Cell Death and Differentiation (1999) 6, 115–123

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Relationships of apoptotic signaling mediated by ceramide and TNF-α in U937 cells

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Received: 2.8.98; revised 9.11.98; accepted 25.11.98
Edited by R.A. Knight

Abstract

It is commonly assumed that ceramide is a second messenger that transduces signaling leading to apoptosis. We tested this hypothesis by investigating the role of ceramide in TNF-α-initiated apoptotic signaling using the histiocytic lymphoma cell line U937. We found considerable differences between cell killing by TNF-α and by ceramide. U937 cells treated with TNF-α are committed early and irreversibly to the apoptotic pathway and start to die 90 min after treatment. U937 cells treated with ceramide start to die 12 h after the initial treatment. The cell death signaling initiated by TNF-α is transduced within minutes of exposure to TNF-α and it is irreversible. Exogenous ceramide increases the intracellular level of ceramide rapidly, significantly, and well above the physiological levels, within minutes, but cellular commitment to death does not occur until after the first 6 h of incubation. Furthermore, the endogenous ceramide in U937 cells treated with TNF-α increases well after the commitment to the apoptotic pathway. The differences between ceramide and TNF-α in the kinetics and the commitment to the apoptotic pathway suggest that, (a) ceramide is not a second messenger in the apoptotic signaling of TNF-α, (b) ceramide elevations, in TNF-α treated cells, are a consequence rather than a cause of apoptosis and (c) exogenously added ceramide and TNF-α kill cells via different pathways.

Keywords: apoptosis; ceramide; tumor necrosis factor alpha; sphingolipids; cell death signaling

Abbreviations: TNF-α, tumor necrosis factor alpha; D-*e*-C_8-Cer, *N*-octanoyl-D-*erythro*-sphingosine; D-*t*-C_8-Cer, *N*-octanoyl-D-*threo*-sphingosine; DG kinase, diacylglycerol kinase

Introduction

Ceramide is a sphingolipid generated along with phosphocholine upon the hydrolysis of sphingomyelin by neutral (magnesium-dependent and -independent) and acidic sphingomyelinases.[1–6] The discovery of multiple ceramide targets that are involved in cell growth, cell cycle, cell death, HIV replication, IL-6 production and differentiation, suggests the physiological importance of ceramide in cellular homeostasis and metabolism.[3,7–14]

One important role of ceramide is presumed to be the regulation and activation of the cell death pathway.[6,11,12,15–18] This assumption is based on the following criteria: (a) The endogenous ceramide concentration increases under various conditions that lead to cell death, for instance, ionizing radiation,[19] withdrawal of serum,[10] ultraviolet-C radiation, heat shock,[18] chemotherapeutic agents such as daunorubicin[20] and oxidative stress.[21] (b) Addition of synthetic ceramide analogs to a variety of cells in culture (such as U937 cells) induces apoptosis.[10–12,15] (c) Lipids structurally related to ceramide, such as oleic acid, dioctanoylglycerol and phorbol 12-myristate 13-acetate and dihydroceramides fail to kill cells.[15,22] The specificity of ceramide induced apoptosis suggests that ceramide is a second messenger in the activation of cell death.

Several laboratories have presented evidence that ceramide mediates the apoptotic signaling by TNF-α and Fas.[5,6,15,16,22–24] These two members of the TNF superfamily are major components in the physiological regulation of apoptosis.[25–28] TNF-α association with its receptors generates ceramide,[29,30] suggesting that ceramide mediates the cytotoxicity of TNF-α.[15,18,22,31] This hypothesis is supported by studies showing that exogenously added synthetic ceramides, which bypass the ligand-receptor cell signaling, mimic the cytotoxicity of TNF-α and kill cells.

Although ceramide elevation has been shown in many instances of cell death, it is still possible that the rise in ceramide is a consequence rather than a cause of apoptosis. To examine this possibility we: (a) investigated the kinetics by which ceramide and TNF-α kill cells (b) determined the time at which ceramide and TNF-α treated cells commit themselves to the apoptotic pathway and (c) established the time at which ceramide is generated in relation to the commitment to the apoptotic pathway.

Results

Kinetics of TNF-α and D-*e*-C_8-Cer induced cell killing

We examined the time required by TNF-α and exogenous D-*e*-C_8-Cer to induce apoptosis in U937 cells. We postulated that if the ceramide generated by TNF-α mediates apoptosis in U937 cells, then addition of exogenous ceramide should activate the apoptotic pathway with timing similar to that of TNF-α.

U937 cells were exposed to 10 ng/ml of TNF-α for 30, 60, 90, 120, 180 and 360 min. At the end of the incubation period equal numbers of cells were lysed and the degree of apoptosis was assessed by agarose gel electrophoresis of

the isolated low mass DNA. DNA fragments were first detected at 90 min (Figure 1A). DNA fragmentation was thereafter detected in cells treated for as long as 360 min (Figure 1A). Contrary to the rapid induction of cell death by TNF-α, ceramide required incubation times that were substantially higher than those of TNF-α to kill U937 cells. Using agarose gel electrophoresis of low molecular mass DNA, we detected nucleosomal fragments in cells treated with 10 μM of D-*e*-C_8-Cer only after 12 and 18 h (Figure 1B). We did not detect nucleosomal fragmentation in cells treated with D-*e*-C_8-Cer for 3 and 6 h (Figure 1B). Thus, the time required for killing U937 cells is substantially less for TNF-α than for D-*e*-C_8-Cer. These observations were confirmed with nuclear staining of chromatin using the DNA fluorochrome bis-benzimide (data not shown).

A possible explanation for the difference in the kinetics of cell killing by TNF-α and D-*e*-C_8-Cer could be that TNF-α increases the ceramide levels rapidly, whereas exogenously added ceramide enters U937 cells more slowly. We therefore, measured the intracellular ceramide level in cells treated with TNF-α and compared this level to the ceramide level of cells treated with D-*e*-C_8-Cer. U937 cells were treated with 10 ng/ml of TNF-α for 2, 7.5, 30, 60, 180 and 360 min. At the end of each incubation time the intracellular level of ceramide was measured as described in Materials and Methods. We did not detect ceramide elevations in cells treated with TNF-α for the first 60 min. We detected a small (0.02-fold) increase in ceramide over the control after 1 h of incubation with TNF-α (Figure 2A), 30 min before DNA fragmentation was detected (Figure 1A). The ceramide continued to increase gradually and by the end of a 6 h incubation time the ceramide level increased 0.45-fold above the control (Figure 2A).

