Impairment of the DNA Repair and Growth Arrest Pathways by p53R2 Silencing Enhances DNA Damage–Induced Apoptosis in a p53-Dependent Manner in Prostate Cancer Cells

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Abstract

p53R2 is a p53-inducible ribonucleotide reductase that contributes to DNA repair by supplying deoxyribonucleotide triphosphates in response to DNA damage. In this study, we found that p53R2 was overexpressed in prostate tumor cell lines compared with immortalized prostatic epithelial cells and that the protein was induced upon DNA damage. We investigated the effects of p53R2 silencing on DNA damage in LNCaP cells (wild-type p53). Silencing p53R2 potentiated the apoptotic effects of ionizing radiation and doxorubicin treatment as shown by increased sub-G1 content and decreased colony formation. This sensitizing effect was specific to DNA-damaging agents. Comet assay and γ-H2AX phosphorylation status showed that the decreased p53R2 levels inhibited DNA repair. Silencing p53R2 also reduced the levels of p21WAF1/CIP1 at the posttranscriptional level, suggesting links between the p53-dependent DNA repair and cell cycle arrest pathways. Using LNCaP sublines stably expressing dominant-negative mutant p53, we found that the sensitizing effect of p53R2 silencing is mediated by p53-dependent apoptosis pathways. In the LNCaP sublines (R273H, R248W, and G245S) that have defects in inducing p53-dependent apoptosis, p53R2 silencing did not potentiate DNA damage–induced apoptosis, whereas p53R2 silencing was effective in a LNCaP subline (P151S) which retains the ability to induce p53-dependent apoptosis. This study shows that p53R2 is a potential therapeutic target that could be used to enhance the effectiveness of ionizing radiation or DNA-damaging chemotherapy in a subset of patients with prostate cancer. (Mol Cancer Res 2008;6(5):808–18)

Introduction

Carcinoma of the prostate is the most commonly diagnosed cancer and the second leading cause of cancer-related death in American men (1). Patients with localized disease are candidates for ionizing radiation therapy. Patients with metastatic carcinoma of the prostate are usually treated with androgen ablation therapy. After further disease progression, they may be treated with chemotherapy including docetaxel and doxorubicin (2, 3).

Ionizing radiation and doxorubicin are DNA-damaging agents that induce double-strand breaks in DNA. This leads to the activation of DNA damage checkpoints. One of the key proteins in these pathways is the tumor suppressor p53, which triggers cell cycle arrest and induces the repair of DNA damage, responses needed for cell survival, or alternatively, apoptosis (4-6). p53 exerts its functions mainly through transactivational activity, including the induction of CDKN1/p21, 14-3-3 proteins, and p53AIP1, PERP, and CD95 for apoptosis (12, 13). Considerable evidence indicates that the choice made by p53 to activate cell cycle arrest and DNA repair pathways or the apoptosis pathway after DNA damage is dependent on the extent of unrepaired or misrepaired double-strand breaks in the DNA (14, 15). Blocking the p53-induced DNA repair could therefore prove to be an efficient approach to enhancing the efficacy of DNA-damaging agents.

p53R2 is a p53-inducible ribonucleotide reductase small subunit 2 (hRRM2) homologue. It participates in p53-induced DNA repair by supplying nuclear deoxyribonucleotide triphosphates in response to various types of DNA damage caused by ionizing radiation, UV irradiation, and anticancer drugs (11, 16). Inactivation of p53R2 by antisense oligonucleotides, small interfering RNA (siRNA), or mutation sensitizes several types of cancer cells to DNA-damaging anticancer agents or to ionizing radiation (11, 16, 17). In the present study, we explored the effect of inhibiting p53R2 expression on sensitizing prostate cancer cells to DNA-damaging agents. We used RNA interference to silence p53R2 in LNCaP cells and found that this treatment sensitizes the cells to DNA-damaging agents. The
molecular mechanisms that underlie this sensitizing effect were identified. Silencing p53R2 not only blocked DNA repair in LNCaP cells, it also repressed the induction of p21. Because silencing p53R2 did not enhance cell killing after DNA damage in LNCaP sublines stably expressing dominant-negative mutant p53 that have defects in inducing p53-dependent apoptosis, this indicates that p53-dependent apoptosis pathways are required for p53R2-dependent sensitization of prostate cancer cells to be affected by DNA-damaging agents.

Results
Overexpression of p53R2 in Prostate Cancer Cell Lines
Increased expression of the basal levels of p53R2 protein was observed in prostate cancer cell lines including LNCaP (wild-type p53), DU145 (mutant p53), CWR22rv1 (mutant p53), and PC3 (effectively p53-null) compared with that in immortalized, nonmalignant prostate epithelial cells (PZ-HPV-7 and RWPE-1; Fig. 1A). The high expression levels of p53R2 in PC3 and DU145 cells indicates that p53 is not required for its basal expression.

