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Resistance to CD95-mediated apoptosis of CD40-activated chronic lymphocytic leukemia B cells is not related to lack of DISC molecules expression

Daniela de Totero*[,1], Mariapina Montera[2], Ombretta Rosso[1], Marino Clavio[2], Enrico Balleari[2], Robin Foa[3] and Marco Gobbi[2]

[1]*Immunopharmacology Lab, National Institute for Cancer Research, Genoa, Italy;* [2]*Chair of Hematology and Oncology, Department of Internal Medicine, University of Genoa, Italy;* [3]*Department of Cellular Biotechnologies and Hematology, 'La Sapienza' University, Rome, Italy*

In B-cell chronic lymphocytic leukemia (CLL), accumulation of neoplastic B cells may be the result of dysregulated apoptosis. One of the major molecules triggering apoptosis, CD95 (FAS), is not expressed on CLL B cells at resting conditions. However, CD40 triggering of CLL B cells upregulates receptors belonging to the tumor necrosis factor (TNF) superfamily, like CD95. In the present study, we analyzed in B cells from 20 CLL patients the effect of CD40/CD40L interaction on: (i) CD95 modulation; (ii) CD95-mediated apoptosis and (iii) mRNA and protein expression of the death-inducing signaling complex (DISC) molecules.

CD40 activation of CLL B cells was carried out by coculture with CD40L-transfected cells and cytofluorimetric analyses were performed to study CD95 modulation and apoptosis induction by an anti-CD95 moAb. Despite strong CD95 upregulation on the membrane of all the cases studied, only a minority of cases analyzed (3/20) proved weakly responsive to CD95-mediated apoptosis. Multiplex RT-PCR was used to analyze FLICE, FAS, FADD and TRADD mRNAs before and after CD40 triggering. In agreement with the cytofluorimetric data, FAS mRNA appeared significantly increased after CD40 triggering; the other molecules involved in DISC formation and in CD95-mediated apoptosis were also expressed without relevant differences between resting and activated conditions. Western blot analyses further confirmed FLICE and FADD protein expression by resting and activated CLL cells.

Our findings demonstrate that, following CD40 triggering, CLL B cells are resistant to CD95-mediated apoptosis despite a strong CD95 upregulation on the membrane and a normal mRNA or protein expression of the DISC components.

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the abnormal expansion of CD5+ mature B lymphocytes.[1] Defects in the apoptosis-inducing pathways can eventually lead to the accumulation of neoplastic B cells. In CLL, many factors have been suggested to contribute to prolong B cell survival, like Bcl-2 overexpression[2,3] or enhanced production of anti-apoptotic cytokines, such as IL4 and IFNγ[4,5] by residual T cells[6] or IL8 by the neoplastic cells themselves.[7] Moreover, the CD95 (FAS) antigen, a 45-kDa membrane protein belonging to the tumor necrosis factor receptor (TNFR) superfamily,[8–10] mainly involved in apoptosis induction, is not expressed on leukemic CLL cells under resting conditions,[11] suggesting that a reduced FAS pathway utilization might contribute to the relentless accumulation of malignant cells. The interaction between CD95 and its ligand, CD95L (FASL), mediates deletion of activated T cells in the periphery and elimination of virally infected or malignant cells by cytotoxic T lymphocytes (CTL).[12] Furthermore, CD95 plays a key role in positive and negative selection of B cells during development, and the expression of functional CD95 on premalignant or malignant cells may potentially provide a mechanism of tumor suppression. Triggering of apoptosis by CD95 ligation results in the recruiment of the adapter molecule Fas-associated death domain protein (FADD) and

*Correspondence: Daniela de Totero, c/o Immunopharmacology Lab, National Institute for Cancer Research, Largo Rosanna Benzi n. 10, 16132 Genoa, Italy; Tel.: 39-0105737373
fax: 39-0105737374
E-mail: detotero@yahoo.com
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procaspase-8 (FLICE) to form the death-inducing signaling complex (DISC).[13] The formation of DISC leads to the release of activated caspase-8, cleavage of caspase-3 or amplification of the apoptotic signal via mitochondria[14] and, finally, to apoptosis.

