The role of TACI expression in chronic lymphocytic leukemia

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Abstract

Transmembrane activator and CAML interactor (TACI) was identified as a receptor for BAFF and APRIL, two members of TNF ligand family. The significantly lower TACI expression on B cells from CLL patients than from normal controls raised the question of the importance of TACI in CLL pathogenesis. We investigated the relationship between TACI expression and pro- and antiapoptotic proteins expression in CLL cells. Moreover, we were interested in a possible relationship between TACI expression and prognostic factors such as: CD38 antigen or ZAP-70 protein expression, which are known poor prognosis factors in B-CLL. Our results confirm the significance of apoptosis deregulation in CLL and suggest the possible relationship between TACI expression and the clinical course of the disease.

Key words: *CLL, TACI, ZAP-70, PAR-4, DAXX, ZIPK, apoptosis.*

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Introduction

Many members of TNF (tumor necrosis factor) superfamily and their receptors play critical role in the homeostatic regulation of immune effector cells [1]. One member of the TNF superfamily, BAFF (B-cell activating factor of the TNF family) also called BLyS (B lymphocyte stimulator) is a critical molecule for B cell survival, maturation and tolerance [2, 3]. BAFF promotes the survival of both activated and resting B cells. BAFF binds to three receptors: BCMA (B-cell maturation antigen), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BAFF receptor (BAFF-R/BR3) [3]. TACI and BCMA also bind APRIL (a proliferation inducing ligand) the structural homolog of BAFF. Defects in the synthesis of these molecules or expression of their receptors have been associated with various B cell malignancies [3, 4]. Emerging information on the function of TACI in B-cell survival suggest that TACI plays a significant negative regulatory role in B-cell homeostasis and autoimmunity. It has been shown, that TACI-deficient mice have an accumulation of splenic B cells and increased serum immunoglobulins levels [5, 6]. In addition, TACI-deficient mice are predisposed to the development of B cell lymphomas [5, 6]. The observed phenotype of lymphoproliferation and autoimmunity in TACI$^{-/-}$ mice suggests that this receptor may be able to promote apoptosis in activated B cells [6]. These findings suggest that TACI may be involved in the pathogenesis of chronic lymphocytic leukemia (CLL). In the present study, we compared membrane TACI expression in patients with CLL and normal persons. We were interested in a possible relationship between TACI expression and prognostic factors such as: CD38 antigen or ZAP-70 protein (zeta-associated protein of 70 kD) expression, which are known poor prognosis factors in CLL [7, 8]. Moreover, the aim of our study was the examination of the relationship between membrane TACI expression with expression of pro- and antiapoptotic proteins such as PAR-4, ZIP kinase, DAXX, BCL-2 and BAX. PAR-4 (prostate apoptosis response-4) is a cancer cell-selective pro-apoptotic protein that functions intracellularly in the cytoplasmic and nuclear compartments as a tumor suppressor [9]. It has been found that PAR-4 promotes the Fas apoptosis pathway and parallel NF-κB inhibition [10]. In lymphatic cells, increased levels of PAR-4 protein were fol-

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lowed by BCL-2 protein downregulation and cleavage of poly(ADP-ribose) polymerase (PARP) [11]. PAR-4 cooperates with other proapoptotic proteins, including the nuclear ZIPK (zipper interacting protein kinase) and DAXX (death-associated protein) [12, 13]. DAXX was identified as a proapoptotic protein that binds to the death domain of the CD95 death receptor [14]. ZIPK is a proapoptotic protein kinase initiating a nuclear apoptotic pathway in collaboration with PAR-4 and DAXX proteins [13]. We raised the question of whether aberrant TACI expression in leukemic B cells might also be related with apoptosis deregulation in CLL.

