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Expression of Glucosylceramide Synthase, Converting Ceramide to Glucosylceramide, Confers Adriamycin Resistance in Human Breast Cancer Cells*

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Multidrug-resistant cancer cells display elevated levels of glucosylceramide (Lavie, Y., Cao, H. T., Volner, A., Lucci, A., Han, T. Y., Geffen, V., Giuliano, A. E., and Cabot, M. C. (1997) *J. Biol. Chem.* 272, 1682–1687). In this study, we have introduced glucosylceramide synthase (GCS) into wild type MCF-7 breast cancer cells using a retroviral tetracycline-on expression system, and we developed a cell line, MCF-7/GCS. MCF-7/GCS cells expressed an 11-fold higher level of GCS activity compared with the parental cell line. Interestingly, the transfected cells demonstrated strong resistance to adriamycin and to ceramide, whereas both agents were highly cytotoxic to MCF-7 cells. The EC_{50} values of adriamycin and ceramide were 11-fold ($p < 0.0005$) and 5-fold ($p < 0.005$) higher, respectively, in MCF-7/GCS cells compared with MCF-7 cells. Ceramide resistance displayed by MCF-7/GCS cells closely paralleled the activity of expressed GCS with a correlation coefficient of 0.99. In turn, cellular resistance and GCS activity were dependent upon the concentration of the expression mediator doxycycline. Adriamycin resistance in MCF-7/GCS cells was related to the hyperglycosylation of ceramide and was not related to shifts in the levels of either P-glycoprotein or Bcl-2. This work demonstrates that overexpression of GCS, which catalyzes ceramide glycosylation, induces resistance to adriamycin and ceramide in MCF-7 breast cancer cells.

Glucosylceramide synthase (GCS)[1] transfers glucose from UDP-glucose to ceramide and produces GC. GC serves as the core structure for more than 300 glycolipids (1). Recently, it has been shown that human GCS is a glycoprotein containing 394 amino acids encoded from 1182 nucleotides, including a G+C-rich 5′ untranslated region of 290 nucleotides (2). A large body of literature shows that ceramide, the substrate of GCS, exerts an important role mediating myriad biological activities. Ceramide is a pleiotropic cellular activator capable of inducing two mutually exclusive cellular functions, cell proliferation and cell death. Ceramide is now recognized as a messenger of signaling events that originate from different cell surface receptors, including interferon-γ, TNF-α, interferon-1β, CD95 (Fas/APO-1), nerve growth factor receptor, and CD28 (3–5). Ceramide is also involved in the action of protein kinase C ζ, *vav* protooncogene, 1α-25-dihydroxy vitamin D_3, dexamethasone, ionizing radiation, and chemotherapeutic agents (3–5). There have been several lines of evidence suggesting that loss of ceramide production is one cause of cellular resistance to apoptosis induced by either ionizing radiation, TNF-α, or adriamycin (6–13).

Glycolipids, in addition to being essential membrane structural elements, are putatively involved in cell proliferation (14), differentiation (15–17), and oncogenic transformation (18, 19). GC has recently been shown to be associated with resistance to chemotherapy (12, 13, 20, 21). Accumulation of GC is a characteristic of some MDR cancer cells and tumors derived from patients who are less responsive to chemotherapy (20, 21). Further studies have shown that MDR modulators of varying chemical structure inhibit the production of GC (12, 21). Although these studies document the accumulation of GC in multidrug-resistant cancer, little is known about the expression of GCS in MDR cells and its relationship to drug resistance. In particular, it is not known whether increased levels of GC are due to GCS gene expression or to other drug resistance factors that play a role in modulating GCS activation or GCS degradation.

The retroviral Tet-off/Tet-on vector is a highly regulatory, versatile mammalian expression system (22–26). Expression of a target gene inserted in multiple cloning sites under CMV promoter control can be mediated simply by withdrawal of tetracycline or addition of doxycycline. Utilizing a retroviral Tet-on system, we established the MCF-7/GCS cell line from MCF-7 breast adenocarcinoma cells. The MCF-7/GCS-transfected cells express high levels of GCS activity, and they are resistant to cytotoxicity imparted by adriamycin and ceramide.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]UDP-glucose (40 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). EcoLume (liquid scintillation mixture) was from ICN (Costa Mesa, CA), and [α-^{32}P]dCTP (6,000 Ci/mmol) was from Amersham Pharmacia Biotech. C_6-Ceramide (*N*-hexanoylsphingosine) was purchased from LC Laboratories (Woburn, MA). 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 (Alabaster, AL). C219, the monoclonal antibody against human P-glycoprotein, was from Signet Laboratories (Dedham, MA), and Bcl-2 (Ab-1) monoclonal antibody against human Bcl-2 was from Oncogene Research Products (Cambridge, MA). Hygromycin B was purchased from Boehringer Mannheim. Doxycycline hydrochloride, adriamycin (doxorubicin hydrochloride), and other chemicals were purchased from Sigma. FBS was purchased from HyClone (Logan, UT). RPMI 1640 medium and Dul-

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[1] The abbreviations used are: GCS, glucosylceramide synthase (ceramide glucosyltransferase, UDP-glucose:*N*-acylsphingosine D-glucosyltransferase (EC 2.4.1.80)); GC, glucosylceramide; FBS, fetal bovine serum; MCF-7/GCS, glucosylceramide synthase-transfected cell line; Tet, tetracycline; rtTA, reverse tetracycline transactivator; TNF-α, tumor necrosis factor-α; Bcl-2, B-cell leukemia oncoprotein, MDR, multidrug resistance; CMV, cytomegalovirus.

