CD95/Fas-induced Ceramide Formation Proceeds with Slow Kinetics and Is Not Blocked by Caspase-3/CPP32 Inhibition*

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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 onset. Subsequently, the cytosolic caspase-3/CPP32 to the CD95 death-inducing signaling complex, where it is activated (7, 8). Activation of caspase-3/CPP32 (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 TNFα-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 Amersham; C2-Cer was from Biomol; Bacillus cereus SMase, Type III Cer, N-stearyl-N-sphingosine, N-nercovonoyl-D-sphingosine, N-nercovonoyl-D-sphinganine, dimethylaminopiryridine, and tamoxifen (free base) were from Sigma; Silica G60 TLC plates and cyclohexane Lichrosolv® were from Merck; Silica HPLC column (250 × 4.6 mm) and HPLC sample filters were from Chrompack can cleave CPP32 in vitro (10), it is still unclear whether these caspases directly interact in vivo.

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§ The abbreviations used are: TNF, tumor necrosis factor; Cer, ceramide; C2-Cer, N-acetyl-D-sphingosine; SM, sphingomyelin; SMase, sphingomyelinase; FACs, fluorescence-activated cell sorting; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; d+MAPP, d-erythro-2-(N-myrystoylamin)-1-phenyl-1-propanol; PPMP, d-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol; mAb, monoclonal antibody; DEVD-CHO, Ac-Asp-Glu-Val-Asp aldehyde.

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The Netherlands; anti-CD95 monoclonal antibody (CH-11) was from Immunotech (Marseille, France). Polyonal antisera against a glutathione S-transferase fusion protein of human caspase-3/CPP32 was prepared by Dr. G. Gil-Gomez in our institute. DEVD-CHO was from Calbiochem and PFFMP from Matreya, b-MAFP was kindly provided by Dr. H. Merrill Jr. (Emory University School of Medicine, Atlanta, GA).

Cell Culture and Stimulation—The J16 wild type clone was derived by limited dilution from the human T-cell line Jurkat and selected for high sensitivity to CD95-induced apoptosis. It was cultured in Iscove'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 × 106 cells/ml in Yssel's medium in a 24-well culture plate, and stimulated with CH-11 mAb, C2-Cer (prepared as 10 μg stock in Me2SO), or SMase for various time periods at 37 °C, 5% CO2.

Apoptosis Assay—For apoptosis measurements, cells were seeded at 1 × 106 cells/ml, 200 μl/ well in round-bottomed, 96-well microtiter plates in Yssel's medium (24). Cells were lysed by 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml 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.

Lipid Extraction and Preparation of Cer Benzoate-derivatives—Cells (1 × 106) were collected by brief centrifugation and washed twice with phosphate-buffered saline. Lipids were extracted with chloroform/methanol (1:2, v/v) and phases separated (26). Benzoate-derivatives were prepared essentially as has been reported for mono- and diglycerides (27). Dried lipid extracts of 1 × 106 cells were dissolved in 0.25 ml of toluene containing 8 mg of benzoic anhydride and 3 mg of 4-(dimethylamino)pyridine and allowed to stand at room temperature for 2 h. Under these conditions, no amide acylation occurs (28). Samples were cooled on ice, and 2 ml of 25% NH4OH was added dropwise. Benzoate-derivatives were extracted three times with 2 ml of n-hexane. The samples were dried, reconstituted in 1 ml of chloroform, passed through a 0.2-μm HPLC sample filter, dried under N2, and dissolved in a small volume (usually 50 μl) of carbon tetrachloride.

High Performance Liquid Chromatography—HPLC was performed using a Waters 600E System Controller. Separation of benzoylated lipids was accomplished using isocratic HPLC with a ChromSpher silica analytical column (4.6 × 250 mm, 5-μm pore size) based on published methods (29, 30). Samples of 15 μl, corresponding to lipids from 3 × 106 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 (Fig. 1c).

Cer Quantification Using [14C]Serine Labeling—Cells (1 × 106/ml) in Yssel's medium (24) were labeled with [14C]serine (0.2 μCi/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/25% NH4OH (50/50/21, v/v). Plates were dried under nitrogen and chromatographed to the top of the plate in CHCl3/MeOH/H2O/25% NH4OH (90/10/0.5/0.5, v/v). Under these conditions, Cer, glucosylceramide, sphingosine, SM, and glycerocephospholipids are well separated. Radioactive lipids were visualized and quantitated using a Fujix BAS 2000 TR phosphorimager (exposure time 1–2 days). For chromatographic reference, radiolabeled Cer was prepared from endogenous [14C]SM isolated by TLC and sonicated in 1.5 ml of 0.1 M Tris-HCl, pH 7.4, 0.1% Triton X-100, 6 mM MgCl2, by incubation overnight with B. cereus SMase (1 units/ml) at 37 °C. [14C]Cer, extracted with diethyl ether, yielded a doublet on TLC corresponding to Cer species containing either C22–24 (upper spot) or C16–18 (lower spot) fatty acid chains, as was confirmed by thin-layer chromatography on brain Cer (mainly C24:1 and C18:0 species, C24:1-Cer, and C18:0-Cer as commercial standards (data not shown)).

