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Down-Regulation of Ceramide Production Abrogates Ionizing Radiation-Induced Cytochrome *c* Release and Apoptosis

STEVEN J. CHMURA, EDWARDINE NODZENSKI, SURENDER KHARBANDA, PRAMOD PANDEY, JOSE QUINTANS, DONALD W. KUFE, and RALPH R. WEICHSELBAUM

Department of Radiation and Cellular Oncology, Division of Biological Sciences, University of Chicago, and the Pritzker School of Medicine, Chicago, Illinois (S.J.C., E.N., J.Q., R.R.W.); Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts (S.K., P.P., D.W.K.)

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ABSTRACT

Previous work has demonstrated that down-regulation of ceramide production after selection of cells with *N*-oleoylethanolamine (OE), an inhibitor of ceramidase, results in resistance to DNA damage-induced apoptosis. We report here that acute exposure of WEHI-231 cells (murine B-cell lymphoma) to OE activates neutral sphingomyelinase, induces ceramide production and increases intracellular reactive oxygen species. OE exposure also induces mitochondrial permeability, cytochrome *c* release, and apoptosis. Cells selected for resistance to OE exhibit little if any change in reactive oxygen species and cytochrome *c* release when exposed either to OE or to toxic doses of ceramide. Importantly, the OE resistant cells are also resistant to ionizing radiation-induced cytochrome *c* release and apoptosis. These findings demonstrate that down-regulation of neutral sphingomyelinase activity is associated with decreased DNA-damage-induced apoptosis. In addition, the data suggests that agents that modify extranuclear targets responsible for ceramide production select for cells resistant to ionizing radiation-induced apoptosis through alterations in mitochondrial function.

Sphingomyelin (SM) hydrolysis and subsequent ceramide generation play key roles in the regulation of cell life and death. Ceramide production results from either the de novo synthesis by ceramide synthase (Kolesnick, 1994) or through the hydrolysis of SM by at lease five types of sphingomyelinases (SMase). The currently identified SMases include the acidic, acidic zinc-dependent, neutral magnesium-dependent, neutral magnesium-independent, and alkaline SMase, and they differ primarily in their tissue distribution and regulation. Current efforts have focused on differentiating the effects of neutral and acidic SMases with regard to apoptosis (Merrill and Jones, 1990; Liu et al., 1997). Ceramide is reported to initiate the apoptotic program in cells exposed to diverse types of stress, including ionizing radiation (IR) (Haimovitz-Friedman et al., 1994; Chmura et al., 1997b). A direct relationship between resistance to IR- induced apoptosis and the failure of cells to accumulate ceramide immediately after IR has been reported in several Burkett lymphoma cell lines, a glioma line (Michael et al., 1997), normal endothelial cells (Billis et al., 1998), and a mouse lymphoma (WEHI-231) line (Chmura et al., 1997b).

Acid SMase has been implicated in radiation-induced apoptosis. Acidic SMase knockout mice demonstrated resistance to IR-induced apoptosis in the lymphocytes (Santana et al., 1996; Billis et al., 1998). This work is supported further by the finding that lymphoblasts derived from patients with Niemann-Pick disease, i.e., lacking acidic SMase function, are resistant to IR-induced apoptosis. Other studies, however, implicate neutral SMase as a mediator of apoptosis (Haimovitz-Friedman et al., 1994; Chmura et al., 1997a,b; Zhang et al., 1997). These data suggest that differences in SMase activity may be accounted for both by cell line and by tissue specificity.

