Ceramide Induces Cell Death in the Human Prostatic Carcinoma Cell Lines PC3 and DU145 but Does Not Seem to Be Involved in Fas-Mediated Apoptosis

Andreas Gewies, Oskar W. Rokhlin, and Michael B. Cohen

From the Departments of Pathology (AG, OWR, MBC) and Urology (MBC), The University of Iowa, and Veterans Affairs Medical Center (MBC), Iowa City, Iowa

SUMMARY: Treatment of cells with synthetic C2-ceramide has been reported to induce apoptosis in several cell systems, and endogenously formed ceramide has been proposed to act as a second messenger, activating signaling pathways which contribute to the execution of apoptotic cell death after Fas ligation or tumor necrosis factor receptor-1 ligation. In this study, we examined the effect of exogenously administered C2-ceramide on the human prostatic carcinoma cell lines PC3 (Fas-sensitive) and DU145 (Fas-resistant). In both cell lines, C2-ceramide induced cell death in a dose-dependent manner, whereas a structural analog, C2-dihydroceramide, did not. The pan-caspase inhibitor zVAD-fmk did not prevent C2-ceramide–induced cell death but did prevent C2-ceramide–induced DNA fragmentation, indicating that apoptotic and non-apoptotic mechanisms are involved in C2-ceramide–induced death. Interestingly, cycloheximide prevented C2-ceramide–induced DNA fragmentation, indicating that ceramide-induced apoptosis in PC3 and DU145 requires new protein synthesis. In addition, because cycloheximide converts Fas-resistant DU145 to Fas-sensitive as assessed by DNA fragmentation, ceramide does not seem to play a major role in the Fas-mediated pathway in this cell line. We also determined the levels of endogenous sphingomyelin after Fas ligation in PC3. No decrease of sphingomyelin levels could be detected after Fas activation. We conclude that sphingomyelinase-generated ceramide does not play a role in Fas-mediated apoptosis in PC3, and that there are fundamental differences in the mechanisms of cell death induced by C2-ceramide and Fas ligation. (*Lab Invest 2000, 80:671–676*).

T he Fas (CD95) receptor is a type I transmembrane protein and belongs to the tumor necrosis factor receptor family (Ashkenazi and Dixit, 1998; Nagata and Golstein, 1995). Ligation of the Fas receptor results in the activation of various signaling pathways that ultimately lead to apoptosis (Li et al, 1998; Medema et al, 1997; Muzio et al, 1997). It has recently been reported that stimulation of cell surface receptors, including Fas, results in the rapid generation (minute range) of endogenous ceramide (Gulbins et al, 1995, 1996; Tepper et al, 1995). Because treatment of cells with cell-permeable analogs of ceramide such as C2-ceramide or C6-ceramide was shown to induce apoptosis (Jarvis et al, 1994; Jayadev et al, 1995; Obeid et al, 1993), ceramide was thought to be a second messenger in cell death signaling. In most cases, the increase in ceramide levels after Fas ligation was explained by the activation of cellular sphingomyelinases (SMase) which hydrolyze sphingomyelin (SM) to yield phosphorylcholine and ceramide (Hannun, 1996; Hofmann and Dixit, 1998). Both neutral SMase and acidic SMase (aSMase) are activated by the Fas receptor (Cifone et al, 1995; Gulbins et al, 1995; Tepper et al, 1995), although it is aSMase that is

thought to generate ceramide during apoptosis (Wiegmann et al, 1994). The regulatory mechanisms of SMase activation by the Fas receptor are unknown (Brenner et al, 1998), and studies of Fas-mediated apoptosis in human cells deficient in aSMase have yielded conflicting results. One report concluded that aSMase is required for Fas-mediated apoptosis (De Maria et al, 1998) whereas another report concluded that aSMase is not required (Cock et al, 1998). Moreover, in another study, neither treatment of lymphocytes with inhibitors of SMase or with inhibitors of de novo ceramide synthesis prevented Fas-mediated apoptosis (Gamen et al, 1998). In addition to these contradictory findings, other studies have reported different ceramide responses to the same inducer of apoptosis (Hannun, 1996), and thus the role of ceramide as a second messenger in apoptosis has been questioned (Hofmann and Dixit, 1998; Skowronski et al, 1996; Watts et al, 1999). One important observation that emerged was that the diacylglycerol kinase assay for measuring cellular ceramide is misleading because it generates artificial results (Watts et al, 1997). Thus, reports of signal-induced ceramide generation should be verified using a different assay for ceramide (Hofmann and Dixit, 1998). The use of alternative assays, such as mass spectrometry or HPLC, generally have not shown increases of endogenous ceramides after Fas ligation; at least not during the early stages of the apoptotic response (Sillence and Allan, 1997; Tepper et al, 1997; Watts et al, 1997, 1999). Thus, ceramide

might not be involved in the early phase of Fas-mediated apoptosis (Sillence and Allan, 1997; Tepper et al, 1999).

