Ionizing Radiation-inducible Apoptosis in the Absence of p53
Linked to Transcription Factor EGR-1*

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The tumor suppressor protein p53 is a pivotal regulator of apoptosis, and prostate cancer cells that lack p53 protein are moderately resistant to apoptotic death by ionizing radiation. Genes encoding the transcription factor early growth response-1 (EGR-1) and cytokine tumor necrosis factor-α (TNF-α) were induced upon irradiation of prostate cancer cells, and inhibition of EGR-1 function resulted in abrogation of both TNF-α induction and apoptosis. Induction of the TNF-α gene by ionizing radiation and EGR-1 was mediated via a GC-rich EGR-1-binding motif in the TNF-α promoter. Because TNF-α induces apoptosis in prostate cancer cells, these findings suggest that, in the absence of p53, ionizing radiation-inducible apoptosis is mediated by EGR-1 via TNF-α transactivation.

Prostate cancer in men is the most common malignancy and the second most leading cause of cancer deaths (1). Radiation therapy that causes growth inhibition and programmed cell death (apoptosis) of tumors is a well established mode of treatment for both primary and metastatic prostate cancer (2). However, despite using high doses of radiation, about 50–60% of prostate cancer patients show persistent local disease (3–5). A major reason for failure to eradicate local disease is the intrinsic radioresistance of the tumors. One of the molecular determinants regulating the response to ionizing radiation is the tumor suppressor protein p53 that serves as a pivotal component of the apoptosis pathways in diverse cell types (6, 7). Wild-type p53 protein confers radiation responsiveness, but loss of p53 function owing to either mutation(s) or deletion of p53 alleles confers radioresistance (8–13). Because p53 protein is mutated and non-functional in a large number of prostate tumors (14, 15), it is imperative to identify other proapoptotic genes that can function via a p53-independent mechanism and further to design novel approaches to induce the expression of such genes for the control of radio-resistant tumors.

Previous studies have shown that EGR-1 protein, encoded by the immediate-early gene Egr-1, is induced by ionizing radiation in a wide spectrum of tumor cell types (16, 17). EGR-1 is a member of the Egr (early growth response) family of transcription factors that includes EGR-2, EGR-3, NGFI-C, the tumor suppressor Wilms’ tumor gene product WT1, and EGR-α (18–20). The Egr family members show a high degree of homology in the amino acids constituting the zinc finger domain and bind to the same GC-rich consensus DNA sequence (21–23). Functional studies have suggested that EGR-1 is an anti-proliferative signal for tumor cells (24, 25) and that it acts to increase the potency of apoptotic agents (26, 27). In human melanoma cells that contain wild-type p53, abrogation of EGR-1 function confers radioresistance despite induction of p53 (17), suggesting that EGR-1 may function by a p53-independent mechanism. A p53-independent mechanism for apoptotic death of prostate cells in p53-null mice after testosterone ablation effected by orchiectomy has also been suggested (28). Incidentally, EGR-1 is induced early in the rat prostate after orchiectomy (29), but its function or mechanism of action in prostatic cells is not known.

Another gene that is induced by radiation in a wide range of cell types is tumor necrosis factor-α (TNF-α) that encodes a cytokine with pleiotropic effects (30). The induction of TNF-α gene expression represents an important aspect of cellular response to ionizing radiation that causes both autocrine and paracrine tumor cell killing (30–32). Ionizing radiation causes a transient increase in TNF-α mRNA followed by a corresponding increase in TNF-α protein (30). TNF-α protein is a well characterized cytokine that induces apoptosis in different cell types by binding to the TNF-R1 receptor (33, 34). Binding to the TNF-R1 receptor triggers the sequential recruitment and activation of a cascade of death domain-containing proteins, which further activate cysteinyl-aspartate-specific proteinases (caspases) such as interleukin-converting enzyme and those of the interleukin-converting enzyme-related family (33–38). The caspases then cleave substrates, such as poly(ADP-ribose) polymerase, nuclear lamins, actin, protein kinase C-δ, and fodrin, that are essential for cell survival, subsequently leading to apoptotic cell death (33–36). The present study used a prostate cancer cell line, PC-3, that lacks functional p53 protein (9, 39, 40) to determine the role of EGR-1 in radiation-induced apoptosis. We demonstrate here that ionizing radiation-inducible apoptotic death is caused by EGR-1 despite the absence of p53 protein and that EGR-1 action involves the up-regulation of the TNF-α gene.

