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Citation for published version (APA):
Tepper, A. D. (2000). The role of ceramide in apoptosis

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CD95/Fas-induced ceramide formation proceeds with slow kinetics and is not blocked by caspase-3 / CPP32 inhibition.

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reprinted from The Journal of Biological Chemistry
(1997) 272:24308
The current confusion regarding the relevance of endogenous ceramide in mediating CD95/Fas-induced apoptosis is based mainly on (i) discrepancies in kinetics of the ceramide response between different studies using the same apoptotic stimulus and (ii) the observation that late ceramide formation (hours) often parallels apoptosis onset. We investigated CD95-induced ceramide formation in Jurkat cells, using two methods (radiolabeling/thin layer chromatography and benzoylation/high performance liquid chromatography), which, unlike the commonly used diglyceride kinase assay, discriminate between ceramide species and de novo formed dihydroceramide. We demonstrate that ceramide accumulates after several hours, reaching a 7-fold increase after 8 h, kinetics closely paralleling apoptosis induction. No fast response was observed, not even in the presence of inhibitors of ceramide metabolism. The majority (~70%) of the ceramide response remained unaffected when apoptosis was completely inhibited at the level of caspase-3/CPP32 processing by the inhibitor peptide DEVD-CHO. Exogenous cell-permeable C2-ceramide induced the proteolytic processing of caspase-3, albeit with somewhat slower kinetics than with CD95. DEVD-CHO dose-dependently inhibited C2-ceramide- or exogenous sphingomyelinase-induced apoptosis. The results support the idea that ceramide acts in conjunction with the caspase cascade in CD95-induced apoptosis.

Important physiological triggers for apoptosis include ligation of the CD95 (Apo-1/Fas) cell surface receptor, which belongs to the tumor necrosis factor (TNF) receptor superfamily. A cascade consisting of several cysteine proteases of the ICE/CED-3 family (caspases; Ref. 1) has been implicated in apoptosis in different cell types (2, 3). Upon stimulation, the CD95 receptor trimerizes and recruits caspase-8/Flice/Mch5 (4–6) to the CD95 death-inducing signaling complex, where it is activated (7, 8). Subsequently, the cytosolic caspase-3/CPP32/Yama becomes activated (9, 10). There is little insight into the mechanism by which the cascade is regulated. Although Flice can cleave CPP32 in vitro (10), it is still unclear whether these caspases directly interact in vivo.

A second pathway leading to apoptosis involves activation of sphingomyelinase (SMase), which hydrolyzes sphingomyelin (SM) to ceramide (Cer). SM hydrolysis is evoked not only by triggering of the TNF or CD95 receptor (11–14) but also by other apoptotic inputs such as oxidative stress, UV, and γ-irradiation (15–17). A specific role for Cer in mediating apoptotic signals is suggested by the apoptotic effect of exogenous short-chain Cer, the structurally closely related compound dihydroCer being inactive. Likewise, treatment with bacterial SMase or drugs that inhibit metabolic conversion of Cer causes apoptosis, supposedly via elevated endogenous Cer levels (18). There is currently much confusion about the role of endogenous Cer in apoptosis, inasmuch as kinetics and magnitude of the Cer response differ widely among various studies (18). Some investigators observe a response within minutes after stimulation, which never exceeds 200% (12, 14), whereas others, using the same stimulus, only measure significant elevation after several hours up to 5–7-fold above basal (19–21).

One may question whether late ceramide formation is a consequence rather than a cause of apoptosis, inasmuch as kinetics often parallels the onset of apoptosis (19, 21). On the other hand, overexpression of Bcl-2 in ALL-697 cells did not affect vincristine- or TNF-α-induced delayed ceramide formation whereas it completely prevented apoptosis (22, 23), demonstrating that late ceramide is not necessarily a result of cell death. Activation of both SMase and caspases has been implicated in apoptosis, especially when induced by members of the TNF receptor family, but the connection between both pathways has not been elucidated.

