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Molecules and Cells

FLIP is Constitutively Hyperexpressed in Fas-resistant U266 Myeloma Cells, but Is Not Induced by IL-6 in Fas-sensitive RPMI8226 Cells

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Despite the expression of Fas, some clones of myeloma cells are resistant to Fas-mediated apoptosis. To define a cellular factor involved in the resistance, we performed a comparative study using two clones of myeloma cells, RPMI8226 and U266. These cells were reported to express cell surface Fas at similar levels, but only RPMI8226 cells lost their viability upon anti-Fas treatment. The resistance of U266 cells to anti-Fas did not appear to reflect dysregulation of Bcl-2, Bcl-X_L, and Bax, because these proteins were expressed in both RPMI8226 and U266 cells to similar levels. Moreover, levels of those proteins were not significantly altered by treating RPMI8226 cells with IL-6, a cytokine which suppresses the Fas-mediated death of RPMI8226 cells. Interestingly, mRNA levels of $FLIP_L$, an endogenous inhibitor of Fas signaling, were constitutively elevated in U266 cells. Consistent with this observation, U266 cells expressed both $FLIP_L$ protein and its truncated 43 kDa product which is seen in $FLIP_L$-overexpressing cells. The truncated form of $FLIP_L$ protein was not detected in RPMI8226. Moreover, the levels of truncated $FLIP_L$ in U266 cells were considerably higher than those of pro-$FLIP_L$ in RPMI8226. The overall data indicate that $FLIP_L$ is constitutively hyperexpressed in U266 cells. However, IL-6 failed to enhance the protein levels of FLIP molecules in either of the tested cells. It appears, therefore, that $FLIP_L$ plays a role in the intrinsic resistance of U266 cells to the apoptotic action of Fas, but is not involved in the protective action of IL-6.

Keywords: Apoptosis; Fas; FLIP; IL-6; Myeloma Cells.

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Introduction

Multiple myeloma is a B cell malignancy resulting from the uncontrolled expansion of a single clone of plasma cells (myeloma cells) in the bone marrow. Although this outgrowth of neoplastic clones reflects either their enhanced capacity to proliferate or a decreased rate of cell death (apoptosis), this latter mechanism may be particularly important in slowly proliferating tumors such as multiple myeloma. Indeed, many, but not all, clones of myeloma cells can withstand a lethal stimulus such as an agonistic anti-Fas antibody (anti-Fas) (Shima *et al*., 1995; 1996; Westendorf, *et al*., 1995). Moreover, when the cells are exposed to IL-6, the Fas-mediated death of other susceptible clones is suppressed (Chauhan *et al*., 1997; Shima *et al*., 1995; Xu *et al*., 1998). To date, however, the mechanisms underlying the intrinsic and IL-6-induced ability of myeloma cells to survive against anti-Fas have been poorly described.

Fas (APO-1/CD95) is a member of the TNF/nerve growth factor receptor family, and is expressed in a wide variety of normal and transformed cells (Itoh *et al*., 1991). Upon binding to its ligand, Fas rapidly undergoes oligomerization, followed by recruitment of an adaptor molecule FADD/MORT1 to the ligated receptor (Chinnaiyan *et al*., 1996). FADD, in turn, associates with caspase-8 (FLICE/MACH/MCH5) through death effector domains (DEDs) present at the C-terminus of FADD and the N-terminus of caspase-8, forming the death-inducing signaling complex (DISC) (Boldin *et al*., 1996). Recruitment of caspase-8 to the DISC leads to its proteolytic activation, which then triggers a downstream signaling cascade that culminates in apoptosis (Ashkennazi and Dixit, 1998).

FLIP (FLAME-1/MRIT/CLARP) is a recently identified cellular protein which can interfere with the

Fas-induced activation of caspase-8 (Irmler *et al.*, 1997; Srinivasula *et al.*, 1997). Human cells express two alternatively spliced isoforms of FLIP, $FLIP_s$ (28 kDa) and $FLIP_L$ (55 kDa) (Irmler *et al.*, 1997). They both contain two DEDs, which are, in the case of $FLIP_L$, followed by a caspase-like domain at the C-terminus. Nevertheless, $FLIP_L$ is believed to be catalytically inactive because the active-center cysteine residue is substituted by a tyrosine residue. $FLIP_s$ and $FLIP_L$ can interact with FADD and caspase-8 through DEDs, resulting in blockage of the caspase-8 activation at the DISC. Consequently, cells stably overexpressing FLIP molecules have been shown to resist Fas-mediated apoptosis (Scaffidi *et al.*, 1999).

