Sphingosine Enhances Apoptosis of Radiation-resistant Prostate Cancer Cells

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ABSTRACT

Ceramide has been implicated as an important component of radiation-induced apoptosis of human prostate cancer cells. We examined the role of the sphingolipid metabolites—ceramide, sphingosine, and sphingosine-1-phosphate—in susceptibility to radiation-induced apoptosis in prostate cancer cell lines with different sensitivities to γ-irradiation. Exposure of radiation-sensitive TSU-Pr1 cells to 8-Gy irradiation led to a sustained increase in ceramide, beginning after 12 h of treatment and increasing to 2.5- to 3-fold within 48 h. Moreover, irradiation of TSU-Pr1 cells also produced a marked and rapid 50% decrease in the activity of sphingosine kinase, the enzyme that phosphorylates sphingosine to form sphingosine-1-phosphate. In contrast, the radiation-insensitive cell line, LNCaP, had sustained sphingosine kinase activity and did not produce elevated ceramide levels on 8-Gy irradiation. Although LNCaP cells are highly resistant to γ-irradiation-induced apoptosis, they are sensitive to the death-inducing effects of tumor necrosis factor α, which also increases ceramide levels in these cells (K. Kimura et al., Cancer Res., 59: 1606–1614, 1999). Moreover, we found that although irradiation alone did not increase sphingosine levels in LNCaP cells, tumor necrosis factor α plus irradiation induced significantly higher sphingosine levels and markedly reduced intracellular levels of sphingosine-1-phosphate. The elevation of sphingosine levels either by exogenous sphingosine or by treatment with the sphingosine kinase inhibitor N,N-dimethylsphingosine induced apoptosis and also sensitized LNCaP cells to γ-irradiation-induced apoptosis. Our data suggest that the relative levels of sphingolipid metabolites may play a role in determining the radiosensitivity of prostate cancer cells, and that the enhancement of ceramide and sphingosine generation could be of therapeutic value.

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer deaths in men (1). Radiation therapy that causes growth inhibition and apoptosis is often used for treatment of both primary and metastatic prostate cancer. However, despite using high doses of radiation, about 20–25% of prostate cancer patients with noninvasive disease (stages T1, T2) relapse. A major reason for failure to eradicate local disease is the intrinsic radioresistance of the tumors. Ionizing radiation mediates cell death, in part, through chromosomal damage and also by the induction of apoptosis. Although apoptosis seems to be less prevalent than clonogenic cell death, one mechanism by which cancer cells become resistant to radiation or chemotherapy is by the disruption of pathways leading to apoptosis.

Abundant evidence suggests that the sphingolipid metabolite, ceramide, is a critical component of ionizing radiation-induced apoptosis (2–5). This apoptotic pathway is initiated by hydrolysis of sphingomyelin, a membrane lipid, attributable to the activation of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases SMases (6, 7) to generate ceramide. Ceramide, in turn, can activate several pathways important for the induction of apoptosis (reviewed in Refs. 6, 7). Both neutral and acidic SMases, distinguishable by their pH optima, have been reported to be involved in the induction of apoptosis after ionizing radiation (reviewed in Refs. 8, 9). Acidic SMase may play an essential role in radiation-induced apoptosis because lymphocytes from individuals with Niemann-Pick disease (who have an inherited deficiency of acidic SMase) and from acidic SMase-deficient mice, do not generate ceramide and have defective apoptotic responses to ionizing radiation (4). These deficits are reversible on restoration of acidic SMase activity, which further substantiates the obligatory role for ceramide generation in these apoptotic responses. However, ionizing radiation-triggered apoptosis of sensitive, but not resistant, human myeloid leukemic cell lines correlated with sphingomyelin hydrolysis and ceramide generation through activation of neutral, but not acidic, SMase (10). Similarly, loss of ceramide production from a neutral SMase confers resistance to radiation-induced apoptosis of lymphocytes (5). Moreover, depletion of glutathione, an endogenous inhibitor of neutral SMase (11), may also contribute to its activation, because glutathione depletion occurs in a variety of cells during radiation-induced apoptosis (12, 13). In addition, it has been suggested that de novo synthesis of ceramide as a result of increased ceramide synthase activity may also be involved in apoptosis (14), particularly in radiation-insensitive LNCaP prostate cancer cells that are induced to die by the phorbol ester PMA (15). LNCaP cells express androgen receptor, and their growth is increased by androgen. However, because they do not undergo apoptosis after androgen withdrawal, these cells can be used as an in vitro model to study strategies for treating prostate cancers that are resistant to androgen ablation. LNCaP cells are highly resistant to apoptosis induced by γ-irradiation, although somewhat sensitive to the death-inducing effects of TNF-α. Recently, we have shown that TNF-α sensitizes LNCaP cells to γ-irradiation-induced apoptosis by elevating ceramide levels (16). Moreover, exogenous C2-cer also sensitized LNCaP cells to irradiation, which lends further support to the notion that ceramide generation might be important for radiation-induced apoptosis in human prostate cancer.

