Tumor Necrosis Factor-α Sensitizes Prostate Cancer Cells to γ-Irradiation-induced Apoptosis

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ABSTRACT

LNCaP prostate cancer cells are highly resistant to induction of programmed cell death by γ-irradiation and somewhat sensitive to the death-inducing effects of tumor necrosis factor (TNF)-α. Simultaneous exposure of LNCaP cells to TNF-α and 8 Gy of irradiation was synergistic and resulted in a 3-fold increase of apoptotic cells within 72 h compared to TNF-α alone. It appeared that TNF-α sensitized the cells to irradiation because, when cells were irradiated 24 h after exposure to TNF-α, increased cell death was observed. In contrast, irradiation delivered 24 h prior to TNF-α exposure did not result in more cell death than after TNF-α alone. TNF-α induced expression of its own mRNA, but TNF-α mRNA induction was neither induced nor enhanced by irradiation. Activation of the transcription factor nuclear factor κB can be induced by TNF-α and has a modulating antiapoptotic effect. But enhancement of TNF-α-induced cell death by irradiation did not result from altered activation of nuclear factor κB. TNF-α treatment of LNCaP cells resulted in partial activation of caspase-8 and -6 but not caspase-3. There was only minimal poly(ADP-ribose) polymerase cleavage seen in LNCaP cells after exposure to both TNF-α and irradiation at 72 h, a time when 60% of the cells were apoptotic. Experiments with peptide inhibitors of cysteine and serine proteases suggested that caspases were the predominant mediators of apoptosis induced by TNF-α alone but that serine proteases contributed significantly to cell death induced by TNF-α plus irradiation. TNF-α increased production of ceramide in LNCaP cells 48 h after exposure. Although irradiation alone had no effect on ceramide production in LNCaP cells, TNF-α plus irradiation induced significantly more ceramide than TNF-α alone. Ceramide production did not occur immediately after exposure to TNF-α, but rather was delayed such that ceramide levels were increased only 24 h after exposure to apoptotic stimuli. Moreover, nontoxic levels of exogenous C2-ceramide sensitized LNCaP cells to irradiation similarly to TNF-α, suggesting that one mechanism by which LNCaP cells were sensitized to irradiation was by increased intracellular ceramide. Hence, ceramide generation is a critical component in radiation-induced apoptosis in human prostate cancer cells. Inhibition of ceramide generation may provide a selective advantage in the development of radioresistance in prostate cancer.

INTRODUCTION

One mechanism by which cancer cells become resistant to radiation or chemotherapy is disruption of the pathways that lead to apoptosis (1, 2). Radiation is an important treatment modality for prostate cancer, and it kills cells by inducing apoptosis, generating free oxygen species, and causing DNA strand breaks (3–6). γ-Irradiation activates acidic sphingomyelinase to produce ceramide, a catabolic product of membrane sphingolipids that is a cell death signal (7–11). Ceramide can also be generated de novo by ceramide synthase, as was shown in LNCaP cells induced to undergo apoptosis after exposure to 12-O-tetradecanoylphorbol-13-acetate (12). Although ceramide appears to function as a second messenger for stress signaling (13–17), there is still some controversy about the precise role that ceramide plays in apoptosis (7, 18–21).

LNCaP is a hormone-dependent human prostate cancer cell line that is moderately sensitive to induction of apoptosis by TNF-α, an inflammatory cytokine that can induce a diverse range of biological responses (22, 23). Signaling by TNF-α is initiated by binding to tumor necrosis factor receptor 1, which causes the association of an adapter protein TNF receptor-associated death domain with the intracellular death domain of the tumor necrosis factor receptor 1 molecule (24). TNF receptor-associated death domain mediates the subsequent recruitment of an adapter protein Fas-associated death domain to form a DISC, which initiates apoptosis through activation of caspase-8, a proximal element in the cascade of cysteine proteases (18, 25–30). TNF-α can induce ceramide production, which, in turn, induces activation of caspase-3 and -7, which cleave PARP, and of caspase-6, which cleaves lamain B, thus contributing to dissolution of the nuclear envelope (19, 31–33). Signaling by TNF-α also activates antiapoptotic cell signals via activation of the transcription factor NF-κB, which may override the effects of apoptosis pathways in some cells (34–36).

