Defective DNA Strand Break Repair after DNA Damage in Prostate Cancer Cells:
Implications for Genetic Instability and Prostate Cancer Progression

Rong Fan,1 Tirukalikundram S. Kumaravel,1 Farid Jalali,1 Paula Marrano,1 Jeremy A. Squire,1,3,4 and Robert G. Bristow1,2,4

1Ontario Cancer Institute/Princess Margaret Hospital, University Health Network; and Departments of 2Radiation Oncology, 3Pathology, and 4Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

Together with cell cycle checkpoint control, DNA repair plays a pivotal role in protecting the genome from endogenous and exogenous DNA damage. Although increased genetic instability has been associated with prostate cancer progression, the relative role of DNA double-strand break repair in malignant versus normal prostate epithelial cells is not known. In this study, we determined the RNA and protein expression of a series of DNA double-strand break repair genes in both normal (PrEC-epithelial and PrSC-stromal) and malignant (LNCaP, DU-145, and PC-3) prostate cancer cell cultures. Expression of genes downstream of ATM after ionizing radiation-induced DNA damage reflected the p53 status of the cell lines. In the malignant prostate cell lines, mRNA and protein levels of the Rad51, Xrcc5, Rad52, and Rad54 genes involved in homologous recombination were elevated 2–5-fold in comparison to normal PrEC cells. The XRCC1, DNA polymerase-β and -δ genes were also elevated. There were no consistent differences in gene expression relating to the nonhomologous end-joining pathway. Despite increased expression of DNA repair genes, malignant prostate cancer cells had defective repair of DNA breaks, alkali-labile sites, and oxidative base damage. Furthermore, after ionizing radiation and mitomycin C treatment, chromosomal aberration assays confirmed that malignant prostate cells had defective DNA repair. This discordance between expression and function of DNA repair genes in malignant prostate cancer cells supports the hypothesis that prostate tumor progression may reflect aberrant DNA repair. Our findings support the development of novel treatment strategies designed to reinstate normal DNA repair in prostate cancer cells.

INTRODUCTION

The genetic determinants of prostate tumor progression are still poorly understood (1). However, numerous models of prostate carcinogenesis suggest that increasing chromosomal instability with the acquisition of mutations and chromosomal aberrations drives progression from preneoplasia to neoplasia (2). Furthermore, increased levels of chromosomal aberrations can be associated with decreased telomere length in high-grade prostate intraepithelial neoplasia, and acquired centrosome dysfunction is associated with prostate cancer progression and dissemination (3, 4). Human cells have therefore evolved complex signaling responses to both endogenous and exogenous DNA damage to preserve genomic integrity. Tumor progression in a number of epithelial malignancies has been associated with the sequential loss of function of genes that normally protect against DNA damage. For example, in response to DNA double-strand breaks, the ATM (ataxia telangiectasia mutated) protein is activated and stabilizes the p53 tumor suppressor protein. This leads to the up-regulation of p53-dependent genes (e.g., p21WAF1, Bax, and Gadd45) and posttranslational modifications of the CHK2, BRCA1, and NBS1 proteins. These genes act together to induce G1, S, and G2 cell cycle arrest; DNA repair; and/or activation of cell death pathways (e.g., apoptosis, mitotic catastrophe, or terminal growth arrest) depending on the cellular context (5–7).

Defective DNA repair as a determinant of prostate cancer progression has not been extensively studied. Several groups have observed defective mismatch-repair in prostate cancer cells (8, 9). Other data support DNA polymorphisms in the Xrcc1, Ogg1, and DNA polymerase-β genes (involved in base excision repair or DNA single-strand break repair) as risk factors for prostate cancer (8, 10, 11). However, data are lacking concerning the homologous recombination and nonhomologous recombination (i.e., end-joining) pathways, which are involved in the repair of DNA double-strand breaks.

