

Tumor necrosis factor- α and ceramide induce cell death through different mechanisms in rat mesangial cells

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Guo, Yan-Lin, Baobin Kang, Li-Jun Yang, and John R. Williamson. Tumor necrosis factor- α and ceramide induce cell death through different mechanisms in rat mesangial cells. *Am. J. Physiol.* 276 (*Renal Physiol.* 45): F390–F397, 1999.—It has been proposed that ceramide acts as a cellular messenger to mediate tumor necrosis factor- α (TNF- α)-induced apoptosis. Based on this hypothesis, it was postulated that resistance of some cells to TNF- α cytotoxicity was due to an insufficient production of ceramide on stimulation by TNF- α . The present study was initiated to investigate whether this was the case in mesangial cells, which normally are insensitive to TNF- α -induced apoptosis. Our results indicate that although C₂ ceramide was toxic to mesangial cells, the cell death it induced differed both morphologically and biochemically from that induced by TNF- α in the presence of cycloheximide (CHX). The most apparent effect of C₂ ceramide was to cause cells to swell, followed by disruption of the cell membrane. It is evident that C₂ ceramide caused cell death by necrosis, whereas TNF- α in the presence of CHX killed the cells by apoptosis. C₂ ceramide did not mimic the effects of TNF- α on the activation of c-Jun NH₂-terminal protein kinase and nuclear factor- κ B transcription factor. Although mitogen-activated protein kinase [extracellular signal-related kinase (ERK)] was activated by both C₂ ceramide and TNF- α , such activation appeared to be mediated by different mechanisms as judged from the kinetics of ERK activation. Furthermore, the cleavage of cytosolic phospholipase A₂ during cell death induced by C₂ ceramide and by TNF- α in the presence of CHX showed distinctive patterns. The present study provides evidence that apoptosis and necrosis use distinctive signaling machinery to cause cell death.

apoptosis; mitogen-activated protein kinase; c-Jun NH₂-terminal protein kinase; nuclear factor- κ B

CELL DEATH CAN OCCUR through two very different ways, termed apoptosis (or programmed cell death) and necrosis. Apoptosis is characterized by such morphological changes as cell shrinkage, cytoplasmic blebbing, chromatin condensation, and DNA fragmentation, whereas necrosis is defined by cell swelling, destruction of organelles, and cell lysis resulting from disruption of the cell membrane (3, 34). Apoptosis occurs generally in a highly regulated manner and is thought to be a physiological form of cell death for the purpose of removing unwanted or damaged cells. It is important for such processes as embryogenesis and the immune response. On the other hand, necrosis is considered to represent a degenerative cell death induced by direct

toxic, chemical, or physical injuries. It is usually involved in processes such as inflammatory reactions (26, 34).

Tumor necrosis factor- α (TNF- α) is a cytokine produced by many cell types. It was originally identified on the basis of its cytotoxic effect on certain cells. It has been found to elicit a wide range of biological responses including inflammatory reaction, cell proliferation, and differentiation in addition to its ability to induce cell death. Which response is elicited appears to depend on the cell type and its state of differentiation (3). Many tumor cells are sensitive to TNF- α -induced apoptosis, but normal cells are usually resistant. Some cells lose their viability only when they are treated with TNF- α in the presence of other agents or when the cells are damaged (27, 37). Although cell death by apoptosis and necrosis have been well characterized by morphological criteria, the initial events in the signal transduction pathways responsible for the later phases of cell death are poorly understood. Among the several signaling pathways stimulated by TNF- α , production of ceramide through the sphingomyelin cycle is thought to be critical for the initiation of apoptosis (18, 24). This hypothesis was advanced because exogenous ceramide derivatives can mimic the effect of TNF- α in activating the signaling pathways leading to apoptosis in certain cells (32, 33, 41). It is supported by the findings that the resistance of some cells to TNF- α cytotoxicity is due to defects in the sphingomyelin cycle because restoring this pathway resulted in the cells becoming susceptible to TNF- α -induced apoptosis (4, 7, 30). Furthermore, sphingomyelinase knockout mice failed to hydrolyze sphingomyelin and generate ceramide and also showed defective apoptotic responses (24). Although these findings strongly support the role of ceramide as a cellular messenger for TNF- α -induced apoptosis in certain cells, there are a number of recent reports that indicate that this is not a universal response. For instance, in human vascular endothelial cells and neutrophils, ceramide did not mediate TNF- α -induced gene expression (36, 43). In leukemia ML-1 cells, it was found that ceramide is necessary but not sufficient for TNF- α -induced apoptosis (21). In other cells, although both TNF- α and ceramide were cytotoxic, they seemed to cause cell death through different mechanisms (1, 12, 36). It is evident that the role of ceramide in cellular processes is likely to be cell-type dependent. A general role for ceramide as a second messenger for TNF- α -induced apoptosis is clearly debatable.