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becco's modified Eagle's medium (high glucose) were from Life Technologies, Inc., and cultureware was from Corning-Costar (Cambridge, MA).

Cell Lines and Culture Conditions—Human breast adenocarcinoma cells, MCF-7 cells, and MCF-7 adriamycin-resistant cells (MDR clone) were kindly provided by Drs. Kenneth Cowan and Merrill Goldsmith (National Institutes of Health, NCI, Bethesda, MD). Cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 584 mg/liter L-glutamine. Cells were cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator and subcultured weekly using a trypsin-EDTA (0.05%-0.53 mM) solution. Transfected cells, MCF-7/GCS, were cultured in RPMI 1640 medium containing 10% FBS and 200 μg/ml hygromycin in addition to the above components.

pTRE-GCS Expression Vector Construction and Transfection—pCG-2, a Bluescript II KS containing GlcT-1 (see Ref. 2 for terminology for GCS) in the *Eco*RI site was kindly provided by Drs. Shinichi Ichikawa and Yoshio Hirabayashi (Institute of Chemical and Physical Research, RIKEN, Saitama, Japan). The gene encoding human glucosylceramide synthase was immune-selected by monoclonal antibody M2590 from a human melanoma cell (SK-Mel-28) library (2). The full-length cDNA of human GCS was subcloned into the *Eco*RI site in the pTRE, Tet-repressible expression plasmid. The Tet-on gene expression system was purchased from CLONTECH (Palo Alto, CA). This system contains three vectors, pTet-on, pTRE, and pTK-Hyg. The pTet-on vector (pUHD17–1neo) expresses a doxycycline-controlled rtTA that is a fusion protein of a reverse Tet repressor and the C-terminal domain of protein 16 of herpes simplex virus, constitutively expressed under control of human CMV promoter (22, 26). The pTRE vector (pUHD10–3) contains a multiple cloning site to accept any cDNA to be expressed followed by an SV40 polyadenylation sequence (24). The promoter region upstream from the multiple cloning site contains a minimal human CMV promoter (P_{minCMV}) with heptamerized tet operators. This promoter is silent in the absence of binding of rtTA to the tet operators. However, when the reverse Tet repressor of the rtTA binds to the tet operators, the virion protein 16 domain of the rtTA can activate P_{minCMV} activity to a very high level and switch on expression of the target gene, GCS. Binding of doxycycline to the reverse Tet repressor domain of the rtTA can almost completely activate rtTA binding to the promoter (22, 26).

Sense orientation of the GCS cDNA was analyzed with Vector NTI 4.0 and doubly checked by restriction enzyme digestion with *Hin*dIII and with *Xho*I plus *Not*I. The pTK-Hyg vector, which has a hygromycin-resistant gene under control of the mouse β-globin promoter, was used to select the stable transfectants. When MCF-7 cells reached 20% confluence, pTet-on DNA (10 μg/ml, 100-mm dish) was introduced by co-precipitation with calcium phosphate (Mammalian Transfection Kit, Stratagene, La Jolla, CA). The transfected cells were selected in RPMI 1640 medium containing 10% FBS and 400 μg/ml G418. Each G418-resistant clone was screened by luciferase assay, after transient transfection with pTRE-Luc vector containing the reporter gene, luciferase. pTK-Hyg (10 μg DNA) and pTRE-GCS (10 μg DNA) were introduced into the selected MCF-7 Tet-on cells by co-precipitation with calcium phosphate. The GCS-transfected cells were primarily selected in RPMI medium containing 10% FBS and 200 μg/ml hygromycin. As a control for transfection, MCF-7 Tet-on cells were co-precipitated with pTK-Hyg and pTRE plasmid without GCS cDNA.

Transient Transfection and Luciferase Assay—This procedure was performed as described previously (22, 24, 25). After MCF-7 cell transfection with pTet-on vector, each G418-resistant clone was grown for 16 h in 6-well plates (4000 cells/well) in 10% FBS RPMI medium, then shifted to 10% FBS Dulbecco's modified Eagle's medium. After a 6-h incubation, pTRE-Luc (1.5 μg of DNA) was introduced by co-precipitation with calcium phosphate. After culturing in 10% FBS Dulbecco's modified Eagle's medium for 18 h and in 10% FBS RPMI medium for 48 h, luciferase activity was measured using a commercial luciferase assay system according to the instruction manual (Promega, Madison, WI). Incubation for 48 h in 3.0 μg/ml doxycycline was used to induce expression of rtTA protein. Cellular extracts (100 μg of protein) from each clone were used. The activity of luciferase was measured by scintillation spectroscopy 2 min after the addition of substrate. MCF-7 cells transfected with pTRE-Luc were used as controls.

Glucosylceramide Synthase Assay—To determine the expression of GCS in the hygromycin-resistant clones, a modified radioenzymatic assay was utilized (12, 27). After incubation in the absence or presence of doxycycline (3 μg/ml for 48 h), cells were homogenized by sonication in lysis buffer (50 mM Tris-HCl, pH 7.4, 1.0 μg/ml leupeptin, 10 μg/ml aprotinin, 25 μM phenylmethylsulfonyl fluoride). Microsomes were isolated by centrifugation (129,000 × *g* for 60 min). The enzyme assay, containing 50 μg of 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 C_6-ceramide (1.0 mM), phosphatidylcholine (3.6 mM; molecular weight, 786.15), and brain sulfatides (0.9 mM; molecular weight, 563). The liposomal substrate was prepared by mixing the components, evaporating the solvents under a stream of nitrogen, and sonicating in water over ice for 1 min using a microtip at 50% output (Kontes, Micro Ultrasonic Cell Disrupter). Other reaction components included sodium phosphate buffer (0.1 M), pH 7.8, EDTA (2.0 mM), $MgCl_2$ (10 mM), dithiothreitol (1.0 mM), β-nicotinamide adenine dinucleotide (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 Na_2SO_4 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 of the upper phase, which contained GC, was withdrawn and mixed with 4.5 ml of EcoLume for analysis of radioactivity by liquid scintillation spectroscopy.