Recent studies have demonstrated that the mitochondrial permeability transition ($\Delta\Psi$m) is a key event in apoptosis (Marchetti et al., 1996a,b; Zamzami et al., 1996) that precedes release of cytochrome *c* into the cytoplasm and activation of the apoptotic machinery. Apoptosis-inducing stimuli, such as toxic doses of exogenous ceramide, induce $\Delta\Psi$m that is blocked by overexpression of Bcl-2 gene family members (Decaudin et al., 1997; Susin et al., 1997). Increased expression of antiapoptotic genes and decreased expression of proapoptotic genes are mechanisms of resistance to IR and chemotherapy-induced apoptosis (Thompson, 1995). IR also induces the production of reactive oxygen species (ROS) and $\Delta\Psi$m associated with disruption of membrane integrity. Thus, in addition to Bcl-x_L or Bcl-2 overexpression (Datta et

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ABBREVIATIONS: SM, sphingomyelin; SMase, sphingomyelinase; IR, ionizing radiation; $\Delta\Psi$m, mitochondrial permeability transition; ROS, reactive oxygen species; OE, *N*[*cis*-9-octadecenoyl]ethanolamine; MMP, mitochondrial membrane potential; DAG, 1,2-diacylglycerol; FACS, fluorescence-activated cell sorter; SO, superoxide.

al., 1995), tumor cells may develop resistance to IR-mediated apoptosis by acquiring alterations in ceramide metabolism or resistance to $\Delta\Psi m$. However, no studies to date have demonstrated that selective pressures in a tumor cell microenvironment that alters mitochondrial membrane potential (MMP) result in resistance to IR-induced apoptosis.

N-Oleoylethanolamine (OE) is a ceramide analog that is a potent inhibitor of ceramidases (Sugita et al., 1975; Coroneos et al., 1995). OE is highly soluble in lipid bilayers and is an inhibitor of mitochondrial swelling (Broekemeier et al., 1985). Previous work has demonstrated that treatment of WEHI-231 cells with OE produces a rapid rise in intracellular ceramide and apoptosis (Wiesner et al., 1997). Selection of WEHI-231 JM cells in OE resulted in the WEHI-231 OE cell line that is defective in IR-induced ceramide production and displays an apoptosis-resistant phenotype. To define the mechanisms by which selection of WEHI-231 JM cells with OE produces an apoptosis resistant phenotype, we studied OE effects on $\Delta\Psi m$ and the production of ROS in WEHI-231 JM and OE cells. We report here that exposure of wild-type WEHI-231 (JM) cells with OE results in ceramide production from activation of neutral SMase and increased mitochondria membrane permeability. We also show that resistance to OE is associated with defects in cytochrome *c* release and apoptosis after IR exposure. Moreover, the resistance to IR-induced apoptosis is not attributable to overexpression of Bcl-x_L.

Experimental Procedures

Materials. TP, sphingosine-1-phosphate, DL-*threo*-dihydrosphingosine, and propidium iodide were obtained from Sigma Chemical Corp. (St. Louis, MO). C2-ceramide and OE were obtained from Matreya Chemicals (Pleasant Gap, PA). Reagents for the terminal transferase assay were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). TLC plates were obtained from Whatman (10 × 10-cm LHP-K TLC plate; Tewksbury, NY). Autoradiography film was obtained from DuPont (Wilmington, DE). [γ-^{32}P]ATP was purchased from DuPont NEN (Boston, MA). All solvents were HPLC grade.

Selection of OE Variants. WEHI-231 JM cells (1×10^7) were treated with two successive treatments of 100 μM and 125 μM OE. Cells were grown for 96 h in medium containing OE. Individual resistant cells were isolated by serial dilutions. Every 2 weeks, cells were reselected with 125 μM OE.

Assays for Ceramide Production and SMase Activity. The mixed micellar assay using ^{14}C-labeled SM to quantitate neutral and acidic SMase activity was performed as described previously with minor modifications (Wiegmann et al., 1994; Chmura et al., 1996). Data (mean ± S.E.) were derived from three independent experiments with two or more determinants.

