Attenuated Response of p53 and p21 in Primary Cultures of Human Prostatic Epithelial Cells Exposed to DNA-damaging Agents

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Abstract

The multifocal origin of prostate cancer suggests a pan-organ defect in a tumor suppressor pathway. Although structural mutations in the p53 gene have been implicated in late-stage prostate cancer, little is known about the p53 response to genotoxic stress in normal human prostatic epithelial cells from which adenocarcinomas originate. We found that the majority (10 of 12) of epithelial cell cultures derived from histologically normal tissues of radical prostatectomy specimens failed to exhibit p53 accumulation in response to ionizing radiation. Epithelial cell cultures derived from benign prostatic hyperplasia and a primary prostatic adenocarcinoma also failed to accumulate p53 in response to ionizing radiation. In contrast, cultures of prostatic stromal cells derived from normal, benign prostatic hyperplasia, or adenocarcinoma tissues exhibited a 3-9-fold induction of p53 within 1-3 h after irradiation. Since p53 regulates a cell cycle checkpoint through the induction of the cyclin-cdk inhibitor p21, we examined p21 accumulation and cell cycle arrest following exposure to ionizing radiation. With one exception, epithelial cells that did not display increased p53 or p21 induction did not demonstrate a significant G1-S arrest in response to ionizing radiation, whereas stromal cells that accumulated p53 and p21 exhibited a large cell cycle arrest. These results indicate a functional difference between the DNA damage response of epithelial and stromal prostatic cells and suggest a possible mechanism for the increased susceptibility of prostatic epithelial cells to accumulate genetic alterations.

Introduction

Prostate adenocarcinoma is the most common noncutaneous malignancy in men and the second leading cause of male cancer deaths in the U.S. (1). Despite the prevalence of the disease, little progress has been made in our understanding of the molecular mechanisms that lead to prostate tumor development, and no single oncogene or tumor suppressor gene has been convincingly linked to the initial stages of the malignancy. The role of alterations of the tumor suppressor gene p53 in prostate cancer has been the focus of numerous studies in the last few years. Mutations of the p53 gene or elevated expression of the p53 protein (which is often the result of p53 gene mutations) have been reported in a small percentage (10–20%) of prostate tumors as well as in several tumor-derived cell lines (2, 3). However, almost all of the p53 mutations are associated with high-grade, metastatic, or hormone-refractory tumors (2–4). Furthermore, mutations of p53 in BPH are rare (5, 6). These results suggest that p53-inactivating mutations are late events in prostate tumor development (7) but that they are less frequent than in some other types of cancer.

The finding that treatments of cells with DNA-damaging agents result in accumulation of p53 protein (8, 9) sheds new light on the role of p53 as a tumor-suppressor gene. Data from several laboratories indicate that the observed G1-S cell cycle arrest in cells exposed to DNA-damaging agents such as IR is mediated by a p21-mediated accumulation of p53 protein and by subsequent transcriptional induction of downstream genes like p21CIP/WAF-1 and GADD 45 (9–12). The products of these genes were found to interact with and negatively regulate the activity of components that are involved in cell cycle progression, such as cyclin-dependent protein kinases and proliferating cell nuclear antigen (12–14). Activation of this p53-regulated pathway in response to DNA damage leads to a G1-S cell cycle arrest, which presumably allows the cell increased time to repair or restore its DNA damage before DNA replication in S (15, 16).

When the function of p53 is abolished either by inactivating mutations or by targeted degradation of the protein, DNA alterations necessary for the initiation or progression of cells into a malignant phenotype can accumulate. For example, there is considerable evidence linking infection of epithelial cells by certain types of HPV to cervical carcinoma (17, 18). It has been demonstrated that the E6 gene product from high-risk HPV types 16 and 18 forms complexes with wild-type p53 and targets it for proteolytic degradation via an ubiquitin-dependent pathway (19–21). In contrast, the E6 gene product from low-risk HPV types 6 and 8 binds poorly to p53 and is rarely associated with cervical carcinoma (22). However, since high-risk HPV has only been identified in approximately 5% of human prostate cancers, it does not seem to be strongly associated with the etiology of prostate carcinoma (23).

