

Fas, ceramide and serum withdrawal induce apoptosis via a common pathway in a type II Jurkat cell line

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Abstract

Ceramide is a key mediator of apoptosis, yet its role in Fas-mediated apoptosis is controversial. Some reports have indicated that ceramide is either a primary signaling molecule in Fas-induced cell death, or that it functions upstream of Fas by increasing FasL expression. Other studies have suggested that ceramide is not relevant to Fas-induced cell death. We have approached this problem by studying ceramide-induced apoptosis in unique Jurkat cell clones selected for resistance to membrane-bound FasL-induced death. Resistance of the mutant Jurkat cells was specific for FasL killing, since the mutant clones were sensitive to other apoptotic stimuli such as cycloheximide and staurosporine. We tested the effects of serum withdrawal, one of the strongest inducers of ceramide, and of exogenous ceramide on apoptosis of both wild-type and FasL-resistant clones. Wild-type Jurkat cells were remarkably sensitive to serum withdrawal and to exogenous ceramide. In contrast all FasL-resistant mutant clones were resistant to these apoptosis-inducing conditions. In contrast to previous work, we did not detect an increase in FasL in either wild-type or mutant clones. Moreover activation of stress-activated protein kinases (JNK/SAPKs) after serum withdrawal and exogenous ceramide treatment was detected only in the wild-type and not in the resistant clones. Because of the parallel resistance of the mutant clones to Fas and to ceramide-induced apoptosis, our data support the notion that ceramide is a second messenger for the Fas/FasL pathway and that serum withdrawal, through production of ceramide, shares a common step with the Fas-mediated apoptotic pathway. Finally, our data suggest that activation of JNK/SAPKs is a common mediator of the three pathways tested.

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Keywords: Fas; ceramide; starvation; JNK/SAPKs; Jurkat cells

Abbreviations: JNK/SAPK, c-Jun N terminal protein kinase/stress-activated protein kinases; SW, serum withdrawal; FasL, Fas ligand

Introduction

Programmed cell death is a tightly regulated mechanism that can be induced by a variety of stimuli, which often rely on the expression of membrane receptors and their natural ligands.¹ Fas (APO-1/CD95), an important member of the tumor necrosis factor receptor superfamily,² is expressed in many different tissues and it has been shown to play a pivotal role in immune privileged sites such as the eye.³ Fas also play a fundamental role in the homeostasis of the immune system. In fact, Fas-signaling deficiency can lead to autoimmune disease in both mice and humans.^{4–6} Finally it has also been proposed that Fas and its natural ligand (FasL) play an important role in the apoptotic elimination of cells undergoing environmental trauma.^{7,8}

Triggering of Fas by FasL prompts the formation of the death-inducing signaling complex (DISC) composed by the adaptor molecules FADD and pro-caspase 8, followed by the release of active caspase 8. In many cell types (type I) caspase-8 directly activates the effector caspases⁹ and Bcl-2 or Bcl-x_L cannot inhibit this cascade of events. In other cell types (type II) Fas triggering induces little DISC formation, inadequate to directly activate effector caspases, yet sufficient to initiate the mitochondrial apoptotic machinery that activates effector caspases.^{10,11} Finally Yang and coworkers have shown that Fas triggering can induce apoptosis by activation of JNK/SAPK through DAXX.¹² Overall these data show that the complexity of Fas signaling is greater than previously thought. Evidence of such complexity is the long lasting debate on whether^{13–17} or not^{18–22} ceramide is involved as second messenger in Fas-induced apoptosis.

In mammalian cells ceramide can be generated by two mechanisms: (1) from sphingomyelin by activation of acidic or neutral sphingomyelinase (aSM and nSM respectively); and (2) from *N*-acetylation of dihydrosphingosine by ceramide synthase.²³ Ceramide formation can be induced by serum starvation, UV irradiation, γ -irradiation, oxidative stress, TNF receptor (for extensive review see reference²⁴), and T²⁵ and B²⁶ cell receptor engagement.