Analysis of Ceramide and Glucosylceramide—Analyses were performed as described previously (12). Cellular lipids were radiolabeled by incubating cells with $[^3H]$palmitic acid (2.5 μCi/ml culture medium) for 24 h. After removal of medium, cells were rinsed twice with phosphate-buffered saline (pH 7.4), and lipids were extracted (12). After nitrogen evaporation of solvents, total lipids were resuspended in 100 μl of chloroform/methanol (1:1, v/v), and aliquots were applied to TLC plates. 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). Commercial lipid standards were co-chromatographed. After development, lipids were visualized by iodine vapor staining, and areas of interest were scraped into 0.5 ml of water. EcoLume counting fluid (4.5 ml) was added, the samples were mixed, and radioactivity was quantitated by liquid scintillation spectrometry.

RNA Analysis—RNA was extracted from cells using the single-step method described by Chomczynski and Sacchi (28). Equal amounts of total RNA (15 μg) were denatured in 59% formamide/2.2 M formaldehyde, size-separated by electrophoresis on 1% agarose-formaldehyde, and then blotted onto NitroPure nitrocellulose transfer membrane (29). GCS cDNA was prepared from pCG-2 plasmid, digested with *Eco*RI, and *Hin*dIII (Stratagene). The 1.1-kilobase pair fragment was then isolated by 1% low melt agarose electrophoresis using a commercial agarose gel DNA extraction kit (Boehringer Mannheim). Probing of ^{32}P-GCS cDNA was performed by nick translation according to the instruction manual (Boehringer Mannheim). Nitrocellulose-plus membranes were hybridized with the ^{32}P-GCS probe at 68 °C for 18 h. The filters were exposed at –70 °C for autoradiography. For even gel loading, 28 S RNA was used.

Cytotoxicity Assay—The assay was performed as described previously (12). Briefly, after culture in the absence or presence of 3.0 μg/ml doxycycline for 48 h, cells were harvested and seeded in 96-well plates (2,000 cells/well), in 0.1 ml of RPMI 1640 medium containing 10% FBS in the absence or presence of 3.0 μg/ml doxycycline. Cultures were incubated at 37 °C for 24 h before addition of drug. 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. Absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay reader (Molecular Devices, San Diego, CA).

Western Blot Analysis—Western blots were performed using a modified procedure (30, 31). Confluent cells were washed twice with phosphate-buffered saline containing 1.0 mM phenylmethylsulfonyl fluoride, and detached with trypsin-EDTA solution. Cells were pelleted by centrifuging at 500 × *g* for 5 min. Cell pellets 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 mM phenylmethylsulfonyl fluoride, 1% aprotinin) for 60 min with shaking. The insoluble debris was excluded by centrifugation at 12,000 × *g* for 45 min at 4 °C. The detergent soluble fraction was loaded in equal aliquots by protein and resolved using 4–20% gradient SDS-polyacrylamide gel electrophoresis. The transferred nitrocellulose blot was blocked with 3% fat-free milk powder in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) at room temperature for 1 h. The membrane was then immunoblotted with monoclonal antibodies C219 (5 μg/ml) or Bcl-2 (Ab-1) (2.5 μg/ml) in Tris-buffered saline containing 0.5% bovine serum albumin (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) at 4 °C for 18 h. Detection was performed using ECL (Amersham Pharmacia Biotech).

