

Glycosylation of Ceramide Potentiates Cellular Resistance to Tumor Necrosis Factor- α -Induced Apoptosis

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Ceramide, as a second messenger, initiates one of the major signal transduction pathways in tumor necrosis factor- α (TNF- α)-induced apoptosis. Glucosylceramide synthase (GCS) catalyzes glycosylation of ceramide and produces glucosylceramide. By introduction of the GCS gene, cytotoxic resistance to TNF- α has been conferred in human breast cancer cells. MCF-7/GCS-transfected cells expressed 4.1-fold higher levels of GCS activity and exhibited a 15-fold ($P < 0.0005$) greater EC₅₀ for TNF- α , compared with the parental MCF-7 cell line. DNA fragmentation and DNA synthesis studies showed that TNF- α had little influence on the induction of apoptosis or on growth arrest in MCF-7/GCS cells, compared to MCF-7 cells. These studies reveal that TNF- α resistance in MCF-7/GCS cells is closely related to ceramide hyperglycosylation, a hallmark of this transfected cell line, and resistance was not aligned with changes in TNF receptor 1 expression. This work demonstrates that GCS, which catalyzes ceramide glycosylation, potentiates cytotoxic resistance to TNF- α . © 1999 Academic Press

Key Words: tumor necrosis factor- α ; ceramide; glycosylation; drug resistance; breast cancer.

INTRODUCTION

Tumor necrosis factor- α (TNF- α)² is one of the most pleiotropic cytokines, acting as a host defense factor in myriad immunological and inflammatory responses and antitumor activity [1–3]. The cytotoxic effects of TNF- α are primarily mediated through TNF-R1 and

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² Abbreviations used: TNF- α , tumor necrosis factor- α ; TNF-R1, tumor necrosis factor receptor 1; GCS, glucosylceramide synthase (ceramide glucosyltransferase, UDP-glucose:*N*-acylsphingosine D-glucosyltransferase, EC 2.4.1.80); GC, glucosylceramide; DEVD-AFC, acetyl-Asp-Glu-Val-Asp- α -(7-amino-4-trifluoromethyl coumarin); FBS, fetal bovine serum; MCF-7/GCS, glucosylceramide synthase transfected cell line; Tet, tetracycline; pTRE, Tet-repressible expression plasmid; MDR, multidrug resistance/resistant.

the receptor-associated proteins, TNF-R1-associated death domain protein (TRADD) and Fas-associated death domain (FADD/MORT1) [3–5].

It has been revealed that ceramide generation and caspase activation represent potential regulation points of apoptotic signaling by TNF- α [6, 7]. Activation of sphingomyelinase, resulting in ceramide formation, represents an early event in the apoptotic cascade of TNF- α [6, 8, 9]. In MCF-7 breast cancer cells, ceramide is one of the essential molecules in TNF- α -induced apoptosis [10, 11]. In mammalian systems, ceramide induces apoptosis directly through the effector caspases, such as caspases-3 and -7 [12–14]. Loss of ceramide production is a cause of cellular resistance to apoptosis induced by TNF- α [15], and increased ceramide generation by the P-glycoprotein blocker, PSC 833, can restore TNF- α -induced apoptosis in KG1a leukemia cells [16].

Compared to our knowledge of TNF- α signaling cascades, little is known regarding the biological fate of ceramide in modulation of TNF- α cytotoxicity. In the metabolism of ceramide, glucosylceramide synthase (GCS) transfers glucose from UDP-glucose to ceramide and produces glucosylceramide (GC) [17]. GCS has recently been cloned [18] and shown to be associated with resistance to cytotoxicity of C₆-ceramide [19]. We hypothesized that glycosylation of ceramide can mediate TNF- α cytotoxicity. In the present study, we observed the effects of ceramide glycosylation on apoptosis and cell growth arrest induced by TNF- α in GCS-transfected human breast carcinoma cells. Here, we document that GCS, catalyzing ceramide glycosylation, potentiates cellular resistance to TNF- α .

