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Research Article

# Ceramides induce apoptosis in HeLa cells and enhance cytochrome c-induced apoptosis in *Xenopus* egg extracts

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**Abstract.** Ceramide has been reported to induce typical apoptotic changes in nuclei incubated in a cell-free system, and that the addition of ceramide bypasses the requirement for mitochondria. Here, we explore the possible pathways by which ceramide induces apoptosis either in intact cells or in a cell-free system which we have developed. We found that in the cell-free system, C2-ceramide is not able to induce apoptosis in nuclei whereas cytochrome c does, but it is able to induce HeLa cells to undergo apoptosis. Ceramide is also not able to induce apoptosis when added into the cell-free system together with purified mitochondria. Further investigation showed that C2-ceramide at certain concentrations greatly increases nuclear apoptosis caused by cytochrome c in the cell-free system. From these results we conclude that the induction of apoptosis by ceramide may require intact cells in which some unknown signal transduction pathways are involved.

**Key words.** Ceramide; HeLa cell; apoptosis; egg extract; cytochrome c; nuclei.

Ceramide is a form of lipid second messenger that belongs to the sphingomyelin family and it functions in multiple cell responses to external stimuli. Since the recent discovery that it functions as an intracellular effector during the process of cell apoptosis, its function and regulation have received increasing attention.

Many apoptosis-inducing factors, such as tumor necrosis factor (TNF), Fas, $\gamma$- or ultraviolet (UV) radiation, and heat shock, have been reported to result in rapid ceramide generation and that cells undergo apoptosis [1–3]. Ceramide is generated via multiple pathways in vivo, two of which are mainly involved in apoptosis. One is the de novo pathway via serine and palmitoyl-CoA, in which ceramide synthase and dihydroceramide reductase are involved. The activation of ceramide synthase will cause excessive generation of ceramide and induce cells into apoptosis [4]. The other way for cells to produce ceramide is via the sphingomyelin (SM) pathway, which involves the activation of sphingomyelinase (SMase). SMase hydrolyzes SM to yield the lipid second messenger ceramide and phosphorylcholine. Ceramide generated by neutral Smase (N-SMase) at the plasma membrane directs the activation of ceramide-activated protein kinase (CAPK) that phosphorylates and activates Raf-1 kinase [5, 6]. Activated Raf-1 in turn phosphorylates and stimulates a dual-specificity mitogen-activated kinase (MEK-1), which eventually phosphorylates and activates extracellular signal-regulated kinases (ERKs), leading to cell growth, differentiation, and upregulation of vital cellular functions [7]. The downstream targets of acid SMase (a-SMase) have yet to be defined and may include protein kinase PKC$\zeta$, JNK, and caspases [8–10].

Ceramide can signal diverse cellular functions, including cell proliferation, differentiation, the inflammatory reaction, and apoptosis. These may be brought about, at

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least in part, by triggering the SM pathway at distinct subcellular sites. Studies on the TNF receptor suggest a model that might clarify how ceramide acts to signal both the proinflammatory and apoptotic effects of TNF. They demonstrate that specific receptor domains of TNF link to different SMases. The proinflammatory responses may be downstream of N-SMase, while the activation of A-SMase corresponds to apoptosis.

The direct downstream targets of ceramide in the regulation of apoptosis are far from clear. Ceramide has been reported to signal apoptosis by at least two independent and perhaps coordinately regulated mechanisms: one involves transcription through SAPK/JNK [11] and the other is mediated by direct effects on mitochondrial homeostasis [12]. More recently, Zhou et al. [13] reported that ceramide also regulates apoptosis by decreasing anti-apoptotic signaling in cells by inhibiting Akt, a key anti-apoptotic kinase.

Martin et al. [14] reported that ceramide is able to induce typical apoptotic changes in nuclei incubated in the egg extracts of *Xenopus laevis*, and the addition of ceramide bypasses the requirement for mitochondria. Based on the extracts of normal eggs of *X. laevis*, we have developed a cell-free system to study apoptosis in vitro. In this paper, we explore the possible pathways for ceramide to induce apoptosis either in intact cells or in cell-free systems. Our investigations show that C2-ceramide is able to induce HeLa cells to undergo apoptosis but is unable to induce apoptosis in nuclei in the egg extracts we have developed, whereas cytochrome c can. Further, ceramide cannot induce apoptosis when added into the cell-free system together with purified mitochondria. Further investigation shows that C2-ceramide at certain concentrations greatly increases nuclear apoptosis caused by cytochrome c in the egg extracts. From these results, we conclude that the induction of apoptosis by ceramide may require intact cells in which some unknown signal transduction pathways are involved. Intracellular ceramide accumulation itself is not enough to initiate apoptosis, and some components in the plasma membrane are also required, maybe because the production of ceramide and the molecule that responds to it occur in the plasma membrane.

## Materials and methods

**Materials.** C2-ceramide, hydroxy-ceramide, and nonhydroxy-ceramide were obtained from Sigma. C2-ceramide was dissolved in ethanol, hydroxy-ceramide and nonhydroxy-ceramide in dodecane and ethanol (2:98). Cytochrome c was obtained from Sigma and dissolved in distilled water (100 μM). The caspase inhibitors AC-DEVD-CHO and AC-YVAD-CHO were obtained from Sigma and dissolved in DMSO (10 mM).