Another member of the TNFR superfamily, the CD40 antigen, expressed on all B cells, is principally involved in regulating B-cell activation and differentiation following interaction with its ligand (CD40L).[15,16] Defective CD40L (CD154) expression can result in severe immunodeficiency and individuals with a genetic defect in the X-linked gene encoding CD154 show recurrent bacterial infections and demonstrate deficiency in antigen-specific secondary antibody responses.[17–20] Similar defects are also present in CLL and the observed CD40L downmodulation on activated T cells[21] may account for the immune defects often recorded in these patients. However, CD40 triggering of CLL B cells, which induces upregulation of CD80/CD86[22] and of CD95,[23–25] renders these cells competent as antigen presenting cells capable of activating allogeneic CTL.[26] Moreover, CLL B cells transduced with CD40L gene by a replication-defective adenovirus vector (Ad-CD40L) are highly effective in stimulating an *in vitro*, and more importantly, an *in vivo* autologous T-cell response leading to significant reductions in leukemic cell counts and lymph node size.[27] It is still unclear whether apoptosis of neoplastic B cells by CTL is driven by CD95/CD95L B/T interaction or whether it involves other factors and receptors. Studies concerning CD95-mediated apoptosis in CD40-activated CLL B cells have, in fact, given conflicting results. Kitada *et al*.[25] failed to observe apoptosis when an agonistic anti-CD95 moAb was used to trigger CD95 receptor on activated cells. Williams *et al*.,[28] instead, reported that CD95L+ T cells are capable of inducing apoptosis of autologous malignant B cells in CLL after their preventive culture with type I cytokines (IL12 and IFNα), which upregulate the expression of the CD95 molecule.

In the present study we determined, in 20 CLL cases, CD95 expression and sensitivity to CD95-mediated apoptosis of leukemic B cells before and after CD40 triggering. We further analyzed, in resting and in activated cells, mRNA and protein expression of molecules that compose the DISC complex (FAS, FASL, FLICE, FADD and TRADD), which participate mainly in the CD95 apoptotic pathway.

Materials and methods

Patients' samples

Peripheral blood samples from 20 patients fulfilling the clinical, morphologic and immunophenotypic criteria of CLL were collected at the Hematology Department, University of Genoa and blood samples obtained after informed consent. Seven patients were males and 13 females, with a mean age of 71 years (range 55–88). According to Rai's staging system,[29] 6 patients were in stage 0, 10 in stage I, 3 in stage II and 1 in stage III. The median white blood count was 30×10^9/l (range: 7–300×10^9/l), with a median lymphocyte count of 21×10^9/l (range: 5–216×10^9/l). At the time of the study, 14 patients had never been treated, while the remaining 6 had received only chlorambucil therapy and had been off treatment for at least 3 months.

B-lymphocytes enrichment

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation by lymphoprep (ICN, Costa Mesa, CA, USA), washed twice with RPMI 1640 medium (Biowhittaker Europe, Verviers, Belgium) and resuspended in the same medium containing 10% fetal calf serum (FCS) (Euroclone, UK), L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel) and penicillin–streptomycin (Mascia-Brunelli, Milano, Italy). B cells were purified by negative selection with antibody-coated magnetic beads, when residual non-B cells exceeded 10%. Briefly, after 1 h of incubation at 37°C on plastic to remove monocytes, non-adherent cells were incubated for 30 min at 4°C with magnetic polystyrene anti-CD2 immunobeads (Dynal, Oslo, Norway) at a bead/cell ratio of 4:1 in phosphate-buffered saline (PBS) (Euroclone) with 5% FCS (Euroclone). Rosette-forming CD2+ cells (T and NK cells) were removed using a cobalt–samarium magnet and the purity of B cells obtained was checked by staining with fluorescein isothiocyanate conjugated (FITC) CD19, CD2 and CD3 monoclonal antibodies (moAbs) (Coulter-Immunotech, Miami, FL, USA) and flow cytofluorimetric analysis (EPICS XL, Coulter).

Immunofluorescence analysis of cell surface antigen expression

Flow cytometric analyses of total CLL cells and of B-cell enriched fractions were performed by two-color immunofluorescence, as previously described.[30] A panel of moAbs was used to study the phenotype of CLL B cells both at resting conditions and after coculture with CD40L transfectant cells: FITC-CD19 (Coulter-Immunotech), -CD20, -CD22 (Becton Dickinson, San Jose', CA, USA), -CD40, -CD95, (Pharmingen, San Diego, CA, USA), moAbs and phycoeritrin conjugated (PE) -CD19, (Coulter-Immunotech), -CD40L (Pharmingen), -CD5, -CD23, (Caltag, Burlingame, CA, USA) moAbs. FITC and PE goat anti-mouse IgG1, IgG2a, IgG2b (Southern Biotechnology, Birmingham, AL, USA) were also included as negative controls. Briefly, 1×10^5 cells were incubated with FITC- or PE-moAbs for 30 min at 4°C. After two washes in PBS (Euroclone) with 2% of FCS (Euroclone), cells were resuspended in washing solution and analyzed by flow cytometry.