Based on the lack of correlation between p53 mutations and early-stage prostate cancer, we hypothesized that abrogation or attenuation of p53 activity after DNA damage may be an early event in the development of prostate carcinomas. It is conceivable that failure to induce p53 transcriptional enhancing activity in response to DNA damage would greatly enhance the probability of a cell accumulating the necessary genetic alterations for the formation of a carcinoma. This abrogation of p53 function would in turn result in the introduction and propagation of mutations in the genome, giving rise to genomic instability and eventually to malignancy. Therefore, according to this hypothesis, the failure to accumulate p53 or p21 in response to DNA damage would represent a much earlier event in prostate tumorigenesis than the occurrence of p53-inactivating mutations. Furthermore, cell lines derived from individuals affected with ataxia telangiectasia have been found to have the above described characteristics due to a defect in a signal transduction pathway for p53 and not due to inactivation or mutation of p53, establishing a precedent for this hypothesis (9).

To test this hypothesis, we studied the IR-induced accumulation of p53 and p21 proteins as well as the p53-dependent, IR-induced cell cycle arrest in human prostate cells that were obtained from radical prostatectomy specimens. These prostate cells were shown to have wt-p53 by methods described previously (24). The levels of the p53

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2 Both T. G. and C. K. contributed equally to this work.

3 To whom requests for reprints should be addressed.

4 The abbreviations used are: BPH, benign prostatic hyperplasia; IR, ionizing radiation; HPV, human papillomavirus; wt, wild type; AT, ataxia telangiectasia.

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protein in untreated cells in these studies were low, indicating that p53 possesses a short half-life in these cells. We found that while in all stromal cell strains p53 and p21 levels were increased after IR treatments, the majority of epithelial strains (12 of 14) failed to show a similar induction. The attenuated induction of p53 and p21 in prostatic epithelial cells correlated with loss of a G1/S checkpoint after IR. These results suggest a possible role for a signaling defect in the IR pathway for p53 induction as a predisposing event for prostate cancer.

Materials and Methods

Cell Culture. Tissue samples were dissected from normal peripheral zone, normal central zone, BPH, and adenocarcinomas of radical prostatectomy specimens (25). None of the patients had received any prior hormonal, radiation, or chemotherapy. Following overnight digestion of the tissue with collagenase, epithelial cell cultures were derived according to protocols published previously (26). Primary and serial cultures of epithelial cells were maintained in medium PFMRAA (27) supplemented with 10 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, 10 µg/ml bovine pituitary extract, 4 µg/ml insulin, 1 µg/ml hydrocortisone, 0.1 mM phosphoethanolamine, 3 X 10^-4 M selenous acid, 2.3 X 10^-9 M α-tocopherol, 3 X 10^-11 M retinoic acid, and 100 µg/ml gentamicin. Dishes coated with type I collagen were used routinely. The epithelial nature of cultures obtained by these methods was verified as described previously (26). Epithelial cell strains are denoted as “E,” followed by the tissue of origin (peripheral zone, central zone, BPH, or adenocarcinoma) and the strain number. Fibroblastic cell strains were established by incubating collagenase-digested tissue into MCDB 105 (Sigma Chemical Co., St. Louis, MO) supplemented with fetal bovine serum (10% v/v) and gentamicin (100 µg/ml). The fibroblastic nature of cells derived by this protocol has been described previously (28). Fibroblastic cell strains are denoted as “F,” followed by the tissue of origin and strain number. LNCP, an established prostate cancer cell line, was obtained from the American Type Culture Collection and served as a positive control for wt-p53 induction after IR.

Cell Treatment. Cells (75% confluent) were treated with 6 Gy of ionizing radiation from a 137Cs source. Following treatments, cells were returned to a 37°C incubator for varying times until harvested for immunoblot or cell cycle analysis.