**Cell culture.** HeLa cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% newborn calf serum, 10 mM glutamine, and 100 units/ml each of streptomycin and penicillin in a humidified incubator at 5% $CO_2$.

**Induction and assay of apoptosis in HeLa cells.** HeLa cells were maintained in serum-free DMEM-F12 for 24 h prior to experiments. Insulin (5 μg/ml) and transferrin (5 μg/ml) were added to culture media before C2-ceramide; hydroxy-ceramide and nonhydroxy-ceramide were diluted into serum-free DMEM-F12 at the indicated concentrations. The final concentration of ethanol did not exceed 0.25%. Cell viability was determined using trypan blue exclusion. Blue-excluding cells were scored as apoptotic or normal based on morphological criteria: small, round, condensed cells were scored as apoptotic; all other cells were scored as healthy. For each sample, about 300 cells were scored in a blind manner, and experiments were repeated three times.

The DNA fragmentation assay was performed as follows. Cells were lysed in NDB solution (Tris-HCl 10 mM, pH 8.2, NaCl 0.4 M, EDTA 2 mM, SDS 0.5%, proteinase K 0.1 mg/ml), overnight at 37 °C. NaCl was then added to a final concentration of 1.5 M, and the cells were centrifuged at 5000 g for 15 min. Supernatants were transferred to a new tube, and ethanol was added to precipitate the DNA. DNA pellets were washed with 70% ethanol, air-dried, and dissolved in 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. Prior to electrophoresis, DNA samples were treated with DNase-free RNase A (0.2 mg/ml) for 50 min at 37 °C. DNA was then analyzed on a 1.5% agarose gel.

For electron microscopy, cells were collected and fixed with 2% glutaraldehyde in PBS (pH 7.4) for 2 h, washed with PBS and fixed with 1% $OsO_4$ for 1 h. Thin sections were prepared and stained with uranyl acetate and lead citrate. Transmission electron micrographs were taken using a JEM 1010.

**Preparation of *Xenopus* egg extracts s-200 [15].** Healthy *X. laevis* females were injected with 600 units of human chorionic gonadotropin (Sigma) on the night before use and were placed in 5 l of 100 mM NaCl. Eggs were dejellied with 2% cysteine (pH 7.8), rinsed twice in MMR (0.1 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA) and three times with three volumes of a solution containing 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 100 mg/ml cycloheximide, 5 μg/ml cytochalasin B, and 1 mM dithiothreitol. The eggs were crushed at 10,000 g, 4 °C for 15 min. The material between the lipid top layer and yolk pellet was removed and centrifuged again at 10,000 g, 4 °C for 15 min. The supernatant was supplemented with aprotinin to 6 μg/ml and leupeptinin to 8 μg/ml, and further separated into cytosol, membrane-rich, and gelatinous pellet fractions by ultracentrifugation at

200,000 g for 2 h in a HITACHI 55p-72 ultracentrifuge. The cytosol produced was stored in aliquots under liquid nitrogen.

**Preparation of mouse liver nuclei.** Mouse liver nuclei were prepared as described previously [16]. In brief, minced mouse liver was homogenized in homogenization buffer (250 mM sucrose, 10 mM Hepes, pH 7.4, 15 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.5 mM PMSF, 0.5 mM $\beta$-mercaptoethanol, 2 mM cytochalasin B). After filtration through a layer of 200-mesh silk screen, 2 vol of homogenization buffer containing 2.3 M sucrose was added, mixed thoroughly and centrifuged (RPS 50-2 rotor, 4 °C, 30 min, 124,000 g) into a cushion consisting of homogenization buffer plus 2.3 M sucrose. The pellet was resuspended in nuclei stock solution (10 mM Pipes, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, 50% glycerol) at a concentration of $5 \times 10^4$ nuclei/μl and stored under liquid nitrogen.

**Isolation and purification of mitochondria [17].** Livers from mouse were rinsed with ice-cold PBS, minced and homogenized in three volumes of H-buffer [0.07 M sucrose, 0.21 M D-mannitol, 2 mM Hepes-KOH, pH 7.4, 0.05% BSA-V (w/v) (defatted)]. The homogenates were centrifuged at 1100 g for 3 min and the supernatants were centrifuged at 6780 g for 15 min. Pellets were resuspended in 1/4 volume of H-buffer and centrifuged at 20,200 g for 15 min. The sediments were crude mitochondria. These were resuspended in 1/8 volume of H-buffer, centrifuged at 3000 g for 3 min, and the supernatant was retained. The sediments were resuspended and centrifuged once more. The two supernatants were pooled and centrifuged at 20,200 g for 20 min. The upper fluffy pellet was removed. Then, 1/8 volume of H-buffer was added to the tube, and mitochondria were collected by centrifugation at 20,200 g for 20 min. They were re-suspended in H-buffer, counted and store in liquid nitrogen.