We have previously shown that in the human prostatic carcinoma cell lines PC3 and DU145 the pathway(s) leading to Fas-mediated apoptosis are intact (Rokhlin et al, 1997). PC3 is sensitive to treatment with a monoclonal antibody (mAb) to Fas, whereas DU145 is only sensitive under combined treatment with an anti-Fas mAb and cycloheximide (CHX). CHX is necessary to convert DU145 from Fas-resistant to Fas-sensitive due to a labile dominant inhibitory protein(s), presumably acting at the apex of the apoptotic cascade (Rokhlin et al, 1997, 1998). We have also demonstrated that the pathways leading to Fas-mediated apoptosis in these cell lines involve the activation of caspase-8 and caspase-7 (Rokhlin et al, 1998), as well as cytochrome c release into the cytosol, and activation of caspase-9 (Gewies et al, 2000). In this study, we examined the effect of exogenously administered C2-ceramide on PC3 and DU145. We found that C2-ceramide induces cell death by non-apoptotic and apoptotic mechanisms in both cell lines. We then asked whether ceramide signaling plays a role in Fas-mediated apoptosis of PC3 and DU145, and whether defective ceramide signaling might be in-volved in the resistance to Fas ligation in DU145. Our data, which are consistent with recent reports in other cell systems, show that ceramide does not play a role in Fas-mediated apoptosis in human prostatic carcinoma cell lines.

Results

C2-Ceramide Induces Cell Death That Is Not Inhibited by the Caspase Inhibitor zVAD-fmk in PC3 and DU145

To determine whether human prostatic carcinoma cell lines are susceptible to ceramide-induced cell death, we measured the viability of PC3 and DU145 after 24 hours of treatment with different concentrations of the cell-permeable ceramide analog C2-ceramide. In response to C2-ceramide, PC3 and DU145 died in a dose-dependent manner (Fig. 1, A and C). In contrast, the structurally related C2-dihydroceramide induced a lower level of cell death that was not dose-dependent. Upon treatment of PC3 with 40 μM C2-ceramide, or DU145 with 60 μM C2-ceramide, the cells rounded-up and finally detached from the plastic surface; whereas even 100 μM C2-dihydroceramide did not result in any visible change (data not shown). C2-ceramide–induced cell death could not be prevented by the
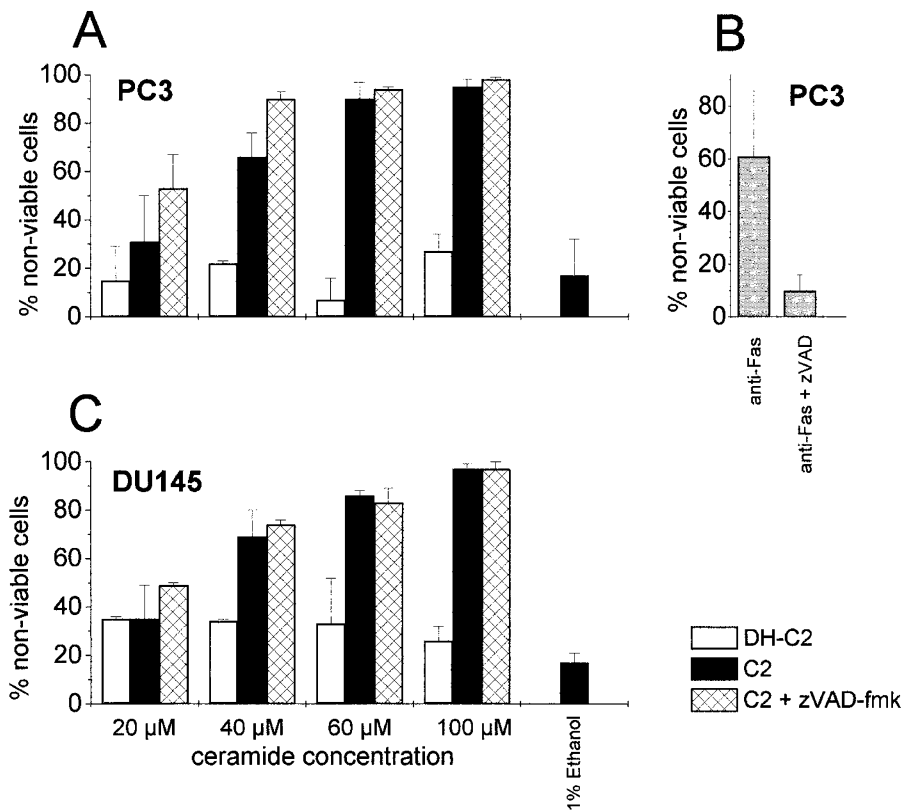

