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Phytosphingosine can overcome resistance to ionizing radiation in ionizing radiation-resistant cancer cells

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ABSTRACT

Although the majority of cancer cells are killed by inonizing radiation, certain types show resistance to it. We previously reported that phytosphingosine also induces apoptotic cell death in caspase dependent pathway in human cancer cells. In the present study, we examined whether phytosphingosine could overcome radiation resistance in the variant Jurkat clones. We first selected radiation-resistant Jurkat clones and examined cross-responsiveness of the clones between radiation and phytosphingosine. Treatment with phytosphingosine significantly did not affect apoptosis in all the clones, indicating that there seemed to be cross-resistance between radiation and phytosphingosine. Nevertheless, combined treatment of phytosphingosine with radiation synergistically enhanced killing of radiation-resistant cells, compared to radiation or phytosphingosine alone. The pan-caspase inhibitor z-VAD-fmk did not completely inhibit the synergistic cell killing induced by combined treatment of ionizing radiation and phytosphingosine. These results demonstrated that apoptosis induced by combined treatment of radiation and phytosphingosine in radiation-resistant cells was associated with caspase independent pathway. We also found that apoptotic cell death induced by combined treatment of ionizing radiation and phytosphingosine correlated to the increases of ROS. The enhancement of ROS generation induced the loss of mitochondria transmembrane potential. In conclusion, ROS generation in combined treatment of phytosphingosine with radiation significantly induced the translocation of AIF to nucleus from mitochondria, suggesting a potential clinical application of combination treatment of radiation and phytosphingosine to radiation-resistant cancer cells.

INTRODUCTION

Exposure of cells to ionizing radiation (IR) gives rise to a variety of cellular responses including both DNA and membrane damage. DNA damage leads to a coordinate network of signal transduction pathways involved in cell-cycle arrest, apoptosis, stress response, and activation of DNA repair processes (1). DNA damage monitoring and signaling systems are responsible for either resistance to or delay of apoptosis depending on the cell type and on the dose administered (2). Phytosphingosine (PS) is one of the most widely distributed natural sphingoid bases, which is abundant in fungi and plants, and also found in animals including humans. Moreover, PS is structurally very similar to sphingosine: PS processes a hydroxyl group at C-4 of the sphingoid long chain base, whereas sphingosine has a trans-double bond between C-4 and C-5 (3). Many reports pronounced that a variety of exogeneous stimuli such as TNF-1, anti-Fas antibody, IL-8b, and ionizing radiation, rapidly increase intracellular levels of ceramide and sphingosine through sphingomyelinase, leading to modulation of various cellular events, including apoptosis, proliferation, and differentiation (4). However, the physiological roles of PS are largely unknown. Mitochondrial membrane permeabilization is considered to be one of the initial events of the apoptotic process, including cell death induced by chemotherapeutic drugs. Opening of the mitochondrial permeability transition pore, which is under the control of members of the Bcl-2 family, can result in the permeabilization of the outer mitochondrial membrane and the release of potentially apoptogenic proteins such as cytochrome c and AIF from the intermembrane space (5). Cytosolic cytochrome c binds to Apaf-1 in a ternary complex with caspase-9, leading to activation of caspase-9, which in turn activates caspase-3 (6). Cleavage of ICAD (inhibitor of the caspase-activated DNase) by caspase-3 leads to activation of CAD and cleavage of DNA into characteristic oligonucleosomal-length fragments (7).

AIF (apoptosis-inducing factor) was more recently cloned and identified as a mitochondrial intermembrane space protein with homology to bacterial NADH oxidoreductases. In response to the apoptotic stimuli, AIF is released, translocates to the nucleus and participates in the induction of chromatin condensation, the exposure of phosphatidylserine in the outer leaf of the plasma membrane, and the dissipation of the mitochondrial transmembrane potential. These effects seem to be caspase-independent, since none of them are prevented by the broad spectrum caspase inhibitor Z-VAD.fmk and are independent of the apoptosome complex (8, 9).