MATERIALS AND METHODS

Materials. [³H]UDP-glucose (40 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). EcoLume (liquid scintillation cocktail) was from ICN (Costa Mesa, CA), and [α -³²P]dCTP (6000 Ci/mmol) was from Amersham (Arlington Heights, IL). Sulfatides (ceramide galactoside 3-sulfate) were from Matreya (Pleasant Gap, PA), and phosphatidylcholine (1,2-dioleoyl-sn-glycero-3-phosphocholine) was from Avanti Polar Lipids (Alabas-

ter, AL). Anti-TNF-R1 antibody, htr 9, was from Bachem Bioscience (King of Prussia, PA). Anti-GCS antiserum from rabbit was kindly provided by Drs. D. L. Marks and R. E. Pagano, Mayo Clinic and Foundation (Rochester, MN). Human recombinant TNF- α (yeast, 10^5 units/ μ g) and hygromycin B were purchased from Boehringer Mannheim (Indianapolis, IN). Doxycycline hydrochloride and other chemicals were purchased from Sigma (St. Louis, MO). FBS was purchased from HyClone (Logan, UT). RPMI 1640 medium and DMEM medium (high glucose) were from GibcoBRL (Gaithersburg, MD), and cultureware was from Corning Costar (Cambridge, MA).

Cell lines and culture conditions. Human breast adenocarcinoma cells, MCF-7 and MCF-7 AdrR cells (MDR clone), were kindly provided by Dr. Kenneth Cowan and Dr. Merrill Goldsmith (National Cancer Institute, Bethesda, MD). Cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 584 mg/liter L-glutamine. Cells were cultured in a humidified, 5% CO₂ atmosphere tissue culture incubator and subcultured weekly using trypsin-EDTA (0.05%–0.53 mM) solution. Transfected cells, MCF-7/GCS, were cultured in RPMI 1640 medium containing 10% FBS and 200 μ g/ml hygromycin in addition to the above components. The transfected cell line (MCF-7/GCS) was used in all experiments at passages no greater than 15.

pTRE-GCS expression vector construction and MCF-7/GCS cells. The full-length cDNA of human GCS [18] was subcloned into the *EcoRI* site of a Tet-repressible expression plasmid (pTRE). pTRE-GCS (10 μ g DNA) was introduced into the selected MCF-7 Tet-on cells by coprecipitation with calcium phosphate [19]. The MCF-7/GCS-transfected cells were selected from the hygromycin-resistance clones, using radioenzymatic GCS assay. The high expression of GCS was induced in cells incubated with 3 μ g/ml doxycycline [19].

Glucosylceramide synthase assay. To determine the expression of GCS in the hygromycin-resistant clones, a modified radioenzymatic assay was utilized [19, 20]. Cells were homogenized by sonication in lysis buffer (50 mM Tris-HCl, pH 7.4, 1.0 μ g/ml leupeptin, 10 μ g/ml aprotinin, 25 μ M PMSF). Microsomes were isolated by centrifugation (129,000g, 60 min). The enzyme assay, containing 50 μ g microsomal protein, in a final volume of 0.2 ml, was performed in a shaking water bath at 37°C for 60 min. The reaction contained liposomal substrate composed of C₆-ceramide (1.0 mM), phosphatidylcholine (3.6 mM), and brain sulfatides (0.9 mM). Other reaction components included sodium phosphate buffer (0.1 M), pH 7.8, EDTA (2.0 mM), MgCl₂ (10 mM), dithiothreitol (1.0 mM), β -NAD (2.0 mM), and [³H]UDP-glucose (0.5 mM). Radiolabeled and unlabeled UDP-glucose were diluted to achieve the desired radiospecific activity (4700 dpm/nmol). To terminate the reaction, tubes were placed on ice and 0.5 ml isopropanol and 0.4 ml Na₂SO₄ were added. After brief vortex mixing, 3 ml *t*-butyl methyl ether was added, and the tubes were mixed for 30 s. After centrifugation, 0.5 ml upper phase, which contained GC, was withdrawn and mixed with 4.5 ml EcoLume for analysis of radioactivity by liquid scintillation spectroscopy.

Analysis of ceramide and glucosylceramide. Analyses were performed as previously described [19, 21]. Cellular lipids were radiolabeled by incubating cells with [³H]palmitic acid (2.5 μ Ci/ml culture medium) for 24 h. After removal of medium, cells were rinsed twice with PBS (pH 7.4), and lipids were extracted [21]. Ceramide was resolved using solvent system I, which contained chloroform/acetic acid (90/10, v/v). GC was resolved using solvent system II, which contained chloroform/methanol/ammonium hydroxide (70/20/4, v/v). After development, lipids were visualized by iodine vapor staining, and areas of interest were scraped and quantitated by liquid scintillation spectrometry.

