Effect of Ceramide on Intracellular Glutathione Determines Apoptotic or Necrotic Cell Death of JB6 Tumor Cells

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Selective induction of cell death is a means to remove unwanted cell populations from a tissue or organ. Understanding the signaling events responsible for mediating cell death by cytokines, such as tumor necrosis factor-alpha (TNFα) are key to the development of pharmacologic inducers of this response. Ceramide has been implicated as a secondary messenger for TNFα-induced cell death, but many of the intracellular effects of ceramide are not fully understood. Recent reports suggest that ceramide signaling may involve oxidative stress. To explore the relationship between TNF sensitivity and ceramide signaling, two genetic variants of mouse JB6 RT101 epidermal tumor cells, one resistant and one sensitive to TNFα-induced cytotoxicity, were treated with C2- ceramide. Treatment with 20 μM ceramide induced apoptosis and this was quickly followed by oncotic necrosis in the TNFα-sensitive JB6 (TNFs) cells. The same concentration of ceramide induced apoptosis, but not oncotic necrosis of the TNFα-resistant JB6 (TNFr) cells. The basal level of glutathione was significantly higher in TNFr cells than in TNFs cells. Treatment with 20 μM ceramide decreased cellular glutathione in TNFs cells by 50%, in contrast to an insignificant decrease in the TNFr cells. A significant increase in reactive oxygen was noted in TNFs cells treated with 10 or 20 μM ceramide. Furthermore, pretreatment with the antioxidant N-acetylcysteine or with glutathione monoethylester delayed the onset of ceramide-induced oncotic necrosis, but did not inhibit apoptosis. Our results suggest that the severity of the decrease in glutathione appears to determine whether cells undergo just apoptosis or also oncotic necrosis. They also suggest that ceramide-induced oncotic necrosis is modulated by a decrease in cellular glutathione and an elevation of reactive oxygen. These results suggest that a decrease in cellular redox potential determines susceptibility to ceramide-dependent killing pathways.

Key Words: epidermal cells; cell death; ceramide; glutathione; apoptosis.

Tumor necrosis factor alpha (TNFα) induces intracellular signals that mediate cell death. One major signal induced by TNFα is activation of neutral and acidic sphingomyelinases which catalyze the degradation of sphingomyelin to ceramide (Hannun, 1996; Testi, 1996). Although ceramide has been proposed to be an intracellular mediator for many of the early cellular responses elicited by TNFα, including apoptosis (Cai et al., 1997; Schutze et al., 1994; Verheij et al., 1996; Westwick et al., 1995), the intracellular events that connect ceramide to these cellular responses remain unclear. However, alteration of nuclear factor kappa B (NFκB) expression of mitogen activated protein kinase (MAPK) activities and of protease activities are 3 of the potential intracellular events that may connect ceramide to cell death (Dbaibo, 1997; Hannun, 1996; Hartfield et al., 1997; Kitajima et al., 1996; Mizushima et al., 1996; Pronk et al., 1996; Shirakabe et al., 1997). Another important and immediate cellular effect of ceramide may be generation of reactive oxygen (ROS, which generally refers to hydroxyl anion, hydrogen peroxide, singlet oxygen, or other oxygen radicals). Indeed, many of the intracellular effects of ceramide may be attributable to modifications in thiols that act as direct targets for reactive oxygen. Moreover, the effect of ceramide on the major intracellular thiol, glutathione is of vital importance since glutathione maintains the reduced state of most intracellular proteins.

The objective of this study was to examine the relationship between TNF sensitivity, ceramide signaling, and ceramide-induced oxidative stress. We hypothesized that the cytotoxic effects of exogenous ceramide are attributable to ceramide-induced oxidative stress. To connect the effect of ceramide on cellular redox to TNFα sensitivity, we used variants of mouse JB6 epidermal cells (Singh et al., 1995) that show differential sensitivity to TNFα-induced cell death. The JB6 tumor cell variant RT101 TNFr, passage ≥200 cells is relatively insensitive to TNFα-induced cell death, while the other variant, RT101 TNFs, passage ≤90, is highly sensitive to TNFα cytotoxicity (Singh et al., 1995; Sun et al., 1992). If ceramide via ROS is a mediator of TNFα killing, then the TNFr and the TNFs variants should show differential responses to exogenous ceramide and this response to ceramide should be mediated by...
changes in cellular redox status. We report here that the TNFs variants are more sensitive to ceramide-induced cytotoxicity than the TNFr variants. The TNFr cells have a higher basal level of GSH than the TNFs cells, which may protect these cells from the acute cytotoxic effects of ceramide. The results suggest that increased ROS and decreased GSH mediate the cytotoxic effects of and cellular susceptibility to ceramide.

