Possible Role of Ceramide as an Indicator of Chemoresistance: Decrease of the Ceramide Content via Activation of Glucosylceramide Synthase and Sphingomyelin Synthase in Chemoresistant Leukemia[1]

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ABSTRACT

We investigated the possibility of the proapoptotic lipid ceramide as an indicator of chemoresistance in leukemia. Doxorubicin (DOX) increased the ceramide level and apoptosis in drug-sensitive HL-60 cells but not in drug-resistant HL-60/ADR cells, under the condition that the uptake of DOX was not different between the two cell lines. In addition, exogenous *N*-acetylsphingosine (C2-ceramide) enhanced DOX-induced apoptosis in HL-60/ADR cells without affecting the expression of multidrug resistant-1 protein (MDR 1) and the uptake of DOX. A lower level of ceramide with higher activities of glucosylceramide synthase (GCS) and sphingomyelin synthase (SMS) was detected in HL-60/ADR cells than in HL-60 cells. In contrast, HL-60/GCS cells, overexpressing GCS, significantly inhibited DOX-induced ceramide increase and apoptosis. These observations suggest the involvement of ceramide regulation in drug resistance of leukemia cells. *In vivo*, the level of ceramide was lower in chemoresistant leukemia patients (6.4 ± 1.8 pmol/nmol phosphate; *n* = 14) than in chemosensitive patients (9.5 ± 2.7 pmol/nmol phosphate; *n* = 9), and the activities of GCS and SMS were more than 2-fold higher in chemoresistant leukemia cells than in chemosensitive cells. MDR-1 protein was faintly expressed in one of four chemoresistant patients, but Bcl-2 were clearly detected in four patients. Therefore, it is suggested that a decrease of the ceramide level via activation of GCS and SMS is associated with the chemoresistant condition in leukemia, probably in relation to Bcl-2 but not to MDR-1 expression.

INTRODUCTION

In the past decade, significant progress in chemotherapy to treat leukemia has led to the achievement of 5-year disease-free survival in more than 50% of treated patients (1–3). However, even among patients treated with allogeneic bone marrow transplantation after high-dose chemotherapy, a large number of cases are still unable to be completely cured. Resistance to anticancer drugs is a critical mechanism by which the outcome in patients with leukemia is affected (4). The MDR[3] phenotype caused by overexpression of the *mdr-1* gene has been intensively investigated as a cause of chemoresistance (5–7), but the clinical implications of a drug-efflux system are still controversial in patients with leukemia (8–13). Leith *et al.* (14, 15) recently showed that in elderly patients with AML, the expression of the *mdr-1* gene was significantly higher than in younger patients with AML and that, although CR rate in the patients with AML was negatively associated with expression of the *mdr-1* gene, overall or disease-free survival was not affected by this expression. In addition, one randomized study using cyclosporine A suggested a survival advantage to block the efflux function of MDR-1, but many clinical studies to recover chemosensitivity have been limited by exacerbating the toxicities of chemotherapy (16). Therefore, to improve the outcome in patients with refractory leukemia, other kinds of novel mechanisms for overcoming MDR should be taken into consideration.

Thus, even if anticancer drugs reach their sites of action, some cells still survive via the inhibition of proapoptotic signaling (17). It has been well known *in vitro* and *in vivo* that multidrug-resistant cells show cross-resistance not only to chemical drugs but also to proapoptotic stresses including anti-Fas antibody cross-linking, tumor necrosis factor a, irradiation, and serum starvation in a manner not related to the drug-efflux

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[3] The abbreviations used are: MDR, multidrug resistance; C2-ceramide, *N*-acetylceramide; AML, acute myelogenous leukemia; CR, complete remission; GlcCer, glucosylceramide; GCS, GlcCer synthase; SM, sphingomyelin; SMS, SM synthase; CML, chronic myelogenous leukemia; ACL, acute lymphocytic leukemia; TLC, thin-layer chromatography; DAPI, 4′,6 diamidine-2′-phenylindole dihydrochloride; FACS, fluorescence-activated cell sorter; cs-L, chemosensitive leukemia; cr-L, chemoresistant leukemia; BC, blastic crisis; AL, acute leukemia; DAG, diacylglycerol; NBD, 6-(N-(7-nitrobeng-2-oxa-1,3-diazol-4-yl)amine); DGK, diacylglycerol kinase.

Table 1 Characteristics and outcome of chemosensitive and chemoresistant patients with leukemia

Patient no.	Age (yr)	Disease	Stage	Blast (%)	Prior chemotherapy	Outcome (2 mo after examination)
Chemosensitive cases						
1	25	AML(M$_2$)	Fresh	83	B^{a}DMP	CR
2	84	AML(M$_1$)	Fresh	100	BDMP	CR
3	53	AML(M$_1$)	Fresh	92	DCMP	CR
4	54	AML(M$_1$)	Fresh	90	DCMP	CR
5	55	AML(M$_5$)	Fresh	78	Alda	CR
6	82	AML(M$_5$)	Fresh	93	Alda	CR
7	76	AML(M$_0$)	Fresh	94	Alda	CR
8	81	ALL	Fresh	90	BDOMPCy	CR
9	62	ALL	Fresh	94	BDOMPCy	CR
10	36	ALL	Fresh	94	BDOMPCy	CR
11	23	ALL	Fresh	93	BDOMPCy	CR
12	56	AML(M$_5$)	Relapse	67	Alda	CR
13	21	AML(M$_2$)	Relapse	77	Alda	CR
14	20	ALL	Relapse	96	BDOMPCy	CR
Chemoresistant cases						
1	56	AML(M$_5$)	Refractory	98	BDMP, MitEC, Ida, *etc.*	Death
2	68	AML(M$_2$)	Refractory	90	BDMP, Ida+E, Ida+C, EAC, *etc.*	Death
3	29	AML(M$_4$)	Refractory	93	BDMP, Ida, V, C11, Ac, EAMit, *etc.*	Death
4	27	ALL	Refractory	92	BDOMPCy, ODMit, EOP, *etc.*	Death
5	47	ALL	Refractory	90	CyODOX × 2, BmitE × 2, highDA, *etc.*	Death
6	35	ALL	Refractory	100	TVP, BmitEP, highDA, BmitEP, *etc.*	Death
7	56	CML	BC	73	H+IFNα, PHO+E, *etc.*	Death
8	70	CML	BC	76	Bu, H, MH, APMit, VIP, *etc.*	Death
9	19	CML	BC	81	Bu, ODOXP, H+IFNα, OdoAMeP, *etc.*	Death

