Reversal of Radiation Resistance in LNCaP Cells by Targeting Apoptosis through Ceramide Synthase

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ABSTRACT

Cell lines derived from human prostate cancer are regarded as relatively resistant to both radiation-induced clonogenic death and apoptosis. Here we attempted to modulate the response of LNCaP prostate cancer cells to radiation therapy (XRT) by pretreatment with 12-O-tetradecanoylphorbol acetate (TPA), a known apoptogenic agent in LNCaP cells. Using plateau-phase cultures, we investigated the response of these cells to XRT, TPA, and a combination of XRT and TPA. LNCaP irradiation did not result in ceramide generation or apoptosis. However, pretreatment with TPA enabled XRT to generate ceramide via activation of the enzyme ceramide synthase and signal apoptosis. Apoptosis was abrogated by the combination of the two resulted in substantial (20–25%) apoptosis within 24 h. There was an additional benefit associated with this regimen because TPA pretreatment protected the adjacent rectum from radiation-induced apoptosis. This represents the first description of signaling-based therapy designed to overcome one form of radiation resistance expressed preferentially in LNCaP human prostate cancer cells.

INTRODUCTION

Prostate cancer is the most prevalent cancer in men, resulting in approximately 42,000 deaths annually in the United States (1). Although radiation is capable of permanently eradicating localized prostate tumors, nearly 30% of patients treated with potentially curative doses relapse at the sites of the irradiated tumors (2–4). These data indicate that prostate tumors vary in sensitivity to ionizing radiation. Furthermore, clinical data show that in patients who relapse locally after irradiation, initial treatment eliminates the great majority of the tumor cells, whereas a small fraction of tumor clonogens survive the lethal effects of radiation and eventually repopulate the irradiated site. This observation indicates that there are variations in clonal sensitivity to the lethal effects of radiation even within a given tumor. Thus far, there have been no criteria for predicting the presence or prevalence of radiation-resistant tumor clones, nor is there an effective approach to modulate the radiation response of human prostate tumor cells. Improved understanding of pathways of radiation-induced cell death and signaling systems that regulate these pathways may yield opportunities for pharmacological modulation of radiation resistance in prostate cancer.

The predominant mechanism by which radiation kills mammalian cells is the reproductive (also known as clonogenic) death pathway. DNA is the target, and double-stranded breaks in the DNA are regarded as the specific lesions that initiate this lethal response (5, 6). Whereas most radiation-induced DNA double-stranded breaks are rapidly repaired by constitutively expressed DNA repair mechanisms, residual unrepaired or misrepaired breaks lead to genetic instability and to increased frequency of mutations and chromosomal aberrations (5–7). Lethal mutations or dysfunctional chromosomal aberrations eventually lead to progeny cell death (7, 8), usually after several mitotic cycles (9, 10).

Radiation can also signal apoptosis, although it appears to be significantly less prevalent than clonogenic cell death (11). Apoptosis is an inducible death pathway of sequential biochemical events that are constitutively expressed in an inactive form in most, if not all, mammalian cells (12). A variety of physiological or environmental stresses impact distinct cellular targets to initiate cell type-specific apoptotic signaling pathways (12, 13). The various upstream signaling cascades converge downstream to activate a common final caspase-dependent effector mechanism for dismantling the dying cell (12). Radiation was shown to target either the cell membrane or the nucleus to activate different apoptotic pathways (14–18). In endothelial cells, radiation signals the hydrolysis of plasma membrane sphingomyelin, generating the proapoptotic second messenger ceramide via activation of SMase (14). Alternately, in thymocytes, radiation initiates p53-mediated apoptosis via recognition of DNA damage (17, 18). Whole body irradiation of p53 and acid SMase knockout mice proved that these death pathways are distinct and independent (15).

An alternative mechanism to SMase-mediated generation of ceramide in response to stress is a pathway that involves de novo synthesis of ceramide. Ceramide synthesis is catalyzed by the enzyme ceramide synthase, which localizes to the endoplasmic reticulum and mitochondria (19–21). Ceramide synthase, once activated, catalyzes the condensation of sphinganine and fatty acyl-CoA to form dihydrosphingosine, which is rapidly oxidized to ceramide (19). Recent studies demonstrated that in different cells, radiation may activate either of these mechanisms for ceramide generation, sometimes activating both within a single cell type (16).

