The Journal of Immunology

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J Immunol 1998; 161:3936-3942; ;
http://www.jimmunol.org/content/161/8/3936

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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

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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.**

Tumor necrosis factor receptor family members transmit death or survival signals and are involved in the regulation of tissue homeostasis (1). In the immune system, the Fas (CD95)/Fas ligand (FasL, CD95L)[3] system plays a critical role in the deletion of activated T cells in the periphery (2, 3) and in the elimination of virus-infected or transformed cells by CTL (4–8). Moreover, in immune-privileged sites such as the eye and testis, activated inflammatory cells are thought to be killed through FasL, which is expressed on these organs, thus avoiding tissue destruction (9–11). FasL also plays a role in the immune privilege of tumors. Tumor cells such as melanoma cells express FasL and can counterattack infiltrating T cells (12–14).

Fas contains a death domain (DD), which is essential for transmitting apoptotic signals (1). The adapter protein FADD (15, 16), which contains a DD and a death effector domain (DED), binds to Fas through a DD-DD interaction. The DED of FADD is used to bind to caspase-8 (FLICE) and caspase-10 (17–19), which both contain two DEDs and a caspase domain. Upon activation, Fas recruits this set of intracellular proteins to form a death-inducing signaling complex (DISC) (17, 20, 21). When incorporated in the DISC, caspase-8 is proteolytically activated (21), possibly in the form of a dimeric complex, $(p10/p20)_2$, as observed in caspase-1 (22, 23), and subsequently cleaves downstream caspases and target proteins such as caspase-3 and poly(ADP-ribose) polymerase (17–19, 21). In addition to FADD, Daxx has recently been identified to bind to the Fas DD and to activate the Jun N-terminal kinase pathway, resulting in apoptosis (24).

Fas is widely expressed in various tissues and cell lines. However, susceptibility to Fas does not necessarily correlate with its cell surface expression (25, 26), suggesting that cellular inhibitors of Fas-mediated signaling pathways exist. We and other groups (27–29) have reported that several γ-herpesviruses and the tumorigenic human molluscipoxvirus encode a family of viral inhibitors (v-FLIPs (FLICE-inhibitory proteins)). v-FLIPs consist of two DEDs and can interact with FADD, thereby preventing apoptosis in a dominant-negative manner (27–29). More recently, a cellular homologue of the viral proteins, designated FLIP (Casper/I-FLICE/FLAME-1/CASH/CLARP/MRIT) has been described (30–36). The long form of FLIP ($FLIP_L$) contains two DEDs and an inactive caspase domain, whereas the short form, $FLIP_S$, contains only two DEDs. Both $FLIP_L$ and $FLIP_S$ can interact with FADD, caspase-8, and possibly caspase-10 and thereby specifically inhibit apoptosis mediated by all currently known death receptors (e.g., Fas, TRAIL-R, TNFR-1, TRAIL-R2, TRAMP).

The potent inhibitory activity of FLIP on death receptor signaling enabled us to investigate the effect of FLIP on various stimuli proposed to induce apoptosis via the FasL/Fas system. We found that FLIP inhibits activation-induced cell death (AICD) in T cells and FasL-dependent CTL-mediated target cell lysis. In contrast, FLIP does not prevent apoptosis through anti-cancer drugs and gamma irradiation.

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

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Received for publication April 1, 1998. Accepted for publication June 15, 1998.

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[3] Abbreviations used in this paper: FasL, Fas ligand; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; FLIP, FLICE-inhibitory protein; v-FLIPs, viral FLIPs; hFLIP, human FLIP; mFLIP, mouse FLIP; rhsFasL, recombinant human soluble FasL; JFL2, Jurkat cells stably transfected with $hFLIP_L$; UdR, 2′-deoxyuridine; z-VAD-fmk, Z-Val-Ala-DL-Asp-fluoromethylketone; pfu, plaque-forming unit; AICD, activation-induced cell death.