MATERIALS AND METHODS

Plasmid Constructs—Plasmid pCMV-WT1-EGR1, which encodes a dominant-negative mutant of EGR-1, contains a WT1-EGR-1 chimera downstream of the cytomegalovirus (CMV) promoter in vector pCB6 (41). The plasmid CMV-EGR-1, which encodes full-length EGR-1 protein, contains EGR-1 cDNA downstream of the CMV promoter in the vector pCB6 (41). The reporter construct, CAT-CAT (41), contains three EGR-1 binding sites (CGCCCCCGG) placed in tandem.
upstream of a minimal c-Fos promoter and chloramphenicol acetyltransferase (CAT) cDNA. The TNF-α promoter region from −470 to +102 was generated from normal human genomic DNA template by PCR (42). The sense primer TNFp−470F: 5′-TTTTCCTTAGATTTC-CCTCCAACCGGTTC-3′, and antisense primer TNFp+102L: 5′-TAAGCTTCAGGGGATGTGGCGTCTGAGG-3′, contained built-in sites (underlined) for XbaI and HindIII, respectively, and they generated a 589-base pair fragment that contained the EGR-1-binding site 5′-CGCCCCCGC-3′. The EGR-1 binding site in the 589-base pair fragment was mutated to 5′-GGTAA-3′ by using PCR-directed mutagenesis. The 589-base pair fragments representing wild or mutant EGR-1 binding sites were cloned in pG-CAT, a vector for CAT reporter, and the fidelity of PCR reactions and subcloning was confirmed by nucleotide sequencing.

**Cell Cultures**—Human prostate cancer cells PC-3 that lack functional p53 protein (9) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Pools of transfected PC-3 clones, PC-3 vector (P1 or P2) containing the pcB6 vector, PC-3/WT1-EGR1 (P1 or P2) expressing the chimera, or PC-3/EGR1-1 (P1 or P2) expressing the cDNA of Egr-1, were grown in medium supplemented with G418 sulfate (300 μg/ml).

**DNA Transfections and CAT Assays**—Transfections were performed as described previously (27) by using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP) (Boehringer Mannheim). CAT assays were performed by thin-layer chromatography as described previously (27).

**Irradiation**—A 100 kV industrial x-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm Al plus 1-mm Be filter was 1.85 Gyu min at a focus-surface distance of 30 cm.

**Assay for 3HThymidine Incorporation and Colony Formation Assay**—The 3Hthymidine incorporation experiments were performed as described previously (17). For clonogenic cell survival studies, two different cell concentrations in quadruplicate sets were used for each radiation dose. Parental PC-3 cells and PC-3 transfectants were left untreated or exposed to 1–6 Gy dose of radiation. After incubation for 10 or more days, each flask was stained with crystal violet, and the colonies containing more than 50 cells were counted. The surviving fraction (SF) was calculated as a ratio of the number of colonies formed and the plating efficiency. The curve was plotted using X Y log scatter (Delta Graph®4.0) and fitted by single hit multi-target model (43) to obtain D0, n, SF0 values (44). D0 is the dose required to reduce the fraction of cells to 37%, indicative of single-event killing; the n value of the curve is a measure of the width of the shoulder, indicative of multiple-event killing; and SF0 is the survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy.