The pleiotropic nature of death pathways induced by radiation suggests that radiation resistance is likely to be regulated by a variety of mechanisms, each of which is associated with a specific death pathway. Whether radiation resistance of human prostate tumor clones is associated with a single mechanism or a spectrum of mechanisms is unknown. There are only a few immortalized human prostate cell lines because it is unusually hard to establish primary cultures or xenographs from surgical specimens of human prostate tumors, even at short term (22). The best characterized lines include the PC-3, DU-145, and LNCaP cell lines established from metastatic human tumor lesions (23–25). In general, these cell lines are among the most radiosensitive human tumor cells, as assessed by the clonogenic assay (26–29). However, the dose-survival data do indicate distinct differences between these cell lines, as expressed by the Dq, D0, SF-2, and

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The abbreviations used are: SMase, sphingomyelinase; XRT, radiation therapy; TPA, 12-O-tetradecanoylphorbol acetate; FB1, fumonisin B1; DG, diacylglycerol; PSA, prostate-specific antigen; Vmax, maximal velocity; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PKC, protein kinase C; CPT, carnitine palmitoyltransferase; ATM, ataxia telangietasia.

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the linear quadratic α and β exponents. There are also differences in the apoptotic response to radiation. Several studies reported lack of apoptosis in PC-3 cells up to 72 h after exposure to doses of 10–30 Gy (30, 31) and an incidence of 10–15% apoptosis in DU-145 cells at 72 h after 10–12 Gy (30, 32). However, one study reported 40% apoptosis in PC-3 cells at 72 h after 20 Gy (33). This study is also the only study that has thus far reported apoptosis in LNCaP cells, occurring at a rate of 35% at 72 h after exposure to 20 Gy (33). Altogether, these observations indicate clone-specific sensitivities of human prostate tumor cells to radiation. These data also suggest that an approach to reduce radiation resistance clinically might require the use of combinations of chemical and biological modifiers to cover a spectrum of resistance mechanisms that may operate in prostate cancer.

In a recent study, we reported that protein kinase C (PKC) activation by TPA induced ceramide synthase activation in LNCaP cells (34). Ceramide generation was rapid, detectable by 1 h after TPA treatment, and progressive for 12 h. This was followed by a delayed form of apoptosis that reached maximal levels at 48 h. Investigations into the mechanism of TPA-induced ceramide generation revealed that acid and neutral SMase activities were not enhanced. In contrast, TPA induced an increase in ceramide synthase activity that persisted for at least 16 h. Treatment with FB1, a natural competitive inhibitor of ceramide synthase, abrogated both TPA-induced ceramide production and apoptosis. Thus, ceramide synthase activation appears to be required for TPA-induced apoptosis in LNCaP cells.

In the present study, we demonstrate that ceramide synthase activation also appears to be required for radiation-induced apoptosis in LNCaP cells. Whereas LNCaP cells failed to respond to radiation with ceramide generation and apoptosis, pretreatment with TPA converted this pattern, enabling radiation to signal ceramide synthase activation and apoptosis. Furthermore, treatment of nude mice with i.v. TPA before radiation also resulted in a synergistic apoptotic response in orthotopically implanted LNCaP tumors, sensitizing the tumors to the effects of radiation in vivo. This represents the first description of a signaling-based therapy designed to overcome one form of radiation resistance expressed preferentially in human prostate cancer cells.

**MATERIALS AND METHODS**

**Materials.** Fatty acid-free BSA, TPA, palmitoyl-CoA, bis-benzimidazole (Hoechst 33258), ceramide type III, FB1, DMSO, propidium iodide, Staphylococcus aureus SMase, and sphinganine were purchased from Sigma Chemical Co. C2-ceramide and Escherichia coli DG kinase were from Biomol. Cardiolipin was purchased from Avanti Polar Lipids. [1-14C]Palmitoyl-coenzyme A (57.1 mCi/mmol) and [γ-32P]ATP (3000 Ci/mmol) were obtained from DuPont (New England Nuclear). Octyl-β-D-glucopyranoside was from Calbiochem. C2-ceramide was stored at −20°C and dissolved in DMSO (final concentration, 0.4%) just before each experiment. FB1 was diluted in sterile saline (0.9%) and stored at 4°C until use. TPA was dissolved in DMSO, aliquoted, and stored at −20°C until use.

**Cell Cultures, Mice, and Irradiation.** LNCaP cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. For apoptosis and lipid studies, cells were plated at a density of 5.0 × 10^5 cells/ml onto either 6- or 12-well plates and treated with 0.05% trypsin. Of note, this cell density was significantly greater than the density used in our previous report (34). For ceramide synthase assays, cells were plated at a similar density onto 100-mm dishes. Irradiation of cultured cells was carried out at 25°C in a gamma-cell 40 chamber containing two sources of 137Cs (Atomic Energy of Canada) at a dose rate of 100 Gy/min. One hour before irradiation, the culture medium was changed to RPMI 1640 containing 0.2% human serum albumin.