[a] B, enocitabin; D, daunorubicin; M, mercaptopurine; P, prednisolone; A, cytarabine; B, bleomycin; C, dacarbazine; C11, irinotecan; Cy, cyclophosphamide; E, etoposide; Ida, idarubicin; H, hydroxyurea; Me, methotrexate; Mi, mitoxanthrone; O, vincristine; T, pirarubicin.

system (18). These suggest the possibility that dysregulation of apoptotic signaling plays a more substantial role in causing MDR than an increase of drug-efflux function in leukemia cells (19).

What kinds of apoptosis-inducing molecules are involved in the mechanisms of chemoresistance? Mutation of the *p-53* gene and an increase of Bcl-2 expression were reported to be prognostic indicators of poor clinical outcome in AML (20, 21), although a final conclusion about clinical implications of bcl-2 in drug-resistant leukemia is still controversial (16, 22). Proapoptotic sphingolipid ceramide-induced cell differentiation and apoptosis (23–27) and the levels of intracellular ceramide were shown to be increased by anticancer drugs and stresses in leukemia cells (25, 28). Recently, a decrease of the ceramide content was found in drug-resistant breast cancer MCF-7 cells because of enhancement of the activity of GCS, which converts ceramide to GlcCer by adding a glucose moiety (29). We also suggested that not only GCS but also another ceramide-metabolizing enzyme, SMS, which generates SM from ceramide and phosphatidylcholine, are closely involved in the induction of apoptosis under the drug-resistant condition because increasing the ceramide content by simultaneous inhibition of GCS and SMS restored drug susceptibility in drug-resistant human leukemia HL-60/vinc cells (30).

Therefore, to test the clinical implication of ceramide as an indicator of chemoresistance, we investigated, here, whether the levels of intracellular ceramide and the activities of GCS and SMS were related to the chemoresistant condition of leukemia cells *in vivo* as well as *in vitro*. We found that not only in drug-resistant HL-60/ADR cells but also in the patients with cr-L, the levels of intracellular ceramide content were sustained lower than in drug-sensitive cells and chemosensitive patients because of increased activities of GCS and SMS, and it is discussed whether the regulation of ceramide might be related with other drug-resistant factors including MDR-1 and Bcl-2 under drug-resistant or chemoresistant condition.

MATERIALS AND METHODS

Cell Culture and Reagents. Human leukemia HL-60 cells and HL-60/ADR cells were kindly provided by Dr. M. Saito (National Cancer Institute, Tokyo, Japan) and Dr. M. S. Center (Kansas State University, Kansas City, KS), respectively (31). The cells were maintained in RPMI 1640 containing 10% fetal bovine serum and kanamycin sulfate (80 ng/ml) at 37°C in a 5% CO2 incubator. HL-60 cells in the exponential growth phase were resuspended in 10% serum-containing medium at an initial concentration of 2.75×10^5 cells/ml, and then treated as indicated in the text. Viable cell numbers were assessed by the 0.025% trypan blue dye exclusion method under microscopic observation. C2-ceramide was purchased from Matreya, Inc. [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Other chemicals, if not mentioned, were obtained from Sigma Co. LTD.

Patient Materials. The patients were admitted to Kyoto University Hospital or other hospitals and referred to us for characterization of leukemia from 1998 to 1999. As shown in Table 1, our study of the ceramide content, and the activities of GCS and SMS was performed in a total of 23 cases including 12 cases of AML, 8 cases of ALL, and 3 cases of CML. The clinical outcome for anticancer drugs was determined after the

first course of induction therapy at the time of diagnosis or of the first relapse, or after the several courses of intensive chemotherapy. Accordingly, 14 cases were subgrouped into cs-L based on the following criteria for CR: marrow with <5% leukemic blasts concomitant with a neutrophil count of >1000/µl; and a platelet count of >100,000/µl. Nine of the refractory leukemia cases were determined as chemoresistant ones when the patients did not get into a CR state and died within 2 months after the final chemotherapy. After informed consent was obtained from each patient, the cells from bone marrow or peripheral blood were collected before chemotherapy (for chemosensitive cases) or after the recovering time from the nadir period by chemotherapy (for chemoresistant cases). The average percentage of leukemic blasts in the specimen was 88 ± 8%. Mononuclear cells were collected by density gradient centrifugation (Ficoll-Paque) and washed twice in PBS as described in the manufacturer's protocol. The cells were frozen at −80°C until the following examinations were performed.

