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FLIP Prevents Apoptosis Induced by Death Receptors But Not by Perforin/Granzyme B, Chemotherapeutic Drugs, and Gamma Irradiation

Takao Kataoka,* Michael Schröter,* Michael Hahne,* Pascal Schneider,* Martin Irmler,* Margot Thome,* Christopher J. Froelich,† and Jürg Tschopp2*

FLICE-inhibitory protein, FLIP (Casper/I-FLICE/FLAME-1/CASH/CLARP/MRIT), which contains two death effector domains and an inactive caspase domain, binds to FADD and caspase-8, and thereby inhibits death receptor-mediated apoptosis. Here, we characterize the inhibitory effect of FLIP on a variety of apoptotic pathways. Human Jurkat T cells undergoing Fas ligand-mediated apoptosis in response to CD3 activation were completely resistant when transfected with FLIP. In contrast, the presence of FLIP did not affect apoptosis induced by granzyme B in combination with adenovirus or perforin. Moreover, the Fas ligand, but not the perforin/granzyme B-dependent lytic pathway of CTL, was inhibited by FLIP. Apoptosis mediated by chemotherapeutic drugs (i.e., doxorubicin, etoposide, and vincristine) and gamma irradiation was not affected by FLIP or the absence of Fas, indicating that these treatments can induce cell death in a Fas-independent and FLIP-insensitive manner. The Journal of Immunology, 1998, 161: 3936–3942.

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Materials and Methods

Cells

hFLIP stably transfected Jurkat cells (JFL2) (30) and wild-type Jurkat cells were maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS and an antibiotic mixture (Life Technologies, Paisley, U.K.; 50 µg/ml of penicillin, 50 µg/ml of streptomycin, and 100 µg/ml of neomycin). A20 cells and Fas-negative variant A20R cells (12) were maintained...
in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 10 mM HEPEs (pH 7.4), 50 μM 2-ME, and the antibiotic mixture described above.

Reagents

hFas-Fc (37), hTRAILR2-Fc (38), hTNFR1-Fc (39), and rhFasL (37) were constructed as described previously. Etoposide was kindly provided by Dr. S. Gasser (Swiss Institute for Experimental Cancer Research (ISREC), Epalinges, Switzerland). Human anti-CD3 TR66 was kindly provided by Dr. S. Valitutti (Institute of Biochemistry, Epalinges, Switzerland). Other reagents were purchased from commercial suppliers.

Assay for AICD

Flat-bottom ELISA microtiter plates (Nunc, Roskilde, Denmark) were coated with PBS containing anti-human CD3 TR66 for 3 h at 37°C. Before use, the plates were washed twice with PBS and once with the RPMI 1640 medium. Jurkat cells (5 × 10⁶/ml, 100 μl) were distributed to each well, then centrifuged (200 × g, 3 min) and incubated for 24 h at 37°C. Cell viability (OD₅₉₀) was measured by the nonradioactive cell proliferation kit (Promega, Madison, WI). DNA fragmentation (OD₅₉₀) was measured by the cell death detection ELISA kit (Boehringer Mannheim, Mannheim, Germany).

Assay for granzyme B-mediated DNA fragmentation

The method was basically performed as previously described (40) with a slight modification. Jurkat cells were labeled with [¹²⁵I]UDR (Amersham, Buckinghamshire, U.K.) for 2 h at 37°C and washed three times with the medium (RPMI 1640 supplemented with 0.5% BSA and the antibiotic mixture). [¹²⁵I]UDR-labeled Jurkat cells (2 × 10⁶ cells/ml, 100 μl) were incubated with granzyme B together with 100 pfu/cell of replication-defective adenovirus or sublytic concentrations of perforin (160 U/ml) for 4 h. The cells were then lysed with an equal volume of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0) buffer containing 0.2% Triton X-100 and centrifuged (800 g, 3 min) and incubated (800 × g, 5 min). Supernatants were removed and counted in a gamma counter. Specific DNA fragmentation (%) was calculated by the following formula: ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100.