Induction and Nuclear Translocation of p53R2 upon DNA-Damaging Treatments in LNCaP
Cells were treated with 10 Gy of ionizing radiation or with 0.5 μg/mL of doxorubicin. Using these treatments, p53 and p21 were induced as anticipated (Fig. 1B, i). p53R2 protein was also stably induced in a time-dependent manner (Fig. 1B, i). Doxorubicin treatment (0.5 μg/mL) caused a stronger induction of p53 than that of 10 Gy of ionizing radiation. Likewise, p53R2 induction was stronger after doxorubicin treatment compared to treatment with 10 Gy of ionizing radiation (Fig. 1B, ii). In contrast, hRRM2 protein was transiently induced at 6 hours post-10 Gy or 12 hours post-doxorubicin treatment and then decreased sharply by 24 and 48 hours posttreatment.

Immunocytochemistry staining revealed that in untreated cells, p53R2 was mainly localized in the cytoplasm, whereas after 10 Gy of ionizing radiation, it had translocated into the nucleus within 1 hour (Fig. 1C). To determine whether p53R2 protein induction is p53-dependent, PC3 cells were treated with 10 Gy of ionizing radiation or 0.5 μg/mL of doxorubicin. In this cell line, p53R2 levels remained unchanged, indicating that p53 is required for p53R2 induction (Fig. 1D).

In vitro Inhibition of Ionizing Radiation or Doxorubicin-Induced p53R2 Induction by siRNA in LNCaP
Anti-p53R2 siRNA successfully down-regulated p53R2 protein to 20% of its basal level (Fig. 2). Silencing p53R2 prevented the induction of p53R2 after treatment with 10 Gy of ionizing radiation or 0.5 μg/mL of doxorubicin (Fig. 2). The
same treatments induced p53R2 expression in LNCaP cells that had been transfected with a control siRNA. Because hRRM2 has an 80% sequence homology to p53R2, we analyzed the protein levels of hRRM2. Treatment with anti-p53R2 siRNA did not reduce the expression of hRRM2, indicating that the anti-p53R2 siRNA is p53R2-specific (Fig. 2).

p53R2 Silencing Increases Ionizing Radiation- and Doxorubicin-Induced Apoptosis and Decreases Colony Formation

In LNCaP cells, treatment with ionizing radiation alone did not effectively induce apoptosis (3.04 ± 0.55% versus 0.91 ± 0.35% in untreated controls) at 48 hours post-ionizing radiation. In contrast, it caused a strong G2-M arrest (39.29 ± 0.37% versus 17.95 ± 0.25% in untreated controls; Fig. 3A). Although LNCaP cells that had been transfected with the anti-p53R2 siRNA had a moderate increase in apoptosis (sub-G1 content, 4.66 ± 0.66%), the combined treatment of these cells with 10 Gy of ionizing radiation and anti-p53R2 siRNA further enhanced their sub-G1 content to 24.08 ± 1.66%, a ~5-fold increase over the control siRNA-treated cells that were subjected to the same combined treatment (4.83 ± 0.32%; Figs. 3A and 7B, i). The combination treatment of 10 Gy ionizing radiation and anti-p53R2 siRNA also caused a significant reduction in the percentage of cells in the G2-M phase (16.56 ± 2.19%) compared with the control (30.70 ± 0.71%). To confirm the occurrence of apoptotic events at the molecular level, PARP-1 cleavage was examined. As shown in Fig. 3C, increased PARP-1 cleavage was observed in the combined anti-p53R2 siRNA and 10 Gy treatments. To determine the long-term effects of the combined treatment with ionizing radiation and p53R2 silencing, colony assays were done using two dosages of ionizing radiation (2 and 4 Gy). The control LNCaP cells were treated with ionizing radiation and the control siRNA. Treatment with anti-p53R2 siRNA and 2 or 4 Gy of ionizing radiation reduced colony formation to 46 ± 1.3% (2 Gy; P < 0.0005) and 36 ± 1.2% (4 Gy; P < 0.0005) of their controls (Fig. 3B, i).

To determine whether silencing of p53R2 sensitizes LNCaP to DNA-damaging agents, LNCaP cells were treated with a combination of doxorubicin and anti-p53R2 siRNA. Although 0.5 μg/mL of doxorubicin induced apoptosis in LNCaP cells (18.18 ± 0.79% versus 0.91 ± 0.35% in untreated controls) at 48 hours posttreatment, the combination of doxorubicin and anti-p53R2 siRNA further enhanced their sub-G1 content by ~2-fold (55.29 ± 4.31%) compared with doxorubicin combined with the siRNA control (25.72 ± 2.91%; Figs. 3A and 7B, ii). PARP-1 cleavage analysis showed increased cleavage in the combined anti-p53R2 siRNA and doxorubicin treatment (Fig. 3C). Colony formation assays showed that treatment with anti-p53R2 siRNA and 0.01 or 0.05 μg/mL of doxorubicin reduced the number of colonies to 57 ± 2% (P < 0.03) and 46 ± 0.6% (P < 0.0002), of their respective controls (Fig. 3B, ii). We conclude, therefore, that silencing p53R2 expression sensitizes LNCaP cells to DNA-damaging agents.