Intracellular Ceramide Measurement. The cells were seeded at a cell density of 2.75×10^5/ml in 2 ml of RPMI 1640 supplemented with 10% fetal bovine serum in 30-mm dishes and cultured at 37°C for 24 h. Then, the media were replaced with serum-free media containing 2 µl of L-[U-^{14}C]serine (150 µCi/mmol/100 µl), and the cells were incubated and harvested at various times up to 48 h. The mixture of cells and supernatant was centrifuged at 3000 rpm for 5 min. Lipids were extracted from the pelleted cells by the Bligh-Dyer method and separated on TLC plates with a solvent system containing methylacetate/propanol-1/chloroform/methanol/0.25% KCl (25/25/25/10/9; v/v/v/v/v). Radioactive lipids on the TLC plates were quantitated by using a BAS2000 Image Analyzer (Fuji).

Ceramide mass measurement was performed by using the *Escherichia coli* DAG kinase method as described elsewhere (23). Ceramide and DAG were converted to ceramide-1-phosphate and phosphatidic acid, respectively, then separated on TLC plates in a solvent system containing chloroform/acetone/methanol/acetic acid/H_2O [10:4:3:2:1 (v/v/v/v/v)].

GCS and SMS Assays. The cells were lysed by passing through a 27-gauge needle in a buffer containing 20 mM Tris/HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2.5 µg/ml leupeptin. Complete cell lysis was confirmed by microscopic examination. The lysate was centrifuged at $800 \times g$ for 5 min at 4°C. The supernatant was collected and used as the whole-cell extract. Protein concentrations were determined by using a Protein Assay kit (Bio-Rad). Fifty µg of protein was added to a reaction buffer [250 µM UDP-glucose, 5 mM Tris/HCl (pH 7.5), 500 µM EDTA, 10 µg/ml C_6-NBD-ceramide, and 100 µg/ml phosphatidylcholine; total volume, 100 µl] and incubated at 37°C for 2 h. The reaction was stopped by the addition of 400 µl of H_2O and 1 ml of chloroform/methanol (2:1, v/v), mixed well and centrifuged. The lower phase was collected, and the solvent was evaporated. Aliquots were applied to TLC plates, and the reaction products were resolved in a solvent system that contained chloroform/methanol/12 mM $MgCl_2$ in H_2O (65:25:4, v/v/v). C_6-NBD GlcCer and SM were visualized by UV illumination and were measured by using a TLC scanner with a fluorometer (excitation = 475 nm, emission = 525 nm). The rate of flow values of ceramide, GlcCer, and SM were 0.75, 0.53, and 0.07, respectively.

Western Blotting Analysis. The samples (50 µg) were denatured by boiling in Laemmli's sample buffer for 5 min, subjected to SDS-PAGE using a 7.5% running gel, and electroblotted to Immobilon-P Transfer Membrane (Millipore) (26). Nonspecific binding was blocked by incubation of the membrane with PBS containing 5% skim milk and 0.1% Tween 20 for more than 1 h. Then the membrane was washed in PBS containing 0.1% Tween 20 (PBS-T) for 15 and 5 min, and incubated with a 1: 200 dilution of anti-MRP antibody in PBS-T for 1 h. The membrane was washed in PBS-T for 15 and 5 min, and incubated with 1:4000 dilution of antimouse or antirat immunoglobulin peroxidase conjugate in PBS-T for 1 h. After washing the membrane three times for 5 min each time in PBS-T, detection was performed using ECL Western blotting detection reagents (Amersham) according to the manufacturer's protocol (26).

Detection of Apoptosis by DAPI. Changes in cellular nuclear chromatin, were evaluated by staining with DAPI. The cells were washed and fixed with 1% glutaraldehyde for 30 m, and labeled with 2 µg/ml DAPI. After labeling, apoptotic cells were visualized with a fluorescent microscope (BX60–34FFB1; Olympus). At least 200 cells in one determination were counted.

FACS Analysis Using Propidium Iodide Staining. Flowcytometric DNA analyses were performed for measuring cell death by apoptosis. Because of DNA degeneration and subsequent leakage from cells, apoptotic cell could be detected by diminished staining with DNA-specific fluorochromes. In brief, 2.5×10^6 cells were harvested, washed with PBS, and resuspended in PBS containing 0.5% paraformaldehyde and 0.5% saponin for fixation of cells. The cells were then washed and resuspended in fluorochrome solution containing 50 µg/ml propidium iodide and 1 mg/ml RNase (Bachem California, Torrance, CA). Red fluorescence was measured with a FACScan (Becton Dickinson Advance Cellular Biology, San Jose, CA). We assessed the number of subdiploid cells (apoptotic cells) and cell cycle phases with more than diploid DNA content (nonapoptotic cells).

Measurement of Intracellular DOX concentration. For determination of the intracellular DOX concentration, aliquots of cell suspension were cooled on ice and then centrifuged at $150 \times g$ for 3 min. The cells were washed and resuspended in 1.0 ml of ice-cold PBS, and then mixed for 30 s with 5.0 ml of chloroform-methanol (4:1, v/v) and centrifuged at $1200 \times g$ for 15 min. The concentration of drug in the organic phase was determined with a fluorescence spectrophotometer, Hitachi F3000 (Hitachi Ltd., Tokyo; excitation, 470 nm; emission, 585 nm; Ref. 32).

Expression Vector Construction and Transient Transfection. The full-length cDNA of human GCS gene *GluT-1*, which was cloned by expression cloning using monoclonal antibody M2590 from a human melanoma cell library, was subcloned to pCG-1 as described previously (33). HL-60 cells were transfected with the vector by electroporation method (GenePulser; Bio-Rad; 320 V, 950 µF) as described elsewhere (34). The transfected cells were kept in 10% FCS-containing media at least 48 h after transfection and then treated with C2-ceramide or DOX.