We next measured the rise in the intracellular ceramide in cells treated with D-*e*-C_8-Cer. U937 cells were treated with 10 μM D-*e*-C_8-Cer for 2, 7.5, 15, 30, 60 and 180 min and at the end of each incubation time the ceramide level was quantitated as above. In D-*e*-C_8-Cer treated cells, ceramide increases rapidly and within the first 2 min of incubation we detected a significant increase (0.6-fold) over the control (the ceramide concentration in control cells was about 50 pmoles/1 $\times 10^6$ cells) untreated cells, (Figure 2B). With a 15 and a 60 min incubation time the increase in ceramide was approximately 2- and 8-fold above the control, respectively (Figure 2B and C). At the end of a 3 h incubation the levels of ceramide rose 18-fold above the control and beyond the linear range of ceramide

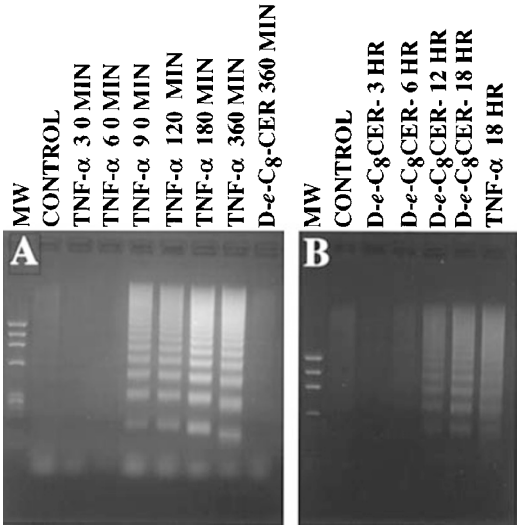

Figure 1 Time course of DNA fragmentation induced by TNF-α and D-*e*-C_8-Cer. Agarose gel electrophoresis and ethidium bromide staining of low molecular mass DNA extracted from U937 cells (5×10^6) treated with 10 ng/ml TNF-α for 30, 60, 90, 120, 180 and 360 min or with 10 μM D-*e*-C_8-Cer for 3, 6, 12 and 18 h. (**A**) DNA fragmentation ladders from U937 cells treated with TNF-α for 30, 60, 90, 120, 180 or 360 min. The last lane contains DNA from U937 cells treated with 10 μM D-*e*-C_8-Cer for 360 min. Cells incubated with media containing 1% fetal bovine serum for 360 min were used as control. (**B**) DNA fragmentation ladders from U937 cells treated with D-*e*-C_8-Cer for 3, 6, 12 and 18 h. The last lane contains DNA from cells treated with TNF-α for 18 h. Cells treated with 0.1% ethanol were used as control. MW, molecular mass markers (ϕX174 DNA/*Hae*III)

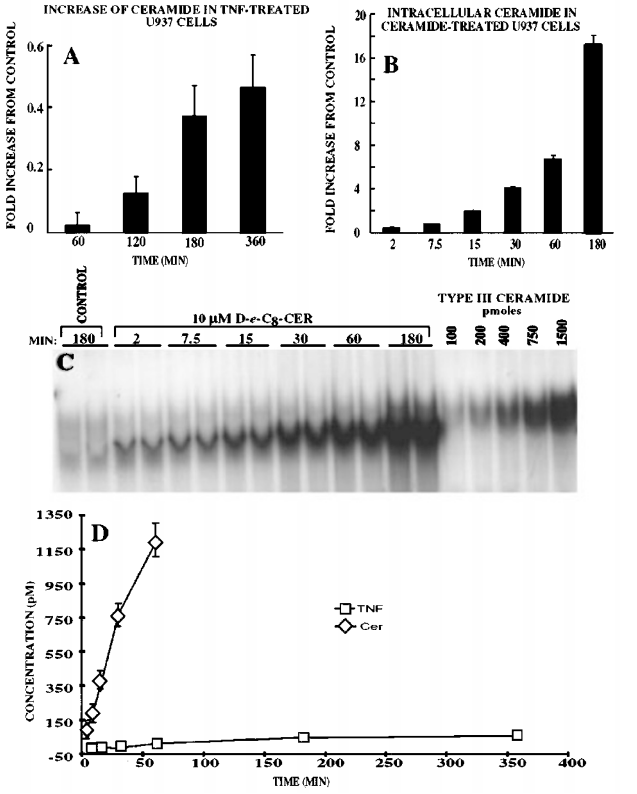

Figure 2 Endogenous levels of ceramide in cells treated with TNF-α or D-*e*-C_8-Cer. Labeling and quantitation of the intracellular ceramide levels in U937 cells treated with 10 ng/ml TNF-α or 10 μM D-*e*-C_8-Cer for various times. Ceramide bands were scraped from the silica gel plates and counted in a scintillation counter. (**A**) U937 cells treated with TNF-α for 60, 120, 180 and 360 min. Increase in endogenous ceramide is expressed as fold increase from control (untreated) cells. In control cells the ceramide concentration was approximately 50 pmoles per 1×10^6 cells. (**B**) Intracellular ceramide levels in U937 cells treated with 10 μM D-*e*-C_8-Cer for 2, 7.5, 15, 30, 60 and 180 min. (**C**) The autoradiogram of the TLC plate containing the ceramide bands that were scraped and counted in a scintillation counter shown in **B**. (**D**) Comparison of the ceramide levels in cells treated with 10 ng/ml TNF-α or 10 μM D-*e*-C_8-Cer. Concentration (in pmoles) is expressed as the difference from control. □ pmoles of ceramide from cells treated with TNF-α. ◇=pmoles of ceramide from cells treated with D-*e*-C_8-Cer

quantitation (50–2000 pmoles[32]). Thus treatment of U937 cells with TNF-α induces a delayed and modest increase in the ceramide concentration, whereas exogenously added ceramide increases the intracellular ceramide concentration considerably and within 1 min of incubation (Figure 2D). Therefore, our data suggest that the differences in the killing time between ceramide and TNF-α could not be attributed to slow internalization and accumulation of ceramide within cells.