MATERIALS AND METHODS

Cells and treatments. The JB6-derived transformed mouse RT101 epidermal cell variants with differential sensitivity to TNFs-induced cell death used in these studies have been previously described (Singh et al., 1995; Sun et. al., 1992). Sixty mm dishes containing 4 × 10^5 cells were maintained in Eagles minimal essential medium (EMEM) containing 5% fetal bovine serum, 200 μM glutamine, penicillin (100 U/ml), amphotericin B (0.25 μg/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO2 in air. Treatment of TNFr and TNFs cells was carried out in EMEM (Paragon) containing 0.25% serum minus antibiotics, in the same environment. Four 60-mm dishes of cells per treatment group were treated with 10 or 20 μM N-acetyl-N-sphingosine (C1-ceramide, Biomol with purity and stability [assessed by thin layer chromatography]) for specified time points. In some experiments, cells were pre-treated with 200 μM N-acetyl-cysteine (NAC, Fluka) or 2 mM γ-glutamylcysteinylethyl ester (γ-GCE, glutathione ester; BACHEM) in EMEM containing 0.25% serum for 6 h. Cell extracts were prepared for GSH analysis as described in the methods. Three independent experiments were performed for GSH analysis with each pre-treatment, with indicated concentrations of ceramide.

Glutathione (GSH) Analysis. Cell extract preparation and HPLC analysis of thiols were carried out as outlined in Faris and Reed (1987). Briefly, cells in 60-mm dishes were washed with Hanks Balanced Salt solution (HBSS) without magnesium or calcium and collected in 10% perchloric acid containing 0.25% serum minus antibiotics, in the same environment. Four 60-mm dishes of cells per treatment group were treated with 10 or 20 μM N-acetyl-N-sphingosine (C1- ceramide, Biomol with purity and stability [assessed by thin layer chromatography]) for specified time points. In some experiments, cells were pre-treated with 200 μM N-acetyl-cysteine (NAC, Fluka) or 2 mM γ-glutamylcysteinylethyl ester (γ-GCE, glutathione ester; BACHEM) in EMEM containing 0.25% serum for 6 h. Cell extracts were prepared for GSH analysis as described in the methods. Three independent experiments were performed for GSH analysis with each pre-treatment, with indicated concentrations of ceramide.

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Measurement of reactive oxygen (ROS) generation. Dihydroyhodamine-123 (DHR123) is a cell-permeable fluorescent indicator for reactive oxygen species generation, mainly H2O2 and O2- (Emmendorfer et al., 1990). The oxidation of the non-fluorescent DHR123 by generated reactive oxygen species results in anionic species of DHR123, which is quenched by the mitochondria. Cells were grown in 12-well plates at 3 × 10^5 cells/well. Two dishes were used for each treatment protocol and 2 columns of wells were used for each treatment. Ceramide was added to 2-day cultures at concentrations of 10 and 20 μM. Treatment of cells with 1-mM H2O2 was used as a positive control. At various time points, cells were loaded with 20 μM DHR123 in medium for 30 min. After loading, the cells were washed twice with medium and fluorescence was measured with the Cytofluor system described above, with an excitation of 490 nm and emission of 520 nm. Three independent experiments were used for statistical evaluation.

Statistical analysis. In some cases, data were analyzed with a paired or unpaired, 2-tailed Student’s t-distribution using QuatroTM Pro V. 6.0 for Windows. In experiments in which multiple comparisons were made, ANOVA was used. Data are expressed as mean ± SE for n = 3 independent experiments.

RESULTS

Differential Cytotoxicity to TNFr and TNFs Cells of Ceramide

Treatment with 10 μM ceramide caused cell death of the TNFs cells but not TNFr cells after 24 h (Fig. 1A). When treated with 20 μM ceramide, TNFs cells showed a significant increase in propidium iodide (PI) uptake at 6 h, followed by a major increase in PI uptake at 24-h post treatment, indicating an oncotic/necrotic response (Fig. 1B). There was no significant increase in PI uptake in TNFr cells when treated with 10 or 20 μM ceramide at any time point measured (Figs. 1A and 1B). TNFs cells treated with 20 μM ceramide for 6 h showed morphologic changes of oncotic/necrotic cell death, such as swelling and large fluid-filled blebs by phase contrast microscopy (Fig. 2A, panel c). However, TNFr cells treated with 20 μM ceramide showed morphologic changes consistent with apoptosis (such as cell shrinkage and small dense blebs with little loss in cell membrane integrity) 6 h after treatment (Fig. 2A, panel f). Treatment with 10 μM ceramide for 24 h induced a mixture of oncotic necrosis and apoptotic morphology in the TNFs cells (Fig. 2A, panel b) but no apparent morphologic change in the TNFr cells (Fig. 2A, panel c). Thus, while both TNFs and TNFr cells are sensitive to ceramide-induced apoptosis, only TNFs cells are sensitive to ceramide-induced oncotic necrosis.