To investigate whether this sensitizing effect is specific to DNA-damaging agents, LNCaP cells were treated with a combination of 4 nmol/L of docetaxel, an antimicrotubule agent, and anti-p53R2 siRNA. Docetaxel used alone or combined with the siRNA control induced strong apoptosis (~40.21% and ~42.11%, respectively) compared with the untreated control (Fig. 3A). However, in combination with anti-p53R2 siRNA, docetaxel treatment resulted in a smaller sub-G1 content (~22.42%; Fig. 3A). We conclude, therefore, that silencing p53R2 antagonizes, rather than sensitizes, LNCaP cells to docetaxel. The probable explanation of this effect is that silencing p53R2 induces G1 arrest, thereby preventing cells from entering G2 where docetaxel exerts its major antimicrotubule effects. Taken together, our data indicate that silencing p53R2 sensitizes LNCaP cells to DNA-damaging agents but not to chemotherapeutic agents that use alternative mechanisms to induce cell death.

Silencing p53R2 Impairs DNA Repair in LNCaP

Previous studies reported that upon DNA damage, p53R2 complexes with the large M1 subunit of ribonucleotide reductase, forming a holoenzyme that plays an important role in supplying deoxynucleotide triphosphates for DNA repair (18, 19). We anticipated that silencing p53R2 would inhibit DNA repair, resulting in an increased number of double-strand breaks after ionizing radiation or doxorubicin treatment. LNCaP cells were treated using a combination of 10 Gy of ionizing radiation and the anti-p53R2 siRNA. At 2 and 6 hours post-radiation, the phosphorylation levels of H2AX (i.e., γ-H2AX), an indicator of DNA double-strand breaks, were strongly enhanced in the “10 Gy + anti-p53R2 siRNA” treatment group compared with the “10 Gy + siRNA control” treatment group (Fig. 4A). A complementary immunocytochemical assay was used to detect γ-H2AX levels after the same treatments. We found that the combination treatment of 10 Gy ionizing radiation and anti-p53R2 siRNA enhanced γ-H2AX mean fluorescence in
these cells at both the 2-hour (49.94 ± 1.92 versus 37.77 ± 1.56; 
P < 0.0001) and the 6-hour (30.54 ± 1.30 versus 17.57 ± 1.39; 
P < 0.0001) time points following ionizing radiation (Fig. 4B, 
i and ii).

To further confirm that silencing of p53R2 impairs DNA 
repair, we used a neutral comet assay that detects double-strand 
breaks in the DNA of single cells. This assay was done at 0, 0.5, 
1, and 2 hours post-10 Gy ionizing radiation. Tail moments, 
accounting for both the amount of broken DNA that migrates 
from the nucleus and the distance traveled, were analyzed in a 
minimum of 50 single cells per treatment. LNCaP cells treated 
with 10 Gy and either anti-p53R2 siRNA or the siRNA control 
showed a maximum of mean tail moment at 0.5 hours post-
10 Gy ionizing radiation. There was a higher mean tail moment 
in the 10 Gy + anti-p53R2 siRNA group (12.54 ± 0.74 versus 
7.28 ± 0.51, P < 0.0001; Fig. 4C, i and ii). The mean tail 
moment was reduced at 1 and 2 hours post-radiation, with the 
10 Gy + anti-p53R2 siRNA group continuing to have higher 
mean tail moments than the control group (11.59 ± 0.90 versus 
5.67 ± 0.31 and 5.32 ± 0.52 versus 1.04 ± 0.10, respectively, 
P < 0.0001; Fig. 4C, i and ii). These data indicate that 
inhibition of p53R2 disrupts DNA repair kinetics in LNCaP 
cells, underscoring the importance of p53R2 in p53-mediated 
DNA repair.

Silencing p53R2 Represses p21 Induction after DNA 
Damage in LNCaP

A recent study indicated that p53R2 interacts physically with 
p21 and facilitates p21-induced G1 arrest after UV irradiation 
(20). Because of this cross-talk between p53R2 and p21, we 
investigated the response of p21 to the silencing of p53R2 in 
LNCaP cells. The protein levels of p21 were examined by 
Western blotting analysis in cells treated with a combination of 
anti-p53R2 siRNA and either 10 Gy of ionizing radiation or 
0.5 μg/mL of doxorubicin compared with cells treated with 
the combined siRNA control and ionizing radiation or doxo-
rubin (controls). We found that p21 induction was severely 
repressed over the time course of treatment in the cells treated

