Upregulation of c-FLIP_{S+R} upon CD40 stimulation is associated with inhibition of CD95-induced apoptosis in primary precursor B-ALL

Short title: CD40-mediated upregulation of c-FLIP_{S+R} in BCP-ALL

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Abstract

Previous studies on apoptosis defects in acute lymphoblastic leukemia (ALL) have focused on chemotherapy-induced, primarily mitochondrial death pathways. Yet, immunological surveillance mechanisms including sensitization to apoptotic signals mediated via the death receptor CD95 might contribute to leukemic control. Here, we show that primary B-cell precursor ALL cells from children escape from receptor-dependent cell death in two ways: Resting ALL blasts are protected from receptor-mediated apoptosis due to the absence of CD95 surface expression. However, even though CD40 ligation results in upregulation of CD95, ALL blasts, unlike normal B cells, remain resistant to apoptosis. We show that this apoptosis resistance involves the selective upregulation of the short isoforms of the caspase-8 inhibitor c-FLIP acting directly at the CD95 receptor level. Treatment with cycloheximide during CD40-activation prevents upregulation of those c-FLIP isoforms and sensitizes ALL cells towards CD95-mediated apoptosis. We therefore propose that induction of the short c-FLIP isoforms inhibits the onset of CD95-induced apoptosis in primary CD40-stimulated ALL cells despite high CD95 expression.
Introduction

Acute lymphoblastic leukemia (ALL) in children is a malignant disease with a good overall prognosis. Treatment failure in those patients who suffer relapse has to a large part been attributed to drug resistance and defects in apoptosis. Consequently, investigations on the deregulation of apoptosis in ALL have primarily focused on chemotherapy-induced pathways\(^1,2\). Yet, immunological surveillance also contributes to long-term survival.\(^3,4\) The capacity of mature normal B cells to respond to CD40 ligation by upregulation of the CD95 (Fas/APO1) death receptor is critical for their susceptibility to immunological control.\(^5-7\) Primary B cell precursor ALL blasts (BCP-ALL) lack CD95 expression and are resistant to CD95-mediated cell death.\(^8\) Although BCP-ALL blasts express CD40 to variable degrees, there is no information on CD40-dependent modulation of blast sensitivity to apoptotic signals.\(^9\)

Death receptor-mediated pro-apoptotic signals are transmitted via the so called death-inducing-signaling-complex (DISC) formed upon oligomerization of CD95 by interaction with its ligand.\(^10\) The oligomerized death domains (DD) of the receptor associate with the cytoplasmic adapter molecule FADD with subsequent recruitment of procaspase-8 (FLICE) into the DISC. The apoptotic cascade is then initiated by autocatalytic cleavage of procaspase-8 and release of the active enzyme into the cytoplasm. The FLICE inhibitory protein (c-FLIP) can bind to the DISC and interfere with receptor-induced apoptosis.\(^11\) c-FLIP occurs in three different isoforms as a result of alternative splicing: two short isoforms, c-FLIP\textsubscript{Short} (27 kDa) and c-FLIP\textsubscript{Raji} (26 kDa), and a long form c-FLIP\textsubscript{Long} (55 kDa).\(^12,13\) While the short isoforms, thereafter called c-FLIP\textsubscript{S} and c-FLIP\textsubscript{R}, comprise only two death effector domains (DED) critical for interaction with the DISC, the long isoform contains an additional catalytically inactive C-terminal caspase-like domain. Depending on its expression level, c-FLIP\textsubscript{L} can serve as a dual regulator with both pro- and anti-apoptotic function.\(^14,15\) Thus, it has been
shown in adult T cell leukemia that it is the equilibrium between FLIP and caspase-8 that regulates susceptibility to CD95-mediated apoptosis.\textsuperscript{16} On the other hand, the short isoforms c-FLIP\textsubscript{S/R} play an established role as inhibitors of apoptosis preventing cleavage and activation of pro-caspases at the DISC.\textsuperscript{17}

In normal mature B cells most components of the DISC are constitutively expressed, while c-FLIP is tightly regulated.\textsuperscript{18} To evaluate the significance of receptor-induced apoptosis in primary BCP-ALL, we thus examined expression levels of the death receptor CD95 as well as blast sensitization to CD95-mediated apoptosis upon CD40 activation. Here we show that upregulation of c-FLIP\textsubscript{S/R} via CD40 confers resistance of BCP-ALL cells towards CD95-mediated apoptosis.
Material and Methods

The ALL samples were obtained from CoALL study patients. The respective protocols were approved by the ethics committee (IRB) in Hamburg as well as the local ethics committee (IRB) in Duesseldorf including the conductance of respective research projects. Informed consent was obtained from parents or legal guardians of the patients at the time of enrollment.