Recent studies suggest that reactive oxygen species (ROS) may play an important role during apoptosis induction (10). Many stimuli such as TNF-α, anticancer drugs, and chemopreventive agents stimulate cells to produce ROS (11). Generated ROS can directly activate the mitochondrial permeability transition and result in mitochondrial membrane potential (MMP) loss (12). Mitochondrial dysfunction such as loss of MMP results in cytochrome c release from

mitochondrion into cytoplasm. This release of cytochrome c leads to activation of DEVD-specific caspases and to nuclear fragmentation in vitro (13).

In this study, we demonstrate here that PS induces synergically apoptotic cell death of radiation resistant Jurkat T cell clones in combination treatment with ionizing radiation. ROS-generation is necessary for AIF release from mitochondria, which results in caspase-independent apoptotic cell death in combination treatment with ionizing radiation and PS. Moreover, our data provide a potential mechanism for radiosensitizing activity of PS and suggest a potential clinical application of combination treatment with ionizing radiation and PS to radiation-resistant cancer cells.

MATERIALS AND METHODS

Cell Culture. Jurkat, human T cell lymphoma (Type II) was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, GIBCO), penicillin and streptomycin at 37°C in a humidified incubator with 5% CO_2.

Materials. Phytosphingosine was purchased from Avanti (Alabaster, AL, USA). Polyclonal antibody to caspase-3 and monoclonal antibodies to PARP and cytochrome *c* were obtained from Pharmingen (San Diego, CA, USA), and polyclonal antibodies to caspase-8, -9, Bcl-2, Bid and HSP60 were obtained from Santa-Cruse (Santa Cruz, CA, USA). Polyclonal antibodies to , caspase-3 and PARP were obtained from Cell Signaling Technology (Beverly, MA, USA). A broad spectrum caspase inhibitor, z-VAD-fmk was obtained from Calbiochem (San Diego, CA, USA).

Hoechst 33258 Staining. Hoechst 33258 staining was performed as described previously (22). Briefly, cells were fixed with 4% paraformaldehyde for 30 min in room temperature, and then washed once with PBS. Hoechst 33258 (50 ng/ml) was added to the fixed cells, incubated for 30 min at room temperature, and washed with PBS. Cells were mounted and examined by fluorescence microscopy. Apoptotic cells were identified by the condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. At minimum, 500 cells were counted for each treatment.

Flow cytometric analysis of apoptosis. Apoptosis was identified and quantified by flow cytometry with propidium iodide (PI) staining. Both adherent and floating cells were collected after PS treatment, washed with ice-cold PBS and fixed with 70 % ice-cold ethanol overnight at 4C. Fixed cells were washed twice with PBS and treated with 1 mg/ml RNase for 30 min at 37C. Cellular DNA was stained with 50 ng/ml PI in PBS containing 0.05 % Nonidet P-40. Cells were then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). From

the analysis of DNA histograms, the percentages of cells in different phases of cell cycle were evaluated. Cells with DNA content less than the G1 phase (sub-G1) were taken as apoptotic cells.

Western blot Analysis. Western blot analysis was performed as described (23). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris buffered saline and then incubated with primary antibodies for 1 hr at room temperature. Blots were developed by peroxidase-conjugated secondary antibody and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham, Arlington Heights, IL, USA) according to the manufacturer's recommendation.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was determined as the retention of the mitochondria-specific dye $DiOC_6(3)$ (Molecular Probes, Inc., Eugene, OR, USA). Cells were loaded with 30 nM $DiOC_6(3)$ during the last 30 min of PS treatment. After removal of the medium, the cells were washed twice with PBS, and the relative amount of retained $DiOC_6(3)$ was measured by flow cytometric analysis.