0022-1767/98/$02.00

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 rhsFasL (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 (5×10^5/ml, 100 μl) were distributed to each well, then centrifuged ($200 \times g$, 3 min) and incubated for 24 h at 37°C. Cell viability (OD_{490}) was measured by the nonradioactive cell proliferation kit (Promega, Madison, WI). DNA fragmentation (OD_{405}) 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 [^{125}I]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). [^{125}I]UdR-labeled Jurkat cells (2×10^5 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 ($800 \times g$, 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; *Hin*dIII/*Xho*I fragment) was subcloned into the *Hin*dIII/*Xho*I site of pCEP4 vector (Invitrogen, Carlsbad, CA). A20 cells (8×10^6 cells) were transfected with 20 μg of pCEP4 vector or mFLIP-pCEP4 by electroporation (250 V, 960 μF). The cells were cultured for 48 h without selection, then seeded in flat-bottom microtiter plates (2,500–20,000 cells/well), and cultured in the presence of 600 μg/ml of hygromycin B (Calbiochem, San Diego, CA).

Reverse transcriptase-PCR

mRNA was prepared from A20 cells (10^7 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′-GTTAGGTAGCCAGTTGG-3′; reverse: 5′-CCTGC CTTGCTTCAGC-3′) and actin (forward: 5′-ATCAAGATCCTGAC CGAGCG-3′; reverse: 5′-TACTTGCGCTCAGGAGAGGC-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-2^b) were cultured with gamma-irradiated (36 Gy) spleen cells from BALB/c mice (H-2^d) for 5 days. Before use, nonviable cells were removed from samples on a gradient by Ficoll-Paque (Pharmacia Biotech). Target cells were labeled with [^{51}Cr]sodium chromate (Dupont, Boston, MA) for 1 h, then washed three times with RPMI 1640. MLC cells were mixed with target cells (10^4 cells/well) in U-bottomed microtiter plates (final volume, 200 μl), and the plates were centrifuged ($200 \times g$, 3 min). After incubating for 4 h, supernatants were removed and their radioactivity measured. Specific ^{51}Cr release (%) was calculated using the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Assay for chemotherapeutic drug- and gamma irradiation-induced apoptosis

Cells (5×10^5 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 (OD_{490}) and the extent of DNA fragmentation (OD_{405}) 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. 1*A*). 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. 1*B*). In contrast, Jurkat cells stably transfected with FLIP (JFL2) and treated with anti-CD3 showed slightly increased cell viability (Fig. 1*A*), and no effects on DNA fragmentation were observed (Fig. 1*B*). FACS analysis 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. 1*C*), but not by TRAILR2-Fc or TNFR1-Fc (Fig. 1*D*), confirming that Fas-induced apoptosis plays a major role in the AICD of Jurkat cells.

FLIP does not inhibit granzyme B/adenovirus- 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. 2*A*) or sublytic concentrations of perforin (Fig. 2*B*), 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. 3*A*). Western blotting using anti-Flag for detection of Flag-tagged FLIP was unsuccessful because A20 cells express endogenous Igs comigrating with FLIP (data not shown). However, RT-PCR clearly showed that the mRNA expression of FLIP was markedly increased in FLIP transfectants compared with A20 wild-type cells and vector-alone transfectants (Fig. 3*B*). There was no significant difference in Fas expression between these cells as judged by FACS (data not shown). MLC cells generated from *gld* mice (Fas-deficient) equally lysed A20 cells, vector transfectants, and FLIP transfectants (Fig. 3*C*). In contrast, FLIP transfectants were not killed by MLC cells generated from perforin-deficient mice (Fig. 3*D*). These data indicate that FLIP prevents Fas-dependent cytolysis, but not perforin-dependent cytotoxicity of CTLs.