The mice used in this study were 8–10-week-old male Swiss nude (nu/nu) mice obtained from Taconic Farms and housed at the animal core facility of Memorial Sloan-Kettering Cancer Center. This animal housing facility is approved by the American Association for Accreditation of Laboratory Animal Care and is maintained in accordance with the regulations and standards of the United States Department of Agriculture and the Department of Health and Human Services, NIH. For irradiation, mice received whole body irradiation, using a 137Cs irradiator (Shepherd Mark-I, Model 68, SN643) at a dose rate of 270 Gy/min, as described previously (15).

**Cell Cycle Analysis.** Cells were washed with PBS and then trypsinized for 1 min in 37°C. The cells were resuspended in PBS and preserved in cold 70% ethanol. Before analysis, the ethanol was removed, and the cells were resuspended in a propidium iodide solution as described previously (35). DNA content was measured with FACSscan equipped with a Facstaining running CellQuest software (Becton Dickinson). Cell cycle analysis of DNA histograms was performed with Modfit (Phoenix Flow Systems). A minimum of 1 × 10^6 cells were counted per sample.

**Quantiﬁcation of Ceramide.** Ceramide synthase activity. Microsomal membranes were prepared as described previously (34). Briefly, LNCaP cells were washed twice with ice-cold PBS, scraped off the plate, and resuspended in 300 μl of homogenization buffer (25 mM HEPES (pH 7.4), 5 mM EGTA, 50 mM NaF, and 10 μg/ml each of leupeptin and soybean trypsin inhibitor). Cells were disrupted by sonication on ice, and lysates were pelleted at 800 × g for 5 min. The postnuclear supernatant was centrifuged at 250,000 × g for 35 min. The microsomal membrane pellet was resuspended in 0.5 ml of homogenization buffer. Assays of ceramide synthase activity were performed as described previously (34, 36). Briefly, microsomal membrane protein (75 μg) was incubated with 2 mM MgCl₂, 20 μM fatty acid-free BSA, the indicated concentrations of sphinganine, 70 μM unlabeled palmitoyl-CoA, and 0.2 mM of [1-14C]palmitoyl-CoA at 37°C for 1 h. After extraction of the lipids with 2 ml of chloroform:methanol (1:2), diiodohydroceramide was resolved by TLC using a solvent system of chloroform/methanol/3:5 N ammonium hydroxide (85:15:1, v/v/v). Dihydroceramide was detected by iodine vapor staining based on comigration with commercially available ceramide (Sigma Type III) and quantified by liquid scintillation counting. Under the conditions used, the substrate was not rate-limiting, and the reaction was linear for time and enzyme concentration.

**Quantification of Apoptotic Cells.** Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis-benzimide trihydrochloride (Hoechst 33258), as described previously (34). Briefly, LNCaP cell monolayers were detached with 0.25% trypsin and 0.02% EDTA in HBSS and combined with the floating population. The cell pellet was washed in PBS, resuspended in 50 μl of 3% paraformaldehyde, and incubated for 10 min at room temperature. The fixative was removed, and the cells were resuspended in 20 ml of PBS containing 8 μg/ml Hoechst 33258. After a 15-min incubation at room temperature, an 8-μl aliquot was placed on a glass slide, and 500 cells were scored for the incidence of apoptotic chromatin changes under an Olympus BH2 fluorescence microscope using a BH2-DMU2UV Dichroic Mirror Cube filter.

**Orthotopic Transplantation of LNCaP Tumors.** The 8–10-week-old male Swiss nude (nu/nu) mice were implanted orthotopically with LNCaP cells. Immediately before tumor implantation, LNCaP cells grown in culture were trypsinized and resuspended in RPMI 1640 with 10% FBS at a concentration of 20 × 10⁶ cells/ml, and viability was determined by trypan blue exclusion. Only single cell suspensions with >90% viability were used for in vivo injection. Mice were anesthetized with pentobarbital (75 mg/kg i.p.), and orthotopic tumor implantation was performed as described previously (37). Briefly, a low midline abdominal incision was made with a #15 blade (Bard Parker). The peritoneal cavity was entered by sharply incising the linea alba. The bladder and seminal vesicles were identified and gently raised, thus exposing the dorsal lobes of the mouse prostate. Before intraprostatic injection, the LNCaP cells were resuspended to a final concentration of 2.0 × 10⁸
LNCaP cells/ml culture medium. Either lobe was injected with 0.1 ml using a disposable 26-gauge needle. Proper implantation of cell suspension was indicated by bleeding under the prostate capsule. Visceral contents were then replaced into the abdominal cavity, and the wound was closed with surgical clips (Autoclip; Becton Dickinson). Mice were monitored during the postoperative period according to animal care facility guidelines. The injected mice were housed (2–3 mice/cage) in a pathogen-free environment, using filtered, laminar air flow hoods in standard vinyl cages with air filter tops. Cages, bedding, and water were autoclaved before use and cared for in accordance with the institutional and NIH guidelines.