Material and methods

Patients and samples

Peripheral blood (PB) specimens were obtained from 62 untreated CLL patients diagnosed between September 2005 and December 2009 (32 men and 30 women). The median age of patients was 66 years (ranging from 32 to 87 years). CLL diagnosis based on a clinical examination, morphological and immunological criteria [15]. At the time of diagnosis, patients were staged according to the Rai staging system [16] as follows: stage 0 (21 cases), stage 1 (19 cases), stage 2 (12 cases), stage 3 (3 cases) and stage 4 (7 cases). The patients cohort was divided into three groups: patients with Rai stage 0 (21 cases), stage 1-2 (31 cases) and stage 3-4 (10 cases). PB samples were collected into heparinized tubes and immediately processed. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation on Lymphoprep (Nycomed) for 25 minutes at 400 × G at room temperature. Interphase cells were removed, washed twice and resuspended in phosphate-buffered saline (PBS). Control PB samples were obtained from 15 healthy donors (aged from 36 to 65 years, median age of 58 years). The study was approved by the Local Ethical Committee.

Membrane TACI expression staining

Flow cytometry analysis of TACI was performed on fresh PB samples stained with anti-CD19 FITC (BD Pharmingen) and anti-TACI PE (R&D Systems). Cells used for antibody staining were first Fc-blocked by the treatment with FcR-blocking reagent containg human IgG (Miltenyi Biotec). Cells (1×10^6) were then incubated for 30 minutes at 4°C with specified MoAb against surface antigens i.e. CD19 and TACI. Unreacted reagents were then removed by washing cells in PBS, and cells were analyzed by flow cytometry.

Determination of apoptosis by Mito Tracker Red CMXRos

The level of apoptosis was measured by chloromethyl-X-rosamine staining (Mito Tracker Red CMXRos; Molecular Probes). CMXRos is cationic lipophilic fluorochrome that does not accumulate in depolarised mitochondria and can be used to detect disruptions in mitochondrial membrane potential ($\Delta\Psi m$). CMXRos was used in combination with the monoclonal anti-CD19 FITC antibody (BD Pharmingen). Cells were incubated with CMXRos for 30 min at 37°C and after 15 min of incubation, anti-CD19 MoAb was added. $CD19^+$ cells considered to be apoptotic displayed a decrease in mitochondrial membrane potential in CMXRos staining ($\Delta\Psi m^{low}$).

Intracellular analysis of PAR-4, DAXX, ZIP kinase, BCL-2 and BAX

Intracellular PAR-4 staining was performed with anti-PAR mouse IgG_{2a} antibody solution (Santa Cruz Biotechnology) labeled using the *Zenon Alexa Fluor 488 Mouse IgG_{2a} Labeling Kit* (Molecular Probes) according to the manufacturer's instruction. Intracellular DAXX staining was performed with anti-DAXX rabbit monoclonal antibody (EPITOMICS). Intracellular ZIP kinase analysis was performed with anti- ZIPK rabbit monoclonal antibody (Abcam). Anti-DAXX and anti-ZIPK antibodies solution were labeled using the *Zenon Alexa Fluor 488 Rabbit Labeling Kit* (Molecular Probes). Intracellular BCL-2 protein analysis was performed with FITC conjugated anti-BCL-2 mouse monoclonal antibody (DAKO). Intracellular BAX analysis was performed with FITC conjugated anti-BAX mouse monoclonal antibody (Santa Cruz Biotechnology).

For intracellular detection of PAR-4, DAXX, ZIPK, BCL-2 and BAX, PBMC were stained with monoclonal antibodies against cell surface markers, i.e. CD19 PE (20 minutes at RT). Following membrane staining, fixation/permeabilization procedures were performed (Cyto-fix/Cytoperm solution and Perm/Wash buffere, BD Pharmingen). Cells were then incubated with anti-PAR-4, anti-DAXX or anti-ZIPK antibodies solution labeled by Zenon™ Alexa Fluor 488 Labeling Kits or anti-BCL-2, anti-BAX and appropriate isotypic control for 20 minutes at RT. In this study, levels of PAR-4, DAXX, ZIPK, BCL-2 and BAX expression, indicated by mean fluorescence intensity (MFI) were analyzed.