Statistics—All data represent the mean ± S.D. Experiments were

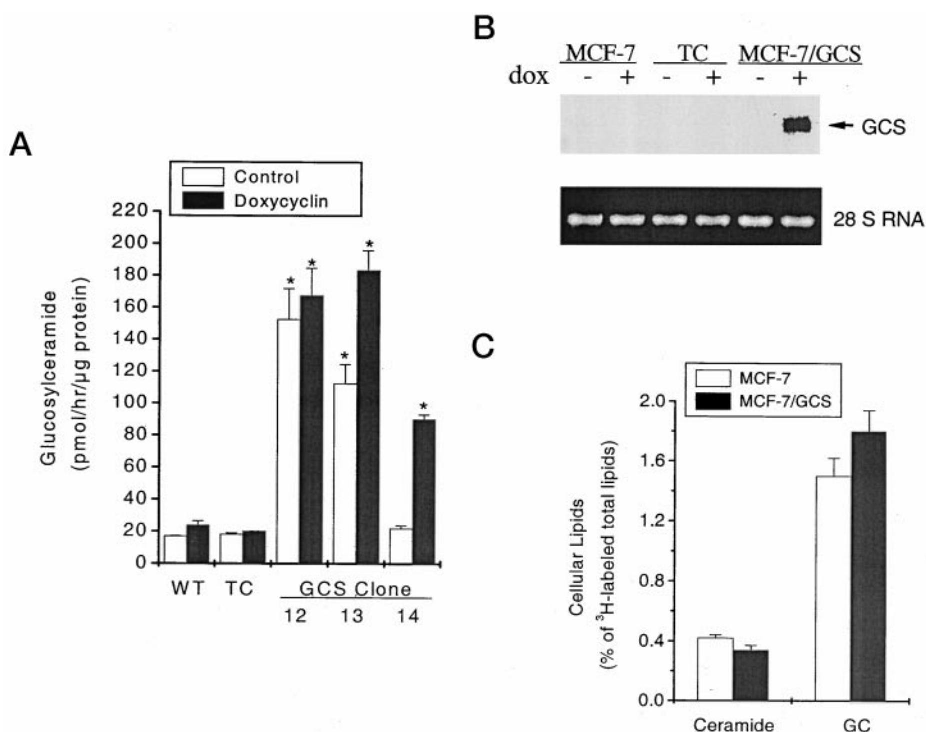

FIG. 1. **Glucosylceramide synthase activity and GCS mRNA expression in MCF-7/GCS cells.** Cells were incubated without (−) or with (+) doxycycline (3 µg/ml) for 72 h. *A,* GCS activity. GCS activity was assayed as detailed under "Experimental Procedures." *WT,* MCF-7 wild type; *TC,* transfected control cells; GCS_{12}, GCS_{13}, and GCS_{14}, subclones of MCF-7/GCS. *, $p < 0.001$ compared with MCF-7 cells. Subclone GCS_{14} was designed as MCF-7/GCS and used in further experiments. *B,* Northern blot analysis of GCS mRNA expression. Total RNA (15 µg/lane) was subjected to agarose-formaldehyde electrophoresis, transblotted to nitrocellulose-plus membrane, and hybridized with GCS cDNA probe. 28 S RNA stained with ethidium bromide was used as control for even loading. *Dox,* doxycycline; *TC,* transfected control. *C,* ceramide and GC levels in MCF-7/GCS cells. Lipids were radiolabeled by incubating cells with [^{3}H]palmitic acid. After lipid extraction, ceramide and GC were resolved by TLC using solvent systems I and II, respectively.

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.

RESULTS

Expression of Glucosylceramide Synthase—MCF-7 cells were transfected with pTet vector and co-transfected with pTRE-GCS and pTK-Hyg. The stable, high expression clones were selected by screening GCS activity using the cell-free enzyme assay and by Northern blot. After transfection of pTet-on in MCF-7 cells, more than 30 G418-resistant clones were collected. Luciferase activity, which is a measure of expression of rtTA in the G418-resistant clones, was analyzed after 3 days of transient transfection with pTRE-luciferase vector. After stimulation with doxycycline, maximal expression of luciferase, 16,000-fold above that of MCF-7 cells, was found in clone 16. Luciferase activity in clone 16 in the absence of doxycycline was 15,000-fold higher than that of MCF-7 cells. Clone 1 demonstrated low basal rtTA expression; however, clone 1 was highly responsive to doxycycline, with induced luciferase activity that was 100-fold over MCF-7 cells. Clones 1 and 16 were selected as the optimal MCF-7 Tet-on clones for expression of rtTA.

After co-transfection of pTRE-GCS and pTK-Hyg into clone 1 and clone 16 of MCF-7 Tet-on cells, 65 hygromycin-resistant clones were selected. Utilizing the [^{3}H]UDP-glucose enzyme assay, we analyzed GCS activity and identified three clones that exhibited 5–11-fold increases in enzyme activity (Fig. 1*A*). Compared with a basal level of 17.2 ± 0.1 pmol of GC in MCF-7 wt cells, doxycycline-induced GCS activity in MCF-7/GCS_{12}, MCF-7/GCS_{13}, and MCF-7/GCS_{14} was 167.4 ± 17.2, 183.3 ± 12.4, and 90.2 ± 2.76 pmol of GC, respectively (Fig. 1*A*). There were no differences in either basal or doxycycline-induced GCS activity in transfection control cells (TC) or in the basal level of GCS in MCF-7 wt cells (Fig. 1*A*). In MCF-7/GCS_{13} and MCF-7/GCS_{14}, the doxycycline-inducible GCS activities were 1.6- and 4.1-fold, respectively, above untreated cells. The MCF-7/GCS_{14} clone was designated MCF-7/GCS, and this clone was used in further experiments.

Doxycycline-induced GCS mRNA was highly elevated in MCF-7/GCS cells compared with doxycycline-naive MCF-7/GCS cells. A representative Northern blot is shown in Fig. 1*B*. Only traces of GCS mRNA were observed in MCF-7 cells, TC, and MCF-7/GCS cells without doxycycline (Fig. 1*B*). The levels of ceramide and GC in MCF-7 and in MCF-7/GCS cells were assessed by steady-state radiolabeling of cultured cells using [^{3}H]palmitic acid. As shown in Fig. 1*C,* transfection with GCS elicited only a moderate decrease in ceramide, compared with MCF-7 cells. The decrease was not statistically significant. GC in MCF-7/GCS compared with MCF-7 cells increased slightly and accounted for 1.8 and 1.5%, respectively, of total cellular radiolabeled lipid.

Adriamycin and Ceramide Resistance in Transfected MCF-7/GCS Cells—Recent work has revealed that effects of therapeutic doses of anthracyclines are closely related to the generation of ceramide, and elevated GC has been shown to be associated with adriamycin resistance in MDR cells (9, 11–13). Adriamycin was used to assess the influence of GCS transfection on cellular response to anthracyclines. After pretreatment with doxycycline for 2 days, MCF-7/GCS cells were incubated with increasing concentrations of adriamycin for 4 days. Fig. 2*A* shows that MCF-7/GCS cells, compared with MCF-7 cells, are resistant to adriamycin toxicity. At 0.5, 1.0, 2.0, and 3.0 µM adriamycin, survival of transfected MCF-7/GCS cells was significantly greater than that of MCF-7 cells ($p < 0.0005$, Fig. 2*A*). As presupposed, it was observed that MCF-7/GCS cells were also resistant to ceramide toxicity. At 2.5 and 5.0 µM C_6-ceramide, MCF-7/GCS cell survival was significantly higher than that of MCF-7 cells ($p < 0.0005$, Fig. 2*B*). The EC_{50} of adriamycin in MCF-7/GCS cells was approximately 11 times greater than the EC_{50} observed in MCF-7 cells (4.01 ± 0.12 *versus* 0.37 ± 0.01 µM, $p < 0.0005$, Fig. 2*C*). However, the EC_{50} in the TC group was nearly identical with that of MCF-7 cells, and there was no statistical difference between the two groups (Fig. 2*C*). The EC_{50} of C_6-ceramide in MCF-7/GCS cells was 4-fold greater than that observed in MCF-7 cells (12.07 ± 1.50 *versus* 3.10 ± 0.50 µM, $p < 0.0005$, Fig. 2*C*), and survival of TC cells was not statistically different from that of the parent cell line, MCF-7 (Fig. 2*C*).