RNA analysis. Extracted RNA (15 μ g) was denatured in 59% formamide/2.2 M formaldehyde, size separated by electrophoresis on 1% agarose-formaldehyde, and then blotted onto nitrocellulose-plus [19]. Nitrocellulose-plus membranes were hybridized with the ³²P-GCS probe at 68°C for 18 h. For even gel loading, 28 S RNA was used.

Cytotoxicity assay. The assay was performed as previously described [19, 20]. Briefly, after culture in the absence or presence of 3.0 μ g/ml doxycycline for 48 h, cells were harvested and seeded in 96-well plates (2000 cells/well) in 0.1 ml RPMI 1640 medium containing 10% FBS in the absence or presence of 3.0 μ g/ml doxycycline. Drugs were added in FBS-free medium (0.1 ml), and cells were cultured at 37°C for the indicated periods. Drug cytotoxicity was determined using the Promega 96 Aqueous cell proliferation assay kit.

DNA fragmentation. The analysis was performed as previously described [22]. Briefly, 500,000 cells were seeded in 10-cm dishes in medium containing 5% FBS. After attachment, cells were treated with varying concentrations of each drug for 72 h. Following harvest by trypsin-EDTA and centrifugation, cells were digested. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) and was precipitated by incubating in one-half volume 7.5 M ammonium acetate plus 2 vol of 100% ethanol at -20°C overnight, followed by centrifugation (10000g, 20 min, 4°C). Contaminating RNA was digested in RNA-digestion buffer (10 mM Tris-HCl, 0.1 mM EDTA, 0.1% SDS, 100 units/ml RNase cocktail). Reextracted DNA (μ g) was analyzed by electrophoresis on a 2% agarose gel in TAE buffer and was visualized with ethidium bromide under UV light.

Caspase-3 assay. Caspase-3 activity was evaluated by DEVD-AFC cleavage, which was analyzed using the ApoAlert caspase-3 assay kit (Clontech, Palo Alto, CA). The assay was performed following kit protocol. Briefly, after treatment with TNF- α , cells were lysed, and the soluble fraction was incubated with 1.0 mM DEVD-AFC at 37°C for 60 min. The free AFC fluorescence was measured at $\lambda_{\text{excitation}}$ 400 nm and $\lambda_{\text{emission}}$ 505 nm using a FL600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT). The caspase-3 inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde was used to exclude non-specific background in enzymatic reaction.

DNA synthesis. Cell proliferation based on DNA synthesis was evaluated using the SPA [³H]thymidine uptake assay system (Amersham). Analysis was performed according to the manual. Briefly, cells (2000/well) were seeded into 96-well plates with 5% FBS RPMI 1640 medium. After 24 h, cells were incubated with 10 ng/ml TNF- α plus 1.0 μ Ci [³H]thymidine, added in 5% FBS medium, for the indicated periods. [³H]DNA, bound to polyvinyltoluene-scintillant beads, was counted with enhancer by liquid scintillation spectroscopy. The incorporated [³H]thymidine in each well was normalized with cell counts in parallel wells.

Immunoprecipitation and Western blot analysis. Assays were performed using modified procedures [19, 23, 24]. Cells were solubilized in 1.0 ml of cold TNT buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1.0 mM PMSF, 1% aprotinin) for 60 min on ice. Insoluble debris were excluded by centrifugation at 100000g for 60 min, at 4°C. Identical amounts (1.5 mg protein) of cell lysate were immunoprecipitated with anti-GCS antiserum (GCS-1.2, 1:1000) or anti-TNF receptor 1 antibody (htr 9, 1:1000) by overnight incubation at 4°C, after the volumes were adjusted to 0.5 ml with NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP40, 1.0 mM EDTA, 0.5% BSA, 1.0 mM PMSF, and 1% aprotinin).

Immunoprecipitated proteins were eluted with Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-HCl, pH 6.8, and 0.001% bromophenol blue) and resolved on 4–20% gradient SDS-PAGE. The transferred nitrocellulose blot was blocked with 3% fat-free milk powder in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h. The membrane was then immunoblotted with anti-GCS antiserum or anti-TNF receptor 1 antibody in TBS containing 0.5% BSA at 4°C for 18 h. Detection was performed using ECL (Amersham).

All data represent the mean \pm SD. Experiments were repeated two or three times. Student's *t* test was used to compare mean values, and linear correlation between variables was tested using Pearson's correlation coefficient.