Here, we show that, although LNCaP cells are highly resistant to γ-irradiation-induced apoptosis, irradiation and TNF-α synergize to induce apoptosis in LNCaP cells. Cell death after exposure to TNF-α plus irradiation is characterized by increased production of ceramide 48–72 h after exposure. Moreover, induction of cell death by the combination of γ-radiation and TNF-α is mediated by the cooperative effects of cysteine and serine proteases, both of which have to be inhibited to block LNCaP cell death completely.

MATERIALS AND METHODS

Cell Culture. Human prostate cancer cell line LNCaP was routinely cultured at 37°C in IMEM (Biofluids, Rockville, MD) supplemented with 5% FCS using standard cell culture procedures (37, 38). Experiments in serum-free medium were performed in IMEM supplemented with either insulin, selenium, and transferrin at 5 μg/ml each or with 1 mg/ml BSA. Twenty-four h before exposure to TNF-α and/or irradiation, the medium was changed to IMEM without phenol red (Biofluids) supplemented with 5% charcoal-stripped calf serum. TNF-α was used at 40 ng/ml to treat LNCaP cells except when dose-response studies were performed. Caspase inhibitors were added 1 h before treatment of cells with either TNF-α or γ-irradiation. Exogenous C2-ceramide was added to cells cultured in serum-free IMEM for 24 h. YVAD was purchased from Bachem Bioscience (King of Prussia, PA). DEVD and zVAD were purchased from Enzyme Systems Products (Livermore, CA). TLCK was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant, human TNF-α, produced in E. coli α and purified by standard chromatographic techniques was purchased from Boehringer Mannheim (Indianapolis, IN).

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3 The abbreviations used are: TNF, tumor necrosis factor; DISC, death-inducing signaling complex; PARP, poly(ADP-ribose) polymerase; NF-κB, nuclear factor κB; IMEM, improved MEM; YVAD, N-acetyl-Tyr-Val-Ala-Asp-chloromethylketone; DEVD, N-acetyl-Asp-Glu-Val-Asp(OMe)-CH 2 F; zVAD, z-Val-Ala-Asp(OMe)-CH 2 F; TLCK, Na-p-tosyl-L-lysine-chloromethylketone; ISEL, in situ end labeling; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase-PCR; GAFDH, glyceraldehyde-3-phosphate dehydrogenase.
IN. The primary structure of recombinant, human TNF-α is identical to that of natural human TNF-α and has a M, of 17,000. According to the manufacturer, the specific activity is 10³ units/mg. C₂-Ceramide was purchased from Biomol Laboratories (Plymouth Meeting, PA). For γ-irradiation, we used a JL Shepherd Mark I Irradiator 137 Cs source with a dose rate of 209 mGy/min.

**Apoptosis Assays.** ISEL assay is routinely used in our laboratory for apoptosis determination and has been described previously(39). To detect DNA ladder formation, we suspended cells in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% sodium-N-lauroylsarcosine. RNase and proteinase K were added to the cell suspension both at the concentration of 10 μg/ml sequentially. After incubation with RNase for 1 h and with proteinase K for 4 h, the samples were extracted several times with phenol-chloroform and precipitated with ethanol and salt. The precipitants were dissolved in TE buffer and resolved on a 1.8% agarose gel.

**Western Blotting.** Two hundred μg of total cellular protein were resolved by electrophoresis on either 8–16% or 10–20% SDS-polyacrylamide gradient gels and transferred onto nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories, Hercules, CA). After blocking with 5% milk in 10 mM Tris-HCl (pH 8.0)–150 mM NaCl with 0.05% Tween 20, membranes were probed with monoclonal antibody to PARP (Enzyme Systems Products, Dublin, CA), rabbit antisera to caspase-3 (a gift from Kristine Kikly, SmithKline Beecham, King of Prussia, PA), caspase-8 antisera (from the laboratory of Peter Krammer), or antibody to lamin B (Calbiochem, La Jolla, CA) and visualized with enhanced chemiluminescence detection (Pierce, Rockford, IL).

