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Differential responses of FLIP_{long} and FLIP_{short} overexpressing human myeloid leukemia cells to TNF α and TRAIL-initiated apoptotic signals

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Abstract

Objective—Clonal marrow cells from patients with *early* myelodysplastic syndrome (MDS) undergo apoptosis in response to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Cells from *advanced* MDS are resistant to TRAIL. Two isoforms of the Flice inhibitory protein, FLIP_{short}(S) and FLIP_{long}(L), which modulate TRAIL signals, showed disease-stage-dependent differential regulation. Therefore, we aimed at characterizing potential differential effects of FLIP_L and FLIP_S, on TRAIL and TNF α -induced apoptosis in model leukemic cell lines.

Methods—Using lentiviral constructs, FLIP_L and FLIP_S, as well as a green fluorescent protein (GFP) control were overexpressed in ML-1 cells, which constitutively express very low levels of FLIP and are highly sensitive to apoptosis induction. Cells were then exposed to TRAIL or TNF α , and effects on the extrinsic and intrinsic pathways of apoptosis induction were assessed.

Results—Overexpression of FLIP reduced TRAIL and TNF α -induced apoptosis in ML-1 cells. However, while FLIP_L completely abrogated apoptosis, FLIP_S allowed for BID cleavage and caspase-3 activation. Concurrently there was a decline of Bcl-xL and XIAP in FLIP_S cells followed by apoptosis. Further, inhibition of NF- κ B activation in TNF α -treated cells resulted in profound apoptosis in FLIP_S, but not in FLIP_L overexpressing cells, consistent with the observations in patients with early stage MDS. Inhibition of NF- κ B had only minimal effects on TRAIL signaling.

Conclusion—Thus, FLIP_L and FLIP_S exerted differential effects in myeloid leukemic cell lines in response to TRAIL and TNF α . It might be possible to therapeutically exploit those differences with effector molecules specific for the FLIP isoforms.

INTRODUCTION

Dysregulation of apoptosis in the bone marrow is a central feature in patients with myelodysplastic syndrome (MDS) [1]. Clonal expansion of autoreactive T lymphocytes is

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observed in many patients [2], and upregulation of tumor necrosis factor (TNF) α and TNF-related apoptosis inducing ligand (TRAIL) occurs in almost all patients [3,4]. TRAIL (also termed Apo2L) induces apoptosis preferentially in neoplastic cells [5,6]. However, many tumors show resistance to TRAIL [7–9], due to various mechanisms. TRAIL cross links agonistic receptors DR4 and DR5, which leads to formation of the death-inducing signaling complex (DISC) and activation of caspase-8 [10]. Signals initiated via death receptors are modulated by several downstream regulators. One of these adaptor molecules, termed FLIP (FLICE inhibitory protein), is a structural homolog of caspase-8 (initially described as FLICE) [11]. At least two splice variants, FLIP long (FLIP_L) and FLIP short (FLIP_S), are expressed in a variety of eukaryotic cells. The full-length FLIP_L (55kDA) contains two death effector domains (DEDs) and a catalytically inactive caspase-like domain and shows overall homology to procaspase-8; the shorter form, FLIP_S (26 kDA), has two tandem DEDs followed by a short C-terminal tail.

Initial studies in patients with MDS provided evidence that FLIP_L protein levels correlated with progression of the disease to a more advanced stage (with predominant proliferation), which was also associated with increasing levels of activation and nuclear translocation of NF- κ B [12]. FLIP_S, in contrast, showed a positive correlation with apoptosis in the early stages of MDS [13]. While differences between FLIP_L and FLIP_S have been described by several investigators [11,14,15], virtually all those studies were carried out in lymphoid cells [11,15, 16], and it has been suggested that FLIP_S completely inhibited cleavage of caspase-8, whereas FLIP_L did not [15]. Based on our data in patients with MDS we were interested in characterizing potential functional differences between the two isoforms in myeloid (rather than lymphoid) cells, and determine whether those differences could explain observations in patients with less advanced and more advanced MDS. Thus, we used leukemia-derived hematopoietic cell lines with different levels of expression of FLIP, including ML-1 cells, which constitutively express very low levels of FLIP and are sensitive to apoptosis induced by various death ligands, as models to determine the effects of constitutive levels and overexpression of FLIP_L and FLIP_S on TRAIL- and TNF α -induced apoptosis.

MATERIALS AND METHODS

Materials

TRAIL (Killer TRAIL™ Apo-2L, soluble [human] recombinant) was obtained from ALEXIS Biochemicals (San Diego, CA). Monoclonal antibodies to human caspase-8 (clone 1C12), caspase-3 (clone 3G2), BID, cytochrome c, XIAP, Bcl-xL, I κ B α and phospho-I κ B α (5A5) were purchased from Cell Signaling Technology (NEB Frankfurt, Germany). The anti-actin antibody was purchased from Sigma (St. Louis, MO). The monoclonal anti-FLIP antibody (NF6) was a kind gift from Dr. P. Krammer (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Monoclonal antibodies to TRAIL receptor 1 (DR4; clone 69036), TRAIL receptor 2 (DR5; clone 71908), TNF receptors 1 (clone 16803) and 2 (clone 22235), and isotype controls were all purchased from R&D Systems, Inc., Minneapolis, MN. The secondary antibodies (anti-rabbit and anti-mouse) were conjugated to horseradish peroxidase (Pierce, Rockford, IL). Signals were detected using the ECL system (Pierce).