Immunoblot Analysis. At the indicated times after treatment, cells were rinsed twice with ice-cold PBS and collected by centrifugation. Cell pellets were resuspended in 3 volumes of lysis buffer (50 mM potassium phosphate, 1 mM phenylmethylsulfonate, 1 mg/ml aprotinin, and 1 mg/ml leupeptin, pH 7.8) and were sonicated on ice. Cell homogenates were centrifuged to pellet cell debris, and the protein concentration for each sample was determined by the bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Fifty µg of total protein was mixed with an equal volume of 2% Laemmli sample buffer (62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.003% bromophenol blue), and samples were heated at 95°C for 5 min. Proteins were separated on 10% polyacrylamide gels for p53 detection or on 15% gels for p21 detection or 15% gels for p21 detection (50 mAh/gel for 1.5 h). Proteins were transferred to Hybond ECL nitrocellulose paper (Amersham, Arlington Heights, IL) using a semidry transfer apparatus (Bio-Rad, Hercules, CA 5 V for 1 h) or by wet-transfer using a Bio-Rad vertical transfer apparatus (50 mA for 3 h). After transfer, the gels and the nitrocellulose membranes were stained with 0.15% Coomasie blue stain and Ponceau S solution (Bio-Rad), respectively, to ensure equal loading and transfer of the protein samples. Membranes were then blocked with TBS-T (20 mM Tris-HCl, 137 mM NaCl, 1% Tween 20, and 2% bovine serum albumin) and were probed with a p53-specific mAb (DO-1: Santa Cruz Biotechnology, Santa Cruz, CA) or a p21-specific polyclonal antibody (sdi-1; Pharmingen, San Diego, CA). Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL; Amersham). Films were scanned with a densitometer (Mikrotek, Torrance, CA), and p53 and p21 immunoreactive bands were quantitated using the NIH Image software program. Results are reported as fold induction by dividing the absorbance after treatment by the absorbance of the untreated control lane. All experiments were performed at least three times.

Cell Cycle Analysis. Cell cycle distribution was performed by determining DNA content alone or by determining both DNA content and DNA synthesis as described previously (29). Briefly, for determination of DNA content alone, cells were fixed by dropwise addition of 70% EtOH that had been kept at -20°C, and after 1 h, the cells were washed twice with PBS. Cells were then incubated with RNase (50 µg/ml) and stained with propidium iodide (25 µg/ml). For analysis of both DNA content and DNA synthesis, cells were pulsed with 10 µM bromodeoxyuridine for 4 h. Cells were then prepared for flow cytometry. Briefly, cells were fixed in 70% methanol as above, resuspended in 0.1 N HCl containing 0.7% Triton X-100 for 100 min on ice, washed with PBS, heated to 97°C for 10 min in 0.0032 N HCl, chilled on ice for 10 min, and washed twice with IFA buffer [10 mM piperrazine-N,N’-bis (2-ethan sulfonic acid) (pH 7.4), 150 mM NaCl, and 4% FCS] containing 0.5% Tween 20. Cells were then incubated with 5 ng of FITC-conjugated anti-bromodeoxyuridine antibody (Boehringer Mannheim) per µl for 30 min on ice, washed twice, treated with 50 units/ml of RNase A at 37°C for 15 min, incubated on ice for 1 h with propidium iodide (25 µg/ml), and analyzed with a FACStarPlus® flow cytometer (Becton Dickinson, San Jose, CA).

Results and Discussion

Induction of p53 levels after IR treatments (6 Gy) was found to be aberrant in 10 of 12 normal epithelial cell strains examined (Table 1). For example, in two epithelial strains, E-PZ-34 and E-PZ-31, p53 failed to accumulate after 4 h of IR. Incubation of cells for longer time periods of up to 24 h after IR still did not result in p53 accumulation. In contrast, the LNCP cell line, derived from metastatic prostate cancer and homozygous for wt-p53, displayed a significant increase in p53 levels 1 h after IR (Fig. 1). In LNCP cells, p53 levels increased within 1 h, peaked at 3 h, and remained increased through 8 h after IR. The identity of the p53-immunoreactive band on the immunoblots was confirmed by electrophoresing purified p53 protein (Oncogene Science, Uniondale, NY) in adjacent lanes (data not shown). Another epithelial cell strain, designated E-PZ-30, exhibited a small (2.5-fold) induction of p53 (Figs. 2A and 3A). However, the induction in E-PZ-30 was not evident until 4 h after IR treatment. p53 was not induced in a strain of epithelial cells derived from BPH or in a strain derived from prostatic adenocarcinoma (Table 1).