Reversibility of D-*e*-C_8-Cer and TNF-α induced cell death in U937 cells

Previous authors have shown TNF-α to induce a transitory increase in the endogenous level of ceramide[4,15,29] and have speculated that the generation of ceramide leads to the activation of the cell death pathway. If the rise of endogenous ceramide is the mediator of the apoptotic signaling of TNF-α, then transient exposure of cells to D-*e*-C_8-Cer should increase the intracellular level of ceramide and trigger apoptosis.

Initially, we asked if exposure of cells to D-*e*-C_8-Cer for 3 h, which increased the intracellular ceramide levels 18-fold over the control (as indicated in Figure 2), was adequate to promote the commitment of U937 cells to the apoptotic pathway, U937 cells (5×10^5/ml) were incubated with 10 μM D-*e*-C_8-Cer for 3 h and then returned to ceramide-free media. The incubation was continued for a further 15 h (a total of 18 h) and cell death was assessed by agarose gel electrophoresis. We did not detect nucleosomal fragmentation in cells treated with ceramide for 3 h and returned to ceramide-free media for 15 h more (Figure 3A). DNA fragmentation was detected only in cells constantly exposed to 10 μM D-*e*-C_8-Cer for 18 h (Figure 3A). Furthermore, ceramide-induced cell death occurred only if cells were incubated in media that contained low concentrations of fetal bovine serum (1% rather than 10%, Figure 3A). Since serum is a source of growth factors the above data suggest that ceramide-induced cell killing is prevented by growth factors. The need for the constant presence of ceramide to kill cells was not restricted to cells treated with the D-*e*-C_8-Cer stereoisomer. U937 cells treated with the more potent stereoisomer D-*t*-C_8-Cer[11] for 3 h and then returned to ceramide-free media for 15 h more also did not die (Figure 3B). Apoptosis and nucleosomal fragmentation was only detected in cells continuously exposed to D-*t*-C_8-Cer (Figure 3B). Our results indicate that exposure of U937 cells to D-*e*-C_8-Cer or D-*t*-C_8-Cer for a considerable amount of time (3 h, which as indicated in Figure 2 increases the intracellular level of ceramide 18-fold over control) was not enough to trigger cell death, and a continuous exposure to these compounds was necessary for cell killing. The reversibility of cell killing by ceramide was also seen in HL-60 cells (data not shown).