**Ceramide Assay.** Lipids were extracted according to the method of Bligh and Dyer (40). Cell lysates were suspended into methanol:chloroform:H₂O (1:1:1). The organic phase was dried under N₂. Lipids were resuspended in 40 μl 7.5% octyl-β-D-glucopyranoside (Calbiochem, Cambridge, MA), 5 mM cardiolipin (Avanti Polar-Lipids, Alabaster, AL), and 10 μM imidazole (pH 6.6) by freezing and thawing several times. Lipids were radiolabeled by the kinase reaction using [γ-32P]ATP (Amersham Life Science, Arlington Heights, IL). One hundred μl of 100 mM imidazole-HCl (pH 6.6), 100 mM NaCl, 25 mM MgCl₂, and 2 mM EGTA; 20 μl of 20 mM EDTA; 30 μl of 6.6 μM ATP; 10 μCi of [γ-32P]ATP; and 10 μg of sn-1,2-diacylglycerol kinase (Calbiochem) and [γ-32P]ATP (Amersham Life Science, Arlington Heights, IL). One hundred μl of 100 mM imidazole-HCl (pH 6.6), 100 mM NaCl, 25 mM MgCl₂, and 2 mM EGTA; 20 μl of 20 mM EDTA; 30 μl of 6.6 μM ATP; 10 μCi of [γ-32P]ATP; and 10 μg of sn-1,2-diacylglycerol kinase were added to each suspended lipid sample. After the reaction mixture was incubated at room temperature for 1 h, the reaction was extracted with 0.5 ml of chloroform:methanol:HCl (100: 100:1) and 50 μl of 2 M KCl. Lipids were separated by TLC, and the radioactive bands were quantitated using a Molecular Dynamics PhosphorImager analyzer (Sunnyside, CA). The amount of ceramide content in each sample was standardized based upon the amount of phospholipids determined by the method of Van Veldhoven and Mannerra(41).

**NF-κB Mobility Shift Assay.** Binding of NF-κB to a consensus DNA sequence was performed as described by Dignam et al. (42). Cells were washed with ice-cold PBS, resuspended in 5 volumes of 1 mM Hepes-HCI (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 0.25 mM DTT, and 1 mM PMSF and incubated on ice for 20 min. Cells were homogenized by 15–20 strokes in a glass homogenizer and centrifuged at 7500 rpm for 6 min at 4°C. The pellets were washed and resuspended in 20 mM Hepes-HCl (pH 7.5), 20% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.25 mM DTT, and 1 mM PMSF, vortexed, and centrifuged at 14,000 rpm for 45 min. Two μg of nuclear extract were assayed for binding to an oligonucleotide containing a radiolabeled NF-κB binding site consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA). Binding was carried out by mixing 1–2 μl of nuclear extract; 2 μl of 50 mM Hepes-HCl (pH 7.5), 5 mM EDTA, 5 mM 2-mercaptoethanol, and 20% glycerol; 1 μl of 1.5 mg/ml poly(dIdC); 1 μl of 20 mM Hepes-HCl (pH 7.9); 20% glycerol, 100 mM KCl, 250 mM EDTA, 500 mM DTT, 500 mM PMSF, and 1% NP40; 1 μl of oligonucleotide; and 3 μl of water. The samples were incubated for 30 min at a room temperature. The samples were resolved on 4% acrylamide gels and analyzed by PhosphorImager.

**RT-PCR.** TNF-α, TNF-β, and control GAPDH mRNA were assayed by PCR amplification of cDNA isolated from LNCaP cells. The primer sequences for PCR amplification are as follows: TNF-α, left, 5’-GGTCCAGGCGGCTGTTGTC-3’; right, 5’-CAAGGCTGTTGCTGTTCCG-3’. TNF-β, left, 5’-GGTCCAGGCGGCTGTTGTC-3’; right, 5’-GAAGGGGCTTCAAGAAGAACAGTA-3’. GAPDH, left, 5’-GGGAAGGTGAAGGTCGCGT-3’; right, 5’-GGTGCATTGATGCCAACAAGA-3’. PCRs were cycled at 94°C for 1 min, 55°C for 1 min, and 72°C for 30 s. For TNF-α, 32 cycles were completed; for TNF-β, 37 cycles were completed; and for GAPDH, 32 cycles were completed.