**Assay of cell-free apoptosis.** The reaction mixture containing 50 μl of egg extract buffer or egg extract s-200 and $\sim 1 \times 10^5$ mouse liver nuclei was supplemented with isolated mitochondria and incubated at 22 °C for the times indicated. In vitro apoptosis was monitored as described previously [15]. After incubation, 10 volumes of buffer D (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, 0.4% SDS, 0.2 mg/ml proteinase K) was added to each reaction and incubated at 37 °C overnight. The DNA was deproteinized with phenol and phenol-chloroform (1:1), precipitated with 2 volumes of ethanol, and then loaded onto a 1.5% agarose gel, electrophoresed, and visualized by staining with ethidium bromide and illumination with short-wave UV light.

## Results

**C2-ceramide induces apoptosis in cultured HeLa cells.** C2-ceramide (50 μM) was administered to cultured HeLa cells. No obvious morphological changes occurred within 3 h. After 6 h, crevices appeared among cells, and some cells were becoming round. With time, more and more cells became round and detached, and many refractive granules, not seen in normal cells, were observed under the microscope. Many membrane-enveloped bodies characteristic of apoptotic bodies were blebbed out of cells (fig. 1D). Within 24 h, more than 90% of cells were detached, while the normal cultured cells grew into a compact monolayer.

HeLa cells treated with 50 μM hydroxy-ceramides also underwent apoptosis, but the apoptosis-inducing effect was less. The apoptotic changes after 12 h in hydroxy-ceramide-treated cells (fig. 1E) corresponded to those treated with C2-ceramide after 7 h (fig. 1B).

Cells treated with nonhydroxy-ceramides at various concentrations (5, 30, 50, and 150 μM) did not show any morphological change, indicating that nonhydroxy-ceramide is not able to induce apoptosis in cultured HeLa cells (data not shown).

Cells stained with DAPI were examined with a fluorescent microscope. Chromatin condensation, margination, and release of apoptotic body-like granules that were finally extruded from nuclei were observed (fig. 2). However, there were some differences between ceramide-induced HeLa apoptosis and cytochrome c-induced nuclear apoptosis, in that the chromatin condensation in cytochrome c-treated nuclei was on a much larger scale: the chromatin marginated and subsequently underwent dramatic hypercondensation into sharply defined spherical domains (fig. 6B).

To confirm that the morphological changes in HeLa cells treated with C2-ceramide reflected apoptosis, cells were fixed with glutaraldehyde and examined under a transmission electron microscope. Membrane blebbing in the cytoplasm, nuclear shrinkage, and chromatin condensation were all observed in C2-ceramide-treated HeLa cells (fig. 3).

Analysis of chromatin DNA from C2-ceramide-treated cells demonstrated that nuclear chromatin was fragmented. The cleavage of DNA increased as the concentration of C2-ceramide increased (fig. 4A). Figure 4B shows that DNA fragmentation increases with time.

**Caspase inhibitors block C2-ceramide-induced HeLa apoptosis.** When caspase inhibitors AC-DEVD-CHO and AC-YVAD-CHO were added to the culture medium before the administration of C2-ceramide, apoptosis was inhibited, to different degrees, with AC-DEVD-CHO having a stronger effect than AC-YVAD-CHO. However, their effects decreased with time. Within 24 h, most cells had died and detached, regardless of the addition of caspase inhibitors (fig. 5).

**C2-ceramide cannot induce nuclear apoptosis in egg extracts of *Xenopus*.** In a cell-free system prepared from egg extracts of *X. laevis*, C2-ceramide at different concentrations (50, 100, 200 μM) was not able to induce nuclei of mouse liver cells to undergo apoptosis. Nuclei treated with C2-ceramide and examined by fluorescent microscopy showed no morphological changes (data not shown). Nuclear DNA exhibited no fragmentation (fig. 6A). However, C2-ceramide did enhance cytochrome c-induced apoptosis in this cell-free system. In egg extracts, 0.1 μM cytochrome c was not sufficient to trigger obvious apoptotic changes in cultured nuclei, and only some degree of chromatin condensation was observed, while no DNA fragmentation was found. After addition of 50 μM C2-ceramide, nuclei were found to undergo obvious apoptosis, with chromatin DNA excessively cleaved (fig. 6A) and many apoptotic bodies (fig. 6B).

The effect of C2-ceramide-treated mitochondria on apoptosis in the cell-free system was examined. Mitochondria from mouse liver were purified and used to induce nuclear apoptosis in the cell-free system. The lowest concentration of isolated mitochondria needed was $2 \times 10^7$/μl (fig. 7A). When $1.6 \times 10^7$/μl mitochondria and 200 μM C2-ceramide were added together into egg extracts, no apoptotic nuclei were found (fig. 7B,C).

## Discussion

Many recent studies suggest that activating sphingomyelinase and subsequent generation of ceramide