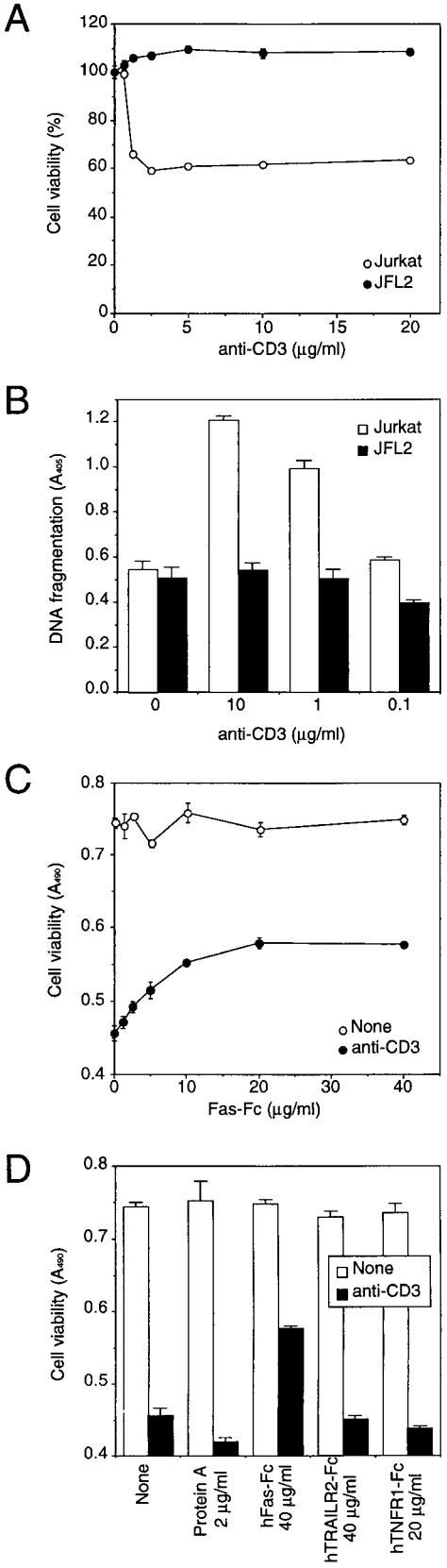

FIGURE 1. FLIP inhibits AICD. *A* and *B*, Jurkat cells (open circles or bars) and FLIP transfectants (JFL2; filled circles or bars) were cultured for 24 h in microtiter plates coated with the indicated concentrations of anti-CD3. Cell viability (%) and DNA fragmentation (OD_{405}) were determined using a cell proliferation assay (*A*) and a histone-DNA complex release assay (*B*). The mean ± SD of triplicate determinations are shown. *C* and *D*, Jurkat cells were cultured in anti-CD3-coated (10 μg/ml; filled circles or bars) or noncoated plates (open circles or bars) for 24 h in the presence of the indicated concentrations of hFas-Fc, hTRAILR2-Fc, or hTNFR1-Fc in the presence of protein A (2 μg/ml). Cell viability (OD_{490}) was determined using a cell proliferation assay. The mean ± SD of triplicate cultures are shown.

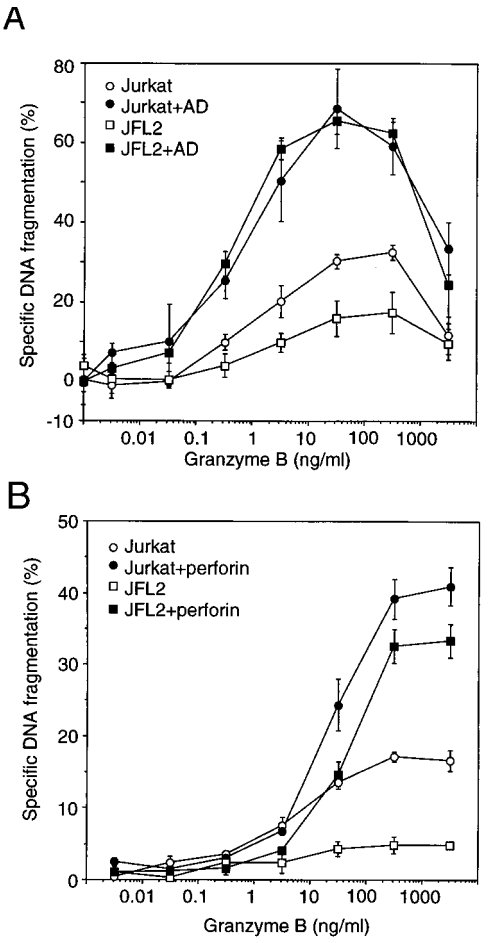

FIGURE 2. FLIP does not inhibit granzyme B/adenovirus- or granzyme B/perforin-mediated apoptosis. *A*, [^{125}I]UdR-labeled Jurkat cells (circles) or JFL2 (squares) were incubated with the indicated concentrations of granzyme B for 4 h in the absence (open symbols) or the presence (filled symbols) of adenovirus (AD). *B*, Jurkat cells (circles) or JFL2 (squares) were incubated with the indicated concentrations of granzyme B for 4 h in the absence (open symbols) or presence (filled symbols) of 160 U/ml of perforin. DNA fragmentation (%) was measured as described in *Materials and Methods*. The mean ± SD of triplicate determinations are shown.

