Although numerous studies document caspase-independent ceramide generation preceding apoptosis upon environmental stress, the molecular ordering of ceramide generation during cytokine-induced apoptosis remains uncertain. Here, we show that CD95-induced ceramide elevation occurs during the initiation phase of apoptosis. We titrated down the amount of FADD transfected into HeLa and 293T cells until it was insufficient for apoptosis, although cycloheximide (CHX) still triggered the effector phase. Even in the absence of CHX, ceramide levels increased rapidly, peaking at 2.7 ± 0.2-fold of control 8 h post-transfection. Dominant negative FADD failed to confer ceramide generation or CHX-mediated apoptosis. Ceramide generation induced by FADD was initiator caspase-dependent, being blocked by crmA. Limited pro-caspase 8 overexpression also increased ceramide levels 2.7 ± 0.2-fold, yet failed, without CHX, to initiate apoptosis. Expression of membrane-targeted oligomerized CD-8 caspase 8 induced apoptosis without CHX, yet elevated ceramide only to a level equivalent to limited pro-caspase 8 transfection. Ceramide elevations were detected concurrently by diacylglycerol kinase and electrospray tandem mass spectrometry. These investigations provide evidence that ceramide generation is initiator caspase-dependent and occurs prior to commitment to the effector phase of apoptosis, definitively ordering ceramide as proximal in CD95 signaling.

The sphingomyelin (SM) pathway is an evolutionarily conserved stress response system linking diverse environmental stresses (UV, heat shock, oxidative stress, and ionizing radiation) to cellular effector pathways (1–3). Ceramide is the second messenger in this system and can be generated either by hydrolysis of SM through SM-specific phospholipases C termed sphingomyelinases (SMases) or by de novo synthesis through the enzyme ceramide synthase. There are at least two classes of SMase, acidic and neutral (ASMase and NSMase), respectively, distinguished by their pH optima. SMase or ceramide synthase activation by stress is cell- and stress type-dependent (4). Either alone, or in combination with other signals, ceramide, once generated, propagates the cellular stress response by coupling to effector systems. Different cells react differently to elevations in ceramide: some cells launch the apoptotic program, others commit to terminal differentiation, or undergo cell cycle arrest, depending on the effector pathways activated. Nonetheless, the most often reported outcome of ceramide signaling is induction of apoptosis (2, 3).

Genetic, biochemical, and pharmacologic evidence support ceramide as an initiator of apoptosis. Investigations have documented that in many systems ceramide generation occurs kinetically early and is caspase-independent (5–9). The role of ceramide generation as a mediator of the apoptotic event was further supported by the use of exogenous addition of ceramide (either analogs or natural (10–12)) or SMase, which elevate cellular ceramide levels by different mechanisms, yet induce apoptosis (2, 3). Selectivity was established by the lack of activity of other lipid second messengers or phospholipases as inducers of apoptosis and the failure of the naturally occurring dihydroceramide to signal death. For agents that use the de novo pathway for ceramide generation, the fungal toxin fumonisin B1, an inhibitor of ceramide synthase, uniformly blocks apoptosis induction (4, 13–16). Furthermore, genetic inactivation of ceramide generation through ASMase provides protection against radiation- and endotoxin-induced apoptosis in vitro and in vivo (17–20). Thus, for many stresses ceramide generation appears to signal apoptosis independent of initiating caspases. (It should, however, be noted that ceramide signaling of apoptosis is cell type-specific as data from ASMase knockout mice treated with total body irradiation indicate that endothelium but not thymocytes utilize this mechanism (17).)

Whether ceramide generation precedes induction of apoptosis upon activation of cytokine receptors or is subsequent to the commitment process is less certain. Some investigators have reported rapid ceramide generation after activation of the 55-kDa TNF receptor or Fas/APO-1/CD95, yet others have been unable to detect early ceramide generation and hence order ceramide downstream in the apoptotic cascade (21, 22). The purpose of the present investigations was to develop models in which ceramide generation could be molecularly ordered definitively with respect to CD95 signaling.