Cell culture and apoptosis assays

Fresh purified CLL B cells were cultured in RPMI1640 (Biowhittaker) with 10% FCS (Euroclone) and antibiotics in 24-well plates at a concentration of 1×10^6 cells/ml. The agonistic anti-CD95 moAb (CH11) (500-100 ng/ml) (Coulter-Immunotech) was added at the beginning of the cultures. Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A) (Schering, Berlin, Germany) was used at a concentration of 40 μg/ml to induce apoptosis in CLL B cells as positive control. After 72 h of culture, cells were recovered, washed, treated with Triton (0.1% in PBS) (Sigma Chemicals, St Louis, MO, USA) and stained with DNA prepstain (containing RNAse and propidium iodide (PI) (Coulter) to analyze cell cycle and to determine the percentage of apoptotic cells. Analysis was performed with a dedicated software of the flow cytometer (EPICS XL, Coulter). Early apoptosis induction was also determined by AnnexinV-FITC and PI (MBL, Nakaku, Nagoya, JP) double staining, following the manufacturer's instructions. Briefly, 1×10^6 cells, anti-CD95-treated or untreated, resting or activated, were washed, resuspended in binding buffer 1X and stained with 1 μl of Annexin V-FITC. After 10 min incubation, 1 μl of PI was added and samples were incubated for 5 min.

Coculture of CLL cells with CD40L-transfected cells

Murine L cells stably transfected with the human CD40L (CD154) molecule[31] (a kind gift from Dr R Lemoli, 'Seragnoli' Institute of Hematology and Medical Oncology, University of Bologna, Italy) were cultured in flasks until confluency and used to activate CLL B cells. The 72 h cocultures of CLL B cells with CD40L-transfected cells were set up in 24-well plates at a concentration of 1:100 of transfectants and B cells, respectively. After this incubation time, CLL B cells were collected, washed, resuspended in culture-medium, counted, checked for viability and stained with specific moAbs to determine the upregulation of the CD95 antigen. CD40-activated cells were also recultured for further 72 h in medium alone as controls, or with the anti-CD95 moAb (CH11) (Coulter) in a 24-well plate at a concentration of 1×10^6 cells per well. In order to determine apoptosis of preactivated CLL B cells treated with anti-CD95, cells from each well were then recovered, washed and stained with PI, Annexin V-FITC/PI (MBL), as described above.

Immunofluorescence analysis of Bcl-2 on resting and CD40-activated CLL cells

Bcl-2 expression by resting and CD40-activated CLL B cells was analyzed by flow-cytometry, by staining permeabilized cells with FITC anti-Bcl-2 moAb (Pharmingen). Briefly, resting or activated CLL B cells were dispensed at a concentration of 5×10^5/sample and washed in PBS + 2% FCS. Cells were first stained with PE-CD19, FITC-CD19 or negative controls and incubated at 4°C for 30 min. After two washes with PBS + 2% FCS, cells were fixed and permeabilized with cytofix/cytoperm (Pharmingen) for 20 min at 4°C. Following one wash with Perm/Wash buffer (Pharmingen), cells were stained with FITC-anti-Bcl-2 moAb or a FITC IgG moAb (Pharmingen) as negative control. After 30 min of incubation, samples were washed with Perm/Wash, resuspended in PBS + 2% FCS and analyzed using a Coulter cytofluorimeter.

Intracytoplasmic staining of the FADD molecule in resting and in CD40-activated CLL cells

The expression of the FADD molecule in resting and in CD40-activated CLL cells was studied by intracytoplasmic staining with a specific anti-human FADD moAb and subsequent cytofluorographic analysis. Briefly resting or activated CLL cells (5×10^5 cells/sample) were first permeabilized and fixed with cytofix/cytoperm (Pharmingen) for 20 min at 4°C at dark. After one wash with Perm/Wash buffer (Pharmingen) cells were stained with anti-human FADD (1TB), anti-human FAS (ZB4) (MBL), anti-CD19 moAbs or OKT3 moAb as negative control (Becton Dickinson) and incubated in ice for 30 min. After this time, cells were washed with Perm/Wash and incubated with a secondary goat anti-mouse IgG FITC moAb (Jackson, West Grove, PA) for 30 min at 4°C. After one washing with Perm/Wash, cells were resuspended in PBS + 2% FCS and finally analyzed using a Coulter cytofluorimeter.

RNA isolation and first strand cDNA synthesis

Total cellular RNA was isolated from 5x10^6 purified B cells both at resting conditions and after 72 h coculture with CD40L-transfected cells using Tripure (Boehringer Mannheim, Germany), according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μg of RNA with the cDNA preamplification system (GIBCO-BRL) using SuperScript II reverse transcriptase (RT) and random hexamers.