Since the morphologic alterations induced by 20 μM ceramide differed in the two cell types, we used a cell-death assay which measures nucleosome-bound DNA, as a means to quantitate the induction of apoptosis. The results of this
assay (Fig. 2B) confirm that TNFr variants undergo apoptosis after treatment with 20 μM ceramide. It appears from the results of this assay that internucleosomal DNA fragmentation also occurs in TNFs cells in response to 20 μM ceramide, although they rapidly undergo oncotic necrosis. These results suggest two possibilities: one is that a subpopulation of TNFs cells undergoes apoptosis and the rest undergo oncotic necrosis. A second possibility is that apoptotic cells in culture are not phagocytosed, lose cell-membrane integrity, and swell (showing oncotic necrosis) after apoptotic morphology is exhibited. The TNFr cells showed only an apoptotic response.

Ceramide Causes an Early Decrease in Intracellular Glutathione Which is Inhibited by NAC Pre-treatment

Ceramide decreased intracellular reduced glutathione in TNFs, but not TNFr cells (Fig. 3). Ceramide treatment (20 μM for 6 h) caused a small, statistically insignificant decrease in glutathione (10%) in TNFr cells, whereas it caused a significant decrease in GSH in TNFs cells (48%). Treatment with a lower concentration of ceramide (10 μM for 6 h) did not induce a measurable decrease in GSH in either cell type, but did decrease GSH levels in TNFs cells by 40% after longer treatment times of 16 h (not shown). The basal level of GSH was significantly higher in the TNFr cells than the TNFs cells,
FIG. 2. Cytotoxic effects of ceramide observed by phase contrast microscopy of JB6 TNFr and TNFs cells. (A) Cytotoxic effects of ceramide observed by phase contrast microscopy: a = TNFs control, b = TNFs 10 μM C2 for 24 h, c = TNFs 20 μM C2 for 6 h, d = TNFr control, e = TNFr 10 μM C2 for 24 h, f = TNFr 20 μM C2 for 6 h. (B) An apoptotic cell death ELISA was used to measure apoptotic cell death. The cell death-detection ELISA specifically measures histone-associated DNA fragments measured by absorbance A405 - A490. Values for the ELISA are mean ± SE of n = 3 independent experiments. Values for propidium iodide are means ± SE of n = 3 independent experiments. *Significantly different from control, p < 0.05.
suggested a possible protective effect against onotic necrosis. The higher basal GSH concentration may contribute to the decreased susceptibility of the TNFr cells to undergo necrosis. N-acetyl-cysteine (NAC) functions as a general free-radical scavenger supplying the cell with cysteine. Pre-treatment of either cell variant with 100 μM of the anti-oxidant NAC for 6 h abrogated the ceramide-induced decline in GSH (Fig. 3). These results imply that the ceramide-mediated decrease in GSH may be modulated by an increase in ROS.

Ceramide Causes Generation of Reactive Oxygen Species

Dihydrorhodamine 123 (DHR123) is a cell-permeable fluorogenic marker, which is specifically used to monitor intracellular reactive oxygen generation (Emmendorfer et al., 1990). We observed a significant increase in reactive oxygen generation after treatment with 10- or 20 μM ceramide in TNFs cells (Fig. 4). Pretreatment of cells with NAC decreased the generation of reactive oxygen species (data not shown). These results show that ceramide causes an increase in ROS, which may cause the observed decrease in GSH.

Glutathione Monoethylester Protects against Secondary Oncotic Necrosis but Not Apoptosis

To examine the effect of cellular GSH on ceramide-induced cell death, we pretreated the cells with glutathione monoethylester, which releases GSH intracellularly when cleaved by

[Graph showing GSH levels with different treatments]

**FIG. 3.** Ceramide causes an early decrease in intracellular glutathione in JB6RT101 TNFs cells, but not TNFr cells. Cells were treated with 20 μM C2 for 6 h or pre-treated with 200 μM N-acetylcysteine (NAC), and glutathione was measured in cell extracts by HPLC of thiol derivatized with fluorodinitrobenzene as described in Materials and Methods. Control cells were treated with an equivalent volume of vehicle (DMSO) and cells were treated with NAC alone. Ceramide significantly decreased GSH in TNFs cells. In addition, control TNFr cells have a higher basal level of GSH compared to control TNFs cells; **p < 0.05. Values are means ± SE of n = 5. *Significantly different from control, p < 0.05 using ANOVA.