Figure 1.

C2-ceramide induces cell-death in PC3 and DU145 that is not prevented by zVAD-fmk. C2-ceramide–mediated cell death was estimated using a viability assay as described in "Materials and Methods". The effect of different concentrations of C2-dihydroceramide (*DH-C2*), C2-ceramide (*C2*), and C2-ceramide in the presence of 100 μM zVAD-fmk (*C2 + zVAD-fmk*) on the viability of PC3 (A) and DU145 (C) is shown. As a control, the inhibitory effect of 100 μM zVAD-fmk on Fas-mediated cell death in PC3 was determined (B). Also as a control, the effect of 1% ethanol on PC3 and DU145 was measured. Also, as a control, the inhibitory effect of 100 μm zVAD–fmk on Fas–mediated cell death in PC3 was determined (*B*). The data shown in (A) and (C) are the mean and SD values of at least three independent experiments; the anti-Fas data in (B) are the average of two independent experiments.

addition of the broad-specificity caspase inhibitor, zVAD-fmk (Fig. 1, A and C), whereas zVAD-fmk inhibited Fas-mediated death of PC3, as expected (Fig. 1B). These observations suggest that in both cell lines, PC3 and DU145, C2-ceramide induced a caspase-independent, non-apoptotic mode of cell death.

C2-Ceramide Induces DNA Fragmentation That Is Caspase-Dependent and Can Be Blocked by Cycloheximide

It was recently reported that the killing of Jurkat cells by synthetic ceramide could occur by both apoptotic and non-apoptotic mechanisms (Mengubas et al, 1999a, 1999b). In those studies, zVAD-fmk did not decrease the overall rate of killing of Jurkat cells by C2-ceramide, but did dramatically decrease the induction of apoptosis. Thus, although we did not detect any effect of zVAD-fmk on overall killing by C2-ceramide in PC3 and DU145 (Fig. 1), we could not rule out a partial involvement of apoptotic mechanisms in C2-ceramide–induced killing. Using a quantitative DNA fragmentation assay, we demonstrated that DNA was fragmented in PC3 and DU145 after treatment with C2-ceramide but not after treatment with C2-dihydroceramide (Fig. 2). The broad-specificity caspase inhibitor, zVAD-fmk, effectively inhibited this DNA fragmentation, suggesting the involvement of apoptotic pathways in C2-ceramide–induced cell death. C2-ceramide–induced DNA fragmentation increased in a dose-dependent manner in DU145, but we observed a maximum DNA fragmentation at 40 μM C2-ceramide in PC3. Concentrations of C2-ceramide higher than 40 μM resulted in decreasing levels of DNA fragmentation. These results indicate that apoptotic and non-apoptotic mechanisms of cell death compete, and that, in PC3, concentrations higher than 40 μM result in a shift towards non-apoptotic mechanisms.

Reported studies with neurocytes indicate that inhibition of protein synthesis by CHX blocks or delays ceramide-induced apoptosis (Brugg et al, 1996; Hartfield et al, 1998). This supports the hypothesis that ceramide-induced apoptosis requires new protein synthesis for initiation, at least in some cell systems (Sawai et al, 1995; Westwick et al, 1995). In the case of PC3 and DU145, we found that CHX clearly decreased the degree of C2-ceramide–induced DNA fragmentation (Fig. 2). In contrast, CHX converted DU145 from Fas-resistant to Fas-sensitive as estimated by DNA fragmentation (Rokhlin et al, 1997). This suggests that the ceramide pathway is not essential during the execution of Fas-mediated apoptosis in DU145.