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FIGURE 3. Effects of p53R2 silencing on DNA damage in LNCaP cells. A. Representative cell cycle profiles of cells transfected with anti-p53R2 siRNA or 
control siRNA, and then treated with 10 Gy of ionizing radiation at 48 h posttransfection, or with 0.5 μg/mL of doxorubicin or 4 nmol/L of docetaxel at 
24 h posttransfection. Cells were fixed and subjected to flow cytometry at 48 h post-ionizing radiation or doxorubicin treatment. Apoptosis was measured by 
the sub-G1 content. Comparable results were obtained using Annexin V and propidium iodide staining (data not shown). B. Analysis of the colony formation 
ability by clonogenic assays. Cells were transfected with anti-p53R2 siRNA or control siRNA. At 24 h posttransfection, cells were replated into 100 mm dishes 
at concentrations of (i) 60,000 cells (2 Gy), 120,000 cells (4 Gy), or (ii) 30,000 cells (0.01 and 0.05 μg/mL doxorubicin). On the following day, the cells were 
treated with ionizing radiation or doxorubicin and then left in the culture for up to 14 days. Doxorubicin was washed out 24 h posttreatment (*, P < 0.05; ***, 
P < 0.0005). C. Analysis of PARP-1 cleavage. Cells were treated as described above and harvested for protein extraction at 24 h post-treatment.
with anti-p53R2 siRNA and 10 Gy of ionizing radiation or doxorubicin as compared with the controls (Fig. 5A, i; lanes 4-11). To investigate whether this inhibition of p21 induction occurred at the mRNA level, real-time PCR was carried out. Comparable levels of p21 mRNA induction after DNA damage were observed in LNCaP cells treated with either anti-p53R2 siRNA or with the siRNA control (Fig. 5A, ii; lane 4 versus 5; lane 6 versus 7; lane 8 versus 9; and lane 10 versus 11), suggesting that the inhibition of p21 might occur posttranscriptionally. On the other hand, the protein levels of p53R2 were not affected by p21 silencing. Similar levels of p53R2 induction were observed in LNCaP cells treated with either the combined DNA-damaging agents and anti-p21 siRNA or with the control siRNA (Fig. 5B). We therefore conclude that, in response to DNA damage, both p53R2 and p21 are subjected to regulation by p53 at the mRNA level whereas the stability of p21 protein is also subjected to regulation by p53R2. Taken together, these data indicate that the two p53 pathways, p53-induced DNA repair and p53-induced cell cycle arrest, are controlled in a coordinated manner. When p53-induced DNA repair is disrupted, this also affects the cell cycle arrest induced by p53.

To further investigate whether the sensitizing effect of p53R2 silencing is mediated through p21 suppression, LNCaP cells were treated with a combination of anti-p21 siRNA and 10 Gy of ionizing radiation or 0.5 μg/mL of doxorubicin. The anti-p21 siRNA successfully down-regulated p21 protein to its basal level after DNA damage (Fig. 5B). However, unlike p53R2 silencing, p21 silencing did not significantly increase the amount of apoptosis after either 10 Gy or 0.5 μg/mL doxorubicin treatment (Fig. 5C, i) at 48 hours post-ionizing radiation or doxorubicin. By flow cytometry, the sub-G1 content of the cells treated with anti-p21 siRNA and 10 Gy was 6.68 ± 1.89% (control, 4.83 ± 0.32%; P = 0.2), whereas in the doxorubicin-treated cells it was 28.13 ± 2.40% (control, 25.72 ± 0.91%; P = 0.5). On the other hand, p21 silencing caused a significant

FIGURE 4. Impairment of DNA repair activity by p53R2 silencing in LNCaP cells. A, The phosphorylation of H2AX was detected using the antibody against γ-H2AX-ser 46. B, Representative immunocytochemistry images of γ-H2AX (i). At the indicated time points, cells were fixed and stained with the γ-H2AX-ser 46 antibody followed by FITC-conjugated secondary antibody (green). Analysis of the mean fluorescent intensity of γ-H2AX in cells treated with the combined anti-p53R2 siRNA and 10 Gy of ionizing radiation, or with the combined control siRNA and 10 Gy of ionizing radiation (ii). The mean fluorescent intensities were quantified using Adobe Photoshop software. At least 50 single cells were analyzed for each treatment. C, Representative images of cells with comet tails after 10 Gy of ionizing radiation (i). Analysis of the mean tail moment in cells treated with the combined anti-p53R2 siRNA and 10 Gy of ionizing radiation, or with the combined control siRNA and 10 Gy of ionizing radiation (ii). The mean tail moments were quantified using CometScore software. At least 50 single cells were analyzed for each treatment.
reduction of the percentage of cells in the G2-M phase 48 hours post-10 Gy of ionizing radiation (19.06 ± 1.04% versus 30.70 ± 0.71% in control, \( P = 0.002 \); Fig. 5C, ii). This ablation of G2-M arrest was also observed when cells were treated with anti-p53R2 siRNA and 10 Gy of ionizing radiation (Fig. 5C, ii). Therefore, the enhanced DNA damage–induced apoptosis by p53R2 silencing is coupled with the abolishment of G2-M arrest, and this abolishment of G2-M arrest is regulated through p21 suppression by p53R2 silencing.

