EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 36, No. 5, 411-419, October 2004

# Enhancement of radiosensitivity by combined ceramide and dimethylsphingosine treatment in lung cancer cells

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Accepted 25 August 2004

Abbreviations: DMS, *N*, *N*-dimethyl-D-erythro-sphingosine; FACS, fluorescence-activated cell sorter; IR, irradiation; LLC, Lewis lung carcinoma; MMP, mitochondrial membrane potential ($\Delta\psi m$); PARP, poly-(ADP-ribose) polymerase; TUNEL, TDT-mediated dUTP-biotin nick-end labeling;

## Abstract

**Ceramide generated from sphingomyelin in response to ionizing radiation has been implicated as a second messenger to induce cellular pro-apoptotic signals. Both ceramide and its metabolic inhibitor, *N*, *N*-dimethyl-D-erythro-sphingosine (DMS), might lead to sustained ceramide accumulation in cells more efficiently, thereby sensitizing them to γ-radiation-induced cell death. To delineate this problem, the clonogenic survival of Lewis lung carcinoma (LLC) cells was evaluated following exposure to radiation together with or without C2-ceramide, DMS, or both. The treatment of ceramide/DMS synergistically decreased the survival of the irradiated cells compared with treatment with ceramide or DMS alone. Ceramide/DMS-treated cells displayed several apoptotic features after γ-irradiation, including increased sub $G_1$ population, TUNEL-positive fraction, and poly-(ADP-ribose) polymerase (PARP) cleavage. We also observed ceramide/DMS induced disruption of mitochondrial membrane potential (MMP) and activation of caspase-9 and -3 in a radiation-dose-dependent manner. Furthermore, pretreatment of LLC cells with ceramide/DMS not only increased the protein expression level of Bax, but also decreased Bcl-2 after γ-irradiation. Taken together, the present study indicates that the radiosensitizing activity of ceramide/DMS on LLC cells most likely reflects the dominance of pro-apoptotic signals related to the mitochondria-dependent pathway.**

**Keywords:** apoptosis; ceramide; DMS; radiosensitivity

## Introduction

Ceramide is a key signal-transducing lipid with a role in various regulatory pathways including differentiation, cell cycle arrest, and apoptosis (Cuvillier *et al.,* 2000; Sawada *et al.,* 2000), and induced by not only signals *via* cell-surface receptors for tumor necrosis factor (TNF) or Fas ligand (CD95/Fas/APO-1), but also various stress stimuli such as radiation, heat shock, the depletion of growth factors, and chemotherapeutic agents. The diversity of the ceramide-inducing extracellular stimuli indicates the function of ceramide as a common mediator of various apoptotic mechanisms (Wang *et al.,* 1998; Sawada *et al.,* 2000). In addition, the treatment with exogenous short chain homologues of ceramide mimics endogenously generated ceramide and induces apoptosis. However, the mechanisms that convey activation signals to the enzymes responsible for ceramide production are poorly defined, and the pivotal executioner of ceramide to the apoptotic response still remains arguable (Chmura *et al.,* 1997; Susin *et al.,* 1997; Edsall *et al.,* 1997, 1998; Cuvillier *et al.,* 1998; Kleuser *et al.,* 1998; Kolesnick *et al.,* 1998).

As an important treatment modality for lung cancer as well as many other cancers, the dose of radiation needed to efficiently remove tumor cells was very different (Zhivotovsky *et al.,* 1999; Chinnaiyan *et al.,* 2000). This vast difference of radiosensitivity is influenced by a complex interaction of several factors such as level of oxygen consumption, dividing rate of uncontrolled tumor cells, and ability of cells to repair the radiation-induced DNA damage. The importance of radiotherapy against cancer is especially highlighted, because most cancer patients receive the

radiotherapy at some point in their treatment (Zhivotovsky *et al.,* 1999), therefore, it is imperative to develop efficient radiosensitizers that increase the lethal effects of radiation. Since it is well known that the limitation of radiotherapy is its serious toxicity on normal cells at high dose and minimal therapeutic effect at low dose, identification of biological compounds to increase the sensitivity of tumor cells to radiation has the potential to significantly improve the efficacy of therapy (Dileonardo *et al.,* 1994; Haimovitz-Friedman *et al.,* 1994; Little, 1994; Maity *et al.,* 1997).