Triggering CD95 induces formation of a death-inducing signaling complex, comprised of the adaptor molecules FADD/MORT-1 and caspase 8, resulting in release of active caspase 8 to initiate the apoptotic process (23, 24). Apoptosis, however, ensues only after subsequent steps which commit the cells to effector caspase activation. Recently, Peter and co-workers (25) reported that CD95-induced death may occur in different cells by two separate mechanisms. Type I cells respond to receptor activation with robust initiator caspase activation and apparently signal apoptosis exclusively via a hierarchical caspase...

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cascade. This form of CD95-induced death is resistant to Bcl-2 inhibition and independent of mitochondrial permeability transition and release of mitochondrial apoptogenic factors (i.e. cytochrome c or apoptosis-inducing factor) (26). In contrast, Type II cells have reduced death-inducing signaling complex formation, and effector caspase activation occurs downstream of mitochondrial dysfunction. This latter mechanism was shown to be inhibitable by Bcl-2 and stimulable by ceramide analogs. Consistent with these observations, all published reports show that in cytokine-treated cells, ceramide-mediated apoptosis is Bcl-2-inhibitable (2, 3, 27, 28).

The sensitivity of the Type II cells to ceramide analogs suggests that if ceramide is to play a role in induction of apoptosis it would likely occur under conditions where initiator caspase activation was limiting. In the present studies, we titrated down the amount of transfected FADD or pro-caspase 8 in HeLa and 293T cells to the point where they no longer induced apoptosis. Under these conditions, FADD and pro-caspase 8 still induced maximal ceramide generation, which were inhibitable by CrmA. These investigations provide evidence that ceramide generation is initiator caspase-dependent and occurs prior to commitment to the effector phase of the apoptotic process, definitively ordering ceramide as proximal as CD95 signaling.

MATERIALS AND METHODS

Reagents

Anti-FADD, anti-caspase 8 and anti-Fas (clone CH11) antibodies were from Upstate Biotechnology Inc. Anti-cytochrome c antibody (65981A) was from Pharmingen. Horseradish-conjugated anti-rabbit antibody and enhanced chemiluminescence (ECL) were from Amer sham Pharmacia Biotech. Ceramide type III, Hoechst-33258 and CHX were from Sigma. Escherichia coli diacylglycerol (DAG) kinase was from Biomol.

Cell Culture and Transfection

HeLa and 293T cells were grown in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Life Technologies, Inc.), penicillin, and streptomycin at 37 °C in 5% CO2. For transfection, 2 x 105 cells were seeded per 55-mm tissue culture dish. After 24 h, LipofectAMINE (Life Technologies, Inc.) transfection was performed as per the manufacturer’s instructions using 0.5 μg of total DNA/5 x 105 cells and 5 μl of LipofectAMINE reagent in serum-free medium (Opti-MEM; Life Technologies, Inc.). After 12 h, transfected cells were returned to Dulbecco’s modified Eagle’s growth medium.

Mammalian Expression Vectors

Vectors pCDNA3 AU1-FADD, pCDNA AU1-DNFADD, pCDNA-FLICE, and pCDNA-crmA were kindly provided by Dr. Vishva Dixit. pCDNA-cD8 and pCDNA3-cD8-caspase 8 were kindly provided by Dr. Michael Lenardo.

Immunoblot Assays

Cells were harvested, washed twice in ice-cold phosphate-buffered saline, and lysed in Nonidet P-40 buffer (25 mM phosphate-buffered saline (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM NaVO4). After 20 min, lysates were centrifuged at 8000 g for 10 min and supernatant protein content determined by the BCA assay (Pierce). 50 μg of protein were separated on 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Hybond, Amer sham Pharmacia Biotech) and blocked with 5% nonfat dry milk in TBST (Tris-buffered saline and 0.05% Tween 20). Blots were incubated with anti-caspase 8 or anti-FADD rabbit polyclonal antibody (1:1000 dilution in TBST), followed by horseradish peroxidase-conjugated donkey anti-rabbit antibody. Proteins were visualized by ECL.