**Immunocytochemistry and Western Blot Analysis**—EGR-1 expression
was determined in untreated and irradiated PC-3 cells by immunocyto-chemical analysis, as described by us previously (17), by using anti-EGR-1 antibody, sc-110 (Santa Cruz Biotecnology, Inc., Santa Cruz, CA), and the Elite ABC kit (Vector Laboratories, Burlingame, CA). Total protein extracts from untreated and irradiated PC-3 cells with various time intervals were subjected to Western blot analysis by using sc-110 or the β-actin antibody (Sigma) for a loading control as described (17).

Assays for Apoptosis—For the DNA laddering test, total genomic DNA was prepared from the PC-3 cells exposed to vehicle or recombi-nant TNF-α for 48 h and was subjected to agarose gel electrophoresis as described previously (26). For quantitation of apoptosis, cells were lifted by using non-enzymatic cell dissociation medium (Sigma) and washed with phosphate-buffered saline and stained with Hoehcht (Ho342) and mercocyanin (MC540) and analyzed by flow cytometry using a FACStar Plus cell sorter as described (45).

Fluorescence in Situ Hybridization (FISH) Analysis of Egr-1 Gene—To evaluate the copy number or amplification of the Egr-1 gene, FISH was performed using a spectrum orange-labeled Egr-1 probe (locus: Egr-1 band assignment: 5q23–51.1) obtained from Vysis Inc. (Downers Grove, IL) as described previously (17).

32P-Reverse Transcriptase-Polymerase Chain Reaction (32P-RT-PCR)—Total RNA was extracted from PC-3 and their transfectants using TRIzol reagent (Life Technologies, Inc.). 1 μg of total RNA was reverse transcribed into cDNA using oligo(dT) primers and reverse transcriptase in a 20-μl reaction mix (Perkin-Elmer) as described by us previously (17). The 32P-α cDNA sense primer sequences are from nucleotides 839–860, 5'-AGG CGC TCC CCA AGA AGA CAG-3', and antisense primer sequences are from nucleotides 1039–1060, 5'-AGG CTT GTC ACT CCG GGT TCG-3' (46). In addition, β-actin gene (the sense primer starts at nucleotide 1628 and the antisense primer begins at nucleotide 2379 and generates a 331-base pair PCR product) was used as an internal control (47).

RESULTS

Ionizing Radiation Causes Growth Inhibition and Cell Death in PC-3 cells—Ionizing radiation caused a dose-dependent growth inhibition of PC-3 cells, as demonstrated by [3H]thymi-dine incorporation assays (Fig. 1A). Moreover, colony-forming assays indicated that the survival fraction (SF2) of exponen-tially growing PC-3 cells that were irradiated at the clinically relevant dose of 2 Gy was 0.25 (Fig. 1B). The dose required to reduce the fraction of PC-3 cells to 37% (D0 value, i.e. single-event killing) was 140 cGy. The n value of the curve, which is a measure of the width of the shoulder (indicative of multiple event killing), was 1.03 (Fig. 1B). When compared with previously reported SHMT values for different tumor cell lines (48), those for PC-3 cells in Fig. 1B suggest moderate resistance to ionizing radiation. Clonogenicity inhibition by ionizing radiation was caused because of apoptotic death of the cells as evident from flow analysis, where cultures exposed to ionizing radiation showed a 20–23% increase in apoptotic cells com-pared with untreated cultures (Fig. 1C). These observations suggested that despite the absence of p53 protein, PC-3 cells are susceptible to ionizing radiation-inducible apoptosis.

Ionizing Radiation Causes Induction of EGR-1—We examined whether EGR-1 induction was associated with apoptosis inducible by ionizing radiation. Because Egr-1 maps to chromosome 5 and because PC-3 cells lack the chromosome 5 pair (40), we performed FISH to ascertain that Egr-1 alleles were intact in these cells. FISH analysis confirmed that PC-3 cells have two intact alleles for Egr-1 at 5q23–31.1 (Fig. 2A). Consistent with this observation suggesting that some areas of 5q are intact perhaps in the form of a marker chromosome, another gene, APC, normally located on 5q21, is also expressed as a full-length protein in these cells (49). Western blot analysis further confirmed that PC-3 cells constitutively express modest levels of EGR-1 protein (Fig. 2B). Moreover, after exposure to a 5-Gy dose of radiation, PC-3 cells showed induction of EGR-1 expression with peak levels (3–4 fold) at 45 min (Fig. 2B). These results were corroborated by immunocytochemical analysis that demonstrated a significant increase in nuclear EGR-1 expression levels after 45 min of exposure to radiation (Fig. 2C). The nuclear localization of EGR-1 was particularly important in view of the potential transcriptional activation function of EGR-1 protein.