Nonhomologous end-joining repair requires little or no homology on the ends of the strands being joined and involves two main discrete repair protein complexes (1): the DNA-PK/XRCC4/LigIV complex and (2) the MRE11/RAD50 complex (12). In homologous recombination, extensive homology is required between the region of the DNA double-strand break and the sister chromatid or homologous chromosome from which repair is directed. Homologous recombination involves the BRCA2, RAD51, RAD52, RAD54, RAD55–57, and RPA proteins and the RAD51 paralogs, XRCC2/3 and RAD51B/C/D. The homologous recombination pathway predominates in the late S/G2 phases of the cell cycle and provides relatively error-free repair. In contrast, the nonhomologous end-joining pathway predominates in the G1 phase of the cell cycle (13). Recent data suggest an interplay between the two pathways dependent on cell type and initial versus late times after induction of DNA double-strand breaks (11). Cells defective for either homologous recombination or nonhomologous end-joining show increased rates of mutagenesis and chromosomal instability, which could relate to the propensity for acquired genetic instability during prostate carcinogenesis and tumor progression. Consistent with a possible role for DNA double-strand break repair in prostate cancer progression, BRCA1 and BRCA2 mutations are associated with an increased risk of prostate cancer and development of an aggressive disease course (2, 13–15).

Our laboratory has shown previously that normal and malignant prostate cells preferentially respond to DNA damage by undergoing terminal growth arrest rather than apoptosis (16). This may allow for attempted DNA repair during cell cycle arrests as a response to DNA damage. However, in malignant cells with aberrant cell cycle checkpoint control, defective DNA double-strand break repair could increase genetic instability as part of a “mutator” phenotype (17). We hypothesized that one of the critical steps in prostate tumor progression may be the loss of the normal repair response to DNA damage and that specific defects in DNA double-strand break repair would be associated with prostate malignancy.

Herein, we show that malignant prostate cancer cells have increased
expression of homologous recombination-related and base excision repair-related genes independent of p53 status, G1 cell cycle checkpoint control, and relative cell proliferation. However, despite expressing high levels of DNA repair proteins, malignant cells have a decreased capacity for DNA double-strand break, DNA single-strand break, and base excision repair and acquire discrete chromosomal aberrations after exposure to DNA-damaging agents. Our findings support inappropriate DNA repair as a potential determinant of prostate cancer progression.

MATERIALS AND METHODS

Prostate Cell Cultures and DNA-Damaging Treatments. All of the cell cultures were incubated in vented tissue culture flasks under 5% CO2 and 37°C culture conditions as described previously (16). LNCaP cells were maintained in T-media (Life Technologies, Inc., Gaithersburg, MD) and supplemented with 10% fetal calf serum. PC-3 and DU-145 cells were purchased from American Type Culture collection (Manassas, VA) and supplemented with 10% fetal calf serum in Ham’s F12K or α-Modified Eagles Medium, respectively. PtEC (normal prostate epithelial cells) and PrSC (normal prostate stromal cells) were purchased from Clonetics Corporation (San Diego, CA) and maintained using suggested PrEGM and SGCM medium, respectively. The latter cell cultures have limited proliferative potential in culture and decrease in proliferation after passages 5 to 8 from frozen stock. Approximate doubling times for cell cultures under these conditions were as follows: PtEC, 48 to 72 hours; PrSC, 18 hours; LNCaP, 36 hours; PC-3, 24 hours; and DU-145, 18 hours (16).

Asynchronous cultures were irradiated, or treated with mitomycin C, at 16 to 20 hours after plating to reduce the immediate effects of trypanosin. Cells were either mock irradiated or irradiated with 0 to 10 Gy under aerobic conditions using a 137Cs irradiator at ~1 Gy/min at room temperature (16). Mitomycin C was prepared as a stock solution of 0.5 mg/mL in distilled water before each use.

Quantification of Gene Expression by RNase Protection Assays. Asynchronously growing cells were harvested at 70% to 80% growth confluence either from nonirradiated cultures or at 24 hours after irradiation. RNase protection assays were carried out as per the manufacturer’s instructions (BD Sciences-PharMingen, San Diego, CA). Total RNA was first extracted using Trizol reagent (Life Technologies, Inc.), Antisense riboprobes (Rad50, Mre11, Rad52, Rad54, Rad51, Xrec2, Xrec3, Rad51B, Rad51C, Rad51D, L32 and Gapdh from DBSR1 set; Atm, Nbs1, Xrec2, Xrec3, Xrec9, Ligase IV, Xrec4, Ku70, DNA-PK, Ku86, L32, and Gapdh from DBSR2 set; and Bcl-x, p53, Gadd45, c-fos, p21WAF, Bax, Bcl-2, Mcl-1, L32, and Gapdh from HSTRESS set) were synthesized with multiprobe template sets and purified by MAXIscript (Ambion Inc., Houston, TX). Five micrograms of sample RNA were hybridized with 2,000 counts per minute of the synthesized riboprobes in a single tube with 10 mL hybridization buffer and digested with RNase centrifuged before acrylamide electrophoresis at 1200 to 1500V for 1 to 2 hours. The gel was then dried at 80°C for 1 hour, and the counts per minute values of the individual bands on the gel were counted using a Storm 840 PhosphorImager (Amerham Biosciences, Piscataway, NJ). The statement level of the Gapdh gene was used as an internal control for relative statement levels of other individual genes calculated as a ratio (i.e., the observed total volume for the target gene divided by the volume of Gapdh). Three different RNA extracts were analyzed in triplicate for each cell line. The results of three independent experiments are expressed as the mean value ± 1 SEM. Statistical differences in gene expression were determined using nonparametric Mann-Whitney analyses.