Apoptosis Assays

Bisbenzimide—Nuclear apoptosis was assessed by staining of paraformaldehyde fixed cells with the DNA-binding fluorochrome Hoechst-33258 as described previously (29). A minimum of 400 cells were scored at each point.

Cytochrome c Release—Cell fractionation was performed as described previously (30, 31). Immunoblots for cytosolic and mitochondrial cytochrome c were performed as described above.

Lipid Assays

Ceramide was measured by DAG kinase as described previously (32). Mass Spectrometry

For ceramide analysis by mass spectrometry (MS), cell pellets were transferred to 13 x 100-mm glass test tubes, and 0.5 ml of 0.1 mM KOH in CHCl3-CH2OH (1:2, v/v) was added with 5 nmol of C16-ceramide as an internal standard. The mixture was incubated for 1 h at 37 °C, then diluted with 3.5 ml of CH3OH-H2O (1:1) and spun in a clinical centrifuge to remove precipitated protein. The supernatant was loaded onto a 1-mL RP18 column (pre-equilibrated with 1:1 CH3OH-H2O after washing the resin in 100% CH3OH, 100% C8H8, 100% CH3OH, and 1:1 CH3OH-H2O). The column was washed with 2 ml of CH3OH-H2O (1:1) then eluted with (a) 2 ml of CH3OH-H2O (9:1) and 2 ml of 100% CH3OH, (b) 5 ml of CH3OH-C8H8 (2:1), and (c) 4 ml of CH3OH-C8H8-HOAc (66:33:1). Eluates were dried under vacuum (all fractions were monitored by MS and ceramide was found in fraction “b”).

Cell extracts (from 0.5–1 x 105 cells) were reconstituted in 1 ml of 50% CH3OH/CHCl3. A 100 μl aliquot was diluted to 1 ml with 890 μl of 5% NH4OAc in CH3OH and 10 μl of glacial HOAc. 20 μl of this solution was injected via a Rheodyne 8125 injector into the mass spectrometer at a flow rate of 50 μl min⁻¹.

Experiments were performed on a PE-Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source. Dry N2 was used as the nebulizing gas at a flow rate of 6 liter min⁻¹. The ion spray needle was held at 5500 V, while the orifice and ring voltages were kept low (15 and 180 V, respectively) as was the orifice temperature (50 °C) to prevent collateral decomposition of molecular ions prior to entry into the first quadrupole. Precursor ion spectra were acquired by scanning Q1 from 500a-700b in 0.1 u steps with a dwell time of 1.0 ms. N2 was used to collisionally induce dissociations in Q2, which was offset from Q1 by 40 V. Q3 was then set to pass molecularly distinctive product ions (N⁺ ions) of m/z 264.2. Multiple reaction monitoring scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the five most abundant ceramides found in the precursor ion scans. These transitions occurred at m/z 538.7/264.3, 566.5/264.3, 622.7/264.3, 648.7/264.3, 650.7/264.3, corresponding to ceramides with a d18:1 sphingoid base and C16:0, C18:0, C22:0, C24:1, and C24:0 fatty acid, respectively. Dwell time was 40 ms for each transition.

Statistical Analysis

Statistical analysis was performed by Student’s t test and t test for correlation coefficient. Linear regression analysis was by the method of least squares.