Analysis of ZAP-70 expression in CLL cells

All PB samples were stained for ZAP-70 protein expression. We used a modification of a previously described method for flow cytometric examination of ZAP-70 protein expression [17, 18]. A cut-off point for ZAP-70 positivity in leukemic cells was ≥ 20%.

Detection of CD38 expression

Flow cytometry analysis of CD38 antigen expression was performed on fresh PB samples, as described previously [18]. CLL cells were considered CD38-positive when ≥ 20% of them expressed the membrane antigen.

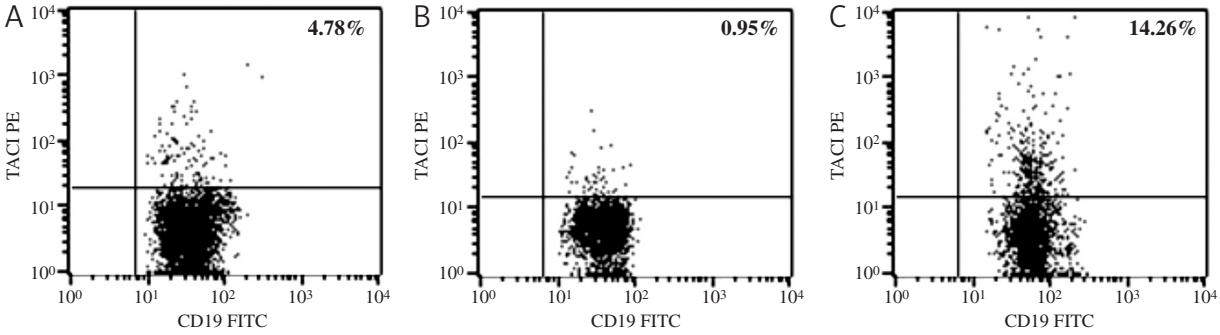

Fig. 1. The dot plots show representative types of TACI expression on the CD19+ cells from ZAP-70-negative (A), ZAP-70-positive (B) CLL patients and on the normal CD19+ cells (C). The number in the upper right quadrant in the dot plots represent the percentage of TACI+/CD19+ cells among CD19+ B lymphocytes

Flow cytometry analysis

Samples were analyzed by two- and three-color flow cytometry using the Becton Dickinson FACS-Calibur instrument. Five data parameters were acquired and stored, i.e. linear forward and side scatter (FSC, SSC), green fluorescence (FL-1), orange-fluorescence (FL-2) and red-fluorescence (FL-3). For each analysis, 10 000 events were acquired and analyzed using the CellQuest software. An acquisition gate was established basing on FSC and SSC that excluded dead cells and debris. Isotype-matched antibody was used to verify staining specificity and as a guide for setting of markers used for delineate positive and negative populations.

Statistical analysis

Differences between two groups were assessed using the U Mann-Whitney test. The Spearman rank correlation coefficient was used in correlation tests. We used Statistica 7.0 PL software for all statistical procedures. Differences were considered statistically significant with P-value ≤ 0.05.

Results

The proportion of leukemic cells expressing TACI above isotype control level ranged from 0.18% to 85.60% with the median of 18.28%. The median percentage of B cells expressing TACI was significantly lower in B-CLL patients than in normal controls ($p = 0.0002$). Likewise, when we compared the levels of membrane TACI expression determined by MFI on CD19+ cells from patients (median: 16.51 MFI) and healthy controls (35.15 MFI, respectively), we found significant differences ($p = 0.036$).