One metabolite of ceramide, sphingosine, formed by ceramidase, has also been implicated in cell growth arrest and apoptosis. Sphingosine is rapidly produced during TNF-α-mediated apoptosis in human neutrophils (17) and cardiac myocytes (18). Recently, it has been shown that sphingosine and other long-chain sphingoid bases induce apoptosis in hepatoma cells by the activation of caspase-3-like proteases (19). Moreover, in androgen-independent human prostatic carcinoma DU-145 cells that express bcl-XL, sphingosine but not its metabolites induced apoptosis by down-regulation of bcl-XL independently of PKC inhibition (20). In contrast to the growth-suppressing and pro-apoptotic roles of ceramide

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The abbreviations used are: SMase, sphingomyelinase; TNF-α, tumor necrosis factor α; C2-cer, C2-ceramide (N-acetylsphingosine); IMEM, Richter’s improved minimal essential medium; ISEL, in situ end labeling; PKC, protein kinase C; SPP, sphingosine-1-phosphate; DM5, N,N-dimethylsphingosine; PMA, phorbol 12-myristate 13-acetate; PARP, poly(ADP-ribose) polymerase; FB1, fumonisin B1.
and sphingosine, SPP, formed from sphingosine by activation of sphingosine kinase (21), has been implicated in cellular proliferation and survival induced by platelet-derived growth factor, serum, nerve growth factor, and vitamin D3, and protects cells from apoptosis resulting from elevations of ceramide (22–26). In this report, we examined the role of ceramide, sphingosine, and sphingosine kinase in the sensitization of radio-resistant LNCaP prostate cells to γ-irradiation-induced apoptosis.

MATERIALS AND METHODS

Materials. Human TNF-α and poly-D-lysine were purchased from Boehringer Mannheim (Indianapolis, IN). Staphylococcus aureus SMase and FB1 were purchased from Sigma (St. Louis, MO). Sphingosine and DMS were from Biomedical Laboratories (Plymouth Meeting, PA). Escherichia coli diacylglycerol kinase was from Calbiochem (La Jolla, CA). Insulin/transferrin/selenium was from Biofluids (Rockville, MD).

Cell Culture. LNCaP and TSU-Pr1 human prostate cancer cells were maintained at 37°C in IMEM (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (27). LNCaP cells were plated on poly-D-lysine-coated dishes and grown for 5 days. Twenty-four h before treatment, cells were starved in serum-free IMEM medium without phenol red, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml). Cells were treated as indicated without or with γ-irradiation (8 Gy), using a JL Shepherd Mark I Irradiator [137 Cs] source with a dose rate of 209 cGy/min.

Apoptosis Measurement. ISEL was used to determine the extent of apoptosis as described previously (27). In some experiments, apoptotic morphology was also examined by staining cells with Hoechst 33258 (Calbiochem, San Diego, CA) as previously described (24). At least 500 cells were scored to calculate the percentage of apoptotic cells.