Regulation of Ceramide Resistance in MCF-7/GCS Cells—If

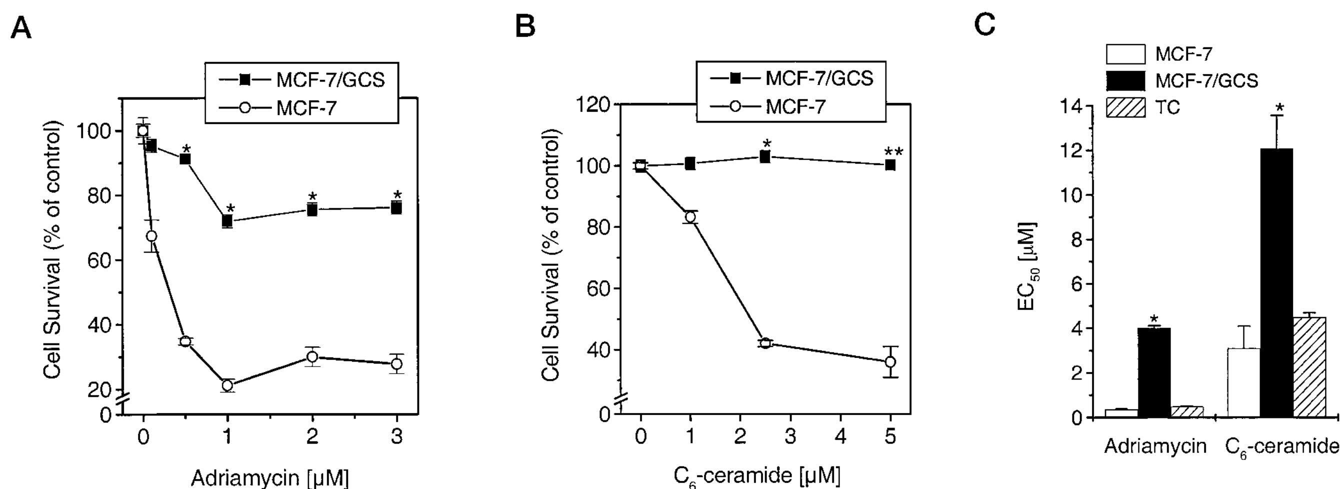

FIG. 2. **Adriamycin and ceramide toxicity in MCF-7 and in GCS-transfected MCF-7/GCS cells.** *A,* cytotoxicity of adriamycin. After a 48-h incubation with doxycycline (3 μg/ml), MCF-7/GCS cells were seeded into 96-well plates and treated the following day with adriamycin at increasing concentrations in 5% FBS RPMI 1640 medium. After a 96-h exposure, cell survival was determined. MCF-7/GCS cells cultured with doxycycline and without adriamycin served as control. The wild type MCF-7 cells were treated without doxycycline and with the adriamycin concentrations shown. Data represent the mean ± S.D. of six replicates from three independent experiments. *, $p < 0.001$ compared with MCF-7 cells. *B,* cytotoxicity of ceramide. The same conditions as cited above were employed, and C_6-ceramide was used in place of adriamycin. *, $p < 0.01$; **, $p < 0.001$ compared with MCF-7 cells. *C,* EC_{50} of adriamycin and ceramide. * $p < 0.001$ compared with MCF-7 cells.

ceramide resistance is induced by GCS expression in MCF-7/GCS cells, the resistance response should be tightly correlated with the level of the inducer, doxycycline. We found that increasing doxycycline concentrations correlated closely with increased expression of GCS, which in turn correlated well with increased resistance of the cells to C_6-ceramide. After cells were exposed to increasing concentrations of doxycycline, higher expression of GCS mRNA was observed in MCF-7/GCS cells with 1.0 and 3.0 μg/ml doxycycline (Fig. 3*A*); the densities were 97 and 256, respectively (GCS band/28 S RNA × 100). In contrast, the mRNA was scarcely detectable at 0 and 0.1 μg/ml doxycycline, with densities measuring 16 and 18, respectively. Only traces of GCS mRNA were found in MCF-7 cells treated with doxycycline (Fig. 3*A*). GCS activity in MCF-7/GCS cells exposed to 0.1, 1.0, and 3.0 μg/ml doxycycline was significantly higher than GCS activity observed in MCF-7 cells ($p < 0.001$, Fig. 3*B*). The r of GCS activity to doxycycline in MCF-7/GCS cells was 0.84. In contrast, increasing amounts of doxycycline did not elevate GCS activity in MCF-7 cells, and the r was 0.48. In concert with enhanced GCS activity, ceramide cytotoxicity in MCF-7/GCS cells was reversed following target gene expression by exposing cells to increasing concentrations of doxycycline. Treatment with C_6-ceramide (5 μM) in conjunction with doxycycline dose escalation effected a dose-dependent increase in survival of MCF-7/GCS cells, and the survival was significantly higher than that of MCF-7 cells ($p < 0.001$, Fig. 3*C*). MCF-7/GCS cell survival upon exposure to ceramide was highly correlated with the concentration of doxycycline in the pretreatment regimen ($r = 0.84$ at 0.1–3.0 μg/ml doxycycline). In comparing Fig. 3*C* with Fig. 3*B*, it is seen that the increase in cell survival mirrored the induction of GCS activity. The correlation coefficient for these biological parameters was 0.99, verifying that cell survival is closely associated with GCS activity.