Ceramide Quantification by 1,2-Diacylglycerol (DAG) Kinase Reaction. Quantitation of ceramide by DAG kinase was similar to that described by Dressler and colleagues (1992). After treatment of cells with OE, lipids were extracted and resuspended by bath sonication in 20 ml of 7.5% *N*-octyl-β-D-glucopyranoside, 5 mM cardiolipin, and 1 mM DETAPAC. Seventy milliliters of a reaction mix was added to give a final concentration of 0.05 M imidazole/HCl, pH 6.6, 0.05 M NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, and DAG kinase at a concentration of 0.7 U/ml. The reaction was started by the addition

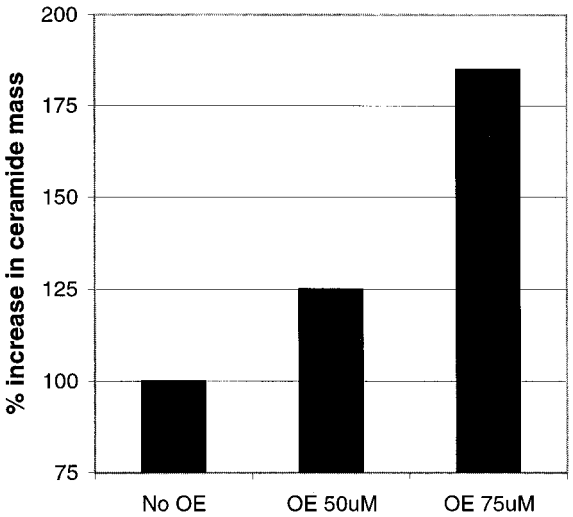

Fig. 1. Ceramide mass increases in WEHI-231 JM cells after exposure to OE. Exponentially growing WEHI-231 JM cells were exposed to increasing concentrations of OE for 12 h. Ceramide mass was then assayed with the DAG kinase reaction as described in *Experimental Procedures* with a representative experiment shown. Bars represent mean percent increase in ceramide mass derived from duplicate determinants compared with control (no OE added).

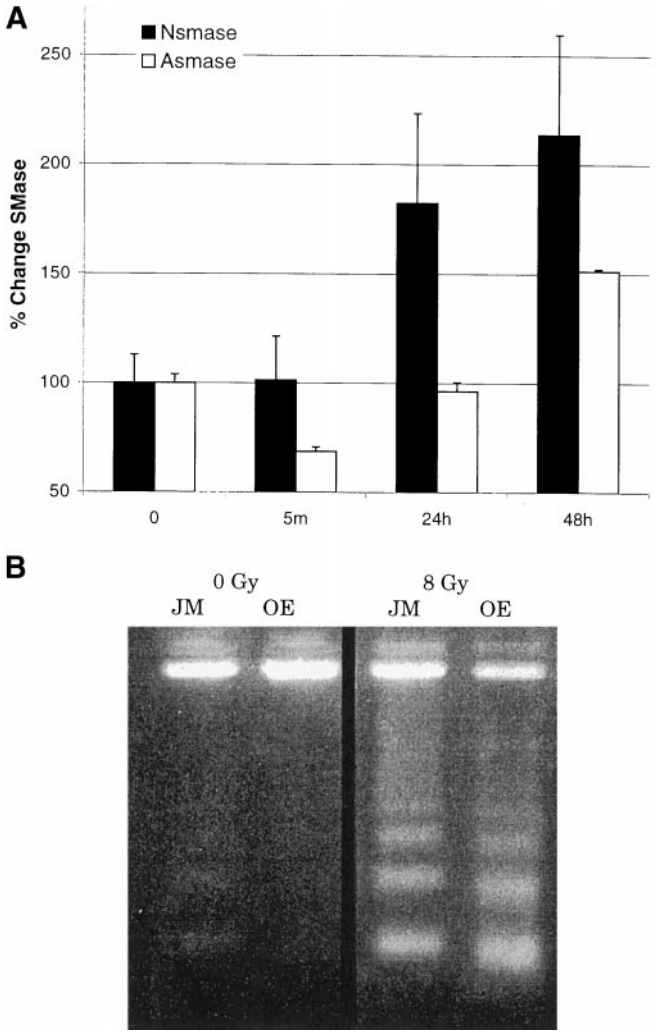

Fig. 2. A, SMase activity is not increased in OE cells until characteristic changes of apoptosis are seen after 8 Gy. WEHI-231 OE cells were irradiated with 8 Gy and assayed for SMase activity at the indicated time points. Bars represent triplicate determinants from at least three independent experiments ± S.D. B, WEHI-231 OE cells show some characteristics of apoptosis 48 h after IR. WEHI-231 JM or OE cells were irradiated with 8 Gy and DNA-analyzed at the appropriate time points.