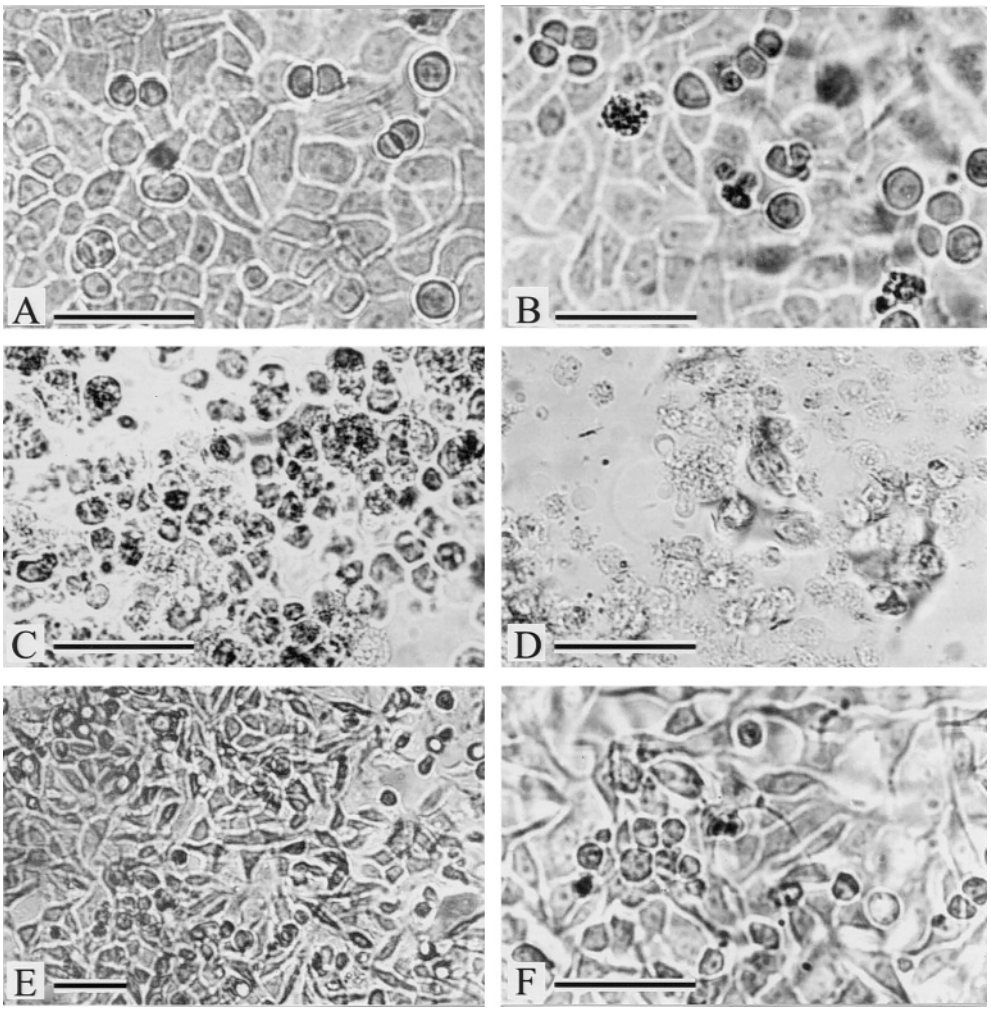


Figure 1. Microscopic observation of C2 ceramide-induced apoptosis. HeLa cells before being treated (*A*); HeLa cells treated with 50 μM C2-ceramide for 6 h (*B*); 12 h (*C*) and 14 h (*D*); HeLa cells treated with 50 μM hydroxy-ceramides for 12 h (*E*); Hela cells cultured in normal medium for 14 h (*F*). Bar, 50 μm.