Adriamycin Induced Hyperglycosylation of Ceramide in MCF-7/GCS Cells—To further define the mechanism of drug resistance in MCF-7/GCS cells, cells were challenged with adriamycin, and the metabolism of ceramide was evaluated (Fig. 4). As illustrated in Fig. 4*A*, after 24- and 48-h exposures to adriamycin, ceramide levels in MCF-7 cells increased 3.4- and 3-fold, respectively; however, in counterpoint, ceramide levels in response to adriamycin in MCF-7/GCS cells increased 1.4- and 1.2- fold at 24 and 48 h, respectively. Examination of GC metabolism (Fig. 4*B*) shows that whereas adriamycin had little impact on GC levels in MCF-7 cells, a time-dependent increase in GC was observed in MCF-7/GCS cells exposed to adriamycin. After 24 and 48 h with adriamycin, GC levels in the GCS-transfected cells increased 1.4- and 2.1-fold, respectively.

To exclude the possibility that allied factors, such as P-glycoprotein or Bcl-2, were responsible for conferring ceramide and adriamycin resistance in the transfected cells, the expression of P-glycoprotein and Bcl-2 was measured. Western blot analysis showed that P-glycoprotein was not detected in either MCF-7/GCS or in MCF-7 cells (Fig. 5*A*), regardless of the absence or presence of doxycycline. Therefore, transfection and inducible expression of GCS did not influence P-glycoprotein levels in MCF-7/GCS cells. Western blot analysis also shows that the phosphorylation/dephosphorylation of Bcl-2 was the same in MCF-7 and in MCF-7/GCS cells, regardless of the absence or presence of doxycycline (Fig. 5*B*).

DISCUSSION

We have introduced GCS cDNA into MCF-7 cells and characterized the resulting MCF-7/GCS cell line. MCF-7/GCS cells highly expressed GCS mRNA (Fig. 1*B*) and demonstrated increased GCS enzymatic activity (Fig. 1*A*). We show that GCS modulates drug resistance in human breast cancer cells. Stable expression of GCS induced adriamycin resistance in MCF-7/GCS cells. Ceramide resistance, also a property of the transfected cells, was highly correlated with induced expression of GCS. MCF-7/GCS cells represent a novel model to study the role of GCS in cell differentiation, programmed cell death, and resistance to anticancer agents that cause ceramide production (9, 11, 13).

Doxycycline-regulated gene expression is different from the tetracycline-regulated systems in the parameters of control. In contrast with the Tet-off vector, target gene expression in the Tet-on system is positively correlated with the amount of doxycycline in the medium (22). MCF-7/GCS cells represent the first cell line that has been developed from MCF-7 cells using the retroviral Tet-on expression system. We found, at concentrations >2 μg/ml, that doxycycline was slightly cytotoxic in MFC-7 cells but had no influence on MCF-7/GCS cells. During transient transfection with pTRE-Luc and stable transfection

