apoptotic cell death upon binding of the natural ligand or agonistic antibodies. CD 95 is expressed on the surface of many transformed cell lines and chronically stimulated T cells (3). CD 95 can mediate apoptosis following ligation with either antibodies or recombinant CD95L (FASL). CD95 can also transduce stimulatory signals to certain B cells and to freshly isolated human T cells and thymocytes. The CD95 antigen is widely expressed on normal tissues and CD95-mediated killing is frequently found on cultured cell lines but not on primary cells. These findings suggest that bcl-2 expression prevents the apoptosis of lymphoid cells induced by the Fas antigen-dependent mechanism and that apoptosis of lymphocytes is exquisitely controlled, at least in part, by regulation of the bcl-2 and Fas genes (8, 5).

MATERIALS AND METHODS

Peripheral blood samples were obtained at diagnosis from 14 patients with B-CLL. The cell surface antigens and bcl-2 mitochondrial oncoprotein in each case were determined on fresh cells at the time of sample submission. Mononuclear cells were isolated by density centrifugation on Lymphoprep (Nycomed, Norway) and washed twice in phosphate buffered saline (PBS) containing 1% bovine serum albumin. Double color immunofluorescence studies were performed using combinations of phycoerythrin (PE) and fluorescein isothyocyanate (FITC) conjugated monoclonal antibodies. Monoclonal antibodies were obtained from Ortho Diagnostic Systems (Germany), Becton Dickinson (Germany), or Dako (Denmark). The following antibody combinations were used: FITC IgG1/IgG2a PE negative control antibody, CD 3 PE, CD 95. Goat anti-mouse Ig FITC was used as a secondary antibody. 10^6 cells were incubated with antibodies for 30 min at 4°C and washed twice with PBS afterwards. For the immunodetection of the intracellular bcl-2 protein 10^6 cells were fixed and permeabilized with 0.25% paraformaldehyde (15 min at room temperature) and cold (4°C) 70% methanol for 60 min at 4°C before incubation with FITC conjugated bcl-2 mouse monoclonal antibody or IgG1 FITC negative control (1). All samples were measured on a Cytoron flow cytometer (Ortho Diagnostic Systems). 10,000 cells were analysed per test. In order to quantitate the levels of fluorescence, the mean fluorescence intensity and fluorescence signal strength of the bcl-2^+ and CD 95^+ cells were calculated. The mean fluorescence signal strength of bcl-2 and CD 95 histogram were measured from the upper limit of the negative control.

RESULTS

Peripheral blood cells from 14 patients with CLL (in st. 1 or 2 Raji) were studied for bcl-2 and CD 95 expression. All patients were not treated. All patients expressed significant levels of bcl-2 protein, 99.3% of cells expressed the bcl-2 protein. Mean fluorescence intensity of bcl-2 was 108.91± 9.02 (min. 114.10 – max. 142.90). On the other hand, intra-cellular bcl-2 proteins measured as mean fluorescence intensity were significantly diminished in the $CD3^+$ T cells (109.31± 12.56) compared with the $CD\ 19^+$ leukaemic B cells (124.55± 8.69) (Fig. 1 and 2). The results have shown that the bcl-2 protein is strongly expressed in human leukaemic CLL cells