RESULTS

Initial studies examined the effect of overexpression of the gene coding for FADD on ceramide levels in HeLa cells. Prior studies (23, 33, 34) reported that transfection with 5–10 μg pCDNA3 AU1-FADD/10⁶ cells signaled apoptosis. For our studies, we reduced the quantity of the plasmid 5–10-fold (1 μg of pCDNA3 AU1-FADD/10⁶ cells) to attenuate the induction of apoptosis (see below). HeLa Cells normally manifest low levels of FADD. Transfection with small amounts of pCDNA3 AU1-FADD significantly increased FADD expression within 2 h of resuspension into growth medium, and a 10-fold increase in FADD levels above control was measured by 12 h (not shown). Concomitantly, ceramide levels rose peaking at 8 h (Fig. 1A; p < 0.05 versus pCDNA at all times after 2 h (n = 3)). Ceramide elevation was maintained for at least 24 h post-transfection. In contrast, transfection of a truncated variant of FADD lacking the N-terminal DED domain, which serves dominant negative function (DN-FADD) (35), did not lead to ceramide generation (Fig. 1B).

Fig. 1C shows that FADD-mediated ceramide generation after limited FADD transfection was not associated with expression of apoptosis. Limited expression of FADD failed to induce apoptosis at any time up until 12 h after resuspending cells into growth medium as measured by bisbenzimide staining (Fig. 1C) or by cytochrome c release (Fig. 1C, inset). Other
FIG. 1. Ceramide generation by FADD precedes induction of apoptosis. A, kinetics of ceramide generation in HeLa cells transfected with FADD. Cells were transfected with pCDNA3-FADD or vector using LipofectAMINE and transferred after 12 h into serum-containing medium (time
experiments showed that apoptosis was not detected for as long as 48 h under these conditions (not shown). In contrast, when cells were transfected with higher amounts of the FADD vector (4 μg of pCDNA3 AU1-FADD/10^6 cells), we, like other investigators (23, 33, 34), observed as much as 50–60% apoptosis by 48 h (n = 3). As ceramide levels peaked in our system at 8 h after limited FADD transfection, we arbitrarily chose to add CHX (10 μg/ml) at 12 h to allow for induction of apoptosis. Fig. 1C shows that addition of CHX to these FADD overexpressing cells, but not to cells transfected with DN-FADD or the pCDNA vector alone (not shown), rapidly induced apoptosis as determined by bisbenzimide staining (Fig. 1C) and confirmed by cytochrome c release into cytosol (Fig. 1C, inset). A reciprocal reduction in mitochondrial cytochrome c content was detected (not shown). CHX addition had no effect on ceramide levels in pCDNA- or DN-FADD-transfected cells and induced no further elevation of ceramide levels in FADD-transfected cells. HeLa cells were also resistant to C₂-ceramide (50 μM)-induced apoptosis measured by bisbenzimide staining or the use of the fluorogenic caspase substrate Z-DEVD-AFC, as described previously (36). In contrast, 46 ± 6% of CHX pretreated HeLa cells underwent apoptosis within 6 h of C₂-ceramide addition (n = 3), resulting in a 8.3-fold increase in DEVDase activity. These studies show that active FADD induces ceramide generation independent of the effector phase of apoptosis.

We used the same strategy to explore the association of ceramide generation after FADD transfection to induction of apoptosis in 293T cells. At 24 h post-transfection with limited amounts of pCDNA3 AU1-FADD (1 μg of pCDNA3 AU1-FADD/10^6 cells), 293T cells transfected with pCDNA3 AU1-FADD, but not pCDNA, displayed a 2.5 ± 0.2-fold increase in ceramide levels (Fig. 1D; p < 0.02 versus pCDNA). Nonetheless, the morphologic appearance of the 293T monolayer (fibroblastoid with dendritic-like processes) was unaltered. Furthermore, there was no evidence of apoptosis as measured by bisbenzimide staining (Fig. 1E) or cytochrome c release into the cytosol (Fig. 1E, inset). Hence, like HeLa cells, 293T cells expressing small amounts of FADD failed to undergo apoptosis. In contrast to HeLa cells, however, CHX (10 μg/ml) addition to 293T cells expressing limited FADD was insufficient to induce apoptosis, unless cells were concomitantly treated with the proapoptotic anti-Fas antibody clone CH11 (100 ng/ml; Fig. 1E). Furthermore, minimal apoptosis occurred in response to anti-Fas antibody CH11 in 293T cells lacking FADD overexpression. FADD-expressing cells, when treated with anti-Fas antibody CH11 and CHX, rounded up by 6 h and began to lift off the dish. At 24 h after treatment, nearly 75% of cells were apoptotic as measured by bisbenzimide staining (Fig. 1E) and confirmed by cytochrome c release into the cytosol (Fig. 1E, inset). However, treatment of FADD-expressing cells with antibody CH11 and CHX induced no further increase in ceramide accumulation, which remained at 2.7 ± 0.3-fold of control. These studies show that overexpression of FADD induces ceramide elevation independent of the effector phase of apoptosis in both 293T and HeLa cells.