Detection of Human GCS mRNA by Reverse Transcription-PCR. Total RNA was extracted from wild HL60 cells, transfected HL60 cells with mock vector, and GCS cDNA vector using TRlzol (Life Technologies, Inc., Japan). The purified total RNA (4 μg) was reverse transcribed into cDNA using the First-strand cDNA Synthesis kit (Pharmacia). The final volume of the cDNA synthesis reaction was 33 μl. The reverse-transcribed sample (1 μl) was used for a 40 μl of PCR reaction. The PCR primers used were hGCS-sense, 5'-TTTTTCATGT-GTCATTGCCTGGCA-3', and human Glc T-antisense, 5'-TCTAGGATTTCCTCTGCTGTACCC-3'. Primers for amplification of β-actin cDNA were also used so that β-actin cDNA could be used as an internal control. PCR was performed with an initial denaturation step of 1 min at 95°C, and then 25 cycles were run as follows: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C. The reaction products were electrophoresed on an agarose gel.

Analysis of Statistical Significance. Results were shown as means ± 1 SD of more than three experiments, and significance was determined by Student's t test if not described specifically.

RESULTS

Apoptosis and Ceramide Increase Induced by DOX, and Uptake of DOX in Drug-Sensitive HL-60 Cells and Drug-Resistant HL-60/ADR Cells. When the cells were treated with 0.1 μM DOX for 24 h, the proportions of DAPI-positive cells were 33% and 2% in HL-60 and HL-60/ADR cells, respectively (Fig. 1, A and B). To eliminate the possibility that the lack of uptake of DOX in HL-60/ADR cells caused no induction of apoptosis, we measured the concentrations of DOX in the cells after the treatment with various concentrations of DOX. As shown in Fig. 1C, the content of DOX was not significantly different after treatment with 0.1 μM DOX for 24 h in HL-60 and HL-60/ADR cells, although at higher concentrations, DOX uptake was reduced in HL-60/ADR cells. The ceramide levels, which were measured by the [^{14}C]serine labeling method, were increased in HL-60 cells in a dose-dependent manner, but not in HL-60/ADR cells (Fig. 1D). These results suggest that regulation of intracellular ceramide is closely related to drug resistance and DOX-induced apoptosis.

Enhancement by C2-ceramide of DOX-induced Apoptosis without Affecting MDR-1 Expression and DOX Uptake in HL-60/ADR Cells. In HL-60/ADR cells, the addition of various concentrations of C2-ceramide simultaneously with 0.1 μM DOX enhanced the induction of apoptosis as judged by the DAPI method in a dose-dependent manner. As shown in Fig. 2A, simultaneous treatment with 5, 10, and 20 μM C2-ceramide and 0.1 μM DOX increased the percentage of apoptotic cells from 8% to 13%, 18%, and 34%, respectively, in HL-60/ADR cells when C2-ceramide alone at concentrations of 5, 10, and 20 μM induced 4, 6, and 16% of apoptosis, respectively. Next, we examined whether C2-ceramide affected the expression of MDR-1 to increase DOX levels in the cells. As shown in Fig. 2B, MDR-1 expression was not inhibited by 20 μM C2-ceramide. In fact, when we measured DOX concentrations after the addition of C2-ceramide, the uptake of DOX was not changed. These results suggest that ceramide action may overcome the
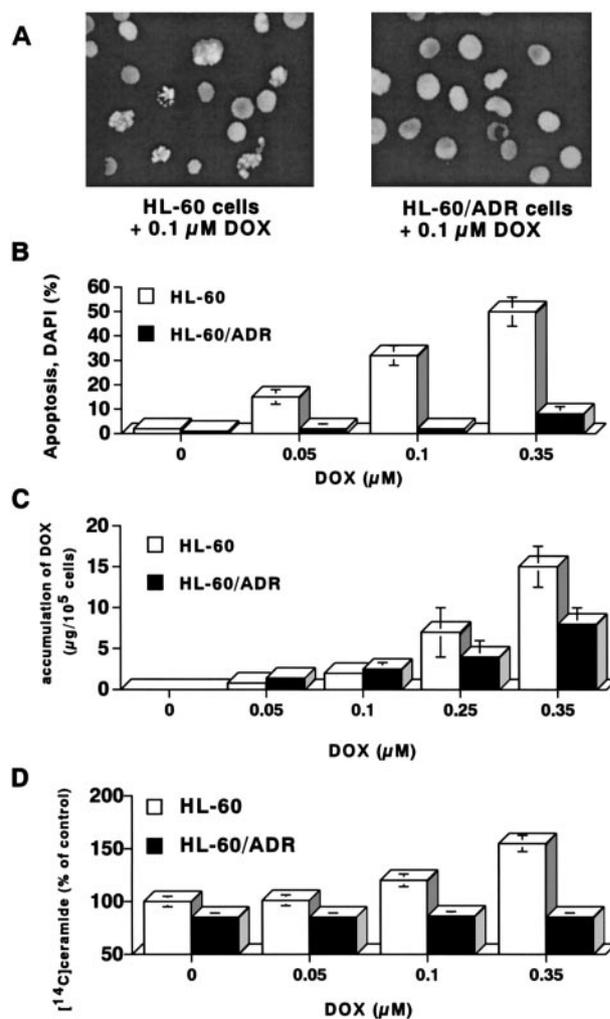

Fig. 1 Apoptosis and an increase of the ceramide level induced by various concentrations of DOX and uptake of DOX in HL-60 and HL-60/ADR cells. *A* and *B*, the cells were treated with various concentrations of DOX for 24 h and then stained by the DAPI method. The cells with nuclear fragmentation and condensation were counted as apoptotic cells under a fluorescent microscope. *C* and *D*, after labeling the cells with or without [^{14}C]serine for 24 h, they were treated with various concentrations of DOX for 24 h. Then, uptake of DOX and [^{14}C]ceramide were measured as described in "Materials and Methods."

drug resistance without an increase of intracellular DOX concentration via MDR-1 in HL-60/ADR cells.