Fas triggering can also induce generation of ceramide, but contrasting reports have called into question its importance in Fas-induced apoptosis. Watts and colleagues have shown by mass spectrometry that Fas-induced cell death was independent by ceramide generation.²⁷ In contrast, Kirschnek and coworkers have shown that aSM-deficient mice are resistant to Fas-induced apoptosis indicating that generation of ceramide is key.¹⁶ The use of SM deficient human B cells has given conflicting results. De Maria and coworkers reported resistance to Fas-induced cell death in B cells derived from patients with Niemann-Pick disease, which have low level of acidic sphingomyelinase.¹⁴ In contrast Boesen-de Cock *et al.* have reported no protection.²⁸ Also debated is whether

ceramide formation upon Fas activation precedes²⁹ or follows³⁰ commitment to cell death. Finally Lin and co-workers have shown that ceramide might serve as second messenger for Fas signaling only in certain tissues.³¹

To help elucidate whether or not ceramide is involved in Fas-mediated apoptosis, we thought to utilize a system in which the Fas/FasL system was defective.⁷ We generated a type II cell line truly resistant to Fas triggering. Here we show that type II cells resistant to FasL are also resistant to exogenous ceramide (C2-ceramide), but retain sensitivity to Fas-independent stimuli. Moreover, these cells are also refractory to apoptosis induced by serum withdrawal (SW), a condition that triggers generation of endogenous ceramide.³² Our results indicate SW and Fas-triggering, through generation of ceramide, share a common step in the apoptotic pathway.

Results

E6-1 wild-type and mutant clones

E6-1^{R2} clones were generated by continuous culture of Jurkat cells with NIH-3T3-FasL transfected cells (7 and Figure 1). Cells resistant to membrane-bound FasL killing were cloned by limited dilution and clone c8, c12 and c45 were selected based on their equivalent Fas expression compared to the wild-type cell line (Figure 1A). Resistance to FasL killing was repeatedly tested for each clone. After 5 h incubation with the NIH-3T3-FasL cell line, wild-type and mutant clones were harvested and stained with PI for apoptosis detection. As shown in Figure 1B, 90% of wild-type cells were apoptotic, yet only spontaneous apoptosis was detectable in the mutant clones. In order to understand if resistance to FasL killing was specific, E6-1^S and E6-1^{R2} clones were treated with cycloheximide or staurosporine, which are known to induce apoptosis in a Fas-independent manner. As shown in Figure 2, a similar degree of apoptosis was detectable for all the mutant clones and wild-type cells, demonstrating that FasL resistance of the E6-1^{R2} clones was specific and that different apoptotic pathways were still functional. To further characterize the mutant clones we also tested PARP and caspase 3 cleavage after FasL, cycloheximide and staurosporine treatment (data not shown). After FasL treatment there was no PARP or caspase 3 cleavage, suggesting that the lesion in apoptosis in E6-1^{R2} cells lies upstream of caspase 3; in contrast, after cycloheximide and staurosporine treatment both molecules were cleaved suggesting that the apoptosis executioner steps were still functional (data not shown).

Ceramide and serum withdrawal (SW) induce apoptosis in wild-type but not in mutant clones

Conflicting data concerning ceramide involvement in the Fas/FasL system have been reported.^{13–20,22,30} In order to test if in our system ceramide and Fas trigger a common apoptotic pathway, E6-1^S and E6-1^{R2} clones c8, c12 and c45 were treated with exogenous ceramide (C2-ceramide, 30 μ M) for 10 h. Apoptosis was detected by PI staining, in which the apoptotic population appears in the <2N DNA peak. As expected, the wild-type clone showed marked susceptibility,