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

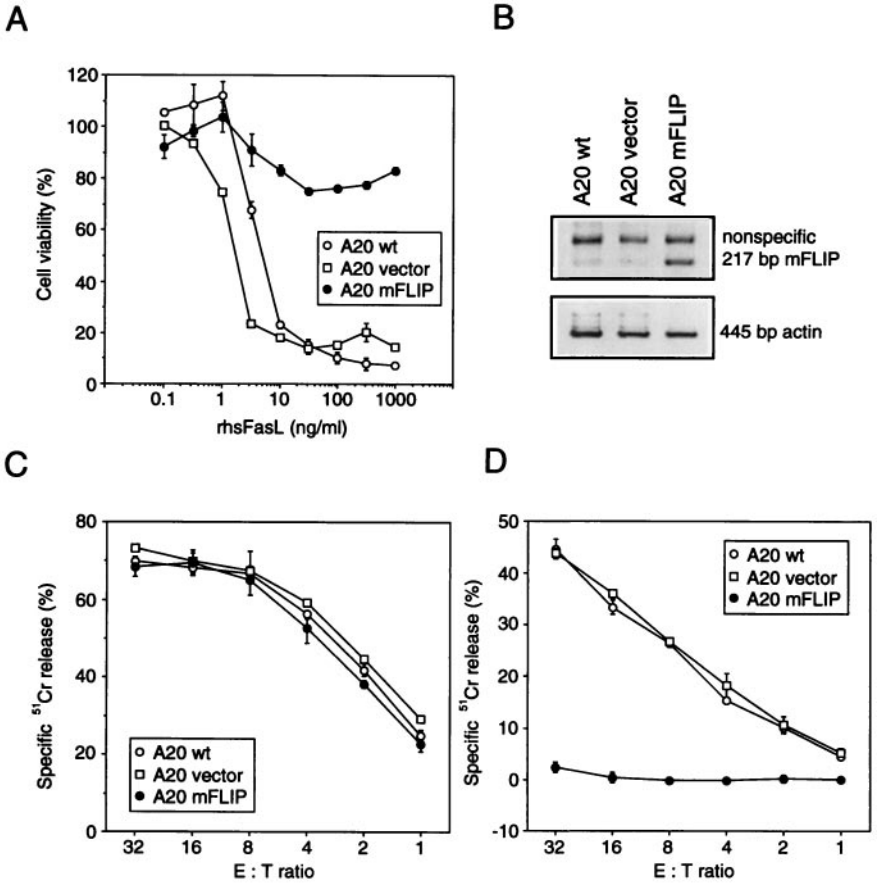

FIGURE 3. FLIP inhibits Fas-dependent, but not perforin-dependent, CTL-mediated target cell lysis. *A*, A20 wild-type cells (open circles), vector transfectants (open squares), and mFLIP transfectants (filled circles) were treated with serially diluted Flag-tagged rhsFasL plus 1 μg/ml of anti-Flag for 4 h. Cell viability (%) was measured using a cell proliferation assay. The mean ± SD of triplicate cultures are shown. *B*, Expression of mFLIP and actin was measured by RT-PCR. *C* and *D*, Spleen cells from *gld* (*C*) or perforin-deficient mice (*D*) were cultured with gamma-irradiated spleen cells for 5 days. MLC cells were incubated with ^{51}Cr-labeled A20 wild-type cells (open circles), vector transfectants (open squares), or mFLIP transfectants (filled circles) for 4 h. Radioactivity released into the culture supernatant was measured. The mean ± SD of triplicate cultures are shown.

(Fig. 4*G*). 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. 5*A*). Again, doxorubicin, etoposide, and vincristine killed FLIP transfectants as efficiently as they did vector transfectants (Fig. 5*A*). 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. 5*B*). 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. 6*A*) and induced DNA fragmentation (Fig. 6*B*). 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.