of 10 μl of [γ-^{32}P]ATP (1.0 μCi/tube) in 5 mM ATP and incubated at room temperature for 30 min and stopped by the extraction of lipids with 450 μl of $CHCl_3$:CH_3OH (1:2) and 20 μl of 1% $HClO_4$. The monophase was mixed and after 10 min, 150 μl of $CHCl_3$ and 150 μl of 1% $HClO_4$ were added and the tubes vortexed and centrifuged at 5000*g* for 5 min. The lower organic phase was washed twice with 1% $HClO_4$ and then dried in a vacuum apparatus. The phosphorylation products, ceramide-1-phosphate and phosphatidic acid were resolved on a 10 × 10-cm LH P-K TLC plate (Whatman). Data was calculated as picomoles of ceramide per 10^6 cells by scintillation counting.

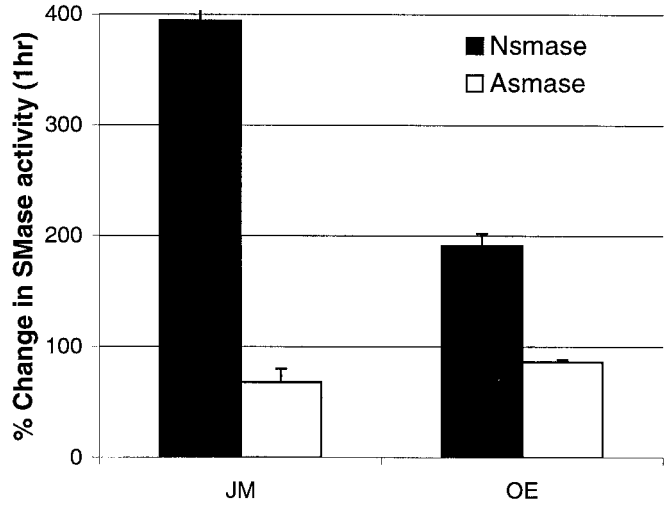

Fig. 3. OE activates SMase in in vitro cell extracts. WEHI-231 JM or OE complete cell extracts were treated with 75 μM OE and incubated at 37°C degrees for 1 h. Bars represent the mean of at least three independent experiments ± S.D.

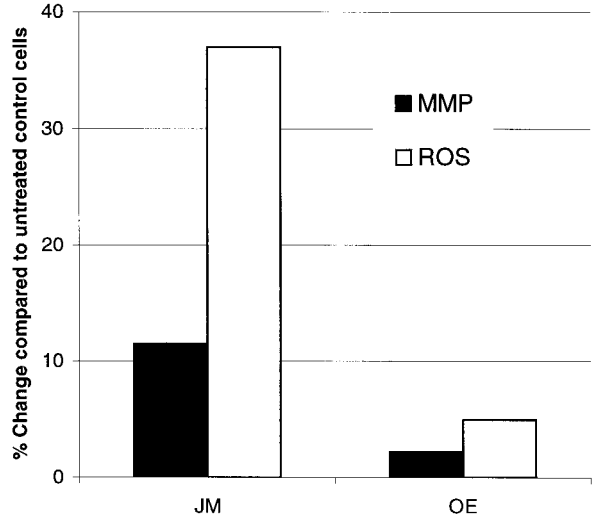

Fig. 4. MMP and production of ROS after OE. WEHI-231 cells were treated with 100 μM OE and assayed 1 h after incubation at 37° for MMP or ROS changes by FACS and flourometric dyes as described in *Experimental Procedures*.

Assays for Apoptosis and Cell Viability. To detect DNA fragmentation, cells were collected and lysed in 0.5 ml of lysis buffer (0.6% SDS + 10 mM EDTA, pH 7.0). NaCl was added to 1 M and mixed by inversion and left for 12 h in 4°C and spun at 14,000*g* for 30 min. Samples were chloroform- and ethanol-precipitated (1:1) and run in a 3% agarose gel stained with ethidium bromide. For viability staining, 2.5 to 3.5 × 10^5 cells were treated with varying concentrations of C2-ceramide, irradiation, or both. At the indicated times, cells were harvested, washed once in PBS, resuspended in PBS containing 25 ng/ml propidium iodide, and analyzed using flow cytometry fluorescense-activated cell sorter (FACS).