Fas Ligation Does Not Result in a Decrease of SM Levels

Crosslinking of the Fas receptor has been reported to trigger the generation of endogenous ceramide during the early stages of apoptosis; ie, within 10 to 15 minutes after Fas ligation (Brenner et al, 1998; Cifone et al, 1994; Gulbins et al, 1995). However, recent findings provide evidence against an involvement of ceramide during the initiation phase of Fas-mediated apoptosis (Hannun, 1996; Hofmann and Dixit, 1998; Sillence and Allan, 1997; Tepper et al, 1997, 1999). In most cases, increased levels of ceramide are thought to be mediated by the activation of SMase that catalyze the hydrolysis of SM to ceramide and phosphorycholine (Brenner et al, 1998; Cifone et al, 1994; Hofmann and Dixit, 1998). To determine whether SMase-generated ceramide is an early response in Fas-mediated apoptosis of prostatic carcinoma cell lines, we measured SM levels after Fas ligation of the Fas-sensitive cell line PC3. As shown in Figure 3, we did not detect differences between the SM levels of untreated or anti–Fas-treated PC3 cells during the first 40 minutes of stimulation. These results suggest that ceramide does not play an early signaling role in Fas-mediated apoptosis in human prostatic carcinoma cell lines.

Discussion

Our data show that C2-ceramide induces cell death in the human prostatic carcinoma cell lines PC3 and DU145. Cell death induced by C2-ceramide seems to be executed by apoptotic and non-apoptotic mechanisms, because overall cell death can not be inhibited by the pan-caspase inhibitor zVAD-fmk (Fig. 1); whereas apoptotic DNA fragmentation is inhibited by zVAD-fmk (Fig. 2). The simultaneous occurrence of apoptotic and non-apoptotic mechanisms in ceramide-mediated killing has been reported previously (Mengu-
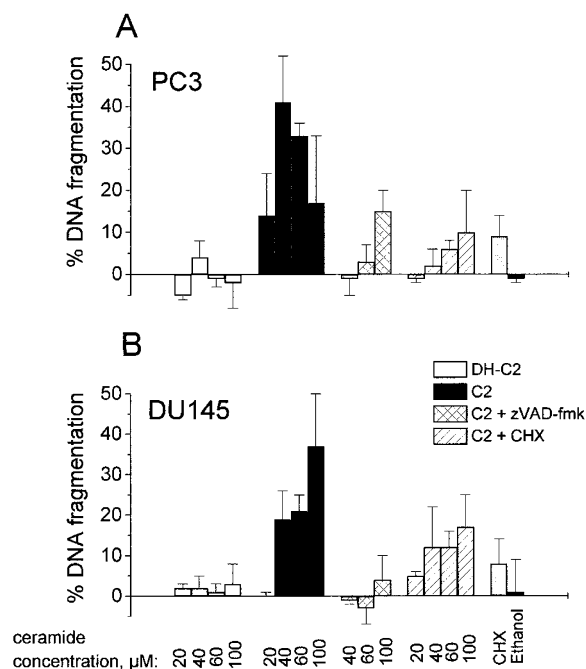

Figure 2.

DNA fragmentation induced by C2-ceramide is inhibited by zVAD-fmk and cycloheximide (CHX). DNA fragmentation was estimated after treatment of PC3 (A) and DU145 (B) with C2-dihydroceramide (*DH-C2*), C2-ceramide (*C2*), C2-ceramide with 100 μM zVAD-fmk (*C2 + zVAD-fmk*), or C2-ceramide with 10 μg/ml of CHX (*C2 + CHX*). As a control, the effects of 1% ethanol or 10 μg/ml of CHX alone were measured. The percentage of DNA fragmentation was determined as described in "Materials and Methods". The data shown are the mean and SD values of at least three independent experiments.
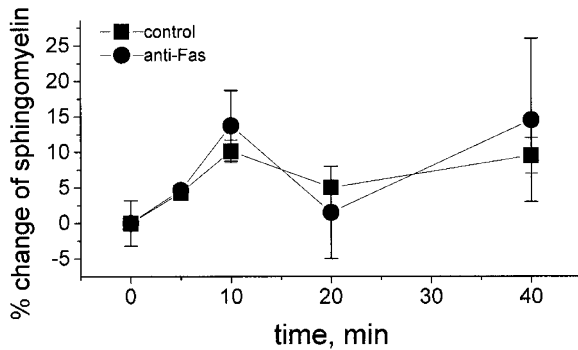
Figure 3.