Mesangial cells are a prominent cell type of the kidney glomerulus. They are smooth muscle-like contractile cells that take part in the regulation of the glomerular microcirculation, ultrafiltration, and im-

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immune response. Like many primary cells, mesangial cells are normally resistant to TNF- α cytotoxicity, but they rapidly undergo apoptosis when treated with TNF- α in the presence of the protein synthesis inhibitor cycloheximide (CHX) (14). These data support the hypothesis that a TNF- α -inducible factor is responsible for the protective effect against further TNF- α cytotoxicity in resistant cell types (40). Previous studies by Guo et al. (15) and others (10) indicated that mitogen-activated protein (MAP) kinase phosphatase-1 may act as such a protective factor by repressing a sustained c-Jun NH₂-terminal protein kinase (JNK) activity. Although our hypothesis provided a plausible explanation for the resistance to TNF- α -induced cell death in mesangial cells, we cannot rule out the possibility that this resistance could also be due to an insufficient production of ceramide stimulated by TNF- α as demonstrated in some cell types (7, 30). Here, we present evidence that C₂ ceramide, a cell-permeable ceramide analog, did not mimic the effects of TNF- α on the activation of MAP kinases and nuclear factor- κ B (NF- κ B) transcription factor. Although C₂ ceramide is toxic to mesangial cells, it causes cell death through a mechanism distinct from TNF- α plus CHX-induced apoptosis.

METHODS

Materials. Recombinant TNF- α was obtained from Chemicon International (Temecula, CA). Anti-cytosolic phospholipase A₂ (cPLA₂), anti-I κ B α , and anti-NF- κ B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated anti-extracellular signal-related kinase (ERK) was from Promega (Madison, WI). C₂ ceramide (*N*-acetyl-D-erythro-sphingosine) and C₂ dihydroceramide (dihydro-*N*-acetyl-D-erythro-sphingosine) were obtained from Calbiochem (San Diego, CA). Hoechst 33258, propidium iodide, and fluorescein-labeled anti-rabbit IgG were from Molecular Probes (Eugene, OR).

Cell culture and cell viability assays. Rat mesangial cells were isolated from male Sprague-Dawley rats under sterile conditions with the sieving technique as previously described (25). The cells were maintained in RPMI 1640 medium containing 20% FCS and 0.6 U/ml of insulin at 37°C in a humidified incubator (5% CO₂-95% air). Cells from 5 to 20 passages were used. After the cells were grown to 80–90% confluence, they were made quiescent by incubation for 16–18 h in insulin-free RPMI 1640 medium containing 2% FCS.

For cell viability assays, mesangial cells were grown in 12-well plates. The quiescent cells were treated with reagents for the indicated times. Uptake of neutral red dye was used as a measurement of cell viability (14). At the end of the incubations, the medium was removed, and the cells were incubated in DMEM with 2% FCS and 0.001% neutral red for 90 min at 37°C. Uptake of the dye by viable cells was terminated by removing the medium, washing the cells briefly with 1 ml of 4% paraformaldehyde in PBS (pH 7.4), and solubilizing the internalized dye with 1 ml of a solution containing 50% ethanol and 1% glacial acetic acid. The absorbencies, which correlate with the amount of live cells, were determined at 540 nm.

Morphological analysis of apoptosis and necrosis. Cells were grown on 25-mm glass coverslips in six-well plates to 80% confluence. After treatment with various reagents as

indicated, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4). The morphological features of the cells were examined under a light microscope at different time intervals during incubation. Apoptosis and necrosis were analyzed according to the protocols described by Yoshimura et al. (44) with some modifications. Briefly, the fixed cells were stained with 10 μ M Hoechst 33258 and 10 μ M propidium iodide for 30 min and analyzed under a fluorescence microscope, with excitation at 340 nm. Hoechst 33258 stains nuclei of viable and apoptotic cells with a blue color, whereas propidium iodide stains nuclei of cells with a disrupted cell membrane with a red color. Therefore, nuclei of viable, apoptotic, and necrotic cells were identified as blue intact nuclei, fragmented nuclei, and intact red nuclei, respectively (44).

Immunodetection of NF- κ B. Cells grown on 25-mm glass coverslips in six-well plates were fixed with 4% paraformaldehyde in PBS (pH 7.4) after treatment with various reagents as indicated. The coverslips were incubated with anti-NF- κ B (65-kDa subunit) antibodies [1:200 in Tris-buffered saline and 0.1% Triton X-100 (TBS-T) containing 5% BSA] for 2 h at room temperature, washed with TBS-T three times, and incubated with fluorescein-conjugated anti-rabbit IgG for 1 h at room temperature. After three washes with PBS, the coverslips were mounted on slides. The NF- κ B was localized with fluorescence microscopy.