Cell transfection

Flag-tagged mouse FLIP (mFLIP; HindIII/Xhol fragment) was cloned into the HindIII/Xhol site of pCEP4 vector (Invitrogen, Carlsbad, CA). A20 cells (8 × 10⁵ cells) were transfected with 20 μg of pCEP4 vector or mFLIP-pCEP4 by electroporation (250 V, 960 μF).

Reverse transcriptase-PCR

mRNA was prepared from A20 cells (10⁵ cells) using a mini-message maker kit (R&D Systems, Minneapolis, MN) and reverse transcribed to cDNA using the T-primed first-strand kit (Pharmacia Biotech, Uppsala, Sweden). cDNA was amplified by PCR using the following primers: mFLIP (forward: 5'-GGATTTAGTACAGCCATGTGG-3'; reverse: 5'-CTCTGAGTACATTGCTACGC-3'), which gave a 217-bp and a 445-bp product, respectively. Conditions for PCR were: 94°C for 5 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and the last cycle of 72°C for 10 min. The products were analyzed on a 2% agarose gel.

Assay for the cytolytic activity of the MLC cells

Responder spleen cells from perforin-deficient or gld C57BL/6 mice (H-2b) were cultured with gamma-irradiated (36 Gy) spleen cells from BALB/c mice (H-2b) for 5 days. Before use, nonviable cells were removed from samples on a gradient by Ficoll-Paque (Pharmacia Biotech). Target cells were labeled with [³⁵Cr]sodium chromate (Dupont, Boston, MA) for 1 h, then washed three times with RPMI 1640. MLC cells were mixed with target cells (10⁴ cells/well) in U-bottomed microtiter plates (final volume, 200 μl), and the plates were centrifuged (200 × g, 3 min). After incubating for 4 h, supernatants were removed and their radioactivity measured. Specific ³⁵Cr release (%) was calculated using the following formula: ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100.

Assay for chemotherapy-induced drug- and gamma irradiation-induced apoptosis

Cells (5 × 10⁵ cells/ml, 100 μl) were incubated with doxorubicin, etoposide, or vincristine (all from Sigma, St. Louis, MO) for 24 h. Alternatively, cells were treated with gamma irradiation and then incubated for 24 h. Cell viability (OD₅₉₀) and the extent of DNA fragmentation (OD₅₉₀) were measured using commercial kits.

Results

FLIP prevents AICD in T cells

Fas-mediated apoptosis has been shown to be involved in the AICD of T cells (41–43). Since FLIP potently inhibits apoptosis induced by death receptors such as Fas, we first asked whether FLIP inhibits AICD. Human Jurkat T cells are known to undergo apoptosis after TCR activation. Thus, when Jurkat cells were treated with immobilized anti-CD3, cell viability was markedly decreased (Fig. 1A). The reduction of cell viability was due to apoptosis, since histone-associated DNA fragments were significantly augmented at anti-CD3 concentrations, which decreased cell viability (Fig. 1B). In contrast, Jurkat cells stably transfected with FLIP (JFL2) and treated with anti-CD3 showed slightly increased cell viability (Fig. 1A), and no effects on DNA fragmentation were observed (Fig. 1B). FACS analyses showed that there was no significant difference in the expression of Fas and CD3 between Jurkat cells and JFL2. FasL levels were also identical (data not shown). Anti-CD3-induced apoptosis was antagonized by Fas-Fc in a dose-dependent manner (Fig. 1C), but not by TRAILR2-Fc or TNFRI-Fc (Fig. 1D), confirming that Fas-induced apoptosis plays a major role in the AICD of Jurkat cells.