Induction of apoptosis in primary B cells and ALL blasts

After ethics committee approval and receipt of informed consent, normal and malignant B cells were isolated from the mononuclear cell fraction of peripheral blood or bone marrow by standard Ficoll gradient-centrifugation. All BCP-ALL samples employed exhibited a CD19/10 double positive preB- or c-ALL phenotype and contained >80% leukemic blasts. No proB-ALL samples were included. In normal B cells >80% purity was achieved by magnetic activated cell sorting using CD19 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). CD95 receptor expression was assessed by flow cytometry using CD95-PE coupled antibody (Becton Dickinson, San Jose, USA). For activation BCP-ALL blasts or normal B cells were cultured for 72 h on wild-type or transduced murine fibroblasts expressing human CD40-ligand (CD40L). Following crosslinking of 10^6 cells with 1 µg/ml anti-CD95 (CH-11, Immunotech, Marseille, France), CD95-specific apoptosis was determined by flow cytometry after staining with annexin V-FITC and propidium iodide for 10 min according to the manufacturer's protocol (Immunotech). Specific apoptosis was calculated as [experimental apoptosis(%)–spontaneous apoptosis(%)] / [100%–spontaneous apoptosis(%)] x 100.

Western blot analysis

Post-nuclear supernatant equivalents of 10^6 cells were separated by 12% SDS-PAGE and blotted onto nitrocellulose membranes as described. The following primary antibodies were employed: anti-c-FLIP-mAb NF6, anti-caspase-8 (Alexis Biochemicals, San Diego, USA).
and anti-FADD (BD Transduction Laboratories, San Jose, USA). Horseradish peroxidase-coupled isotype-specific secondary antibodies (Southern Biotech, Birmingham, USA) were used in combination with the chemiluminescence-based system (Chemicon, Temecula, USA) for detection.

Caspase activity assay

Induction of caspase-8-like and caspase-3/7-like activity was measured by a luminescent substrate (Caspase-Glo) assay according to manufacturer’s instructions (Promega, Madison, USA). After CD95 crosslinking for 4 h and 16 h respectively, cell lysates were incubated with the specific luminescent substrates which upon caspase cleavage and in conjunction with luciferase result in light emission that was measured in a luminometer.

Statistics

Expression levels and specific apoptosis rates in different cell types were compared by Mann-Whitney test. All statistical analyses were performed with the SPSS program version 12.0. A p-value of <0.05 was considered significant.
Results and Discussion

In order to investigate the sensitivity of primary B cell precursor blasts (BCP-ALL) towards death receptor–mediated apoptosis, we first assessed CD95 expression by flow cytometry. On primary BCP-ALL blasts and normal peripheral B cells, constitutive expression of CD95 was negligible. Following activation via CD40, a similar upregulation of CD95 cell surface expression was observed in both cell types (p=0.68) (Figure 1A). Yet, while levels of CD95 expression were comparable in CD40-activated BCP blasts and normal B cells, receptor-specific apoptosis was significantly lower in BCP-ALL (p=0.001) (Figure 1A, B). In keeping with this observation, induction of initiator caspase-8- and effector caspases-3/7-like activity remained unchanged in CD40-stimulated blasts upon CD95 crosslinking, while caspase activity increased in activated normal B cells (Figure 1C).

As inhibition of caspase-8 in CD40-stimulated blasts suggests a proximal block in the CD95-pathway, molecules participating in formation of the death-inducing signaling-complex (DISC) were assessed by Western blot analysis. FADD, procaspase-8 and the long isoform of c-FLIP were constitutively expressed in ALL as well as in normal B cells and expression remained largely unchanged upon CD40 stimulation (Figure 2A). In contrast, the anti-apoptotic short isoforms c-FLIP_S and c-FLIP_R were barely detectable in both non-activated cell types. Interestingly, in ALL blasts pronounced upregulation of c-FLIP_S+R was observed following ligation of CD40, while in normal B cells expression of the short isoform c-FLIP_S remained weak and c-FLIP_R was absent (Figure 2A). Specific upregulation of the anti-apoptotic isoforms c-FLIP_S+R in CD40-activated ALL blasts with c-FLIP_S+R exceeding c-FLIP_L expression was subsequently confirmed in five individual patients by quantitative densitometry (Figure 2B).
To further delineate the role of c-FLIP_{S+R} expression in resistance of BCP-ALL blasts towards CD95-mediated apoptosis, protein synthesis was inhibited by addition of cycloheximide. Indeed, cycloheximide prevented upregulation of both short isoforms of c-FLIP in CD40-activated blasts, particularly c-FLIP_{R} (Figure 2C) and sensitized BCP-ALL blasts to CD95-mediated cell death following CD40-ligation (Figure 2D). This demonstrated that modulation of c-FLIP_{S+R} expression was closely associated with resistance and sensitivity to CD95-mediated apoptosis in primary BCP-ALL.

