Functional p53 Increases Prostate Cancer Cell Survival After Exposure to Fractionated Doses of Ionizing Radiation

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ABSTRACT

External beam radiation therapy is an effective therapy for localized prostate cancer, although failures occur at high rates. One variable that may affect the radiosensitivity of prostate tumor cells is their p53 status, because this gene controls radiation-induced cell cycle arrest, apoptosis, and the repair of DNA damage. Using a system in which p53 function was conditionally restored to p53-null PC3 prostate cancer cells by stable transfection with a human temperature-sensitive p53 mutant allele, we tested the hypothesis that functional p53 increases cell cycle arrest and contributes to increased clonogenic survival after ionizing radiation (IR) of prostate carcinoma cells. Cell cycle arrest and clonogenic survival in response to single and multiple daily exposures to clinically relevant 2-Gy doses of IR were examined. Whereas the temperature-sensitive p53 protein was activated by phosphorylation after IR exposure at both the permissive and restrictive temperatures, CDKN1/p21 (G1 arrest) and p53-homologous protein (p16 INK4A) (G2 arrest) were induced by functional p53 (at the permissive temperature). In the presence of functional p53, the maintenance of G2 arrest was significantly longer (P < 0.01), and a small increase in cell survival measured by clonogenic assay was seen after exposure to a single 2-Gy dose of IR. However, functional p53 significantly increased clonogenic survival (P < 0.01) after exposure to multiple doses of 2 Gy of IR and contributed to a more sustained G2 arrest and increased G1 arrest in response to the multifractionation regimen. These studies implicate the presence of wild-type p53 with increased survival of prostate carcinoma cells after fractionated exposure to radiation. Additionally, the data provide evidence that wild-type p53 in prostate tumor cells may reduce the effectiveness of radiation therapy.

INTRODUCTION

CaP is the second leading cause of cancer death among American men, exceeded only by lung cancer (1, 2). Nearly 90% of patients will present with presumed localized disease, making them candidates for potential curative local therapy, which currently includes radiation therapy, radical prostatectomy, and, in some cases, active surveillance. As a treatment option, EBRT is effective for localized disease. However, using prostate-specific antigen levels as a marker for tumor control, patients who present with organ-confined disease have a 5-year failure rate of 10–40% after EBRT (3, 4).

Exposure to IR results in DNA double-strand breaks and other cellular injuries. Cellular responses to radiation-induced DNA damage include cell cycle arrest and DNA damage repair, responses needed for cell survival, and cell death, which occurs through both mitotic and apoptotic processes when damage is either unreparable or mis-repaired (5–7). Thus, signaling pathways and control of cell cycle progression are closely linked to the intrinsic radiosensitivity of cells (8, 9). These pathways may include targets that can be exploited to enhance the efficacy of radiation therapy, particularly for those tumor types such as CaP that are considered to be relatively radio-resistant.

The p53 status of prostate tumor cells may affect their radiosensitivity. p53 is involved in cell cycle arrest, apoptosis, and the repair of DNA damage (10–14). It has been well established that the p53 protein contributes to both G1 and G2 arrests through transcriptional regulation that includes the induction of CDKN1/p21 (G1 arrest) and p53-homologous protein (p16 INK4A) (G2 arrest) (15–19). Some studies have demonstrated that cells lacking p53 or having a mutated p53 show more radioresistant phenotypes (20–23). Explanations for the increase in radioresistance in cells with mutant p53 include the lack of cell cycle checkpoint arrest and p53-dependent apoptosis, each of which can increase radioresistance (24, 25). Additionally, some mutations in p53 result in a gain of function, which may also result in an increase in radiation resistance (26). However, other investigators have reported that cell lines that lack both wild-type p53 alleles or express mutant p53 either do not show significant changes in their radiosensitivity or are more sensitive to radiation (27–31). Thus, relationships between the p53 status of cells and their radiosensitivity are less than clear and may vary as a function of cell type, the specific p53 mutation, or the profile of genomic alterations present in the different tumor cells.

