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CD40L and IL-4 stimulation of acute lymphoblastic leukemia cells results in upregulation of mRNA level of FLICE - an important component of apoptosis

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Abstract: The use of cancer vaccines based on dendritic cells (DC) presenting tumor antigens can be a promising tool in the treatment of leukemia. The functional characteristics of leukemia derived DC is still to be elucidated. CD40 promotes survival, proliferation and differentiation of normal B cells. CD40 triggering was used to enhance the poor antigen-presenting capacity of leukemic B-cells. Since it is still unclear whether CD40 ligation drives neoplastic B-cells to apoptosis or not, we assessed the mRNA expression of FLICE, FAS, FADD and TRADD - important components of apoptosis machinery, using real-time PCR in acute lymphoblastic leukemia cells before and after CD40 and IL-4 stimulation. ALL cells stimulated with CD40L/IL-4 expressed dendritic cell phenotype at mRNA and protein levels (upregulation of main costimulatory and adhesion molecules noted in real-time RT PCR and flow cytometry); they also expressed higher amounts of mRNA for FLICE, TRADD and FADD after CD40L/IL-4 stimulation. However differences statistically significant comparing cells cultured with CD40L/IL-4 and medium alone regarded only FLICE. Concluding, we showed upregulation of important elements of apoptosis at mRNA level in ALL cells after CD40 ligation.

Key words: Acute lymphoblastic leukemia - CD40L - Dendritic cells - apoptosis - FAS - FADD - TRADD - FLICE

Introduction

Acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) are the most common types of leukemia in children and in adults, respectively. ALL can be cured in more than 80%, but some patients suffer from refractory or recurrent disease and cannot be cured with conventional chemotherapy [18]. Therefore new treatment approaches, like effective and safe immunotherapy, are needed. The use of cancer vaccines based on dendritic cells (DC) presenting tumor antigens can be a promising tool. Dendritic cells play a pivotal role in the immune response as well as in the induction of specific antitumor reaction. They take up antigens, present them to T cells as peptides bound to both MHC class I and II products and control the type of T cell response [15]. Dendritic cells can be generated from peripheral blood monocytes with the use of various stimuli (preferably GM-CSF, TNF-α and

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Abbreviations: ALL - acute lymphoblastic leukemia, AML - acute myeloid leukemia, APC - antigen presenting cell, CLL - chronic lymphocytic leukemia, DC - dendritic cells, DISC - death-inducing signalling complex, FADD - Fas-associated death domain protein, FLICE - caspase 8/a, TRADD - TNFRSF1A-associated via death domain.

IL-4) and media (RPMI 1640 supplemented with 2% human serum albumin, 2% TCH, X-VIVO 15 or Panserin 501) [24]. An interesting approach, intensively examined in the last few years involves the differentiation of leukemic cells into DC which could combine both APC function and expression of tumor antigens [16]. Our findings and data reported by other authors seem to demonstrate that such approach is feasible, but the functional characteristics of leukemia derived DC is still to be elucidated [3,14]. Immunotherapy could be a clinical option in a group of patients with high risk of recurrence of the disease. CD40, a member of TNF family, promotes survival, proliferation and differentiation of normal B cells. The CD40-CD40 ligand (CD40L) system is of pivotal importance in the immune response via interactions between T cells and antigen-presenting cells. CD40 triggering was used to enhance the poor antigen-presenting capacity of leukemic B-cells. Even in heavily pretreated patients, including children with ALL and AML, autologous tumor vaccine expressing CD40L and IL-2 resulted in clinical and laboratory response [21]. Intravenous infusion of leukemic cells after CD40 ligation in adult patients with B-CLL resulted not only in a reduction in leukemic cell counts and lymph node size but also caused an increase in T-cell counts [26].

Modulation of signal transduction pathways also represents a promising tool for treating hematologic malignancies. Disregulation of cell death mechanism may contribute to oncogenesis. Apoptosis is a type of programmed cell death involved in many biological processess and many diseases such as cancer, AIDS and autoimmunity, are associated with either too much or too little apoptosis. It also plays a special role in the immune system [13]. Apoptosis forms the basic mechanism for the action of chemotherapeutic agents on cancer cells. FAS (CD95) is the best-known death receptor. CD95 ligation results in the enrollment of Fas-associated death domain protein (FADD) and procaspase-8 (FLICE), forming together death-inducing signaling complex (DISC) [5,13]. The formation of DISC leads to the release of caspase-8, cleavage of caspase-3 or amplification of the apoptotic signal via mitochondria and finally to apoptosis. FADD and caspase 8 are also essential for the DISC formation with TRAIL-R1 and R2 and play a central role in signaling via death receptors. Caspases are the proteases necessary for execution of apoptosis and could also be a potential target for the new cancer therapies. Inhibition of caspases prevents cell death but there also exists a caspase-independent mechanism of apoptosis.