Based on the inhibitory role of FLIP, it seems possible that the cellular level of FLIP may be a factor influencing the susceptibility/resistance of myeloma cells to Fas-mediated apoptosis. To address this question, we performed a comparative study using two clones of myeloma cells, RPMI8226 and U266, which have been shown to express Fas to similar levels (Shima *et al.*, 1996). In contrast to RPMI8226 which undergoes death upon Fas ligation (Shima *et al.*, 1995), U266 cells are resistant to anti-Fas treatment. Interestingly, the $FLIP_L$ protein was found to be hyperexpressed in U266 cells. However, IL-6 failed to elevate the protein levels of either $FLIP_L$ or $FLIP_s$ in RPMI8226 cells. Therefore, $FLIP_L$ appears to play a role in the intrinsic resistance of U266 cells to Fas-mediated apoptosis, but does not appear to be involved in the protective action of IL-6.

Materials and Methods

Materials The culture medium and its supplements were purchased from Bio Whittaker (Walkersville, MD). All the antibodies were raised against human proteins, and were purchased from the following sources; anti-FLIP and -Bax, Pharmingen (San Diego, CA); anti-Bcl-2, DAKO (Carpinteria, CA); anti-Bcl-X_L, Transduction Laboratories (Lexington, KY); anti-Fas (IgM), Upstate Biotechnology (Lake Placid, NY). Promega (Madison, WI) provided deoxynucleotide triphosphate (dNTP), RNasin, avian myeloblastosis virus reverse transcriptase, and random hexamer.

Cell culture and treatments RPMI8226 and U266 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS and gentamicin (50 μg/ml). To treat cells with anti-Fas and IL-6, the cell density was adjusted to 3×10^5/ml. The untreated control and treated cells were maintained at 37°C in 5% CO_2 and high humidity for the periods of time indicated.

Analysis of viability Incubated cells received propidum iodide (PI) (5 μg/ml), followed by analysis on a FACScan flow cytometer (Becton Dichinson, Mountain View, CA) to simultaneously monitor PI uptake (FL-2 channel) and cell size (forward light scatter). The cells that displayed both a reduction in cell size and a high permeability to PI were understood to be dead, as previously defined (Mangan *et al.*, 1991).

Western blotting Cell lysates were prepared as described previously (Lee and Um, 1999). Equal amounts of proteins (25–100 μg) were separated by SDS-PAGE, then electrotransferred to Immobilon membranes (Millipore, Bedford, MA) which were subsequently blotted using the indicated antibodies and visualized by chemiluminescence (ECL; Amersham, Arlington Heights, IL).

RT-PCR analysis Total RNA from indicated cells was extracted using Tri-reagent (Sigma, St. Louis, MO). First-strand cDNA was synthesized at 37°C for 1 h in 50 μl of reaction mixture containing 3 μg of total RNA, 1.5 mM of each dNTP, 0.5 μg of random hexamer, 10 units of avian myeloblastosis virus reverse transcriptase, and 25 units of RNasin. PCR reactions were then performed in a total volume of 25 μl containing 5 μl of the transcribed cDNA, 1 unit of *Taq* DNA polymerase (Takara, Shiga, Japan), 0.2 μM of each dNTP, 0.5 μCi of [α^{32}P]-CTP, and 25 pmole of each primer. Two sets of primers were used for the amplification of FLIP cDNA; one (5′-ATG TCT GCT GAA GTC ATC CAT C-3′, and 5′-TGC TCC TTG AAC AGA CTG C-3′) specific to the N-terminal region of FLIP which is commonly shared by $FLIP_L$ and $FLIP_s$, and the other (5′-TCA GAG CAT ACC TGA AGA G-3′, and 5′-AGA GTG TGC TGC AGC CAG AC-3′) specific to the C-terminal extension of $FLIP_L$. For an internal control, β-actin cDNA was amplified by using 5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-TCC TTA ATG TCA CGC ACG ATT TC-3′. The optimal number of cycles for RT-PCR was determined as previously described (Becker *et al.*, 1991), and we performed the amplification at 23 cycles for β-actin, at 25 cycles for the shared N-terminal region of FLIP, and at 27 cycles for the C-terminal extension of $FLIP_L$. Each cycle included denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 1 min. Ten μl of the reaction mixture was separated on 5% acrylamide gels, and the relative levels of amplification were compared by autoradiography of the gels.