Sphingomyelin levels after anti-Fas treatment. The Fas-sensitive cell line PC3 was labeled with [methyl-³H]choline, and then treated for the indicated times with 2 µg/ml of agonistic anti-Fas mAb. The sphingomyelin level was determined by extraction and thin layer chromatography as described in "Materials and Methods". The data shown represent at least two independent experiments.

bas et al, 1999a, 1999b). The lack of a protective effect of zVAD-fmk on C2-mediated cell death was accompanied by a slight increase in overall cell killing in the presence of zVAD-fmk (Fig. 1). This effect has been described previously in C2-ceramide–induced killing of Jurkat cells, and it has been proposed that blockage of caspase activation switches ceramide killing from apoptotic to non-apoptotic pathways (Mengubas et al, 1999a). It also has been reported that zVAD-fmk can block characteristic biochemical and morphologic events associated with apoptosis, but can not block cell death by non-apoptotic cell death (necrosis) (Hirsch et al, 1997; McCarthy et al, 1997). Importantly, C2-dihydroceramide–induced cell death is lower than that induced by C2-ceramide, and there is no dose-dependent increase of killing with C2-dihydroceramide, in contrast to the dose-dependent increase seen with C2-ceramide (Fig. 1). This indicates that C2-ceramide–induced cell death, mediated by both apoptotic and non-apoptotic mechanisms, might be the result of a specific biologic activity of C2-ceramide; eg, by interaction of C2-ceramide with specific cellular targets. Alternatively, C2-ceramide might induce cell death via perturbation of the mitochondrial membrane, resulting in the induction of a mitochondrial permeability transition. Permeability transition has been proposed to be involved in apoptotic and necrotic modes of cell death (Hirsch et al, 1997).

The mechanisms by which ceramide mediates apoptosis are incompletely understood, but one of the downstream pathways is the stress-activated protein kinase cascade (Ariga et al, 1998). By an unidentified coupling mechanism, ceramide activates a protein kinase cascade, eventually resulting in the activation of c-Jun, which represents a major component of the AP-1 transcription factor complex. Direct or indirect inhibition of cJun has been reported to abolish the apoptotic responses to ceramide (Sawai et al, 1995; Verheij et al, 1996). Thus, ceramide-mediated apoptosis would be expected to require new protein synthesis. Indeed, there are reports that indicate that inhibition of protein synthesis by either CHX or actinomycin D inhibits ceramide-induced apoptosis (Brugg et al, 1996;

Hartfield et al, 1998; Vento et al, 1998). We found that ceramide-induced DNA fragmentation in PC3 and DU145 was inhibited in the presence of CHX (Fig. 2).

Thus, ceramide-mediated apoptosis in PC3 and DU145 seems to require new protein synthesis. The Fas-resistant cell line DU145 is converted to Fas-sensitive by the inhibition of protein synthesis (Rokhlin et al, 1997), which may indicate that the ceramide pathway does not play an essential role in Fas-mediated apoptosis in this cell line. However, this conclusion is based on the unproven assumption that the exogenous C2-ceramide has the same properties as physiologically generated ceramide.

There are controversial publications with respect to the rapid generation of ceramide after crosslinking of the Fas receptor. Several studies report the generation of ceramide and the concomitant decrease of SM levels within 10 minutes of Fas ligation (Brenner et al, 1998; Cifone et al, 1994; Gulbins et al, 1995), whereas several other studies provide evidence against a rapid accumulation of ceramide after Fas activation (Sillence and Allan, 1997; Tepper et al, 1997, 1999 ; Watts et al, 1997). We analyzed the levels of endogenous SM levels after Fas ligation of the Fas-sensitive cell line PC3 (Fig. 3). We did not find any difference between SM levels in untreated or Fas-treated cells within the first 40 minutes of Fas ligation. This result provides further evidence against a role of SMase-generated ceramide in the initiation of Fas-mediated apoptosis in human prostatic carcinoma cell lines.