Preparation of cytosolic and mitochondrial protein fractions for the measurement of cytochrome c and AIF release. Cells were collected and washed twice in ice-cold PBS, resuspended in S-100 buffer [20 mM HEPES, pH 7.5, 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, mixture of protease inhibitors] and incubated on ice for 20 min. After 20 min incubation on ice, the cells were homogenized with a Dounce glass homogenizer and a loose pestle (Wheaton, Millville, NJ, U.S.A.) for 70 strokes. Cell homogenates were spun at 1,000 g to remove unbroken cells, nuclei, and heavy membranes. The supernatant was spun again at 14,000 g for 30 min to collect the mitochondria-rich (the pellet) and the cytosolic (the supernatant) fractions. The mitochondria-rich fraction was washed once with the extraction buffer, followed by a final resuspension in lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA) containing protease inhibitors for western blot analysis.

RESULTS

Selection of IR-resistant and –sensitive Jurkat clones

We selected variant clones from parental Jurkat T cells for sensitivity or resistance to IR-mediated apoptosis. As shown in Fig. 1, clones #1, #11, #6 and #16 showed resistance to IR, compared with parental Jurkat T cells. When irradiated with 10Gy IR for 48hr, ionizing radiation effectively killed parental Jurkat T cells. However, IR-resistant clones #1, #11, #6 and

#16 showed an effective resistance to IR. Among the IR-resistant clones, #6 is more resistant to IR than the others.

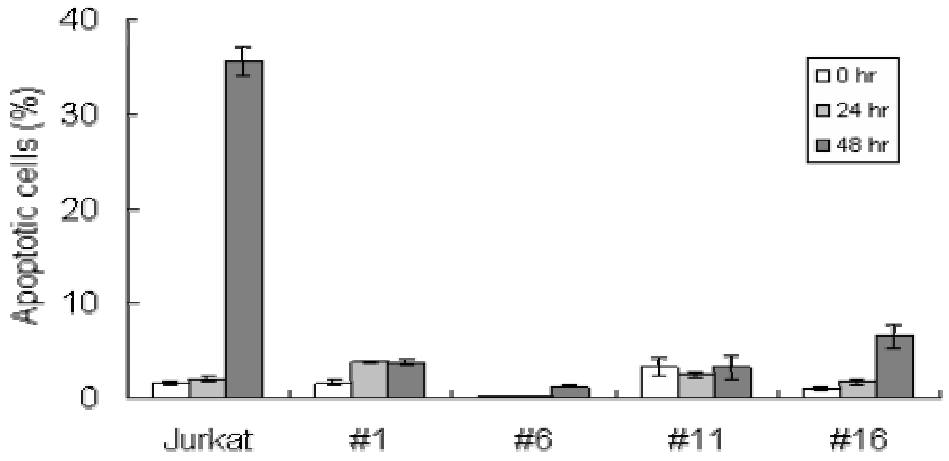

Figure 1. Selection of IR-resistant Jurkat clones. Single cells were incubated in 96 well plate for 2 months and then cells were treated with 10Gy IR for 24 or 48hr. Cells were stained with Hoechst 33258, and apoptotic cells were analyzed by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number measured. Results from three independent experiments are shown as means; bars, ±SEM

PS sensitizes IR-resistant cells to ionizing radiation-induced apoptosis in caspase-independent manner

Recent studies demonstrated that DNA damaging chemotherapeutic agents such as doxorubicine, cisplatin or etoposide enhanced ionizing radiation sensitivity in certain cancer types (1), therefore, we next examined whether combination treatment of PS with IR also had a sensitizing effect on cell death of IR-resistant cells. As shown in fig. 2A, combination treatment indeed synergically enhanced the killing of IR-resistant clones. We next investigated whether caspases were involved in enhancement of IR-resistant cell killing by combination treatment with IR and PS. As shown in fig. 2B, a broad-spectrum caspase inhibitor, z-VAD-fmk did not attenuate the cell death induced by combination treatment of IR and PS in IR-resistant clones. These findings suggest that PS in combination with IR modulates the sensitivity to IR in IR-resistant cells, resulting in synergistic enhancement of the cell death in caspases-independent manner.