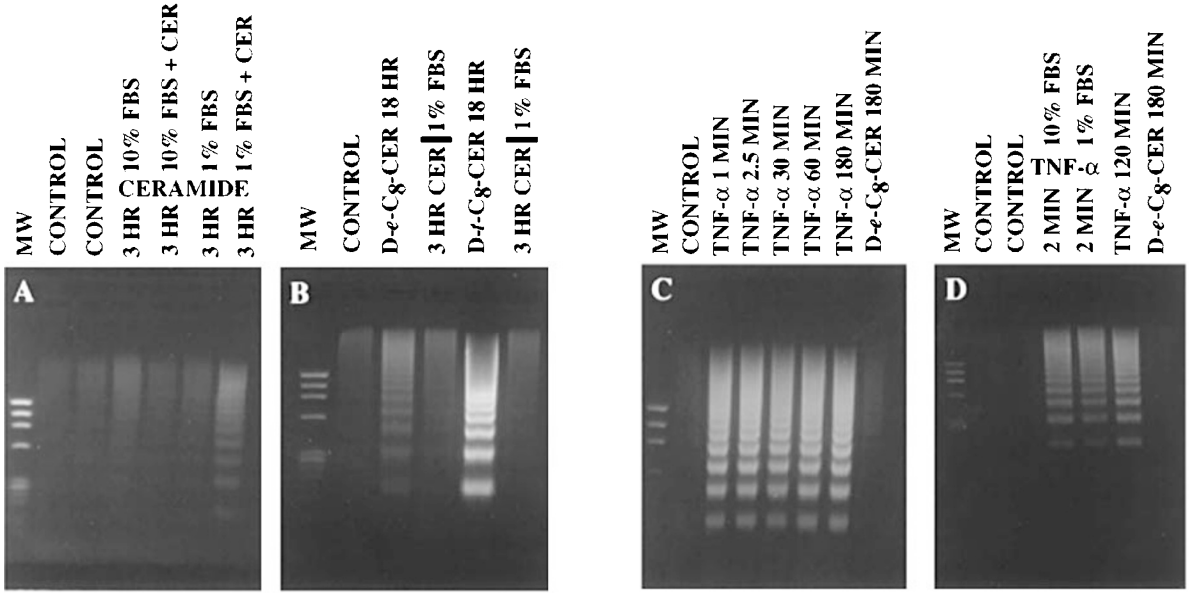

Figure 3 Agarose gel electrophoresis of DNA isolated from U937 cells transiently exposed to TNF-α, D-*e*-C_8-Cer or D-*t*-C_8-Cer. Agarose gel electrophoresis and ethidium bromide staining of low molecular mass DNA extracted from U937 cells (5×10^6) transiently exposed to 10 μM D-*e*-C_8-Cer, 10 μM D-*t*-C_8-Cer or to 10 ng/ml TNF-α. (**A**) U937 cells were treated with 10 μM D-*e*-C_8-Cer for 3 h, and returned to media containing 1 or 10% fetal bovine serum with or without 10 μM D-*e*-C_8-Cer. The incubation was continued for another 15 h before the isolation of DNA. Cells treated with 0.1% ethanol for 3 h were returned to media containing 1 or 10% fetal bovine serum and used as controls (first 2 lanes, respectively). (**B**) U937 cells were treated with 10 μM D-*e*-C_8-Cer or D-*t*-C_8-Cer for 18 h or transiently exposed to 10 μM D-*e*-C_8-Cer or D-*t*-C_8-Cer for 3 h and returned to media containing 1% fetal bovine serum. Cells transiently exposed to ceramide were further incubated for 15 h before the extraction of DNA. Control cells were exposed to 0.1% ethanol for 18 h. (**C**) U937 cells treated with TNF-α for 1, 2.5, 30 and 60 min and returned to TNF-α-free media for a total of 180 min before the isolation of DNA. The last two lanes contain DNA from cells treated with 10 ng/ml TNF-α or 10 μM D-*e*-C_8-Cer for 180 min, respectively. Cells incubated in media containing 1% fetal bovine serum for 180 min were used as controls. (**D**) DNA from U937 cells treated with TNF-α for 2 min and returned to TNF-α-free media containing 10 or 1 min fetal bovine serum media for a total of 120 min. The last two lanes contain DNA from cells treated with TNF-α or D-*e*-C_8-Cer for 120 min respectively. Cells treated with 0.1% ethanol for 2 min were returned to media containing 1 or 10% fetal bovine serum and used as controls (first 2 lanes, respectively). MW, molecular mass markers (ϕX174 DNA/*Hae*III)

Treatment of cells with TNF-α produced a different result. U937 cells were treated for just 1 min with 10 ng/ml TNF-α and returned to TNF-α-free media containing 1% fetal bovine serum. Cells were incubated in a TNF-α-free media for a total of 2 h and apoptosis was assessed by DNA isolation and agarose gel electrophoresis. We detected DNA fragments in U937 cells treated for only 1 min with TNF-α (Figure 3C). The induction of cell death by TNF-α was unaffected by high concentrations of fetal bovine serum (10%) in the media suggesting that an increase in the growth factor concentration does not prevent cell killing initiated by TNF-α (Figure 3D).

To determine what happens to accumulated ceramide once it is internalized, we exposed U937 cells to 10 μM D-*e*-C_8-Cer for 3, 6, 12 and 18 h. At the end of the incubation the cells were either lysed for determination of the intracellular level of ceramide or returned to ceramide-free media. Cells returned to ceramide-free media were kept in the incubator for a total of 18 h, lysed and the endogenous level of ceramide was determined. The endogenous levels of ceramide in cells treated with D-*e*-C_8-Cer for 3, 6, 12 and 18 h increased 18, 24, 30 and 22-fold above the control, respectively (Figure 4, shaded bars). The endogenous levels of ceramide in cells treated with D-*e*-C_8-Cer for 3, 6 and 12 h and then returned to ceramide-free media decreased to 2, 5 and 14-fold above the control, respectively (a crude estimate is that $t_{½}$ for the disappearance of ceramide is approximately 9 h, indicating that ceramide is slowly degraded or modified into other sphingolipids, Figure 4, white bars). Thus, continuing the incubation of cells initially exposed to D-*e*-C_8-Cer in ceramide-free media decreases the intracellular levels of ceramide reflecting the degradation or modification into other sphingolipids not phosphorylated by DG kinase within the cells. The decrease of ceramide (Figure 4, from 18-fold to twofold above the control) appears to be associated with the lack of nucleosomal DNA ladders (as indicated in the previous experiment, Figure 3). These results suggest that U937 cells can tolerate considerable increases in ceramide for hours without activating the apoptotic pathway.

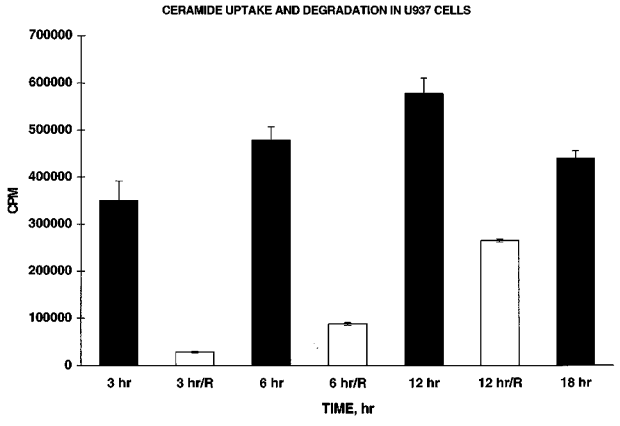

Figure 4 Internalization and degradation of ceramide in U937 cells treated with D-*e*-C_8-Cer. Internalization and degradation of ceramide in U937 cells treated with 10 μM D-*e*-C_8-Cer for 3, 6, 12 and 18 h. Shaded bars, intracellular ceramide in cells treated with D-*e*-C_8-Cer for 3, 6, 12 and 18 hr. Open bars, intracellular ceramide in cells treated with 10 μM D-*e*-C_8-Cer for 3, 6 and 12 h and then returned to ceramide-free media containing 1% FCS for 15, 12 and 6 h, respectively

Exogenous ceramide does not change the time required by TNF-α to induce apoptosis in U937 cells

Although the above studies suggest that D-*e*-C_8-Cer and TNF-α kill U937 cells with different kinetics, we detected a small increase of ceramide 1 h after treatment with TNF-α (Figure 2), and 30 min prior to the initiation of DNA fragmentation, (Figure 1). We therefore could not rule out the possibility that ceramide despite its late generation plays a role in TNF-α cell killing. To address whether ceramide is necessary for the initiation of the TNF-α cell killing or, as appears from our findings, a secondary factor, we increased the cellular levels of ceramide prior to the addition of TNF-α. We assumed that if ceramide is critical in the initiation of apoptosis by TNF-α, then providing U937 cells with ceramide prior to TNF-α exposure should decrease the time required for TNF-α cell killing.