We found an inverse correlation between TACI membrane expression and Rai disease stage (R = –0.317; $p = 0.040$). We observed a significantly lower median percentage of CD19+ lymphocytes with TACI expression in patients in Rai stage 3-4 as compared to those in early B-CLL stages (39.00% vs. 25.41%) ($p = 0.010$). The proportion of CD19+/CD5+ cells expressing ZAP-70 inverse correlated with the percentage of leukemic cells with membrane TACI expression (R = –0.385; $p = 0.032$). The percentage of CD19+/TACI+ was significantly lower in ZAP-70+ (median: 14.02%) compared with ZAP-70- patients (median: 20.56%) ($p = 0.047$). Representative plots are shown in Fig. 1A-C. Likewise, we observed a significantly lower percentage of B cells with membrane TACI expression in CD38+ patients (12.94%) than in CD38- patients (21.27%) ($p = 0.012$).

The membrane expression of TACI showed an inverse correlation with WBC count (R = –0.398; $p = 0.043$). There was also an inverse correlation between the number of CD5+ B cells and TACI membrane expression (R = –0.236; $p = 0.045$). We identified a positive correlation between TACI expression and the percentage of CD19+/$\Delta\psi m^{low}$ cells (R = 0.219; $p = 0.047$). The membrane expression of TACI correlated inversely with the BCL-2 protein expression (R = –0.385; $p = 0.0001$) (Fig. 2). There was also an inverse correlation between the expression of TACI and the BCL-2/BAX ratio (R = –0.251; $p = 0.007$). We found a positive relationship between TACI and PAR-4 expression (R = 0.268; $P = 0.037$) (Fig. 3). Additionally, there was a positive correlation between TACI and both DAXX and ZIPK protein expression (R = 0.523; $p = 0.037$ and R = 0.389; $p = 0.027$, respectively).

Discussion

Transmembrane activator and CAML interactor (TACI) was identified as a receptor for BAFF and APRIL, two members of TNF ligand family. Both BAFF and APRIL induce proliferation, activation and survival of B cells [19]. In our previous study, we have shown that BAFF and APRIL proteins are aberrantly expressed by B cells in patients with CLL [20]. It has been found that B cells isolated from BAFF transgenic mice expressed elevated levels of anti-apoptotic Bcl-2 protein [21]. Likewise, it was reported that

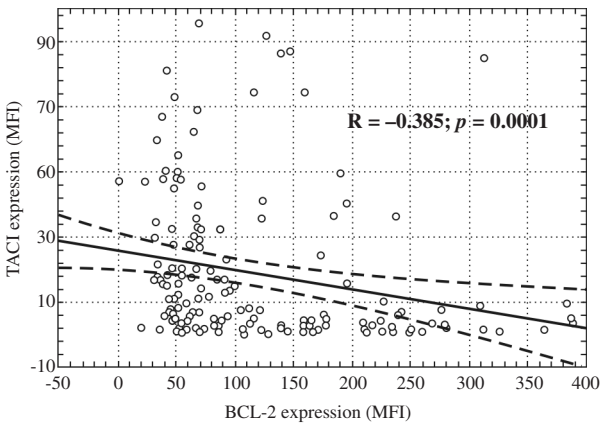

Fig. 2. The relationship between TACI and BCL-2 protein expression. MFI-mean fluorescence intensity

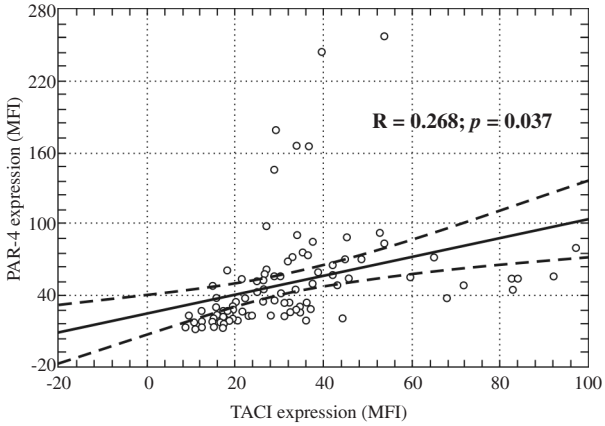

Fig. 3. The relationship between TACI and PAR-4 protein expression. MFI-mean fluorescence intensity