Results

Anti-Fas kills RPMI8226, but not U266 myeloma cells RPMI8226 myeloma cells express Fas on their surface, and thus undergo death upon exposure to anti-Fas (Shima *et al.*, 1995). The cell death was suppressed by the addition of IL-6 (Fig. 1), as reported previously (Shima *et al.*, 1995). It has also been reported that U266 clones of myeloma cells express cell surface Fas to a level similar to that observed in RPMI8226 cells (Shima *et al.*, 1996). However, U266 cells resisted the anti-Fas treatment even in the absence of exogenous IL-6 (Fig. 1). These data suggested that U266 cells constitutively express an anti-death protein(s) which blocks the Fas signaling.

Bcl-2, Bcl-X_L, and Bax are not hyperexpressed in U266 cells Bcl-2 and Bcl-X_L are well characterized anti-death

proteins. Given that overexpression of either Bcl-2 or Bcl-X_L can suppress Fas-mediated apoptosis (Scaffidi *et al.*, 1998), we explored whether these protective proteins were hyperexpressed in U266 cells. However, levels of Bcl-2 and Bcl-X_L in U266 cells were not higher than those in RPMI8226 (Fig. 2). It has been proposed that cellular viability can be determined by the ratio of Bcl-2/Bax (Oltvai *et al.*, 1993). However, both RPMI8226 and U266 cells expressed Bax to similar levels. It appeared, therefore, that the resistance of U266 cells to anti-Fas did not result from dysregulation of the tested members of the Bcl-2 family. Moreover, the exposure of either RPMI8226 or U266 cells to IL-6 did not significantly alter their levels of Bcl-2, Bcl-X_L, and Bax.

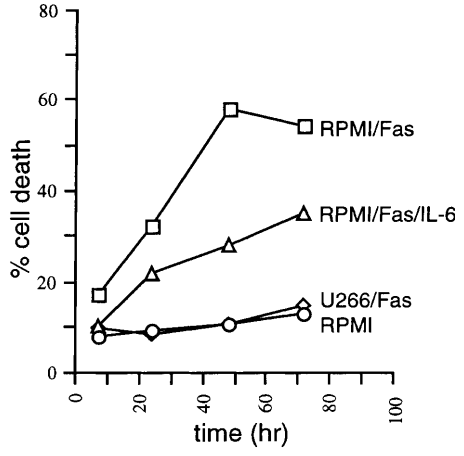

Fig. 1. Differential responses of RPMI8226 and U266 cells to anti-Fas. RPMI8226 and U266 cells were incubated in the following conditions: circle, RPMI8226 in the absence of anti-Fas and IL-6; square, RPMI8226 in the presence of anti-Fas (0.1 μg/ml); triangle, RPMI8226 in the presence of both anti-Fas and IL-6 (1,000 U/ml); diamond, U266 in the presence of anti-Fas. Following the incubation times indicated, cellular viability was determined by flow cytometric analysis. The data is representative of three similar experiments. The viability of untreated U266 cells was almost equal to those treated with anti-Fas.

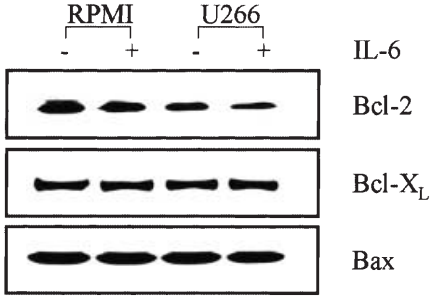

Fig. 2. Expression of Bcl-2, Bcl-X_L, and Bax. RPMI8226 and U266 cells were incubated in the absence or presence of IL-6 (1,000 U/ml) for 24 h. Levels of Bcl-2, Bcl-X_L, and Bax were compared by Western blotting as described in **Materials and Methods**.