U937 cells were either exposed to TNF-α alone, or pretreated with ceramide for 15 min (at which time ceramide levels increase to approximately twofold above the control, Figure 2B) and then exposed the cells to TNF-α. DNA was collected from cells treated with TNF-α for 30, 60, 90 and 120 min and apoptosis was assessed by DNA isolation and agarose gel electrophoresis. Pretreatment of cells with D-*e*-C_8-Cer for 15 min prior to the addition of TNF-α did not alter the time needed for TNF-α to kill cells (Figure 5). DNA fragmentation in cells treated with ceramide and TNF-α is detected after 90 and 120 min of incubation (Figure 5) and it is indistinguishable from DNA fragmentation in cells treated with TNF-α alone (Figure 5). DNA fragmentation was not detected in cells exposed to TNF-α alone or to both D-*e*-C_8-Cer and TNF-α for 30 and 60 min (Figure 5). Thus the increase of intracellular ceramide level prior to the addition of TNF-α did not change the time required by TNF-α to induce DNA fragmentation in U937 cells. A minimum of 90 min incubation was needed for the induction of cell death by TNF-α. Therefore, our results suggest that ceramide elevations are likely to be a consequence rather than a cause of TNF-α cell killing.

Low pH washes and trypsinization do not inhibit cell death promoted by exposure to TNF-α

Exposure of U937 cells to 10 ng/ml (0.57 nM) of TNF-α for just 1 min was sufficient to induce cell death (Figure 3C). In addition, TNF-α killed cells at very low concentrations suggesting that TNF-α-sensitive cells could be eliminated at physiologically relevant concentrations of TNF-α (Figure 6A). Based on these results, we speculated that the apoptotic signaling initiated by TNF-α was an early and irreversible event requiring low concentrations of TNF-α. However, we could not exclude the possibility that complexes formed between TNF-α and its receptor remained intact after the washing with NaCl/P_i. In that case, bound TNF-α could continue signaling suggesting that

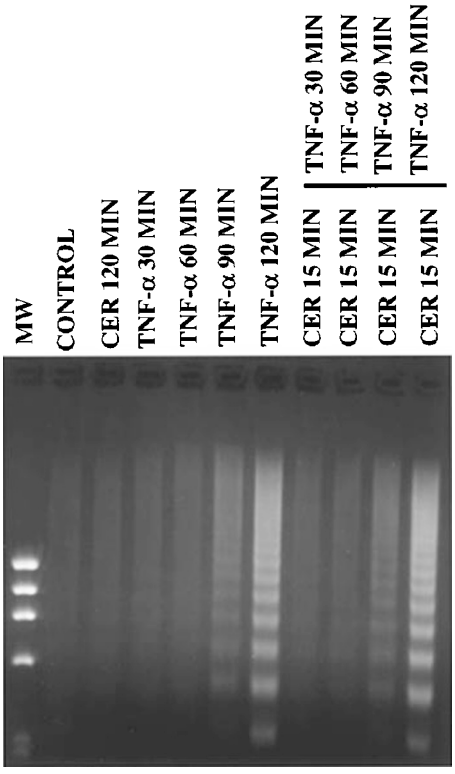

Figure 5 Internalization of exogenous ceramide does not change the time required by TNF-α to kill cells. Agarose gel electrophoresis and ethidium bromide staining of low molecular mass DNA isolated from U937 cells (5×10^6) treated with 10 ng/ml TNF-α or pre-exposed to 10 μM D-*e*-C_8-Cer for 15 min before the addition of 10 ng/ml TNF-α. DNA was collected 30, 60, 90 and 120 min after the addition of TNF-α. Untreated cells or cells treated with 10 μM D-*e*-C_8-Cer for 2 h were used as controls. MW, DNA molecular mass standards (ϕX174 DNA/*Hae*III)

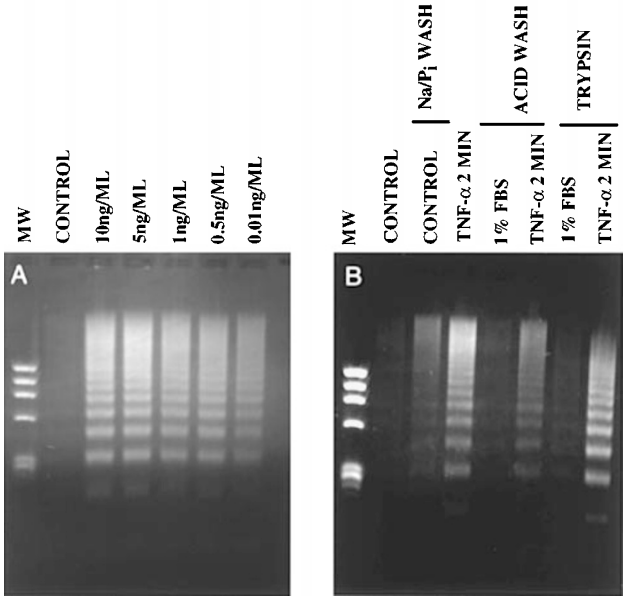

Figure 6 TNF-α dose response, acid wash and trypsinization of U937 cells exposed to TNF-α (**A**) Agarose gel electrophoresis and ethidium bromide staining of low molecular mass DNA isolated from 5×10^6 cells treated with 10 – 0.01 ng/ml TNF-α for 2 h. Control cells were incubated in the absence of TNF-α. (**B**) Agarose gel electrophoresis and ethidium bromide staining of low molecular mass DNA isolated from cells treated with 0.01 ng/ml TNF-α for 2 min and washed in either NaCl/P_i pH 7.4 or NaCl/P_i pH 3.0 (acid wash) or treated with 100 μg/ml trypsin. Cells were returned to TNF-free media containing 1% fetal bovine serum for a total of 2 h before the extraction of DNA. MW, DNA molecular mass standards (ϕX174 DNA/*Hae*III)

the apoptotic signals may arise well after the initial receptor-ligand interactions. Other investigators have disrupted receptor-ligand signaling by washing cells in low pH buffers or by receptor proteolysis.[33,34] Similarly, we exposed U937 cells to very low concentrations of TNF-α so that only a small fraction of the TNF-α receptors is occupied, followed by acid washes or trypsinization. We assumed that dissociation of the ligand-receptor complexes by acid washes and proteolytic cleavage of the TNF receptors could interrupt the apoptotic signaling cascade of TNF-α and prevent cell death. Previous studies with U937 cells showed that a minimum of 10 pM TNF-α (a 17% TNF receptor occupancy) was necessary to translocate NF-κB in U937 cells.[34] The K_D (concentration that gives half-maximal binding) for U937 cells was shown to be 22 pM.[35]

We initially tested if transient exposure of U937 cells to a very low concentration of TNF-α was adequate to promote cell death. U937 cells were treated with 0.01 ng/ml of TNF-α for 2 min. Even at this low concentration of TNF-α, we detected DNA fragmentation (Figure 6). The induction of cell death by such low concentrations of TNF-α suggest that the cell death signaling transduced by the TNF receptor is transmitted with a very small percentage of receptor occupancy.