If a pool of intracellular ceramide elevates in tumor cells/LLC cells exposed to ionizing irradiation (γ-irradiation), it should likely sensitize them to cell death. The treatment of LLC cells with *N*-acetylsphingosine ($C_2$-ceramide), a cell-permeable ceramide analogue, before γ-irradiation, might lead to a sustained ceramide accumulation in the cells. *N*, *N*-dimethylsphingosine (DMS), a competitive inhibitor of sphingosine kinase, might maintain endogenously generated intracellular ceramide level after irradiation. Sphingosine kinase, an enzyme to convert sphingosine into sphingosine-1-phosphate, is known to prevent ceramide-mediated apoptosis. Thus, inhibition of sphingosine kinase may amplify ceramide function by both significantly inducing its accumulation and reducing the sphingosine-1-phosphate level in the cells. In the present study, we explored in detail the radiosensitizing activity of ceramide/DMS treatment and the cellular mechanism of radiation-induced apoptosis in LLC cell, involving caspase-dependent pathways.

## Material and Methods

### Reagents

*N*-acetyl-D-*erythro*-sphingosine ($C_2$-ceramide) and *N, N*-dimethyl-D-*erythro*-sphingosine (DMS) were purchased from Alexis Biochemicals (San Diego, CA). TdT-FragEL™ DNA fragmentation detection kit was from Oncogene Research Products (Boston, MA). Polyclonal antibody to caspase-3 and monoclonal antibody to PARP were obtained from BD PharMingen (San Diego, CA), and polyclonal antibodies to caspase-8, -9, Bcl-2, and Bax were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The broad-spectrum of caspase inhibitor, z-VAD-fmk was purchased from Calbiochem (San Diego, CA). β-actin, Bcl-2, Bcl-xL and Bax primer were from Bioneer Corporation (Seoul, Korea).

### Cell culture

LLC, mouse lung carcinoma cells, was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37°C. Ceramides and DMS were dissolved in dimethylsulfoxide at 2 mM stock concentration and stored at -20°C.

### Clonogenic assay

Cells were seeded into 100-mm dishes at a density to produce approximately 150 colonies per dish in controls and were incubated for 7-12 days. Colonies were fixed with 75% ethanol and 25% acetic acid, and stained with 1% methylene blue. The number of colonies consisting of more than 50 cells was counted.

### Flow cytometric analysis of apoptosis

Apoptosis was identified and quantified by flow cytometry with propidium iodide (PI) staining. Briefly, cells were fixed with 70% ice-cold ethanol and stained at 37°C for 30 min with 1 mg/ml RNase and 50 ng/ml PI in Phosphate buffered saline (PBS, pH 7.4). Cells were then analyzed by a FACScan flow cytometer (Becton Dickinson, Flanklin Lakes, NJ). From the analysis of DNA histogram, the percentages of cells in different phases of cell cycle were evaluated. Cells with DNA content less than the $G_1$ phase (sub $G_1$) were taken an apoptotic cells.

### TUNEL assay

Cells were prepared by cytospin according to the manufacturer's instructions (TdT-FragEL™ DNA fragmentation detection kit; Oncogene Research Products). Briefly, after collection by centrifugation, the cells were fixed for 1 h with 4% paraformaldehyde in PBS. After 2 washes in PBS, the cells were permeabilized with a mixture of 0.1% Triton and 0.1% sodium citrate for 2 min at 4°C. Following another wash, the cells were labeled for 1 h at 37°C in the dark with the TUNEL reaction mixture. For signal conversion, the slides were incubated for 30 min at 37°C in converter-POD, another 10 min incubation in DAB-substrate solution, and evaluated under a light microscope for treatment-induced apoptosis. Nuclei of apoptotic cells appeared brown color. Five or more randomly selected fields encompassing a total $>500$ cells/slide were evaluated to determine the percentage of apoptosis per treatment condition.

### Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\triangle\psi m$) was determined by the cationic lipophilic fluorochrome, 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$, Molecular Probes Inc, Eugene, OR). Cells were loaded with 30 nM $DiOC_6(3)$ during the last 30 min of each treatment.

After removal of the supernatant, the cells were washed twice with PBS, and the concentration of retained $DiOC_6(3)$ was measured by flow cytometric analysis.

### Western blot analysis

To analyze processing of Bcl, Bax, PARP and procaspases, cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-HCl (pH 8.0), 120 mM NaCl, and 0.1% Nonidet-40 (NP-40)] supplemented with protease inhibitors. Proteins (15-30 μg/well) were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (NEN) according to the manufacturer's recommendation.