Extraction of Lipids. Cells were harvested in 1 ml of 25 mM HCl/methanol, and lipids were extracted with 2 ml of chloroform/methanol/acetate acid/water (10:4:3:2:1) and quantified with a Molecular Dynamics Storm phosphoimager exactly as described previously (23). Labeled ceramide-1-phosphate and SPP amounts of ceramide and sphingosine in cellular extracts were measured by the method of Mass Measurement of Total Cellular Phospholipids. Total phospholipids in extracts were quantified as described previously (28).

Measurements of Ceramide, Sphingosine, and SPP Levels. Mass amounts of ceramide and sphingosine in cellular extracts were measured by the diacylglycerol kinase and sphingosine kinase enzyme methods, respectively, exactly as described previously (23). Labeled ceramide-1-phosphate and SPP were resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) and quantified with a Molecular Dynamics Storm phosphoimager (Sunnyvale, CA). SPP levels were measured essentially as described previously (29). Briefly, 500 μl of buffer A [200 mM Tris-HCl (pH 7.4), 75 mM MgCl2, in 2 mM glycerine (pH 9.0)] and 50 units of alkaline phosphatase were added to the aqueous phase containing extracted SPP. After incubating 1 h at 37°C, 50 μl concentrated HCl were added, and sphingosine was extracted and quantitated with sphingosine kinase as described previously (29). For each experiment, known amounts of SPP were used to generate a standard curve.

Sphingosine Kinase Activity. Cells were harvested in buffer B [20 mM Tris (pH 7.4), 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM -glycerophosphate, 0.5 mM 4-deoxypyridoxine, 15 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride] and lysed by freeze-thawing. Supernatants were collected after centrifugation at 100,000 × g for 30 min at 4°C. Cytosolic sphingosine kinase activity was determined as described previously (23).

Immunoblotting. Cells were harvested in buffer C [10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% (v/v) CHAPS, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 20 μg/ml leupeptin] and Western blotting was carried out as described previously (24). Clone 7D3–6 mouse monoclonal anti-PARP (PharMingen, San Diego, CA; 0.5 μg/ml), rabbit polyclonal anti-caspase-7 (Oncogene, Cambridge, MA; 2.5 μg/ml), rabbit anti-caspase-3 (gift of Dr. Donald Nicholson), and mouse anti-caspase-8 (gift of Dr. Markus Peter), were used as primary antibodies. Proteins were visualized with SuperSignal-enhanced chemiluminescent reagent (Pierce, Rockford, IL) using antirabbit or antioimmunohorseradish peroxidase-conjugated IgG (Bio-Rad).

RESULTS

Sphingomyelinase Treatment Sensitizes LNCaP Cells to γ-Irradiation-induced Apoptosis. Previously, we have shown that treatment with C2-cer synergizes with γ-irradiation to induce cell death in LNCaP cells (16). In agreement, we have found that increasing endogenous long-chain ceramide levels in LNCaP cells by pretreatment with SMase induces apoptosis and sensitizes the cells to a dose of γ-irradiation (8 Gy) sufficient to trigger apoptosis of TSU-Pr1 but not of LNCaP cells (Fig. 1). Apoptosis increased in a time-dependent manner, and at least 48 h were required for significant cell death. To confirm the induction of apoptosis, we also examined DNA fragmentation and nuclear condensation by staining with the DNA-specific fluorochrome bisbenzimide (Hoechst 33258). Substantially more DNA ladder formation and fragmented nuclei were seen after 72 h than after 48 h (data not shown).