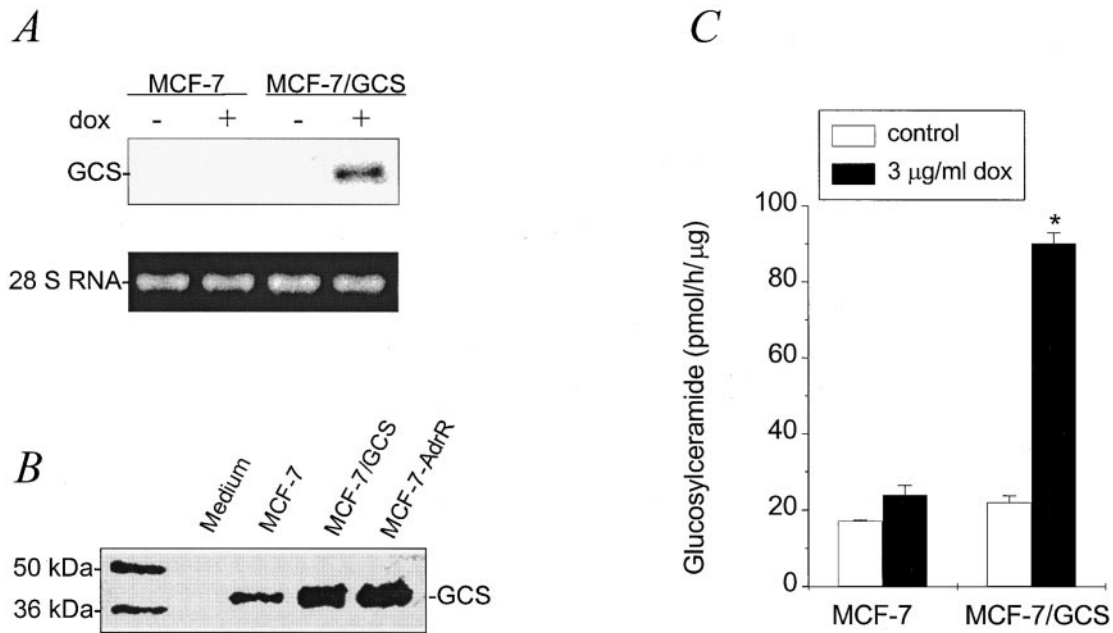


FIG. 1. Expression of glucosylceramide synthase in MCF-7/GCS cells. Cells were incubated without (-) or with (+) doxycycline (3 $\mu\text{g}/\text{ml}$) for 72 h. (A) Northern blot analysis of GCS mRNA expression. Total RNA (15 $\mu\text{g}/\text{lane}$) was subjected to agarose-formaldehyde electrophoresis, transblotted to nitrocellulose-plus membrane, and hybridized with GCS cDNA probe. RNA (28 S) stained with ethidium bromide was used as control for even loading. Dox, doxycycline. (B) Western blot analysis of GCS protein. Detergent-soluble cellular protein (500 μg) was immunoprecipitated with GCS-1.2 antiserum and subjected to 4–20% SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblots were incubated with GCS-1.2 antiserum. Detection was by ECL. Culture medium was used in immunoprecipitation and Western blot controls. (C) GCS activity was assayed as detailed under Materials and Methods. * $P < 0.001$, compared with MCF-7 cells.

RESULTS

Expression of Glucosylceramide Synthase

After MCF-7 cell transfection with pTet-on and pTRE-GCS vectors, stable, high-expression clones were selected by Northern blot and by screening GCS activity using a cell-free enzyme assay. After pTRE-GCS was introduced into MCF-7 Tet-on cells, doxycycline-induced GCS mRNA was highly elevated in MCF-7/GCS cells compared to doxycycline-naive MCF-7/GCS cells. A representative Northern blot is shown in Fig. 1A. Only traces of GCS mRNA were observed in MCF-7 cells and MCF-7/GCS cells that were incubated in the absence of doxycycline (Fig. 1A). Western blot analysis of GCS revealed that MCF-7/GCS cells treated with doxycycline expressed 4-fold greater levels of GCS protein, compared with MCF-7 cells (Fig. 1B). For comparison, the levels of GCS protein were also measured in the adriamycin-resistant clone, MCF-7-AdrR, which we have previously shown contains elevated levels of glucosylceramide and elevated GCS activity [21, 20]. In MCF-7/GCS cells, doxycycline-inducible GCS activities were 4.1-fold above untreated cells (Fig. 1C). Compared with a basal level of 17.2 ± 0.1 pmol GC in MCF-7 cells, doxycycline-induced GCS activity in MCF-7/GCS was 90.2 ± 2.76 pmol GC (Fig. 1C).