**RESULTS**

**Induction of Apoptosis in LNCaP cells by TNF-α and γ-Irradiation.** LNCaP cells are resistant to apoptosis after exposure to 8 Gy of irradiation. TNF-α at a concentration of 40 ng/ml induced ~30% apoptotic cells by 72 h (22, 37). However, simultaneous exposure of LNCaP cells to TNF-α and 8 Gy of irradiation resulted in ~60% apoptosis after 72 h (Fig. 1A). A similar enhancement of cell death by combined TNF-α and irradiation treatment was seen in serum-free medium (Fig. 1B). To determine whether irradiation affected the sensitivity of LNCaP cells to apoptosis induced by TNF-α, we exposed the cells to different concentrations of TNF-α and, simultaneously, to 8 Gy of irradiation. Concentrations of TNF-α of at least 10 ng/ml were required for the induction of apoptosis, whether or not the cells were irradiated with 8 Gy, and the range of effective TNF-α concentrations for apoptosis induction was not affected by γ-irradiation (Fig. 1C). No apoptosis was seen at lower doses of TNF-α. To confirm the induction of apoptosis, we also examined DNA fragmentation. Substantially more DNA ladder formation was seen after treatment with TNF-α and irradiation than after TNF-α alone (Fig. 1D). The degree of DNA ladder formation generally corresponded with the results of the ISEL assay shown in Fig. 1C. We also observed that a higher dose of γ-irradiation (20 Gy) neither caused apoptosis in LNCaP cells nor enhanced the effect of TNF-α any more than did 8 Gy of irradiation (Fig. 1E). The temporal relationship of exposure with TNF-α and irradiation was also shown to be important for the enhancement of TNF-α-induced apoptosis. If LNCaP cells were exposed to TNF-α 24 h after 8 Gy of irradiation, no enhancement of apoptosis was seen compared to exposure to TNF-α alone. In contrast, if the cells were first exposed to TNF-α and irradiated 24 h later, the synergistic effect of the two treatments was seen (Fig. 1F). The combined effect of TNF-α and irradiation was seen when cells were irradiated at 12 or 24 h but not at 36 h after treatment with TNF-α (data not shown).

**Expression of Endogenous TNF-α.** Because TNF-α can induce its own mRNA, we asked whether irradiation affected the induction of TNF-α message and, thereby, enhanced cell death activated by exogenous TNF-α. Fig. 2 shows a semiquantitative RT-PCR assay for TNF-α mRNA under different conditions used to treat LNCaP cells. TNF-β (lymphotoxin) and GAPDH were used as controls for mRNA induction and loading. Whereas TNF-α induced its own mRNA, irradiation did not induce TNF-α mRNA. Moreover, combined TNF-α and irradiation did not change the expression of TNF-α mRNA that was induced by TNF-α alone.

**Activation of NFκB in LNCaP Cells Exposed to TNF-α and γ-Irradiation.** NFκB is an antiapoptotic transcription factor that is released from a cytoplasmic complex with its inhibitor protein, IκB, after binding of TNF-α to its receptor (34–36, 43). After TNF-α treatment, there were no differences in nuclear NFκB in the presence or absence of 8 Gy of irradiation (Fig. 3). NFκB was found to be present in the nucleus at 6 h after treatment with TNF-α and sustained at the same level throughout 48 h of exposure to TNF-α with or without irradiation (data not shown).

**Caspase Activation in LNCaP Cells Exposed to TNF-α and γ-Irradiation.** Proteolytic cleavage and activation of caspase-8 is believed to result from interaction between caspase-8 and the DISC after TNF-α binds to the TNF receptor (27). Caspase-8 cleavage peptides p42 and p20 were generated by 72-h treatment with 40 ng/ml TNF-α, with or without irradiation (Fig. 4). There was a slight induction of caspase-8 cleavage induced by 10 ng/ml TNF-α after 8
Gy of irradiation. Caspase-3 is downstream of caspase-8 and is a critical effector protease for cleavage of PARP and for activation of the endonuclease that generates characteristic DNA ladder formation seen in some forms of cell death (44, 45). We observed essentially no caspase-3 activation in LNCaP cells treated with 40 ng/ml TNF-α and 8 Gy of irradiation. In contrast, exposure to 30 nM okadaic acid, a known apoptotic agent for these cells, resulted in robust caspase-3 activation (Refs. 46 and 47; Fig. 4).