In summary, C2-ceramide induces cell death in PC3 and DU145 by apoptotic and non-apoptotic mechanisms. The apoptotic mode of cell death induced by C2-ceramide can be inhibited by CHX, and thus is dependent on protein synthesis. This result provides evidence that in DU145, which becomes Fas-sensitive in the presence of CHX, the ceramide-signaling pathway does not play a role in Fas-mediated apoptosis. We also did not find changes in SM levels after Fas ligation in the Fas-sensitive cell line PC3. Thus, ceramide is not likely to play a role during the early stages of Fas-mediated apoptosis in human prostatic carcinoma cell lines.

Materials and Methods

Cell Culture and Treatment Conditions

The human prostatic carcinoma cell lines were cultured as described previously (Rokhlin et al, 1997). The RPMI medium with 10% fetal calf serum (FCS) was exchanged at least 2 hours before cells were treated with the respective agent.

Reagents

Ten millimolar stock solutions of C2-dihydroceramide (Calbiochem, La Jolla, California) and C2-ceramide (Calbiochem or Cayman Chemical, Ann Arbor, Michigan) were prepared in ethanol. The stock solution of CHX (Sigma, St. Louis, Missouri) was prepared at 25 mg/ml in ethanol. zVAD-fmk (Calbiochem) was dissolved in dimethyl sulfoxide at a concentration of 10 mM.

Quantitative Viability Assay

To determine the percentage of non-viable cells during drug treatment, cells were seeded at 5000 cells/well in 96-well flat-bottomed plates in the presence or absence of the indicated concentration of C2-dihydroceramide, C2-ceramide, C2-ceramide + 100 μM zVAD-fmk, anti-Fas mAb (IPO-4), anti-Fas mAb + 100 μM zVAD-fmk, zVAD-fmk alone, or ethanol. In the inhibition studies, 100 μM zVAD-fmk was added to the cells 1 hour before the ceramides were added. After incubation for the indicated times, the viability of the cells was determined using the Calcein AM assay (Molecular Probes, Eugene, Oregon). Each data point in each experiment was represented by a group of quadruplicate samples. The percentage of dead cells was calculated as described previously (Rokhlin et al, 1997).

Quantitative DNA Fragmentation Assay

The method was described previously (Rokhlin et al, 1997). Briefly, to measure DNA loss during apoptosis, cells were labeled overnight with [^{3}H]thymidine and then cultured in 96-well flat-bottomed plates (5000 cells/well) in the presence or absence of the indicated concentration of C2-dihydroceramide, C2-ceramide, C2-ceramide + 100 μM zVAD-fmk, C2-ceramide + 10 μg/ml of CHX, 10 μg/ml of CHX alone, or 1% ethanol. In case of inhibition studies, zVAD-fmk was added 1 hour before ceramide was added to the cells, and CHX was added 3 hours before ceramide was added to the cells. Radioactivity was measured by liquid scintillation counting in quadruplicate samples. The percentage of DNA fragmentation was calculated as described previously (Rokhlin et al, 1997).

Analysis of SM Levels

Cellular SM content was determined as described previously (Brenner et al, 1998) with some modifications. In T25 flasks, 150,000 PC3 cells were metabolically labeled by incubation with 1 μCi/ml of [methyl-^{3}H]choline chloride (75 Ci/mmol; DuPont-New England Nuclear, Boston, Massachusetts) for 48 hours, in 5 ml of complete RPMI medium with 10% FCS. The medium was then replaced by 5 ml of fresh complete RPMI medium with 1% FCS. After incubation for 2 hours, the medium was replaced by 1.5 ml of complete RPMI medium with 1% FCS containing or not containing 2 μg/ml of anti-Fas mAb. After different times of incubation, Fas stimulation was stopped by the addition of 1.5 ml of 0.22 N HCl, and cells were harvested using a cell scraper. The cells were transferred to a glass tube, spun down at 800 $\times$g, and the pellet was resuspended in 200 μl of PBS. Lipids were extracted by addition of 2.7 ml of CHCl$_3$/CH$_3$OH (2:1, v/v), 0.9 ml of CHCl$_3$, and 0.9 ml of KCl (1M). The organic phase was collected after mixing, and centrifugation for 15 minutes at 800 $\times$g, dried en vacuo, and resuspended in 50 μl of CHCl$_3$/CH$_3$OH (1:1, v/v). Twenty-five microliters of lipid extract was separated on G60 silica gel thin layer chromatography plates (Redi Plate, 20$\times$20 cm; Fisher Scientific, Pittsburgh,