**FIG. 4.** Ceramide causes generation of reactive oxygen species in JB6 TNFs and TNFr cells. Cultured JB6 RT101 TNFr and TNFs cells were treated with 10 or 20 μM C2 for 3 h in MEM. Cells were loaded with dihydorhodamine123 for 30 min and fluorescence 490 nm ex and 520 nm, measured using a Cytofluor plate reader as described in Materials and Methods (1-mm H2O2 was used as a positive control). (A) Values are expressed as means ± SE of 3 independent experiments. (B) Shows fluorescence microscopy of JB6RT101 cells labeled with DHR as outlined above: a = TNFs control; b = TNFs treated with 20 μM ceramide for 3h; c = TNFr control; d = TNFr cells treated with 20 μM ceramide for 3 h. Bars with * are significantly different from their respective controls (p < 0.05) with ANOVA.
esterases. Glutathione monoethyl ester pre-treatment produced a notable decrease in oncotic necrosis (Figs. 5A and 6). The ceramide-induced decrease in cellular GSH appears to mediate the onset of oncotic necrosis of the TNFs cells and appears to augment ceramide-induced cytotoxicity. The phase-contrast micrographs of the TNFs cells in Figure 6 demonstrate the protective effects of glutathione monoethylester on ceramide-induced cytotoxicity. The data also imply that the decrease in GSH occurs after ROS production since glutathione monoethylester specifically increased GSH and did not decrease ROS generation (not shown). NAC pre-treatment resulted in a similar decrease in the onset of oncotic necrosis. However, glutathione monoethyl ester pre-treatment did not protect the TNFs or TNFr cells from ceramide-induced apoptosis (Fig. 5B). Ceramide-decreased GSH occurred in TNFs cells not TNFr cells, thus correlating with sensitivity to ceramide.

**FIG. 5.** Exogenous glutathione protects JB6RT101 TNFs cells from ceramide-induced cell death. Cells were pretreated with 200 μM N-acetyl-cysteine (NAC; Fluka) or 2-mM α-glutamyl-cysteinylethyl ester (GCE; BACHEM ) in EMEM containing 0.25% serum for 6 h prior to 20 μM ceramide (C2) treatment for 6 h. (Panels A and B) GCE pretreatment inhibits onset of secondary oncotic necrosis (panel A) in TNFs, but does not completely protect the TNFs or TNFr cells from ceramide-induced apoptosis as shown in panel B measured by internucleosomal fragmentation.

**FIG. 6.** Protective effects of GCE on ceramide-induced oncotic necrosis of JB6RT101 cells. Phase contrast microscopy of JB6RT101 cells treated with ceramide shows the protective effects of GCE on ceramide-induced oncotic necrosis. (a) control TNFs; (b) TNFs treated with 20 μM C2; (c) TNFs pretreated with GCE, then treated with 20 μM C2.

**DISCUSSION**

The results outlined above established that ceramide-induced cell death is mediated by a pathway that includes ROS generation and decreased GSH. The results of the present study show that cellular susceptibility to ceramide parallels that of TNFα. The TNFr cells were resistant to ceramide-induced oncotic necrosis and underwent apoptosis when treated with 20 μM ceramide, while the TNFs cells underwent rapid oncotic
necrosis. The intracellular responses of TNFr and TNFs cells also differed. Ceramide caused an elevation in ROS and a significant decrease in GSH in TNFs but not in the TNFr variants. In this model, preventing a decrease in GSH appears to inhibit the onset of oncotic necrosis but does not appear to inhibit apoptosis. The TNFr cells have a higher basal level of GSH, which may be protective against the acute induction of ceramide-induced oncotic necrosis. It appears, however, that the induction of apoptosis can occur when the intracellular GSH concentration is not significantly decreased. Furthermore, a decrease in the reducing capacity of the cell may mediate the genetic susceptibility to ceramide seen in the sensitive variants.