TNF- α Resistance in Transfected MCF-7/GCS Cells

TNF- α signals cell death through a ceramide-mediated pathway. Recent work has shown that deficient ceramide generation contributes to TNF- α resistance [10, 11]. Because MCF-7/GCS cells, through gene transfection, have the capacity to shunt ceramide into GC [19], thereby limiting ceramide buildup, we hypothesized that the cells would be resistant to TNF- α -induced cytotoxicity. To test this, MCF-7/GCS cells were incubated with increasing concentrations of TNF- α , and cell survival was evaluated. Fig. 2A shows that MCF-7/GCS cells, compared to MCF-7 cells, were more resistant to TNF- α toxicity. At 10, 25, 50, and 100 ng/ml TNF- α , survival of MCF-7/GCS cells was significantly greater than that of MCF-7 cells ($P < 0.0005$, Fig. 2A). The EC_{50} of TNF- α in MCF-7/GCS cells was approximately 15-fold greater than the EC_{50} observed in MCF-7 cells (155.3 ± 3.0 vs 10.5 ± 0.6 , Fig. 2B).

In DNA fragmentation studies, it was found that MCF-7/GCS cells demonstrated resistance to apoptosis induced by TNF- α exposure. After treatment for 72 h, TNF- α induced marked apoptotic DNA laddering in MCF-7 cells (Fig. 3A, Lane 2); however, under the same conditions, we did not detect apoptosis-typical oligonucleosomal fragmentation in transfected MCF-7/GCS cells (Fig. 3A, lane 4). In accordance, we also

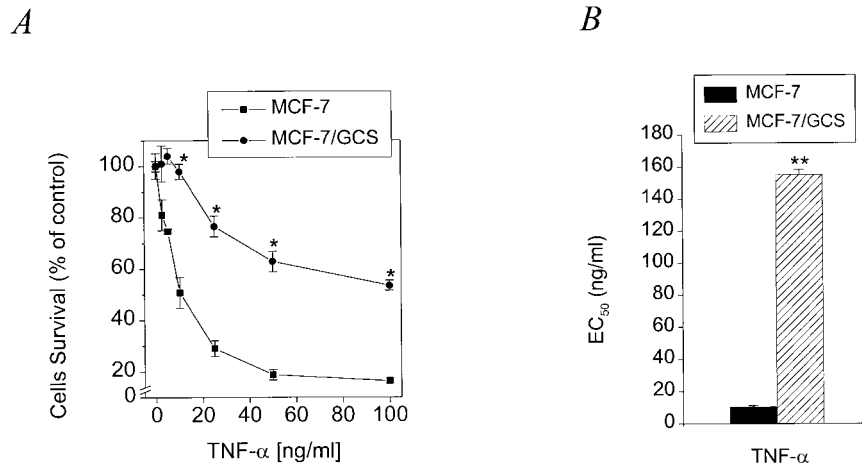


FIG. 2. TNF- α toxicity in MCF-7 and in GCS-transfected MCF-7/GCS cells. (A) Cytotoxicity of TNF- α . After a 48-h incubation with doxycycline (3 μ g/ml), MCF-7/GCS cells were seeded into 96-well plates and treated the following day with TNF- α at increasing concentrations in 5% FBS-RPMI 1640 medium. After a 96-h exposure, cell survival was determined. MCF-7/GCS cells cultured with doxycycline and without TNF- α served as control. Wild-type MCF-7 cells were treated with the TNF- α concentrations indicated, in the absence of doxycycline. Data represent the mean \pm SD of six replicates from three independent experiments. * P < 0.001 compared with MCF-7 cells. (B) EC₅₀ of TNF- α . * P < 0.0001 compared with MCF-7 cells.

observed that after TNF- α treatment (30 ng/ml), caspase-3 activity was reduced by 50% in MCF-7/GCS cells (199 \pm 32 vs 106 \pm 16% of control at 24 h; 362 \pm 11 vs 178 \pm 33% of control at 48 h in MCF-7 and in MCF-7/GCS cells, respectively, Fig. 3B).