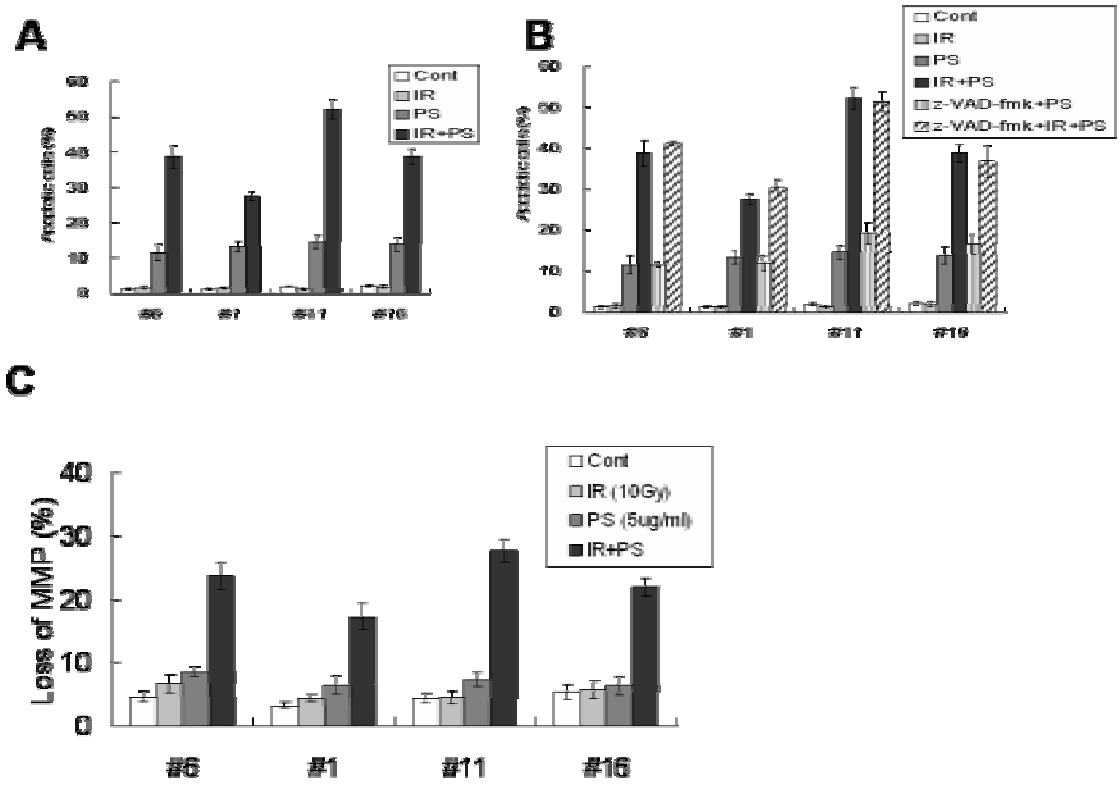

Figure 2. Phytosphingosine enhances ionizing radiation-induced cell death in IR-resistant Jurkat clones. Cells were treated with IR of 10Gy and 5 μg/ml of phytosphingosine for 3 hrs. A) Cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number counted. Results from three independent experiments are shown as means ± S.E.M. The data represent a typical experiment conducted three times with similar results. B) Cells were treated with IR of 10 Gy and 5 μg/ml of phytosphingosine for 3 hrs in the presence or absence of 30 μM z-VAD-fmk. Cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number counted. Results from three independent experiments are shown as means ± S.E.M. The data represent a typical experiment conducted three times with similar results. C) Mitochondrial transmembrane potential of these cells was determined by retention of DioC6 (3) added during the last 30 min of phytospingosine treatment. After removal of the medium, PI was added and the amount of retained DioC6 (3) uptake were measured by flow cytometry.