### RT-PCR analysis

Bcl-2, Bcl-xL, Bax and β-actin mRNAs were assayed by PCR amplification of cDNA isolated from LLC cells. RNA (1 μg) was reverse-transcribed in a mixture containing oligo(dT)12-18 primer and superscript reverse transcriptase. The temperatures used for PCR were as follows: denaturation at 95°C for 1 min; annealing at 55-60°C for 1 min; and extension at 72°C for 1 min. The numbers of amplification cycles were determined according to individual primer sets in order to maintain exponential rate of product amplification (30-35 cycles). Amplified DNA fragments were subjected to electrophoresis on 1% agarose gel and visualized by ethidium bromide staining.

### Statistical analyses

All statistical procedures were performed using the GraphPad Prism version 3.02 software (San Diego, CA). Cell viability and survival fractions represent mean and standard deviation (SD) values from a minimum of three replicate experiments. Next, repeated-measures regression techniques were used to estimate the parameters of the linear-quadratic model. For the results of MMP, the differences between treated groups were analyzed by Student's *t*-test.

## Results

### Cell viability after combined treatment of ceramide and DMS in LLC cells

To evaluate the combined effect of exogenous ceramide and DMS on the cell viability, LLC cells were exposed to either ceramide or DMS for 24 h and the viability was measured. As seen in Figure 1, ceramide or DMS induced the loss of LLC cells viability in a dose-dependent manner and the decrease of cell viability by DMS was more effective than ceramide. When cells were treated with both ceramide and DMS, cell death was increased about 20.8% at 5 μM, and up to 84.8% at 20 μM. The significant differences of cell viability were observed in C2 with DMS treated cells compared with C2 or DMS alone treated cells ($P < 0.0001$).

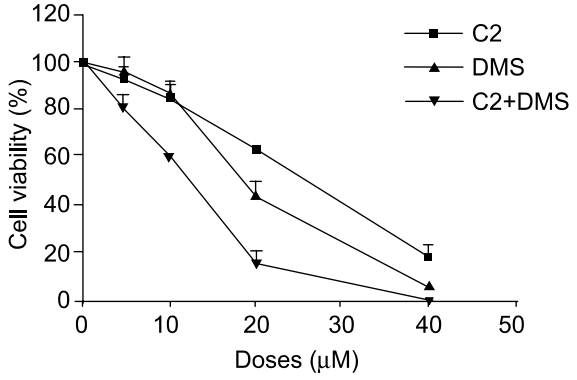


**Figure 1.** Cell viability of ceramide and DMS. LLC cells were treated with indicated concentration of ceramide, DMS, or both, and harvested 24 h later for trypan blue dye exclusion assay. Error bars represent standard error of mean of three independent experiments.

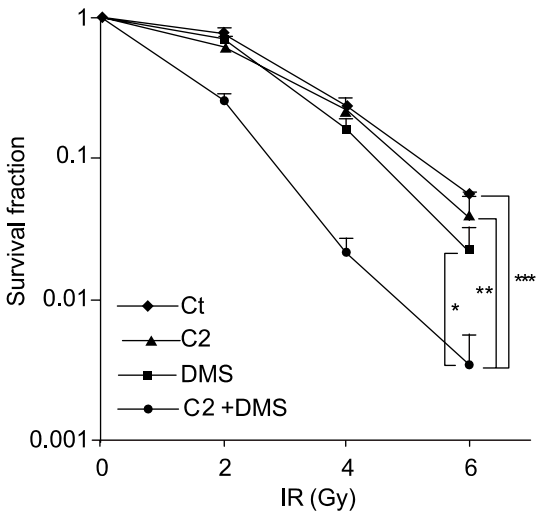


**Figure 2.** Colony-forming ability of ceramide and DMS on LLC cells after γ-irradiation. Five μM each of ceramide, DMS, or both were added into culture medium before various doses of γ-irradiation, and clonogenic survival assay was used to assess their effects on self-renewal capacity of LLC cells. Values are mean ± S.D. from triplicate determinations for separate experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$ represented statistically significant.