In the present study, we have thoroughly investigated CD95-induced Cer formation by two different quantitation methods, other than the commonly used Escherichia coli diglyceride kinase assay. Unlike the latter assay, the new assays based on metabolic radiolabeling/TLC and benzoylation/HPLC, respectively, allow the resolution of Cer with different chain lengths as well as separation from de novo synthesized dihydroCer.

We demonstrate that, in Jurkat T cells, CD95 only induces late Cer formation paralleling caspase-3 activation. Direct inhibition of caspase-3 processing prevents apoptosis but does not block the Cer response. Because exogenous Cer can activate caspase-3, our findings suggest that Cer acts upstream of caspase-3 in the CD95-induced apoptotic signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents- [3-14C]Serine and [methyl-14C]choline chloride were from Amerah; C2-Cer was from Biomol; Bacillus cereus SMase, Type III Cer, N-stearyl-O-sphingosine, N-neronanoyl-O-sphingosine, N-neronanoyl-O-sphingamine, dimethylaminopropidine, benzoic anhydride, and tamoxifen (free base) were from Sigma; Silica G60 TLC plates and cyclohexane/Lichrosolv® were obtained from Merck; Silica HPLC column (250 × 4.6 mm) and HPLC sample filters were from Chrompack.
CHAPTER 2

(19) The Netherlands; anti-C695 monoclonal antibody (CH-11) was from Immunotech (Marseille, France). Polyclonal antisera raised against a glutathione S-transferase fusion protein of human caspase-3/CPP32 was prepared by Dr. G. Gil-Gomez in our institute. DEV-OHD was from Calbiochem and FFMP from Matreya. e-v-MAPF was kindly provided by Dr. A. H. Merrill Jr. (Emory University School of Medicine, Atlanta, GA).

Cell Culture and Stimulation—The J16 wild type clone was derived by limiting dilution from the human T-cell line Jurkat and selected for high sensitivity to C695-induced apoptosis. It was cultured in Isosce's modified Dulbecco's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, and 100 IU/ml penicillin/streptomycin, at 37 °C, 5% CO2. Before cell stimulation, cells were incubated overnight in synthetic Yssel's medium (24), resuspended at 5-10 x 10^6 cells/ml in Yssel's medium in a 24-well culture plate, and stimulated with CH-11 at 37 °C, 5% CO2, serum, 2 mM glutamine, and 100 IU/ml penicillin/streptomycin, at 37 °C, 5% CO2. Serum, 2 mM glutamine, and 100 IU/ml penicillin/streptomycin, at 37 °C, 5% CO2. Before cell stimulation, cells were incubated overnight in synthetic Yssel's medium (24), resuspended at 5-10 x 10^6 cells/ml in Yssel's medium in a 24-well culture plate, and stimulated with CH-11 at 37 °C, 5% CO2, serum, 2 mM glutamine, and 100 IU/ml penicillin/streptomycin, at 37 °C, 5% CO2.

Apoptosis Assay—For apoptosis measurements, cells were seeded at 1 x 10^5 cells/ml, 200 uM well in round-bottomed, 96-well microtiter plates in Yssel's medium (24). Cells were killed in 0.1% sodium citrate, 0.1% Triton X-100, and 50 uM propidium iodide (25). Fluorescence intensity of propidium iodide-stained DNA was determined in 5000 cells on a FACScan (Becton Dickinson, San Jose, CA), and data were analyzed using Lysys software. Fragmented, apoptotic nuclei are recognized by their subdiploid DNA content. Analysis of chromosomal DNA was accomplished using isocratic HPLC with a ChromSpher silica analytical column (4.6 x 250 mm, 5-mu pore size) based on published methods (29, 30). Samples of 15 microg, corresponding to lipids from 3 x 10^6 cells, were injected, and eluates were monitored at 230 nm using a Waters 486 Tunable Absorbance Detector. The attenuation was usually set at a full scale of 0.075 absorbance unit. Data were collected with a Waters 741 Data Module integrator. The mobile phase consisted of cyclohexane, 0.45% (v/v) isopropanol at a flow rate of 1.5 ml/min. This system not only allows the separation of benzoyl-Cer from other benzoylated lipid molecules, it also resolves dihydroCer and Cer molecular species differing in fatty acid chain length.