Pennsylvania) using CHCl$_3$:CH$_3$OH:H$_2$O:acetic acid (50:30:8:4, v/v/v/v) as solvent system. Lipid bands were detected by staining the plate with 2′,7′-dichlorofluorescein (Sigma) and observation under UV light. SM and phosphatidylcholine were identified by co-migration with standards, SM from bovine brain (Sigma) and phosphatidylcholine (Sigma). The SM and phosphatidylcholine bands were separately scraped off the plate, and radioactivity was determined by scintillation counting. The amount of labeled phosphatidylcholine was used as internal control to normalize for equal amounts of loaded material, because phosphatidylcholine levels can be assumed to be constant (Cifone et al, 1995; Schutze et al, 1992).

References

Ariga T, Jarvis WD, and Yu RK (1998). Role of sphingolipid-mediated cell death in neurodegenerative diseases. J Lipid Res 39:1–16.

Ashkenazi A and Dixit VM (1998). Death receptors: Signaling and modulation. Science 281:1305–1308.

Brenner B, Ferlinz K, Grassme H, Weller M, Koppenhoefer U, Dichgans J, Sandhoff K, Lang F, and Gulbins E (1998). Fas/CD95/Apo-I activates the acidic sphingomyelinase via caspases. Cell Death Differ 5:29–37.

Brugg B, Michel PP, Agid Y, and Ruberg M (1996). Ceramide induces apoptosis in cultured mesencephalic neurons. J Neurochem 66:733–739.

Cifone MG, De Maria R, Roncaioli P, Rippo MR, Azuma M, Lanier LL, Santoni A, and Testi R (1994). Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J Exp Med 180:1547–1552.

Cifone MG, Roncaioli P, De Maria R, Camarda G, Santoni A, Ruberti G, and Testi R (1995). Multiple pathways originate at the Fas/APO-1 (CD95) receptor: Sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. Embo J 14:5859–5868.

Cock JG, Tepper AD, de Vries E, van Blitterswijk WJ, and Borst J (1998). CD95 (Fas/APO-1) induces ceramide formation and apoptosis in the absence of a functional acid sphingomyelinase. J Biol Chem 273:7560–7565.

De Maria R, Rippo MR, Schuchman EH, and Testi R (1998). Acidic sphingomyelinase (ASM) is necessary for fas-induced GD3 ganglioside accumulation and efficient apoptosis of lymphoid cells. J Exp Med 187:897–902.

Gamen S, Anel A, Pineiro A, and Naval J (1998). Caspases are the main executioners of Fas-mediated apoptosis, irrespective of the ceramide signalling pathway. Cell Death Differ 5:241–249.

Gewies A, Pokhlin OW, and Cohen MB (In Press, 2000). Cytochrome C is involved in Fas mediated apoptosis of prostate carcinoma cell lines. Cancer Res

Gulbins E, Bissonnette R, Mahboubi A, Martin S, Nishioka W, Brunner T, Baier G, Baier-Bitterlich G, Byrd C, Lang F, Kolesnick R, Altman A, and Green D (1995). FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. Immunity 2:341–351.

Gulbins E, Szabo I, and Lang F (1996). Physiology of Fas-induced programmed cell death. Cell Physiol Biochem 6:361–375.

Hannun YA (1996). Functions of ceramide in coordinating cellular responses to stress. Science 274:1855–1859.

Hartfield PJ, Bilney AJ, and Murray AW (1998). Neurotrophic factors prevent ceramide-induced apoptosis downstream of c-Jun N-terminal kinase activation in PC12 cells. J Neurochem 71:161–169.

Hirsch T, Marchetti P, Susin SA, Dallaporta B, Zamzami N, Marzo I, Geuskens M, and Kroemer G (1997). The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. Oncogene 15:1573–1581.

Hofmann K and Dixit VM (1998). Ceramide in apoptosis—does it really matter? Trends Biochem Sci 23:374–377.

Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA, and Grant S (1994). Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc Natl Acad Sci USA 91:73–77.

Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Push-kareva M, Obeid LM, and Hannun YA (1995). Role for ceramide in cell cycle arrest. J Biol Chem 270:2047–2052.

Li H, Zhu H, Xu CJ, and Yuan J (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491–501.