The first object of our study is to search radio-sensitizing agents, having no harmful effect itself, thus we selected approximately LD10 doses (dose of drug that kills 10% of cells) of ceramide and DMS, 5 μM each. In clonogenic survival assay, the treatment of either ceramide or DMS alone before irradiation

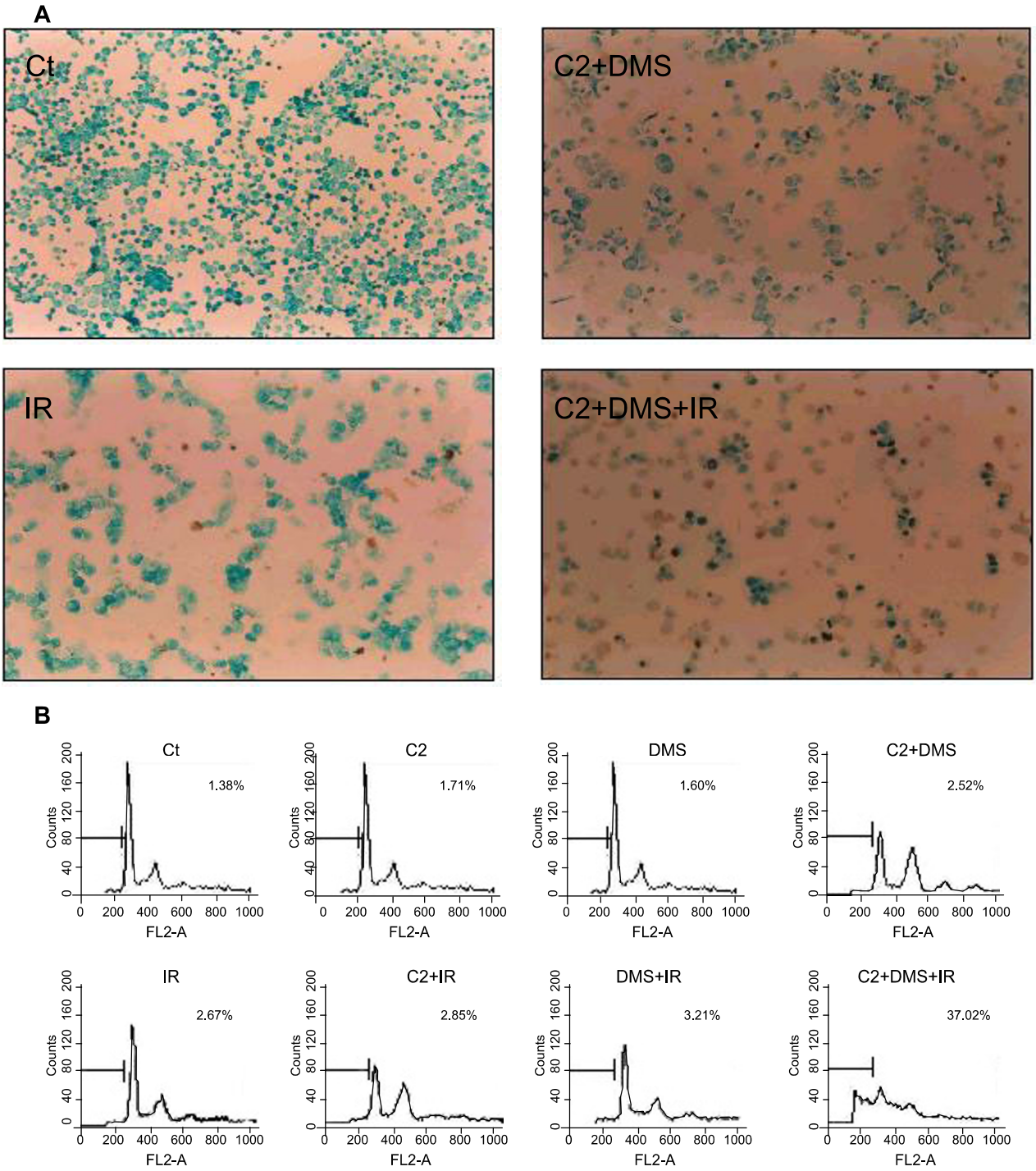


**Figure 3.** Ceramide and DMS enhanced radiation-induced apoptosis. LLC cells were treated with or without 5 μM each of ceramide and/or DMS, respectively, for 18 h, and then incubated for another 6 h followed by 5 Gy of γ-irradiation. (A) Apoptosis was assessed by TUNEL assay. Apoptotic nuclei were identified as brown-yellow stain, whereas normal nuclei are stained blue due to counterstaining with methylgreen. (B) Apoptotic cells were measured by flow cytometric analysis after PI staining. The figure shows a representative result of three independent experiments with similar results.

showed no significant inhibition of the clonogenic growth of LLC cells than the exposure to γ-ray alone ($P$ = 0.24 and 0.23 in C2 and DMS treated group compared with control, respectively (Figure 2). However, when LLC cells were exposed to both ceramide and DMS followed by γ-ray, further decline of clonogenic survival was observed. These results indicated that the treatment with a low dose of both ceramide and one of its metabolic inhibitors, DMS could synergistically reduce the viability of tumor cells.