Tumors were allowed to grow for approximately 4 weeks, at which time tumor size was assessed by serum PSA determination as described previously (37). It has been reported that orthotopically transplanted LNCaP tumors in nude mice produce and secrete PSA that can be detected histochemically in the tumor cells and by radioimmunossays in the mouse serum (37). To assess LNCaP tumor take and tumor volume after intraprostatic transplantation, mice were anesthetized with pentobarbital (50 mg/kg i.p.), and phlebotomy was performed by accessing the retro-orbital venous plexus with a microcapillary pipette (Fisher). Serum PSA determinations were performed by radioimmunoassay (Hybritech) according to the recommendations of the manufacturer. Pilot experiments revealed that prostatic tumor weight correlated with serum PSA values (r = 0.952; Fig. 1). Additional studies demonstrated the PSA doubling time for orthotopically implanted tumors was approximately 10 days (data not shown). This doubling time is markedly prolonged when compared to experiments when PSA levels ranged between 1.1 and 10.4 ng/ml, corresponding to a tumor weight of 45–100 μg.

TPA Infusion into Mice. Preliminary experiments were designed to develop safe and effective systemic delivery of TPA to mice. i.p. infusions of TPA with 0.9% saline as the vehicle were found to be nontoxic at doses up to 0.5 μg/g body weight, but these concentrations did not confer apoptosis in LNCaP tumors after irradiation (data not shown). Additional studies investigated the use of alternative vehicles for i.v. injection. The most effective vehicle was a mixture of polyethylene glycol:ethanol:Tween 80 (6:3:1) used previously in clinical trials for the delivery of bryostatin (38). TPA (Sigma) was dissolved in this vehicle to a final concentration of 1 mg/ml and stored at 20°C. Before each use, this solution was mixed with 0.9% saline (20:1, v/v) and injected into the retro-orbital plexus while the animal was under pentobarbital anesthesia. Acute toxicity, consisting of respiratory distress, petechial skin changes, and weight loss, occurred with doses above 0.1 μg/g body weight. The threshold for acute toxicity varied with different TPA preparations, and toxicity studies were required before the use of each new batch of TPA. With high TPA doses, death occurred with a LD50 of 0.25–0.5 μg/g body weight. To evaluate the possible long-term effects of acutely nontoxic levels of TPA or the polyethylene glycol:ethanol:Tween vehicle, mice received four weekly injections of 0–1 μg TPA/g mouse body weight (two mice per dose) and were followed for 6 months. At the end of this period, the mice were sacrificed, and detailed autopsies were carried out that did not disclose any abnormalities. Experiments on the effects of TPA on the apoptotic response of orthotopically transplanted LNCaP tumors were carried out using a dose of TPA below the toxic range (in the current studies, ≤0.08 μg/g mouse body weight). When radiation was delivered, mice were anesthetized as described above and injected with i.v. vehicle or TPA 20 min before irradiation.

Assessment of Apoptotic Cells in Orthotopically Transplanted LNCaP Tumors. Apoptosis in vivo was assessed by the TUNEL assay, as described previously (39, 40). LNCaP tissue sections from paraffin-embedded blocks (5-μm thick) were adhered to polylysine-treated slides, deparaffinized, and rehydrated. The slides were incubated in 10 mM Tris-HCl (pH 8) for 5 min, digested with 0.1% pepsin, rinsed in distilled water, and treated with 3% H2O2 in PBS for 5 min at 22°C to inactivate endogenous peroxidase. After three washes in PBS, the slides were incubated for 15 min at 22°C in buffer [140 mM sodium cacodylate (pH 7.2), 30 mM Trizma base, and 1 mM CoCl2] and then incubated for 30 min at 37°C in reaction mixture [0.2 unit/μl terminal deoxynucleotidyl transferase, 2 unit biotin-11-dUTP, 100 mM sodium cacodylate (pH 7.0), 0.1 mM DTT, 0.05 mg/ml BSA, and 2.5 mM CoCl2]. The reaction was stopped by transferring the slices to a bath of 300 mM NaCl, 30 mM sodium citrate for 15 min at 22°C. The slides were washed in PBS, blocked with 2% human serum albumin in PBS for 10 min, rewashed, and incubated with avidin-biotin peroxidase complexes. After 30 min at 22°C, cells were stained with the chromogen 3,3′-diaminobenzidine tetrachloride and counterstained with hematoxylin. Nuclei of apoptotic cells appeared brown and granular, whereas normal nuclei stained blue.