To show that the cell death signaling transduced by TNF-α is mediated within the first min of the exposure, we investigated if acid washes and trypsinization, which disrupt the receptor-ligand signaling, could inhibit cell death transduced by TNF-α. Acid washing was performed as described previously[33] and described in the Materials and Methods. As shown in Figure 6B, washing of cells in acidic conditions did not inhibit apoptosis transduced by low concentrations of TNF-α (0.01 ng/ml). Similarly, incubation of U937 cells with 0.01 ng/ml of TNF-α for 2 min followed by trypsinization (100 μg/ml in serum-free media), did not prevent cells from dying (Figure 6B). Therefore, acid washing and trypsinization did not inhibit apoptosis mediated by TNF-α. The inability to prevent cell death, by disrupting the receptor-ligand complexes, by acid washes and trypsinization strongly suggests that TNF-α treated cells (unlike ceramide treated cells) commit themselves to the apoptotic pathway within seconds of exposure to TNF-α.

Discussion

Apoptosis triggered by TNF-α,[15,22] Fas antigen,[23,36] ionizing radiation, ultraviolet radiation, and heat shock[18] is hypothesized to be mediated by the second messenger ceramide. The data from these studies suggest that the intracellular ceramide accumulation is associated with the receptor-ligand complex formation and the activation of the apoptotic signaling.[37,38] Although there is little controversy over the

generation of ceramide under conditions that lead to cell death, the requirement of ceramide for the activation of the apoptotic pathway is speculative.

In the current study we investigated mechanisms by which ceramide and TNF-α kill U937 cells. U937 cells were used extensively for the elucidation of the cell death signaling of TNF-α and ceramide.[15,22,31] Our study attempts to extend these initial observations and clarify the role of ceramide in the cell death signaling of TNF-α in a system that has been previously established. We hypothesized that if ceramide is the mediator of cell death by TNF-α, then exogenously added ceramide and TNF-α should require similar exposure times to kill cells. In addition, we assumed that a direct relationship between ceramide elevation and the commitment to cell death should have been present in ceramide and TNF-α treated cells. This was not the case (Figures 1 and 2). U937 cells started to die within 90 min of exposure to TNF-α, whereas ceramide required at least 12 h to kill. The difference in cell killing was not attributed to the slow internalization of the exogenously added ceramide (Figure 2). Short chain ceramides were shown previously to saturate cellular membranes quickly[39] and activate ceramide targets within 1 min.[40–42] For instance, 10 min exposure of A431 cells to 10 μM C_8-Cer was adequate to induce maximal phosphorylation of the epidermal growth factor receptor.[41] Thus, rapid and massive increases in intracellular ceramide were not adequate to cause early cell killing or commit cells to the apoptotic pathway (Figures 1–3).

In contrast, TNF-α killed U937 cells rapidly, within 90 min of exposure, with late and modest elevations of endogenous ceramide (Figures 1 and 2). Thus ceramide and TNF-α kill cells with different kinetics and this difference in cell killing is not governed by intracellular ceramide elevations. These observations strongly suggest that exogenously added ceramide and TNF-α kill cells via different pathways.

Previous studies with the U937 variant cell line U9-TR indicated that the resistance of cell killing by TNF-α was associated with the lack of ceramide generation.[31] Furthermore, addition of exogenous ceramide induced apoptosis in U9-TR cells suggesting that the block in TNF-α cytotoxicity was probably due to the failure to hydrolyze sphingomyelin and generate ceramide. However, these observations are suggestive because concrete evidence for the involvement of ceramide as the mediator of the apoptotic signaling of TNF-α is lacking. If indeed ceramide and TNF-α kill cells via a different pathway, then addition of exogenous ceramide will kill cells irrespectively of whether there is a block in the apoptotic signaling of TNF-α.

The ability of TNF-α to activate apoptosis at low concentrations (0.01 ng/ml) and within the first 2 min of treatment, even when cells were acid-washed or exposed to trypsin (Figure 6) suggests that cell death induced by TNF-α is (a) transduced rapidly and within minutes of exposure to TNF-α, (b) irreversible and (c) effectuated by a very low receptor occupancy. The ability of TNF-α to transduce signals rapidly and at low concentrations is not restricted to the activation of apoptosis. Similarly, picomolar concentrations of TNF-α were shown to translocate NF-κB within a few minutes.[34,43,44] It is therefore likely that the apoptotic signaling cascade mediated by TNF-α is transduced with the same rapidity as the signaling that translocates NF-κB. More importantly, ceramide generation in U937 cells begins 1 h post-treatment with TNF-α and well after the 2 min needed by cells to commit themselves to the apoptotic pathway indicating that ceramide generation is not required for the activation of cell death. Another line of evidence supporting that ceramide may not mediate the apoptotic signaling of TNF-α is that the provision of D-*e*-C_8-Cer prior to the hydrolysis of the sphingomyelin pools by TNF-α did not alter the timing needed by TNF-α to kill cells (Figure 5). Under these conditions, the availability of ceramide did not change the kinetics of cell killing initiated by TNF-α.