FACS Analysis of ΔΨm Production of ROS. The fluorochromes 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and hydroethidine were used to measure Δψm and mitochondrial superoxide anion production as described previously (Rottenberg and Wu, 1998). Cells were collected at the appropriate time points and fluorochromes added before analysis using FACS.

Assay for Cytosolic Cytochrome *c*. For cytochrome *c* release assays, WEHI-231 cells were washed twice with PBS, and the pellet was suspended in 5 ml of ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin, aprotinin, and pepstatin A) containing 250 mM sucrose. The cells were homogenized by being run through a Dounce homogenizer (Promega Corp., Madison, WI) 14 times with a sandpaper-polished pestle. After centrifugation for 5 min at 4°C, the supernatants were centrifuged at 105,000*g* for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction. Proteins were separated by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose membranes, and probed with anti-cytochrome *c* (Pharmingen, San Diego, CA). Membranes were developed using the enhanced chemiluminescence detection system (NEN Life Science Products). Autoradiographs were quantitated using a Hewlett Packard 30-bit scanner and NIH Image software (public domain program developed at the National Institutes of Health and available from the Internet via FTP from zippy.nimh.nih.gov).

Results

Selection of WEHI-231 JM Cells with OE Results in Increased Ceramide Mass. To demonstrate that treatment of WEHI-231 JM with OE increased intracellular ceramide mass, we measured ceramide accumulation with the DAG kinase reaction 12 h after exposure of cells to increasing concentrations of OE. As shown in Fig. 1, there is an increase in ceramide mass in a dose-dependent manner, demonstrating that OE increases intracellular ceramide mass during the selection process.

SMase Activation in Response to IR Is Decreased in OE-Resistant Cells. To identify mechanisms associated with resistance to OE, we studied WEHI-231 JM and WEHI-

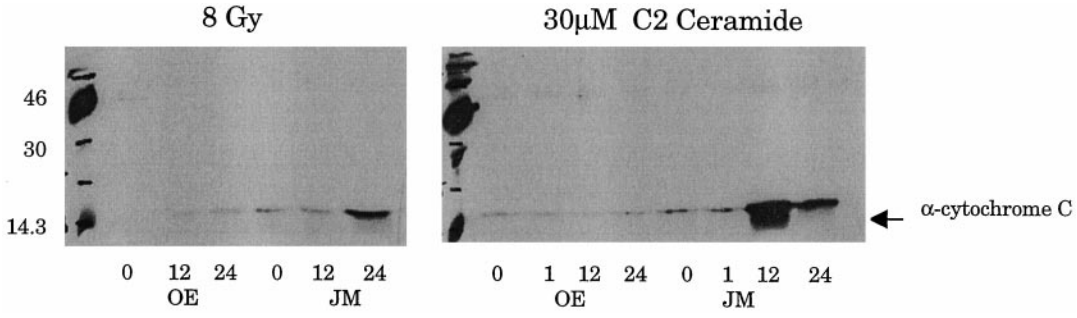

Fig. 5. Cytoplasmic cytochrome *c* release after 8 Gy and 30 μM C2-ceramide. WEHI-231 JM or WEHI 231 OE cells were irradiated with 8 Gy or treated with 30 μM C2-ceramide. Cell extracts were prepared at the indicated time points. Gels represent one of four independent experiments.

231 OE cells for induction of SMase activity and cell death in response to IR. Consistent with data reported previously, N-SMase is activated within seconds of IR treatment in JM (140% of control) but not OE cells (104% of control) after a single dose of 8 Gy. Ceramide mass was found to increase to a maximum of 150% of control within 30 min after IR in the JM line. No detectable increase in ceramide mass in OE cells was measured (Chmura et al., 1997b). Twenty-four hours after IR, N-SMase increased to 182% of baseline in the OE cells and peaked 48 h after IR (214% of baseline). By contrast, A-SMase activity decreased in WEHI-231 OE cells until 48 h after IR (Fig. 2A). These data suggest that increased A-SMase activity represents a late event in OE cell death that occurs after WEHI-231 cells exhibit qualitative characteristics of cell death including DNA fragmentation (Fig. 2B).