Dominant-negative Mutant of Egr-1 inhibits EGR-1-depend-
ent Transcriptional Activation via the GC-rich Binding Site in Parental PC-3 and Stably Transfected PC-3 Cells—To ascertain that the WT1-EGR-1 chimera, which has been demonstrated in other cell types to act as a dominant-negative mutant of EGR-1, abrogates EGR-1 function in PC-3 cells, we performed transient cotransfections with a reporter construct EBS-CAT that contains three tandem EGR-1-binding sites, an EGR-1 expression construct CMV-EGR-1, and the chimera. As seen in Fig. 3A, transactivation of the reporter construct by CMV-EGR-1 was inhibited by transient cotransfection of PC-3 cells with CMV-WT1-EGR-1. These results confirmed that the chimera functioned as a dominant-negative mutant of EGR-1 in the PC-3 cell background.

We next tested PC-3 cells stably transfected either with the chimera or with an empty vector as a control for abrogation of EGR-1 function. In these experiments, we performed transient cotransfections with a reporter construct EBS-CAT that contains three tandem EGR-1-binding sites, an EGR-1 expression construct CMV-EGR-1, and the chimera. As seen in Fig. 3A, transactivation of the reporter construct by CMV-EGR-1 was inhibited by transient cotransfection of PC-3 cells with CMV-WT1-EGR-1. These results confirmed that the chimera functioned as a dominant-negative mutant of EGR-1 in the PC-3 cell background.

The Dominant-negative Mutant of Egr-1 Confers Resistance to Ionizing Radiation-inducible Growth Inhibition and EGR-1 Confers Enhanced Radiosensitivity—To determine the functional role of EGR-1 in radiation-inducible apoptosis of PC-3 cells, we transfected PC-3 cells with plasmid CMV-EGR-1, CMV-WT1-EGR-1, or an empty vector (pCB6) and obtained stable transfectant clones by selection with G418 sulfate (27, 41). Pools of about 200 transfected clones were designated P1 or P2, and at least two different pools of clones from each transfection were used in this study. Initially, we determined the effect of abrogation of EGR-1 function in PC-3 cells expressing the chimera in response to ionizing radiation. In colony-forming assays, the SF2 value of exponentially growing irradiated PC-3/vector.P1 cells was 0.2 with a D0 value of 124 cGy, and n value was 1.01 (Fig. 3C). On the other hand, the SF2 for PC-3/WT1-EGR-1.P1 was 0.45 with the D0 value of 227 cGy, and n value was 1.1, suggesting that compared with vector-transfected cells, the cells transfected with the chimera were significantly resistant (p < 0.0001) to ionizing radiation. Then, we tested the effect of EGR-1 overexpression in PC-3 cells expressing the chimera in response to ionizing radiation. In colony-forming assays, the SF2 for PC-3/EGR-1.P1 was 0.095 with a D0 value of 86 cGy, and n value was 1, suggesting enhanced sensitivity (p < 0.0001) than the vector-transfected cells to ionizing radiation. Consistent with this observa-