Combination with IR and PS enhances cell death by AIF translocation to nucleus

Since the disruption of the mitochondrial membrane potential is a critical step occurring in

cells undergoing apoptosis (21), we evaluated whether combined treatment with IR and PS had any effect on the mitochondrial membrane potential. Fig. 3A shows that treatment of IR-resistant clones with IR or PS alone, no substantial changes of the mitochondrial membrane potential were detected. However, when the cells were treated with IR and PS in combination, a marked loss of mitochondrial membrane potential was observed. Involvement of the mitochondria in apoptosis has been associated with cytochrom c release to cytosol, where it initiates caspase activation (21), and AIF translocation from mitochondria to nucleus, which is involved in caspase independent pathway (21). Indeed, in the combination treatment of IR-resistant cells with IR and PS, the release of cytochrome c (data not shown) or AIF release was observed (Fig 3B). Released AIF mostly translocates to the nucleus and participates in the induction of chromatin condensation (8). Furthermore, experiment with RNAi for AIF showed that concomitant treatment with IR and PS-induced cell death was effectively blocked by the transfection of RNAi for AIF in IR-resistant #6 clones (Fig 3C). These results suggest that concomitant treatment with IR and PS dramatically promotes the loss of mitochondrial membrane potential, which results in translocation of AIF release from mitochondria and subsequently caspase independent cell death was induced.

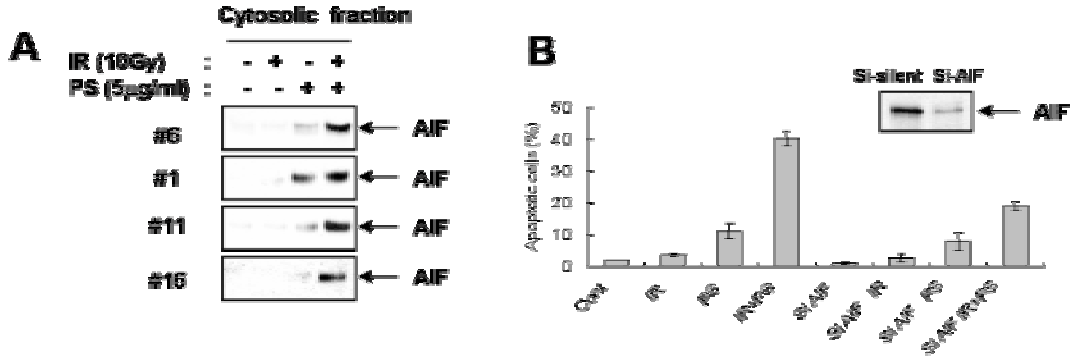

Figure 3. Cells were treated with IR of 10Gy and 5 μg/ml of phytosphingosine for 3 hrs. A) Cytosolic fraction was prepared, and AIF c was detected by Western blot analysis using anti-AIF antibody. B) Small interference RNA for AIF was used to transfect Jurkat T cells. The #6 clones transfected with small interference RNA for AIF were treated with 10 Gy of IR and 5 μg/ml of phytosphingosine for 3 hr. And then cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number counted. Results from three independent experiments are shown as means ± S.E.M. The data represent a typical experiment conducted three times with similar results.

Important Role of ROS in combination treatment with IR and PS-induced Cell Death in IR