Statistical Analysis. Statistical analysis was performed by Student’s t test and the r test for the correlation coefficient. Linear regression analysis was performed by the method of least squares.

RESULTS

Radiation Does Not Induce Ceramide Generation or Apoptosis in Plateau-phase LNCaP Cells. A previous study reported that radiation induces apoptosis in exponentially growing LNCaP cells (33), although the mechanism of this response was not defined. In the present studies, we explored the apoptotic response of plateau-phase, rather than exponentially growing, LNCaP cells, because this culture condition more closely resembles the state of the majority of tumor cells in vivo. For these studies, 5 × 105 cells were plated in 12-well plates and irradiated when the cultures reached approximately 80–90% confluence. Cell cycle analysis confirmed a decreased rate of proliferation of plateau-phase cultures as compared to log-phase cells. The proportion of cells in S phase in log-phase cultures was 15.6 ± 1.2%, compared to 5.5 ± 1.2% in plateau-phase cells (P > 0.05). Radiation doses up to 20 Gy did not result in apoptotic cell death in plateau-phase cells for up to 5 days after exposure as measured by bis-benzamide staining to assess nuclear morphology, generation of a sub-G0/G1 apoptotic fraction by fluorescence-activated cell-sorting analysis, or by DNA ladder formation (data not shown). Furthermore, there was no evidence of ceramide generation in these cells at any point in time after exposure to radiation doses of 10–20 Gy. To explore whether plateau-phase LNCaP cells were inherently resistant to the proapoptotic effects of ceramide, the cells were treated with the cell-permeable analogue of ceramide, C2-ceramide. Similar to previously published data in exponentially growing cells (34), concentrations as low as 25 μM of C2-ceramide were capable of inducing apoptosis in plateau-phase cells (data not shown), and 62% apoptosis was observed after 48 h of incubation with 100 μM C2-ceramide. To determine whether ceramide produced naturally via activation of SMase mimics the effect of C2-ceramide, plateau-phase LNCaP cells were treated with 10 μl of bacterial SMase. At 48 h of this treatment, nearly 70% of the cells underwent apoptosis. These data indicate that whereas ceramide is capable of signaling apoptosis in plateau-phase LNCaP cells, failure to generate ceramide after ionizing radiation may be associated with lack of an apoptotic response.
TPA Modulates the Response of LNCaP Cells to Radiation.

Our previous studies demonstrated that TPA induces sequential ceramide synthase activation, ceramide generation, and apoptosis in exponentially growing LNCaP cells (34). In those experiments, $1.5 \times 10^5$ cells were plated in 6- or 12-well plates, and when they reached 40–50% confluence, the cells were treated with 10 ng/ml TPA, leading to ceramide generation by 3 h and apoptosis by 12 h (34). The present studies showed that plateau-phase LNCaP cells exhibited delayed and reduced ceramide generation and apoptosis in response to treatment with 10 ng/ml TPA. Fig. 2 shows initial evidence of ceramide elevation from a baseline of 643 ± 12 to 749 ± 65 pmol/10^6 cells was detected at 10 h, reaching a level of only 866 ± 44 pmol/10^6 cells at 16 h. Apoptosis remained undetectable in TPA-treated cells up to 12 h and reached a level of only 18 ± 5% at 24 h. However, exposure of plateau-phase LNCaP cells to both 10 ng/ml TPA and 20 Gy of irradiation resulted in an accelerated and enhanced generation of ceramide and apoptosis. Fig. 2 shows that cells treated with both TPA and radiation already reached a near maximal level of ceramide elevation (from 643 ± 12 to 941 ± 13 pmol/10^6 cells) by 6 h. Similarly, 18 ± 5% of the cells showed evidence of apoptosis at 12 h. There was a dose-response relation for TPA in sensitizing the cells to apoptosis after irradiation. Doses of 0.5–10 ng/ml TPA were used, and apoptosis was assessed in 1000 cells at 24 h after exposure to 20 Gy. Whereas 1.5 ng/ml TPA given alone had no significant effect on apoptosis (2.1 ± 0.4%), a small but statistically significant synergistic effect (8.4 ± 2.4%) was observed when it was given in combination with radiation ($P < 0.05$). Higher TPA doses (3.0 and 10.0 ng/ml) induced an apoptotic response even when given alone (6.1 ± 3.3% and 22.9 ± 2.8%, respectively), but apoptosis was significantly enhanced when the cells were also exposed to 20 Gy of irradiation (13.4 ± 1.0% and 36.2 ± 3.8%, respectively; $P < 0.05$). Because radiation alone did not result in the induction of either cellular ceramide or apoptosis (Fig. 2), these data indicate that TPA modulates the resistance of LNCaP cells to irradiation, leading to synergistic effects with the combination. It should be noted that the level of the effect of TPA on LNCaP cells varied with different TPA preparations. Therefore, it was necessary to establish the optimal concentration of TPA required to confer the effects described above for each new batch of TPA.