FIG. 3. Effect of ionizing radiation on transfected PC-3 cell lines. A and B, the WT1-EGR-1 chimera competitively inhibits EGR-1-mediated transactivation. Parental PC-3 cells (A) or pooled clones of stably transfected cells PC-3/vector.P1 or PC-3/WT1-EGR-1.P1 (B) were transiently cotransfected with 4 μg of EBS-CAT and the indicated amounts (μg) of CMV-WT1-EGR-1 and CMV-EGR-1. CAT activity was assayed and expressed as percent conversion of [14C]chloramphenicol to acetylated forms as described (27). C and D, EGR-1 protein increases radiosensitivity of PC-3 cells, and dominant-negative mutant of EGR-1 protects PC-3 cells from radiation-inducible growth inhibition and apoptosis. PC-3/EGR-1.P1, PC-3/WT1-EGR-1.P1, or PC-3/vector.P1 cells were left unexposed or exposed to the indicated doses of radiation, and either cell survival was assayed by colony-forming ability and expressed by using SHMT model (C) or apoptosis was quantified after 48 h by flow analysis (D). Each data point in SHMT model is a mean of three separate experiments, and error bars represent ± S. D. The relative apoptotic index values represent the mean of three independent flow cytometry experiments.
Fig. 4. EGR-1 regulates TNF-α gene expression. A, TNF-α causes apoptosis in PC-3 cells. PC-3 cells were either untreated (UT) or treated with 100 units/ml of TNF-α for 48 h, and total DNA from the cells was examined for a DNA laddering. B and C, 32P-RT-PCR analysis of TNF-α mRNA expression in untreated and irradiated cells. Parental (B) or transfected (C) PC-3 cells were left untreated (UT) or treated with radiation (5 Gy), and total RNA was extracted at the indicated time points. PCR was performed by using the products of reverse transcription reaction and the upstream and downstream primers flanking the TNF-α gene (sense primer at nucleotides 839–860 and antisense primer at nucleotides 194 and 186 upstream of the cap site of TNF-α gene (42)). This gene has not been demonstrated to mediate radiation-induced apoptosis as vector transfected PC-3 control cells (Fig. 3D). Together these findings suggest that EGR-1 is required for ionizing radiation-inducible apoptosis and that when overexpressed EGR-1 potentiates the effects of ionizing radiation.

TNF-α as Downstream Target of EGR-1—To identify potential downstream targets that might mediate the proapoptotic action of EGR-1, we conducted a GenBankTM/EBI search for genes that contain the EGR-1 consensus binding sites in their promoter regions, focusing on genes that satisfied the following stringent criteria: (i) they should be inducible by ionizing radiation; (ii) they should be functionally involved in apoptosis; and (iii) they should induce apoptosis via a p53-independent pathway. One of the genes that met these criteria was TNF-α. We first ascertained that TNF-α protein could cause apoptosis in the PC-3 cells. Cells were left untreated or treated with 100 units/ml of exogenous recombinant TNF-α, and total DNA was examined for DNA laddering that is characteristic of apoptosis. TNF-α caused nucleosomal DNA fragmentation in PC-3 cells (Fig. 4A), suggesting that the cell killing by TNF-α is mediated through apoptosis.

Next, we determined whether TNF-α was inducible by ionizing radiation in PC-3 cells. TNF-α mRNA levels increased about 2–3-fold over basal levels in cells after 30 min of exposure to a 5-Gy dose of ionizing radiation (Fig. 4B). We tested whether EGR-1 regulated the radiation-inducible expression of TNF-α by using 32P-RT-PCR analysis in transfected PC-3 cells. A dose of 5 Gy radiation elevated TNF-α mRNA about 2–3-fold in 30 min in PC-3/vector.P1 cells (which was similar to parental PC-3 cells) but caused down-regulation of TNF-α mRNA levels (to 0.08% of those in untreated cells) in PC-3/WT1-EGR-1.P1 cells (Fig. 4C). Thus, the dominant-negative mutant of EGR-1 abrogates ionizing radiation-inducible TNF-α expression. Because the dominant-negative mutant acts by inhibiting the function of EGR-1, these findings indicate that EGR-1 is essential for ionizing radiation-inducible TNF-α expression.