It is generally thought that the arrest maintenance role of p53 in response to radiation-induced DNA damage is, at least in part, to allow sufficient time for DNA damage repair before DNA replication (G1 arrest) or before entering mitosis (G2 arrest). If the cell cycle arrest functions of p53 are protective, they may be particularly significant for cell types in which apoptosis has a minor role in the overall response to IR. This may be the case in prostate cancer, as suggested by the low numbers of radiation-induced apoptotic cells detected in CaP cell lines (32–34). Additionally, these survival mechanisms may be especially relevant to IR treatment of the ~65% of primary prostate tumors that retain wt p53 (35). Thus, we hypothesized that functional p53 increases cell cycle arrest and contributes to increased clonogenic survival after IR of CaP cells. A model system was used in which p53 function was conditionally restored by stable transfection with a temperature-sensitive p53 mutant allele (P223L) isolated from a human prostate cancer cell line. This study demonstrates the restoration of p53 function in prostate cancer cells at the permissive temperature of 32°C and uses the PC3p53+/- mutant prostate cancer model to examine cell cycle arrest and clonogenic survival in response to clinically relevant doses of IR.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The prostate cancer cell line PC3 (p53 null) used in this study was obtained from the American Type Culture Collection. The PC3 cells are hemizygous for chromosome 17p, and their single copy of the p53 gene has a bp deletion at codon 138 that has caused a frameshift and a new in-frame stop codon at position 169 (36). As a result, PC3 cells do not express p53 protein (37). The PC3V2 vector control cell line (PC3-vector) containing an empty pCR3.1 vector (Invitrogen, Carlsbad, CA)
was a generous gift from Dr. Xu-Bao Shi (University of California Davis Cancer Center). The PC3<sup>neo</sup> stable transfectants were created as described below. Cells were routinely cultured in Ham’s F-12 media (BioWhittaker, Walkersville, MD) supplemented with 7% heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, CA), t-glutamine, vitamins, fungizone, penicillin, and streptomycin. G418 antibiotic (500 μg/ml) was added to the medium to maintain selection for the transfected plasmids. Cells were maintained at 37°C in 5% CO₂ in air for routine culture.

**Stable Transfection of the P223L Allele into PC3 Cells.** PC3 cells were transfected with the pCR3.1 vector (Invitrogen) that contains the pCMV reagent complexed with 6 ml of DNase-free RNase (Roche Molecular Biochemicals, Indianapolis, IN) was added to a final concentration of 0.05 mg/ml, and samples were allowed to stand at room temperature, protected from light, for a minimum of 15 min. Cell aggregates were removed by filtration into Falcon polystyrene tubes fitted with cell-strainer caps (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). Propidium iodide fluorescence as an indicator of relative DNA content/cell was measured with the Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL). At least 10,000 cells were analyzed per sample with doublet discrimination. The percentages of cells in the G₁, S, and G₂-M phases of the cell cycle were estimated from the DNA content histogram obtained from each cell sample using the Phoenix Multicycle computer software (Phoenix Flow Systems, San Diego, CA). Unirradiated control cells were harvested at a cell culture time equivalent to the 24 h postirradiation time point or, in some experiments, at approximately the same time as the irradiation and thus represent logarithmically growing cells. Preliminary studies revealed that the cell cycle distribution of an untreated cell culture obtained at these times in any given experiment was essentially identical. All cell cycle experiments were performed at least twice.

**Mitotic Trapping.** Cells were cultured in T-25 flasks to 50–70% confluence and then irradiated with a single dose of 2 Gy. Nocodazole was added to the culture media 30 min after irradiation or temperature down-shift (unirradiated cells) at a final concentration of 0.1 μg/ml culture medium. The cells were then cultured in the presence of nocodazole for 24 h. Because mitotic cells can become easily detached from the culture flasks, the culture medium from each flask was removed and saved before the remaining cells were harvested by trypsinization to ensure that these cells were not lost from the analyses. Once the cells were detached from the culture flask, the saved medium was used to collect the trypsinized cells and transferred to tubes, and the cells were pelleted by centrifugation. The pelleted cells were washed once in 1× PBS and fixed and stained in a solution containing 3.7% formaldehyde, 0.1% Igepal, and 10 μg/ml Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) in PBS. Fifty μl of the stained cell suspension were placed on a microscope slide, and a coverslip was placed on top. Nuclei with condensed, evenly staining chromosomes were scored as mitotic. At least 300 cells were counted for each treatment from each experiment using fluorescent microscopy to visualize the nuclei.