Multiplex polymerase chain reaction amplification of FLICE, FAS, FASL, FADD and TRADD

The mRNA expression of FLICE, FAS, FASL, FADD and TRADD was evaluated using the multiplex polymerase chain reaction (MPCR) kit for human apoptosis genes (hAPO4G from MBL). This kit provides a quick method to analyze human FLICE, FAS, FASL, FADD and TRADD gene expression, and normalize their expression against GAPDH expression. In addition to the positive control included in the kit, we also used the Jurkat cell line that expresses all the above-mentioned PCR products with the exception of FASL. PCR reaction samples (50 μl) were obtained according to the manufacturer's instructions with 5 μl of first strand

cDNA template with the following amplification profile: 96°C for 1 min and 60°C for 4 min (2 cycles); 94°C for 1 min and 60° for 90 s (30 cycles). The MPCR products were visualized by gel electrophoresis on a 2.5% agarose gel stained with ethidium bromide. The gel image was acquired by the Chemilmager™ 5500 Imaging System and analyzed by Gel-Pro-Analyze 3.1 software (Alpha Innotech Corporation, San Leandro, CA, USA) to measure directly band intensity by densitometry on the agarose gel. The normalized level of expression of FAS mRNA was given as the ratio (FAS, FADD, TRADD optical density/GAPDH optical density) and standardized results were expressed in arbitrary units. In addition, FAS mRNA expression was compared by Student's *t*-test between CLL B cells under resting or activated conditions.

Western blot analysis of FLICE and FADD expression in resting and in CD40-activated CLL cells

Purified CLL B cells (5×10^6 cells/sample), at resting conditions or after 72 h coculture with CD40L transfected cells, were resuspended in lysis buffer (10 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.02% NaN_3; 1% NP40; 0.1% SDS; 1 mM phenylmethyhlsulfonyl fluoride; 100 U/ml aprotinin) and, after 20 min incubation in ice, lysates were cleared by centrifugation at 12 000*g* for 20 min at 4°C. Protein concentrations of cleared lysates were measured by the Bio-Rad protein assay (Bio-Rad SA, Ivry sur Seine, France). A total of 25 μg of boiled and reduced CLL lysates were loaded on Novex precast 10% polyacrylamide gel (Invitrogen, Groningen, The Netherlands) and separated by running at 4°C in a Xcell *SureLock* Mini-Cell apparatus for 50 min at 200 V, using 1X MES Buffer plus NuPage antioxidant (Invitrogen). Full Range Rainbow (Amersham, Milan, Italy) was run as standard molecular weight marker. The gels were blotted onto a Hybond-C membrane (Amersham) at constant 200 mA for 1 h at 4°C and the blots were saturated overnight at 4°C in 1X Tween–Tris buffered saline (TTBS) containing 10% nonfat dry milk.

For the immunostaining, blots were incubated at room temperature for 1h with mouse anti-human FLICE 5D3 (1 μg/ml) or anti-human FADD 1TB (2 μg/ml) moAbs (MBL) diluted in 1X TTBS containing 5% nonfat dry milk. After washings with 1X TTBS, the blots were stained with peroxidase-conjugated anti-mouse Ig antiserum (DAKO, Glostrup, Denmark) at 1:1500 dilution for 1 h at room temperature.

Bands were revealed by the ECL chemiluminescence system according to the manufacturer's instructions (Amersham).

Statistical analysis

The results are reported as the mean of percentage ± standard deviation (s.d.) of the mean, unless not expressively indicated. The Student's paired *t*-test was used for statistical analysis. Only *P*-values <0.05 were considered as statistically significant.

RESULTS

Upregulation of the CD95 (FAS) receptor following CD40 triggering of CLL cells

After a preliminary phenotypic characterization to confirm CD5, CD23 and CD40 positivity on purified B cells from 20 CLL patients, we studied CD95 (FAS) expression before and after CD40 triggering. CD95, faintly detected in only one (No. 8) of the 20 cases analyzed at resting conditions, was significantly upregulated after 72 h coculture with CD40L-transfected cells in all the samples studied ($N=20$) (Figure 1). The mean percentage of CD95 expression on cells at resting conditions was 3.1 ± 1.94 (mean ± s.d.) and increased to 68.7 ± 17.93 after CD40 activation ($P<0.0001$).