The Sensitizing Effect of p53R2 Silencing Is Mediated by p53-Dependent Apoptosis Pathways

Because p53R2 is a p53-regulated protein, we examined the effects of the combined treatment of p53R2 silencing and DNA-damaging agents on LNCaP variants expressing mutant p53 alleles. Four LNCaP sublines that were stably transfected with dominant-negative mutant p53 (G245S, R248W, R273H, and P151S) were examined for their response to the combined treatments. In our previous studies, we reported that G245S, R248W, and R273H were also gain-of-function (GOF) mutants, whereas P151S is a loss-of-function (LOF) mutant (21-23). Although the p53 basal levels were higher in these mutant sublines, no significant differences in p53R2 basal levels were observed among them (Fig. 6A). The induction of p53R2 was observed in all four sublines after DNA damage (Fig. 6B). We speculated that the induction of p53R2 in these sublines was due to their residual endogenous wild-type p53 activity. To test this hypothesis, we used a siRNA targeting the 3' untranslated region of p53 to knock down endogenous wild-type p53 expression but not ectopically introduced expression (which lacks the 3' untranslated region) in the R273H and P151S sublines. When the endogenous wild-type p53 was downregulated, the induction of p53R2 was suppressed after doxorubicin treatment (Fig. 6C), indicating that induction of p53R2 in these sublines could be attributed to their endogenous content of wild-type p53.

Previous reports have shown that p53 GOF mutations may confer therapeutic resistance (24, 25). Flow cytometry was used to assess the sensitivity of the four LNCaP sublines to ionizing radiation or doxorubicin treatment alone. Judging the extent of

**FIGURE 5.** Repression of p21 induction by p53R2 silencing after DNA damage in LNCaP cells. A. Cells were transfected with anti-p53R2 siRNA or control siRNA, and then treated with 10 Gy of ionizing radiation at 48 h posttransfection, or with 0.5 \( \mu \)g/mL of doxorubicin at 24 h posttransfection (i). Cells were harvested at 12 and 24 h post-ionizing radiation or doxorubicin treatment and subjected to Western blotting. Cells were treated and harvested as mentioned above. Real-time PCR was done to detect the induction of p21 mRNA (ii). B. The anti-p21 siRNA down-regulated p21 to its basal level. Cells were transfected with 100 nmol/L of anti-p21 siRNA or control siRNA. Transfected cells were treated with 10 Gy of ionizing radiation at 48 h posttransfection, or with 0.5 \( \mu \)g/mL of doxorubicin at 24 h posttransfection. Cells were then harvested for Western blotting at 24 h post-ionizing radiation or doxorubicin treatment. C. Analysis of apoptotic cells by sub-G0 content (i). Cells were treated as above, fixed, and subjected to flow cytometry at 48 h after ionizing radiation or doxorubicin treatment. Unpaired Student’s t test was done between the combined control and the combined treatment. p21 silencing did not significantly increase the sub-G0 content after DNA damage, whereas p53R2 silencing significantly enhanced the sub-G0 content. Analysis of the G2-M cell cycle (ii). Both p21 silencing and p53R2 silencing caused significant reductions of the percentage of cells in the G2-M phase (***, \( P < 0.0005 \)).
apoptosis by the sub-$G_1$ content and by analysis of the clonogenic assays, the G245S, R248W, and R273H mutant p53 sublines were resistant to ionizing radiation or doxorubicin treatment compared with their parental LNCaP cells (data not shown). The expression of Bax, a p53 target that promotes apoptosis, was examined in the parental LNCaP and mutant sublines. Although the mutant sublines showed enhanced Bax basal levels compared with the parental LNCaP (Fig. 7A), they also had enhanced Bcl2 basal levels (data not shown). After DNA damage, Bax was induced in the parental LNCaP cells but not in the three mutant sublines (Fig. 7A). The resistance of these GOF mutants to anticancer treatment can therefore be attributed, in part, to defects in inducing p53-dependent apoptosis. To determine whether the silencing of p53R2 sensitizes these mutant p53 sublines to DNA damage, they were transfected with anti-p53R2 siRNA followed by 10 Gy of ionizing radiation or 0.5 $\mu$g/mL of doxorubicin. Although anti-p53R2 siRNA was able to inhibit p53R2 induction as efficiently in these sublines as in LNCaP parental cells (data not shown), silencing p53R2 did not enhance apoptosis (Fig. 7B, i and ii) nor did it reduce colony-forming potential (data not shown), indicating that p53-dependent apoptosis pathways are required for the sensitizing effect of p53R2 silencing.