Some authors have found a relationship between the expression of molecules involved in apoptosis and survival in patients with hematologic malignancies; the expression of FLIP (Fas-associated death domain-like interleukin 1 beta-converting enzyme-like inhibitory protein) was a positive prognostic factor in the treatment of elderly AML patients [12]. In another study, low Fas expression was a predictor for poor outcome in ALL [23]. Lack of the Ki67 antigen expression, one of the most sensitive markers of proliferation, may represent an unfavourable prognosis in children with AML [17]. Survivin, an inhibitor of apoptosis could also serve as a prognostic factor in non-small cell lung cancer - its overexpression was correlated with shorter survival [10].

Lately the treatment with CD40L and IL-10 resulted in reconstitution of IgA secretion in IgA-deficient humans. It has been indicated that this mechanism is associated with protection of CD20+ B cells from apoptosis (*e.g.* increase in IAP-2 inhibitor of apoptosis expression) [9].

Since it is still unclear whether CD40 ligation drives neoplastic B-cells to apoptosis or not, we decided to assess the mRNA expression of FLICE, FAS, FADD and TRADD - important elements of apoptosis machinery, using real-time PCR in acute lymphoblastic leukemia cells before and after CD40L and IL-4 stimulation.

Materials and methods

Patients. Children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) were prospectively enrolled into the study (N=25, 13 boys=52% and 12 girls=48%, aged 2-17 years, mean 6.84 ± 4.6). The patients were treated in departments of pediatric hematology and oncology in Białystok, Warszawa, Bydgoszcz, Chorzów and Zabrze according to the protocol recommended by the Polish Pediatric Leukemia/Lymphoma Study Group, ALL IC-BFM 2002 (September 2005 to January 2006). Peripheral blood samples or bone marrow were taken at the time of diagnosis, before any treatment. Bone marrow was obtained when more than 80% of blast infiltration was present, peripheral blood - when the leukocyte count was more than 10×10^9/l and blasts accounted for over 90% of mononuclear cells in differential white blood cell count. In the study group the white blood count range was 2.5-36 $\times 10^9$/l, mean 10.47 ± 9.08, the percentage of peripheral blasts was 1-92%, mean 42.4 ± 27.4. The study was approved by the institutional review board and informed consent was obtained from patients and their parents.

Cells and culture. Mononuclear cells from bone marrow or peripheral blood samples were obtained via density-gradient centrifugation, frozen immediately in fetal calf serum (FCS, Sigma) containing 10% dimethyl sulphoxide (DMSO, Sigma) and stored in liquid nitrogen. Thawed ALL cells (>80% vital cells) were resuspended in medium (RMPI-1640/FBS 90%/10%), cultured in 24-well flat-bottom plates at a concentration of 1×10^5/ml and stimulated (or not) with CD40L (3 μg/ml, a kind gift of Immunex/Amgen, USA) and IL-4 (80 ng/ml, Sigma). The cells were incubated in a total volume of 1ml/well at 37°C in 5% CO_2 for 96 hours. After the culture, ALL cells were gently removed and used for flow cytometry and mRNA isolation. The trypan blue (Sigma) exclusion assay was used to study cell viability. All assessed samples had at least 80% viable cells before and after the culture.

Real-time quantitative PCR. The mRNA was isolated from mononuclear cells using Dynabeads mRNA Direct Micro Kit

Table 1. Results obtained from real-time PCR for molecules involved in apoptosis (mean mRNA expression of FAS, FLICE, FADD and TRADD).