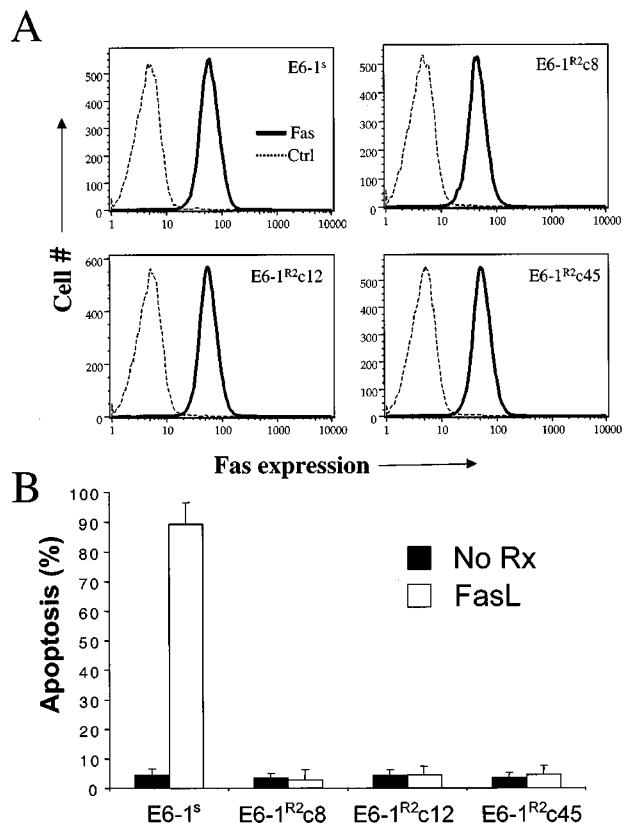


Figure 1 Description of E6-1^{R2} clones. The E6-1^{R2} cell line were originally generated by continuous culture with NIH-3T3-FasL transfected cell line. (A) E6-1^S and three different E6-1^{R2} clones were stained with anti-Fas-FITC (solid lines) and isotype control (dashed lines). Fas expression in wild-type and mutant is similar. (B) Cells were cultured for 5 h with NIH-3T3-FasL (open bars) or NIH-3T3-pSR (close bars) cell line and were then stained with PI. Percentage of FasL killing is shown according to PI staining results. The E6-1^S, as expected, was very sensitive to FasL killing (close bars). E6-1^{R2} cells failed to undergo apoptosis when cultured with FasL transfected 3T3 cells. Means+standard deviations are shown

while in contrast all three FasL-resistant clones were remarkably resistant to the treatment (Figure 3A).

To test whether these mutant clones were also resistant to apoptotic stimuli which trigger synthesis of endogenous ceramide, we investigated the effects of serum withdrawal (SW). SW induces strong production of endogenous ceramide through activation of sphingomyelinase³² and is a potent inducer of apoptosis.³³ As shown in Figure 3B, while E6-1^S after 24 and especially after 48 h of serum free culture showed a large percentage of apoptosis, all three mutant clones tested were significantly resistant to SW. Nevertheless the mutant clones underwent significant residual killing. These results can be explained by the hypothesis that whereas ceramide is essential when apoptosis is induced through Fas engagement, multiple pathways are involved when apoptosis is induced by SW.³²

FasL expression after ceramide treatment and serum starvation

It has been proposed that ceramide induces cell death by promoting expression of FasL and by subsequent autocrine or