To determine the mechanism by which EGR-1 may up-regulate TNF-α, we examined the TNF-α gene promoter for consensus EGR-1-binding sites. Interestingly, a sequence 5'-GGGCCCCGC-3' that conforms exactly with that first shown (21) to bind EGR-1 protein was detected between nucleotides 194 and 186 upstream of the cap site of TNF-α (42). This sequence in TNF-α gene has not been demonstrated to mediate transactivation by EGR-1. To determine the mechanism by which the dominant-negative mutant protein of EGR-1 blocked
radiation-inducible TNF-α expression, we performed CAT assays by using a reporter construct, TNFp-CAT that contains the TNF-α promoter region from −470 to +120. PC-3 cells were transiently cotransfected with TNFp-CAT, CMV-EGR-1, and either the dominant-negative mutant CMV-WT1-EGR-1 or the empty vector pCB6. Cells cotransfected with TNFp-CAT and vector showed a modest background level of CAT activity (Fig. 4D). The CAT activity was increased significantly when the cells were cotransfected with TNFp-CAT, CMV-EGR-1, and vector. On the other hand, when cotransfection was performed with TNFp-CAT, CMV-EGR-1, and CMV-WT1-EGR-1, CAT activity was severely reduced (Fig. 4D). These findings indicated that CMV-EGR-1 can transactivate, and the dominant-negative mutant WT1-EGR-1 can transrepress, the TNF-α promoter.

We also studied the reporter activity of TNFp-CAT construct in cells exposed to ionizing radiation. Ionizing radiation induced CAT activity from TNFp-CAT within 1 h of the exposure (Fig. 4E). Induction of TNFp-CAT expression by ionizing radiation was significantly reduced when cells were cotransfected with the dominant-negative mutant of EGR-1 (Fig. 4E). These findings suggest that inducible expression of the TNF-α promoter by ionizing radiation required functional EGR-1. When a mutation, which abolished the ability of EGR-1 protein to bind to the TNF-α promoter (42), was introduced into the TNFp-CAT construct, basal expression driven by the promoter was attenuated, and radiation-inducible CAT activity was abolished (Fig. 4E). As expected, ectopically expressed EGR-1 caused induced expression of CAT activity from the promoter construct that contained wild-type EGR-1-binding site but not from the construct that contained the mutation EGR-1-binding site (Fig. 4E). Together, these findings suggest that EGR-1 transactivates TNF-α promoter through the EGR-1-binding site and that this site is required for radiation-inducible TNF-α expression.

**DISCUSSION**

Wild-type p53 has been shown to be functionally necessary for growth inhibition and apoptosis following exposure to ionizing radiation (6), and p53 mutations have been reported to increase resistance to apoptosis (8). In agreement with these observations, the present study has shown that the absence of wild-type p53 protein in prostate cancer cells renders them moderately resistant to ionizing radiation-induced apoptosis. The expression of genes encoding EGR-1 and TNF-α that induced apoptosis was up-regulated by ionizing radiation in the PC-3 cells, and inhibition of EGR-1 transactivation function by the dominant-negative WT1-EGR-1 chimera abrogated ionizing radiation-inducible TNF-α induction and apoptosis. Consistent with these observations, ectopically expressed EGR-1 enhanced ionizing radiation-inducible TNF-α expression and apoptosis. These findings suggest that EGR-1 is an upstream modulator of TNF-α induction and apoptosis in the pathway evoked by ionizing radiation. Moreover, EGR-1 causes transcriptional activation of the TNF-α promoter via a consensus EGR-1-binding site providing a mechanism for EGR-1-inducible expression of the TNF-α gene. Thus, EGR-1 is an important mediator of radiation responsiveness in prostate cancer cells that lack functional p53 protein. Because p53 protein is mutated in prostate tumors (14, 15), EGR-1, which functions by a p53-independent pathway, is of critical importance in the apoptotic death of the tumors. Both EGR-1 and TNF-α are induced by ionizing radiation in diverse tumor types, and future studies may design approaches to further exploit this novel pathway for the containment of radio-resistant tumors.