Since use of the DAG kinase assay to quantify ceramide has been questioned recently (37), increases in ceramide were confirmed by tandem MS. For these analyses, the ceramides of 293T cells were first determined to be sphingosine-containing species comprised mainly of palmitic acid (C16:0) and lesser amounts of C18:0, C22:0, C24:0, and C24:1 fatty acids, as shown in Fig. 2A. With this information, both the amounts and types of cellular ceramides were measured (Fig. 2B) for vector only (A), FADD (B), or pro-caspase 8 (C). Shown are duplicate injections of each sample (the time abscissa in the lower panel represents the arbitrary times at which each sample was injected into the mass spectrometer).

Fig. 2. Mass spectrometric analysis of ceramides in 293T cells. A, a representative analysis of the major ceramide species of 293T cells is shown. Most were comprised of sphingosine backbones (based on the N⁴-fatty fragment ion of m/z 264) and the fatty acids: C16:0 (m/z 539), C18:0 (m/z 567), C22:0 (m/z 623), C20:1 (m/z 649), and C24:0 (m/z 651). B displays the relative ion abundance of these ceramides upon injection into the electrospray ionization/MS/MS of lipid extracts of cells transfected with vector only (A), FADD (B), or pro-caspase 8 (C). Shown are duplicate injections of each sample (the time abscissa in the lower panel represents the arbitrary times at which each sample was injected into the mass spectrometer).

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FIG. 3. Ceramide generation after FADD overexpression is dependent on initiator caspase activity. A, crmA inhibits FADD-induced ceramide generation. 293T cells were co-transfected with pCDNA3-FADD, and pCDNA-crmA or pCDNA, and handled as in Fig. 1A. Data (mean ± range) are from one of three experiments performed in duplicate. Western analysis was performed at 24 h. B, left panel, overexpression of pro-caspase 8 induces ceramide generation. For these studies, 293T cells were transfected with pCDNA-FLICE, and after 24 h ceramide was...
the mean of the differences between the results by DAG kinase assay and MS was only 15 ± 9%, n = 6), thus, confirming that expression of these gene products increases ceramide amounts in the cells. Differences in ceramide species were not found in FADD- and pro-caspase 8-overexpressors when compared with controls.

To determine whether ceramide generation induced by FADD is caspase-dependent, we co-transfected 293T cells with the initiator-caspase inhibitor crmA, and FADD. Co-transfection of crmA did not affect expression of FADD (Fig. 3A, bottom panel). However, crmA completely blocked FADD-induced ceramide generation (Fig. 3A). These investigations provide evidence that FADD-induced ceramide generation is initiator caspase-dependent.

To address this issue directly, we overexpressed a small amount of pro-caspase 8a in 293T cells (1 µg of pCDNA-FLICE/106 cells). Under the conditions of our study, pro-caspase 8a was detected to a level 2–3-fold of control, which resulted in the generation of the 43- and 41-kDa processed forms described in the literature (Fig. 3B, right panel) (38, 39). (Note: lower molecular weight forms were not detected under these conditions.) Overexpression of pro-caspase 8 resulted in a 2.7 ± 0.2-fold increase in ceramide levels as measured using the DAG kinase assay (Fig. 3B, left panel; p < 0.05 versus pCDNA), which was confirmed by tandem MS (Fig. 2). As with limited FADD overexpression, which induced similar ceramide elevation in 293T cells, limited pro-caspase 8 overexpression failed to elicit apoptosis (Fig. 3C). Again, addition of CHX (10 µg/ml) with anti-Fas antibody CH11 (50 ng/ml), but not CHX alone, resulted in rapid apoptosis of 293T cells specifically expressing limited pro-caspase 8. Limited pro-caspase 8 overexpression also induced a 2.4 ± 0.2-fold increase in ceramide content in HeLa cells and conferred CHX-dependent apoptosis (not shown). These studies provide direct evidence that ceramide generation is initiator caspase-dependent and independent of the effector phase of apoptosis.