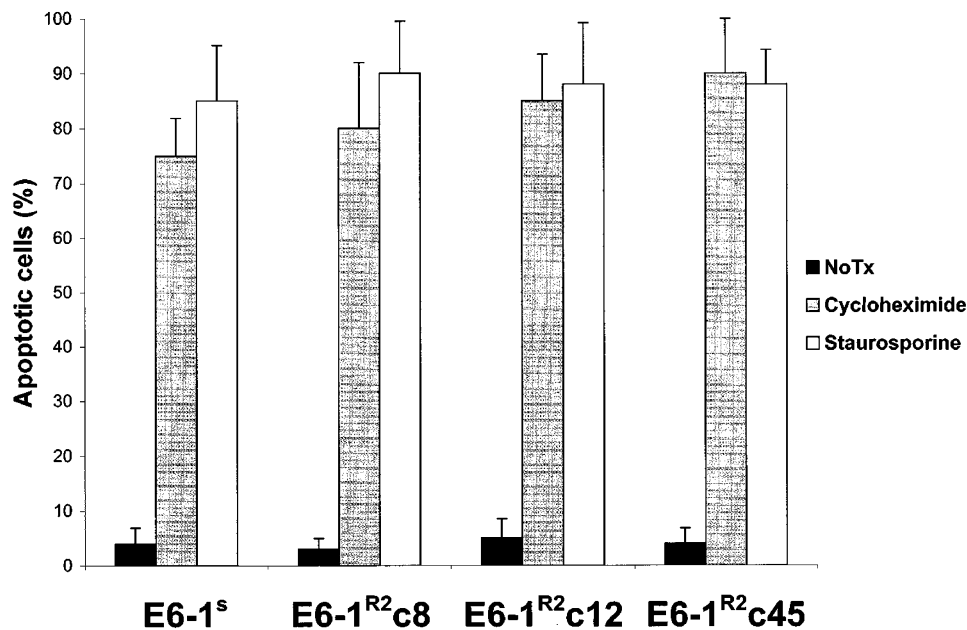


Figure 2 E6-1^{R2} clones are sensitive to FasL-independent killing. E6-1^S and E6-1^{R2} clones were treated with cycloheximide (60 μ g/ml) and staurosporine (1 μ M) for 10 h and 8 h respectively. Then cells were harvested and apoptosis measured by PI staining. Treatment with cycloheximide or staurosporine (Fas-independent killing) induced similar degrees of apoptosis in both wild-type and resistant clones similar to that seen with FasL. These data show that the mutant clones are still capable to undergo apoptosis when Fas-independent stimuli are used. Data shown are representative of three different experiments

paracrine Fas-mediated apoptosis.¹ Although our clones were selected for resistance to FasL, we wanted to determine if the exogenous ceramide (C₂-ceramide) and SW treatments were inducing FasL expression. Both wild-type and mutant clones were treated with C₂-ceramide (10 h) or cultured for 48 h in serum free media. Cells were lysed and FasL expression was measured by Western blot. Under neither condition did wild-type or E6-1^{R2} clones increase FasL expression (Figure 4). Although increased FasL after ceramide treatment has been reported,^{34,35} in our system both exogenous and endogenous ceramide appeared to act downstream of the Fas pathway and not through FasL expression.

Activation of JNK/SAPKs after ceramide treatment, serum starvation and anisomycin

Ceramide serves as a second messenger leading to the induction of the stress-activated protein kinases (JNK/SAPKs).³⁶ JNK/SAPKs are also strongly activated during stressful conditions such as serum withdrawal.^{37,38} In order to test if the ceramide-JNK/SAPKs pathway was involved in our system, E6-1^S and E6-1^{R2} clones c8, c12 and c45 were incubated for 10 h with 30 μ M of C₂-ceramide or under serum free conditions for 48 h. After treatment, cells were harvested, washed, lysed and JNK/SAPKs activation was tested by Western blot with antibodies directed against active JNK. Both C₂-ceramide treatment and serum free conditions induced increased JNK/SAPKs activity in E6-1^S cells, as shown in Figure 5. In contrast, the FasL resistant clones showed no or little activation after C₂-ceramide treatment, while after serum starvation only mild activation was detected,

probably accounting for the relative amount of apoptosis induced by SW through multiple pathways (Figure 3B).³² To determine if the JNK/SAPKs pathway was still functional in our mutant clones, cells were treated with anisomycin (1 μ g/ml, 30 min), which is a potent activator of JNK.³⁹ Cell extracts were then analyzed for JNK activation by Western blot. As shown in Figure 5, both wild-type and mutant clones activated JNK/SAPKs upon stimulation with anisomycin.

Discussion

The central observation of this report is that clones selected for resistance to FasL-induced cell death are also protected from ceramide-induced apoptosis. Our mutant clones were derived from a type II cell, and according to Peter and coworkers CD95 sensitive type II cells have reduced DISC formation and preferentially use mitochondrial machinery to reach the executioner phase of apoptosis.¹⁰ We therefore infer that ceramide in type II Jurkat cells not only shares an obligatory step downstream of Fas triggering, but that ceramide is also necessary for the execution of the mitochondrial cell death pathway.