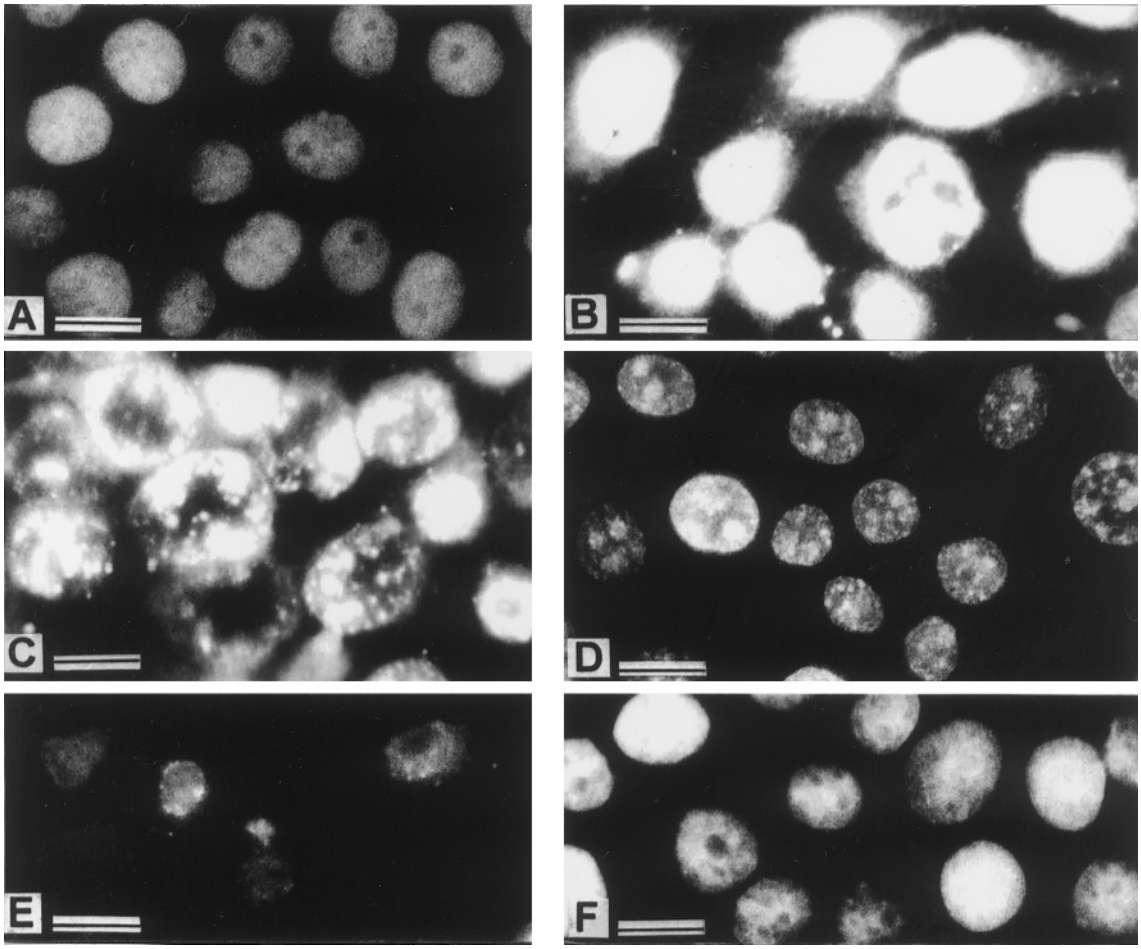


Figure 2. Fluorescent micoscopy of ceramide-induced apoptosis. HeLa cells were cultured in DMEM-F12 with 5 mg/l insulin and 5 mg/l transferrin. Ethanol (*A*) or 50 μM C2-ceramide was added to the media for 6 h (*B*), 12 h (*C*), 14 h (*D*), or 24 h (*E*). (*F*) Untreated HeLa cells at 24 h. Bar, 10 μm.

play an important role in regulating apoptosis in many systems. Apoptosis induced by a variety of initiators such as tumor necrosis factor, Fas ligand, daunorubicin, removal of growth factors, UV irradiation, and x-irradiation is associated with increased ceramide production, which indicates a strong correlation exists between production of ceramide and subsequent cell death. Moreover, cell-permeable ceramide analogues mimic these agents and induce apoptosis in many different cell types. These observations are consistent with that ceramide is a lipid second messenger that functions as part of a ubiquitous signaling system to link cell surface receptors and environmental stresses to the apoptotic pathway. However, signal transduction pathways mediating ceramide-induced apoptosis are largely unknown, except for a recent study showing that the proapoptotic stress-activated protein kinase/c-Jun N-terminal kinase plays an important role in ceramide-induced apoptosis.

Clearly, definition of direct ceramide targets remains a major goal that will provide important clues to the involvement of ceramide in specific signaling pathways and its biological significance in general. Whether the accumulation of ceramide directly initiates apoptosis by activating caspases or indirectly leads to apoptosis by interacting with other cellular components, the plasma membrane for example, remains unclear. There are almost no membrane vesicles in the egg extracts after centrifugation at 200,000 g for 2 h. They are composed strictly of soluble components. In this extract, cytochrome c can induce nuclear apoptosis efficiently, while C2-ceramide at various concentrations cannot, indicating that the accumulation of ceramide alone is not sufficient to activate the caspase cascade.

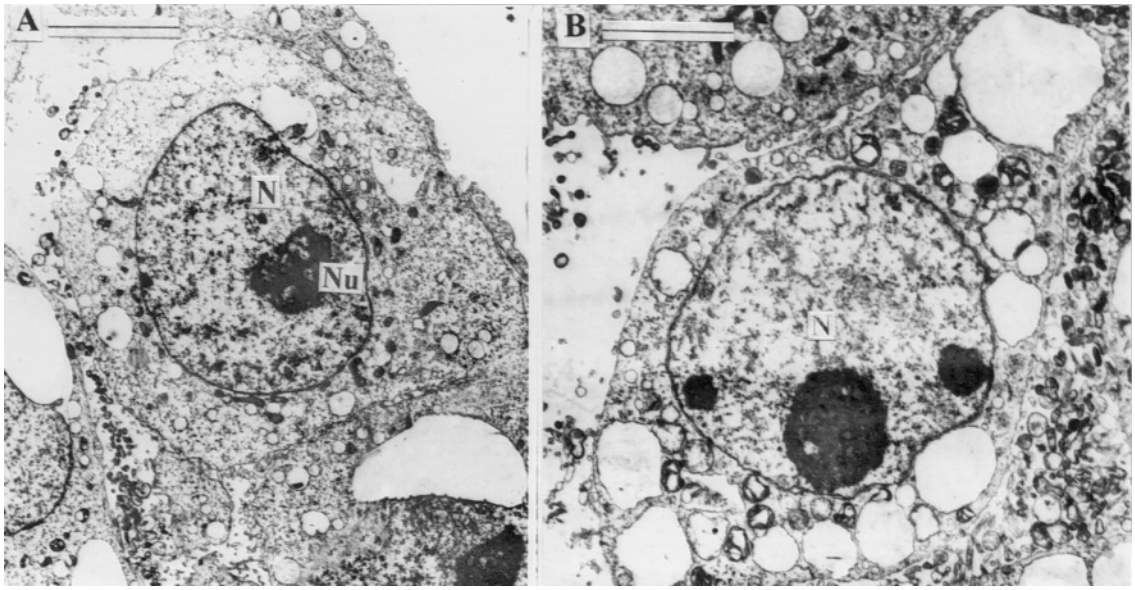


Figure 3A, B. Electronic microscopy of apoptosis. HeLa cells were treated with 50 μM C2-ceramide. Electronic microscopy showed that there was obvious transformation in the nuclei and cytoplasm of HeLa cells, including the shrinkage of nuclei, abnormal condensation of chromatin, and cytoplasm blebbing. N, nuclei; Nu, nucleolus. Bar, 5 μm. (*A*) Normal HeLa cell. (*B*) Apoptotic HeLa cells.