### Increase of apoptosis after combined treatment of ceramide and DMS in LLC cells

The cell death caused by ceramide and DMS in irradiated LLC cells was analyzed by TUNEL assay (Figure 3A). Either ceramide/DMS treatment or radiation alone exhibited similar magnitude of apoptosis about 10%, however, combined treatment of ceramide/DMS and radiation significantly increased apoptosis at 6 h after irradiation. Likewise, combined treatment of ceramide/DMS and radiation increased cells with sub-$G_1$ contents of DNA, as evidenced by flow-cytometric cell death analysis (Figure 3B).

### Caspase activation of ceramide/DMS-increased radiation-induced apoptosis

Likely participation of caspases in the ceramide and DMS-induced apoptosis of LLC cells was analyzed by

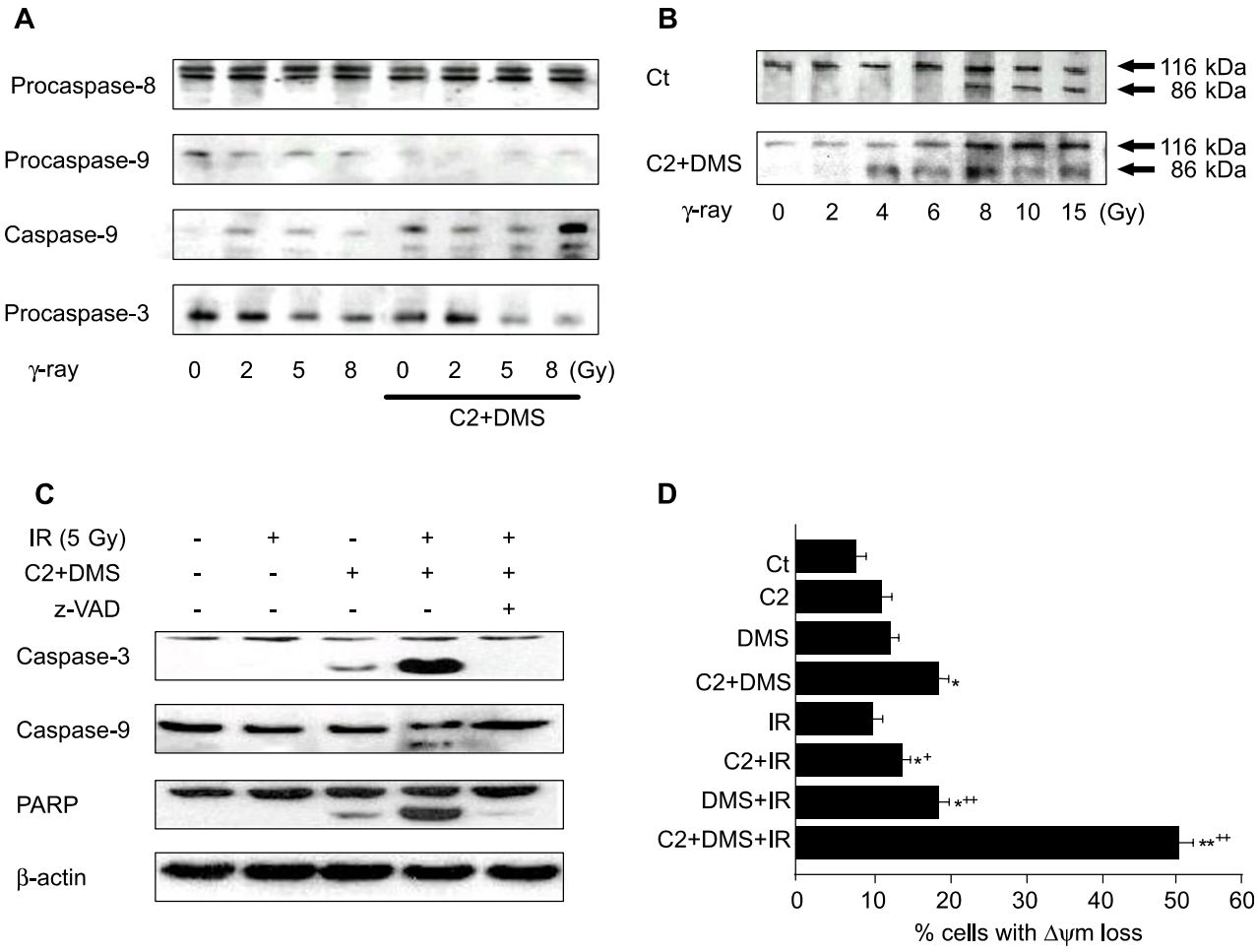


**Figure 4.** Caspase activities of irradiated LLC cells with or without the combination of ceramide and DMS. LLC cells were pretreated for 18 h with or without both 5 μM each of ceramide and DMS. Six hours after irradiation, cell lysates were prepared, and assessed by Western blot analysis. (A) Cellular proteins were immunoblotted with antibodies against caspase-3, -8, and -9. Results are a representative of three separate experiments with compatible outcomes. (B) PARP cleavage induced by γ-irradiation in LLC cells is increased by the pretreatment with both ceramide and DMS. Similar results were obtained in three independent experiments. (C) LLC cells were treated with ceramide/ DMS and radiation in the presence or absence of 30 μM z-VAD-fmk for 3 h before harvest. Cell lysates were prepared and assessed by Western blot