FLIP does not inhibit granzyme B/adeno virus- or granzyme B/perforin-mediated apoptosis

Granzyme B was shown to have a crucial role in inducing apoptosis in perforin-dependent CTL-mediated cytotoxicity, as granzyme B-deficient CTLs still retain potent lytic activity but lack the ability to induce acute DNA fragmentation (44). Thus, we questioned whether FLIP renders resistance to granzyme B-induced apoptosis. Granzyme B enters target cells independently of perforin, and perforin or adenovirus can initiate apoptosis (40, 45). In combination with adenovirus (Fig. 2A) or sublytic concentrations of perforin (Fig. 2B), granzyme B induced a marked level of DNA fragmentation in Jurkat cells in a dose-dependent manner. However, there was no significant difference in DNA fragmentation between Jurkat cells and JFL2 under either condition.

FLIP prevents Fas-dependent, but not perforin-dependent, target cell lysis in CTL-mediated cytotoxicity

To clarify whether FLIP inhibits perforin- and FasL-dependent cytolytic pathways of CTLs, we stably transfected A20 cells with mFLIP. Several of the hygromycin B-resistant clones were markedly resistant to FasL (Fig. 3A), confirming that Fas-induced apoptosis plays a major role in the AICD of Jurkat cells.

FLIP prevents Fas-dependent, but not perforin-dependent, target cell lysis in CTL-mediated cytotoxicity

Cells generated from A20 wild-type cells and vector-alone transfectants (Fig. 3B) showed equally lysed A20 gld (Fas-deficient) and vector transfectants, and FLIP transfectants (Fig. 3C). In contrast, FLIP transfectants were not killed by MLC cells generated from perforin-deficient mice (Fig. 3D). These data indicate that FLIP prevents Fas-dependent cytolyis, but not perforin-dependent cytotoxicity of CTLs.
FLIP does not block apoptosis induced by chemotherapeutic drugs

It was reported that apoptosis induced by chemotherapeutic drugs is mediated by Fas (46, 47). If this is the case, FLIP transfectants should be insensitive to these treatments. Three anti-cancer drugs, i.e., doxorubicin, etoposide, and vincristine, which have different cellular targets, were tested. However, these drugs decreased the cell viability of JFL2 cells as well as Jurkat cells during a 24-h incubation (Fig. 4, A–C). The decrease in cell viability was due to apoptosis, because these drugs markedly induced DNA fragmentation (Fig. 4, D–F). Drug-induced apoptosis was completely blocked by z-VAD-fmk, but not by Fas-Fc (Fig. 4, D–F), although the same amount of Fas-Fc totally blocked FasL-induced apoptosis.

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Similar results were obtained when cells were exposed to doxorubicin for 3 days (data not shown). We also examined the effects of anti-cancer drugs on A20 FLIP transfectants (Fig. 5A). Again, doxorubicin, etoposide, and vincristine killed FLIP transfectants as efficiently as they did vector transfectants (Fig. 5A). To further confirm that drug-induced apoptosis is independent of Fas, a Fas-negative A20 variant cell line (A20R) was used (12). No difference in sensitivity was observed between A20 cells and A20R cells (Fig. 5B). Thus, these data clearly indicate that chemotherapeutic drug-induced apoptosis is Fas-independent and FLIP-insensitive in the two cell lines tested.

**FLIP does not block apoptosis induced by gamma irradiation**

Previous reports have shown that γ-irradiation-induced apoptosis is mediated by Fas (48). Jurkat cells and JFL2 cells were irradiated with different doses of gamma ray and incubated for 24 h (Fig. 6). Gamma irradiation decreased the cell viability in a dose-dependent manner (Fig. 6A) and induced DNA fragmentation (Fig. 6B). Again, there was no difference in sensitivity observed between Jurkat cells and FLIP transfectants (JFL2). Akin to the anti-cancer drugs, gamma irradiation-induced apoptosis was completely suppressed by z-VAD-fmk, but not by Fas-Fc.

**Discussion**

We have characterized the antiapoptotic activity of FLIP on various apoptotic pathways. FLIP inhibited AICD in T cells and Fas-dependent CTL-mediated target cell lysis. However, FLIP neither prevented perforin-dependent CTL-mediated target cell lysis nor apoptosis induced by anti-cancer drugs and gamma irradiation.