To examine whether there was a correlation between ceramide levels and radiation sensitivity, we measured changes in ceramide levels after irradiation of these two cell lines. Exposure of radiation-sensitive TSU-Pr1 cells to 8 Gy irradiation led to acute, but small, increases in ceramide levels within 30 min, followed by a sustained elevation in ceramide 12 h after irradiation, which reached a 3-fold increase by 48 h (Fig. 2A). This generation of ceramide preceded the appearance of nuclear fragmentation that was evident only after 48 h (Fig. 1). In contrast, ceramide levels did not change in radio-resistant LNCaP cells (Fig. 2A). In addition, irradiation induced a rapid and sustained decrease in sphingomyelinase activity in TSU-Pr1 cells but not in LNCaP cells (Fig. 2B). A surge in sphingosine levels was detected in TSU-Pr1 cells in correspondence with the inhibition of sphingomyelinase activity (Fig. 2C). SPP levels in the radiosensitive TSU-Pr1 cells were below the detection limit (<0.01 pmol/nmol phospholipid), and we were, thus, unable to detect any increases after irradiation. Interestingly, levels of SPP in TSU-Pr1 cells were much lower than in LNCaP cells (0.12 pmol/nmol phospholipid), which are more resistant to γ-irradiation. Thus, there seems to be a reciprocal relationship between ceramide/sphingosine and sphingosine kinase in radiation-sensitive prostate cancer cells.

Sphingosine Generation in Apoptosis-sensitized LNCaP Cells. Because it has been suggested that sphingosine, a breakdown product of ceramide, might also be a mediator of programmed cell death (19), it was of interest to determine whether sphingosine could be involved in the sensitization of LNCaP cells to apoptosis that was induced by irradiation. Treatment with bacterial SMase, which markedly sensitizes these cells to radiation (Fig. 1), as expected, induced a rapid 4- to 5-fold elevation in ceramide levels, which remained elevated at 24 h and declined thereafter (Fig. 3A). Interestingly, SMase treatment
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gamma-irradiation (Fig. 5B), although in agreement with a recent study (15), it almost completely blocked the increase in PMA-induced apoptosis in LNCaP cells. These results suggest that the de novo ceramide generation pathway does not play a role in the apoptosis that is induced by TNF-alpha and gamma-irradiation. The increase in ceramide preceded that of sphingosine, which suggested that sphingosine might arise from ceramidase-catalyzed metabolism of ceramide. This is a

Fig. 2. gamma-Irradiation-induced ceramide elevation and decreased sphingosine kinase activity in TSU-Pr1 but not in LNCaP cells. LNCaP cells (■) or TSU-Pr1 cells (□) were seeded at 2 × 10^6 per well and grown in 10-cm dishes. At the indicated time after exposure to 8 Gy of irradiation, ceramide levels (A), sphingosine kinase activity (B), and sphingosine levels (C, □) were measured as described in “Materials and Methods.” Results are means ± SD of triplicate determinations from a representative experiment and are expressed as fold-changes relative to zero time. The basal sphingosine level in TSU-Pr1 cells was 0.23 ± 0.07 pmol/nmol phospholipid. Similar results were found in three independent experiments. However, the small acute ceramide increase at 30 min observed in A was not statistically significant.

Fig. 3. Changes in ceramide and sphingosine levels after gamma-irradiation of sphingomyelinase-treated LNCaP cells. Cells were seeded and grown as described in Fig. 2 and treated without or with SMase (100 mU/ml; ■) and then irradiated with 8 Gy as indicated (□). At the indicated times, ceramide, sphingosine, and phospholipid levels were determined as described in “Materials and Methods.” Data are expressed as fold-increases relative to untreated controls, and are means ± SD of triplicate determinations from a representative experiment.

Fig. 4. Changes in levels of the sphingolipid metabolites ceramide, sphingosine, and SPP after treatment of LNCaP cells with gamma-irradiation and TNF-alpha. Cells were seeded and grown as described in Fig. 2. Cells were treated without (control) or with TNF-alpha (100 ng/ml) for 1 h before irradiation with 8 Gy as indicated. Ceramide (A), sphingosine (B), SPP (C), and phospholipid levels were measured after 6, 24, and 48 h as described in “Materials and Methods.” Results are means ± SD of triplicate determinations from a representative experiment and are expressed as fold-changes relative to control. Levels of ceramide, sphingosine, and SPP in untreated LNCaP cells were 19 ± 2, 0.23 ± 0.03, and 0.29 ± 0.06 pmol/nmol phospholipid, respectively. ■ control; □ gamma-irradiation (8 Gy); ■, TNF-alpha; □, TNF-alpha + 8 Gy.