Caspase-8 inhibitor Z-IETD-FMK was purchased from BD Pharmingen (San Diego, CA); EnzChek Caspase-3 Assay Kit #1 *Z-DEVD-AMC substrate* from Molecular Probes, Inc (Eugene, OR); Caspase-8 Assay Kit, Fluorimetric, from Sigma (St. Louis); AnnexinV-PE apoptosis detection Kit from BD Pharmingen, and Platinum SybrGreen qPCR Supermix UDG from Invitrogen (Carlsbad, CA).

Cell cultures and sample preparation

The human myeloid leukemia cell line ML-1, derived from a patient with acute myeloid leukemia, the monocytoid cell line U937, derived from a histiocytic lymphoma patient, and the myeloblastic cell line KG-1a, a subclone of a cell line derived from the bone marrow of a patient with acute myeloid leukemia were maintained in RPMI 1640 medium, containing 10% heat-inactivated fetal bovine serum (FBS), in a humidified 5% CO₂ environment at 37°C. The myeloblastic cell line HL-60, derived from a patient with acute promyelocytic leukemia, was maintained in Iscove's Modified Dulbecco's Medium (obtained from ATCC), containing 10% heat-inactivated FBS in a humidified 5% CO₂ environment at 37°C. ML-1, U937 and HL-60 cells stably expressing FLIP_L, FLIP_S, or the control vector (Neo.GFP) were cultured under the same conditions as the wild-type cells.

Lentivirus constructs

To overexpress FLIP, we used the lentiviral vector pRRLMSCV.ires2eGFP.sin. The vector plasmid was digested with *AgeI* and *BamHI*. cDNA was prepared from total RNA extracted from KG-1a cells, which show constitutively high FLIP expression. *AgeI* and *BglII* sites were added to the 5' and 3' ends, respectively, of the PCR product prepared with the use of Hifi Taq polymerase. PCR products were gel purified and digested with the above enzymes, ligated into the vector, and checked for sequence integrity.

FLIP_S was cloned into the *EcoRI/BamHI* sites of the same lentiviral vector. The FLIP_S coding sequence was obtained as a PCR product engineered with *EcoRI/BglII* sites. The template for FLIP_S was the vector construct Flag-hFLIP short in PCR-3 kindly provided by P. Schneider, University of Lausanne (Switzerland). As control, the irrelevant gene Neomycin (*Neo*) was cloned into the same vector as an *EcoRI/BglII* PCR fragment.

Lentiviral stock generation and transduction of cell lines

To produce lentivirus, 293T-cells were co-transfected with the overexpression constructs along with the constructs containing the gag/pol and the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotype lentiviral envelope using calcium phosphate precipitation as previously described [17]. Lentiviral supernatants were collected at 18, 30, and 42 hours after cotransfection in DMEM (containing 10% heat inactivated FBS 1% P/S + 20mM HEPES), filtered through a 0.22 µm filter, concentrated 100-fold by centrifugation for 24 hours at 7200×g, and then resuspended in 1/100 of the volume in Iscove's medium. Cells were transduced with concentrated viral stocks expressing FLIP or control vector (Neo.GFP).

Treatment of cells and determination of apoptosis

Cells were treated with TRAIL over a dose range from 100 ng/ml to 500 ng/ml in increments of 100. After 24 hours, treatment was stopped by washing with cold PBS containing 2% BSA. Cells were then stained with Annexin V-PE and 7AAD according to the directions of the supplier, analyzed by FACScan (Becton Dickinson, Mountainview, CA), and results were examined using CellQuest software (Becton Dickinson).

Western blot analysis

Total cell lysates were obtained using Chaps Cell Extract Buffer (Cell Signaling Technology, NEB, Frankfurt, Germany) according to the manufacturer's protocol. Protein concentrations were determined using the Bradford assay (BIO-RAD, Hercules, CA). Equal amounts of protein (50 µg) were separated by 4%–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF; BIO-RAD, Hercules, CA). After blocking with 5% non-fat dry milk at room temperature for 1 hour, membranes were incubated overnight at 4°C with the primary antibody. Monoclonal antibodies for

caspase-8, caspase-3, cytochrome c, XIAP, I κ B α , phospho-I κ B α , and actin were used at concentrations of 1:1000. Antibodies to BID, Bcl-xL and FLIP were used at concentrations of 1:500. The secondary antibodies (anti-rabbit and anti-mouse) were used at dilutions of 1:20,000 and 1:10,000, respectively. Signals were detected using the ECL system. Appropriate loading controls were included in all experiments.