OE Activates Neutral SMase in Cell Free Extracts. To determine potential mechanisms by which selection of WEHI-231 JM cells with OE produces an apoptosis-resistant phenotype, we examined whether OE directly alters SMase activity (Fig. 3). OE, in a dose that induces apoptosis in 50% of WEHI-231 JM cells (75 μM), was added to WEHI-JM cell extracts. Under these experimental conditions, OE increased N-SMase activity to 394 ($\pm$103%) of control at 1 h. In contrast, A-SMase was inhibited to 68 ($\pm$12% of control (Fig. 3). Using WEHI-231 OE cell extracts, OE treatment activated N-SMase to a maximum of 191 $\pm$ 11% of control and A-SMase was inhibited to 86 $\pm$ 2% of control at 1 h. These studies demonstrate that OE increases N-SMase activity in WEHI-231 JM cell extracts and that this increase in N-SMase is abrogated in part in extracts from WEHI-231 OE cells.

WEHI-231 OE Cells Show a Reduction in Mitochondrial Permeability in Response to OE and Exogenous Ceramide When Compared with WEHI-231 JM Cells. Stability of the MMP was assessed by adding $DiOC_6$ and monitoring the production of superoxide (SO) using hydroethidine cell staining and FACS. WEHI-231 JM cells treated with 100 μM OE for 1 h exhibited an 11.5-fold decrease in MMP as quantitated by $DiOC_6$ and a 37-fold increase in SO anions using hydroethidine. In contrast, WEHI-231 OE cells show a 2.2-fold decrease in MMP and a 5-fold increase in SO anions (Fig. 4). To determine whether the attenuated response of WEHI-231 OE cells extends to other apoptotic stimuli, we tested with 30 μM exogenous ceramide, a dose that induces apoptosis in 90% of WEHI-231 JM cells. Ceramide-induced $\Delta\Psi$m is 12-fold increased in WEHI-231 OE compared with WEHI-231 JM cells. These results demonstrate that OE selection results in cells with reduced SO production and altered $\Delta\Psi$m compared with the parental line after exposure to OE and exogenous ceramide. Consistent with previous reports suggesting that shirt-chain ceramides may induce apoptosis in a mitochondrial-independent and -dependent manner (Arora et al., 1997; Quillet-Mary et al., 1997; Degli Esposti and McLennan, 1998; Scaffidi et al., 1999), OE cells were found to be equally sensitive to C2-ceramide-induced apoptosis as described previously (Chmura et al., 1997b), suggesting that short-chain ceramides induce apoptosis through a mitochondrial-dependent and -independent manner.

WEHI-231 OE Cells Demonstrate Decreased Cytochrome *c* Release After IR When Compared with WEHI-231 JM Cells. Disruption of $\Delta\Psi$m and ultimately the opening of the $\Delta\Psi$m pore (MTP) may represent a key component in the apoptotic response after IR. To determine whether selection with OE and subsequent alterations in mitochondrial membrane permeability are associated with changes in IR-induced cytosolic cytochrome *c* release, we irradiated WEHI-231 JM and OE cells (8 Gy) and assayed cytoplasmic cytochrome *c* levels at 0, 12, and 24 h after treatment (Fig. 5). The results demonstrate that JM cells exhibit a greater than 5-fold increase in cytoplasmic cytochrome *c* levels 24 h after IR. By contrast, cytochrome *c* release was less than a 2-fold increase in OE cells. At 24 h after IR, >50% of JM cells were apoptotic (Fig. 6), compared with <20% of the OE cells as determined using propidium iodide and FACS. Treatment of JM cells with 30 μM exogenous ceramide also increased cytochrome *c* release within 12 h, whereas there was no detectable change in ceramide-treated OE cells. These results demonstrate that reduced $\Delta\Psi$m after IR is associated with a decrease in cytoplasmic cytochrome *c* release.