**Clonogenic Assays.** Cell survival after IR was measured by clonogenic assay. For all experiments, single cells were seeded into 60-mm culture dishes on day 0 and allowed to attach for 24 h at 37°C. The cells were then irradiated at the desired dose (day 1), immediately returned to the incubator, and cultured for approximately 6 doubling times. Controls were handled identically to the irradiated test cells, with the exception of the radiation exposure. Thus, in the experiments in which p53 function was restored by temperature down-shift, both the irradiated cells and unirradiated controls were incubated at 32°C for 24 h after the IR exposure and then incubated at 37°C for the remainder of the assay (starting at day 2). Colonies were fixed in 1.0% crystal violet and 0.5% glacial acetic acid in ethanol, and visible colonies containing approximately 50 or more cells were counted. The SF at 2 Gy (SF2) was calculated relative to the unirradiated control cells for each temperature to account for the plating efficiency of each cell line in each experiment (number of IR colonies/number of colonies in control).

**Survival after Three Daily Doses of 2 Gy (3 × 2 Gy).** Cells were plated as described for the single-dose assays. The cells were then irradiated daily with 2 Gy of radiation (approximately 24 h between each dose) for 3 consecutive days. For cells undergoing temperature down-shift, both the irradiated and unirradiated controls were incubated at 32°C for 3 days beginning after the first 2-Gy dose. After the last dose of irradiation, the cells were incubated to the 50-cell colony stage. At this point, the cells were fixed, stained, and counted as above. The SF3 × 2 was calculated relative to the untreated controls maintained under the same temperature conditions to account for plating efficiencies (as described above for single-dose experiments).

**RESULTS**

To study the contribution of the p53 protein to the radiation response in prostate cancer cells, the p53-null PC3 cells were transfected with the P223L mutant p53 allele that encodes a temperature-sensitive protein. This approach was chosen because of the difficulties encountered in establishing a stable line that expresses wild-type p53.
Clone 5 was used in these radiation studies and renamed PC3tsp53. Among the five clones, two of the five clones expressed the tsp53 protein, as shown in Fig. 1 at 35°C. From the transfect PC3 cells, five G418-resistant clones were isolated and screened for expression of p53 by Western blotting. As shown in Fig. 1A, two of the five clones expressed the tsp53 protein. Clone 5 was used in these radiation studies and renamed PC3tsp53 (unfortunately, Clone 4 was lost in a liquid nitrogen freezer failure). Because the Cdk inhibitor Cdkn1/p21 (p21) is transcriptionally regulated by p53, p21 protein expression was assessed by Western blot analysis as a marker of restored function (Fig. 1B; Refs. 45 and 46). Culturing PC3tsp53 at 32°C resulted in an induction of p21 protein as early as 1 h after the temperature down-shift and increased in a time-dependent manner (1.1-fold at 1 h to 2.4-fold at 5 h compared with the 37°C control). This was in contrast to the PC3-parental and PC3-vector cells, each of which showed no induction of p21 during the same time period after temperature down-shift (data not shown). Thus, these data demonstrate that the transcriptional function of the tsp53 protein was rapidly restored when the cells were incubated at 32°C, and thus established it as the permissive temperature for the PC3tsp53 cell line. Additional Western blot analysis using phospho-specific antibodies showed that both serines 15 and 20 of the tsp53 protein were phosphorylated after exposure to IR, confirming that this protein is activated in response to radiation-induced damage (data not shown).

**Temperature Effects on Cell Cycling.** Because the model chosen for these radiation studies required the PC3tsp53 cell line to be cultured at the nonphysiological temperature of 32°C (permissive temperature) to restore function to the tsp53 protein, it was important to establish what effects this lower culturing temperature would have on the distribution of cells in the G1, S, and G2-M phases of the cell cycle. PC3tsp53 cells were incubated at 32°C for up to 24 h, with cells harvested at 6-h intervals for flow cytometry analysis of cell cycle distribution. Representative histograms and mean percentages of cells in each cell cycle phase derived from multiple experiments are shown in Fig. 2A. The histogram labeled Control-37°C, against which the cells grown at 32°C were compared, represents logarithmically growing cells at 37°C. Incubation at 32°C with restoration of functional p53 resulted in little change in the percentage of cells in G1 over the time points examined. The predominant effects were seen in the percentage of cells in both S phase, which decreased to a nadir at 18 h followed by a recovery by 24 h, and G2-M, which increased and remained elevated from 18 to 24 h. Mitotic trapping using the microtubule inhibitor nocodazole revealed no statistical difference in the percentage of PC3tsp53 cells attempting cell division in a 24-h period for those cells cultured at 32°C compared with similarly treated cells cultured at 37°C (data not shown; Refs. 15 and 17). Whereas mitotic trapping showed that the effects of culturing the PC3tsp53 cells at 32°C were relatively transient over the 24-h period examined, there was some delay of cells moving through the G1-S transition, and an accumulation in the percentage of G2-M cells. Thus, these data establish that it is important for PC3tsp53 to be compared with itself at the two culturing temperatures for the purpose of the experiments presented here, which examine the role of functional p53 after exposure of the cells to IR.