Interestingly, the LOF mutant (P151S) exhibited a different pattern of susceptibility to ionizing radiation or doxorubicin than the GOF mutants. Although p151S cells were as resistant to ionizing radiation as the GOF mutants, their susceptibility to doxorubicin was similar to that of the parental LNCaP cells. When P151S cells were treated with combined p53R2 silencing and DNA-damaging agents, silencing p53R2 did not sensitize P151S to ionizing radiation but it enhanced doxorubicin-dependent apoptosis by $\sim 1.5$-fold (Fig. 7B, ii). Reasoning that the doxorubicin-induced apoptosis was mediated by p53-dependent apoptosis pathways, we transfected P151S cells with anti-p53 siRNA to knock down the expression of the endogenous wild-type p53. As we anticipated, down-regulation of wild-type p53 abolished the sensitivity of P151S cells to doxorubicin treatment (Fig. 7C). These results show that in mutant p53-containing cells that retain active p53-dependent apoptosis pathways, silencing p53R2 can effectively sensitize cells to DNA-damaging agents.

**Discussion**

Ribonucleotide reductase is the enzyme that catalyzes the conversion of ribonucleotide diphosphates to their corresponding deoxyribonucleotides, the precursors of deoxynucleotide triphosphate that are required for DNA synthesis and repair (26). Thus far, three ribonucleotide reductase subunits have been found in humans: M1, M2, and p53R2. M1 is the large subunit, whereas M2 and p53R2 are small subunits that share 80% sequence homology. M1 interacts with either M2 or p53R2 to become the catalytically active form of eukaryotic ribonucleotide reductase (18). It is well established that the M1-M2 complex provides deoxynucleotide triphosphate pools for normal DNA replication in proliferating cells (26). This enzyme activity is tightly regulated by the cell cycle–specific availability of the M2 subunit, whereas the M1 protein level remains relatively constant throughout the cell cycle (27). It has been proposed that repair of DNA damage is dependent on the M1-p53R2 holoenzyme rather than on the M1-M2 enzyme (11, 16, 18). Our data show that the M2 protein levels are dramatically reduced when cells enter $G_1$ or $G_2$ arrest after DNA damage, whereas p53R2 is stably induced at those cell cycle stages. This is consistent with the suggestion that M1-p53R2 is the main enzyme that provides deoxynucleotide triphosphate pools for DNA repair during cell cycle arrest. In recent years, several ribonucleotide reductase inhibitors, including the M1 inhibitors triapine, gemcitabine and GTI-2501, and the M2 inhibitor GTI-2040 have entered clinical trials (28-33). Targeting p53R2 for therapeutic intervention would be advantageous because this approach would avoid the undesirable side effects caused by the disruption of normal DNA synthesis in noncancerous cells.
Several studies have suggested a role for p53R2 in tumor progression and resistance to chemotherapy and radiation. Uramoto et al. reported that p53R2 expression detected by immunohistochemistry in NSCLC patients was correlated with the pathologic stage of their disease (34). p53R2 expression levels also correlated with disease progression and resistance to chemoradiation in esophageal squamous cell carcinoma (35). However, in human gastric carcinoma or cell lines derived from this cancer, p53R2 expression levels were not significantly different from those of normal tissues (36). In this study, we found that p53R2 expression was significantly elevated in prostate cancer cell lines compared with immortalized, nontumorigenic prostate epithelial cell lines.

In previous studies of cells having a p53-dependent DNA damage checkpoint, it was reported that inhibition of p53R2 expression led to reduced ribonucleotide reductase activity and reduced cell survival after exposure to various genotoxins (11, 16, 17, 36, 37). Kimura et al. noted that p53R2 knockout mice showed severe renal failure, growth retardation, and early mortality (37). They found that p53 was stabilized and the expression of several p53 targets including Bax, Pmaip, and Trp53inp1 were elevated in kidney, indicating the activation of p53-dependent apoptosis pathways. Our studies suggest a possible mechanism to explain this phenomenon. Using γ-H2AX analysis and comet assays, we showed that silencing p53R2 in LNCaP cells led to reduced DNA repair after radiation treatment. Impairment of the DNA repair pathway prolongs the DNA damage signal, resulting in continued activation of the p53-dependent apoptosis pathways. We found that apoptosis was enhanced and colony formation was reduced in LNCaP cells treated with a combination of p53R2 silencing and radiation.

There is increasing evidence showing that, after DNA damage, p21 mediates cell cycle arrest allowing time for DNA repair and protecting cells from p53-dependent apoptosis.
Several p53 point mutants that are unable to transactivate the p21 gene are more potent inducers of apoptosis than wild-type p53 to treatment with ionizing radiation (38, 39), UV (40), or doxorubicin (41). Other studies have reported that over-expression of p21 protects cells from chemotherapy-induced cytotoxicity (42, 43). In the present study, we found that after DNA damage, p53R2 silencing in LNCaP cells showed repressed p21 induction and reduced G2-M arrest. Therefore, the result of p53R2 inhibition is not only retardation of DNA damage repair and activation of p53-dependent apoptosis but also the elimination of p53-mediated G2-M growth arrest induced by p21. Using anti-p21 siRNA, we found that abolishment of G2-M arrest alone did not enhance DNA damage–induced apoptosis in LNCaP cells. Therefore, silencing p53R2 has a more potent effect than silencing p21, suggesting that targeting p53R2 may be a better strategy because p53R2 silencing exerts a dual effect and thereby promotes entry into p53-mediated apoptosis pathways. The mechanism of p21 inhibition by p53R2 has not yet been well characterized. Xue et al. reported that p53R2 physically interacts with p21 and facilitates p21 induction of G1 arrest upon UV radiation. They observed considerably less nuclear accumulation of p21 in MEF p53R2−/− cells compared with MEF p53R2+/+ cells (20). Our study complements this report and supports the concept that there is a biochemical link between the p53-dependent cell cycle arrest and DNA repair pathways that coordinates the cellular response to DNA damage. The result from this study warrants further investigation.