The relationship of Fas-induced cell death to ceramide production remains controversial. Cuvillier and coworkers have shown that Fas triggering induced an increase of ceramide and sphingosine, a ceramide metabolite, and that the subsequent apoptosis was induced in a mitochondria-dependent fashion in Jurkat cells.⁴⁰ Moreover they and others have found that Bcl-x_L inhibited the Fas-ceramide apoptotic pathway.^{40,41} Other investigators have also shown increased ceramide production after Fas triggering, although there is controversy regarding its role.^{13–20,22,30}

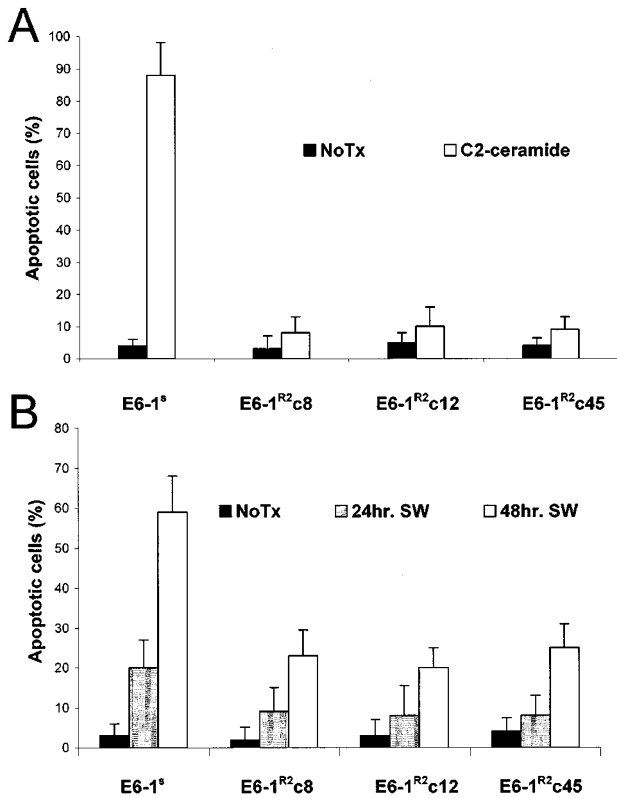


Figure 3 E6-1^{R2} clones are resistant both to ceramide and serum withdrawal (SW). (A) E6-1 wild-type and the mutant clones were incubated for 10 h in the presence of exogenous ceramide (C2 ceramide, 30 μ M). Then cells were fixed in ethanol and stained with PI. As expected the wild-type showed ~90% of apoptosis, while the mutant clones were resistant to C2-ceramide treatment. These data support the cross-talk between the cellular stress pathway initiated by ceramide and the Fas pathway of apoptosis. (B) Since one of the strongest inducers of endogenous ceramide is serum withdrawal, which is known to be a strong apoptosis inducer, we tested if E6-1^{R2} clones c8, c12, c45 were also resistant to SW. Mutants and the wild-type cell line were cultured in serum free medium for 24 and 48 h. Apoptosis was measured by light scatter. While E6-1^s after 24 and especially after 48 h of serum free culture showed a large percentage of apoptosis, all three mutant clones tested were remarkably resistant to the treatment. A residual ~20% of cell death was still appreciable in the mutant clones, probably accounting for the activation of multiple pathways when apoptosis is induced by serum starvation. Means+standard deviations are shown

Interpretation of the data has been complicated by the discovery that the ceramide increase is bimodal (i.e. in early and late phases of apoptosis). It has been suggested that the bimodal increase is dependent on the cell line and dose used, and that the early increase may be the initiator of the apoptotic cascade, while the late increase would function as an amplifier.⁴⁰ Our results clearly demonstrate that in type II Jurkat cells, ceramide and Fas share a common mandatory step toward the executioner phase of apoptosis.

In the last few years several laboratories have shown that ceramide increases after a variety of stress stimuli, such as anticancer drugs, heat shock, ultraviolet and gamma irradiation, tumor necrosis factor and growth factor withdrawal.^{24,42} Serum starvation has been shown to correlate in time with the accumulation of intracellular