**Functional p53 Increases the Duration of the IR-Induced G2 Arrest.** To examine the effects of functional p53 on the response to IR exposure, the PC3tsp53 cells were exposed to a single 2-Gy dose of IR and cultured at either the permissive (Fig. 2A, IR +37°C) or restrictive (Fig. 2A, IR +32°C) temperature for up to 24 h post-IR. Cell cycle responses were assessed every 6 h as described above, and the results are shown in Fig. 2A. The predominant effect of exposure to IR in the presence of functional p53 (IR +32°C) was a sustained G2-M accumulation (+ ~23% versus control) from 12 to 24 h compared with the cells incubated at the restrictive temperature post-IR (IR +37°C). At the restrictive temperature, the percentage of cells in G2-M was also elevated by 12 h; however, this accumulation quickly declined so that by 24 h, G2-M content approached that seen in the unirradiated control. At both the permissive and restrictive temperatures, exposure to 2 Gy of IR resulted in an initial decrease in G1 cells compared with the control. This decrease persisted in the cells cultured at 32°C up to 12 h, but by 24 h, the percentage of G1 cells was equivalent to control. In contrast, in the cells maintained at 37°C, the fraction of cells in G1 began to increase at 12 h, so that by 18 h and persisting to 24 h, the percentage of G1 cells exceeded that seen in the control (~60% versus ~46%, respectively). Reductions in S phase were seen at both temperatures; however, the loss of S-phase cells was more substantial at 24 h when p53 was functional, demonstrating the contribution, albeit somewhat delayed, of p53 to G2 arrest. The post-IR cell cycle changes seen after a single dose of IR suggest that the most potent influence of functional p53 is a more persistent G2 arrest. To further explore the contribution of functional p53 to the maintenance of IR-induced G2 arrest, mitotic trapping of the irradiated PC3tsp53 cells was performed as described above for the unirradiated cells. The PC3tsp53 cells were exposed to 2 Gy of IR followed by culturing at the permissive or restrictive temperature in the presence of nocodazole for 24 h. The percentage of cells that exited G2 and entered mitosis was significantly reduced (~23%
P < 0.01) in the presence of functional p53 compared with the cells incubated at 37°C (Fig. 2B). Thus, these data provide evidence that, when p53 was functional in PC3 tsp53 cells, the IR-induced G2 arrest was more durable. These results likely explain the lower percentage of cells in G1 12–24 h post-IR at the permissive temperature because more cells are inhibited from completing the cell cycle and entering G1 than in the absence of functional p53. Additionally, these data are consistent with the reported role of p53 in the maintenance of G2 arrest after DNA damage (15, 16, 18, 19, 47–49).

**Functional p53 Increased Clonogenic Survival After Exposure to Fractionated IR.** The ability of prostate cancer cells not only to survive but also to retain reproductive potential after radiation underlies the problem of tumor recurrences seen in patients. To test the hypothesis that functional p53 contributes to increased clonogenic survival after IR of CaP cells, the PC3 tsp53 and PC3-vector cells were exposed to a single dose of 2 Gy of IR and to a regimen of three consecutive daily doses of 2 Gy in separate experiments. Clonogenic survival was assessed using a standard colony formation assay that measures the loss of reproductive integrity after exposure to IR (50). After IR, the cells were either cultured at 37°C for the entire assay or to a regimen of three consecutive daily doses of 2 Gy in separate experiments. Clonogenic survival was assessed using a standard colony formation assay that measures the loss of reproductive integrity after exposure to IR (50). After IR, the cells were either cultured at 37°C for the entire assay or to a regimen of three consecutive daily doses of 2 Gy in separate experiments. Clonogenic survival was assessed using a standard colony formation assay that measures the loss of reproductive integrity after exposure to IR (50).