Contrary to TNF-α, D-*e*-C_8-Cer and D-*t*-C_8-Cer failed to activate apoptosis even under prolonged incubations (3 h, Figure 3A and B). Continuous incubation with D-*e*-C_8-Cer or D-*t*-C_8-Cer in media containing low fetal bovine serum concentrations (1%) was essential for the detection of DNA fragmentation ladders (Figure 3A and B). The intracellular ceramide levels in cells treated with D-*e*-C_8-Cer surpassed significantly the ceramide levels generated by TNF-α (Figure 2), without activating apoptosis (Figures 1 and 2). It is therefore possible that unlike TNF-α, cell death induced by D-*e*-C_8-Cer and the other ceramide stereoisomers to arises from toxic effects due to the sustained accumulation of unphysiologically high concentrations of ceramide within cells and the prolonged activation of ceramide targets such as the CAPK and CAPP (shown to be maximally activated by 10 μM ceramide).[40,42] The use of ceramide concentrations exceeding 10 μM (25–100 μM)[10,18,45] may test the cell tolerance to unphysiologically high ceramide concentrations, but may not necessarily clarify the importance of ceramide in the cell death signaling initiated by TNF-α. The ceramide concentration generated by TNF-α is significantly less than the intracellular concentration of ceramide in cells treated with D-*e*-C_8-Cer (Figure 2).

Several studies indicated that sphingomyelin pools are found in different subcellular compartments and their selective hydrolysis has been associated with different cellular responses.[4,46,47] Interestingly, the location of the sphingomyelin pool hydrolyzed by TNF-α is controversial. Several investigators have proposed that the TNF-α-sensitive pool resides in the external leaflet of the plasma membrane.[48] Others proposed that the sphingomyelin pool hydrolyzed by TNF-α is localized in the endolysosomal compartments[4] or the inner leaflet of the plasma membrane.[47,49] One could argue that exogenous ceramide kills cells with different kinetics, because of its differential accumulation in subcellular compartments that are different from those generated by TNF-α. Studies with synthetic fluorescent ceramide analogs have shown ceramide to accumulate in the Golgi apparatus and sequentially in other membrane compartments[39] suggesting that cellular membranes are saturated quickly in the presence of exogenous ceramide. Ethanol has been used extensively as the delivery system for ceramide[11,12,15,22] and there is no evidence that different ceramide delivery vehicles can target ceramide to particular subcellular compartments. If

TNF-α and other stress signals generate ceramide in subcellular compartments that are inaccessible to exogenously added ceramide, then the use of exogenous ceramides to study the cell death mechanisms of TNF-α and other factors that lead to cell death may be irrelevant.

Recent studies revealed that TNF-α-receptor complex recruits the cytoplasmic factors FADD and TRADD that may assist in the propagation of the cell death signaling to the apoptotic apparatus.[50] The ability of exogenous ceramide to activate or recruit these factors in a way similar to TNF-α has not been confirmed. It is therefore possible that the differences in cell killing between TNF-α and exogenous ceramide to arise from the inability of exogenous ceramide to assemble the apoptotic apparatus in a manner similar to TNF-α. Although this is an existing possibility requiring further investigation, our data strongly indicates that exogenous ceramide alone is not adequate to promote apoptosis in U937 cells with the same kinetics as TNF-α.

Another important consideration is that ceramide generation has not been exclusively associated with apoptosis. Ceramide was shown to stimulate cellular proliferation in fibroblasts.[8] In other studies, the generation of ceramide by interleukin-1β in dendrocytes was not associated with apoptosis, although exogenously added ceramide killed dendrocytes.[51] Furthermore, ceramide was not found to be an upstream messenger in the cell death signaling mediated by Fas. In these studies, ceramide generation was regarded as a consequence rather than a cause of apoptosis.[52] All these observations suggest that the cellular generation of ceramide may not necessarily lead to cell death and that ceramide may be the consequence rather than the cause of apoptosis.

Materials and Methods

Materials

RPMI, penicillin, streptomycin, fetal bovine serum, and L-glutamine were obtained from Biofluids, Inc. [γ-^{32}P]dATP (3000 Ci/mmol) were from Amersham Corp. Diethylenetriamine-pentaacetic acid, natural ceramide (type III from bovine brain) and Hoechst 33258 stain were purchased from Sigma. Cardiolipin (beef heart) was from Avanti Polar Lipids. *N*-Octyl-β-D-glucopyranoside and *Escherichia coli* diacylglycerol (DG) kinase were from Calbiochem. DNA molecular mass markers (ϕx 174 DNA/*Hae*III fragments) were from Gibco BRL. TLC plates (6 nm silica gel plates of 0.25 mm thickness) were from Whatman. *N*-octanoyl-D-*erythro*-sphingosine (D-*e*-C_8-Cer) was purchased from Biomol. *N*-octanoyl-D-*threo*-sphingosine (D-*t*-C_8-Cer) was kindly provided by Dr. Robert Bittman, Queens College. TNF-α was from R&D systems. RNase A was purchased from Boehringer and Mannheim. Trypsin was from Gibco BRL. U937 cells (human histiocytic lymphoma) were purchased from American Type Culture Collection (ATCC).

Staining of U937 cells with bis-benzimide

To assess the degree of apoptosis in ceramide and TNF-α-treated cells, we stained U937 cells with the DNA fluorochrome bis-benzimide (Hoechst 33258) as described previously.[11] Briefly, at the end of the incubation with ceramide or TNF-α, the cells were washed with NaCl/P_i and fixed in 3% paraformaldehyde. Subsequently, the cells were washed in NaCl/P_i and the nuclei were stained with 16 μg/ml bis-benzimide. Nuclei with supercondensed chromatin at the nuclear periphery or nuclei fragmented into smaller dense bodies were considered apoptotic. Nuclei with evenly dispersed chromatin were considered as not apoptotic.

Tissue culture cells and treatment

U937 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Before treatment, U937 cells were washed once in phosphate-buffered saline (NaCl/P_i, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) and then resuspended (5×10^5 cells/ml) in RPMI containing 1% fetal bovine serum, antibiotics and glutamine. D-*e*-C_8-Cer was dissolved in ethanol and added to the cells as ethanolic solution (final concentration of ethanol not exceeding 0.1%). Lyophilized TNF-α was reconstituted in 0.1% bovine serum albumin (10 ng/μl). Cells were incubated in the presence of 10 μM D-*e*-C_8-Cer or different concentrations of TNF-α for specified times. At the end of the incubation, cells were pelleted, washed once with NaCl/P_i, pH 7.4 and lysed for low molecular mass DNA isolation.