Effect of TNF- α on sphingomyelin metabolism. Mesangial cells were grown to 60% confluence in 60-mm dishes. To label sphingomyelin in vivo, [³H]serine (20 μ Ci/ml) was added to the culture medium. After 48 h, the medium was replaced with insulin-free RPMI 1640 medium containing 2% FCS, and the cells were incubated overnight. The cells were stimulated with 10 ng/ml of TNF- α . Lipids were extracted from the cells according to the method described by Bligh and Dyer (5) and were separated on silica gel thin-layer plates. Thin-layer chromatography was carried out by the method of Gomez-Munoz et al. (13). Sphingomyelin and ceramide were identified based on the migration of authentic standards. The matrix on the appropriate spots was scraped and transferred to scintillation vials. Radioactivity was determined by scintillation counting.

Cell lysate preparation. The quiescent cells were treated with reagents for the indicated times, washed twice with ice-cold PBS, and scraped into cell lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM Na₃VO₄, 50 mM pyrophosphate, 100 mM NaF, 1 mM EGTA, 1.5 mM MgCl₂, 1% Triton X-100, 10% glycerol, 10 μ g/ml of aprotinin, 10 μ g/ml of leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cells were incubated for 10 min on ice, lysed by sonication (25 pulses, output control 3) in a sonicator (Sonifier 450, Branson), and centrifuged at 15,000 *g* for 15 min. The supernatant was designated as whole cell lysate. Protein concentration was determined with the method of Bradford (6), with BSA as standard.

Protein kinase assays. JNK activity was measured with a solid-phase kinase assay method. Glutathione *S*-transferase (GST)-c-Jun(1–79) (GST-Jun) fusion protein was isolated from bacterial cells expressing pGEX-c-Jun plasmid. JNK activity was determined with GST-Jun as substrate as previously described (14). Briefly, 50 μ g of cell lysate were incubated with 2 μ g of GST-Jun agarose beads at 4°C for 2 h with rotation and centrifuged at 10,000 *g* for 1 min. The beads were washed three times with washing buffer [25 mM HEPES, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.05% (vol/vol) Triton X-100, 5 μ g/ml of aprotinin, 5 μ g/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β -glycerophosphate, and 10 mM NaF]. The beads were then resuspended in 10 μ l of kinase buffer containing (final concentra-

tions) 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM Na₃VO₄, 20 mM β-glycerophosphate, 5 mM NaF, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 40 μM ATP, and 1 μCi of [γ -³²P]ATP. After incubation at room temperature for 20 min, the reaction was terminated by adding SDS sample buffer followed by heating at 100°C for 3 min. The proteins were separated by SDS-PAGE, and the phosphorylated proteins were detected by autoradiography. ERK activation was determined by Western blot analysis with anti-ERK antibodies that only recognize the phosphorylated forms of ERK1 and ERK2 (15).

Western blot analysis. The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T and incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. The immunoblots were visualized with an enhanced chemiluminescence kit obtained from Amersham Pharmacia Biotech.

RESULTS

TNF-α does not induce production of ceramide in mesangial cells. The cells were labeled *in vivo* with [³H]serine, a commonly used method for the study of sphingolipid metabolism. In one experiment, 15 × 10³ counts/min were incorporated into ceramide and 34 × 10³ counts/min were incorporated into sphingomyelin after the cells were labeled for 48 h. During the first 45 min after TNF-α stimulation, ceramide and sphingomyelin contents remained at levels indistinguishable from baseline values. A second experiment gave essentially the same results.

Effect of C₂ ceramide on the viability of mesangial cells in presence and absence of TNF-α. Treatment of mesangial cells with TNF-α at concentrations from 10 to 50 ng/ml, which effectively induced apoptosis in susceptible cells (37, 39), caused neither detectable cell death (14) nor measurable production of ceramide in mesangial cells. These results indicate that resistance to TNF-α-induced apoptosis exhibited by normal mesangial cells could be due to insufficient generation of ceramide. To test this possibility, the cells were treated with C₂ ceramide (a synthetic cell-permeable ceramide analog) alone and in combination with TNF-α. As shown in Fig. 1, treatment of the cells with 12 μM C₂ ceramide alone, which is sufficient to induce apoptosis in susceptible cells, or in combination with 10 ng/ml of TNF-α did not affect their viability within a 24-h period of incubation (Fig. 1A). However, >50% of the cells lost their viability within 4 h when they were incubated with 50 μM C₂ ceramide. This toxicity was not significantly potentiated by TNF-α or CHX. The same concentration of C₂ dihydroceramide showed no apparent effect on cell viability (Fig. 1B). A dose response of the cells to ceramide toxicity is shown in Fig. 2. Cell death was prominent when C₂ ceramide concentrations were >18 μM during 24 h of incubation.