Estimation of Caspase-3/CPP32 Activation by Western Blotting—Cells (2 × 105) were lysed for 30 min at 4 °C in 30 μl of 10 mM triethanolamine HCl, pH 7.8, 0.15 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, containing 1% Nonidet P-40, 0.02 mg/ml trypsin inhibitor, and 0.02 mg/ml leupeptin. Lysates were centrifuged, and supernatants were taken up in reducing SDS sample buffer and separated on 12% SDS-polyacrylamide gels (equivalents of 0.8–1 × 106 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-Id (10 μg/ml) in phosphate-buffered saline, 0.1% Tween 20, 1% nonfat dry milk, followed by a 1:7500 dilution of horseradish peroxidase-conjugated swine anti-rabbit Ig (Dako A/S, Glostrup, Denmark); and developed with enhanced chemiluminescence (Amersham).

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 at 37 °C 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 trans double 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, 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 determination 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 monolonal 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 treatment 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-diethyithiazol-2-yl]-2,5-diphenyl tetrazolium bromide viability assay (19) or biobenzamide 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 3h) Cer responses, but these were never detected (data not shown). Alternatively, we metabolically labeled SM with [14C]choline (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 its rapid attenuation through the action of ceramidase or glucosyltransferase. Cer degradation to sphingosine by neutral ceramidase is inhibited.
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FIG. 1. CD95-induced apoptosis parallels Cer formation, which is most prominent for species with intermediate-chain fatty acids. Jurkat cells were treated 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.

by d-e-MAPP (34), whereas PPMP (35) and tamoxifen (36) block conversion of Cer to glucosylceramide. Interestingly, these agents have been reported to be inducers of apoptosis, presumably via increasing endogenous Cer levels (18). After [14C]serine labeling of sphingolipids, cells were pretreated overnight with 500 nM PPMP or 10 μM tamoxifen, which indeed increased the steady-state ratio Cer/glucosylceramide (data not shown), or with 10 μM d-e-MAPP. Subsequent CD95 triggering in the presence of these agents, however, did not reveal any acute Cer formation, whereas all agents induced apoptosis at higher concentrations (results not shown).

To exclude the possibility that [14C]serine might have selectively labeled a subpopulation of SM molecules inaccessible to possible rapid CD95-induced hydrolysis, we also developed an HPLC method to quantify Cer mass. In this assay, total cellular lipids were subjected to a derivatization procedure to yield chromophoric, nonpolar benzoate-derivatives. Exposure of Jurkat cells to anti-CD95 mAb caused Cer accumulation, which, again, was most prominent for the second peak of a doublet, corresponding to C16–18 Cer species (Fig. 2). Also with this assay, kinetics of Cer formation closely paralleled apoptosis and no acute Cer formation was detected, in agreement with the radiolabeling assay.

From these extensive analyses, we have to conclude that CD95 triggering does not evoke rapid activation of SMase, but 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-IgM (38) were reported to lack Cer formation. However, whether this really is the case, as was suggested, rather than a consequence of their resistance remains a question. In the case of TNFα-induced apoptosis in MCF-7 cells, ceramide accumulation is also slow but clearly precedes apoptosis (23).

Activation of the ICE/Ced-3-like cysteine protease caspase-3/CPP32 was previously shown to be required for CD95-induced apoptosis (39). Fig. 3 shows the kinetics of proteolytic activation of caspase-3 as triggered by CD95 in Jurkat cells. A first significant decline in the 32-kDa proform with concomitant appearance of a proteolytic fragment is observed after 4 h. The caspase inhibitor DEVD-CHO (40) efficiently blocked caspase-3/CPP32 activation (Fig. 3) and apoptosis (Fig. 6). However, this inhibitor did not prevent CD95-induced Cer formation (Fig. 4A). Interestingly, Fig. 4B shows that inhibition of caspase-3 and apoptosis hardly affected the kinetics of Cer formation, although its magnitude is reduced by approximately 30%. These data suggest that CD95-induced ceramide formation occurs upstream of caspase-3 activation. In a different system, i.e. TNFα-stimulated MCF-7 cells, Dbaibo et al. (23) also concluded that Cer was generated upstream of caspases involved in the “execution stage” of apoptosis, inasmuch as Bcl-2 overexpression did not interfere with TNFα-induced ceramide generation, while it provided protection from exogenous

FIG. 2. Cer mass analysis by normal-phase HPLC. Lipids were converted to benzoate-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 C24:1-dihydroCer was found to elute before and sphingosine 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 15 min.
Cer-induced poly(ADP-ribose) polymerase cleavage and apoptosis (22, 23). Our data are at variance with the work by Gamen et al. (41), wherein DEVD-CHO completely prevented CD95-induced Cer generation. However, they measured only \(^{14}C\)stearic acid labeled Cer at 16 h and showed no kinetics of Cer formation or apoptosis. Their 16-h timepoint might well reflect an end point 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 SSMase. Thus, our results support the model in which the SSMase 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.
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