Effect of CD40 triggering on apoptosis induction via CD95 (FAS)

CD95-dependent apoptosis, as determined by PI staining, was never detected when CLL B cells were analyzed at resting conditions, in agreement with the lack of CD95 expression in 19 of the 20 CLL cases studied (Figure 1). Despite the high CD95 upregulation after CD40 triggering, activated CLL B cells still failed to undergo a significant degree of apoptosis in the presence of the agonistic anti-CD95 moAb (CH11) for 72 h. We, in fact, could observe very low levels of apoptosis induction (range: 15–18%) in only three of the 20 cases studied (No. 2, 4, 8), as documented by the representative CLL case (No. 2) illustrated in

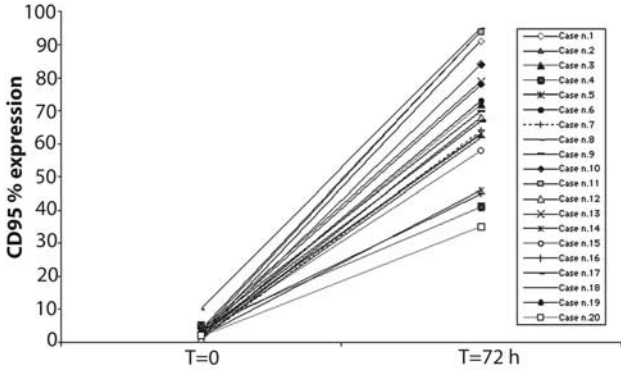

Figure 1 Upregulation of the CD95 (FAS) antigen in CLL B cells after CD40 triggering. CD95 (FAS), negative or very weakly expressed at resting conditions ($T=0$), was strongly upregulated after 72 h of CD40-activation ($T=72$ h) in all cases analyzed ($N=20$), as demonstrated by staining with specific moAb and subsequent cytofluorographic analysis.

Figure 2. The same concentration of moAb (100 ng/ml), however, induced a 68% of apoptosis in Jurkat cells, used as positive control (Figure 2). Moreover, higher concentrations of anti-CD95 moAb (500 ng/ml) failed to increase apoptosis in the three weakly sensitive CLL cases (data not shown).

Externalization of phospatydilserine residues on the membrane, a feature typical of cells in early apoptosis, was further assessed by double Annexin-V-FITC/PI staining and confirmed that, after CD95 triggering, only a small percentage of CD40-activated B cells are in apoptosis (22% in Figure 3a: CD4072h + 72haCD95), as shown for one of the three CLL cases (No. 4) weakly responsive to CD95-mediated apoptosis. We, however, could observe high levels of apoptosis when B cells from the same patient were exposed to fludarabine *in vitro* (69% in Figure 3a: CD4072h + 72hFLU).

In addition, the analysis of Bcl-2 modulation in patient No.4, carried out through intracytoplasmic staining with a specific anti-Bcl-2 moAb, confirmed previous data of a moderate apoptosis induction of CLL B cells to CD95 triggering. As shown in Figure 3b, Bcl-2, already downmodulated on all the B cells following CD40 activation, proved further decreased after 72 h culture *in vitro* with medium alone (CD4072h + 72hCTR), and displayed a bimodal peak of fluorescence suggesting that a small percentage of cells (11%) with a lower mean fluorescence intensity has undergone spontaneous apoptosis. Moreover, the percentage of cells with a lower Bcl-2 expression increased further after treatment with the anti-CD95 moAb (CD4072h + 72haCD95 = 34% MFI = 5), thus indicating that in this case CD95 triggered into apoptosis only a minority of cells. However, fludarabine, used as control, significantly downregulated Bcl-2 in all cells (CD4072h + 72hFLU; MFI = 3). A double Bcl-2/CD19 staining of CD40-activated cells allowed us to further demonstrate that all cells analyzed were CD19 +, thus excluding that the low percentage of apoptotic cells detected could be represented by residual T cells highly expressing CD95 (Figure 3b).

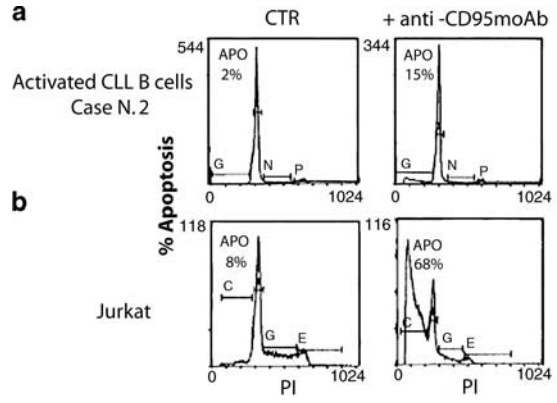

Figure 2 Apoptosis induction by the agonistic anti-CD95 moAb in 1 of 3 CD95-weakly sensitive CLL cases. In CD40-activated CLL B cells, the agonistic anti-CD95 moAb induced weak apoptosis in only a minority of cases studied as shown hereby in one representative case (No. 2). On the contrary, the Jurkat cell line was strongly sensitive to the anti-CD95 moAb CH11, and was used here as a positive control. Apoptosis was determined by PI staining and subsequent cytofluorographic analysis.