also increased intracellular sphingosine after 24 h (Fig. 3B). It is likely that this increase in sphingosine results from degradation of ceramide, inasmuch as the ceramide increase preceded that of sphingosine. It should be pointed out that the elevation of these two sphingolipid metabolites precedes the onset of apoptosis (Fig. 1). As previously shown (16), we found that TNF-alpha also sensitized LNCaP cells to irradiation with a concomitant increase in ceramide levels (Fig. 4A and 5A). Although irradiation alone did not increase sphingosine levels in LNCaP cells, irradiation together with TNF-alpha induced significantly higher sphingosine levels than TNF-alpha alone (Fig. 4B). The increase in sphingosine was detected 24 h after treatment, coinciding with elevation in ceramide levels and preceding nuclear fragmentation that was evident 48 h after treatment (16). In LNCaP cells that were sensitized to die by irradiation and TNF-alpha, there was a marked decrease in SPP levels 48 h after treatment, whereas no significant changes in SPP levels were detected in cells treated with gamma-irradiation alone (Fig. 4C). It should be noted that SPP levels in LNCaP cells (0.12 ± 0.01 pmol/nmol phospholipid) are greater than in other normal and cancer cells (29), including HL60 cells (0.01 ± 0.001 pmol/nmol phospholipid), PC12 pheochromocytoma cells (0.02 ± 0.001 pmol/nmol phospholipid), and human breast cancer MCF7 cells (0.05 ± 0.001 pmol/nmol phospholipid), which might explain the high resistance of LNCaP cells to apoptotic stimuli.

De novo synthesis of ceramide has been implicated in the apoptosis of LNCaP cells induced by treatment with phorbol ester (15). Thus, it was of interest to examine whether the increased ceramide levels was attributable to stimulation of ceramide synthase. The mycotoxin, FB1, a known inhibitor of ceramide synthase, alone did not induce apoptosis, nor did it affect the extent of apoptosis induced by TNF and gamma-irradiation (Fig. 5B), although in agreement with a recent study (15), it almost completely blocked the increase in PMA-induced apoptosis in LNCaP cells. These results suggest that the de novo ceramide generation pathway does not play a role in the apoptosis that is induced by TNF-alpha and gamma-irradiation. The increase in ceramide preceded that of sphingosine, which suggested that sphingosine might arise from ceramidase-catalyzed metabolism of ceramide. This is a
likely possibility because sphingosine is not synthesized de novo, and can only be produced from ceramide (30, 31). However, N-oleoylethanolamine, a proposed acidic ceramidase inhibitor (32), by itself, even at a relatively low concentration (0.1 mM), markedly induced LNCaP cell death. It should be pointed out that N-oleoylethanolamine may not be a specific acidic ceramidase because it did not inhibit acidic ceramidase activity in an in vitro assay.

**Sphingosine and DMS Sensitize LNCaP Cells to γ-Irradiation-induced Apoptosis.** Sphingosine, but not ceramide, induced apoptosis of the androgen-independent human prostatic carcinoma cell line DU-145 (17, 20). To further examine whether sphingosine generation might also be important to sensitize LNCaP cells to irradiation, we used exogenously added sphingosine, which is efficiently taken up by cells. Significant apoptosis was induced by treatment with 20 μM sphingosine that was detectable only after 72 h (Fig. 6). Sphingosine also markedly sensitized LNCaP cells to γ-radiation in a dose-dependent manner (Fig. 6), which was evident even at 48 h. After irradiation in the presence of 20 μM sphingosine, most (>60%) of the cells were apoptotic by 72 h.

Another means to increase sphingosine levels is by the inhibition of sphingosine kinase. Recently, we (33) and others (34) have shown that DMS is a specific competitive inhibitor of sphingosine kinase which is effective at concentrations that do not inhibit PKC. DMS, at concentrations of 10 and 20 μM, induced 20 and 60% apoptosis, respectively, after 72 h (Fig. 7). Moreover, DMS also sensitized LNCaP cells to apoptosis induced by γ-irradiation in a dose-dependent manner.