In contrast to the results with epithelial cells, almost all stromal cells exhibited a robust induction of p53 following IR treatments. For example, the fibroblast cell strains F-PZ-12 and F-PZ-11 exhibited 10- and 5-fold inductions of p53, respectively (Fig. 2B). Increases ranged from a 2.8-fold induction in the F-CA-3 strain to a 10-fold induction in the F-PZ-12 strain. In most cases, p53 induction was evident within 1 h after IR (Fig. 3B).

Table 1. Induction of p53, p21, and cell cycle arrest in prostate cells following IR

<table>
<thead>
<tr>
<th>Cell type</th>
<th>wt-p53 induction</th>
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<th>Cell cycle arrest</th>
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<tr>
<td>LNCP</td>
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* Induction of p53 by 6 Gy ionizing radiation and assaying from 30 min to 6 h later.
+ Induction of p21 by 6 Gy ionizing radiation and assaying from 1 to 6 h later.
ND Cell cycle arrest as determined by the G1/S ratio of cells before and after 6 Gy of IR.  
+ Cell cycle arrest; +/- weak or delayed induction; - no induction or no cell cycle arrest; ND, not determined.
Fig. 1. Effect of IR on p53 levels in prostatic epithelial cells. Cells were exposed to 6 Gy of IR and were lysed at the times indicated after treatment. Whole-cell extracts from untreated cells (c) or after successive time intervals following IR were blotted and probed with the anti-p53 mAb (DO-1). E-PZ-34 and E-PZ-31 are epithelial strains. LNCaP is a homozygous wt-p53 cell line derived from metastatic prostate adenocarcinoma and is included for comparison of the damage-induced accumulation of p53.

In cell lines with wt-p53, accumulation of p53 after IR is followed by an increase in p21 mRNA and protein levels. To investigate whether epithelial and stromal strains also exhibited differences in the induction of genes downstream of p53, we looked at the accumulation of p21 protein after IR treatments. Only two epithelial cell strains (E-PZ-32 and E-CZ-9) exhibited a significant p21 induction (Table 1). It is noteworthy that in E-PZ-32 cells, p21 induction occurred by a mechanism that did not require induction of p53 levels. p21 was also induced less than 3-fold in three other epithelial cell strains (E-PZ-30, E-PZ-36, and E-CZ-7), but in E-PZ-30, this response was evident only after 6 h after IR (Figs. 1C and 4). As was the case with the induction of p53, all stromal cells exhibited a strong induction of p21 after IR. Increases ranged from 7–9-fold and were usually evident within 3 h after IR (Figs. 2D and 4).

It is possible that the observed differences in the responses of induction of p53 and p21 following IR treatments between stromal and epithelial cells could be due to differences between subjects and may not reflect a physiological difference between the two cell types. To investigate this possibility, we examined the induction of p53 and p21 in stromal and epithelial cells that were isolated from the same prostatectomy specimen (F-BPH-8 and E-PZ-35). Induction of the levels of both p53 and p21 was greater than 5-fold in the F-BPH-8 cells, but induction of p53 was significantly attenuated (less than 2-fold) in the E-PZ-35 cells (Figs. 3 and 4). The lack of p53 and p21 induction in epithelial cells and the normal induction of these genes in stromal-derived samples from the same individual suggest that differences in the induction of the two proteins following IR are due to an underlying cell type-specific variation and not to intersample differences.