Morphological features of cell death induced by C₂ ceramide and TNF-α in presence of CHX. For comparison, time courses of cell death caused by C₂ ceramide and TNF-α in the presence of CHX are shown in Fig. 3. Both treatments caused an ~80% loss in cell viability after 4–5 h. These conditions provided us with a

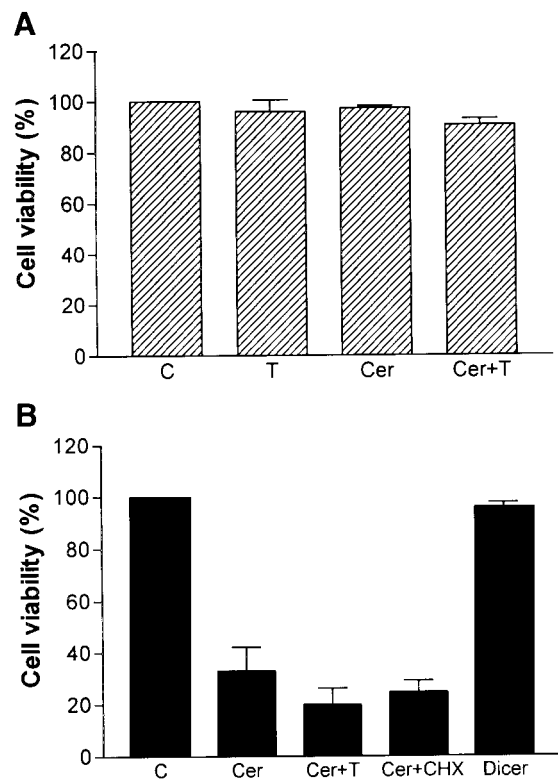


Fig. 1. Effects of C₂ ceramide [Cer; 12 (A) and 50 (B) μM] and tumor necrosis factor-α (TNF-α; T; 10 ng/ml) on viability of mesangial cells. C, control; Dicer, C₂ dihydroceramide (50 μM); CHX, cycloheximide (5 μg/ml). Cells were incubated with indicated reagents for 24 (A) or 4 (B) h. Cell viability was determined with neutral red assay method. Results are means ± SE of 3 experiments.

convenient time frame in which to study the morphological changes and their correlation with pertinent signaling pathways during cell death. Normal mesangial cells exhibited typical stellate morphology, with large round nuclei when they were subconfluent (Fig. 4, top, A). The cells began to increase in cell volume and lose their fiberlike structures after 30 min of incubation with 50 μM C₂ ceramide. Thereafter, the cell membrane was progressively disrupted while the nuclei remained intact, exhibiting typical features of necrosis (Fig. 4,

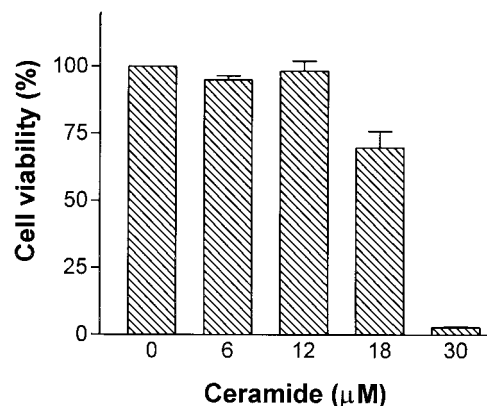


Fig. 2. Dose response of mesangial cells to Cer toxicity. Cells were incubated with indicated concentrations of Cer for 24 h. Cell viability was determined with neutral red assay method. Results are means ± SE of 3 experiments.

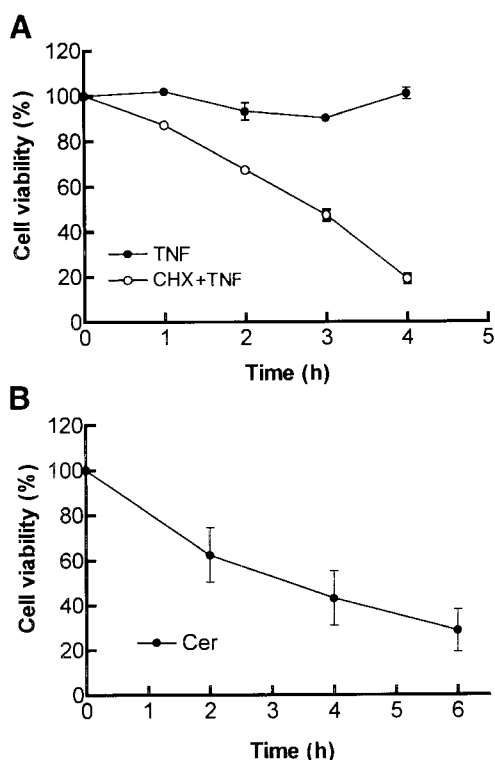


Fig. 3. Time course of cell death induced by TNF- α and Cer. Cells were incubated with TNF- α (10 ng/ml) alone and in presence of CHX (5 μ g/ml; A) or with C₂ ceramide (50 μ M; B) for indicated times. Cell viability was determined by neutral red assay method. A: results are representative of at least 3 experiments. B: results are means \pm SE of 3 experiments.