**Activation of Caspases in Sphingosine and DMS-induced Sensitization of LNCaP Cells to γ-Irradiation.** The broad-specificity tetrapeptide caspase inhibitor z-VAD-fluoromethyl ketone blocks apoptosis induced by sphingosine or DMS in HL-60 (35) and Hep3B hepatoma cells (19), which suggests that a protease of the caspase-3 subfamily is activated. Thus, we examined whether elevation of sphingosine resulted in the activation of caspases-3 and -7, which drive the effector phase of apoptosis by cleaving key proteins, particularly the DNA repair enzyme PARP. As previously shown (16), treatment with 30 nm okadaic acid for 48 h resulted in apoptosis and PARP cleavage in LNCaP cells (Fig. 8). In agreement with our previous studies (16), when compared with okadaic acid, sphingolipid

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**Fig. 5.** Effect of FB1 on TNF-α and γ-irradiation-induced apoptosis. In A, cells were treated without (■) or with 100 ng/ml TNF-α (▲, ○) followed by irradiation (△, ●), and apoptosis was assayed by ISEL at the indicated times. ■, TNF-α + 8 Gy; ○, TNF-α; ▲, γ radiation (8 Gy); △, control. B, cells were treated with the indicated concentrations of FB1 in the absence or presence of TNF-α and/or 8 Gy irradiation as indicated, and apoptosis was determined after 72 h. □, 0 μM FB1; ●, 50 μM FB1; ■, 100 μM FB1; ▲, 150 μM FB1. Inset, the effect of FB1 (100 μg/ml) on PMA-induced apoptosis 48 h after treatment.

**Fig. 6.** Sphingosine sensitizes LNCaP cells to γ-irradiation-induced apoptosis. LNCaP cells were treated without or with the indicated concentrations of sphingosine for 1 h and then either not irradiated (□) or irradiated with 8 Gy (●). Apoptotic cells were assayed by ISEL at the indicated times. Data are means ± SD of triplicate determinations from one of three representative experiments.

**Fig. 7.** The sphingosine kinase inhibitor, DMS, induces apoptosis and sensitizes LNCaP cells to γ-irradiation-induced apoptosis. LNCaP cells were treated without or with the indicated concentrations of DMS for 1 h and then either not irradiated (□) or irradiated with 8 Gy (●). Apoptotic cells were assayed by ISEL at the indicated times. Data are means ± SD of triplicate determinations from one of three representative experiments.
metabolites were less effective in inducing PARP cleavage, even in the presence of radiation. Barely detectable PARP cleavage was seen after cells were exposed to sphingosine alone at 20 μM, a concentration that induced 30% apoptosis at 72 h (Fig. 6). PARP cleavage activity was increased in cells treated with sphingosine and 8 Gy irradiation after 72 h (Fig. 8), in agreement with the enhanced apoptosis. Interestingly, DMS alone was able to induce PARP cleavage in LNCaP cells without irradiation (Fig. 8), in agreement with its ability to induce apoptosis. Proteolytic processing of procaspases-3 and -7 was examined by Western blotting using antisera specific for caspase-3 and the active p20 caspase-7 subunit, respectively (Fig. 8). In agreement with our previous study (16), the procaspases-3 and -7 were cleaved into their active forms after treatment with okadaic acid, whereas no significant activation of caspase-3 could be detected in extracts from irradiated cells treated with sphingosine or DMS (Fig. 8). However, the activation of caspase-7 in the presence of sphingosine and DMS, especially after irradiation, was detected by the appearance of the M₁ 20,000 large subunit (Fig. 8). In contrast, no activation of the initiator caspase-8 could be detected after treatment with sphingosine or DMS, in the absence or presence of irradiation (Fig. 8).