Ceramide Level and the Activities of GCS and SMS in HL-60/ADR and HL-60 Cells. In addition to the measurement of ceramide using the [^{14}C]serine labeling method, we used a DAG kinase assay to determine the mass of ceramide. As shown in Fig. 3A, the basal level of ceramide in HL-60/ADR cells (6.0 ± 0.4 pmol/nmol phosphate) was significantly lower than that in HL-60 cells (7.1 ± 0.3 pmol/nmol phosphate), which agreed well with the data in Fig. 1D showing that the level of [^{14}C]ceramide in HL-60/ADR cells was ~80% of that in HL-60 cells. We examined whether ceramide-synthesizing enzymes, neutral sphingomyelinase (N-SMase) and acid sphingomyelinase (A-SMase), or ceramide-metabolizing enzymes
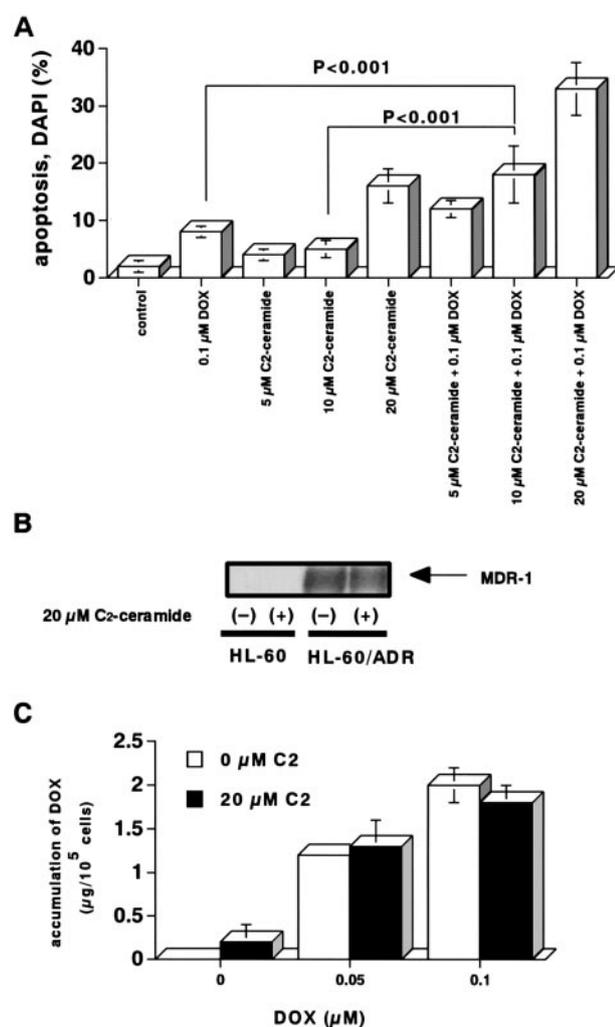
Fig. 3 Lower level of ceramide and higher activities of GCS and SMS in HL-60/ADR cells than in HL-60 cells. Lipids and proteins were extracted from the cells without treatment as described in "Materials and Methods." The content of ceramide was measured by converting ceramide to ceramide-1-phosphate according to the protocol for the DAG kinase assay (*A*) and the activities of SMase (*B*), GCS, SMS, and ceramidase (C) were determined as described in "Materials and Methods."

Fig. 2 Enhancement of DOX-induced apoptosis by C2-ceramide without affecting MDR-1 expression and uptake of DOX in HL-60/ADR cells. The cells were treated simultaneously with or without 0.1 μM DOX and the indicated concentrations of C2-ceramide. After incubation for 24 h the proportion of apoptotic cells (*A*), expression of MDR-1 protein (*B*), and uptake of DOX (*C*) were examined by the DAPI staining method, Western blotting analysis and fluorescence spectrophotometry, respectively, as described in "Materials and Methods."

such as GCS, SMS, and ceramidase, were involved in a decrease of the ceramide level in HL-60/ADR cells. The results showed no differences of the activities of N-SMase, A-SMase, and ceramidase between HL-60 and HL-60/ADR cells (Fig. 3*B*), but the activities of SMS and GCS were 2.2-fold and 2.0-fold higher, respectively, in HL-60/ADR cells as compared with HL-60 cells (Fig. 3*C*), suggesting that an increase of SMS and GCS is involved in a decrease of the ceramide level in drug-resistant leukemia cells.