Martin et al. [14] have reported that 50–150 μM C2-ceramide causes typical nuclear apoptosis in their cell-free system, also prepared from *Xenopus* eggs: the addition of C2-ceramide bypasses a requirement for a heavy membrane fraction (mitochondria). Our results appear to be incompatible. However, in the preparation of egg extracts, there are important differences between the two systems. Our *Xenopus* egg extracts are prepared from normal eggs, while those of Martin et al. [14] were prepared from apoptotic eggs and had been in the oviduct for a sufficiently long time after maturation that the signals for apoptosis in eggs were already initiated when laying. Nuclei incubated in these apoptotic extracts will finally undergo apoptosis without addition of any other components, if incubated long enough [14]. In fact, the function of C2-ceramide in such extracts is only to accelerate the execution of apoptosis, which is consistent with our results (fig. 6).

AC-DEVD-CHO is an inhibitor of caspase-3 and Ac-YVAD-CHO is an inhibitor of caspase-1 and -4. Our result in figure 5 clearly shows that AC-YVAD-CHO has no effect on C2-ceramide-induced apoptosis and that AC-DEVD-CHO only slows down this effect. These results suggest that caspase-3 is, while caspase-1 and -4 are not, involved in the process of ceramide-induced apoptosis, and that a caspase-3 independent pathway in this process also exists. Ceramide-induced apoptosis was recently reported to occur independently of caspases in U937 and MCF7 cells [18]. So the pathway of ceramide-induced apoptosis may involve a caspase-independent pathway in different cell lines.

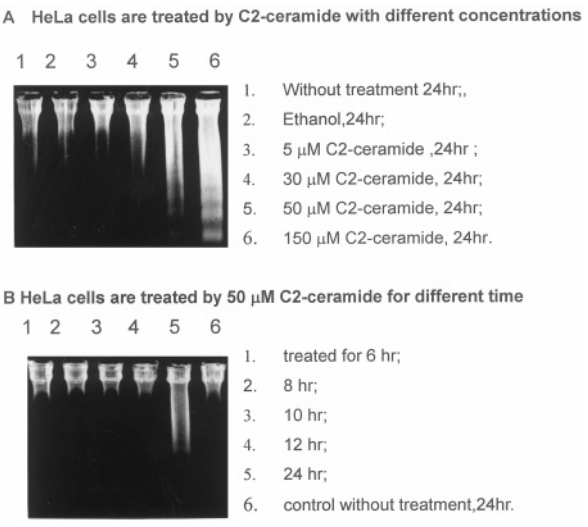


Figure 4A, B. DNA fragmentation in HeLa cells treated with C2-ceramide. (*A*) HeLa cells treated with C2-ceramide at different concentrations: 1, without treatment at 24 h; 2, ethanol at 24 h; 3, 5 μM C2-ceramide at 24 h; 4, 30 μM C2-ceramide at 24 h; 5, 50 μM C2-ceramide at 24 h; 6, 150 μM C2-ceramide at 24 h. (*B*) HeLa cells treated with 50 μM C2-ceramide for various times: 1, treated for 6 h; 2, 8 h; 3, 10 h; 4, 12 h; 5, 24 h; 6, control without treatment at 24 h.

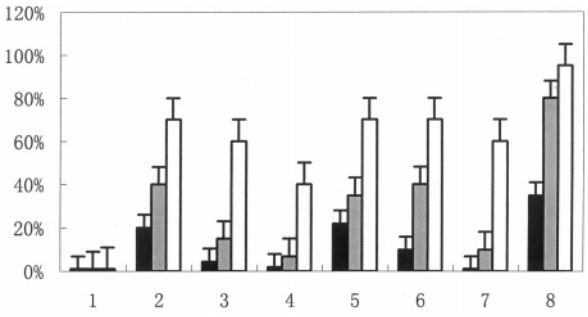


Figure 5. Effects of caspase inhibitors on C2-ceramide-induced apoptosis. 6 h (closed bars), 10 h (shaded bars), and 24 h (open bars) after treatment: 1, control; 2, 50 μM C2-ceramide; 3, 50 μM C2-ceramide + 1 μM AC-DEVD-CHO; 4, 50 μM C2-ceramide + 10 μM AC-DEVD-CHO; 5, 50 μM C2-ceramide + 10 μM AC-YVAD-CHO; 6, 50 μM C2-ceramide + 100 μM AC-YVAD-CHO; 7, 50 μM C2-ceramide + 10 μM AC-DEVD-CHO + 100 μM AC-YVAD-CHO; B, 150 μM C2-ceramide. Apoptotic cells in the culture were quantified as described in Materials and methods and are shown as percentages. For each sample, at least 300 cells were obtained in triplicate in each experiment. Experiments were repeated at least three times. Error bars show SD.