To compare levels of ceramide generated independent of apoptosis with those associated with apoptosis, we expressed CD8-caspase 8 in HeLa cells. Prior studies showed that expression of this membrane-targeted oligomerized form of caspase 8 efficiently induced apoptosis in Jurkat cells. In contrast to pro-caspase 8, CD8-caspase 8 expression in HeLa cells induced apoptosis independent of CHX (Fig. 3D, right panel; p < 0.05 versus CD8). Nevertheless, CD8-caspase 8 induced a 2.7 ± 0.3-fold elevation in ceramide in HeLa cells (Fig. 3D, left panel; p < 0.05 versus CD8), a level similar to that induced by pro-caspase 8 expression in the absence of CHX (see above). These studies indicate that generation of ceramide in this system is initiator caspase-mediated and occurs independent of whether apoptosis progresses to the effector stage.

**DISCUSSION**

While in several stress response models ceramide generation clearly precedes apoptosis and is not inhibitable by caspase blockade, it has been questioned whether ceramide elevation during CD95 signaling occurs prior to or after the commitment step to apoptosis (21, 22, 40). The present studies resolve this issue. We show that HeLa and 293T cells overexpressing amounts of FADD or pro-caspase 8 insufficient for an apoptotic response require CHX treatment to activate the effector phase of apoptosis. Nevertheless, ceramide elevation occurs in these cells independent of CHX, indicating that ceramide is an early stage product of CD95 signaling and independent of the effector stage of apoptosis entirely. Our data confirm previous reports (41, 42) that ceramide generation depends on initiator caspase action, as evidenced by ceramide elevations in response to caspase 8 expression and its inhibition by crmA. Even if a minute amount of apoptosis did occur, undetectable by our assays, these studies nonetheless show that the ceramide response is exquisitely sensitive to initiator caspase action. Consistent with this observation, pro-caspase 8, which in the absence of CHX delivered measurable apoptosis, and CD8-caspase 8, which induced marked apoptosis independent of CHX, induced equivalent ceramide elevations. Whether unprocessed pro-caspase 8 is the active form in our studies, as has been suggested for pro-caspase 9 (43), or whether the small amount of processed enzyme induces the ceramide elevation is unknown. Although these studies do not address the role of ceramide in CD95-induced apoptosis, they molecularly order ceramide proximal in CD95 signaling. Nevertheless, the requirement for CHX for low levels of FADD, caspase 8, and exogenous ceramide to signal apoptosis in these cells is consistent with endogenous ceramide serving as an amplification signal for CD95-induced death. Furthermore, these investigations do not indicate whether acid or neutral SMase mediates CD95-induced ceramide elevation, as both mechanisms have been reported downstream of initiator caspase activation (44–46).

Ceramide elevation as detected by the DAG kinase assay was validated in the present study by the use of tandem mass spectrometry. Gill and Windebank (47) have also measured ceramide generation by both DAG kinase and MS within 12 h of treatment of primary cultures of dorsal root ganglion neurons with the antineoplastic agent suramin. Apoptosis in their study was not manifest until 36 h, hence ceramide generation preceded apoptosis by many hours. This and the present study confirm the utility of measuring ceramide elevation by DAG kinase.