Because p53 is frequently mutated in a majority of human tumors, we investigated the effect of p53R2 silencing in LNCaP sublines containing mutant p53. We found that the sensitizing effect of p53R2 silencing on DNA damage–mediated apoptosis is mediated by p53-dependent apoptosis pathways. It is noteworthy that p53R2 does not effectively sensitize LNCaP sublines that stably express the G245S, R248W, and R273H p53 mutants to DNA damage–induced apoptosis. These GOF mutants promote cell survival due to defects in the induction of the proapoptotic gene (Bax) and suppression of an antiapoptotic gene (Bcl2; ref. 21). Additionally, they have acquired new properties that include androgen-independent growth, tumorigenicity, and transactivation of MDR-1 or PCNA, genes which are not regulated by wild-type p53 (21-23). In contrast, the cells expressing the LOF p53 mutant were as sensitive to doxorubicin treatment as the parental LNCaP cells. Using siRNA targeting the endogenous p53, we found that the endogenous wild-type p53 played a major role in mediating the doxorubicin-induced apoptosis, which indicates that these cells retain the ability in inducing p53-dependent apoptosis. Consequently, these cells were effectively sensitized to doxorubicin-induced apoptosis by p53R2 silencing. Our present study thus supports the concept that inhibition of p53R2 may prove useful for adjuvant treatment of patients who have prostate tumors that retain wild-type p53 activity.

Materials and Methods

Cell Lines and Cell Culture

Prostate cancer cell lines (LNCaP, CWR22rv1, DU145, and PC3) and prostate immortalized cells (RPWE-1 and PZ-HPV-7) were acquired from the American Type Culture Collection. Cells (except PZ-HPV-7) were maintained in RPMI medium supplemented with 10% fetal bovine serum, 1-glutamine, vitamins, and penicillin and streptomycin. PZ-HPV-7 cells were maintained in keratinocyte-SFM medium. LNCaP sublines stably expressing the p53 mutant alleles (R248W, R273H, G245S, and P151S; ref. 21) were grown in the same medium as the parental cells with the addition of 500 mg/mL of G418 antibiotic (Life Technologies) to maintain selection for cells containing the transfected vectors that contain a neomycin resistance gene (neo*) in addition to the genes being tested. Cells were incubated at 37°C in 95% air/5% CO2.

Treatments

For radiation treatments, cells were irradiated using a 6 MeV linear accelerator (Varian) at room temperature in the Department of Radiation Oncology at the UC Davis Cancer Center. Doses ranging from 2 to 10 Gy were delivered at a dose rate of 2.3 Gy/min. The cells were returned to an incubator after the irradiations and maintained at 37°C until further use. For drug treatments, doxorubicin and docetaxel were diluted immediately prior to treatment.

Western Blotting

Cell pellets were lysed in radioimmunoprecipitation assay buffer with freshly added inhibitors (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and 10 μg/mL apro- tinin). Soluble proteins were then quantitated using a modified Bradford analysis (Bio-Rad Laboratories). SDS-PAGE analysis was done using a minigel apparatus (Bio-Rad Laboratories). Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Inc.) at a constant 15 V for 1 h using semitransfer equipment (Bio-Rad Laboratories). Blots were incubated overnight at 4°C with the appropriate antibodies [anti-p53R2, anti-p21, and anti–PARP-1 (Santa Cruz Biotechnology); anti-p53 and anti-Bax (EMD Chemicals, Inc.); and anti–γ-H2AX (Cell Signaling Technology, Inc.)] followed by incubation with the appropriate secondary antibody in a 1:1,000 dilution for 1 h. After incubation with enhanced chemiluminescence detection reagents (Amersham) for 1 min, membranes were exposed to Kodak XAR films. β-Actin levels were used as a standard to evaluate equivalent protein loading. Band intensity was quantified using Quantity One software.

Transient Transfection

For analysis of RNA interference–mediated suppression of gene expression, 3 × 105 LNCaP cells were seeded into 60-mm culture dishes. Cells were transfected 24 h after plating in serum-containing medium (without antibiotics) with 100 nmol/L of anti-p53R2 siRNA, anti-p21 siRNA, anti-p53, or control siRNA (Dharmacon) using LipofectAMINE2000 Reagent (Invitrogen Life Technologies) according to the protocol described by the manufacturer.