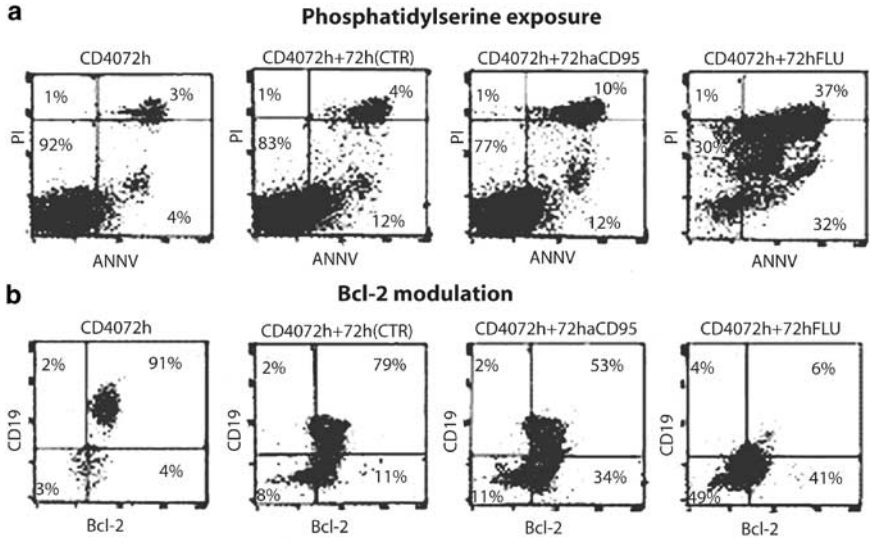

Figure 3 Determination of apoptosis by Annexin-V-FITC/PI (A) and of Bcl-2 downmodulation by intracytoplasmic staining (B) in 1 (No 4) of the 3 CLL patients weakly sensitive to CD95-apoptosis induction. Exposure of phosphatidyl-serine residues, a feature of cells in early apoptosis, was determined by double ANN-V/PI staining in CD40-activated CLL B cells (CD4072h), in CD40-activated cells cultured for further 72 h with only medium (CD4072h + 72h(CTR)), or with the anti-CD95 moAb (CD4072h + 72haCD95), or with fludarabine as control (CD4072h + 72hFLU). The percentage of cells in apoptosis (ANN-V + /PI- and ANN-V + /PI +) increased from 16 to 22% after treatment with the αCD95 moAb and to 69% after treatment with fludarabine. In agreement, the percentage of cells downmodulating Bcl-2 and CD19 was increased after CD95-induced apoptosis (34 *versus* 11%).

FLICE, FAS, FASL, FADD and TRADD mRNA expression in resting and in CD40- activated CLL cells

In order to investigate whether a deregulated expression in CLL B cells of molecules involved in the formation of the DISC (FAS, FLICE, FADD)[32] could be responsible for their resistance to CD95-triggered apoptosis, we performed mRNA analyses by multiplex PCR assay, on purified leukemic B cells from eight CLL cases (No. 1, 2, 6, 7, 8, 16, 19, 20) before and after CD40-activation. In agreement with the cytofluorimetric data, FAS mRNA (321 bp), negative or faintly expressed at resting conditions, was significantly upregulated after CD40 triggering (Figure 4). Through gel densitometry analysis we calculated, at resting conditions, a FAS mRNA expression mean intensity value of 0.36 ± 0.35 that was significantly enhanced to 1.87 ± 1.16 after CD40 activation ($P = 0.005$). FLICE (caspase-8) mRNA (405 bp) was clearly expressed in all cases and analyzed without relevant differences between resting and activated conditions. Also, FADD (205 bp) and TRADD (150 bp) mRNAs, although expressed at low levels, were detectable without significant differences between resting and activated conditions (FADD: 0.55 ± 0.45 *versus* 0.49 ± 0.36, $P = 0.80$; TRADD: 0.34 ± 0.20 *versus* 0.32 ± 0.27, $P = 0.87$). In the Jurkat cell line, here used as a positive control because of its sensitivity to CD95-mediated apoptosis, the levels of FADD mRNA expression appeared slightly higher (1.58) than in CLL cells, while those of TRADD (0.49) were very similar.