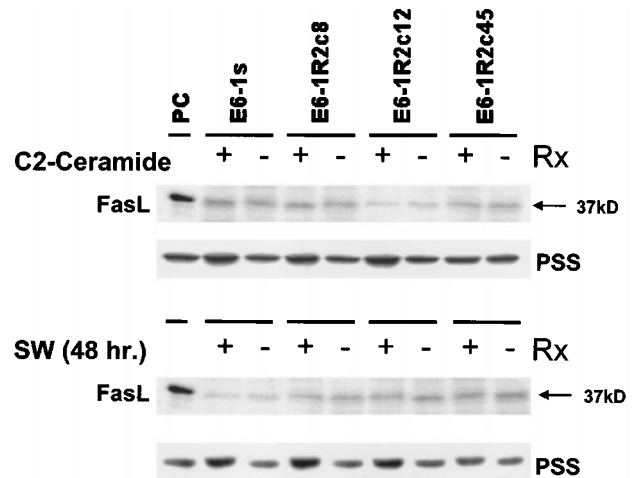


Figure 4 FasL expression and apoptosis induced by endogenous and exogenous ceramide. E6-1^s and E6-1^{R2} clones c8, c12 and c45 were treated with C2-ceramide (30 μ M) for 10 h or cultured for 48 h in complete or serum free medium. Cell extracts were analyzed by Western blot with antibody anti-FasL. In both conditions no FasL increase was detected, suggesting for ceramide a direct involvement in Fas downstream pathway. PC=positive control (NIH-3T3-FasL cell line). Ponceau S staining (PSS) is shown for protein loading control. Arrows indicate FasL molecular weight

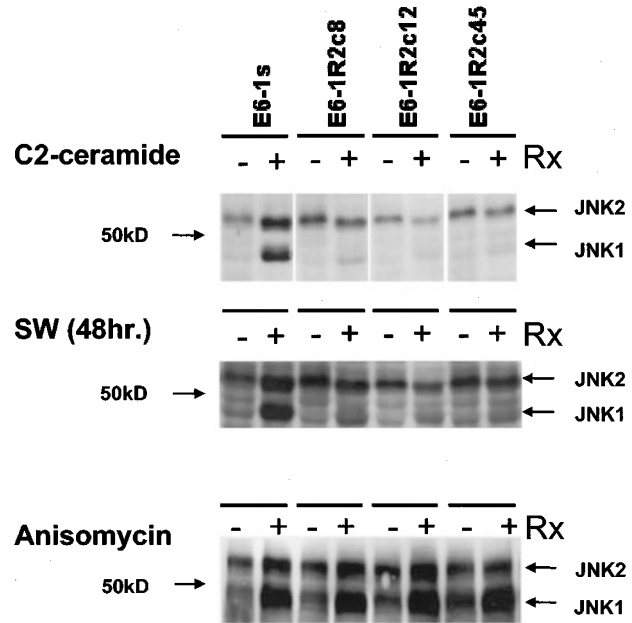


Figure 5 Activation of JNK/SAPKs after C₂-ceramide treatment, serum starvation and anisomycin. Activation of JNK/SAPKs, was tested in our wild-type E6-1^s cells and FasL resistant cells E6-1^{R2} (clones c8, c12 and c45). After 10 h (C₂-ceramide treatment) or 48 h (serum withdrawal), cells were harvested, lysed and subjected to high-speed centrifugation. Aliquots of each extract were analyzed by SDS-PAGE (10%) and transferred to nitrocellulose membranes. The membranes were probed with anti-active JNK. Activation of JNK/SAPKs for both conditions was detected only in the wild-type clone. Finally, to determine if JNK/SAPK pathway was functional, wild-type and mutant clones were treated with anisomycin, a classic activator of JNK. Arrows indicate molecular weight marker, phosphorylated JNK1 and JNK2

ceramide, leading to cell cycle arrest and apoptosis.⁴³ Moreover serum starvation has been considered to be among the strongest inducers of intracellular ceramide formation.³²

In the present study we used serum withdrawal to investigate if our FasL-resistant clones were also resistant to endogenous ceramide. Our experiments show that E6-1^{R2} mutant clones were remarkably resistant to this treatment, supporting the idea that ceramide is a second messenger of Fas in type II Jurkat cells. Moreover our results show that Fas triggering and serum starvation through production of ceramide share a common step in the apoptotic pathway.