	I	II	III	Statistics		
	after thawing $2^{-\Delta\Delta CT}$ median (25^{th}-75^{th} centile)	cultured with medium + CD40L + IL-4 $2^{-\Delta\Delta CT}$ median (25^{th}-75^{th} centile)	cultured with medium $2^{-\Delta\Delta CT}$ median (25^{th}-75^{th} centile)	I vs II	I vs III	II vs III
FAS	0.37 (0.19-0.83)	1.01 (0.49-1.83)	0.46 (0.26-1.80)	ns	ns	p=0.07
FLICE	4.47 (2.03-10.02)	27.83 (15.76-80.16)	2.00 (1.00-3.00)	p=0.003	p=0.01	p=0.04
FADD	12.00 (4.61-19.05)	59.32 (35.31-138.86)	68.43 (12.14-210.81)	p=0.01	ns	ns
TRADD	129.96 (101.26-183.92)	322.68 (229.92-1742.56)	461.12 (240.68-2674.40)	p=0.002	ns	ns

(Dynal) according to the producer's instructions. The first strand of cDNA was synthesized using random hexamers as primer and High Capacity cDNA Archive Kit by Applied Biosystems. Six different costimulatory/adhesion molecules at mRNA level (CD1a, CD40, CD54, CD80, CD83, CD86) and four - involved in apoptosis (FAS, FLICE, TRADD, FADD) were determined by real-time PCR technique with the TaqMan chemistry using ready-to-use Low Density Arrays for Gene Expression by Applied Biosystems. It contained target-specific primers and probe and TaqMan Universal Master Mix, containing AmpErase uracil-N-glycosylase (UNG) to prevent the re-amplification of carryover PCR products. The PCR amplification and fluorescence data collection were performed with ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). To normalize the amount of expressed mRNAs, the internal housekeping gene GAPDH was used and each complementary DNA (cDNA) product was tested in quadruplicates for each of ten molecule mRNA and GAPDH mRNA. To calculate our data we used Comparative Ct method for relative quantification (Ct method) which describes the change in expression of the target gene in a test sample relative to a calibrator sample and provides accurate comparison between the initial level of template in each sample. As a calibrator sample we used Total Raji RNA by Applied Biosystems which was processed in the same way as the test samples. Data were analyzed with Sequence Detector System (SDS) software version 2.1 (Applied Biosystems).

Flow cytometry. Immunophenotyping was performed as previously described [14]. Briefly, cultured cells were incubated for 20 min with monoclonal antibodies, then samples were washed twice with PBS and analyzed with Beckman Cytomics FC 500 MPL. The following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibodies (and isotype controls) were used: CD1a, CD10, CD11c, CD19, CD40, CD45, CD54, CD80, CD83, CD86, CD123, HLA class I, HLA-DR (all purchased from Beckton Dickinson, USA). All gates were set using appropriate isotype control antibodies. Percentages of positive cells were calculated. A minimum of 10^4 events was acquired for each analysis. Results are expressed as percentage of positive cells (%) among stained cells. The purity of leukemic cells was always higher than 90% (determined by flow cytometry).

Statistical analysis. Statistical analysis was performed using Access '97 and Statistica 6.0 for Windows. The results were not normally distributed and are expressed as median and 25th-75th percentile. Significance levels were calculated according to the nonparametric Wilcoxon test (comparison between results obtained before and after stimulation with CD40L and IL-4 and between groups stimulated with cytokines and medium alone). In all analyses, p value less than 0.05 was considered significant.

Results

ALL cells stimulated with CD40L and IL-4 express phenotype of dendritic cells at mRNA and protein level

After the culture with CD40L and IL-4, a statistically significant rise in all assessed cell populations was noted, i.e. in HLA class I and II, $CD1a^+$, $CD11c^+$, $CD40^+$, CD83+, $CD54^+$, $CD80^+$, $CD86^+$ and $CD123^+$ cells. For example, leukemic cells with CD80+ and $CD86^+$ coexpression increased from 5% and 14% to 16% and 30%, respectively (p=0.0001, p=0.0005). An increase in was also observed in mRNA levels for CD40, CD54 and CD80 after CD40L and IL-4 stimulation (data not shown, differences were statistically significant). There was also a tendency for the higher median mRNA levels for CD83 and CD86 after the culture with CD40L than before the culture (statistically insignificant). Results obtained from real-time PCR and flow cytometry show that CD40 ligation induced up-regulation or de novo expression of critical