Further work at the nuclear level showed that DNA synthesis upon TNF- α challenge was also significantly different in MCF-7 and MCF-7/GCS cells. Although exposure to TNF- α over a 3-day period resulted in a time-dependent decrease in DNA synthesis in both cell lines, DNA synthesis was nearly halted in MCF-7 cells compared to MCF-7/GCS cells (Fig. 3C). After 24 h in

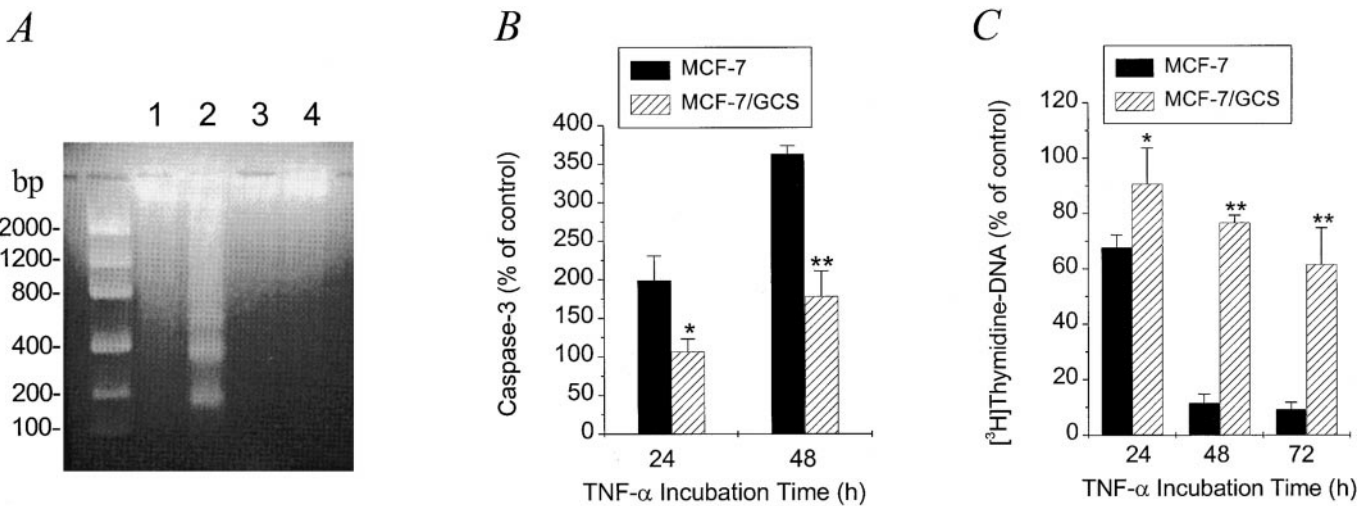


FIG. 3. The effect of TNF- α on apoptosis and DNA synthesis in MCF-7 and in MCF-7/GCS cells. (A) DNA fragmentation. Cells (500,000 cells/100-mm dish) were exposed to TNF- α (30 ng/ml) for 72 h, and cellular DNA was extracted and resolved by agarose gel electrophoresis. A low DNA mass ladder, used as a standard, is shown on the left. Lane 1, MCF-7 cells; Lane 2, MCF-7 cells treated with TNF- α ; Lane 3, MCF-7/GCS cells; Lane 4, MCF-7/GCS cells treated with TNF- α . (B) Caspase-3 activity. Cells (500,000/100-mm dish) were exposed to TNF- α (30 ng/ml) for 24 and 48 h. The soluble fraction obtained after cell lysis was incubated with DEVD-AFC substrate at 37°C for 60 min. The fluorescence of cleaved AFC was measured at 505 nm. * P < 0.05, compared to MCF-7 cells incubated with TNF- α for 24 h; ** P < 0.01 compared to MCF-7 cells incubated with TNF- α for 48 h. (C) DNA synthesis in MCF-7 and MCF-7/GCS cells treated with TNF- α . Cells were cultured in 5% FBS medium containing TNF- α (10 ng/ml) and [³H]thymidine (1.0 μ Ci/well) in 96-well plates for the indicated times. After lysis, [³H]thymidine-DNA was adsorbed onto scintillate beads, and tritium was measured by scintillation counting. * P < 0.05 and ** P < 0.001, compared to that of MCF-7 cells treated with TNF- α .