Although a role for ceramide in regulating cell cycle arrest, cell death, and cell senescence has been suggested, the identity of the specific secondary messengers for ceramide in these responses remains unclear. In the mouse JB6 variants, the ceramide-mediated cell-death response is likely due to a depletion of GSH, which potentiates the elevation in ROS such as H₂O₂. Since, N-acetyl-L cysteine functions as a general free-radical scavenger, its observed effects on ceramide-induced ROS may occur independently of glutathione depletion. The fact that GSH monoethylester also protected TNFs cells from oncotic necrosis suggests that the intracellular effects of ceramide can be directly modulated by a decrease in GSH. The protective effects of GSH may involve an increased supply of cysteine through disulfide exchange and maintenance of cell membrane integrity. It has been reported that GSH inhibits neutral magnesium-dependent sphingomyelinase, but not acid sphingomyelinase in some cell types, implying that depletion of cellular GSH can result in the hydrolysis of SM and additional generation of ceramide (Liu and Hannun, 1997). Thus, loss of GSH may provide a positive feedback loop between ceramide generated from the acid-sphingomyelinase pool and the neutral sphingomylinase. Ceramide has also been reported to activate the transcription factor NFKB, which is also regulated by an intracellular redox state (Dbaibo et al., 1993). However, the acute loss of cell viability (onset of oncotic necrosis) is likely to occur independent of transcriptional regulatory pathways, while apoptotic pathways can be mediated by transcription factors. Cellular redox may be the primary determinant of which cellular response path the cell will take. The distinction between oncotic necrosis and apoptosis is also of great importance in vivo because the pathway of death will determine the extent of tissue damage and inflammation.

A decrease in GSH in organelle pools may be more specifically related to differential sensitivity to ceramide. A large decrease in the cytosolic pool of GSH, such as the one observed in the TNFs cells, will likely decrease GSH in the mitochondria. A decrease in mitochondrial GSH will cause alterations in mitochondrial Ca²⁺ homeostasis and loss of activity of respiratory chain complex IV (Casini et al., 1987; Richter et al., 1995). Recently, Garcia-Ruiz et al. (1997) have reported a direct effect of ceramide on mitochondrial electron transport in isolated rat hepatocytes, which was potentiated by depletion of GSH. Therefore, our observed decrease in cellular GSH may be part of a series of intracellular responses to ceramide-induced oxidative stress that involves mitochondrial dysfunction. Indeed, Quilet-Mary and coworkers (1997) reported that C6 ceramide induced H₂O₂ production in human myeloid leukemia cells, which appeared to be generated at the ubiquinone site of the mitochondrial respiratory chain. C₂-ceramide was shown to increase H₂O₂ generation and decrease the function of rat liver mitochondria (Arora et al., 1997; Cai et al., 1997; Garcia-Ruiz et al., 1997; Gomez et al., 1996). Considering these reports and our observed GSH decrease in TNFs cells, the difference in the observed sensitivity of the TNFs and TNFr cells to ceramide may lie at the level of the mitochondria.

Exogenous short-chain ceramides have been used to examine the multiplicity of intracellular responses elicited by ceramide generation. The exogenous ceramide used in this study is equivalent to endogenous ceramide generated in the acidic (lysosomal/endosomal) compartment, which is released by acidic sphingomyelinase (Higuchi et al., 1996; Santana et al., 1996), and not the neutral sphingomyelinase-generated ceramide (cytosol/inner and outer leaflet of cell membrane; Zhang et al., 1997). The distinct pool of acidic sphingomyelinase-released ceramides has been implicated as the “Cell Death Pool” of endogenous ceramide (Pena et al., 1997). The reason for two distinct biological responses to the alternate sphingomyelinase pools is not clear. As implied by our results, loss of GSH may determine the pathway of death for the cell (apoptosis vs. oncotic necrosis) and may contribute to the mechanism of these distinct biological responses. Furthermore, access of generated ceramides to cellular GSH pools may also explain the distinct biological responses to alternate sphingomyelinase pools. Distinct GSH pools can be found in the mitochondrion, nucleus, cytoplasm, and endoplasmic reticulum (Smith et al., 1996). Depletion of these pools will have different consequences for the cell (Mithofer et al., 1992; Reed and Fariss, 1984). Therefore, the effect of ceramide on each of the distinct pools of GSH will be important to determine.

In summary, our observations demonstrate the coupling of cellular redox potential to ceramide-activated cell death. Genetic variants sensitive to ceramide killing show elevated ROS and decreased GSH while the sensitive cells show neither. Since the redox potential differs between cell types and because GSH contributes to the regulation of Redox potential, it follows that susceptibility to ceramide-induced cell killing will be regulated in part by cellular glutathione levels. Indeed, specific elevation of GSH decreases both the ROS generation and the cell killing induced by ceramide. The effect of accumulating ceramide during systemic stress in disease, tissue injury, or ischemia may be to potentiate the cytotoxic effects of inflammatory cells. Depending on the cells microenvironment (i.e., solid vs. soft tissue or lymphatic tumor, oxygen tension, endogenous metabolism, GSH levels) ceramide may have a major impact on cell survival in vivo.
REFERENCES