FLIP is hyperexpressed in U266 cells Based on the report that Fas-mediated apoptosis is blocked by overexpression of FLIP (Irmler *et al.*, 1997; Scaffidi *et al*, 1999; Srinivasula *et al.*, 1997), we next compared the levels of FLIP molecules in RPMI8226 and U266 cells. To do so, RT-PCR was conducted by using a set of primers that was specific to the N-terminal region of both $FLIP_s$ and $FLIP_L$ mRNA. As shown in Fig. 3A, a greater amplification was obtained using cDNA from U266 cells, suggesting higher levels of FLIP mRNAs in the cells. Similar results were obtained using the other set of primers that was specific to the C-terminal extension of $FLIP_L$ mRNA. Therefore, the FLIP mRNAs which were up-regulated in U266 cells involved $FLIP_L$ mRNA. When levels of FLIP proteins were compared by Western analysis, we did not detect the 28 kDa $FLIP_s$ protein in either of the cells (Fig. 3B). While the two cells expressed the 55 kDa $FLIP_L$ protein, the U266 cells displayed an additional protein band corresponding to 43 kDa. This protein represents the proteolytically truncated form of $FLIP_L$ protein, which has been commonly observed in cells overexpressing $FLIP_L$ (Irmler *et al.*, 1997; Scaffifi *et al.,* 1999). Importantly, the levels of the 43 kDa form in U266 cells were much higher than those of the 55 kDa form in RPMI8226 cells. These data suggested that expression of the $FLIP_L$ protein was enhanced in U266 cells to the

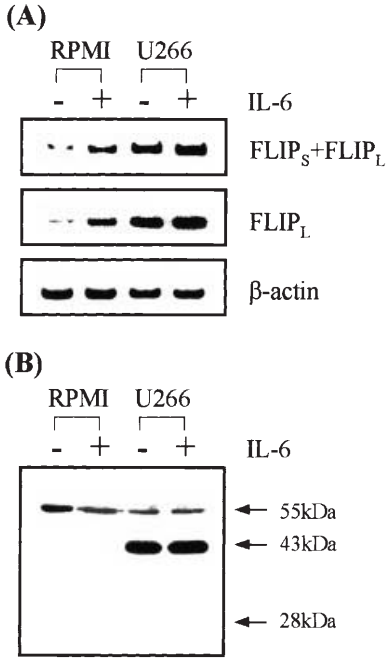

Fig. 3. Expression of FLIP mRNA and proteins. RPMI8226 and U266 were incubated in the absence or presence of IL-6 (1,000 U/ml). **A.** At 8 h after the treatment, total RNA was extracted, and the levels of FLIP mRNA were compared by RT-PCR. **B.** After 24 h, the levels of FLIP proteins were analyzed by Western Blotting.

level that the protein could undergo its proteolytic cleavage.

IL-6 induces FLIP mRNA, but not its protein, in RPMI8226 cells To explore whether the protective effect of IL-6 is accompanied by the induction of FLIP, we treated RPMI8226 cells with IL-6 for 8 h, and analyzed the levels of FLIP mRNA. As shown in Fig. 3A, IL-6 dramatically enhanced the levels of FLIP mRNA. This effect of IL-6 was observed using both sets of primers. However, IL-6 did not significantly influence the level of the 55 kDa $FLIP_L$ protein (Fig. 3B). Moreover, we did not detect any induction of the 28 kDa $FLIP_s$ protein and the truncated form of $FLIP_L$ by treating RPMI8226 cells with IL-6. This was true when we analyzed the FLIP proteins from 4 h up to 48 h after the treatment. These results indicated that levels of FLIP mRNA, but not its protein, are under the control of IL-6 in RPMI8226 cells.

In the case of U266, levels of FLIP mRNA did not significantly respond to IL-6 (Fig. 3A), suggesting that FLIP mRNA was in a saturated level without the IL-6 treatment. Similarly, the levels and expression patterns of FLIP proteins were not altered by treating U266 cells with IL-6 (Fig. 3B).