resistant cells

It has been reported previously that sphingolipids have been implicated in ROS accumulation (24, 25, 26), and that ROS regulate apoptosis and proliferation in response to a variety of stimuli, including tumor necrosis factor-, UV and –irradiation, and anthracylines(16). To see whether ROS is increased under the concomitant treatment with IR and PS in IR-resistant cells, we stained the cells with CM-H2 DCFDA, a cell-permeable fluorescence dye that reacts to a broad spectrum of ROS. As shown in Fig. 4A, ROS levels were dramatically increased in the concomitant treatment with IR and PS in IR-resistant clones and elevated ROS were specifically blocked by NAC, which is a specific ROS scavenger. Then, we tested whether ROS accumulation is required for the concomitant treatment with IR and PS-induced dysfunction of mitochondrial membrane potential and cell death by pretreatment of the cells with a ROS scavenger NAC. As shown in fig4. B and C, NAC effectively suppressed concomitant treatment –induced loss of mitochondrial membrane potential and cell death. These data suggested that ROS plays an important role in enhancement of IR-induced cell death by PS. Furthermore, NAC effectively blocked AIF release from mitochondria induced by concomitant treatment with IR and PS in IR-resistant #6 clone (Fig 4D). These observation suggest that ROS contributes to mitochondria mediated caspase-independent cell death in concomitant treatment of IR-resistant cells with IR and PS.