Overexpression of GCS Induced the Resistance to DOX-caused Ceramide Increase and Apoptosis in HL-60 Cells. To confirm the direct involvement of ceramide and its regulation in the development of drug resistance, we examined whether overexpression of GCS in HL-60 cells affects DOX-
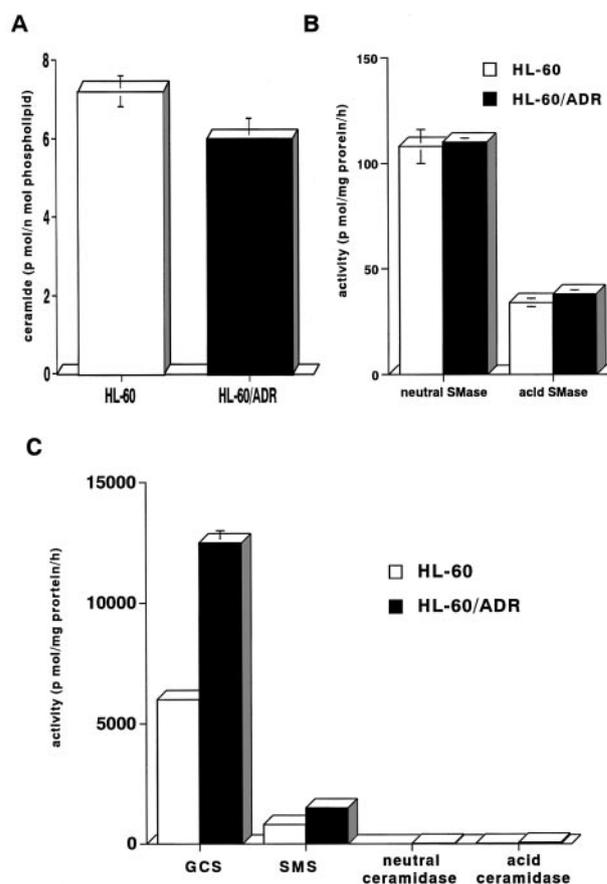
induced apoptosis. Transfected HL-60/GCS cells showed a significantly higher level of expression of GCS mRNA than did wild HL-60 and HL-60/Mock cells when the levels of actin mRNA were equally detected (Fig. 4*A*). Ceramide level in HL-60/GCS cells was slightly lower than that in HL-60 cells and HL-60/Mock cells. Treatment with 0.35 μM DOX increased the ceramide level from 7.5 to 11.8 pmol/nmol phosphate and apoptosis from 17 to 67% in HL-60/Mock cells as compared with no DOX treatment (Fig. 4, *B* and *C*). However, the same treatment of DOX induced no increase of ceramide level and only 20% increase of apoptosis in HL-60/GCS cells (Fig. 4, *B* and *C*). These results suggest that drug-sensitive cells acquired the resistant condition to DOX-caused apoptosis by sustaining a lower ceramide level through the activation of GCS.

Patients Overall. To investigate the involvement of ceramide and its metabolic regulation in chemoresistance *in vivo*, we next examined whether the levels of ceramide, SMS, and GCS were different among the patients with cs-L and those with cr-L. The patients with leukemia were subgrouped into cs-L and
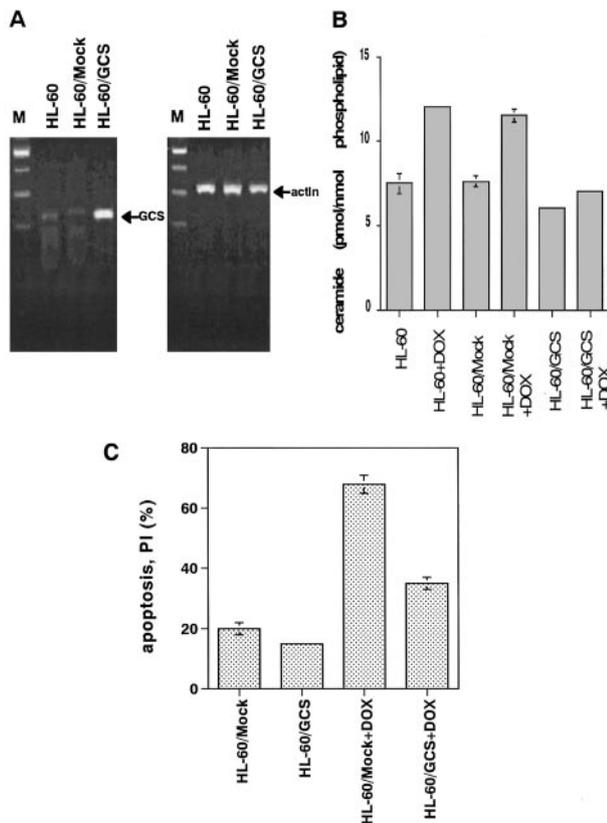
Fig. 4 Overexpression of GCS in HL-60 cells inhibited DOX-caused ceramide increase and induced the resistance to DOX. The cells were transfected with pCG1 subcloned from cDNA of human GCS gene *GluT-1* by electroporation method as described in "Materials and Methods." The expressions of *GluT-1* and the β-*actin* gene were confirmed by the reverse transcription-PCR method (*A*), and ceramide levels were measured by DGK assay method (*B*) in the transfected cells with GCS gene (*HL-60/GCS*), in the control cells transfected by the vector (*HL-60/Mock*), and in the parent cells (*HL-60*). HL-60/GCS cells were treated with 0.35 μM DOX for 24 h, and then FACS analysis using propidium iodide staining was performed for apoptosis measurement (*C*). Data in Fig. 6, *A* and *C*, were representative of three independent experiments.