Our result shows that high concentrations of C2-ceramide in the cytoplasm cannot initiate apoptosis, indicating that ceramide itself is not able to activate the apoptotic machinery, including caspases, in this cell-free system. Ceramide can, however, improve the cytochrome c-induced apoptosis in the cell-free system, suggesting there may be an accessory pathway that can enhance the apoptosis triggered by cytochrome c. Since most of the membrane components have been sedimented out of cytosol s-200, membrane components are unlikely involved in this accessory pathway. Schwandner et al. [19] reported that accumulation of ceramide can be dissociated from caspase-8-induced apoptosis. Overexpression of caspase-8 readily induced apoptosis of 293 cells regardless of ceramide level. On the other hand, they also postulate an accessory pathway in which cytoplasmic ceramide is involved. In fact, the role of ceramide in accelerating apoptosis induced by various stimuli in diverse cell types remains an open issue, and its precise delineation requires further extensive investigation [19].

Ceramide-induced cell apoptosis and cytochrome c-induced nuclear apoptosis clearly differ. In nuclei treated with cytochrome c, chromatin condensation is much more extensive than in those treated with ceramide, indicating that the pathways through which each regulates apoptosis may differ.

Ghafourigar et al. [20] have reported that ceramide-induced cytochrome c release takes place when cytochrome c is oxidized but not when it is reduced. They applied antimycin A to block the mitochondrial respiratory chain complex III and keep cytochrome c in an oxidized state. Thus, they observed that ceramide induce cytochrome c release under nonphysiological conditions. Cytochrome c in the presence of cytosolic extracts is in reductive state [21]. Our results show that ceramide does not enhance the release of cytochrome c from isolated mitochondria in the cell-free system which is in the reductive state. There is no contradiction between our results and the results of Ghafourifar et al. [20].

Leupeptin has been reported to inhibit ceramide-induced apoptosis to some degree [18]. Some leupeptin-related proteases seem to be involved in the pathway of ceramide-initiated apoptosis. To stabilize our extracts, 8

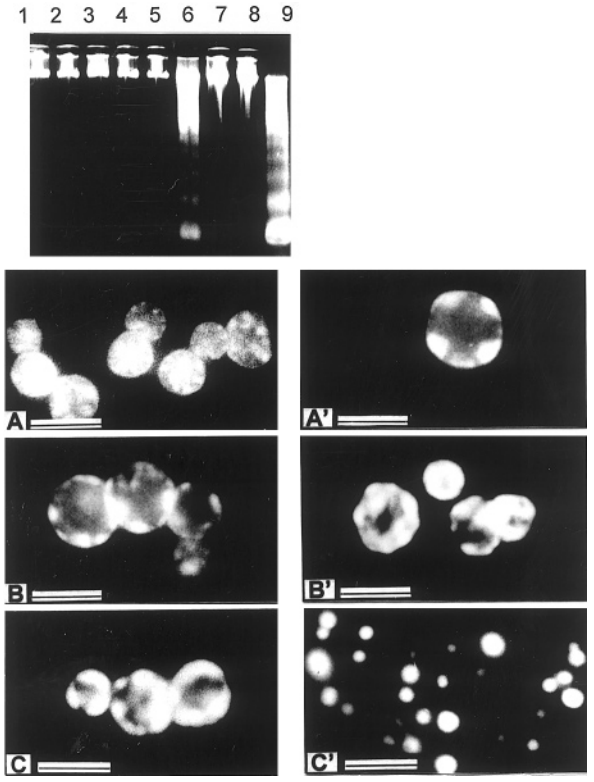


Figure 6A, B. Cytochrome c-induced nuclear apoptosis is greatly enhanced by C2-ceramide. (*A*) Analysis of chromatin DNA in incubated nuclei. Mouse liver nuclei were incubated in egg extract s-200, and nuclear DNA was analyzed after 2 h. 1, control; 2, 50 μM; 3, 100 μM; 4, 200 μM C2-ceramide; 5, 0.1 μM cytochrome c; 6, 0.1 μM cytochrome c + 50 μM C2-ceramide; 7, 0.1 μM cytochrome c + 100 μM C2-ceramide; 8, 0.1 μM cytochrome c + 200 μM C2-ceramide; 9, 2 μM cytochrome c. (*B*) Morphological changes of nuclei. Mouse liver nuclei were added to *Xenopus* egg extracts containing 0.1 μM cytochrome c (A, B, C) or 50 μM C2-ceramide and 0.1 μM cytochrome c (A′, B′, C′), and incubated for 10 min (A, A′), 30 min (B, B′), and 2 h (C, C′). Extracts with C2-ceramide induce nuclear apoptosis much more efficiently. Bar = 10 μm.