These findings are in agreement with Olivera *et al.*, who observed that overexpression of sphingosine kinase, the enzyme responsible for the formation of sphingosine-1-phosphate (SPP), which has been proposed to be an antagonist of ceramide and sphingosine,⁴⁴ led to intracellular accumulation of SPP and consequent resistance of Jurkat cells to exogenous and endogenous ceramide.³³

Cell death induced by endogenous or exogenous ceramide could be due to subsequent expression of FasL and ultimately paracrine and/or autocrine FasL cell death.⁴⁵ Conflicting data regarding FasL expression after ceramide treatment have been reported.^{34,40} In our experimental conditions, no FasL increase was detected after both ceramide treatment and serum starvation, nevertheless both treatments induced apoptosis in E6-1^S cell line. We could detect no increase in FasL in our mutant clones as well. These data suggest that in type II Jurkat cells ceramide serves as a second messenger which directly involves the mitochondrial machinery and subsequent activation of caspase 8, 3 and ultimately the final executioners. Our data are in agreement with a model recently proposed, in which ceramide produced during both Fas activation or other stimuli is followed by mitochondrial changes, caspase activation and finally DNA condensation and degradation.²⁴

We acknowledge that others have shown that withdrawal of survival factors leads to apoptosis through FasL up-regulation. Such discrepancy with our findings might be due to the different cell type used in other conditions. For example Le-Niculescu and coworkers have shown increase of mRNA FasL in PC12 cells after survival factor withdrawal and that primary neuronal cultures from *gld* mice, which express a non-functional FasL, were resistant to the treatment.³⁸

In the last few years both ceramide and Fas induced apoptosis have been linked to the JNK/SAPK kinase cascade,^{36,46,47} although the extent of JNK/SAPK kinase involvement differs among cell types.⁴⁸ Increase in ceramide induced by a variety of stimuli precedes the activation of JNK/SAPK pathway and overexpression of dominant negative JNK/SAPK mutants suppressed apoptosis but not ceramide formation.⁴⁹ JNK/SAPK are also activated after Fas triggering but before the mitochondrial disruption, suggesting a pivotal role in Fas induced cell death pathway.⁵⁰

We therefore tested in our FasL resistant clones if both endogenous and exogenous ceramide could still activate

this pathway or if their resistance to ceramide correlated with the lack of JNK/SAPK activation. FasL resistant clones showed no activation of JNK/SAPK kinase after C2-ceramide treatment, while some activation was detectable after serum starvation. The latter result is in agreement with the fact that serum starvation in our mutant clones induced some apoptosis and that withdrawal of survival factors leads to JNK/SAPK activation through multiple pathways.³² Nonetheless our results are consistent with the notion that JNK/SAPK activation is a common step in the apoptotic pathway of Fas, ceramide and serum starvation. The absence of JNK/SAPK activation in our FasL resistant clones after exogenous ceramide treatment is in agreement with a previous report in which apoptosis induced by anti-Fas antibody or C6-ceramide treatment was prevented by transfection of transdominant inhibitory elements and pharmacological block of JNK/p38-K family JNK/p38-K inhibition.⁴⁷ We have extended these results by showing that FasL resistant type II cells were also resistant to endogenous ceramide and that the resistance correlated with lack of JNK/SAPK activation. Moreover, despite the JNK/SAPK activation, we have shown that FasL expression was not upregulated after apoptosis induced by both exogenous and endogenous ceramide, suggesting that JNK/SAPK activation is directly upstream of the final steps of apoptosis. We also excluded the possibility that this pathway was in general blocked in the mutant clones, by treating the clones with actinomycin, a classic JNK activator.³⁹

In this report we utilized a type II Jurkat cell line resistant to FasL (E6-1^{R2}) to test the relevance of ceramide as a second messenger in the death pathway induced by Fas triggering.

E6-1^{R2} clones expressed surface Fas, yet they were resistant to membrane-bound FasL. We have previously shown that clones derived from E6-1^{R2} cell line are truly resistant to FasL.⁷ In fact clones derived from Jurkat cell lines selected for resistance to anti-Fas antibodies were still sensitive to FasL killing, suggesting that anti-Fas antibodies do not completely mimic Fas triggering by the natural ligand and that FasL resistant clones are more suitable to investigate the Fas pathway.⁷

We are currently investigating the fault in the Fas signaling pathway in E6-1^{R2} cells. Our data suggest that the defect(s) lies upstream of caspase 3 and that is because exposure to FasL did not cleave caspase 3 (data not shown). Nevertheless, death can still be induced by other apoptotic stimuli such as cycloheximide or staurosporine. And when these two stimuli are used, DNA fragmentation is preceded by both caspase 3 and PARP cleavage, demonstrating that the final steps of apoptosis are still functional.