TCR-mediated signals trigger not only IL-2 production and proliferation, but also cell death. TCR activation leads to the up-regulation of Fas and FasL, and T cells are killed by Fas/FasL interaction in an autonomous manner (41–43). In agreement with these results, CD3 activation induced a marked cell death in Jurkat cells, whereas FLIP transfectants were completely insensitive to such treatment. FLIP is expressed in the early stage of T cell activation and disappears when T cells become sensitive to FasL-mediated apoptosis (30); thus, FLIP may regulate the fate of mature T cells in the periphery.

FasL-dependent CTL-mediated target cell lysis was prevented by FLIP. Ag receptor-stimulated B cells are resistant to Fas-dependent Th1-mediated killing, whereas CD40L-stimulated B cells are highly Fas sensitive (26). Thus, it is possible that FLIP also plays a role in the regulation of mature B cells in the periphery.

In the perforin-dependent pathway, granzyme B was shown to be a major inducer of DNA fragmentation (44). Although granzyme B can cleave and activate caspase-3 and caspase-6–10 in vitro (17, 19, 49–54), caspase-10 was reported to be primarily activated by granzyme B in granule-mediated killing (55). In our reconstituted systems using purified proteins, FLIP failed to inhibit DNA fragmentation induced by granzyme B in combination with adenovirus or perforin. Likewise, in CTL-mediated target cell lysis by primary MLC cells, FLIP was unable to protect against perforin-dependent killing. Thus, FLIP is able to inhibit only one of the two major lytic pathways of CTLs.
Anti-cancer drugs and gamma irradiation were reported to induce apoptosis in leukemia and hepatoma cells by Fas/FasL system (46–48). Three anti-cancer drugs with different modes of action induced apoptosis not only in Jurkat and A20 cells, but also in FLIP-transfected cells. Anti-cancer drug-induced apoptosis was not blocked by soluble Fas-Fc and also occurred in the absence of Fas surface expression. Likewise, gamma irradiation-induced apoptosis was not prevented by FLIP expression and soluble Fas-Fc, at least in the cell lines examined in this report. Thus, anti-cancer drug- or gamma irradiation-induced apoptosis can proceed in a Fas-independent and FLIP-insensitive manner. This finding is consistent with recent reports showing that these apoptotic pathways are independent of Fas (56, 57).

**FIGURE 4.** FLIP does not inhibit chemotherapeutic drug-induced apoptosis. A–C, Jurkat cells (open circles) and JFL2 (filled circles) were treated with the indicated concentrations of doxorubicin (A), etoposide (B), or vincristine (C) for 24 h. Cell viability (%) was measured using a cell proliferation assay. The mean ± SD of triplicate cultures are shown. D–F, Jurkat cells (open bars) and JFL2 (filled bars) were treated with the indicated concentrations of the drugs for 24 h in the absence or the presence of 100 μM z-VAD-fmk (Bachem, Bubendorf, Switzerland) or 20 μg/ml of hFas-Fc plus 2 μg/ml of protein A. G, Jurkat cells were treated with 100 ng/ml of rhsFasL for 24 h in the presence of 100 μM z-VAD-fmk or 20 μg/ml of hFas-Fc plus 2 μg/ml of protein A. DNA fragmentation (OD 405 ) were measured using a histone-DNA complex release assay (D–G). The mean ± SD of duplicate cultures are shown.
Bcl-2 family proteins can block cell death signaling pathways triggered by diverse stimuli including anti-cancer drugs and gamma irradiation. In Fas-mediated apoptosis, FADD and Daxx are two distinct downstream mediators that bind to Fas-DD (24). FADD activates the caspase-8 pathway, whereas Daxx activates the Jun N-terminal kinase pathway, which is sensitive to Bcl-2 (24). Since Fas-mediated apoptosis is rarely inhibited by Bcl-2, the FADD-mediated pathway that is highly sensitive to FLIP seems to predominate.

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References