top, D). Incubation with C₂ dihydroceramide showed no apparent effect on cell morphology (Fig. 4, top, B). On the other hand, when cells were treated with TNF- α plus CHX, they underwent apoptosis, exhibiting characteristic nuclear condensation and cytoplasm blebbing (Fig. 4, top, C). The nuclei of cells under these conditions were analyzed further by double staining with Hoechst 33258 and propidium iodide. Hoechst 33258 stains nuclei of viable and apoptotic cells with a blue color, whereas propidium iodide stains nuclei of cells having a disrupted cell membrane with a red color. As shown in Fig. 4, bottom, the nuclei of cells treated with C₂ dihydroceramide were stained blue (B), similar to the nuclei of control cells (A). Nuclei of cells treated with TNF- α plus CHX were disrupted and fragmented, exhibiting typical features of apoptosis (Fig. 4, bottom, C). In these cells, the fragmented nuclei were still confined in the membrane vesicles. Although propidium iodide could enter the vesicles, its red color was overshadowed by the blue color of a larger amount of Hoechst 33258; therefore, the fragmented nuclei appear as a bright blue color. On the other hand, after treatment with C₂ ceramide, the nuclei were stained red (Fig. 4, bottom, D), indicating that although these cells had intact nuclei, their membrane systems were disrupted. The characteristics of nuclei staining correlated well with the observed cell morphological changes for apoptosis and necrosis as defined for other cells (44). DNA, isolated from the cells treated as described in

Fig. 4, was analyzed by agarose gel electrophoresis. Only the cells that were incubated with TNF- α plus CHX showed DNA fragmentation (data not shown). These results clearly demonstrate that cell death induced by TNF- α plus CHX and by C₂ ceramide was through different mechanisms, i.e., apoptosis and necrosis, respectively.

It was noted that although C₂ ceramide did not cause significant cell death at concentrations < 18 μ M after a period of 24 h of incubation (Fig. 2), it induced apparent morphological changes, most notably cell swelling starting at 30 min of incubation, whereas TNF- α alone did not show any observable effect on cell morphology. Cell death caused by 18 and 30 μ M C₂ ceramide (Fig. 2) showed the same necrotic features as seen in cell death caused by 50 μ M C₂ ceramide (Fig. 4D). Likewise, cell death caused by higher concentrations of TNF- α (20 ng/ml) and CHX showed the same apoptotic features as seen in cell death caused by 10 ng/ml of TNF- α plus CHX (Fig. 4C). There was no indication that the concentrations of C₂ ceramide and TNF- α caused a shift between the two modes of cell death.

Effects of C₂ ceramide on ERK and JNK activities. A selective activation of JNK compared with ERK has been considered as major evidence for a role of ceramide in mediating apoptosis induced by TNF- α , ultraviolet light, and Fas ligand (39, 41). However, further studies have shown that the selectivity of activation of MAP kinase family members by ceramide seems to vary depending on the cell type (12). In our case, C₂ ceramide did not activate JNK to a detectable level (Fig. 5A) but strongly activated ERK (Fig. 5B). TNF- α plus CHX strongly activated both JNK and ERK

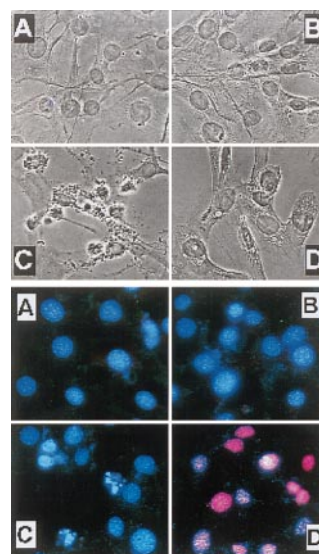


Fig. 4. Morphological features of apoptosis and necrosis in mesangial cells. A: control (DMSO). B and D: Dicer (50 μ M) and Cer (50 μ M), respectively, for 5 h. C: TNF- α (10 ng/ml) plus CHX (5 μ g/ml) for 3 h. After treatment, 1 set of samples was examined under phase-contrast microscopy (top, A–D). The other set of samples was stained with 10 μ M Hoechst 33258 and 10 μ M propidium iodide for 30 min and analyzed under a fluorescence microscope with excitation at 340 nm (bottom, A–D). Hoechst 33258 stains nuclei of viable and apoptotic cells with a blue color (A–C), whereas propidium iodide stains nuclei of cells with a disrupted cell membrane with a red color (D).