The present investigations bring up the question of why some investigators have found it difficult to show ceramide elevations preceding apoptosis. Although it is possible that the use of this mechanism is confined to specific cells, recent studies provide another explanation. Merrill and co-workers (48) showed that interleukin 1β induced a 40% reduction in SM mass within 15 min of stimulation of primary cultures of rat hepatocytes but no ceramide elevation. Since SMase is the only known enzyme capable of catalyzing SM degradation in mammalian cells, these investigators tracked ceramide metabolites. They showed that concomitant with SMase action, interleukin 1β also rapidly activated ceramidase, which degraded the generated ceramide to free sphingoid bases. Thus to rule out a contribution of the SM pathway, ceramide determination must be accompanied by measurement of SM content. Consistent with this observation, Henk van den Bosch and co-workers showed that nitric oxide (NO) induced sustained ceramide elevation and apoptosis in rat renal mesangial cells whereas TNFα induced only a transient early ceramide elevation without apoptosis (49). Nevertheless both agents induced prolonged SMase activation. Further examination of the pattern of enzyme activation revealed that TNFα, but not NO, simulta-

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**Quantified values:**

Data (mean ± S.E.) are from three experiments performed in triplicate. Right panel, Western analysis was performed at 24 h after transfection. C, pro-caspase 8 induces apoptosis only upon treatment with anti-Fas antibody CH11 and CHX. For these studies, cells were transfected as in Fig. 3B and apoptosis assessed by bisbenzimid staining as in Fig. 1E. Data (mean ± S.E.) are from three experiments performed in duplicate. D, CD8-caspase 8 induces ceramide generation and apoptosis independent of CHX treatment. For these studies, cells were transfected with pCDNA3-CD8 or pCDNA3-CD8-caspase 8 and ceramide generation and apoptosis assessed 10 h post-transfection as in Fig. 3. A and C, respectively. Data (mean ± S.E.) are from three experiments each performed in duplicate.
neously increased ceramidease activity, attenuating the ceramide rise. Most importantly, pharmacologic inactivation of ceramidease using N-oleoyl-ethanolamine, converted the ceramide elevation in response to TNFα to the sustained pattern and conferred apoptosis. Similarly, the rapid conversion of ceramide to glucosylceramide via the enzyme glucosylceramide synthase appears to attenuate apoptosis in response to diverse chemotherapeutic agents in cells displaying the multidrug resistance phenotype and pharmacologic or genetic inactivation of glucosylceramide synthase results in sustained ceramide elevation and sensitization to chemotherapy-induced apoptosis (50–53). These studies show that ceramide, once generated, is subject to regulated metabolism, which results in decreases in ceramide mass via a variety of different enzymatic mechanisms. Based on this information, we suggest that many of the studies that concluded that the SM pathway is not involved in the early action of cytokines and other stresses, based solely on the inability to detect ceramide elevation, should be reevaluated.

While the present studies demonstrate that CD95 signals ceramide generation during the initiation phase of apoptosis, they do not address a role, if any, for ceramide in CD95-induced apoptosis. Recent in vivo studies, however, define a requirement for ceramide in one form of CD95-induced death.2 Whereas intravenous injection of the Jo2 anti-Fas antibody (3 g/25 g mouse body weight) into acid SMase knockout mice simultaneously increased ceramidase activity, attenuating the ceramide-mediated pathway failed to effect hepatocyte apoptosis or death, massive hepatic disintegration during the terminal, effector phase of the apoptotic response.

Whereas intravenous injection of the Jo2 anti-Fas antibody (3 g/25 g mouse body weight) into acid SMase knockout mice simultaneously increased ceramidase activity, attenuating the ceramide-mediated pathway failed to effect hepatocyte apoptosis or death, massive hepatic disintegration during the terminal, effector phase of the apoptotic response.

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CD95(Fas/APO-1) Signals Ceramide Generation Independent of the Effector Stage of Apoptosis
Carsten Grullich, M. Cameron Sullards, Zvi Fuks, Alfred H. Merrill, Jr. and Richard Kolesnick

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