Flow Cytometry

Cells were trypsinized, washed with PBS, resuspended in 70% ethanol, and incubated at 20°C overnight. Cells were then
centrifuged, washed in PBS, resuspended in 450 μL of PBS and 10 μL of DNase-free RNase (10 μg/mL; Roche) and then incubated at 37°C for 45 min. Following RNase treatment, 50 μL of propidium iodide (Boehringer Mannheim Corp.) was added. Cells were incubated at room temperature for 10 min and protected from light. Cell aggregates were removed by filtration prior to analysis. Cell cycle analysis was done on the Coulter Epics XL flow cytometer (Beckman Coulter). The percentages of cells in each of the sub-G1, G1, S, and G2 phases were determined on 50,000 cells with doublet discrimination. Analysis of cell cycle position was done using the Phoenix Multicycle software (Phoenix Flow Systems).

**Clonogenic Assay**

For the combined treatment of anti-p53R2 siRNA and DNA damage, cells were transfected as described above, then replated at 24 h posttransfection as single cells (60,000 cells for 2 Gy ionizing radiation; 120,000 cells for 4 Gy ionizing radiation; 30,000 cells for 0.01 or 0.05 μg/mL doxorubicin treatment) into 100 mm culture dishes. At 24 h after replating, cells were irradiated or treated with doxorubicin at the desired doses. The medium containing doxorubicin was washed out 24 h after the treatment. Cells were cultured for approximately six doubling times. Colonies were fixed and stained with 1% crystal violet in 0.5% glacial acetic acid in ethanol. Colonies containing approximately 50 or more cells were counted.

**Immunocytochemistry**

LNCaP cells were plated on polylysine-coated, four-well chamber slides (3,000 cells/chamber). After treatment, cells were fixed in 4% paraformaldehyde in PBS and rendered permeable by treatment for 3 min with 0.1% Triton X-100 in PBS. The cells were covered with blocking solution (1% bovine serum albumin and 0.01% Tween 20 in PBS) for 1 h at room temperature and incubated overnight at 4°C with anti-p53R2 or γ-H2AX antibody diluted 1:500 in blocking solution. The slides were then stained in the dark with secondary antibodies conjugated to FITC, washed with PBS, and viewed with an Olympus BX61 fluorescent microscope.

**Neutral Comet Assay**

Residual DNA damage following irradiation or mock-irradiation was detected using the Trevigen CometAssay Single Cell Gel Electrophoresis Assay following the manufacturer’s protocol (Trevigen). Briefly, treated cells were gently scraped using a rubber policeman, counted, and then pelleted by centrifugation. The cells were washed in ice-cold 1× PBS (Ca²⁺-,Mg²⁺-free) and resuspended in ice-cold medium at a concentration of 1 × 10⁵ cells/mL. The cell suspension was mixed 1:10 v/v with low-melting point agar at 42°C, and immediately pipetted onto the CometSlide area. Slides were incubated at 4°C in the dark for 30 min followed by immersion in chilled lysis solution and then by a second incubation at 4°C for 30 min. Slides were placed in a horizontal electrophoresis chamber and electrophoresed at 1 V/cm for 18 min. Samples were dried and stained with SYBERGreen. Comet tails were imaged by an Olympus BX61 fluorescent microscope and quantitated by CometScore software. A minimum of 50 cells were scored per treatment.

**Real-time PCR**

Total cellular RNA was prepared from LNCaP cells using TRIZOL reagent (Invitrogen Life Technologies). cDNA was synthesized from 1 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Promega Corporation) primed with random hexanucleotide primers under conditions of 23°C for 15 min, 42°C for 30 min, and 95°C for 5 min. cDNAs were diluted 1:4 in double-distilled water and 5 μL of diluted cDNA was added to 20 μL of PCR mixture containing SYBR Green supermix (Bio-Rad Laboratories) and 200 nmol/L of each primer. β-Actin was used as the endogenous expression standard. PCR conditions were a 3-min initial denaturation step at 95°C, 45 cycles at 95°C for 30 s, and 60°C for 1 min followed by an additional 95 cycles starting at 52°C with a 0.5°C increase per cycle for melt curve analysis. Primer sequences were for p21 forward, 5’-GAG CGA TGG AAC TTC GAC TTG G-3’ and reverse, 5’-GCC TTC CTC TTC TTG GAG AATC AG-3’; and for β-actin forward, 5’-GAG CGC GGC TAC AGC TT-3’ and reverse, 5’-TCC TTA ATG TCA CGC ACG ATT T-3’. Data was collected by the i-Cycler (Bio-Rad Laboratories) and analyzed using QGene software.

**Statistical Analysis**

Experiments were repeated independently at least three times. When appropriate, Student’s t test using GraphPad Prism software was conducted to determine whether the treatment group was statistically significant compared with the control (*, P < 0.05; **, P < 0.005; ***, P < 0.0005).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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