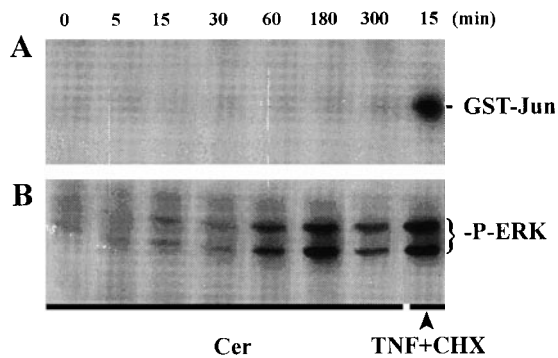


Fig. 5. Effect of Cer on extracellular signal-related kinase (ERK) and c-Jun NH₂-terminal protein kinase (JNK) activities. Cells were treated with Cer (50 μ M) for indicated times. *A*: JNK activity was determined by a solid-phase kinase assay with glutathione *S*-transferase-c-Jun (GST-Jun) as substrate. *B*: ERK activation was determined by Western blot analysis with anti-phospho-ERK (P-ERK) antibodies that recognized both active ERK1 and ERK2.

as described previously by Guo et al. (15). It was used as positive control in this experiment (Fig. 5). It should be pointed out that both TNF- α and ceramide activated ERK, but the activation patterns were different in the two cases; TNF- α only transiently activated ERK at 15 min and the ERK activity declined to the basal level after 30 min (14), whereas C₂ ceramide activated ERK in a sustained manner, with the activation of ERK being apparent at 60 min and lasting for at least 3 h (Fig. 5B).

Effect of C₂ ceramide on activation of NF- κ B. NF- κ B is a transcription factor present as a heterodimer complexed with I κ B in the cytoplasm of unstimulated cells. On cell stimulation, I κ B is degraded, resulting in the release of NF- κ B, which translocates to the nucleus where it initiates transcription activity (2). TNF- α is known to induce NF- κ B activation in many cells, whereas the ability of ceramide to activate NF- κ B varies greatly from cell to cell; in some cells, it mimics the effect of TNF- α (22, 29), but in other cells, it had no effect (12, 36). Degradation of I κ B α has been used as an indirect indication of NF- κ B activation (17). In mesangial cells, TNF- α induced an initial decrease in I κ B α , evident after 15 min, before it returned to initial levels after 2 h (Fig. 6A). CHX did not affect the degradation of I κ B α induced by TNF- α but prevented the de novo synthesis of I κ B α as expected (Fig. 6B). In comparison, treatment of the cells with C₂ ceramide did not cause degradation of I κ B α within 3 h. However, the level of I κ B α decreased after 5 h of incubation (Fig. 6C), at the time when >70% of the cells had lost their viability (Fig. 3).

Translocation of the 65-kDa subunit of NF- κ B was determined by immunolocalization studies with its specific antibodies. When the cells were treated with TNF- α alone or in combination with CHX, the 65-kDa subunit of NF- κ B translocated from the cytosol to the nucleus (Fig. 7, B and C). In contrast, C₂ ceramide showed no effect on the cellular localization of NF- κ B with either short-term or long-term incubations (Fig. 7, D-F). These results are in good agreement with those determined by I κ B α analysis (Fig. 6) and indicate that

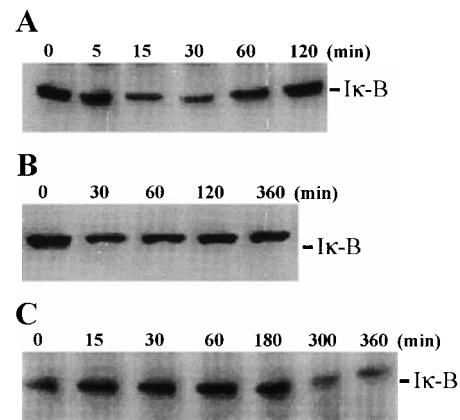


Fig. 6. Effect of TNF- α and Cer on degradation of I κ B. Mesangial cells were treated with various reagents for indicated times. *A*: 10 ng/ml of TNF- α alone. *B*: pretreatment with 5 μ g/ml of CHX for 30 min and then stimulation with TNF- α . *C*: 50 μ M Cer. I κ B α was analyzed by Western blot with anti-I κ B α antibodies. Degradation of I κ B α was indicated by a decrease in intensity of 36-kDa I κ B α band.

C₂ ceramide did not activate NF- κ B transcription activity.