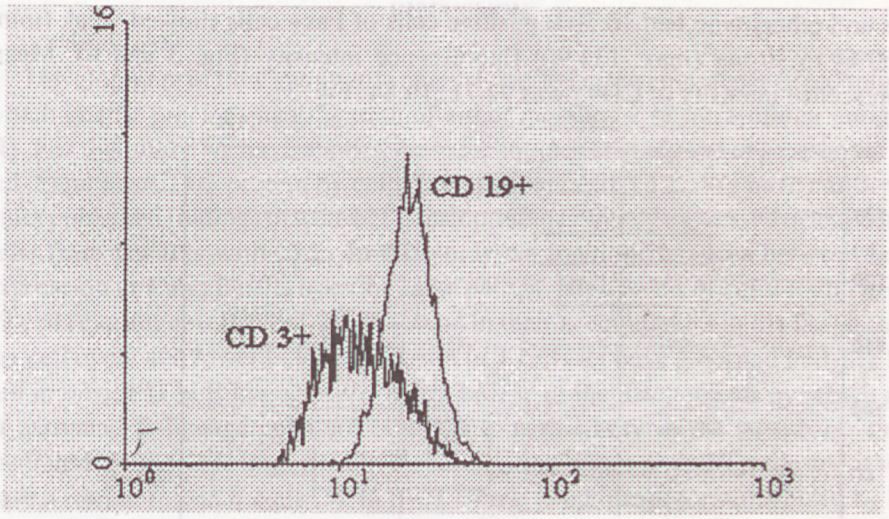

Fig. 1. Expression of bcl-2 on CD 3+ and CD 19+ CLL cells

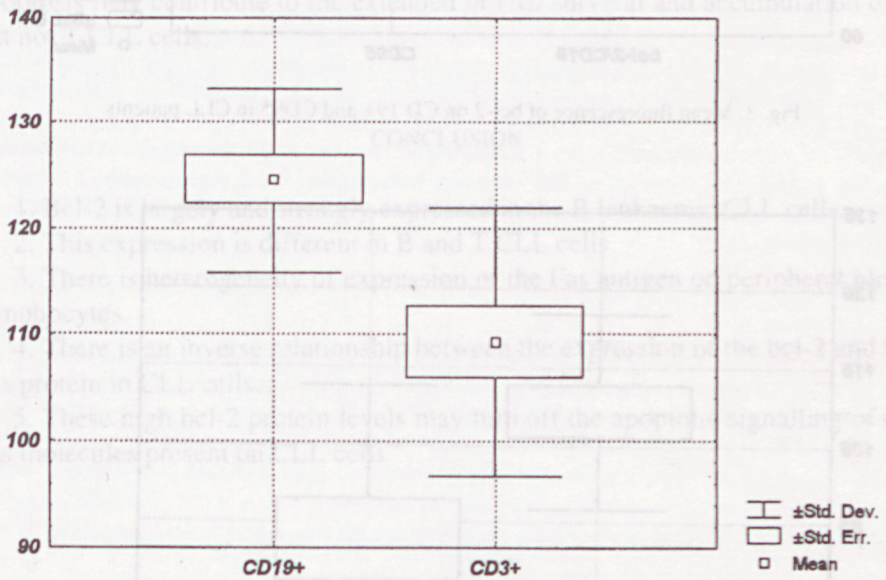

Fig. 2. Mean fluorescence of bcl-2 on CD 3+ and CD 19+ in CLL patients

at the earliest stages of disease. bcl2+ cells include both CD 19 immature B leukaemic cells and more differentiated subpopulation of normal T cells. Fas was expressed on 82% of cells. There is heterogeneity of expression of Fas antigen on peripheral blood lymphocytes, with some CLL patients expressing 98.40% positive

cells and others who had 16.30% positive cells of Fas+ cells showed weak (min. 68.60%) to strong (max. 134.90) fluorescence intensity (Fig. 3 and 4). Mean fluorescence intensity of CD95 was 92.71±19.84 (Fig. 3).