analysis for caspase-3, 9, and PARP. (D) MMP was determined by mitochondria specific dye $DiOC_6(3)$. The percentage of cells with △ψm loss was calculated among the total fractions of cells. Data represent mean ± S.D. (bars) from three separate experiments. $^*P < 0.05$ and $^{**}P < 0.005$ with respect to normal control values, $^+P < 0.01$ and $^{++}P < 0.001$ compared to irradiated controls as determined with Student $t$-test.

Western blot analysis. As shown in Figure 4A, treatment of cells with ceramide and DMS caused activation of caspase-9 and -3 in a dose-dependent manner, as indicated by reduction in the intensity of the proenzymes. However, the expression levels of caspase-8 between untreated and cermiade/DMS-treated cells after γ-irradiation were not altered. In addition, the 85 kDa cleavage product of PARP appeared after 2 Gy irradiation in the cells pretreated with both ceramide and DMS, whereas there was no PARP cleavage in the control LLC cells up to 8 Gy irradiation (Figure 4B). Caspase activation during the course of the ceramide/DMS-induced apoptosis was additionally confirmed by using a broad-spectrum of caspase inhibitor, z-VAD-fmk. As expected, z-VAD-fmk was able to prevent activation of caspase-3, 9 and attenuate apoptosis (Figure 4C). To evaluate the contribution of the mitochondrial pathway to the induction of apoptosis seen after ceramide and DMS treatment, we examined changes in MMP of LLC cells. Figure 4D shows that each treatment of ceramide, DMS or radiation slightly changed MMP, however, the combined treatment of ceramide/DMS with radiation significantly disrupted MMP in LLC cells.