Cer Quatification Using [14C]Serine Labeling—Cells (1 x 10^6) ml) in Yssel's medium (24) were labeled with [14C]Serine (0.2 uCi/ml) for 16-20 h. When inhibitors of Cer metabolism were used, these were added at this point. Cells were washed twice, stimulated, and extracted as described above. Lipid extracts were spotted on TLC silica plates and developed to 70% of the total length in CHCl3/MeOH/H2O/MeOH/H2O (90/10/5.0/5.0, v/v). Under these conditions, Cer, glycosylceramide, sphingosine, SM, and glycerophospholipids are well separated. Radioactive lipids were visualized and quantitated using a Fujix BAS 2000 radiographic image analyzer. The radioactivity was visualized and quantitated using a Fujix BAS 2000 radiographic image analyzer. The radioactivity was visualized and quantitated using a Fujix BAS 2000 radiographic image analyzer.

RESULTS AND DISCUSSION

CD95 Induces Cer Formation after a Few Hours: Lack of a Rapid Response—Previous studies suggested the involvement of acidic SMase, generating Cer, in transmitting signals from the CD95 receptor to the apoptotic machinery (12, 13, 19). However, widely conflicting data regarding kinetics and magnitude of Cer formation have caused much confusion concerning the role of Cer as a mediator of apoptosis as induced by CD95 or other apoptotic stimuli (18). Therefore, we decided to analyze Cer formation by methods alternative to the commonly used procedure for Cer mass measurement involving E. coli diglyceride kinase (31). The major drawback of this assay is that it does not discriminate between Cer and dihydroCer (32), which lacks the C4-C5 bond in the sphingoid backbone and is, therefore, biologically inactive (33). Considering the outstanding question whether Cer plays a role in apoptosis induction, we investigated the kinetics and magnitude of CD95-induced Cer formation in apoptosis-sensitive Jurkat T cells using two different quantitation methods that discriminate between Cer, released from existing sphingolipids, and dihydroCer, produced via stimulated de novo synthesis.

The first method involves metabolic sphingolipid labeling in the sphingoid backbone using [14C]serine and analysis of Cer formation by TLC. Besides a clear distinction between dihydroCer and Cer, this method allows separation of Cer species containing either long (C22-24) or intermediate (C16-18) fatty acids represented by a doublet (Fig. 1A). Exposure of Jurkat T cells to monoclonal anti-human CD95/Fas IgM (CH-11) caused accumulation of Cer, which was most prominent for the intermediate species (lower spot). Analysis of Cer species generated by bacterial SMase treatement of [14C]serine-labeled cells (data not shown) or total isolated endogenous [14C]SM (Fig. 1A) also yielded a doublet with the majority of the radioactivity also residing in the lower spot. Because the Cer species distribution produced upon CD95 stimulation reflects the fatty acid composition of total SM, there is no evidence for the selective hydrolisis of SM with intermediate acyl chains.

Quantification of total Cer showed a first significant increase between 3 and 4 h after CD95 stimulation, which further increased to approximately 7-fold above basal at 8 h (Fig. 1B). Although the accumulation of C16-18 species (referred to as Cer lower spot) may somewhat precede, there is a clear temporal correlation between total Cer formation and the onset of apoptosis measured by nuclear segmentation. Others have shown a similar correlation in SKW6.4 cells, when the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide viability assay (19) or bisbenzamide staining (21) were used to monitor kinetics of CD95-induced cell death. In separate experiments, using several assays (diglyceride kinase, [14C]serine labeling, or HPLC; see below), we have extensively looked for rapid (within 30 min) Cer responses, but these were never detected (data not shown). Alternatively, we metabolically labeled SM with [14C]cholesterol (48 h). Subsequent CD95 triggering did not reveal an acute decrease in [14C]SM, which would be indicative of SMase activity (results not shown).