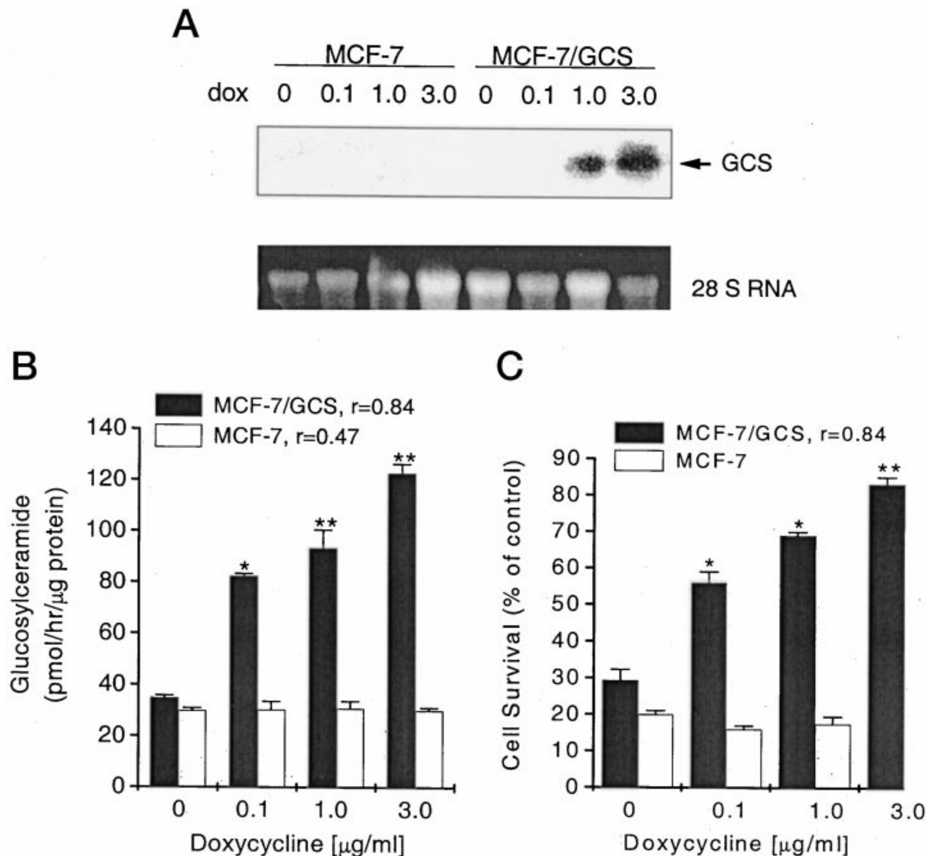

FIG. 3. **Doxycycline-induced GCS activity and ceramide resistance coincide with regulated expression of GCS mRNA.** *A,* regulated expression of GCS mRNA (Northern blot). MCF-7 and MCF-7/GCS cells were incubated for 96 h with the indicated concentrations of doxycycline (*dox*) (μg/ml). Total RNA (15 μg/lane) was subjected to agarose-formaldehyde electrophoresis. After transfer, filters were hybridized with GCS cDNA probe. Ethidium bromide-stained RNA (28 S) was used as a control for even loading. Densities of GCS/28 S RNA (×100) in MCF-7/GCS cells were 16, 18, 97, and 256 at 0, 0.1, 1.0, and 3.0 μg/ml doxycycline, respectively. *B,* dose-response of GCS activity to doxycycline. Cells were incubated for 96 h in medium containing the indicated concentrations of doxycycline. GCS activity was analyzed by radioenzymatic assay. *, $p < 0.01$; **, $p < 0.001$ compared with MCF-7 cells. *C,* doxycycline-induced resistance to ceramide. Cells were pretreated with the indicated concentrations of doxycycline for 48 h, seeded in 96-well plates, and treated the following day with 5 μM C_6-ceramide in RPMI 1640 medium containing 5% FBS. Cell viability was determined after 96 h. Data represent the mean ± S.D. of six replicates from two independent experiments. Control cells were cultured in medium without C_6-ceramide. *, $p < 0.001$ compared with MCF-7 cells; **, $p < 0.001$ compared with MCF-7 cells exposed to 1.0 μg/ml doxycycline.

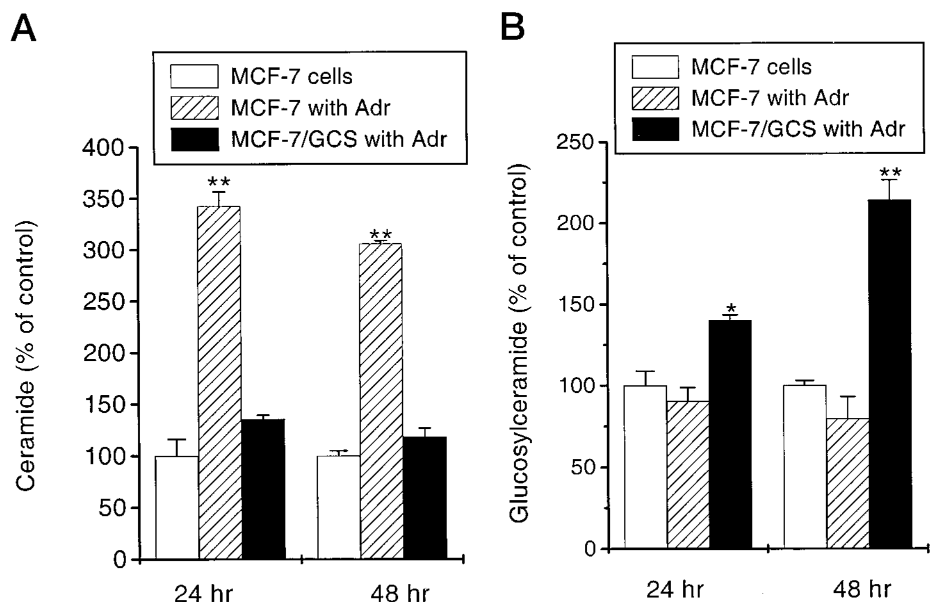

FIG. 4. **Ceramide metabolism in MCF-7 and in MCF-7/GCS cells in response to treatment with adriamycin.** Cells were exposed to adriamycin (1.7 μM) for the times indicated and radiolabeled with [^{3}H]palmitic acid. Ceramide and GC were resolved by TLC of the total cellular lipid extract, and quantitation of tritium was by liquid scintillation spectroscopy. *A,* influence of adriamycin on ceramide metabolism in MCF-7 and in MCF-7/GCS cells. **, $p < 0.01$ compared with MCF-7/GCS cells treated with adriamycin (*Adr*). *B,* influence of adriamycin on GC metabolism in MCF-7 and MCF-7/GCS cells. *, $p < 0.05$; **, $p < 0.01$ compared with MCF-7 cells treated with adriamycin.

with pTRE-GCS, doxycycline exerted tight regulation on target gene expression. We obtained one subclone of MCF-7 Tet-on cells in which rtTA expression was increased 100-fold by doxycycline. In MCF-7/GCS cells, maximum expression of the GCS gene occurred after 48 h with doxycycline (3 μg/ml).