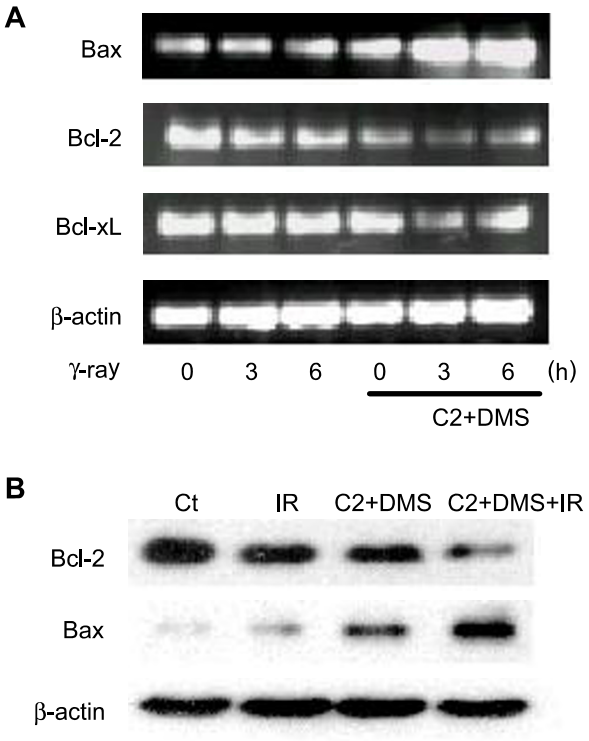


**Figure 5.** Bax: Bcl-2 ratio dictating the relative sensitivity of cells to apoptotic stimuli in LLC cells treated with both ceramide/DMS and γ-ray, or γ-ray alone. (A) Total RNA was isolated from untreated and ceramide/DMS-treated LLC cells 3 h and 6 h after 5 Gy of γ-irradiation and then reverse-transcribed. Similar results were obtained in three independent experiments. (B) Cell lysates were prepared from depicted treatment groups 6 h after 5 Gy of γ-irradiation and assessed by Western blot analysis for Bcl-2 and Bax. The data represented a typical experiment conducted three times with similar results.

### Analysis of Bcl-2 family expression upon ceramide/DMS-increased radiation-induced apoptosis

Bcl-2 family proteins have been implicated in the regulation of two important aspects of mitochondria pathophysiology: (a) mitochondrial permeability transition (PT) pore opening; and (b) release of apoptogenic proteins from mitochondria into the cytosol. To confirm the result of mitochondrial participation in signaling to ceramide/DMS induced caspase pathway, we determined the mRNA expression levels of Bax and Bcl-2, representative proapoptotic and antiapoptotic proteins. As shown in Figure 5A, Bax level in the ceramide and DMS treated cells was significantly increased 3 h after the γ-irradiation concomitant with decreased Bcl-2 level. Moreover, the expression of Bcl-xL in the ceramide and DMS treated cells was slightly decreased as early as 3 h after the γ-irradiation, and then returned to the control level. We also assessed the protein expression levels of Bcl-2 and Bax by Western blot analysis 6 h after irradiation, confirming that Bcl-2 was markedly decreased in ceramide/DMS-treated and irradiated cells and Bax, vice versa (Figure 5B).

## Discussion

Accumulation of ceramide, a lipid second messenger, has been demonstrated in tumor cells exposed to ionizing irradiation. Moreover, a deficiency in any of ceramide-forming enzymes can generate a radioresistant nonapoptotic phenotype in response to ionizing radiation (Little, 1994; Thomberry and Lazebnik, 1998). On the basis of these findings, one can speculate that combining radiotherapy with the regulation of endogenous ceramide levels may have synergistic effects on selected tumors. Thus, modifications in the sphingomyelin/ceramide signaling pathway are expected to be a potential tool to increase the therapeutic efficiency of radiation treatment of tumors (Chmura *et al.,* 1997; Edsall *et al.,* 1997, 1998; Susin *et al.,* 1997; Cuvillier *et al.,* 1998; Kleuser *et al.,* 1998; Kolesnick *et al.,* 1998; Zhivotovsky *et al.,* 1999). Many inhibitors of ceramide synthesis such as DL-PDMP (DL-threo-1-phenyl 2-decanoyl amino-3-morpholino-1-propanol · HCl), B13, and imipramine have already been tried in humans and animals. However, these agents were found to be potentially toxic (Acosta *et al.,* 1984). In the present study, we examined a possibility that induction of intracellular ceramides by a combination

of ceramide and its metabolic inhibitor at relatively low concentrations could increase radiosensitivity in tumor cells. The results showed that the combination of ceramide and DMS worked in concert with radiation to significantly decrease the viability of lung cancer cells.

We chose the concentrations of both agents that affected a minimal toxicity on the LLC cell viability or reproducibility (Figure 1 and 2). However, when these drugs were combined with radiation, potentiated antiproliferative effects through an apoptotic mechanism as evidenced by FACS and TUNEL assay were shown (Figure 3).

Although distinct pathways leading to apoptosis are triggered by different signals, they merge at a common "cytoplasmic regulator" of this multistep process, a family of cysteine proteases called caspases. These key components of the death machinery are linked to signaling pathways that are activated by either ligation of death receptors expressed at the cell surface or intracellular death signals. We obtained that pretreatment of LLC cells with both ceramide and DMS increased the activation of caspase-9 and -3 in radiation dose-dependent manner, while IR did not activate caspase-8 activity even after ceramide/DMS treatment in well accordance with Cuvillier's results (Cuvillier *et al.,* 1998). In addition, overexpression of a caspase-8 inhibitor, FLIP (FLICE inhibitory protein), did not reduce radiosensitivity, suggesting that caspase-8 activation is a marginal event for radiation-induced apoptosis (Kataoka *et al.,* 1998).