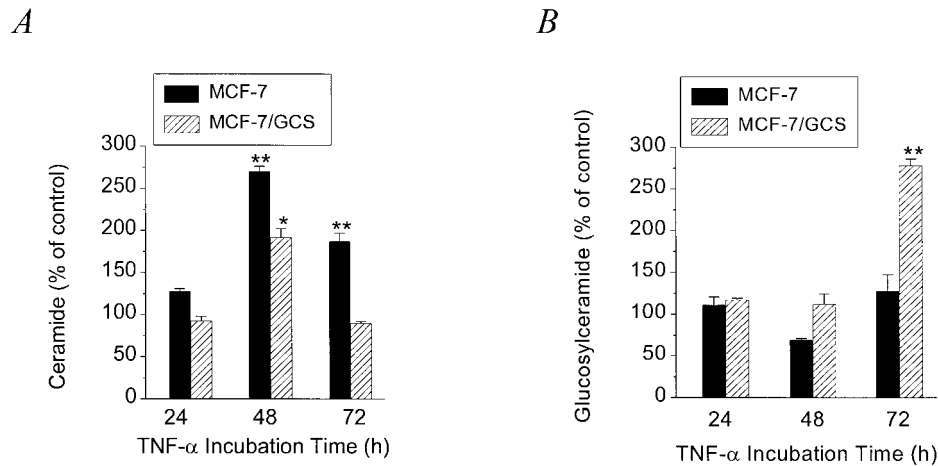


FIG. 4. Ceramide and GC metabolism in MCF-7 and in MCF-7/GCS cells in response to treatment with TNF- α . Cells were exposed to TNF- α (10 ng/ml) for the times indicated and radiolabeled with [3 H]palmitic acid for 24 h. [3 H]Ceramide and [3 H]GC were resolved by thin-layer chromatography and quantitated by liquid scintillation counting. (A) Influence of TNF- α on ceramide metabolism in MCF-7 and in MCF-7/GCS cells. * P < 0.01 compared to MCF-7/GCS cells in the absence of TNF- α ; ** P < 0.001, compared to MCF-7 cells in the absence of TNF- α . (B) Influence of TNF- α on GC metabolism in MCF-7 and MCF-7/GCS cells. ** P < 0.001 compared to MCF-7 cells treated with TNF- α .

the presence of TNF- α , [3 H]thymidine incorporation in MCF-7 cells dropped to 67% of control and by 48 h the incorporation diminished to 10% of control values (Fig. 3C). In contrast, MCF-7/GCS cells exhibited resistance to TNF- α -induced arrest of DNA synthesis. After treatment, [3 H]thymidine DNA levels in MCF-7/GCS cells were 1.5-, 7.2- and 7.8-fold greater than [3 H]thymidine DNA levels in MCF-7 cells at 24, 48, and 72 h, respectively (Fig. 3C).

TNF- α Elicits Increased Glycosylation of Ceramide in MCF-7/GCS Cells

To further define the mechanism of TNF- α resistance in MCF-7/GCS cells, cells were challenged with TNF- α , and the metabolism of ceramide was evaluated. As illustrated in Fig. 4A, ceramide levels in MCF-7 cells, in response to TNF- α exposure, increased 2.7- and 1.9-fold, after 48 and 72 h, respectively. In contrast, ceramide levels in MCF-7/GCS cells in response to TNF- α increased only 1.8-fold at 48 h and exhibited no increase at 72 h. Examination of GC metabolism shows that whereas TNF- α had little impact on GC levels in MCF-7 cells (Fig. 4B), a marked increase in GC was observed in MCF-7/GCS cells (Fig. 4B). After 48 and 72 h with TNF- α , GC levels in the GCS-transfected cells increased 1.6- and 2.2-fold, respectively, compared to GC levels in MCF-7 cells.

TNF-R1

Decreased expression of receptor TNF-R1 in a variant of MCF-7 cells has been shown to be aligned with loss of susceptibility to TNF- α -induced apoptosis [11].

To exclude the possibility that allied factors, such as variation in TNF-R1, were responsible for TNF- α resistance in the transfected cells, the expression of TNF-R1 was measured. Western blot analysis clearly shows that similar levels of TNF-R1 were expressed in MCF-7 and MCF-7/GCS cells (Fig. 5), regardless of the absence or presence of doxycycline and TNF- α . Therefore, transfection of GCS did not alter levels of TNF-R1 in MCF-7/GCS cells.