In the past decade both Fas/FasL system and ceramide have been shown to play pivotal roles in an extraordinary variety of diseases, stress conditions and physiological situations.^{2,23,24,42} For these reasons, they are among the most important strategic targets to control pathological processes or to improve our knowledge of mammalian cellular stress physiology. A better understanding of their inter-relationship will help to realize this task.

Materials and Methods

Cell lines and culture conditions

The Jurkat human T-cell leukemia subclone E6-1 (E6-1^S), constitutively expresses Fas and is sensitive to killing by FasL.⁷ The E6-1^{R2}, a FasL resistant variant of E6-1^S, was selected as described elsewhere.⁷ E6-1^S and E6-1^{R2} cells were maintained at a concentration of 1×10^6 ml in RPMI 1640, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids and 10% fetal bovine serum (FCS) at 37°C in a 5% CO₂/95% air humidified atmosphere. For serum withdrawal treatment, E6-1 cells in log phase growth were washed once with PBS and seeded at 1×10^6 ml in serum free RPMI 1640 or 10% FCS-supplemented RPMI 1640 for 24 and 48 h. NIH-3T3 cell line transfected with FasL (NIH-3T3–FasL) or with the empty vector (NIH-3T3–pSR7) were used as a source of Fas ligand for *in vitro* experiments. Briefly, NIH-3T3 cell lines were cultured in 6-well plates in complete medium. When NIH-3T3 were 90% confluent, the adherent monolayer was washed once with PBS, and 3×10^6 Jurkat cells in fresh medium were added to each well to test FasL induced apoptosis.

Drugs

E6-1^S and E6-1^{R2} clones were treated with C2-ceramide (30 μ M, 10 h; Biomol, Plymouth Meeting, PA, USA), cycloheximide (60 μ g/ml, 10 h; Sigma, St. Louis, MO, USA), staurosporine (1 μ M, 8 h; Sigma), or anisomycin (1 μ g/ml, 30 min, Sigma).

Cell staining for surface markers and apoptosis

For cell staining, fluoresceinated human anti-Fas (clone DX2, isotype: mouse IgG1 κ) and isotype matched FITC-labeled mouse antibody controls were obtained from PharMingen (San Diego, CA, USA). For DNA staining, after permeabilization with 70% ethanol, 0.1 ml of 1 mg/ml RNase A (Sigma) was added per sample, followed by 0.2 ml of 100 μ g/ml propidium iodide (PI) (Sigma). Cells were incubated for at least 30 min in the dark at 4°C and were analyzed with a FACScan (Becton Dickinson) with Cytomation data acquisition software (Fort Collins, CO, USA) for green and orange fluorescence. Detection of apoptotic cells was also made according to the forward-angle light scatter (FSC) and side scatter (SSC) profile after paraformaldehyde fixation.⁵¹ At least 30,000 events were collected per sample in all experiments.

Western blotting for FasL and JNK/SAPK

Samples of 4×10^6 E6-1 cells were washed in PBS and lysed for 20 min on ice in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% Glycerol, 10 mM NaF, 1 mM EDTA, 2 mM Na orthovanadate, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation for 15 min at 14,000 r.p.m., proteins in the supernatant were determined by BCA[®] Protein Assay Reagent (Pierce, Rockford, IL, USA) using bovine serum albumin standards. Supernatants were stored at –20°C. Thirty μ g of proteins in Laemmli buffer were separated per lane on 10% SDS–PAGE. To further determine if equivalent amounts of protein were loaded in each lane, membranes were stained with Ponceau S solution (PSS) for 10 min, scanned for picture collection and destained.

Western blots were then probed with mouse anti-FasL (Calbiochem, Cambridge, MA, USA) and rabbit anti-active-JNK/SAPK (Promega, Madison, WI, USA), that recognizes the dually phosphorylated, active form of JNK. Bound antibodies were detected with goat anti-mouse and anti-rabbit–horseradish peroxidase conjugate using

an enhanced chemiluminescence system (Renaissance, NEN Life Science Products, Boston, MA, USA).

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