During apoptosis, the disruption of $\triangle\psi m$ is an early and decisive event which may result in the activation of caspases. The mitochondrial permeability transition pores are associated with the release of cytochrome c, which induces conformational changes of the adaptor molecule called Apaf-1 that recruits and activates procaspase-9 (Li *et al.,* 1997; Kwon *et al.,* 2003). Procaspases in radiation-induced apoptosis are also tightly regulated by many factors such as Bcl-2 family proteins. It has been suggested that the antiapoptotic members of Bcl-2 family such as Bcl-2 and Bcl-xL block the release of mitochondrial cytochrome c, or suppress the formation of reactive oxygen species (ROS) as antioxidant. On the other hand, the release of mitochondrial proteins is promoted by proapoptotic members such as Bax, Bak, or Bid which open mitochondrial voltage-dependent channel. Therefore, the ratio among the amounts of these proapoptotic and antiapoptotic proteins seems to determine whether or not a cell will respond to an apoptotic stimulus, and involvement of the Bcl-2 family is now considered to be a hallmark for mitochondrial participation in the apoptotic pathway. In the present study, exposure of LLC cells to ceramide/DMS and radiation induced marked dissipation of MMP and increased Bax level concomitant with decreased Bcl-2 level, suggesting the molecular ratio of Bax: Bcl-2 was increased and thus accelerated the radiation-induced apoptosis (Figure 4 and 5). The molecular mechanism of changes in Bcl-2 family expressions by ceramide/DMS in apoptotic process remains unclear and further investigation is required. However, the dose of radiation itself used in this experiment did not alter the caspase activation or Bax: Bcl-2 interaction in LLC cells, these results suggest that cells treated with both agents respond very rapidly and intensively to radiation-induced apoptotic stimuli.

Apoptosis can be induced via two distinct signaling pathways, the death receptor-mediated or the mitochondrial activation-mediated pathway (Smith *et al.,* 1994; Chen *et al.,* 1996; Ibrado *et al.,* 1997; Vogt *et al.,* 1998). The former pathway is characterized by the formation of the death-inducing signal complex (DISC) following receptor ligation, upon which procaspase-8 is activated (Peter and Krammer, 1998; Reed, 1998; Scaffidi *et al.,* 1998). Recently, it has been proposed that the amount of procaspase-8 recruited to the receptor may determine whether the apoptotic pathway is mitochondrion-dependent (type II cells) or not (type I cells) (Tepper *et al.,* 1999). In type I cells, the activated caspase-8 cleaves and activate caspase-3, which then executes a series of apoptotic events. In contrast to the death receptor pathway, the amount of caspase-8 is very small in type II cells, and apoptosis in these cells depends on the release of mitochondrial constituents Bcl-2 family regulates (Farrow and Brown 1996; Yang *et al.,* 1996; Chauhan *et al.,* 1997; Kroemer *et al.,* 1997; Cuvillier *et al.,* 2000). During non-receptor-mediated apoptosis including radiation-induced death, the release of mitochondrial proteins and the activation of procaspase-9 seem to play a key role (Li *et al.,* 1997; Yang *et al.,* 1997; Bossy-Wetzel *et al.,* 1998; Zou *et al.,* 1997, 1999). Our finding that the ceramide and DMS treatment enhanced radiation-induced apoptosis might be related with the disruption of MMP, the increase of Bax/Bcl-2 ratio, caspases-9, -3 activity and PARP cleavage as estimated by FACS and Western blot analysis, respectively (Figure 4 and 5). These molecular changes, all of which were not induced by exposure to $\gamma$-ray alone, are biochemical characteristics of mitochondria-dependent apoptosis (Ito *et al.,* 1999; Tepper *et al.,* 1999; Cartee *et al.,* 2000).

Further understanding of the ceramide pathway during ceramide/DMS-increased radiation-induced apoptosis, especially identification of the upstream and downstream molecules, may shed light on molecular mechanisms of apoptosis induced by chemotherapeutic agents, and provide us a novel approach to treat malignant tumors (Sawada *et al.,* 2000). Combining radiotherapy with intracellular ceramides in-

ducing agents may be an effective and promising strategy to kill tumor cells.

**Acknowledgement**

This work was supported by Nuclear R&D program from the Ministry of Sciences and Technology in Korea.

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