Quantitative real-time PCR

cDNA was prepared from total RNA from transduced ML-1 cells using Oligo-dT20 as primers and Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA). PCR primers for each gene were designed as previously reported for FLIP_L, FLIP_S, and β 2-microglobulin [13], with melting temperatures at 52–56°C and resulting products of 124, 93, and 338 bp, respectively. Each PCR was carried out in triplicate in a 25 μ L volume using SYBR Green Master Mix (Invitrogen, Carlsbad, CA) for 2 minutes at 50°C, 2 minutes at 95°C, and 50 cycles of 95°C for 30 seconds and 60°C for 45 seconds in the ABI Prism 7700 sequence Detection System. Cloning of FLIP_L and FLIP_S cDNA into pcDNA3.1 plasmids was performed as previously described [13] to construct standard curves for each transcript analyzed. β 2-microglobulin was cloned into pcr2.1 TOPO Vector (Invitrogen, Carlsbad, CA) using a standard protocol. Values for the genes tested were normalized to expression levels of β 2-microglobulin.

Caspase-3 fluorimetric assay

Cells in triplicates (1×10^6 cells each) were treated with TRAIL at 300 ng/ml for 4 hours (conditions determined in ancillary studies) to induce apoptosis. The reaction was stopped by washing twice with cold PBS. The cells were then resuspended in 50 μ L of lysis buffer, and lysed by freeze/thaw. After centrifugation, 50 μ L of the supernatant was used in the assay. To one set of TRAIL-treated cells, the inhibitor Ac-DEVD-CHO (for Caspase-3) was added to assess background activity of Caspase-3 in individual cell lines. Fifty μ L of 2 $\times$ substrate (z-DEVD-AMC, containing a Caspase-3 specific cleavage site, thus releasing fluorometrically active AMC) was added to each sample including controls and incubated for 30 minutes. Readings were obtained on a fluorometer (excitation: 360 nm; emission: 460 nm). For calibration, a standard 7-amino-4-methylcoumarine (AMC) curve was constructed.

Caspase-8 fluorimetric assay

To induce apoptosis, triplicates of 1×10^6 cells each were treated with TRAIL at 300 ng/ml for 0.5 to 3 hours. The reaction was stopped by washing with cold PBS. The cells were then lysed in 50 μ L of lysis buffer and used in the assay. To one set of cells treated with TRAIL for 3 hours, the inhibitor Ac-IETD-CHO (for Caspase-8) was added to assess background activity of Caspase-8 in individual cell lines. Ten microliters of substrate and 40 μ L of Assay Buffer were added to each sample and incubated for 30 minutes. As for caspase-3, readings were obtained on a fluorometer (excitation: 360 nm; emission: 440 nm).

Nitrogen cavitation

Nitrogen cavitation for cell disruption to obtain mitochondria from cultured cells was carried out as described by Gottlieb et al [18]. Following treatment in the respective experiments, 10×10^6 cells, they were washed twice with cold PBS, and the pellets were stored at -80°C . On the day of nitrogen cavitation, pellets were resuspended in 2 ml of homogenization buffer (1 mM EGTA, 0.25 M Sucrose, 10 mM Tris, pH 7.4 + protease inhibitors). Samples were introduced into a vessel designed for lysis of cells (the “bomb”), and subjected to high pressure. Lysates were centrifuged twice at $800\times g$ for 5 minutes to remove nuclei and intact cells, then at $10,000\times g$ for 30 minutes to isolate mitochondria. The mitochondria pellet was resuspended in wash buffer containing 2% Chaps supplemented with protease inhibitors. The supernatant

containing the cytosolic fraction was concentrated through centrifugation by microcon YM-10. All steps were carried out at 4°C.

Statistics

Student's t-test was used for comparison of results. Bar graphs represent results as mean $\pm$ 1 standard deviation.

RESULTS

Constitutive expression of FLIP_L and FLIP_S in myeloid cell lines

Constitutive levels of FLIP expression were determined in ML-1, U937, HL60, and KG-1a cells (Figure 1A). The lowest levels of FLIP were observed in HL60 and ML-1 cells, while U937 expressed moderate and KG-1a high levels of FLIP. As shown in Figure 1B, apoptotic responses of these cells to TRAIL exposure varied. U937 showed the highest apoptotic index (despite measurable levels of FLIP) followed by ML-1 and HL60, while KG-1a exhibited little or no response. These data are consistent with a role of FLIP in TRAIL-mediated apoptosis, although the findings in U937 cells indicate that additional factors are involved, possibly in a lineage dependent fashion.