For the studies testing the reversibility of induction of cell death induction, U937 cells were plated in RPMI media containing 1% fetal bovine serum, antibiotics and glutamine. Cells treated with 10 μM D-*e*-C_8-Cer for 3 h were transferred into fresh media containing 10% or 1% fetal bovine serum with or without 10 μM D-*e*-C_8-Cer. The cells were incubated for an additional 15 h (total incubation 18 h), washed once with NaCl/P_i, pH 7.4 and lysed for DNA isolation.

For the studies testing the reversibility of induction of cell death with TNF-α, U937 cells were treated with the indicated concentrations of TNF-α for various times, washed once with NaCl/P_i, pH 7.4 and returned to TNF-α-free media (RPMI) supplemented with 10% or 1% fetal bovine serum. The incubation was continued for a total of 2 h and the low molecular mass DNA was extracted and electrophoresed as above to assess DNA fragmentation. All experimental procedures were repeated at least three times.

DNA isolation

U937 cells (5×10^6) were lysed in 600 μl cell lysis buffer (10 mM Tris-HCl, 0.2% Triton X-100 and 10 mM EDTA, pH 7.5). The cell lysate was incubated on ice for 10 min and then centrifuged at 4°C at 12 000 $\times$ *g* for 15 min. The supernatant, containing the low molecular mass DNA was incubated for 1 h with 100 μg/ml RNase A and then extracted twice with 600 μl of phenol:chloroform:isoamyl alcohol (24 : 24 : 1) and once with 600 μl of chloroform:isoamyl alcohol (24 : 1). The DNA was precipitated with 300 mM NaCl and 2.5 volumes ethanol at -20°C overnight. The next day the samples were centrifuged at 16 000 $\times$ *g* for 30 min and the DNA pellet was washed once with 75% ethanol, air dried, and suspended in 20 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (T.E.). The degree of DNA fragmentation was analyzed by agarose gel electrophoresis as described previously.[11]

Ceramide labeling and quantitation by diacylglycerol (DG) kinase

The intracellular level of ceramide was determined by the *E. coli* DG kinase assay[53] as described previously.[11] Briefly, U937 cells were washed with NaCl/P_i, pH 7.4 and plated at a concentration of 5×10^5 cells/ml in media containing 1% fetal bovine serum. After treatment

with ceramide or TNF-α, the cells were washed once with NaCl/P_i, pH 7.4 and lysed in 1 ml chloroform/methanol/1 N HCl (100/100/1, v/v/v). The organic phase was separated with the addition of 270 μl of buffered saline solution (BSS, 135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose and 10 mM HEPES, pH 7.2), and 30 μl of 100 mM EDTA. The lower organic phase was dried under a stream of N_2.

To destroy glycerophospholipids, the extracted total lipids were subjected to mild alkaline hydrolysis (0.1 M methanolic potassium hydroxide for 1 h at 37°C). The organic phase was isolated and dried under N_2. Cellular lipids were solubilized by bath sonication and labeled by DG kinase as described previously.[11] Each TLC lane contained ceramide extracted from 3×10^6 cells. The TLC plates were subjected to autoradiography for 6–24 h, depending on the intensity of the labeled ceramide bands, and the silica from the TLC plates containing the ceramide was scraped and quantitated by liquid-scintillation counting. Ceramide type III was labeled the same way as lipids extracted from cells and used as standard.

Determination of the fate of internalized D-*e*-C_8-Cer in U937 cells

To determine the fate of ceramide internalized by U937 cells, two separate flasks of cells (6×10^6 cells/flask) were exposed to 10 μM D-*e*-C_8-Cer. The cells were incubated in the presence of ceramide for different times. At the end of the incubation period the cells from one of the two flasks were washed once with NaCl/P_i, pH 7.4, lysed, and the lipids were collected for the assessment of the intracellular level of ceramide. The cells of the second flask were centrifuged, returned to ceramide-free media containing 1% fetal bovine serum and allowed to incubate for a total of up to 18 h. At the end of this incubation, the cells were washed with NaCl/P_i, pH 7.4 and lysed. The intracellular level of ceramide was ascertained as described above.

Acid wash and trypsinization of TNF-α treated cells

To interrupt the propagation of the signals transduced by the TNF receptors we washed U937 cells in low pH NaCl/P_i (pH 3.0) or proteolytically cleaved the TNF receptors with trypsin. Low pH NaCl/P_i (pH 3.0) washes, which disrupt receptor-ligand associations and stop the receptor signaling, were done as described previously.[33] Briefly, 5×10^6 U937 cells were exposed to 0.57 pM of TNF-α (0.01 ng/ml) for 2 min, and washed once with ice cold NaCl/P_i, pH 7.4 and then the cell pellet was resuspended in 2 ml of ice cold NaCl/P_i, pH 3 for 2 min on ice. The cells were washed with NaCl/P_i, pH 7.4 and returned to TNF-α free media containing 1% fetal bovine serum. The incubation was continued for a total of 2 h. Control cells were carried through the same procedure, but without TNF-α treatment. Low molecular mass DNA was extracted to assess the degree of apoptosis.

Another means of interrupting the TNF receptor signaling is by proteolytic cleavage of the receptors with proteases, such as trypsin. Trypsinization was performed as described previously.[34] U937 cells (5×10^6) were exposed to 0.57 pM of TNF-α for 2 min and then washed in ice cold NaCl/P_i, pH 7.4. The cell pellet was suspended in 10 ml of fetal bovine serum-free media containing 100 μg/ml trypsin. The cells were further incubated for a total of 2 h. Cells not exposed to TNF-α were treated in a similar manner and used as controls.

Acknowledgements

We would like to thank Dr. Richard Lockshin for the critical reading of this manuscript. This work was supported by the National Institute of Aging Grant- KO4 -AG0031 (to Z.Z.), and the Alzheimer's Association/Allied-Signal Foundation Pilot Research grant (to Z.Z.).

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