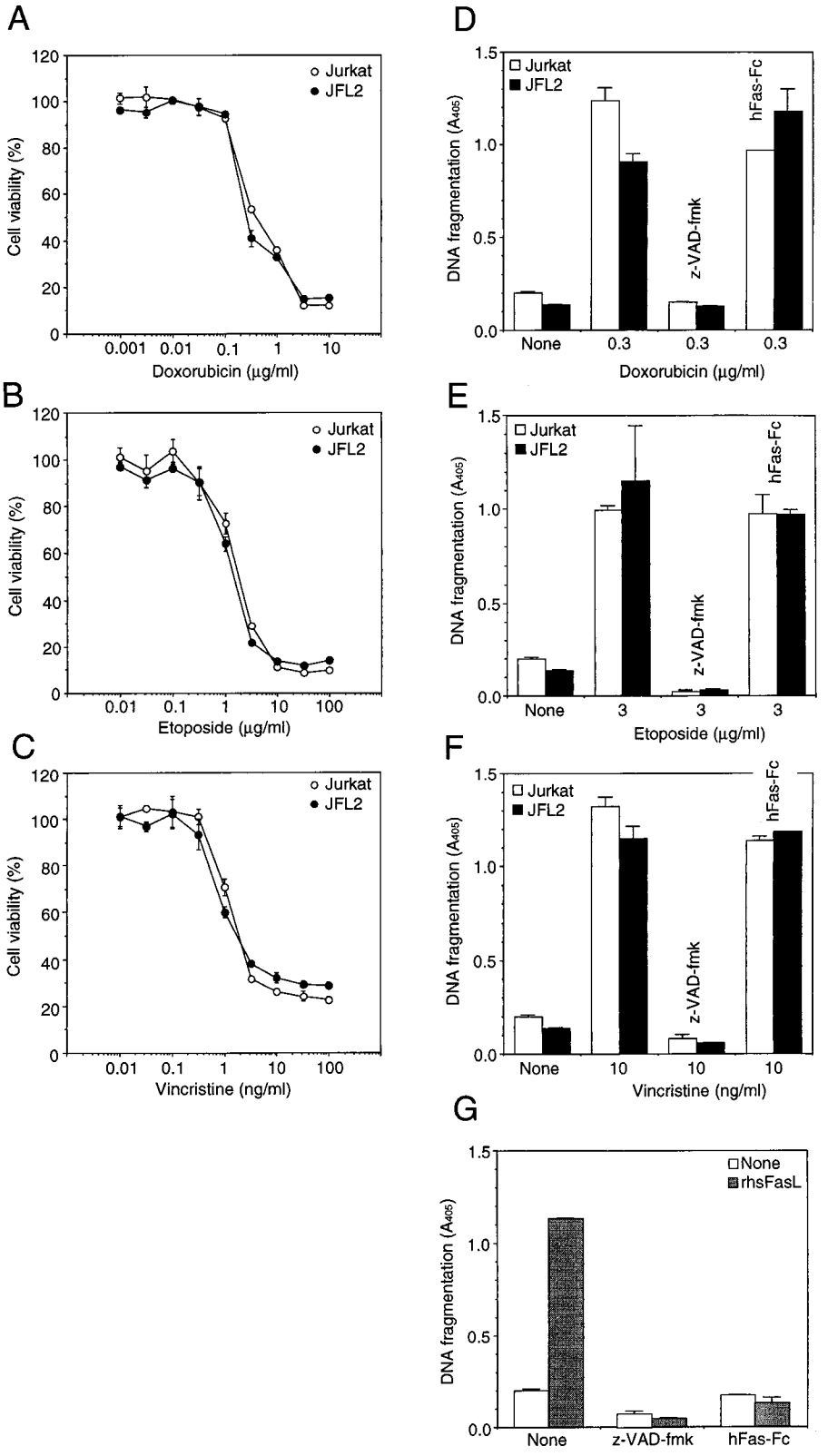

FIGURE 4. FLIP does not inhibit chemotherapeutic drug-induced apoptosis. *A–C*, Jurkat cells (open circles) and JFL2 (filled circles) were treated with the indicated concentrations of doxorubicin (*A*), etoposide (*B*), or vincristine (*C*) for 24 h. Cell viability (%) was measured using a cell proliferation 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.

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).