TRAIL induced apoptosis in ML-1 cells with overexpression of FLIP

ML-1, HL60, and U937 cells were transduced with the appropriate lentiviral vectors to express FLIP_S or FLIP_L. As determined by real-time SYBR Green PCR, mRNA levels for FLIP_L and FLIP_S were increased, with the corresponding vectors (not shown). FLIP_S-transduced cells revealed the expected 26 kDA band (along with smaller cleavage products), while a 55 kDA band representing FLIP_L (along with cleavage products – distinct for each cell line) was present in cells transduced with FLIP_L (Figure 2). Since ML-1 cells expressed the lowest amounts of FLIP among all cell lines tested and were susceptible to apoptosis, ML-1 was chosen as the model cell line. As shown in Figure 3, transfection of ML-1 cells with control vector or FLIP_L or FLIP_S did not significantly alter the expression of receptors for TRAIL (expression of receptor 1 [DR4] was low in all cell lines) nor for TNF α . To determine the effects of FLIP overexpression on apoptosis, transduced ML-1 cells were exposed to TRAIL over a dose range of 100–500 ng/ml. The maximum rate of apoptosis was reached at TRAIL concentrations of 300 ng/ml, with a plateau at higher doses. FLIP_L overexpression provided virtually complete protection of ML-1 cells against apoptosis, while overexpression of FLIP_S allowed for a significant rate of apoptosis, intermediate between FLIP_L and Neo.GFP-overexpressing control cells (Figure 4, panels A and B). Expression of TRAIL receptor 2 (DR5) decreased significantly in response to TRAIL (Figure 3). The pattern of TRAIL responses in U937 and HL60 cells was similar to that in ML-1 cells (Figure 4, panels C and D).

Effects of FLIP overexpression on Caspase-8 and Caspase-3 activation

FLIP_L, FLIP_S and Neo.GFP-transduced ML-1 cells were treated with TRAIL, 300 ng/ml, for 30 minutes to 4 hours, and caspase activity was determined (Figure 5). In Neo.GFP expressing control cells, the active p18 subunit of Caspase-8 was easily detectable by western blot, whereas only trace amounts were present in FLIP_L and FLIP_S overexpressing cells (Figure 5A). However, as absence of p18 cannot be taken as proof of a lack of activation of Caspase-8 [19], we next determined the response of the downstream executioner caspase, Caspase-3. We reasoned that even if small differences at the Caspase-8 level were below detection limits on western blots, they might be amplified in downstream responses. As shown in Figure 5B, Western blots showed clear cleavage of Caspase-3 into the p19/p17 doublet in Neo.GFP control cells, only a faint band in FLIP_S-, but not in FLIP_L-overexpressing cells.

Potential differences between FLIP_L and FLIP_S were further investigated in a fluorometric assay of caspase-3-specific substrate cleavage. As shown in Figure 5C, active caspase-3 levels were two-fold higher in FLIP_S than in FLIP_L-transduced cells, consistent with the Western blot in Figure 2B. These data suggested a quantitative if not qualitative difference between FLIP_L and FLIP_S in TRAIL-initiated apoptosis. However, as caspase-3 is activated via both the extrinsic and the intrinsic pathways, the observed response could not be assigned to one or the other pathway. A similar fluorometric assay to assess active caspase-8 quantitatively failed to show significant differences in the levels of active caspase-8 between the transduced cell lines (results not shown).

BID cleavage correlates with higher rates of apoptosis in FLIP_S cells

Activated caspase-8 mediates downstream apoptotic events that include mitochondrial injury and caspase-3 activation [20,21]. Caspase-3 activation via the intrinsic pathway involves BID cleavage (and generation of tBID), translocation to the mitochondria, and release of cytochrome c into the cytoplasm. We had shown that TRAIL activated the intrinsic pathway in ML-1 cells [22]. To determine to what extent overexpression of FLIP offered protection against apoptosis via the intrinsic pathway, we treated ML-1 cells with TRAIL, performed nitrogen cavitation, and isolated the mitochondrial and cytosolic fractions. As shown in Figure 6, cleavage of BID and formation of tBID in the mitochondrial fraction occurred in Neo.GFP control cells. No band was detectable in FLIP_L-overexpressing cells. The bands of tBID were present in FLIP_S overexpressing cells, albeit very faint. However, together with the cytochrome c data (see below), these results suggested that the presence of FLIP_S did allow for low level activation of the mitochondrial pathway.

Caspase-8-induced cytochrome c release is not inhibited by zIETD-fmk

Since caspase-8 functions upstream of caspase-3, we reasoned that inhibition of caspase-8 in TRAIL-treated ML-1 cells would prevent BID cleavage. As shown in Figure 5, addition of the caspase-8 inhibitor, z-IETD-FMK, 30 minutes or 1 hour before TRAIL exposure prevented or reduced the translocation of tBID into the mitochondria (Figure 6D), although there was no significant effect on cytochrome c release from the mitochondria relative to baseline levels. Conceivably, other effector molecules, including caspase-6 and caspase-7, which need not cleave BID to release cytochrome c, or unidentified proteases were activated [23,24].