Cleavage of cPLA₂ during apoptosis and necrosis. cPLA₂ hydrolyzes membrane phospholipids with the release of arachidonic acid. TNF- α -induced apoptosis has been found to be associated with increased cPLA₂ activity (9, 20). Recently, Wissing et al. (42) found that TNF- α -induced apoptosis was correlated with the proteolytic cleavage of cPLA₂ by caspase-3 and an increase in phospholipase activity. They proposed that a 70-kDa fragment of cPLA₂ was responsible for the increased phospholipase activity and was critical for the induction of subsequent apoptosis. As illustrated in Fig. 8B, the cleavage of cPLA₂ and generation of a 70-kDa

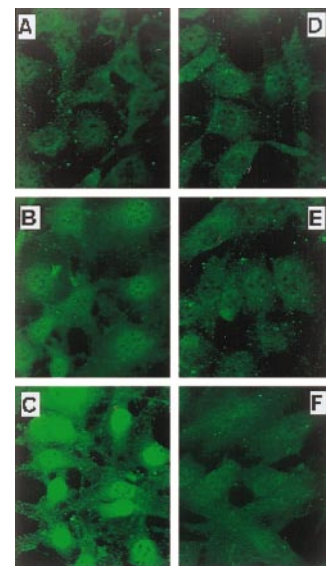


Fig. 7. Effect of TNF- α and Cer on cellular localization of nuclear factor (NF)- κ B. *A*: control. *B*: cells treated with TNF- α (10 ng/ml) for 15 min. *C*: cells pretreated with CHX (5 μ g/ml) for 30 min and then stimulated with TNF- α for 15 min. *D-F*: cells treated 50 μ M Cer for 15, 30, and 60 min, respectively. NF- κ B was detected with anti-NF- κ B (65-kDa subunit) antibodies and localized with fluorescence microscopy.

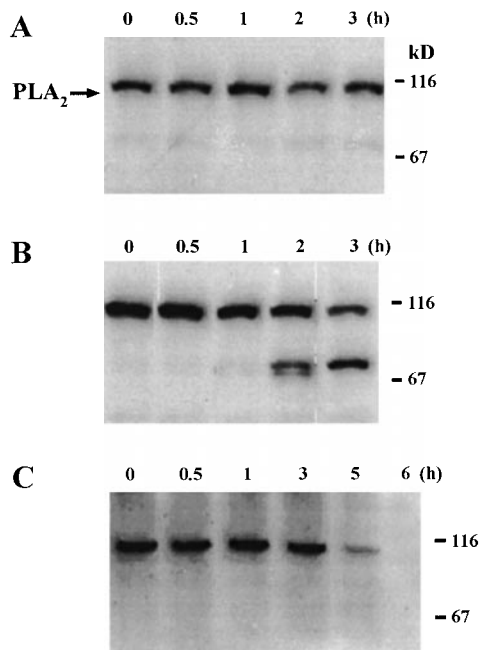


Fig. 8. Cleavage of cytosolic phospholipase A₂ (PLA₂) during apoptosis and necrosis. Mesangial cells were treated for indicated times. *A*: 10 ng/ml of TNF- α alone. *B*: cells pretreated with 5 μ g/ml of CHX for 30 min and then stimulated with TNF- α . *C*: cells treated with 50 μ M Cer. cPLA₂ was identified by Western blot analysis. Nos. on right, molecular mass.

fragment was also observed in mesangial cells at the onset of TNF- α plus CHX-induced apoptosis (\sim 2 h, as shown in Fig. 3). Degradation of cPLA₂ was also associated with C₂ ceramide-induced cell death, but a 70-kDa cleavage product of cPLA₂ was never detected (Fig. 8C). In addition, degradation of cPLA₂ was only observed at the terminal stage of necrosis (\sim 4 h, as shown in Fig. 3). These results indicate that distinct cleavage of signaling molecules was involved in the two modes of cell death.

DISCUSSION

Many tumor cells are sensitive to TNF- α -induced apoptosis, but normal cells are usually resistant. At least three hypotheses have been proposed to explain how some cells achieve resistance to this cytokine-induced apoptosis. First, TNF- α may activate an anti-apoptotic signaling pathway that counteracts the cytotoxicity of the apoptotic pathway (8, 11). Second, TNF- α may elicit the synthesis of a protective factor, with the result that the cells become insensitive to TNF- α cytotoxicity (2, 38). Third, the apoptotic pathway inducible by TNF- α is not activated or is not sufficiently activated to initiate the apoptotic process (21). Each hypothesis has gained some support in certain cell types, but none of them has been fully established to date. Given the complexity of TNF- α signaling pathways, it is not surprising that different cells may use different mechanisms.