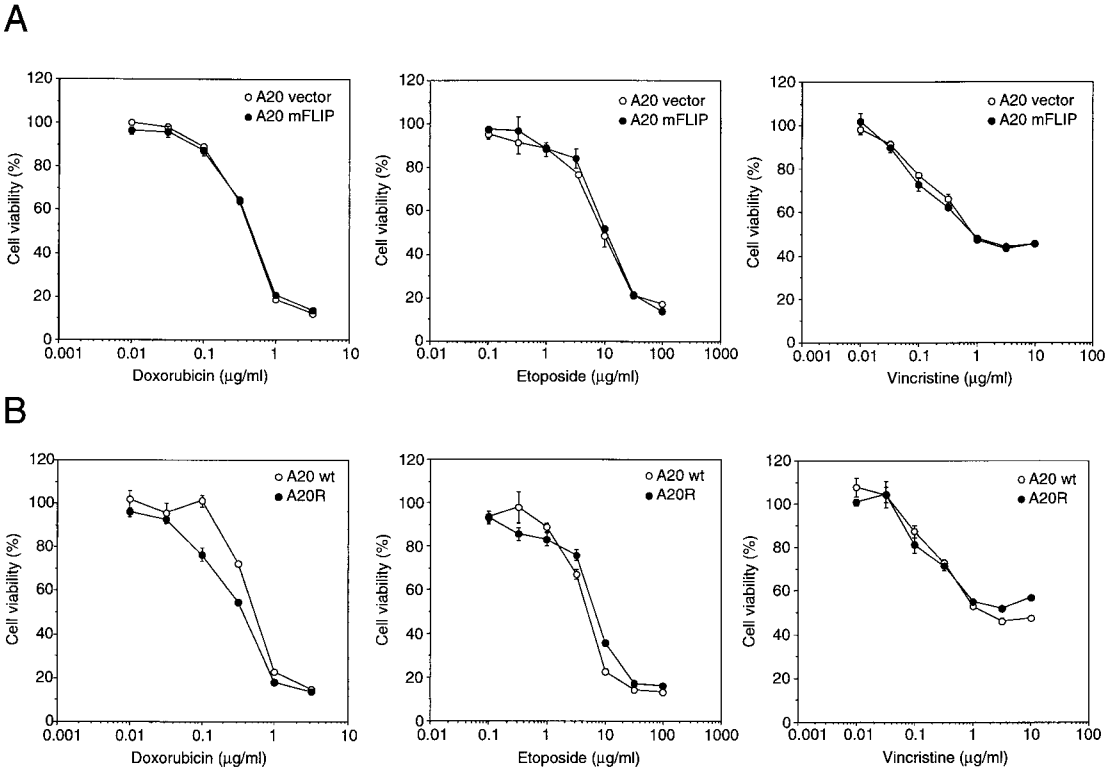

FIGURE 5. Chemotherapeutic drug-induced apoptosis is independent of Fas and is FLIP insensitive. *A*, A20 vector transfectants (open circles) and mFLIP transfectants (filled circles) were treated with the indicated concentrations of doxorubicin, etoposide, or vincristine for 24 h. *B*, A20 cells (open circles) and A20R (filled circles) were treated with the indicated concentrations of doxorubicin, etoposide, or vincristine for 24 h. Cell viability (%) was measured using a cell proliferation assay. The mean ± SD of triplicate cultures are shown.

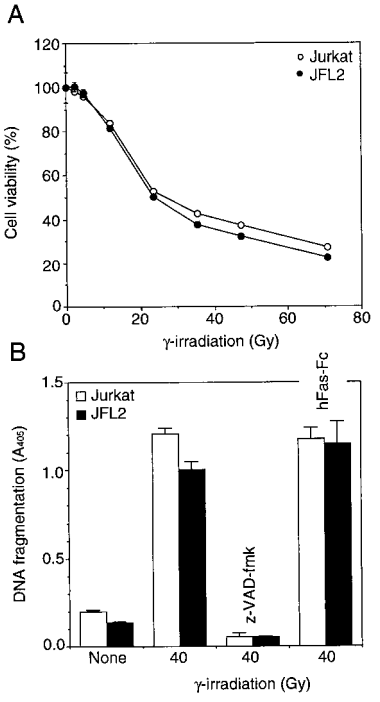

FIGURE 6. FLIP does not inhibit gamma irradiation-induced apoptosis. *A*, Jurkat cells (open circles) and JFL2 (filled circles) were treated with the indicated doses of gamma ray and then cultured for 24 h. Cell viability (%) was measured using a cell proliferation assay. The mean ± SD of triplicate cultures are shown. *B*, Jurkat cells (open bars) and JFL2 (filled bars) were treated with 40 Gy of gamma ray, then cultured for 24 h in the absence of the presence of 100 μM z-VAD-fmk or 20 μg/ml of Fas-Fc plus 2 μg/ml of protein A. DNA fragmentation (OD_{405}) was measured using a histone-DNA complex release assay. The mean ± SD of triplicate 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.

Acknowledgments

We thank Prem Smith at the National Cancer Institute for the adenovirus preparation and Sylvie Hertig for technical assistance. We are grateful to Dr. Sabina Belli for careful reading of the manuscript.

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