Overexpression of FLIP_L prevents downregulation of Bcl-xl and XIAP in TRAIL-exposed ML-1 cells

The above data suggested a contribution of the mitochondrial pathway to TRAIL-mediated apoptosis in ML-1 cells. We attempted to determine, therefore, how FLIP overexpression interfered with the translocation of tBID to the mitochondria and the release of cytochrome c into the cytosol. We examined two representative anti-apoptotic proteins, XIAP and Bcl-xl. XIAP is a member of the inhibitor of apoptosis protein family that selectively binds to and inhibits caspases-3, -7 and -9, but not caspase-8 [25]. Pro-survival factors Bcl-2 and Bcl-xl sequester BH3 domain-only molecules (such as BID) and prevent Bax- and Bak-mediated mitochondrial apoptosis [26]. Bax and Bak undergo allosteric conformational activation including their intramembranous oligomerization in response to translocation of tBID to the mitochondria [27,28]. These reactions were of interest as an amplification loop between FLIP and NF- κ B, the transcription factor involved in Bcl-xl and XIAP regulation, has been recognized [16,29–31]. In control Neo.GFP-transduced ML-1 cells, TRAIL exposure resulted in downregulation of both Bcl-xl and XIAP (Figure 7, panels A and B), while FLIP_L-overexpressing ML-1 cells were basically resistant to the downregulating effect of TRAIL, and both XIAP and Bcl-xl expression remained unchanged. In contrast, FLIP_S-overexpressing ML-1 cells showed downregulation of both Bcl-xl and XIAP, albeit to a lesser degree than

observed in control cells. The level of downregulation of Bcl-xL and XIAP in FLIP_S cells correlated with the release of cytochrome c, which was completely prevented by FLIP_L. These observations were also in agreement with the concept that FLIP employed the NF-κB autoamplification loop to maintain the levels of Bcl-xL and XIAP.

Effect of NF-κB inhibition on TRAIL- and TNFα-induced apoptosis

To further characterize the role of the FLIP/NF-κB amplification loop, we determined the function of NF-κB in the regulation of apoptosis in ML-1 cells. TRAIL induced weak phosphorylation of IκB at 5 minutes, with a second rise beginning at 30 minutes, associated with a decline in total IκB levels (Figure 8A). Pretreatment of cells with IKK4, an NF-κB inhibitor which inhibits IKK2, the kinase primarily responsible for phosphorylation of IκB resulted in an increase in apoptosis, which was significant in FLIP_L- (p=0.011; Figure 8B) but not in FLIP_S-transduced cells. This outcome suggested that this pathway was relevant for FLIP_L modification of apoptosis.

As TNFα is a stronger activator of NF-κB than TRAIL, we also determined the effect of inhibition of NF-κB on TNFα-induced apoptosis. However, TNFα mediates both survival and apoptosis signals, and it is not as strong a mediator of apoptosis as is TRAIL. As shown in Figure 8C, TNFα, similar to TRAIL, induced phosphorylation of IκB in a biphasic pattern; phosphorylation of IκB was stronger than seen with TRAIL. As illustrated in Figure 8D, TNFα induced a low rate of apoptosis basically only in Neo.GFP-expressing control cells, but not in FLIP_S or FLIP_L-overexpressing ML-1 cells. As anticipated, there was a significant decrease in the level of TNF receptor 1 expression following exposure to TNFα (Figure 3C). If NF-κB activation was blocked by IKK4, apoptosis increased significantly in FLIP_S cells (p=3.7 × 10⁻⁸), exceeding, in fact, the rate of apoptosis in control cells, while no apoptosis was seen in FLIP_L-overexpressing cells.

DISCUSSION

Marrow cells from patients with early stage MDS show high rates of apoptosis. However, as the disease progresses, apoptosis declines, proliferation becomes prominent, and leukemia may develop [32–36]. Several death ligands and their receptors, including FasL [3,37,38], TNFα [39], and TRAIL are upregulated in patients with MDS [1,40]. The susceptibility of hematopoietic progenitor cells to apoptosis in patients with MDS is regulated by numerous factors, including an abnormally high ratio of pro- to anti-apoptotic proteins, defects in anti-apoptotic signal transduction pathways or the lack of positive (cytoprotective) signals [36,41, 42]. One central regulator is FLIP [13]. Overexpression of anti-apoptotic gene products such as FLIP may occur via various pathways [43,44]. We characterized previously the expression of the two major splice variants of FLIP, FLIP_L and FLIP_S, in patients with MDS [13]. FLIP_S was prominent in early stages of MDS with high rates of apoptosis, while FLIP_L expression was highest in advanced MDS when cells showed increased resistance to apoptosis [13]. FLIP_S has generally been described as a molecule with antiapoptotic functions, and it was not immediately apparent why levels of FLIP_S in MDS cells should be higher in the context of high rates of apoptosis [13]. However, most previous studies analyzing the two splice variants have been carried out in lymphoid cells or cell lines [11,15].

The objective of the present study was to characterize functional differences between FLIP_S- and FLIP_L-overexpressing myeloid leukemia-derived ML-1 cells in regards to TRAIL- or TNFα-induced apoptosis. Overexpression of both FLIP splice variants in ML-1 cells resulted in decreases in TRAIL-mediated apoptosis. However, while FLIP_L transduction abrogated apoptosis virtually completely, FLIP_S-overexpressing cells allowed for transmission of pro-apoptotic signals. Others have reported that FLIP expression correlated with resistance to death receptor-mediated apoptosis [43,45–49], and, conversely, inhibition of FLIP by siRNA

resulted in restoration of cellular sensitivity to death receptor–induced apoptosis [50]. However, no quantitative differences between FLIP_L and FLIP_S have been reported, although Hietakangas, et al. [51] showed differences between FLIP_L and FLIP_S in regards to their effects on caspase-8 cleavage in K562 cells, and Krueger et al in lymphoid cell lines [15]. While our studies showed slightly lower expression of TRAIL receptor 1 in FLIP_S and FLIP_L overexpressing cells than in wild-type cells, the levels were comparable to those in Neo.GFP transduced cells. No differences in expression were seen for the dominant receptor 2, and it appears unlikely that differences between the two cell lines were related to differences in receptor expression.