McCarthy NJ, Whyte MK, Gilbert CS, and Evan GI (1997). Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J Cell Biol 136:215–227.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, and Peter ME (1997). FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). Embo J 16:2794–2804.

Mengubas K, Fahey AA, Lewin J, Mehta AB, Hoffbrand AV, and Wickremasinghe RG (1999a). Killing of T lymphocytes by synthetic ceramide is by a nonapoptotic mechanism and is abrogated following mitogenic activation. Exp Cell Res 249:116–122.

Mengubas K, Riordan FA, Bravery CA, Lewin J, Owens DL, Mehta AB, Hoffbrand AV, and Wickremasinghe RG (1999b). Ceramide-induced killing of normal and malignant human lymphocytes is by a non-apoptotic mechanism. Oncogene 18:2499–2506.

Muzio M, Salvesen GS, and Dixit VM (1997). FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J Biol Chem 272:2952–2956.

Nagata S and Golstein P (1995). The Fas death factor. Science 267:1449–1456.

Obeid LM, Linardic CM, Karolak LA, and Hannun YA (1993). Programmed cell death induced by ceramide. Science 259:1769–1771.

Rokhlin OW, Bishop GA, Hostager BS, Waldschmidt TJ, Sidorenko SP, Pavloff N, Kiefer MC, Umansky SR, Glover RA, and Cohen MB (1997a). Fas-mediated apoptosis in human prostatic carcinoma cell lines. Cancer Res 57:1758–1768.

Rokhlin OW, Glover RA, and Cohen MB (1998). Fas-mediated apoptosis in human prostatic carcinoma cell lines occurs via activation of caspase-8 and caspase-7. Cancer Res 58:5870–5875.

Rokhlin OW, Hostager BS, Bishop GA, Sidorenko SP, Glover RA, Gudkov AV, and Cohen MB (1997b). Dominant nature of the resistance to Fas- and tumor necrosis factor- alpha-mediated apoptosis in human prostatic carcinoma cell lines. Cancer Res 57:3941–3943.

Sawai H, Okazaki T, Yamamoto H, Okano H, Takeda Y, Tashima M, Sawada H, Okuma M, Ishikura H, Umehara H, and Domae N (1995). Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. J Biol Chem 270:27326–27331.

Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, and Kronke M (1992). TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell 71:765–776.

Sillence DJ and Allan D (1997). Evidence against an early signalling role for ceramide in Fas-mediated apoptosis. Biochem J 324(Pt 1):29–32.

Skowronski EW, Kolesnick RN, and Green DR (1996). Fas-mediated apoptosis and sphingomyelinase signal transduction: The role of ceramide as a second messenger for apoptosis. Cell Death Differ 3:171–176.

Tepper AD, Cock JG, de Vries E, Borst J, and van Blitterswijk WJ (1997). CD95/Fas-induced ceramide formation proceeds with slow kinetics and is not blocked by caspase-3/CPP32 inhibition. J Biol Chem 272:24308–24312.

Tepper AD, de Vries E, van Blitterswijk WJ, and Borst J (1999). Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. J Clin Invest 103:971–978.

Tepper CG, Jayadev S, Liu B, Bielawska A, Wolff R, Yone-hara S, Hannun YA, and Seldin MF (1995). Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. Proc Natl Acad Sci USA 92:8443–8447.

Vento R, Giuliano M, Lauricella M, Carabillo M, Di Liberto D, and Tesoriere G (1998). Induction of programmed cell death in human retinoblastoma Y79 cells by C2-ceramide. Mol Cell Biochem 185:7–15.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, and Kolesnick RN (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380:75–79.

Watts JD, Gu M, Patterson SD, Aebersold R, and Polverino AJ (1999). On the complexities of ceramide changes in cells undergoing apoptosis: Lack of evidence for a second messenger function in apoptotic induction. Cell Death Differ 6:105–114.

Watts JD, Gu M, Polverino AJ, Patterson SD, and Aebersold R (1997). Fas-induced apoptosis of T cells occurs independently of ceramide generation. Proc Natl Acad Sci USA 94:7292–7296.

Westwick JK, Bielawska AE, Dbaibo G, Hannun YA, and Brenner DA (1995). Ceramide activates the stress-activated protein kinases. J Biol Chem 270:22689–22692.

Wiegmann K, Schutze S, Machleidt T, Witte D, and Kronke M (1994). Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell 78:1005–1015.