Overexpression of Bcl-x_L, but not Bcl-2, produces an apoptosis-resistant phenotype in WEHI-231 cells. We examined whether OE selection results in increased Bcl-x_L. There was

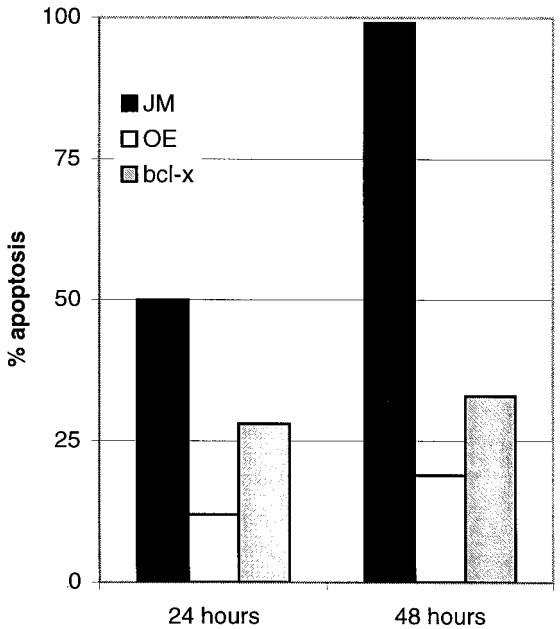

Fig. 6. JM, OE, and Bcl-x_L induced apoptosis after IR. WEHI-231 JM, OE, or Bcl-x_L cells were irradiated with 8 Gy and assayed for apoptosis with propidium iodide and FACS as described in *Experimental Procedures*. Graph represents results of a representative experiment. At least three independent experiments were performed.

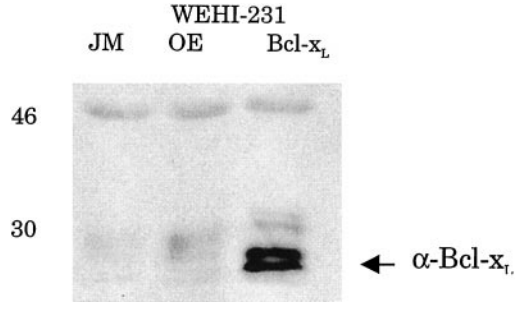

Fig. 7. Bcl-x_L expression is not altered after selection with OE Western blot analysis with α-Bcl-x_L showing relative expression of the protein in WEHI-231 JM, OE, and Bcl-x_L lines, respectively.

little detectable basal Bcl-x_L expression in either WEHI-231 JM or WEHI-231 OE cells compared with the apoptosis-resistant WEHI-231/Bcl-x_L-expressing cells. No up-regulation of Bcl-x_L expression was observed in WEHI-231 OE cells (Fig. 7). These data demonstrate that resistance to IR-induced apoptosis may be mediated through overexpression of Bcl-x_L, a selective pressure against ceramide production, or disruption in $\Delta\Psi m$ with release of cytochrome *c*.

Discussion

We report here for the first time that cells selected for altered ceramide metabolism and mitochondrial membrane permeability show a decrease in cytochrome *c* release after IR and resistance to apoptosis. The activation of N-SMase in WEHI-231 cells may be a necessary component of the apoptotic program in response to IR. In addition, selection for alterations in mitochondrial function and permeability alter the response of cells to IR.

These data suggest that selective pressures in a tumor cell microenvironment may lead to radiation resistance through alterations in both cytoplasmic and mitochondrial membrane responses to IR. Agents that increase radiation-induced production of ceramide and alter mitochondrial response to IR may provide extranuclear targets for tumor cells that have lost the susceptibility to apoptosis induced by nuclear mechanisms.

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Send reprint requests to: Dr. Ralph R. Weichselbaum, M.D., University of Chicago Hospitals, Department of Radiation Oncology, 5841 S. Maryland Ave., MC 1105, Chicago, IL 60637. E-mail: rrw@rover.uchicago.edu