FLICE and FADD protein expression in resting and in CD40-activated CLL cells

To confirm that FLICE and FADD were expressed not only as mRNA but also at the protein level, we performed Western blot analysis of lysates obtained from purified B cells of some CLL cases (No. 4, 7, 20). As shown in Figure 5, a 55 KDa band corresponding to FLICE (procaspase-8) was constitutively expressed at resting conditions and proved slighltly upregulated after CD40-activation (No. 4*, 7*, 20*), or after activation and prolonged culture of CLL B cells in medium alone (No. 20*°). FADD (24 kDa band) was also expressed at high levels already in fresh purified B cells; nonetheless, CD40 stimulation of B cells did not significantly modify the intensity of the signal detected in resting cells. Negative controls for FLICE and FADD protein expression are represented here, respectively, by SHSY5Y human neuroblastoma and BaF3 murine B-lymphoid cells,[33,34] while, as positive control, we used RAJI human Burkitt lymphoma cell.[34]

Moreover, intracytoplasmic staining performed with the specific anti-human FADD moAb(1TB) again confirmed expression of the FADD molecule both in resting (Figure 6a) and in CD40-activated CLL B cells. Figure 6 reports a representative case (No. 8) of the eight analyzed.

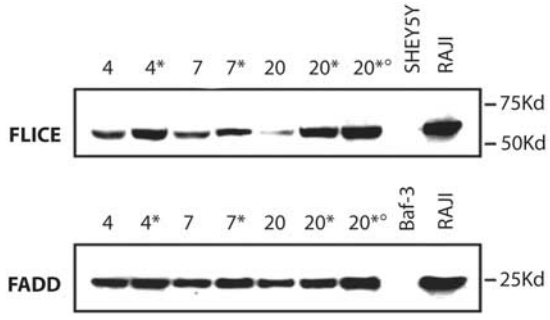

Figure 5 Western blot analysis of FLICE and FADD protein expression in CLL B cells at resting and activated conditions. FLICE (procaspase-8) and FADD proteins expression was analyzed in purified B cells from CLL patients (No. 4, 7 and 20) before and after 72 h CD40 triggering '*in vitro*'. Cellular lysates of the RAJI cell line were used as positive control both for FLICE and FADD immunostaining, while SHEYSY and BaF-3 represented the negative controls for FLICE or FADD respectively. FLICE, expressed in all the cases studied at resting conditions (No. 4, 7, 20), was clearly upregulated after 72 h CD40 activation (No. 4, 7, 20) or after activation and subsequent culture in medium alone for 72 h more (No. 20*°). The FADD protein appeared constitutively expressed in both resting (No. 4, 7, 20) or activated cells (No. 4*, 7*, 20*) without relevant differences between the two conditions, while a slight increase was observed in sample No. 20*°.

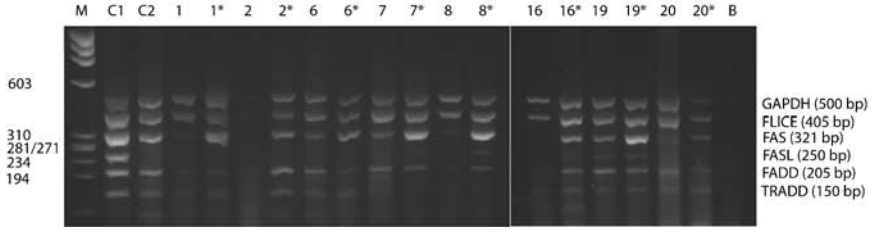

Figure 4 Multiplex mRNA analysis by RT-PCR (MPCR) of molecules comparing the DISC. Here we show mRNA expression of GAPDH (500 bp), FLICE (405 bp), FAS (321 bp), FASL (250 bp) FADD (205 bp), TRADD (150 bp) by MPCR in B cells, resting and CD40-activated (*), from 8 CLL cases. Purified B cells from the eight CLL cases were cocultured with CD40L-tranfected cells for 72 h. Following CD40 triggering, FAS expression was significantly upregulated (cases No. 6*, 7*,19*, 20*) or '*de novo*' expressed (cases No. 1*, 8*,16*) with a $P = 0.005$ value ($n = 7$, Student's *t*-test). FLICE (caspase-8) was present in all the cases without relevant differences between resting and activated conditions. FADD and TRADD were weakly expressed but detectable in almost all the cases without significant differences between resting and activated cells. M:ΦX174, DNA molecular weight. C1: Positive control included in the kit. C2: Jurkat cell line. B: blank PCR control. *CD40-activated cells.

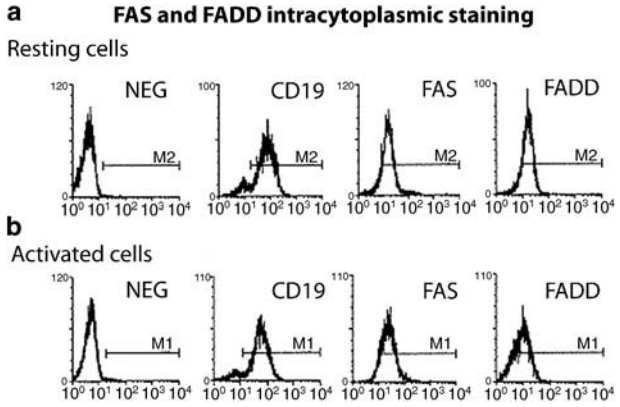

Figure 6 Intracytoplasmic staining of FAS and FADD molecules in a representative case of CLL B cells. Cytofluorimetric analysis of FAS and FADD intracytoplasmic expression by staining with specific moAb further confirmed their expression in resting and activated cells as shown here in one representative case (No. 8).