Previous work from our laboratory has demonstrated that

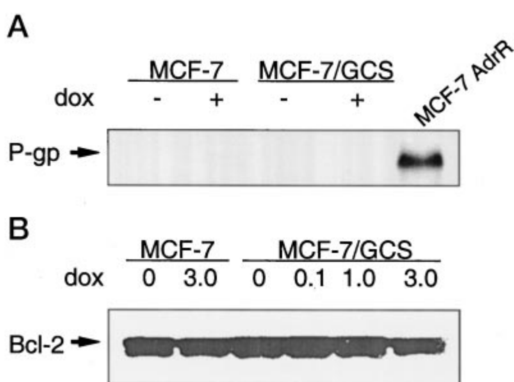

FIG. 5. **P-glycoprotein and Bcl-2 expression in MCF-7 and in MCF-7/GCS cells.** After cell culture without (−) or with (+) the indicated concentrations of doxycycline for 72 h, detergent-soluble cellular protein was isolated and subjected to SDS-polyacrylamide gel electrophoresis (50 μg/lane). Proteins were transferred to nitrocellulose, and immunoblots were incubated with antibody. *A,* P-glycoprotein (*P-gp*) Western blot. The immunoblots were incubated with C219, a monoclonal antibody against human P-glycoprotein. When doxycycline (*dox*) was present, the concentration was 3 μg/ml in the culture medium. MCF-7 adriamycin-resistant (*AdrR*) cells were used as P-glycoprotein positive controls. *B,* Bcl-2 Western blot. The immunoblot was incubated with Bcl-2 (Ab-1), a monoclonal antibody against human Bcl-2. The phosphorylated Bcl-2 (*top band*) and dephosphorylated Bcl-2 (*bottom band*) localized at ~25 kDa. Doxycycline (*dox*) was present at the indicated concentration (μg/ml).

MDR is associated with an accumulation of GC and that reversal of drug resistance by MDR modulators is accompanied by a decrease in the cellular GC component (12, 13, 20, 21). Based on these data, we believed the GC pathway to be an alternative mechanism of drug resistance and that increased expression of GCS would be a key step in this biological event. MCF-7 cells are sensitive to anticancer drugs, ionizing radiation, TNF-α, and CD95 (7, 11–13). After introduction and expression of GCS, MCF-7/GCS cells were resistant to adriamycin and ceramide. The EC_{50} for adriamycin and ceramide was elevated 11- and 5-fold, respectively. Moreover, ceramide resistance was closely correlated with the induced expression of GCS mRNA and GCS enzyme activity. Several studies have shown that the effects of therapeutic doses of adriamycin and daunorubicin are related to the elevation of cellular ceramide (9, 11–13). In this study, we confirmed that adriamycin treatment elicits an increase in cellular ceramide and that induced GCS activity catalyzes the removal of ceramide via glycosylation (Fig. 4). Adriamycin resistance conferred by GCS transfection and expression gives substantially direct evidence for the relationship of ceramide to anthracycline action.

Among the causes of multidrug resistance, P-glycoprotein is the most widely studied (32, 33). P-glycoprotein is an ATP-dependent transporter involved in removal of drug from the cells by efflux pumping (32, 33). It is highly expressed in MCF-7 adriamycin-resistant cells; however, we did not observe elevation of P-glycoprotein in MCF-7/GCS. This implies that in MCF-7/GCS cells, adriamycin resistance induced by GCS was independent of P-glycoprotein. In the ceramide signal transduction pathway of apoptosis, increased Bcl-2, especially dephosphorylated Bcl-2, has strong anti-apoptosis effects (31, 34–37). As analyzed by Western blot, we did not find a significant alteration in Bcl-2 expression and/or phosphorylation/dephosphorylation status in MCF-7/GCS cells. Thus, drug resistance in MCF-7/GCS cells is also divorced from Bcl-2 involvement.

Apoptosis can be induced by cytokines, ionizing radiation, and anticancer agents (4, 5). Ceramide has been shown to mediate apoptosis, acting a cellular second messenger in myriad signal transduction cascades (3–5). Extracellular agents, such as calcitriol, TNF-α, γ-interferon, and interleukin-1 promote ceramide production by hydrolysis of sphingomyelin (4–6). Anthracycline anticancer drugs and the cyclosporine MDR modulator SDZ PSC 833 also mediate cell killing through enhancing the generation of cellular ceramide (9, 11–13, 38, 39). Cell-permeable ceramide analogs can directly induce apoptosis in U937, P388, HL-60, MCF-7, and BL 30A Burkitt's lymphoma cells (6, 9, 11, 13, 40, 41). Ionizing radiation also elicits ceramide production and initiates apoptosis (6, 8, 10, 42); however, loss of ceramide production has been shown to confer resistance to radiation-induced apoptosis (6, 8, 10). Ceramide glycosylation catalyzed by GCS is an effective mechanism for lowering elevations in ceramide caused by exogenous agents. GCS activity contributes to lessening the potential cytotoxic effects of high ceramide, as shown in the experiments in which MCF-7/GCS cells were challenged with either adriamycin or C_6-ceramide.

In addition to accumulation in MDR cells, GC has been shown to exert a regulatory role in cell proliferation (43–46). Administration of conduritol B epoxide, a β-glucocerobrosidase inhibitor, causes an elevation in cellular GC and tissue hyperplasia in mice (43, 44). Intraperitoneal or intracutaneous injection of emulsified GC into mice induces liver hypertrophy and epidermal proliferation (43, 44). These data suggest that elevated GC stimulates hepatocyte proliferation and epidermal mitogenesis through a relatively direct mechanism. Lowering of GC by use of the GCS inhibitor, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, or by inhibiting GC hydrolysis results in proliferation of Madin-Darby canine kidney and other cell types (45). Depletion of endogenous GC causes a cell cycle arrest (46). Only a slight increase in GC was observed in MCF-7/GCS cells compared with MCF-7 cells. This suggests that GC does not contribute to drug resistance; however, we do not know the exact role of GC in adriamycin resistance exhibited in MCF-7/GCS cells. With the direct evidence presented in this paper, we postulate that GCS activity is one of the causes of cellular resistance to adriamycin and resistance to ceramide. The glycosylation of ceramide, in response to *de novo* generation of ceramide elicited by adriamycin treatment, attenuated the cytotoxic response.

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