**DISCUSSION**

Recently, we and others (6, 7, 36) have suggested that the sphingolipid metabolites, ceramide and sphingosine, provide proapoptotic signals, and an additional metabolite, SPP, promotes cell survival and suppresses apoptosis. Dysregulation of this sphingolipid biostat may be important in the acquisition of malignant phenotypes and radioresistance in which transformed cells can circumvent existing apoptotic mechanisms that would normally target the destruction of these cells. Thus, resetting this biostat could potentially be used to enhance apoptosis and overcome resistance to radiation or androgen ablation.

Previously, it has been suggested that LNCaP cells are highly resistant to induction of apoptosis by γ-irradiation attributable in part to a defect in ceramide generation (15, 16). Likewise, resistance to apoptosis involves a defect in ceramide generation in the PC3 prostate cancer cell line (37). However, radiation-induced apoptosis is not solely dependent on ceramide signaling, and there are other ceramide-independent pathways leading to apoptosis. Although in LNCaP cells, irradiation did not result in ceramide generation or apoptosis, pretreatment with PMA not only enhanced radiation-induced apoptosis, but also enabled ceramide generation via activation of ceramide synthase (38). In agreement, apoptosis was abrogated by FB1, a competitive inhibitor of ceramide synthase. Most importantly, when transplanted orthotopically into the prostate of nude mice, LNCaP cells produced tumors that showed the same responses to PMA and radiation therapy (38). However, apoptosis induced by treatment with TNF and γ-irradiation was not mediated by stimulation of de novo ceramide synthesis. Similarly, apoptosis in LNCaP cells induced by the topoisomerase 1 inhibitor camptothecin, ceramide generation was also independent of the de novo pathway (37).

A further metabolite of ceramide, sphingosine, has also been shown to induce apoptosis of androgen-independent human prostate cancer cells (20). Indeed, we found that ceramide production after TNF-α treatment in irradiated LNCaP cells or after SMase treatment is followed by a surge in sphingosine that precedes caspase activation and the onset of apoptosis. Furthermore, whereas addition of exogenous sphingosine induced modest apoptosis by itself, it significantly sensitized LNCaP cells to γ-irradiation. Irradiation of TSU-Pr1 cells, but not LNCaP cells, also produced a marked decrease in the activity of sphingosine kinase, the enzyme that phosphorylates sphingosine to form SPP, with a corresponding increase in sphingosine levels. In addition, a correlation between cell death and decreased SPP levels was observed in LNCaP cells treated with TNF-α plus γ-irradiation. Interestingly, SPP levels also decreased after treatment with TNF-α alone, which induces only modest elevations of sphingosine and ceramide, and which suggests that the balance between these sphingolipid metabolites may regulate LNCaP cell survival. These results raise the possibility that the individual enzymes in sphingolipid metabolism can be differentially regulated. In agreement, it has recently been shown that sphingosine kinase can be activated independently of sphingomyelinase or ceramidase (39). Furthermore, inhibition of sphingosine kinase by DMS blocked the increase in SPP, induced apoptosis, and sensitized prostate cancer cells to γ-irradiation. Thus, the regulation of the sphingolipid biostat may also have important implications for the treatment of prostate cancer, because many therapeutic approaches have been shown to cause accumulation of ceramide and sphingosine, including chemotherapy and ionizing radiation (6, 7, 36).