A small amount of PARP cleavage was seen after cells were exposed to TNF-α alone at 40 ng/ml, a concentration that induced 30% apoptosis at 72 h (Fig. 4). PARP cleavage activity was increased in cells treated with 40 ng/ml TNF-α and 8 Gy of irradiation for 72 h. For comparison, treatment of LNCaP cells with 30 nM okadaic acid for 48 h resulted in pronounced PARP cleavage. Because relatively little PARP cleavage was seen, it was not surprising that another protease that cleaves PARP, caspase-7, was not activated, as is the case after exposure of LNCaP cells to lovastatin (Refs. 48–52; Fig. 4).

Cleavage of lamin B by caspase-6 is important for dissolution of the nuclear membrane during apoptosis (53, 54). Cleavage of lamin B into the characteristic Mr 28,000 fragment was detected under the same conditions that generated caspase-8 activation (Fig. 4). Western blot-
Inhibition of Apoptosis by Protease Inhibitors. Apoptosis in prostate cancer cells may be mediated by both caspases and serine proteases (55). To determine the role of both cysteine and serine proteases in apoptosis induced by TNF-\(\alpha\) and 8 Gy of irradiation, we treated cells with peptide inhibitors specific for either caspases or serine proteases (18, 56). DEVD, an inhibitor of caspase-3, -7, and -8 (21), at 50 \(\mu\)M completely inhibited apoptosis induced by TNF-\(\alpha\) alone but had almost no effect on apoptosis induced by TNF-\(\alpha\) plus 8 Gy of irradiation (Fig. 5A). YVAD, an inhibitor of caspase-1 and related proteases (19), did not show any effect on apoptosis of LNCaP cells after treatment with TNF-\(\alpha\) in the absence or presence of irradiation (Fig. 5B). zVAD, which is a general inhibitor of caspase activity (21), completely inhibited TNF-\(\gamma\)-induced apoptosis in LNCaP cells and reduced apoptosis induced by TNF-\(\alpha\) plus 8 Gy of irradiation by 75\% (Fig. 5C). Because caspase inhibition by zVAD did not completely abrogate apoptosis induced by TNF-\(\alpha\) plus 8 Gy irradiation, we also treated LNCaP cells with an inhibitor of serine proteases, TLCK. TLCK had no effect on apoptosis induced by TNF-\(\alpha\) alone but substantially reduced apoptosis after exposure to TNF-\(\alpha\) and 8 Gy irradiation (Fig. 5D). The importance of both caspases and serine proteases to apoptosis induced by TNF-\(\alpha\) plus 8 Gy irradiation was shown by the combined inhibitory effect of both peptides that was greater than either peptide used alone (Fig. 5E).

To confirm that the peptide inhibitors inhibited caspase activity, caspase-8 and PARP cleavage was determined in the presence of the different inhibitors. Caspase-8 activation was completely blocked by zVAD and DEVD (Fig. 6). The fact that caspase-8 activation was blocked although substantial apoptosis occurred in the presence of caspase inhibitors underscored the fact that serine and other proteases also play a role in apoptosis induction.

Fig. 2. RT-PCR analysis of TNF-\(\alpha\), TNF-\(\beta\), and GAPDH mRNA in LNCaP cells treated with 40 ng/ml TNF-\(\alpha\) with or without irradiation. As a control, 100 nM etoposide was used, which induces partial cell death in LNCaP cells (39) and complete cell death in TSU-Pr1 prostate cancer cells. As can be seen, induction of TNF-\(\alpha\) mRNA occurred both in LNCaP cells and in TSU-Pr1 cells 48 h after treatment with etoposide.

Fig. 3. A mobility shift assay for NF-\(\kappa\)B in LNCaP cells treated with TNF-\(\alpha\) and/or \(\gamma\)-irradiation. LNCaP cells were treated with 40 ng/ml TNF-\(\alpha\) and/or 8 Gy of irradiation, as indicated. The nuclear extracts were made 48 h after the treatment, and a mobility shift assay for NF-\(\kappa\)B was performed. The competitor in Lane 7 (from left to right) was 100-fold excess of cold oligonucleotide with the NF-\(\kappa\)B binding sequence. The p65 and p50 protein components of NF-\(\kappa\)B are indicated on the right.