We next considered the possibility that detection of an acute Cer signal could have been masked by the rapid attenuation through the action of ceramidase or glucosyltransferase. Cer degradation to sphingosine by neutral ceramidase is inhibited

rated on 12% SDS-polyacrylamide gels (equivalents of 0.8-1 x 10^6 cells/ lane). Proteins were transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Blots were blocked with 5% (v/v) nonfat dry milk in phosphate-buffered saline, 0.1% Tween 20; probed with purified anti-lg (10 ng/ml) in phosphate-buffered saline, 0.1% nonfat dry milk, followed by a 1.7500 dilution of horseradish peroxi­
dase-conjugated swine anti-rabbit Ig (DAKO A/S, Glostrup, Denmark); and developed by enhanced chemiluminescence (Amersham).
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by d-e-MAPP (34), whereas PPMP (35) and tamoxifen (36) block conversion of Cer to gucosylceramide. Interestingly, these agents have been reported to be inducers of apoptosis, presumably via increasing endogenous Cer levels (18). After 

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Figs. 1 and 2. Cer mass analysis by normal-phase HPLC. Lipids were converted to benzoic-derivatives and isocratically eluted from a silica column with cyclohexane:0.45% (v/v) isopropanol as described under "Experimental Procedures." A, TLC separation of dihydroCer from a Cer doublet, representing species containing long chain (upper spot) or intermediate chain (lower spot) fatty acids. Lane 1, control cells; lane 2, cells treated with anti-CD95 (CH-11; 1 μg/ml) for 5 h; lane 3, Cer generated from TLC-isolated total cellular 

FIG. 1. CD95-induced apoptosis parallels Cer formation, which is most prominent for species with intermediate-chain fatty acids. Jurkat cells were labeled with [14C]serine and stimulated with anti-CD95 mAb. Lipid extracts of 5 × 10^6 cells were separated by TLC as described under "Experimental Procedures." A, TLC separation of dihydroCer from a Cer doublet, representing species containing long chain (upper spot) or intermediate chain (lower spot) fatty acids. Lane 1, control cells; lane 2, cells treated with anti-CD95 (CH-11; 1 μg/ml) for 5 h; lane 3, Cer generated from TLC-isolated total cellular [14C]SM by B. cereus SMase. B, time course of Cer formation and apoptosis. Cells were treated with CH-11 (1 μg/ml), and, at the indicated times, total Cer (closed circles), lower spot Cer (closed triangles), and percentage apoptosis (open triangles) were determined. Cer formation relative to total radiolabeled polar lipids is expressed as -fold increase relative to control cells (means of three experiments ± S.D.). Apoptosis was read out as nuclear fragmentation by FACS analysis (25). Data are means of at least four experiments.

The caspase inhibitor DEVD-CHO (40) efficiently blocked caspase-3 processing (Fig. 3) and apoptosis (Fig. 6). However, inhibition of this inhibitor did not prevent CD95-induced Cer formation. In a different system, i.e. TNFα-stimulated MCF-7 cells, ceramide accumulation is also slow but clearly precedes apoptosis (23).

only causes a late and sustained elevation of Cer species that predominantly contain intermediate chain fatty acids.

Relation of Cer Formation to Caspase-3 Activation—The relevance of late Cer formation in mediating CD95-induced apoptosis is a topic of debate. In a number of studies, cell lines resistant to apoptosis induced by CD95 (19, 20, 37) and anti- 

FIG. 2. Cer formation analysis by normal-phase HPLC. Lipids were converted to benzoic-derivatives and isocratically eluted from a silica column with cyclohexane:0.45% (v/v) isopropanol as described under "Experimental Procedures." Standard Cer from bovine brain SM was used to identify the position where Cer-benzoates eluted. This yielded a doublet representing species with long chain (region indicated by a) or intermediate chain (indicated by b) fatty acids, as was confirmed by using C24:1- and C18:0-Cer standards (data not shown). Derivatized Cer was found to elute before and spingosine after the Cer doublet. Representative elution profiles are shown of untreated Jurkat cells and cells that were treated with CH-11 (1 μg/ml) for 5 h or B. cereus SMase (300 milliunits/ml) for 5 min.