Radiation Enhances Ceramide Synthase Activation by TPA.

Our previous studies demonstrated that the induction of ceramide generation by TPA was mediated via activation of the enzyme ceramide synthase (34). Whereas neither acidic nor neutral SMase appeared to be activated by 10 ng/ml TPA in exponentially growing LNCaP cells, there was a 60% increase in the $V_{max}$ of ceramide synthase activity after 45 min. The present studies demonstrate that plateau-phase LNCaP cells exhibit attenuated activation of ceramide synthase by TPA. Plateau-phase cells were treated with 10 ng/ml TPA, and microsomal membranes were tested for the kinetics of ceramide synthase activity with increasing sphinganine concentrations. Fig. 3 shows the Eadie-Hofstee transformation of these data. At 3 h, cells treated with TPA alone manifested only a 34% increase in the $V_{max}$ of the reaction (from 116 to 156 pmol/min/mg protein), without a change in the $K_m$. However, there was a significant increase in the $V_{max}$ to 232 pmol/min/mg protein when the cells were exposed to the combination of 10 mg/ml TPA and 20 Gy of irradiation. Radiation alone (20 Gy) did not have any discernible effect on ceramide synthase activity. Similar results were observed in three independent experiments. Because the activity of ceramide synthase can currently be measured only by an enzymatic assay in microsomal membrane preparations, there is significant interassay variation, which does not permit the collation of data from different experiments for statistical analysis. However, when all data were normalized to their own controls, a statistically significant increase in the $V_{max}$ of ceramide synthase was detected only in the TPA + XRT group ($P < 0.05$). These data indicate that TPA confers sensitivity to ceramide synthase activation by ionizing irradiation, leading to a synergistic activation of the enzyme by the combination of TPA and radiation. Ceramide synthase activation appeared to represent the only mechanism for the proapoptotic generation of ceramide, because...
neither radiation, TPA, nor a combination of the two induced the activation of neutral or acidic SMase (data not shown). Baseline activity of neutral and acidic SMase was 122 and 943 pmol/mg protein/min, respectively, and did not change upon cell treatment with TPA, radiation, or the combination of the two.

To further demonstrate the critical role of ceramide synthase in the proapoptotic generation of ceramide in response to TPA plus radiation, experiments were performed using FB1. This natural product of the fungus *Fusarium moniliforme* acts as a specific competitive inhibitor of ceramide synthase (41). Our previous studies showed that in exponentially growing LNCaP cells, FB1 had no effect on basal ceramide, but it markedly reduced TPA-induced ceramide elevation (34). Fig. 4 shows that treatment of exponentially growing LNCaP cells with 100 μM FB1 reduced the induction of apoptosis in response to TPA at 24 h from 28 ± 1% to 11 ± 1% and reduced the induction of apoptosis in response to TPA with XRT from 42 ± 8% to 20 ± 1%. Concomitantly, this concentration of FB1 reduced the baseline level of ceramide from 566 ± 13 to 74 ± 28 pmol/10^6 cells and blocked the increase in response to TPA (10 ng/ml) and to TPA plus 20 Gy of XRT by 78% and 60%, respectively (data not shown). These data support the notion that the synergistic effects of TPA and radiation on ceramide generation and apoptosis are mediated via ceramide synthase activation.