Thus, BCP-ALL cells can escape from receptor-dependent cell death in two ways: Resting ALL blasts are shielded against elimination by receptor-mediated apoptosis via the absence of CD95 surface expression. Upon activation by CD40L, blasts then evade programmed cell death by overexpression of c-FLIP_{S+R} counteracting concomitant upregulation of CD95. c-FLIP isoforms play a critical role in resistance to death-receptor mediated apoptosis in a variety of human cancers including B cell-derived malignancies such as Hodgkin and B-cell lymphomas with high levels of c-FLIP associated with drug resistance, escape from cytotoxic effector mechanism and poor clinical outcome. In adult T-ALL blasts are protected from CD95-mediated apoptosis by HTLV 1-Tax. Here we describe for the first time that in BCP-ALL, strong and selective expression of the two short isoforms of c-FLIP facilitates resistance to receptor-mediated apoptosis. c-FLIP_{S+R} remain stably expressed in ALL cells for six days following CD40-activation (data not shown). This is clearly different from mature normal and malignant B cells where upregulation of c-FLIP following CD40-activation is transient. Activated tonsillar B cells are thus only temporarily protected from CD95-mediated cell death. Similarly in chronic lymphocytic leukemia (CLL), CD40L induces a shift in expression levels of the anti- and pro-apoptotic molecules of the DISC with initial protection but subsequent sensitization of CD40-stimulated CLL cells to receptor-dependent cell death. Moreover, this is the first report on inducible protein expression of the short isoform c-FLIP_{R} in primary leukemic blasts of the B cell lineage. With CD40-induced
expression of c-FLIP<sub>S+R</sub> we thus delineate a novel receptor-associated anti-apoptotic mechanism in BCP-ALL complementing the inhibitors of the mitochondrial apoptosis pathways.
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References


Figures and legends

**Figure 1.** Upregulation of CD95 expression in BCP-ALL blasts following activation via CD40 does not translate into sensitization to apoptosis. (A) Expression of the death receptor CD95 was analyzed by flow cytometry (median; range) in normal peripheral B cells (n=8) and BCP-ALL blasts (n=15) after 72 h culture on non-modified feeder cells or feeder cells transgenically expressing CD40L. (B) For assessment of receptor-mediated cell death, cells were also removed from feeder cells and stimulated for apoptosis induction with an agonistic CD95 monoclonal antibody (mAb) for 16 h. Apoptosis was measured by flow cytometry using annexin V and propidium iodide staining (median; range). The increase in spontaneous apoptosis in the absence of anti-CD95 did not exceed 5% regardless of CD40 activation. Despite equivalent upregulation of CD95 in CD40-activated normal B cells and BCP-ALL, induction of specific apoptosis is significantly lower in ALL blasts (p=0.001). (C) Caspase-8 and caspase-3/7-like proteolytic activities were examined in CD40-activated normal peripheral B cells (n=4) and BCP-ALL blasts (n=4) in a luminescent substrate assay after crosslinking with anti-CD95-mAb for 4 h (caspase-8) and 16 h (caspase-3/7), respectively. The assay was performed in triplicates of 2.5 x 10^4 cells/well for caspase-8 and 1 x 10^4 cells/well for caspase-3/7. The increase in CD95-induced caspase activity in normal B cells and BCP-ALL blasts following activation via CD40 is shown. The increase is presented as the ratio of CD40-activated over resting B cells and blasts (mean±SEM).

**Figure 2.** Pronounced upregulation of the short isoforms of c-FLIP in CD40-activated ALL blasts mediates resistance to death receptor-dependent apoptosis. (A) For assessment of expression levels of molecules that form the death-inducing signaling complex (DISC), cell lysates of resting and CD40-activated normal B cells and ALL blasts were analyzed by Western blotting. (B) The relative expression of c-FLIP_{S,R} and c-FLIP_{L} proteins in CD40-activated normal B cells and ALL blasts was measured by densitometry using Image Java
software program. Results of samples from 5 individual patients and 4 normal B cell controls are represented as the mean ± SEM of FLIP_{SR}/FLIP_{L} peak area ratio. (C,D) CD40-activated ALL cells were treated with anti-CD95 mAb in the absence or presence of 0.1 µg/ml cycloheximide (CHX). After 48 h, expression of the long and short isoforms of c-FLIP was examined by Western blot analysis (C) and CD95-dependent apoptosis was measured by flow cytometry (D). Values represent mean percentage of specific apoptosis ± SEM (n= 7 patients) (t-test ±CD40L; p=0.004; t-test CD40L+CHX; p= 0.01).
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A

CD95 Expression (%)

B cells BCP-ALL B cells BCP-ALL
+CD40L +CD40L

B

Specific Apoptosis (%)

B cells BCP-ALL
+CD40L +CD40L

C

X-fold increase of caspase activity

Caspase 8 Caspase 3

B cells BCP-ALL
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