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These data suggest that CD95-induced Cer accumulation, while it provided protection from exogenous Cer generation, may not contribute to the rapid Cer generation associated with apoptosis.
FIG. 3. DEVD-CHO prevents CD95-induced caspase-3/CPP32 cleavage and apoptosis. After preincubation for 2 h in the absence or presence of DEVD-CHO (100 μM), cells were treated with CH-11 (1 μg/ml) for the indicated times in the absence (left panel) or presence (right panel) of the inhibitor. Total cell lysates were analyzed for caspase-3 processing by immunoblotting. The 32-kDa proform of the inhibitor and apoptosis in the same set of samples was measured by FACS analysis (25).

FIG. 4. DEVD-CHO does not prevent CD95-induced Cer formation. Jurkat cells prelabeled with [3H]serine were preincubated for 2 h in the absence or presence of 100 μM DEVD-CHO, as indicated. Next, they were left untreated or treated with anti-CD95 mAb (CH-11; 1 μg/ml). At indicated times, lipids were extracted and separated by TLC as described under “Experimental Procedures." A, lipid analysis of cells that were left untreated (control) or treated with CH-11 (antiCD95) for 6 and 8 h. Apoptosis (percentage indicated) was quantified in the same set of samples. B, time course of CD95-induced [3H]Cer generation in the open circles or presence (closed circles) of DEVD-CHO. Data (means of three experiments ± S.D.) are expressed as fold increase in total Cer relative to control cells that received no anti-CD95.

FIG. 5. Induction of proteolytic cleavage of caspase-3/CPP32 by exogenous Cer. Jurkat cells were treated for indicated times with vehicle (Me2SO, top panel) or C2-Cer (50 μM, lower panel). Immunoblots showing the 32-kDa proform of caspase-3 and its degradation product of approximately 20 kDa are indicated by arrows. The percentage apoptosis in the same set of samples was measured by FACS analysis (25).

FIG. 6. Caspase-3 activation is required for Cer- and SMase-induced apoptosis. After a 2-h preincubation with DEVD-CHO at varying concentrations, Jurkat cells were exposed to anti-CD95 mAb (CH-11; 500 ng/ml; 8 h), C2-Cer (50 μM; 18 h), B. cereus SMase (300 milliunits/ml; 18 h), or control medium (18 h). Apoptosis was determined by FACS analysis (25).

that Cer is formed in between steps of sequential caspase activation. As the majority of Cer accumulation is not downstream of caspase-3 and because kinetics parallel caspase-3 proteolytic processing, we investigated whether caspase-3 could be a Cer target. Indeed, exogenous C2-Cer induced the proteolytic cleavage of the 22-kDa proform of the protease after 6–8 h (Fig. 5), which is somewhat later than by CD95 stimulation. Notably, in a different system, i.e. C2-Cer-treated Molt-4 cells, and using a different read-out, i.e. proteolytic cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase, Smyth et al. (42) have also found activation of caspase-3 by exogenous Cer. Fig. 6 shows that nuclear fragmentation in response to exogenous Cer and bacterial SMase, like that induced by CD95, was inhibited by DEVD-CHO in a dose-dependent fashion. These results provide evidence for the specific requirement of caspase-3 in mediating the apoptotic effect of both short-chain Cer as well as of naturally occurring Cer species. **Concluding Remarks**—Taken together, we find that CD95 evokes no rapid (<1 h) Cer response but only induces late Cer formation paralleling caspase-3 activation and the appearance of nuclear fragmentation. Direct inhibition of caspase-3 processing by the caspase inhibitor DEVD-CHO completely prevents the apoptotic phenotype but does not block the majority (~70%) of the Cer response. These results indicate that late Cer is not a mere result of CD95-induced apoptosis but rather could be instrumental in the execution of apoptosis. Exogenous Cer was shown to induce caspase-3 processing, and, moreover, caspase-3 activation appeared a requirement for apoptosis induced either by cell-permeable Cer or bacterial SMase. Thus, our results support the model in which the SMase pathway acts upstream of the caspase-3 member of the family of caspases in CD95-induced apoptosis and pose intriguing questions concerning the role of Cer acting in concert with members of the proteolytic cascade.
REFERENCES