**TPA and Irradiation Act Synergistically to Induce Apoptosis of Orthotopically Implanted LNCaP Tumors.** To ascertain whether the mechanisms involved in the induction of apoptosis in LNCaP cell cultures represented a phenotypic response of LNCaP tumors *in vivo*, we implanted LNCaP cells into the prostates of Swiss nude (nu/nu) mice. Mice bearing tumors of approximately 45–100 mg, as determined by serum PSA values, were treated with TPA, XRT, or a combination of the two. Larger tumors were not used because they manifested areas of spontaneous necrosis. TUNEL staining to detect apoptosis was performed at multiple time points from 12–36 h after treatment. As shown in Fig. 5, LNCaP tumors grow as solid neoplasms with few glandular-like structures. TUNEL staining revealed no spontaneous apoptosis. Only incidental apoptosis was manifest at 24 h after treatment with 20 Gy or at other time points from 12–36 h after irradiation (data not shown). TPA induced minimal (1–5%) apoptosis within this time frame. However, in animals treated with TPA followed by 20 Gy, substantial apoptosis (20–25%) was evident. This synergistic effect was detected by 12 h and was manifested for as long as 36 h. Thus, the TPA-mediated sensitization of LNCaP cells to radiation-induced apoptosis observed *in vitro* was recapitulated *in vivo* in LNCaP tumors growing orthotopically within the prostate.

An additional benefit was derived from these studies. In contrast to the resistance of LNCaP tumors to radiation, normal rectal mucosa displayed extensive apoptotic damage (Fig. 6). Although TPA was without effect on the normal rectum, TPA pretreatment almost completely abrogated the damaging effects of radiation on the rectum. Similar protection was observed at all times from 12–36 h. These studies show tissue type-specific transmodulation of apoptosis by TPA.

**DISCUSSION**

These investigations demonstrate that the phorbol ester TPA sensitizes LNCaP prostate cells *in vitro* and *in vivo* to the apoptotic
effects of ionizing radiation. The mechanism of sensitization involves activation of the enzyme ceramide synthase and generation of the proapoptotic lipid second messenger ceramide. Consistent with this observation, elevation of cellular ceramide with ceramide analogues or by treatment with SMase mimicked the effect of TPA plus radiation and directly induced apoptosis. The role of ceramide in this process was confirmed by the use of the competitive inhibitor of ceramide synthase, FB1, which blocked ceramide elevation and apoptosis. It should be noted that the dose of FB1 used in this study only partially blocked the increase of ceramide and apoptosis in response to TPA or TPA plus radiation. Higher doses of FB1 were not feasible due to toxicity. Whether TPA might have a proapoptotic effect on LNCaP cells that is ceramide synthase independent remains unknown. The failure of LNCaP cells to undergo radiation-induced apoptosis via ceramide synthase and its reversal by phorbol ester treatment identify a new form of radiation resistance. Although the prevalence of this resistance to the apoptotic effects of radiation is presently unknown, it is anticipated that it may represent a generic form of radiation resistance that may occur in some prostate cancer clones and tumors of other origins.

The effects of TPA on apoptosis in LNCaP cells are conditional. Our previous study (34) examined apoptosis in log-phase cultures of LNCaP cells. Under these conditions, we found LNCaP cells to be quite sensitive to the apoptotic effect of TPA. In the present studies, we explored the apoptotic response of plateau-phase, rather than exponentially growing LNCaP cells, because this culture condition more closely resembles the state of the majority of tumor cells in vivo. Under these conditions, LNCaP cells are more resistant to TPA-induced apoptosis and manifest slower kinetics of ceramide generation and apoptosis.

The effects of TPA on apoptotic responses are pleiotropic (42). In most cells, TPA provides antiapoptotic protection. In irradiated endothelial cells, TPA confers such an antiapoptotic effect, at least in part, via the inhibition of SMase-mediated generation of ceramide (14). In a few instances, phorbol esters have proven to be proapoptotic. In this regard, EBV-infected Burkitt lymphoma cells display substantial apoptosis in response to phorbol esters, although uninfected Burkitt cells are resistant (43). Immature thymocytes also appear to be susceptible to phorbol ester-induced apoptosis (44). Although the mechanism by which TPA serves as a proapoptotic agent is unknown, prolonged activation of PKC may play a role. de Vente et al. (45) overexpressed PKCα in MCF-7 breast cancer cells, which conferred phorbol ester-mediated cytotoxicity. Furthermore, Powell et al. (46) demonstrated in LNCaP cells that TPA induced a specific increase in PKCα mRNA and prolonged the translocation of this PKC isoform to the membrane before apoptosis. TPA action in LNCaP was also shown to involve retinoblastoma dephosphorylation and G0/G1 arrest (47), events reported to occur in response to ceramide treatment in other cell types (48, 49). Whether ceramide synthase activation is upstream of cyclooxygenase 2 or 5-lipoxygenase inhibition (50, 51), regulation of the levels of Bel-2 family members (52), or Fas/Fas-ligand interaction (53), events involved in LNCaP apoptosis in response to other stresses will require additional experimentation.