**Functional p53 Contributes to Increased G1 Arrest and Sustained G2 Arrest in Response to Fractionated IR.** To examine the relationship between cell cycle response and clonogenic survival after fractionated IR exposure, the PC3 tsp53 cells were exposed to three consecutive daily doses of 2 Gy after a radiation schedule identical to the multifraction survival experiments. Cells were harvested for cell cycle analysis by flow cytometry 24 h after each 2-Gy fraction. Representative histograms are shown in Fig. 4. Cell cycle analysis showed the percentage of G1 cells with functional p53 had increased only after the second and third IR fractions (60% and 55%, respectively, versus 45% in control). The persistent reduction in cells exiting G1 and entering S phase compared with the unirradiated control revealed the influence of functional p53 on G1 arrest. The sustained reductions of S phase (≈30% after three fractions) were coincident with persistent elevations in the percentage of cells in G2-M (≈21%). In contrast, without functional p53, S phase remained comparatively high (≈30%) after each IR exposure, and the fraction of cells in G2-M did not approach the accumulations seen at the permissive temperature. Thus, the presence of functional p53 resulted in more durable

**Fig. 2. Functional p53 increases the duration of the IR-induced G2 arrest.** A, representative histograms of PC3 tsp53 cells exposed to 2 Gy of IR and incubated at 32°C or 37°C for up to 24 h. Cell cycle analysis was performed on cells harvested every 6 h. The panel labeled Control-37° is representative of logarithmically growing cells at 37°C. Percentages of cells in each phase of the cell cycle are the means of at least two experiments for each time point. *p < 0.01), note reduced S phase and persistence of elevated G2-M at 24 h. B, duration of arrest was assessed by trapping the cells that entered mitosis during the 24 h post-IR period using the nocodazole (IR-32° versus IR-37°C). Columns represent the means of duplicate experiments; error bars = SD.
IR-induced G_1 and G_2 arrests. Additionally, a substantially higher percentage of sub-G_1 events were seen at the permissive temperature than at the restrictive temperature (24% versus 7%, respectively; control/H11005 5%) 24 h after the third IR fraction, providing suggestive evidence that p53-dependent apoptosis was also restored.

DISCUSSION

In the studies presented here, a mutant p53 allele (P223L) isolated from a human prostate cancer cell line that produces a temperature-sensitive protein was used to examine the contribution of p53 to cell cycle responses and clonogenic survival after exposure to clinically relevant doses of IR. Significantly increased clonogenic survival after exposure to multiple 2-Gy IR exposures was seen in PC3^{p53} cells when p53 function was restored. This increased survival was associated with a persistent elevation in the fraction of cells in G_2-M, an increased G_1 content, and markedly reduced S phase over the course of the three IR exposures, revealing a p53 component contributing to both G_1 and G_2 arrests. Additionally, the increased survival was not because of a loss of apoptosis as indicated by the higher percentage of sub-G_1 events at the permissive temperature than at the restrictive temperature. Thus, in these cells, functional p53 contributed to both cell cycle arrest, a protective response necessary for repair of IR-induced DNA damage, and the elimination of irreparably damaged cells by apoptosis, with the overall outcome being one of higher clonogenic survival compared with these same cells without functional p53. What was not examined in these investigations was the third arm of the p53 response, DNA damage repair. An interesting finding of note was that exposure to IR resulted in activated p53, as defined by phosphorylation of serines 15 and 20, at both the restrictive (37°C) and the permissive (32°C) temperatures. However, transactivation function, as evidenced by the induction of CDKN1/p21, only occurred at the permissive temperature. This may be the first study to use the P223L mutant p53 allele and a restoration strategy to study the contributions of p53 to the radiation response; the most commonly used temperature-sensitive mutant alleles have been the murine p53, Val-135, or its human equivalent, Ala-143 (51–54).

The data presented here provide evidence that the presence of wt p53 in prostate tumor cells may reduce the effectiveness of radiation therapy and therefore constitute a therapeutic liability. Because in vivo animal model studies cannot be performed using a temperature-sensitive allele, these hypothesis-generating investigations set the stage for further investigations using strategies to down-regulate wt p53 INCREASES SURVIVAL AFTER FRACTIONATED IR Research.


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