myeloma cells treated with BAFF or APRIL upregulate BCL-2 and MCL-1 expression [22]. Interestingly, both multiple myeloma and CLL B cells express elevated levels of BCL-2 and MCL-1 proteins in comparison with normal B cells [23, 24]. BAFF and APRIL bind TACI and both could mediate the negative regulatory effect of this receptor. However, in our study we observed significantly lower TACI expression on B cells in CLL patients than in normal controls. This raised the question of the importance of TACI in CLL pathogenesis. The variable expression of TACI seen in patients with CLL has a potential significance because the receptor has been implicated as a negative regulator of B-cell growth and activation. The observed lymphoproliferation and autoimmunity in TACI-deficient mice suggests that TACI may be able to promote apoptosis in activated B cells [5, 6]. In the present study, we detected a positive correlation between TACI expression on CLL cells and the percentage of apoptotic leukemic B cells. We can hypothesize that decreased TACI expression can reduce the negative regulatory signal to B cells. We correlated TACI expression with expression of pro- and antiapoptotic proteins in CLL cells. The membrane expression of TACI correlated inversely with the BCL-2 protein expression in CLL cells. In our study, there was also an inverse correlation between the expression of TACI and the BCL-2/BAX ratio. It was observed that the expression of BCL-2/BAX correlates with apoptosis and clinical outcome. Decreased BCL-2/BAX ratios are associated with increased sensitivity to cytotoxic drugs *in vitro* and improved responses to chemotherapy in patients [23]. In our study, we found a positive relationship between TACI and PAR-4 expression. Prostate apoptosis response-4 (PAR-4) is unique proapoptotic protein that selective induces apoptosis in cancer cells [25]. PAR-4 protein is suggested to promote apoptosis in various cell types in response to a variety of stimuli, such as chemotherapy, UV-radiation or elevation of intracellular calcium concentration [26, 27]. It was demonstrated that Par-4 exerts its proapoptotic effect by down-regulating the expression of antiapoptotic BCL-2 [11]. Boehrer *et al.* [11] demonstrated that PAR-4 overexpression enhances disruption of mitochondrial membrane potential on stimulation with chemotherapeutic agents. PAR-4 cooperates with other proapoptotic proteins, including the nuclear ZIP kinase and DAXX [12, 13]. In our study, there was observed a positive correlation between TACI and both DAXX and ZIPK protein expression. Boehrer *et al.* [14] found that simultaneous overexpression of DAXX, PAR-4 and ZIPK proteins elicited an over six fold increase in apoptosis compared to control cells.

It has been found that $TACI^{-/-}$ mice showed increased circulating and splenic B cells [5, 28]. It was reported that B cells lacking TACI hyperproliferate in response to various stimuli. Thus TACI may play an inhibitory role in B cell activation [5]. Interestingly, patients with multiple myeloma expressing a low amount of TACI on their malignant cells had worse prognosis than those expressing high amounts of TACI [29]. We observed significantly lower TACI expression on B cells in CLL patients than in normal controls. Seshasayee *et al.* [6], using $TACI^{-/-}$ mice, have shown that loss of TACI results in lymphoproliferation, lymphoma and autoimmune disorders. We indicated an inverse correlation between the number of $CD5^+$ B cells and TACI membrane expression. What is more, the membrane expression of TACI showed an inverse correlation with WBC count. In our study, there was a significantly lower percentage of $CD19^+$ lymphocytes with TACI expression in patients in Rai stage 3-4 as compared to those in early CLL stages. Likewise, we observed a significantly lower percentage of B cells with membrane TACI expression in $ZAP\text{-}70^+$ and $CD38^+$ patients than in $ZAP\text{-}70^-$ and $CD38^-$ patients.

Our results confirm the significance of apoptosis deregulation in CLL and suggest the possible relationship between TACI expression and the clinical course of the disease, which, however needs further investigation.

Acknowledgments

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