TNF- α activates sphingomyelinase, with a concurrent generation of ceramide and a decrease in sphingomyelin in some cells, which usually take place within

5–20 min of stimulation (36, 39). The addition of exogenous C₂ ceramide also mimics the effect of TNF- α on the induction of apoptosis in these cells, supporting the hypothesis that ceramide acts as a second messenger for TNF- α to induce apoptosis. However, under similar conditions, TNF- α failed to cause a measurable production of ceramide in mesangial cells. It has been proposed that resistance of some cells to TNF- α cytotoxicity may be due to insufficient production of ceramide resulting from defects in the sphingomyelin cycle (7, 30). However, data from the present study are not in favor of this possibility in mesangial cells. Exogenous C₂ ceramide at a concentration of 12 μ M, which was sufficient to mimic the effect of TNF- α for the induction of apoptosis in susceptible cells, did not cause the death of mesangial cells either by itself or in combination with TNF- α . These results were not due to an inability of the cells to respond to C₂ ceramide because they exhibited an apparent morphological change (i.e., cell volume increase) on stimulation by C₂ ceramide even at 6 μ M. Furthermore, C₂ ceramide from the same source effectively inhibited thrombin-induced proliferation of CCL-39 cells at concentrations as low as 5 μ M (16). None of these effects was observed when cells were treated with the biologically inactive analog of C₂ ceramide, C₂ dihydroceramide, or with TNF- α . Higher concentrations of C₂ ceramide caused cell death by necrosis rather than by apoptosis as confirmed by various morphological and biochemical criteria.

The signaling pathways activated by C₂ ceramide and TNF- α also showed distinctive patterns in mesangial cells. Neither JNK nor NF- κ B, which was strongly stimulated by TNF- α (14), was activated by C₂ ceramide. Although ERK was activated by both C₂ ceramide and TNF- α , such activation appeared to be mediated by different mechanisms as judged from the kinetics of ERK activation. Activation of caspases, which takes place in a highly controlled manner, is a hallmark of apoptosis induced by various stimuli (31). The cleavage of specific proteins by caspases results in the irreversible commitment to cell death. The partial cleavage of cPLA₂ during TNF- α -induced apoptosis represents an excellent example of this event. It was proposed that a 70-kDa fragment of cPLA₂ generated by caspase-3 was responsible for the increased phospholipase activity, which was critical for TNF- α -induced apoptosis in MCF-7S1 cells (42). A very similar cleavage pattern of cPLA₂ during apoptosis was observed in mesangial cells. On the other hand, C₂ ceramide-induced degradation of cPLA₂ occurred only at the late stage of cell death, and a 70-kDa fragment of cPLA₂ seen during apoptosis was never detected. These results indicate that C₂ ceramide- and TNF- α plus CHX-induced degradation of cPLA₂ was brought about by different proteolytic mechanisms and may have fundamentally different implications for the two types of cell death. Unlike the well-controlled cleavage of cPLA₂ and I κ B α during apoptosis, the degradation of these molecules in C₂ ceramide-induced necrosis is more likely a result of cell lysis rather than of signaling steps that lead to cell death. These results support the notion

that, unlike apoptosis, necrosis seems to be a less-controlled degenerative process characterized by a widespread lysis of cell structures and membrane systems without systematic signaling events (28, 34).

Essentially none of the responses of mesangial cells to C₂ ceramide that were investigated resembled those elicited by TNF- α . A similar disparity between the signaling pathways induced by TNF- α and ceramide has previously been reported in endothelial cells and SW480 cells (23, 36). The effect of CHX to sensitize the cell to TNF- α toxicity is attributed to its effect on inhibiting protein synthesis (2, 38). However, the possibility that ceramide could be produced during TNF- α plus Chx-induced apoptosis and, therefore, may influence the process directly or indirectly cannot be ruled out. Because of complex nonselective effects of CHX on cellular metabolism, it is difficult to ascertain whether generation of ceramide under such conditions is a consequence of cell injury or a cause of cell death. Another study (35) indicated that ceramide did not mediate Fas-induced apoptosis and suggested that the observed generation of ceramide was more likely to be a consequence rather than the cause of apoptosis. It is worth noting that due to the membrane impermeability of natural ceramide, most studies have used synthetic ceramides, mainly C₂ and C₆ ceramides. In most cases, the synthetic ceramides mimicked the effect of natural ones as confirmed by various experimental approaches. One criterion of specificity between the compounds has been that dihydroceramide should have no effect in comparison with ceramide. We saw this in our experiments, which therefore agree with the bulk of data in the literature. However, a recent report (19) indicated that the effects of the synthetic ceramides may be also related to the N-acyl chain length. Together with the potential of synthetic ceramides to cause damage to the membrane, this finding gives cause for concern and caution in interpreting results.

For the past several years, considerable effort has been made to clarify the mechanisms leading to apoptosis. However, the signaling pathways leading to necrosis have not attracted appreciable attention although cell death from necrosis is prominent in many physiological or pathological processes such as inflammation and hypoxic conditions (44). In fact, ceramide-caused necrosis was recently noted in synovial fibroblasts (12) and hepatocytes (1). Thus, in addition to its recognized role as a cellular messenger to mediate apoptosis in some cells, it is clear that ceramide can cause cell death by necrosis in other cell types. The present study provides important evidence that apoptosis and necrosis use distinctive mechanisms to cause cell death. It will be of interest to find out the nature of ceramide targets and how they may mediate the necrotic effect of ceramide. In this respect, activation of the ERK pathway by C₂ ceramide deserves further investigation.

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