Fig. 4. Effect of TNF-\(\alpha\) and/or \(\gamma\)-irradiation on the cleavage of caspase-8, caspase-3, caspase-7, lamin B, and PARP. LNCaP cells were treated with different concentrations of TNF-\(\alpha\) without or with 8 Gy of irradiation. Protein extracts were made after 72 h and subjected to Western blotting. Lane C, positive control protein samples. For caspase-8, the positive control was Jurkat cells treated with anti-FAS antibodies. For the other Western blots, the Lane C contained proteins from LNCaP cells treated with 30 nM okadaic acid for 48 h.
proteases mediated apoptosis induced by the combination of TNF-α and irradiation.

Low levels of PARP cleavage seen in LNCaP cells treated with TNF-α and irradiation were abrogated by treatment with DEVD, zVAD, and also TLCK, suggesting that the low level of PARP cleavage seen after exposure to TNF-α plus irradiation resulted from caspase activation downstream from serine proteases (Fig. 6). Interestingly, lamin B cleavage, which is attributed to caspase-6 (53), was not inhibited by DEVD or TLCK but was blocked by zVAD. Because DEVD inhibited caspase-8 activation but not lamin B cleavage, this suggests that caspase-6 is not directly activated by caspase-8, as has been suggested previously (57). The experiments with caspase inhibitors showed that, although γ-irradiation alone had no effect on protease activation or apoptosis, when combined with TNF-α, γ-irradiation augmented caspase activation and caused serine protease activation. Thus, both caspases and serine proteases are responsible for the execution of cell death in LNCaP cells exposed to TNF-α and irradiation.

Fig. 5. Effect of caspase inhibitors on LNCaP cells treated with 40 ng/ml TNF-α, irradiation, or both. A–D, cells were treated with the indicated concentrations of inhibitors in the absence or presence of 40 ng/ml TNF-α and 8 Gy irradiation. E, after treatment with either zVAD, TLCK, or zVAD plus TLCK, cells were then exposed to 40 ng/ml TNF-α and 8 Gy of irradiation. The percentages of apoptotic cells in A–E were determined after 72 h by the ISEL assay. Bars, SD.
Ceramide Formation after Exposure to TNF-α and/or γ-Irradiation. Both TNF-α and γ-irradiation can induce the production of ceramide in a wide variety of cells (7, 58). Exogenous ceramide itself can induce cells to undergo apoptosis (59, 60). In some cells, ceramide formation was induced as early as 5–10 min after exposure to TNF-α. However, there were no significant changes of ceramide levels during the first 60 min after exposure of LNCaP cells to TNF-α and/or 8 Gy of irradiation (data not shown). Although there was no increase in ceramide production after 6 h, there was a 2-fold increase after exposure of LNCaP cells to 40 ng/ml TNF-α for 24 and 48 h (Fig. 7A). Whereas 8 Gy of γ-irradiation alone did not induce any ceramide production in LNCaP cells, in the presence of TNF-α, there was a marked increase in ceramide levels. However, because no increase in ceramide production was seen within 6 h of exposure to irradiation or TNF-α, it is unlikely that activation of ceramide production is an early event in apoptosis induced by either TNF-α alone or in the presence of 8 Gy of irradiation. In LNCaP cells, YVAD, DEVD, TLCK, and zVAD had very little effect on ceramide production after exposure to TNF-α or to the combination of TNF-α and irradiation (Fig. 7B). The minimal effect of protease inhibitors on ceramide production is in contrast to the marked inhibition of apoptosis (Fig. 5E) and caspase activation (Fig. 6), suggesting that ceramide production is upstream of serine protease and caspase activation.

Because ceramide production induced by TNF-α may possibly sensitize LNCaP cells to induction of apoptosis by γ-irradiation, we exposed LNCaP cells to increasing doses of the cell permeable C2-ceramide and measured apoptosis. At a concentration of 10 μM, C2-ceramide induced 15% apoptosis after 72 h, which was markedly increased to 48% after simultaneous exposure to γ-irradiation (Fig. 8). Therefore, both TNF-α and C2-ceramide sensitized LNCaP cells to irradiation and synergized with irradiation in the induction of cell death.