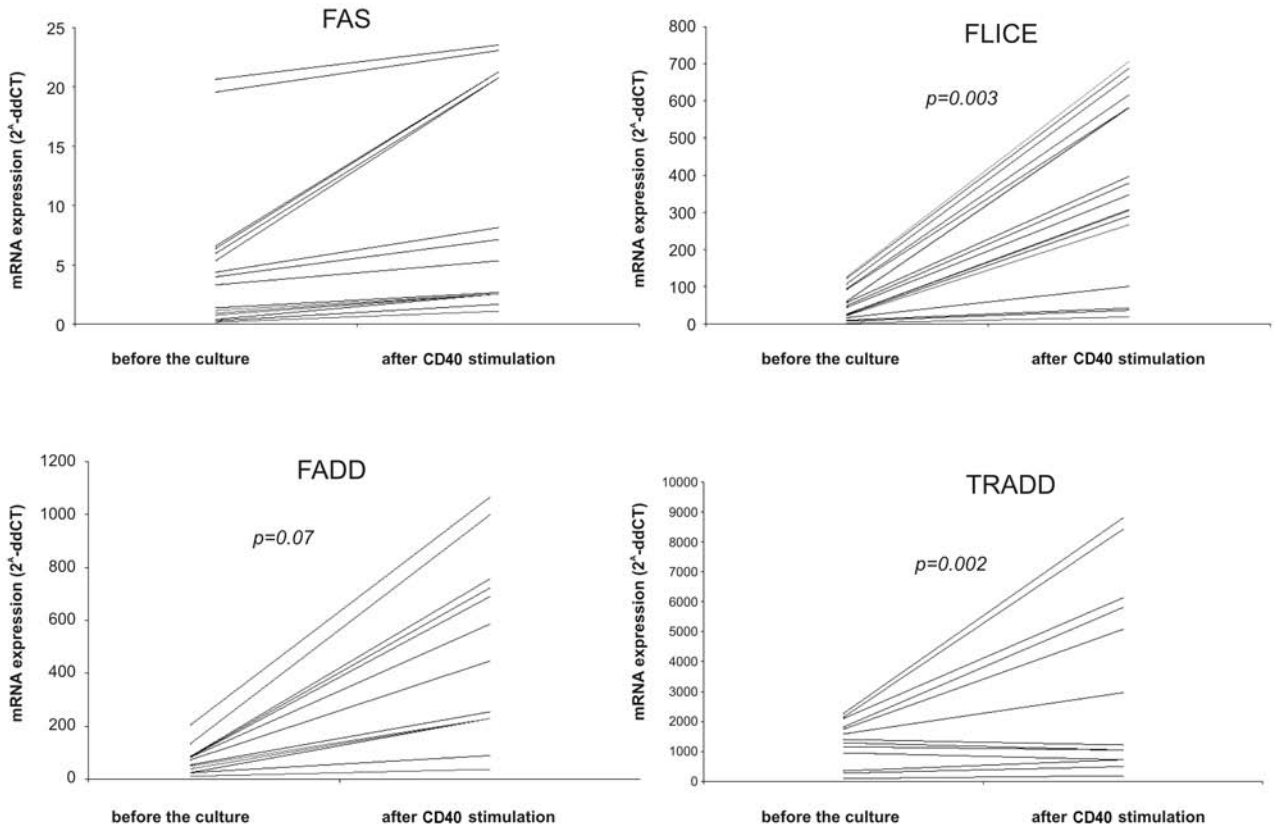

Fig. 1. mRNA expression for FAS, FLICE, FADD and TRADD in ALL cells before and after the culture with CD40L and IL-4.

costimulatory and adhesion molecules on leukemic cells.

ALL cells express higher amounts of mRNA for FLICE, TRADD and FADD after CD40L and IL-4 stimulation

Results concerning the molecules involved in apoptosis, obtained from real-time PCR are presented in Table 1 and Fig. 1. Median expression of mRNA for FLICE and TRADD was higher after CD40 stimulation than before the culture. However only in FLICE the expression after culture with CD40L/IL-4 was significantly higher than after the culture with medium alone (p=0.04). This tendency also referred to FADD molecule, but was not statistically significant (p=0.07).

Discussion

To test the influence of CD40 ligation on apoptosis in leukemic cells we examined ALL blast cells for expression of mRNA for FAS, FADD, FLICE and TRADD before and after CD40L and IL-4 stimulation. As expected, flow cytometry and real-time PCR showing upregulation of critical costimulatory and adhesion molecules confirmed that ALL blasts can be converted into dendritic-like cells. After CD40 stimulation, an increase in mRNA levels for FLICE, FADD and TRADD was noted - these results could indicate activation of apoptosis in leukemic cells. From the clinical point of view, activation of apoptosis in leukemic cells would be a useful effect in cancer immunotherapy.