DISCUSSION

By introducing the GCS gene into cells, we have demonstrated that this enzyme potentiates cellular resistance to TNF- α toxicity. High expression of GCS in MCF-7 cells offers a survival advantage to treatment with TNF- α (Figs. 1, 2). The increased potential for

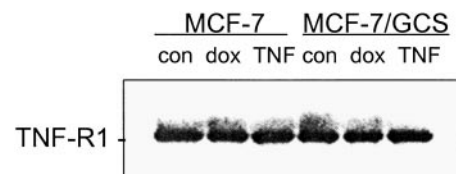


FIG. 5. TNF- α receptor 1 levels in MCF-7 and in MCF-7/GCS cells. Cells were cultured with doxycycline or TNF- α , and detergent-soluble cellular protein (1.0 mg) was immunoprecipitated and subjected to 4–20% SDS-PAGE as described under Materials and Methods. Protein was transferred to nitrocellulose membrane, and immunoblots were incubated with anti-TNFR1 antibody (htr9). Control (con) cells were treated with medium; dox, cells treated with 3 μ g/ml doxycycline for 72 h; TNF, cells treated with doxycycline for 72 h and TNF- α (10 ng/ml) for 48 h.

glycosylation of ceramide in MCF-7/GCS cells (Fig. 4) quenched apoptosis induced by high concentrations of TNF- α and markedly decreased growth arrest induced by low concentrations of TNF- α (Fig. 3).

Ceramide generation is one of the initial events in TNF- α -induced apoptosis [10, 11, 25, 26]. In MCF-7 cells treated with TNF- α , it has been shown that increased ceramide levels result from enhanced sphingomyelinase activity [10]. However, in Kym-1, a human rhabdomyosarcoma cell line, elevated ceramide, in response to TNF- α , is caused by a decrease in sphingomyelin synthase and GCS activities [26]. Previous work from Hirabayashi and colleagues [27, 28] has shown that in TNF- α -sensitive U937 cells, ceramide, which mediates apoptosis induced by TNF- α , was mostly converted to sphingomyelin. In this study, we provide direct evidence demonstrating that GCS glycosylates ceramide and it protects cells from apoptosis induced by TNF- α (Figs. 3A and 4A). The cellular effects of TNF- α were dose- and time-dependent. High concentrations of TNF- α (30 ng/ml) induced apoptosis in MCF-7 cells; however, under like conditions, TNF- α did not cause apoptosis in GCS-transfected cells. The introduction and inducible expression of GCS do not interfere with the expression of Bcl-2 [19], nor do they interfere with TNF-R1 expression (Fig. 5). Therefore, this study demonstrates that GCS contributes exclusively to MCF-7 cell resistance to TNF- α -induced apoptosis.

TNF- α causes growth arrest in human breast carcinoma cells and in human keratinocytes [29, 30]. At present, there is little information on the signaling events that take place between TNF- α receptor binding and cell-cycle-related protein changes. Exogenous C₆-ceramide has been shown to induce dramatic arrest at the G₀/G₁ phase of the cell cycle in Molt-4 cells [31]. We propose, as in the elicitation of apoptosis, that TNF- α induces growth arrest through a ceramide-governed event. In the present study, we show that low-dose TNF- α (10 ng/ml) markedly increased endogenous ceramide in a time-dependent manner (Fig. 4A), and inhibited DNA synthesis in the MCF-7 parental cells (Fig. 3B). In contrast, ceramide levels in transfected cells were lower, and DNA synthesis was dramatically higher in response to TNF- α .

GC has been shown to exert a regulatory role in cell proliferation [32, 33]. Administration of conduritol B epoxide, a β -glucocerebrosidase inhibitor, causes an elevation in cellular GC and epidermal hyperproliferation in mice [32]. Depletion of endogenous GC has been shown to cause cell cycle arrest [33]. In our study, TNF- α elicited a 2.5-fold increase in GC in MCF-7/GCS cells (Fig. 4B), and under these same conditions, DNA synthesis did not increase compared to the untreated MCF-7/GCS cells. Therefore, under these conditions, GC does not appear to impact cell proliferation; how-

ever, we cannot precisely evaluate the influence of GC on cell proliferation in MCF-7/GCS treated with TNF- α due to the dynamics of ceramide metabolism. In summary, GCS transfection potentiates glycosylation of ceramide in MCF-7 cells and offers a survival advantage to cells challenged with the tumor-killing cytokine TNF- α .

We are grateful to Dr. David Marks and Dr. Richard. E. Pagano (Department of Biochemistry and Molecular Biology, Thoracic Diseases Research Unit, Mayo Clinic and Foundation, Rochester, MN) for providing the anti-GCS antiserum, GCS-1.2. This work was supported in part by the Strauss Foundation, Sandra Krause, Trustee; The Fashion Footwear Association of New York (FFANY) Shoes on Sale; the Breast Cancer Fund of the State of California through the Breast Cancer Research Program of the University of California, Grant 0211 to M.C.C.; and National Institutes of Health Grant CA 77632 to M.C.C.

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Received May 14, 1999

Revised version received July 22, 1999