Discussion

Recent studies have demonstrated that certain clones of myeloma cells resist Fas-mediated apoptosis (Shima *et al*., 1995; 1996; Westendorf, *et al*., 1995). To determine the cellular factor involved in the resistance, we have performed a comparative study using RPMI8226 and U266 myeloma cells. Although these two cells express Fas to similar levels (Shima *et al*. 1996), only RPMI8226, but not U266, cells were susceptible to the lethality by anti-Fas. It appears that Bcl-2 and Bcl-X_L are not the constitutive protectors of U266 cells, because levels of those proteins were not enhanced in U266 cells, compared to RPMI8226. Similarly, studies using different sets of myeloma cells have reported that cellular levels of Bcl-2 and Bcl-X_L are not co-related to the responsiveness of myleoma cells to anti-Fas (Egle *et al*., 1997). Although it has been proposed that the cellular level of Bax is a factor determining the susceptibility of certain clones of myeloma cells to anti-Fas (Egle *et al*., 1997), we could not find such a co-relation using RPMI8226 and U266 cells. Therefore, the resistance to anti-Fas appears to be achieved by different mechanisms depending on myeloma cell clones. In the case of U266 cells, we observed up-regulation of $FLIP_L$ mRNA and its truncated form of protein. Although we could not detect the $FLIP_s$ protein in the tested myeloma cells, this does not necessarily indicate that the cells do not express the $FLIP_s$ mRNA. This argument is based on the observation that human peripheral blood T cells highly express $FLIP_s$ mRNA, but not its protein (Irmler *et al*., 1997). Therefore, transcription of $FLIP_s$ does not seem to always guarantee its translation, suggesting a control at the level of post-transcription. Although high levels of $FLIP_L$ protein were detected in malignant melanoma cells (Irmler *et al*., 1997), this is, to our knowledge, the first report that FLIP can be dysregulated in myeloma cells. The role of FLIP in apoptosis was initially controversial: overexpression of FLIP resulted in either suppression or promotion of Fas-mediated cell death depending on experimental conditions (Han *et al*., 1997; Inohara *et al*., 1997; Irmler *et al*., 1997; Srinivasula *et al*., 1997). It was later proposed that the promotion of cell death reflects the non-physiological function of FLIP in transient overexpression systems, and that its stable hypexpression is antiapoptotic (Scaffidi *et al*., 1999). Moreover, overexpression of $FLIP_L$ was shown to elicit a more potent protective effect than that of $FLIP_s$ (Irmler *et al*., 1997). Interestingly, when $FLIP_L$ was overexpressed in BJAB lymphoma cells, only the 43 kDa, but not the 55 kDa, form of $FLIP_L$ was detected in the DISC (Scaffifi *et al*., 1999). This suggests that the 43 kDa form of $FLIP_L$ participates in the blockage of Fas signaling. In this regard, the constitutive hyperexpression of the 43 kDa FLIP protein in U266 cells is consistent with the intrinsic ability of the cells to survive against anti-Fas.

Another important finding of this study is that IL-6 does not regulate the levels of FLIP proteins in the tested myeloma cells. Although levels of $FLIP_L$ mRNA in RPMI8226 cells were enhanced by IL-6, this effect was not reflected in its protein level. This suggests that the expression of $FLIP_L$, like $FLIP_s$, can also be under post-transcriptional control. Certain cytokines such as TNFα and IFNγ were shown to elevate the mRNA levels of FLIP in microglial cells (Spanaus *et al*., 1998). Based on our finding that the levels of FLIP mRNA and its protein can be differentially regulated, FLIP should be analyzed at its protein level as well to confirm the action of the cytokines. We have also investigated the possibility that IL-6 could regulate cellular levels of Bcl-2, Bcl-X_L, and Bax. However, we could not find evidence for this under our experimental conditions. Taken together, the data indicate that IL-6 can suppress the Fas-mediated death of RPMI8226 cells without altering the levels of FLIP proteins and the tested members of the Bcl-2 family. Currently, the mechanism underlying the protective action of IL-6 is unclear. While IL-6 has been reported to suppress the Fas-mediated activation of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Chauhan *et al*., 1997; Xu *et al*., 1998), whether SAPK acts as a mediator of Fas-induced death in myeloma cells remains unanswered. Such a verification appears to be important because the role of SAPK in Fas-mediated apoptosis

varies depending on the cell type (Ashkenazi and Dixit, 1998).

In summary, we have provided evidence that FLIP is dysregulated in Fas-resistant U266 myeloma cells. Therefore, FLIP may be considered as a survival factor imparting at least those specific cells with an intrinsic resistance to lethality by Fas ligation. Given that IL-6 rescues Fas-sensitive RPMI8226 myeloma cells without altering their levels of FLIP proteins, myeloma cells appear to have an alternative control for their response to the Fas ligand.

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