cr-L groups according to the criteria for CR and chemoresistant state as described in "Materials and Methods." As shown in Table 1, the cs-L group showed a first CR after one course of chemotherapy, but the cr-L group died within 2 months after a final chemotherapy and postnadir sampling of leukemia cells. We obtained the specimens from 14 cs-L cases, including 11 freshly diagnosed ALs and 3 relapsed ALs, and from 9 cr-L cases, including 6 refractory ALs and 3 BCs (Table 1). As shown in Figs. 5*A* and 6*A*, the intracellular ceramide levels in cs-L ($n = 14$) and cr-L ($n = 9$) were 9.5 ± 2.7 and 6.4 ± 1.8 pmol/nmol phospholipid, respectively. The percentage of blast cells in cs-L (87%) was similar to that in cr-L (88%). These results showed that ceramide levels were lower in the blasts of chemoresistant cases than in those of chemosensitive cases (Fig. 5*A*) and suggest that the resistance to several anticancer drug-based chemotherapies (Table 1) is linked to the lower levels of ceramide in the leukemic cells. The levels of GCS (0.61 ± 0.27

nmol/mg protein/h) and SMS (0.11 ± 0.05 nmol/mg protein/h) in cr-L cases ($n = 9$) were significantly higher ($P < 0.001$ and <0.004, respectively) than those (0.22 ± 0.12 nmol/mg protein/h and 0.044 ± 0.013 nmol/mg protein/h, respectively) in cs-L cases ($n = 14$; Fig. 4, *B* and *C*), suggesting that an increase of GCS and SMS was involved in a decrease of ceramide level *in vivo* in chemoresistance cases as well as *in vitro*.

To examine whether lower ceramide levels of leukemia cells in chemoresistant cases are related to other determinants of drug resistance, we compared the levels of ceramide with those of MDR-1 and Bcl-2 protein. As shown in Fig. 6, among four chemoresistant cases (cases 3, 4, 8, and 9), in which ceramide levels were lower than among the chemosensitive cases, only case 4 showed MDR-1 expression, and all four of the cases showed prominent Bcl-2 expression. Because the number of cases examined here was small, it remains to be confirmed that there is a relationship between lower levels of ceramide and other drug-resistant factors, but these results, at least in part, suggest that the lower content of ceramide in chemoresistant cases may correlate with the levels of Bcl-2, although not with those of MDR-1.

DISCUSSION

Ceramide is recognized as a proapoptotic lipid mediator because apoptosis-inducing stresses such as anticancer drugs, tumor necrosis factor α, anti-Fas antibody, heat shock, and irradiation increase the level of intracellular ceramide (28). Ceramide has been reported to enhance other proapoptotic molecules such as NH_2-terminal jun kinase, caspase-3, and reactive oxygen species (35, 36) and to suppress antiapoptotic molecules such as heat shock protein-70 (HSP-70) and phosphatidylinositol 3′-kinase (37, 38). Thus, although ceramide-related signals are suggested to be involved in drug resistance (39, 40), the effects of ceramide on the drug-efflux pump, MDR-1, have never been investigated in drug-resistant cells.

We here showed that HL-60/ADR cells that are resistant to the treatment with 0.1 μM DOX showed a faint apoptosis as a result of a diminished increase of the ceramide content because of activation of the ceramide-metabolizing enzymes SMS and GCS, whereas the same concentration of DOX induced HL-60 cell apoptosis with an increase of ceramide content (Figs. 1 and 3). Under this condition, intracellular concentration of DOX was not significantly different between HL-60 and HL/ADR cells, suggesting that the existence of a drug-resistant mechanism is related to ceramide action regardless of a drug-efflux pump like MDR-1. In fact, C2-ceramide enhanced drug susceptibility of HL-60/ADR to DOX without affecting MDR-1 expression and the uptake of DOX (Fig. 2).

Previously, Cabot *et al.* showed *in vitro* that an increase of GlcCer activity and a decrease of the ceramide level were detected in drug-resistant breast cancer cells, but not in wild-type cells, and that overexpression of the *GCS* gene conferred drug resistance to DOX without affecting the level of Bcl-2 protein (29, 41). However, there is no report showing the relationship of ceramide content, its metabolizing enzymes, and clinical chemoresistance in leukemia cells. As shown in Figs. 5*A* and 6*A*, *in vivo* ceramide levels were shown to be significantly lower in cr-L patients (6.4 pmol/nmol phospholipid), who died
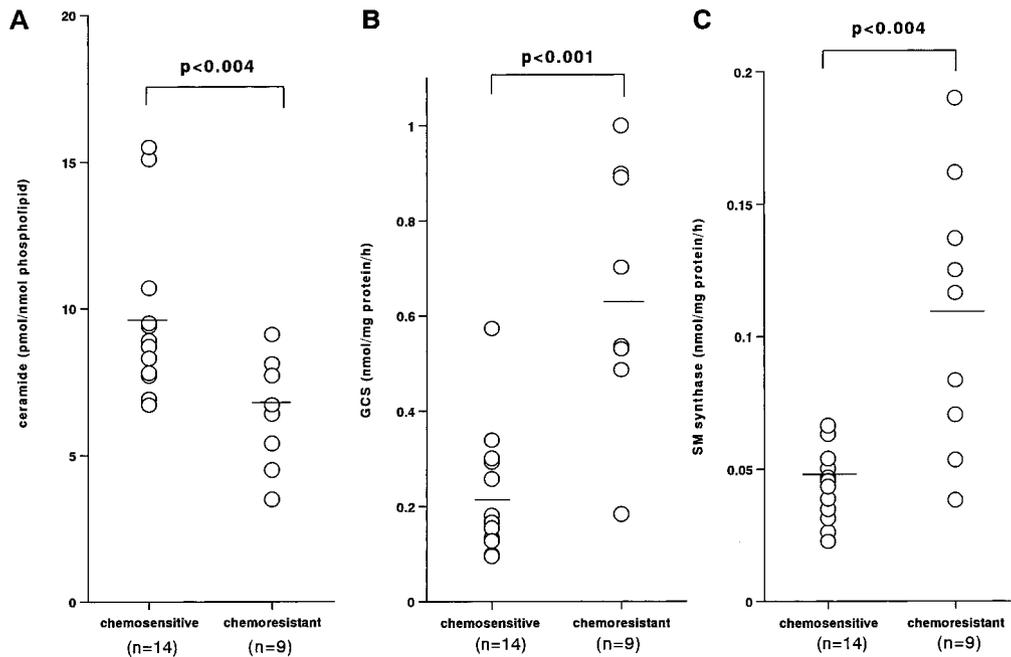
Fig. 5 Levels of the ceramide content, and activities of GCS and SMS in chemosensitive and cr-L patients. Fourteen patients in the chemosensitive condition (9 cases of AML and 5 cases of ALL) and 9 patients in chemoresistant condition (3 cases each of AML, ALL, and CML in BC) were enrolled as shown in Table 1. The mononuclear cells consisting mainly of leukemia blasts, the average proportion of which was ~88% for chemosensitive cases and 87% for chemoresistant cases, were separated from the specimens of peripheral blood or bone marrow; and ceramide content (*A*), GCS activity (*B*), and SMS activity (*C*) in the cells were examined as described in "Materials and Methods."