To define more precisely how downstream events in the TRAIL-mediated apoptosis pathway were affected by overexpression of FLIP_L or FLIP_S, expression of several pro-apoptotic and anti-apoptotic factors was determined. No significant differences in caspase-8 activation were noted between FLIP_L and FLIP_S-overexpressing ML-1 cells; it may be of note in this context that caspase-8 activation via proximity-induced dimerization without cleavage has been reported [52]. Moreover, the association of caspase-8 with FLIP_L at the DISC level is sufficient to directly activate the enzymatic pocket of caspase-8, although caspase-8 activated by this mechanism has altered substrate specificity, presumably because the heterodimer is attached to the membrane while activated caspase-8 homodimers are released into the cytosol [53,54]. This pathway of activation, which occurs independently of cleavage of either caspase or FLIP_L, was not detected by western blot assay [19].

FLIP_S, on the other hand, has been reported to exert only inhibitory function [15,55]. In most cells caspase-8 (or other initiator caspases) are only activated to a low degree and then further amplified by downstream events, involving the generation of ceramide, leading to activation of executioner caspases [56–58]. Therefore, mechanistic differences in the inhibition or activation of caspases by FLIP_L and FLIP_S may cause only minor differences at the levels of active caspase-8 and might be undetectable in our assays. However, the level of active caspase-3 in FLIP_S overexpressing ML-1 cells was two-fold higher than in FLIP_L cells. This observation was consistent with the concept that FLIP_S allows for downstream signaling, apparently through the mitochondrial pathway. That interpretation was supported by the finding that FLIP_S-overexpressing cells also showed cytochrome c release, while FLIP_L-overexpressing cells did not. Preliminary in vivo results also support this concept as tumors of FLIP_S overexpressing ML-1 cells respond to TRAIL treatment, whereas FLIP_L overexpressing cells do not (H.J. Deeg, unpublished).

The release of cytochrome c in the presence of a caspase-8 inhibitor in control cells was unexpected. However, this may have been the result of incomplete inhibition of caspase-8 or the contribution of other initiator caspases. Also, proteolytic processing of caspase-8 may not be required for activity and may be an event following downstream caspase-3 activation [59]. The role of caspase-10 in TRAIL-mediated signaling, the recruitment of caspase-10 to the TRAIL DISC complex [25], and its requirement in apoptosis induction are controversial [60]. Finally, caspase-2 can only function through cleavage of BID in the TRAIL-induced apoptotic pathway in selected Type II cells [59] because it neither processes nor activates executioner caspases directly [61].

Also consistent with differences in the degrees of apoptosis between FLIP_L and FLIP_S-overexpressing cells was the pattern of expression of anti-apoptotic molecules in response to TRAIL: while levels of Bcl-xl and XIAP were basically maintained in FLIP_L cells, they declined in FLIP_S cells. We considered the possibility of Bcl-xl and XIAP being upregulated in FLIP_L cells via a FLIP_L-NF- κ B auto-amplification loop. However, the presence of IKK4, intended to block NF- κ B activation, had no significant effect on Bcl-xl and XIAP levels in comparison to cells treated with TRAIL in the absence of IKK4 (data not shown). In contrast

to findings in TRAIL-treated cells, the response of ML-1 cells to TNF α , a potent activator of NF- κ B, was markedly enhanced by IKK4, and the augmentation of apoptosis in FLIP_S cells exceeded that in control cells. Yet there was no significant increase in apoptosis in FLIP_L cells. This observation indicated that under certain conditions FLIP_S functions differently from FLIP_L in myeloid cells and may actually facilitate apoptosis.

For TNF α to trigger survival signals through NF- κ B, the JNK cascade must be suppressed [62]. Park et al. reported that upon TNF α binding to cells, c-FLIP_S was more rapidly recruited into FADD than FLIP_L, leading to activation of JNK through TRAF-2 [63]. Those data are consistent with the observations that inhibition of NF- κ B activation in TNF α treated cells allows for death via the JNK pathway [64,65]. Since FLIP_S allows for activation of the JNK pathway [63], once NF- κ B is suppressed, apoptosis in these cells can increase to levels observed in control cells. Further, Micheau et al. showed that apoptosis induced via TNF receptor1 (p55/TNFR1) involves two sequential signaling complexes [66]. The initial plasma membrane-bound complex (complex I) consists of TNFR1, the adaptor TRADD, the kinase RIP1, and TRAF2, and rapidly signals activation of NF- κ B. In a second step, TRADD and RIP1 associate with FADD and caspase-8, forming a cytoplasmic complex (complex II). When NF- κ B is activated by complex I, complex II harbors FLIP_L, and cells survive. Thus, the TNFR-1-mediated signal includes a checkpoint where a decision is made as to whether TNF α acts to promote cell survival or apoptosis. Conceivably, FLIP_L overexpressing cells recruit FLIP_L in complex II, which could explain why these cells were highly resistant to TNF α -induced apoptosis, even with NF- κ B blockade. Finally, it has been shown that c-FLIP binds to MKK7 and thereby inhibits the JNK pathway [67]. An additional reason why FLIP_L functions as a more robust inhibitor of caspases than FLIP_S may be the inherent instability of the FLIP_S isoform, which is prone to ubiquitination and has a considerably shorter half-life, apparently determined by its unique 19 amino acid tail [68].