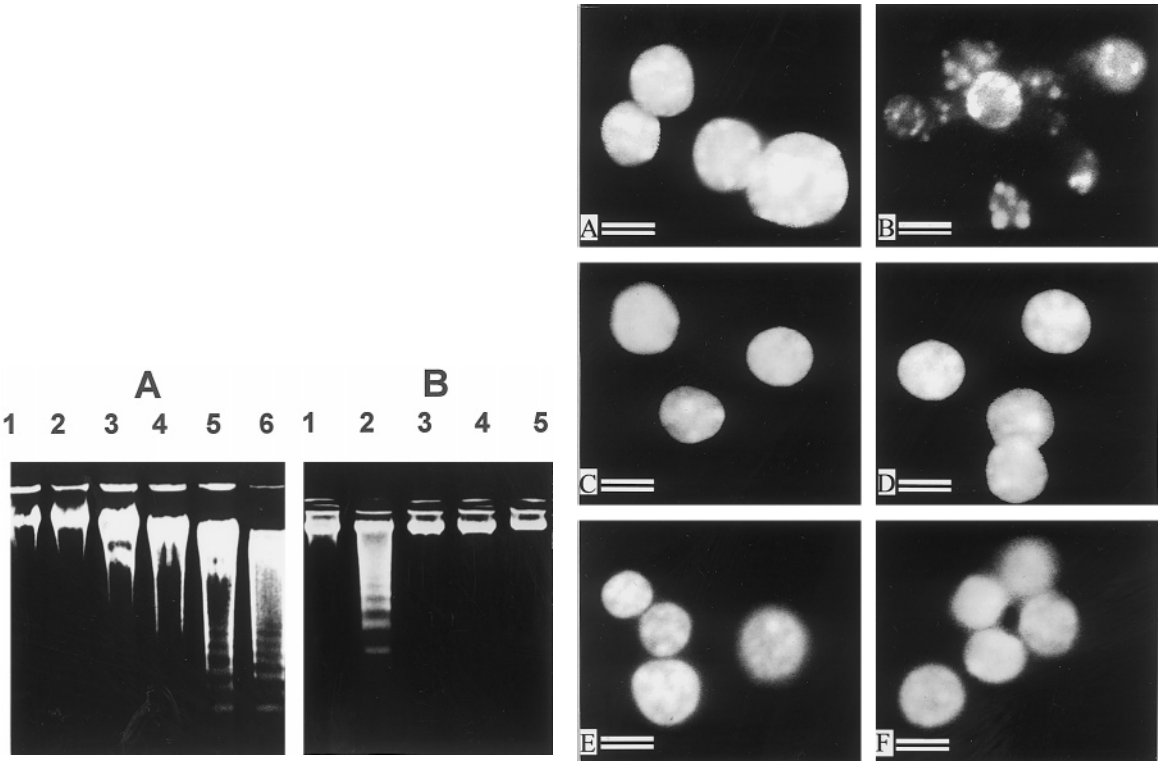


Figure 7A–C. DNA analysis of nuclei treated with mitochondria and ceramide in egg extracts. (*A*) Mitochondria from mouse liver cells were isolated and purified, then added to 50 μl s-200 at final concentrations of $8 \times 10^6$/μl (1), $1.2 \times 10^7$/μl (2), $1.6 \times 10^7$/μl (3), $2 \times 10^7$/μl (4), $2.4 \times 10^7$/μl (5), and $2.8 \times 10^7$/μl (6). After 4 h incubation at 22°C, chromatin DNA was analyzed. Mitochondria are able to induce apoptotic DNA fragmentation in s-200. (*B*) Isolated mitochondria and C2-ceramide at different concentrations were incubated for 4 h in egg extract s-200 with mouse liver nuclei, and nuclear DNA was then analyzed. 1, s-200; 2, s-200 + 2 μM cytochrome c; 3, s-200 + $1.6 \times 10^7$/μl mitochondria; 4, s-200 + 200 μM C2-ceramide; 5, s-200 + $1.6 \times 10^7$/μl mitochondria + 200 μM C2-ceramide. C2-ceramide cannot induce nuclear apoptosis in the presence of exogenous mitochondria in egg extracts. (*C*) Morphological changes in nuclei. Mouse liver nuclei were incubated for 4 h in s-200 (A), s-200 + 2 μM cytochrome c (B), s-200 + $1.6 \times 10^7$/μl mitochondria (C), s-200 + 200 μM C2-ceramide (D), s-200 + $1.6 \times 10^7$/μl mitochondria + 200 μM C2-ceramide (E), and for 10 min in s-200 (F). Bar, 10 μm.

μg/ml leupeptin was added. But the similar results were obtained in egg extracts without leupeptin (data not shown). A ceramide-induced leupeptin-related protease pathway appears not to be activated in our egg extracts.

It is interesting that ceramide induces cultured cells into apoptosis but loses this ability in egg extracts. The latter have no intact cell structure though they contain some dATP, Apaf-1, and caspase-9, which explains why the addition of cytochrome c can induce apoptosis of nuclei [22]. An intact cell structure is apparently necessary for ceramide-triggered cell apoptosis. Since most ceramide is generated in the membrane [2] (N-SMase in the plasma membrane, A-SMase in lysosomes and endosomes) and many downstream targets of ceramide are distributed in membranes, membrane components are necessary for ceramide to initiate apoptosis and accumulation of ceramide alone in the cytosol cannot signal the mitochondria permeability transition. A possible mechanism for ceramide-induced apoptosis is that in intact cells, ceramide accumulation leads to the activation of CAPK which in turn initiates signal transmission including the activation of apoptosis-associated receptors in the plasma membrane, release of cytochrome c from mitochondria, thus finally triggering apoptosis.

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