The majority of experiments on apoptosis in leukemic cells concern CLL in adults, characterized by a slow accumulation rate and availability of tumor cells in peripheral blood and thus represents an ideal target for autologous vaccination immunotherapy since. Results obtained to date are discrepant. Several studies (from one laboratory center - Genoa, Italy) have shown that CLL cells are initially resistant to CD-95 mediated apoptosis. This resistance is probably due to high expression of X-linked inhibitor of apoptosis protein (XIAP) [11], one of the inhibitors of apoptosis proteins (IAPs). Novel XIAP inhibitor, 1540-14, enhances CD95-mediated apoptosis of CLL cells. This effect is mediated by activation of caspases-8 (FLICE) and -3 and could be useful in immunotherapy [11]. According to Farahani et al. it is possible that the anti-apoptotic effect of CD40 ligation on CLL cells is also mediated by VEGF, a well known cell survival promoter [7]. Our study was performed after 96 hours of culture. After such time CLL cells are sensitive to

CD95-mediated apoptosis and this effect is associated with upregulation of proapoptotic protein - Bid B-cell leukemia 2 homology 3 (BH3) interacting with domain death agonist [11]. According to other authors, this sensitization is mediated through an increase in FADD and DAP3 expression [2]. In another study, genes involved in apoptosis, *e.g.* CASP8 and FADD-like apoptosis regulator (FLICE), were upregulated in B-CLL cells after CD40 activation - those results are similar to our observations in ALL [8]. Most recent studies has shown that CD40 ligation sensitizes CLL cells to apoptosis through the activation of p73 (a p53-related transcription factor regulated by c-Abl kinase). CD40 activation also induced CD95 and Bid (BH3-interacting-domain death agonist) expression in leukemic cells [6]. In contrast to our observations, de Totero *et al.* noted that in B-CLL, FAS mRNA was upregulated after CD40 triggering but expression of other DISC molecules, i.e. FLICE, FADD and TRADD did not change in resting and activated cells [5]. They concluded that DISC molecules were not involved in this phenomenon. Despite CD95 upmodulation shown in their study, B-CLL cells were still resistant to CD95-mediated apoptosis. Another approach was presented by Romano *et al.* [20]: T cells from patients with B-CLL, preactivated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, led to higher susceptibility of leukemic cells to apoptosis via CD95 upregulation.

Interesting results concerning apoptosis of normal DC (not derived from neoplasmatic cells) in patients with breast cancer were obtained by Pinzon-Charry *et al.* [19]: supernatants from cancer lines induced apoptosis of DC but this effect could be prevented by ex vivo conditioning of DC with CD40L and/or IL-12. Additionally, DC expressing TRAIL (inducer of apoptosis) were effective for protection against leukemia relapse. TRAIL-transduced DC showed cytotoxicity through the induction of apoptosis in both leukemic cells and alloreactive T cells in graft-versus-host disease (GvHD) [22]. However, anti-CD40 monoclonal antibody led to reduced function of DC in generating an effective T-cell response. This was due to apoptosis via activation of FADD and caspase-8. Authors of that report emphasize this immunosuppressive activity [4].

In infant ALL cells, the low expression of FAS transcripts and low level of FAS protein is considered to be the main mechanism responsible for the resistance of these cells to apoptosis [27]. In another type of leukemia - AML - CD40 engagement rescued leukemic blasts from apoptosis. This effect was shown by reduced expression of APO2.7 and annexin-V binding, and additionally by upregulation of bcl-x (L) protein [1]. CD40 ligation also blocked apoptosis and induced multidrug resistance in non-Hodgkin's lymphoma cells through caspase independent and dependent pathways [25].

Concluding, we showed upregulation of important elements of apoptosis at mRNA level in ALL cells after CD40L and IL-4 stimulation. Understanding of apoptosis machinery in leukemic cells may provide new therapeutic potentials. The findings seem to support therapeutic exploitation of CD40 stimulation in ALL, despite its unclear influence on apoptotic profile of leukemic cells. We hope that our observations may have future implications for the therapy of ALL in children, *e.g.* in sensitizing leukemic cells to chemotherapy.

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