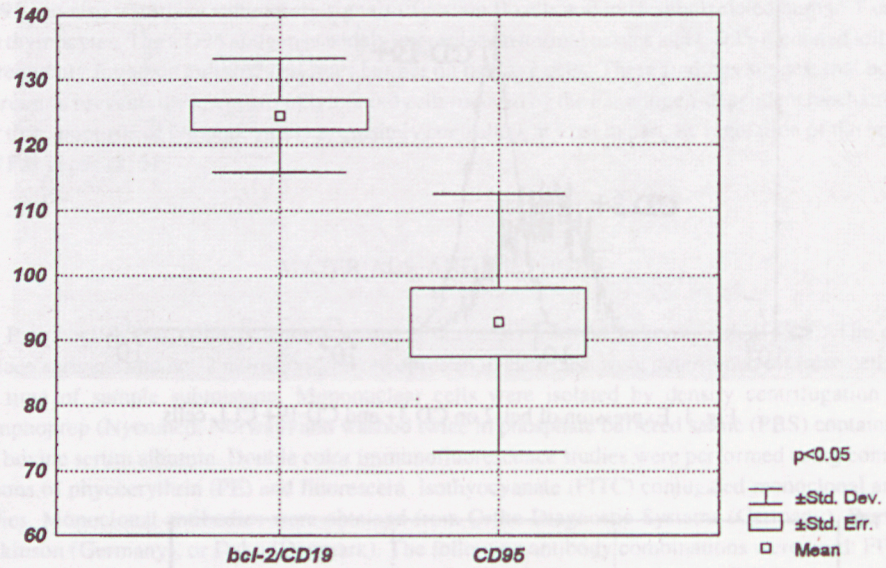

Fig. 3. Mean fluorescence of bcl-2 on CD 19+ and CD95 in CLL patients

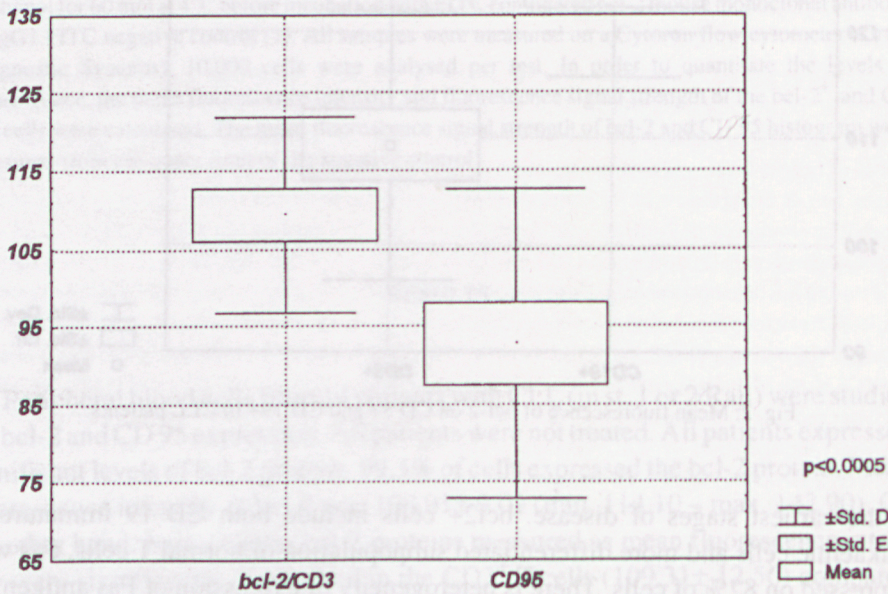

Fig. 4. Mean fluorescence of bcl-2 on CD 3+ and CD95 in CLL patients

DISCUSSION

Development in flow cytometric analysis has led to the emergence of techniques which enable precise quantification of either membrane or intracellular antigens (1). For this study we have applied these techniques to study the expression of the bcl-2 and Fas/CD95 protein by cells from patients with CLL. We have confirmed other published findings that the bcl-2 oncoprotein is widely expressed in leukaemic cells from patients with CLL. In this study we found differences between bcl-2 expression in T and B cells from the same patient. High levels of bcl-2 found in CD 19 cells can suggest that bcl-2 is involved in their accumulation in the G_0 phase of the cell cycle and lower bcl-2 expression in T cells let have a normal half-life this cell *in vivo*. Fas in B lineage cells might be one of the mechanisms by which part of activated B-lineage cells are eliminated, particularly in the periphery. Fas - mediated apoptosis is important in clonal deletion of peripheral T cells and it may have a similar role in B cells (3). In B-CLL cells apoptotic process mediated by Fas can be influenced by anti-apoptotic mechanism, such as bcl-2 (5). It is in contrast normal T cell from patients with CLL where Fas can induce a signal for cell death (7). The combination of high bcl-2 protein levels and resistance to Fas-mediated apoptosis may contribute to the extended *in vivo* survival and accumulation of B but not T CLL cells.

CONCLUSION

1. Bcl-2 is largely and strongly expressed in the B leukaemic CLL cells.
2. This expression is different in B and T CLL cells.
3. There is heterogeneity of expression of the Fas antigen on peripheral blood lymphocytes.
4. There is an inverse relationship between the expression of the bcl-2 and the Fas protein in CLL cells.
5. These high bcl-2 protein levels may turn off the apoptotic signalling of the Fas molecules present on CLL cells.

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STRESZCZENIE

Apoptoza i nerkoza to dwa różne modele śmierci komórkowej. Pierwsza z nich, nazwana również programowaną śmiercią komórek, pełni kluczową rolę między innymi w procesie embriogenezy, dojrzewania limfocytów oraz onkogenezy. Celem naszych badań było określenie roli antygenu CD (APO-1/FAS), należącego do rodziny receptorów czynnika martwicy nowotworów (TNF)–aktywacja, który może prowadzić do apoptozy oraz czynnika przed nią chroniącego – onkoproteiny bcl-2 u chorych na przewlekłą białaczkę limfatyczną B komórkową (B-CLL). Przebadaliśmy grupę 14 świeżo rozpoznanych, nieleczonych przypadków B-CLL stwierdzając podwyższony, zwłaszcza w komórkach CD 19^+, poziom onkoproteiny bcl-2. Korelował on ujemnie z ekspresją antygenu CD 95. Naszym zdaniem zaburzenia równowagi między ekspresją onkoproteiny bcl-2 oraz receptora FAS pełnią kluczową rolę w onkogenezie komórek białaczkowych w przebiegu przewlekłej białaczki limfatycznej B komórkowej.