Ceramide generation in response to apoptotic stimuli is complex (6, 7). In LNCaP cells, neither SMase nor ceramide synthase are activated after irradiation alone (40). In agreement, we failed to detect changes in ceramide levels in γ-irradiated LNCaP cells (Fig. 2 and Ref. 16). However, TNF-α induced an ~2-fold ceramide elevation that was potentiated by irradiation. TNF-α recruitment of the adaptor protein FADD is required for stimulation of acidic SMase (41), which suggests a possible mechanism for ceramide generation in LNCaP cells. Alternatively, ceramide can be generated by the activation of ceramide synthase as has been shown for apoptosis in LNCaP cells mediated by PKC activation induced by the phorbol ester PMA (15). Interestingly, sphingosine, which has been shown to inhibit PKC in
induced robust activation of several caspases while triggering a similar caspase activation (Ref. 16) and Fig. 7), whereas okadaic acid downstream of sphingolipid metabolites. Interestingly, elevation of a study demonstrated that in these cells, TNF-α antagonistic biochemical signaling pathways could regulate the fate of tumor cells or virally infected cells, whereas, in some normal cells, such as in human endothelial cells, TNF-α is not cytotoxic. A recent study demonstrated that in these cells, TNF-α simultaneously and independently regulated sphingomyelinase and sphingosine kinase activity, which leads to the suggestion that the balance of these two antagonist biochemical signaling pathways could regulate the fate of cells in response to TNF-α stimulation (39).

Protease inhibitor studies indicate that ceramide and sphingosine may act independently to induce cell death, and it is argued that sphingosine induces activation of upstream caspases (19, 35). However, ceramide generated by upstream caspases, has been shown to activate additional downstream caspases necessary for apoptosis (6, 43–47). Moreover, ceramide could also induce cell death by a caspase-independent pathway (48). We found that caspase-7, but not caspase-3, was activated in sphingosine-sensitized γ-irradiation-induced apoptosis of LNCaP cells, in agreement with previous reports suggesting that activation of caspase-7, but not caspase-3, is an important step in the execution of apoptosis in LNCaP cells (49). The fact that LNCaP cell lines stably overexpressingcrmA are resistant to sphingolipid-induced-apoptosis may place crmA-inhibitable caspases downstream of sphingolipid metabolites. Interestingly, elevation of ceramide and/or sphingosine in LNCaP cells only had a modest effect on caspase activation (Ref. 16 and Fig. 7), whereas okadaic acid induced robust activation of several caspases while triggering a similar extent of apoptosis in comparison with γ-irradiation combined with TNF-α or with sphingolipid metabolites. This underscores the specificity of the apoptotic response to different stimuli in LNCaP cells and may indicate the participation of other death proteases, such as serine proteases, that cooperate with caspases in the execution of cell death (16, 50). This may be especially important for sensitization of radiation-induced apoptosis by sphingosine and DMS, because they were able to enhance apoptosis with a limited degree of caspase activation.

Different caspases are known to be activated in LNCaP and TSU-Pr1 cells, depending on the apoptotic stimuli. In LNCaP cells, caspases-3, -6, -7, and -8 are activated after okadaic acid treatment. In contrast, caspase-3 is not involved in TNF-α- or γ-irradiation-induced death in LNCaP cells, but is markedly activated in TSU-Pr1 cells. This could explain the fact that LNCaP cells are highly resistant to radiation-induced apoptosis compared with TSU-Pr1 cells (16, 51). Recent studies have demonstrated that caspase-7 and -3 are critical mediators of apoptosis in LNCaP cells. Moreover, overexpression of caspase-7 induced apoptosis even in LNCaP cells that overexpressed the oncoprotein bcl-2 (51). However, in addition to the differential activation of unique caspases (16), our recent study suggests the involvement of additional cell-type specific signaling events in prostate cancer cell death, including activation of the JNK/SAPK pathway (27), which is thought to be involved in ceramide (52) and sphingosine-induced apoptosis (53). Interestingly, expression of bcl-2 not only protected prostate carcinoma cells against the induction of apoptosis by exogenous C2-zer but also blocked its ability to activate JNK1, which indicated that bcl-2 functions at the level of JNK1 or upstream of JNK1 in the ceramide/JNK pathway (54).

Collectively, our results suggest that ceramide and sphingosine generation, together with the inhibition of sphingosine kinase, are critical components in radiation-induced apoptosis in human prostate cancer cells. Preventing ceramide and sphingosine generation and/or stimulation of sphingosine kinase may provide a selective advantage in the development of radioreistance of prostate tumors. Therefore, development of agents that specifically regulate levels of sphingolipid metabolites may provide new tools to use in conjunction with radiation therapy.

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