of leukemia progression because of chemoresistance within 2 months after sampling the specimen, as compared with those in cs-L patients (9.5 pmol/nmol phospholipid), who achieved CR after a first induction chemotherapy. Moreover, the activities of GCS and SMS in cs-L (0.22 nmol/mg protein/h and 0.044 nmol/mg protein/h, respectively) were much lower in cr-L (0.61 nmol/mg protein/h for GCS and 0.11 nmol/mg protein/h for SMS; Fig. 5, *B* and *C*). These data suggest that the suppression of the ceramide content is attributable to the activation of GCS and SMS in apoptosis-resistant cells *in vivo* as well as *in vitro*. This notion was confirmed by the results in Fig. 4 showing that the drug-sensitive leukemia cells acquired the drug-resistant condition by suppression of DOX-increased ceramide level because of overexpression of GCS.

In general, apoptosis is regulated by the balance between proapoptotic and antiapoptotic signals. Caspases, p-53, BAX, BCL-XS, BAD, and ceramide are well known intercellular proapoptotic molecules, whereas BCL-2, BCL-X$_L$, BCR-ABL, heat shock protein-70 (HSP-70), retinoblastoma protein (Rb), and phosphatidylinositol 3′-kinase are antiapoptotic molecules. It was reported that antisense RNA of bcl-2 increased the sensitivity of AML to 1-β-D-arabinofuranosylcytosine-induced apoptosis (42). In contrast, transfection of the *bcl-2* gene in neuroblastoma cells resulted in resistance to etoposide (43), and high expression of Bcl-2 was associated with a poor outcome of treatment in AML (21). Because Bcl-2 inhibited hypoxia-induced ceramide formation (35) and C2-ceramide was, in contrast, reported to reduce Bcl-2 levels (44), we examined the levels of Bcl-2 as well as MDR-1 in cs-L cases showing lower
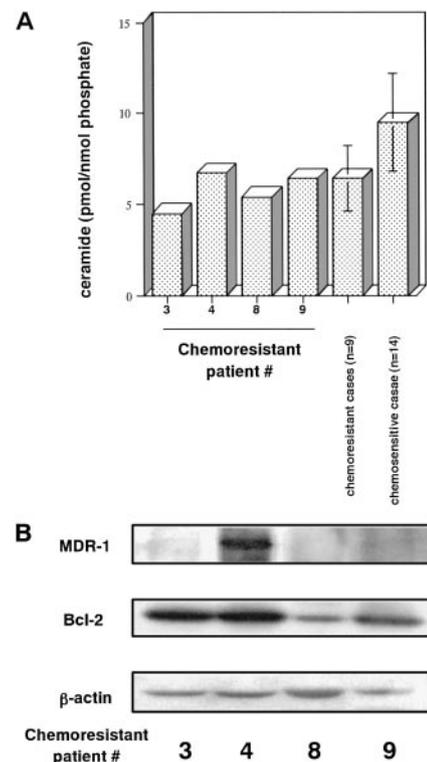

Fig. 6 Ceramide levels and expressions of MDR-1 and Bcl-2 in cr-L cells. Ceramide levels of the leukemia cells were measured as described in Fig. 5. *A*, expression of MDR-1, Bcl-2, and β-actin were detected by Western blotting analysis as described in "Materials and Methods." *B*, data are representative of three independent experiments.

ceramide levels than cr-L cases. As shown in Fig. 6, only one of four chemoresistant patients showed MDR-1 expression, and all four patients expressed *Bcl*II. Thus, the lower level of ceramide in cr-L cases may related to the expression of Bcl-2 but not to MDR-1 expression. Because ceramide formation and Bcl-2 expression are negatively related *in vitro* (35, 44), a decrease of ceramide may enhance Bcl-2 expression in cr-L as well.

In summary, these results suggest a novel possibility of antiapoptotic mechanisms in patients with refractory leukemia, *i.e.,* that ceramide content regulated by GCS and SMS plays an important role in the development of chemoresistance *in vivo* as well as in drug resistance *in vitro*. However, the number of samples studied here was too low to draw definitive conclusions: more clinical trials will be necessary to determine whether the ceramide content can be beneficial as a novel clinical indicator of chemoresistance in refractory leukemia.

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Possible Role of Ceramide as an Indicator of Chemoresistance: Decrease of the Ceramide Content via Activation of Glucosylceramide Synthase and Sphingomyelin Synthase in Chemoresistant Leukemia

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