In summary, the current study provides evidence for differential effects of FLIP_L and FLIP_S in myeloid leukemia cell lines that might explain earlier observations in patients with MDS [13]. High levels of apoptosis in early stage MDS in the presence of high levels of FLIP_S and low NF- κ B activity [12] parallel the situation in our cell line model with overexpression of FLIP_S and NF- κ B inhibition. Conceivably, interventions specific for FLIP isotypes could be used therapeutically.

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Figure 1.

A) Constitutive levels of FLIP expression in leukemia cell lines. The highest levels of FLIP protein were present in KG-1a cells. U937 cells expressed low amounts of FLIP, while barely detectable levels were present in ML-1 and HL-60 cells. **B)** Apoptosis induced by TRAIL in wild-type U937, KG-1a HL60, and ML-1 cells. While KG-1a cells were resistant, U937 cells were highly sensitive to TRAIL, with lesser rates of apoptosis occurring in ML-1 and HL60 cells (n=5). The average percentages of apoptotic cells at baseline/after TRAIL exposure were as follows: KG1a: 2%/4%; U937: 4%/40%; HL-60: 5%/15%; ML-1: 6%/25%.

Figure 2. Over expression of FLIP in leukemia cell lines

A) Western blot for FLIP in KG1a cells and in ML-1 cells transduced with control vector (Neo.GFP), FLIP_{Short} (FLIP_S) or FLIP_{Long} (FLIP_L). Following transduction, FLIP_S and FLIP_L protein were highly expressed in ML-1 cells in comparison to control (Neo.GFP)-transduced ML-1. Additional bands of FLIP_S represent ER modifications, while the lower molecular weight bands in FLIP_L cells represent cleavage products as also reported by Golks [30] and as also seen in KG-1a cells. FLIP protein bands were probed using NF-6 antibody. **B)** Densitometric analysis of FLIP protein by Image J software (NIH, USA) measuring the 26 kDa band for FLIP_S and the 55 kDa band for FLIP_L. **C)** As with ML-1 cells, both HL-60 and U937 cell lines showed the expected band sizes (as determined by probing with NF-6 antibody) corresponding to the constructs used for transduction.

Figure 3. Expression of TRAIL receptors and TNF receptors on wild-type and transfected ML-1 cells

Transfection did not significantly affect the level of receptor expression, although very slight changes were noted for TRAIL R1 (A). As observed previously, significant decreases in expression of TRAIL R2 were noted in response to TRAIL (300 ng/mL) (B), and of TNF R1 in response to TNF α (100 ng/mL) (C), in comparison to control (veh). Changes of TRAIL R1 varied slightly (A), while no changes were seen for TNF R2 (D).

Abbreviations: FLIP_S and FLIP_L = FLIP_{S/L} transduced ML-1 cells; Neo.GFP = control vector transduced ML-1 cells; WT = unmodified ML-1 cells. Note that the scale differs from panel to panel.

Figure 4. Apoptotic responses to TRAIL in FLIP-overexpressing cells

A) ML-1 cells were exposed to TRAIL at doses ranging from 100 ng/mL to 500 ng/mL. Results are shown as fold increase over the baseline (untreated) rate of apoptosis. Neo.GFP control-transduced cells showed the highest rate of apoptosis with a plateau at 300 ng/mL, while FLIP_L-transduced cells were completely resistant to TRAIL-induced apoptosis. FLIP_S overexpressing cells showed lower rates of apoptosis than control cells, but higher rates than observed in FLIP_L overexpressing cells. **B)** At 300 ng/mL of TRAIL, the rates of apoptosis were significantly different between the three cell lines (p-values as determined by paired student t-test). **C and D)** Similar to ML-1 cells, FLIP_L-transduced U937 and HL60 cells were completely protected against apoptosis induced by TRAIL, while FLIP_S-transduced cells showed low rates of apoptosis.