Discussion

In this study, we show that CD95 (FAS) is significantly enhanced on the leukemic B cells of all 20 CLL cases analyzed, after CD40/CD40L interaction. However, despite the high CD95 expression, leukemic cells still failed to undergo apoptosis after CD95 triggering. Following 72 h treatment with an agonistic anti-CD95 moAb we could, in fact, observe a low percentage of apoptotic cells in only three of the 20 CLL cases studied. The levels of apoptosis detected in these three patients were, however, too low (15%) to be considered significant, thus suggesting that CLL B cells are, in general, resistant to CD95-mediated apoptosis. Kitada *et al*.[25] have also previously shown the lack of CD95-mediated apoptosis, in CD40-activated CLL B cells and have correlated the block of the CD95 apoptotic pathway to increased levels of FLIPs (FLICE-inhibitory proteins) observed after CD40-triggering. Recent data from Chu *et al*.[35] have further demonstrated that CD40-activated CLL B cells may acquire sensitivity to CD95-mediated apoptosis only after a prolonged *in vitro* culture (5 days or more), in association with a progressive decline of FLIP expression. However, as reported in this paper, an induced FLIP downregulation by antisense oligonucleotides or pharmacological agents was insufficient to induce CD95-mediated apoptosis in CLL, thus indicating that other factors are possibly involved. The present study demonstrates that FAS mRNA, negative or weakly expressed at resting conditions, is significantly enhanced after CD40 triggering and that FLICE and FADD mRNA are both expressed without relevant differences before and after CD40 activation on CLL B cells. We further show here that FLICE (procaspase-8) and FADD are present as proteins in resting and in CD40-activated cells. Our findings suggest, therefore, that leukemic CLL cells possess a complete machine required to carry out the FAS-triggered apoptotic program. In agreement with our observations, Roue *et al*.[34] previously reported a FADD and FLICE protein expression in resting CLL cells and similarly failed to induce apoptosis of resting or CD40-activated CLL B cells using the anti-CD95 moAb *in vitro*. Altogether these data seem to rule out that apoptosis resistance of CLL cells after FAS-engagement might be due to the deregulated expression of the DISC complex molecules. Williams *et al*.,[28] however, appear to disagree with our results. They, in fact, showed that autologous T cells (FASL+) can efficiently mediate FAS-based lysis of CLL B cells. In addition, Younes *et al*.[36] described that CLL cells could be driven to apoptosis in the presence of the soluble FASL recombinant protein rather than of an anti-FAS moAb. It remains, therefore, still unclear whether the discrepancies found by different groups are due to different reagents or to the different experimental procedures employed. Our observation of a weak apoptosis in only a minority of CLL cases (3/20) may further suggest that CLL cells could show an intrinsic heterogeneity in apoptotic responses induced by CD95 triggering that could lead to conflicting interpretations. It is, however, worth noting that all CLL cases included in our study that proved mostly resistant to CD95-mediated apoptosis were, nonetheless, sensitive to fludarabine apoptosis induction, both when cells were treated at resting conditions or after CD40 activation.[37] These observations demonstrate that other apoptotic pathways, not involving CD95, are preserved and functional in these malignant B cells. It is of further interest to note that the major caspases mediating apoptosis after fludarabine treatment were caspases-6 and -1, while caspases-8 and -3 appeared to be involved only in a limited number of cases (5 of 14 cases studied), as we previously reported.[37] Since caspase-8 is principally activated along the FAS apoptotic pathway,[13] it appears of interest that the three CLL cases showing a weak apoptosis in the presence of the anti-CD95 moAb were among the few cases that activated caspases-8 and -3, in addition to caspases-6 and -1, after fludarabine treatment.[37] This finding could imply that a defective activation of certain caspases (i.e. -8 and -3) may have a role in the block of CD95-induced apoptosis in CLL. This hypothesis, however, needs further investigations to be definitevely proven.

In conclusion, our study demonstrates that the observed resistance of CD40-activated CLL B cells towards CD95-mediated apoptosis is not due to a defective expression of the major molecules that compose the DISC complex and suggests that the reasons for the observed dysregulated apoptosis after CD95 triggering are rather to be searched within a defective activation of given caspases (i.e. caspase-8) or in a defective assembly of molecules composing the DISC complex.

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