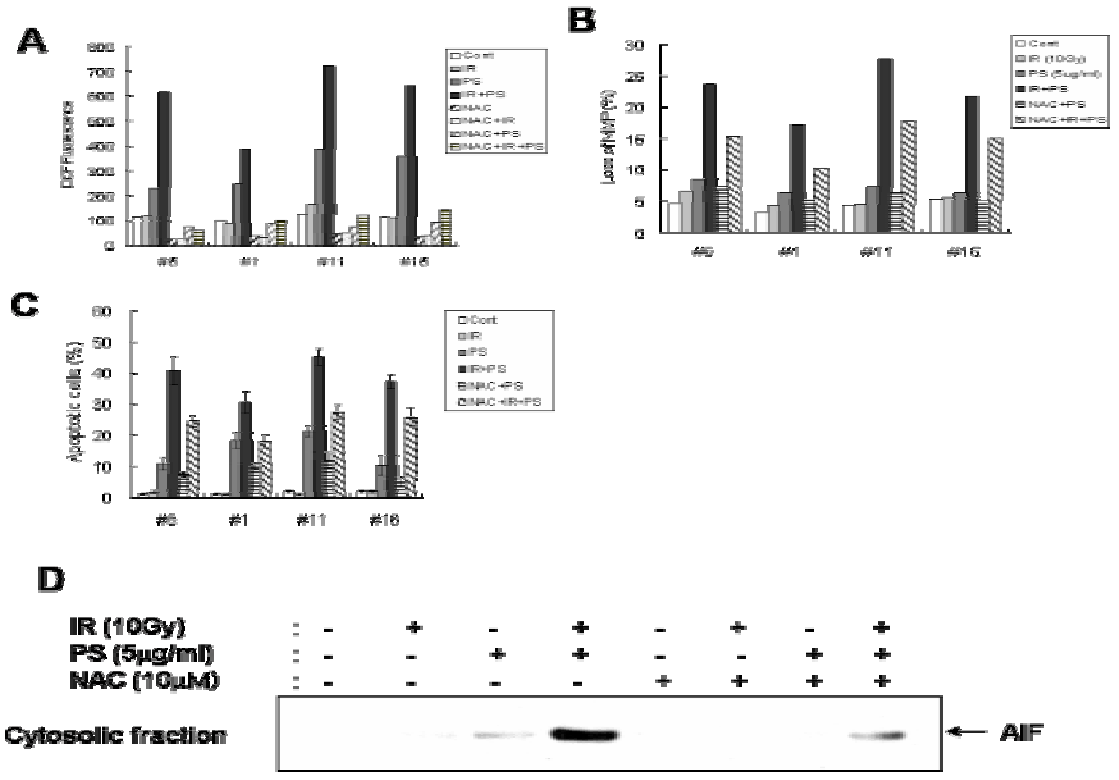

Figure 4. Enhancement of ROS production by combination treatment with IR ans PS. IR-resistant clones were treated with 10 Gy of IR and and 5 μg/ml of phytosphingosine for 3 hrs. A) The harvested cells were then incubated with 10μM of H2DCF-DA for 30 min and analyzed by flow cytometry as described under ‘‘Materials and methods.’’ B) Mitochondrial transmembrane potential of these cells was determined by retention of DioC6 (3) added during the last 30 min of phytospingosine treatment. After removal of the medium, the amount of retained DioC6 (3) uptake were measured by flow cytometry. C) IR-resistant clones were treated with 10 Gy of IR and and 5 μg/ml of phytosphingosine for 3 hrs in presence or absence of 10mM NAC. Cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number counted. Results from three independent experiments are shown as means ± S.E.M. The data represent a typical experiment conducted three times with similar results. D) Cytosolic fraction was prepared, and AIF was detected by Western blot analysis using anti-AIF antibody.

DISCUSSION

In the present study, we describe the mechanism of the concomitant treatment with IR and a member of sphingolipid metabolite, phytosphingosine (PS)-induced cell death in IR-resistant Jurkat T cells. Several studies suggest that acquired defects in ceramide cell death signaling may contribute to the development of radioresistant thymoma cell lines (27). Furthermore, it was suggested that defective radiation-induced ceramide generation and cellular resistance to radiation treatment in sphinomyelinase knockout mice and in patients could be overcame by transfection of the sphingomyelinase gene (28). We previously reported that PS had an anticancer effect on Jurkat T cell lymphoma and NCI-H460 human non-small-cell lung cancer cells. In both cell lines, PS induced apoptosis through the modulation of MAPKs in caspase dependent manner (29). However, in IR-resistant clones, PS did not effectively induce cell death, which means that IR-resistant clones show cross-resistance to PS. Nevertheless, in concomitant treatment with IR and PS, PS strongly increases IR-mediated apoptosis in IR-resistant Jurkat cell clones in caspase independent manners. In despite of pretreatment of a broad spectrum caspase inhibitor, z-VAD-fmk, concomitant treatment with IR and PS-induced did not blocked. Furthermore, in molecular events, AIF translocation to nucleus were detected in concomitant treatment with IR and PS in IR-resistant Jurkat clones. Moreover, concomitant treatment-induced cell death was effectively inhibited by transfection of RNAi targeting for AIF. Taken together, these observations indicate that concomitant treatment with IR and PS-induced cell death is AIF dependent and caspase independent.

ROS production frequently occurs in cells exposed to UV light, ioninzing radiation, H_2O_2, or cytokines (30, 31). In many experimental situations, the induction of apoptosis is accompanied by a rise in intracellular ROS (31). Furthermore, the observed inhibition of apoptosis by different antioxidants such as NAC (32), ascorbate (33), and a-tocophenol (34) suggests that ROS production plays a role in apoptosis in diverse cell lines (32, 33). Moreover, some cancer cells might have an increased expression of antioxidant enzymes leading to resistance against exogeneous stress (36). Our results also show that ROS is involved in combination treatment with IR and PS-induced apoptosis in IR-resistant Jurkat clones. Concomitant treatment with IR and PS-induced cell death is effectively inhibited by pretreatment of NAC, and this inhibition correlated with the decreases of mitochondrial membrane potential and AIF translocation to nucleus by NAC. However, as the concomitant treatment with IR and PS-induced apoptosis is not fully inhibited by NAC, it is possible that undefined signals other than ROS play an important role in combination treatment-induced apoptosis. Taken together, we have shown that PS can overcome the IR-resistance to IR-resistant Jurkat clones through the caspase-independent cell death by enhancement of ROS. The enhancement of ROS by combination treatment promotes the collapse of mitochondria membrane potential and subsequent AIF release. Interestingly, PS appears to utilize ROS to overcome IR-resistance in IR-resistant Jurkat clones. Moreover, our data provide a potential mechanism for radiosensitizing activity of PS and suggest a potential clinical application of combination treatment of radiation and PS to radiation-resistant cancer cells.

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