Figure 5. Activation of caspases in response to TRAIL

A) Activation of caspase-8 in transduced ML-1 cells. Three $\times 10^6$ cells were treated with TRAIL at 300 ng/mL for either 0.5 hours or 3 hours. Cell lysates were prepared and 50 μ g of protein were loaded on the gel (see Methods). As expected, both FLIP_L and FLIP_S cells showed less formation of p18 (active Caspase 8 subunit) than control cells. FLIP_L cells in this case showed slightly earlier formation of the p18 subunit than FLIP_S cells. **B)** Activation of caspase-3 in transduced ML-1 cells (prepared as for caspase-8 determination). The p19/p17 doublet was only present in control cells. No significant amounts of p17 subunit appeared in either FLIP_S or FLIP_L cells. **C)** Caspase-3 activity was also determined (at 4 hours) by the amount of fluorescence product (AMC) released following incubation of individual cell samples with the caspase-3-specific substrate z-DEVD-AMC for 0.5 hours. Baseline activity of caspase-3 was identical in all untreated cells. Following treatment with TRAIL (300 ng/mL), the levels of active caspase-3 rose almost five-fold in control cells, whereas FLIP_L overexpressing cells completely prevented caspase-3 activation. FLIP_S cells, however, did allow for a significant increase in caspase-3 activity.

Figure 6. TRAIL-induced cleavage of BID and cytochrome c release in ML-1 cells

A) Neo.GFP cells showed tBID in the cytosol and appearance of tBID in the mitochondria (Mito). Prominent cytochrome c bands were present in the cytosol (and whole cell lysates) following TRAIL exposure. **B)** FLIP_S cells showed an increase in tBID in whole cell lysates, although tBID was not detectable in the mitochondria. There was also an increase in the amount of cytochrome c in the cytosol following TRAIL treatment. **C)** In FLIP_L cells the amount of tBID following TRAIL treatment was decreased in the cytosol compared to untreated FLIP_L cells. This was accompanied by a decrease in the amount of cytochrome c released into the cytosol. **D)** Pretreatment of Neo.GFP cells with the Caspase-8 inhibitor z-IETD FMK (C8I) decreased the amount of tBID cleavage by TRAIL to the extent that it was no longer detectable in the mitochondrial fraction (compared to cells treated with TRAIL in the absence of inhibitor). It also reduced, but did not completely prevent cytochrome c release. Shown are levels of full length BID, cleaved BID (tBID) and cytochrome c in mitochondria, cytosol and whole cell lysates in untreated cells (untr) and in cells 3hr after exposure to TRAIL (300 ng/ml).

Figure 7. Bcl-xL and XIAP expression in transduced ML-1 cells

A) Levels of Bcl-xL as determined by western blot and densitometric measurement (expressed as ratio of Bcl-xL levels over actin levels [n=3]). Control (Neo.GFP) cells showed a time dependent decrease in Bcl-xL following TRAIL (TR) treatment. No such decrease was seen in FLIP_L-transduced cells, while a slight decrease in Bcl-xL occurred in FLIP_S-transduced cells. **B)** Levels of XIAP as determined by western blot and densitometric analysis (as for Bcl-xL [n=3]). XIAP levels showed a decline in control cells over time as observed for Bcl-xL. A slight decline was observed with FLIP_L, while intermediate XIAP levels were observed at 3 hours in FLIP_S-transduced cells. In all experiments, 3×10^6 cells were treated with TRAIL at 300 ng/mL and harvested at 0.5 hours or 3 hours; 50 μ L of protein were analyzed on the gel.

Figure 8. NF-κB as a modulator of TRAIL and TNFα induced signaling in ML-1 cells
A) Effect of TRAIL on IκB phosphorylation. TRAIL was a weak NF-κB activator, resulting in a faint increase in IκB phosphorylation (at 5 minutes) without a significant decrease in levels of total IκB, and a second, stronger peak at 3 hours; β actin served as control. **B)** Effect of NF-κB inhibition on TRAIL-induced apoptosis. Two × 10⁵ cells were pretreated with DMSO (vehicle) or the NF-κB inhibitor IKK4 for 1 hour at 300 nM and then exposed to TRAIL (TR) at 300 ng/mL for 24 hours. There was a slight increase in baseline apoptosis in the presence of IKK4 in all three cell lines (more so in FLIP_S than in FLIP_L cells). TRAIL treatment in the presence of IKK4 was associated with minimal increases in apoptosis, but the increase was significant only for FLIP_L. **C)** Effect of TNF on IκB phosphorylation. TNFα induced a distinct biphasic response, the first peak occurring at 5 minutes, the second rise beginning at 30 minutes. Total IκB levels decreased significantly concurrently with the initial peak of pIKB. Of note, however, the amount of total IκB increased again in parallel with the second rise in pIKB, presumably related to the fact that NF-κB also regulates transcription of IκB; β actin served as

control. **D)** Effect of NF- κ B inhibition on TNF-induced apoptosis. Two $\times 10^5$ cells were pretreated with DMSO (vehicle) or IKK4 at 300 nM for 1 hour and then exposed to TNF at 100 ng/mL for 24 hours. There was a slight increase in apoptosis in control (Neo.GFP-transduced) cells, but not in FLIP_S or FLIP_L-overexpressing ML-1 cells. However, in the presence of IKK4, the rate of apoptosis increased significantly in FLIP_S-overexpressing cells (n=3; exceeding the extent of apoptosis in control cells), but not in FLIP_L-overexpressing ML-1 cells.