As mentioned above, accumulation of p53 and subsequently of p21 levels following IR results in a cell cycle arrest at G₁-S. We investigated whether the lack of p53 and p21 induction in prostatic epithelial cells correlated with a lack of a G₁-S cell cycle checkpoint. To address this question, we examined the effect of IR on cell cycle distribution in epithelial and stromal cells by FACS analysis of DNA content and DNA synthesis. Four of five epithelial strains analyzed in this way failed to exhibit a G₁-S cell cycle arrest following IR (Fig. 5). Since IR also induces a G₂ block, a G₁-S block is often represented graph-
Fig. 3. Quantitation of levels of p53 protein in epithelial and stromal cells after IR. Quantitation of p53 induction was performed by densitometric scanning of films. Fold induction of p53-immunoreactive bands in epithelial strains (A) and stromal strains (B) is plotted against time after IR.

Fig. 4. Quantitation of levels of p21 protein in epithelial and stromal cells after IR. Quantitation of p21 induction was performed by densitometric scanning of films. Fold induction of p21-immunoreactive bands in epithelial strains and stromal strains after IR treatments is plotted against time after IR.

Table 1. The lack of cell cycle arrest in epithelial strains contrasted sharply with the G1-S arrest (as was evident by the increased G1-S ratio after IR) found in all four stromal strains examined and in the metastatic cell line LNCaP (Fig. 5). Table 1 summarizes the...
results of p53 and p21 induction and cell cycle analysis on all strains examined.

We have found significant differences in the p53 response of prostatic epithelial and stromal cells to IR. Our results lead us to hypothesize that a defective induction of p53 levels following DNA damage may be an underlying mechanism for the epithelial origin of prostate adenocarcinomas. Previous studies have demonstrated that the induction of p21 and cell cycle arrest are closely associated with the transcriptional enhancing activity of p53 that is activated by DNA damage (11, 13, 15, 16). We also found that the attenuated induction of p53 levels in epithelial cells correlated with low p21 levels and an almost absent G1-S cell cycle arrest following exposure to IR.

The mechanism of induction of p53 and p21 in prostatic epithelial cells may be influenced or regulated by cell interactions between fibroblastic and epithelial cells in vivo. Adult fibroblasts have been shown to influence the growth of epithelial cells both in vivo and in vitro by a secretion of factors that act as positive and negative regulators (28, 30, 31). This regulatory network between stromal and epithelial cells could involve defects in the pathway that is activated by DNA damage and is involved in the stabilization of the p53 protein. When we looked at the expression levels and activation of DNA-PK by IR, we found no significant differences between epithelial and stromal cells. This result argues against an involvement of DNA-PK in stabilizing p53 in prostate cells. However, it is still possible, but unlikely, that DNA-PK is involved, not in p53 stabilization, but in activation of p53 as a transcription factor.

In contrast to the results with DNA-PK, we found a significant difference in the levels of MAP kinases (ERK-1 and ERK-2) in epithelial cells compared to stromal cells. MAP kinases are ubiquitous proteins that are involved in the transduction of signals (growth, stress, and others) from the extracellular environment to the nucleus. These kinases are inducible by the same group of stresses that activate p53 and are strong candidates as modulators of p53 stabilization following stress (36, 40). We are currently investigating whether this difference between stromal and epithelial cells in the amounts of ERK-1 and ERK-2 corresponds to a similar disparity in the kinase activity of these proteins. If such a disparity does exist, and if MAP kinases are indeed involved in the in vivo phosphorylation of p53, then the lower level of expression of MAP kinases could explain the defective p53 induction in epithelial cells.

Patients with the hereditary disease AT show a markedly increased predisposition to cancer development. Kastan et al. (9) elegantly showed that the p53 induction mechanism in AT cells is defective; therefore, AT gene product(s) are likely required for the optimal induction of p53 levels following IR treatments. It is possible that defective activation of a kinase in the same pathway that activates p53 in AT in response to IR is involved in the lack of induction of p53 in prostatic epithelial cells. It should be noted that p53 stabilization after IR treatments could be due to a mechanism other than phosphorylation. Whatever the defect in the mechanism of p53 induction in prostatic epithelial cells, we believe that they can serve as useful tools for “dissecting” the pathway(s) that leads to p53 induction after treatments with DNA-damaging agents.

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5 C. Koumenis and A. J. Giaccia, unpublished observations.
References


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