DISCUSSION

Cancer cells develop resistance to programmed cell death as a mechanism of chemotherapy and radiation resistance. LNCaP prostate cancer cells are virtually resistant to induction of programmed cell death by exposures to γ-irradiation as high as 60 Gy. Although DNA damage at high doses of radiation precludes clonogenicity, resistance to apoptosis may permit prostate cancer cells to survive lower doses of γ-irradiation to allow DNA repair and further replication. It is noteworthy that the LNCaP cells used in this study have no mutations of the p53 gene and, therefore, must not be susceptible to p53-mediated apoptosis after irradiation (61). The observation that irradiation did not result in caspase activation or ceramide production suggests that LNCaP cells have a very early block in radiation-induced apoptosis. This block may be attributed to attenuated ceramide production, as our data have suggested, but may also involve other death pathways. Our preliminary investigations have suggested that mitochondrial death pathways are not activated by TNF-α plus irradiation in LNCaP cells.

TNF-α enhanced γ-irradiation-induced apoptosis in LNCaP cells at least in part through the augmentation of pathways involving ceramide. Ceramide production increased 24 h after exposure to TNF-α with or without irradiation. Apoptosis was seen at 48 h. Unlike many other cell lines that undergo cell death within a few hours after exposure to...
after exposure to TNF-α plus irradiation. TNF-α directly activates caspase-8 by signal transduction through the DISC. However, when TNF-α was combined with irradiation, caspase-8 was activated both through the DISC and by serine protease activation. Moreover, although DEVD blocked caspase-8 cleavage, it had minimal effect on apoptosis after exposure to the combination of TNF-α and irradiation.

Our data show that increased ceramide production correlated with enhanced apoptosis in LNCaP cells treated with TNF-α and irradiation. Although irradiation alone did not alter ceramide levels, in the presence of TNF-α, it increased ceramide production. In many cells, ceramide is induced rapidly by both TNF-α and γ-irradiation. In LNCaP cells, we could not detect any change in ceramide content within the first 12 h after TNF-α treatment or irradiation. By 24 h after exposure to TNF-α with or without irradiation, an increase in ceramide content was observed. The ceramide increase at 24 h preceded the onset of substantial apoptosis in LNCaP cells. In cells that manifest both immediate and late peaks of ceramide production, apoptosis correlates with the magnitude of the later rise of ceramide (19). Our data agree with these findings in that the timing and magnitude of ceramide production correlated with the degree of cell death. Moreover, it seems that ceramide generation is a critical component of radiation-induced apoptosis in human prostate cancer cells and suggests that blockage of ceramide generation may provide a selective advantage in the development of radioresistance of prostate tumors. These data are in accordance with recent results indicating that loss of ceramide production confers resistance to γ-irradiation-induced apoptosis in WEHI-231 cells (66).

There appears to be a limit to the amount of apoptosis that can be induced by TNF-α in LNCaP cells. We observed that exogenous TNF-α activated its own mRNA but that irradiation had no effect on TNF-α production. This suggests that limitations in the death response after exposure to TNF-α are probably mediated at the level of interaction between TNF-α and its receptor. In contrast to the failure of LNCaP cells to activate TNF-α mRNA in response to irradiation, radiation-induced death of sarcoma cell lines was mediated, at least in part, by TNF-α acting through an autocrine pathway (67). There is also evidence that cell death induced by radiation or chemotherapy may be accompanied by autocrine production of death ligands (68, 69).

Other death ligands may be activated in response to irradiation. Recent data demonstrated that ceramide rapidly and strongly induced expression of Fas ligand, which interacts with CD95 (APO-1/Fas) to activate cleavage of caspases in fibroblasts (70, 71). Activation of CD95 (APO-1/Fas) signaling by ceramide mediated cancer therapy-induced apoptosis (71). Thus, ceramide, similarly, may link γ-irradiation to induction of the CD95 system of apoptosis to enhance and amplify the death program in an autocrine manner. LNCaP cells express CD95 antigen, but when exposed to FAS antibody in medium containing 10% FCS, they have a minimal apoptotic and antiproliferative response to anti-Fas antibody (72). Our experiments were performed in 5% charcoal-stripped calf serum, conditions that may have been more permissive for the effects of Fas ligand to contribute to cell death after TNF-α and irradiation. However, we saw no difference in death induction when our experiments were done in serum-free medium.

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