The present studies extend an emerging literature that is defining a role for ceramide synthase in stress signaling. Although originally reported as mediating daunorubicin-induced apoptosis in p388, HL-60, and U937 cells (36, 54), recent studies have demonstrated its involvement in multiple forms of apoptotic damage. In primary cultures of cerebral endothelial cells, the sensitizing effect of cycloheximide to tumor necrosis factor-induced apoptosis appeared to require ceramide synthase (55). Furthermore, FB1 abrogated daunorubicin-induced apoptosis in hen granulosa cells (56) and in CPT-11-induced apoptosis in L929 cells (57). Shimabukuro et al. (58) proposed that ceramide synthase-mediated apoptosis might be involved in the pathogenesis of type 1 diabetes. These investigators showed that islets from Zucker fatty diabetic rats, which display a defect in leptin receptor signaling, manifested elevated ceramide levels and apoptosis. In response to a challenge with free fatty acids, these islets displayed reduced fatty acid oxidation and markedly increased incorporation into ceramide, accompanied by apoptosis. FB1 blocked both ceramide generation and apoptosis. Furthermore, transfection of the wild-type leptin receptor restored the ability of leptin to reverse these effects (59). Ceramide synthesis was also found to be involved in mitochondrial functions associated with apoptosis. Paumen et al. (60) isolated CPT I in a screen for genes involved in apoptosis after interleukin 3 withdrawal from LyD9 hematopoietic precursor cells. This enzyme is located in the outer mitochondrial membrane and catalyzes the transfer of long-chain fatty acids into the mitochondria for β-oxidation. In
the presence of a CPT I inhibitor, treatment of cells with fatty acids such as palmitate, which serve as precursors of de novo ceramide synthesis, led to ceramide generation and apoptosis. FB1 blocked these events. CPT I has also been shown to bind bcl-2, suggesting that synthesis by posttranslational activation of ceramide synthase and apoptosis. Ceramide synthase activation was obligatory, because FB1 yielded similar results. EBV-immortalized B cells from ATM patients (16).2,3 These studies demonstrated that one of the functions of ATM is to constrain activation of ceramide synthase, thereby regulating DNA damage-induced apoptosis. Whether LNCaP cells express high levels of the ATM protein or activity and whether TPA converts the LNCaP apoptotic resistance to radiation by affecting ATM are possibilities that require further investigation.

In the present studies, TPA was also found to confer antiapoptotic protection against radiation-induced apoptosis in the rectal mucosa through an unknown mechanism. In a recent study, we reported that lipopolysaccharide-induced apoptosis of intestinal epithelial cells occurs, at least in part, secondary to acid SMase-mediated apoptosis of microvascular endothelial cells in the lamina propria of the intestinal crypts (62). Inactivation of acid SMase by genetic mutation or i.v. injection of basic fibroblast growth factor, which specifically protects the endothelium against stress-induced apoptosis (39, 40), abrogated the apoptotic response in the mucosal epithelial cells (62). Whether the antiapoptotic effect of TPA on radiation-induced apoptosis in the rectal mucosa is mediated via a similar mechanism remains unknown. However, it should be noted that TPA mimics basic fibroblast growth factor in the inhibition of SMase-mediated apoptosis in irradiated bovine endothelial cells (63).

The dual action of TPA, which serves as a radiation sensitizer of LNCaP cells while concomitantly protecting the rectal mucosa against radiation-induced damage, provides a potential for clinical use. Clinical data indicate that the failure of XRT to control prostate tumors results, in many patients, from an inability to deliver sufficient radiation doses to overcome tumor clonal radiation resistance. This inability results in part from the need to include parts of the adjacent and overlapping rectum in the treatment fields to avoid the risk of missing portions of the tumor tissue (4, 64). The rectum is highly sensitive to radiation, thus restricting the maximal doses to the tumor target to suboptimal levels (4, 64). The dual action of TPA on LNCaP and rectal tissue radiosensitivity might provide at least a partial solution to this problem. However, TPA has been shown to act as a tumor promoter on the skin of mice previously initiated with aromatic hydrocarbons, although topical application of TPA alone had no tumorigenic effect (65–67). i.v. TPA therapy, at dose levels similar to those used in our study, has recently been used safely in patients with myelocytic leukemia and other malignancies (68, 69). However, the long-term effects of such therapy are unknown. The search for non-tumor-promoting biological response modifiers of the apoptotic response to radiation that retain the dual effects of TPA on prostatic versus rectal tissues represents a challenge with an important potential for clinical application in prostate cancer therapy.

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Reversal of Radiation Resistance in LNCaP Cells by Targeting Apoptosis through Ceramide Synthase

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