Western Blot Analyses for Relative Expression of Homologous Recombination, Nonhomologous End-Joining, and Base Excision Repair Proteins. Logarithmically growing cells were either mock-irradiated or irradiated and subsequently lysed on ice for 20 minutes with E7 lysis buffer (PBS, 0.1% SDS, and containing protease inhibitors; Roche Molecular Biochemicals, Indianapolis, IN) for Western blotting as described previously (16). SDS-PAGE was performed using up to 7% to 12% acrylamide gels at room temperature. Each well was loaded with 40 μg of total protein plus loading buffer (final concentration 6% glycerol, 0.83% β-mercaptoethanol, 1.71% Tris-HCl (pH 6.8), and 0.002% bromphenol blue). Samples were resolved by electrophoresis at 80 to 110 volts for 1.5 to 2.5 hours and then transferred onto nitrocellulose (Schleicher & Schuell Bioscience, Keene, NH). Prehybridization staining with Ponceau solution confirmed equal loading and transfer between running lanes. After transfer, membranes were rinsed with Tris-buffered saline Tween-20, incubated in appropriate secondary antibodies, and protein bands were detected using ECL Detection Reagent (Amerham Bioscience) before film exposure. Relative expression was determined by densitometry (Molecular Dynamics Computing ImageQuant, Amerham Sciences) and standardized by α-tubulin levels. Primary antibodies were used at dilutions ranging from 1:200 to 1:1,000 as suggested by the supplier and included the following: p21WAF, RAD51, RAD53, and RAD51D (Novus Biologicals, Inc., Littetown, CO); Ku70, RAD52, RAD54, Ku86, DNA polymerase-β, and APE/REF1 (Santa Cruz Biotechnology, Santa Cruz, CA); XRCCh1 (Abcam, Cambridge, United Kingdom); PARP (Biomol, Plymouth Meeting, PA); DNA polymerase-δ (BD Biosciences, San Diego, CA); MRE11 (Genetex, San Antonio, TX); and phospho-H3 (Upstate Technologies, Waltham, MA). Relative protein expression among PrSC, LNCAp, DU-145, and PC-3 cells was normalized to PrEC expression (e.g., arbitrary relative value of one). The Western blots shown are representative of at least two independent experiments.

Single-Cell Gel Electrophoresis (Comet Assay) to Detect DNA Strand Breaks and Base Damage. Repair of DNA double-strand breaks, DNA-single-strand breaks, DNA base damage, and repair of formamidopyrimidine-DNA glycosylase-sensitive sites was quantitated using the Comet assay as previously described (18, 19; www.cometassay.com). For the neutral comet assay, 10 and 25 Gy doses were used; for the alkaline comet and formamidopyrimidine-DNA glycosylase-comet assays, a dose of 6 Gy was used. For the determination of DNA double-strand breaks by the neutral comet assay ~102 cells were admixed with 100 μL of 0.7% of low melting-point agarose (Sigma-Aldrich, St. Louis, MO) at 4°C and spread on regular glass microscope slides precoated with 1% normal melting agarose. Slides were then treated overnight with lysis solution [2.5 mol/L NaCl, 100 mMol/L EDTA, 10 mMol/L Trizma base, 10% DMSO, and 1% Triton X-100 (pH 9.0)] at pH 9.0. The slides were then placed in 1× Tris-buffer boric acid-EDTA and electrophoresed for 15 minutes at 32V (current ~25mA). After electrophoresis, the slides were dried and stored until scoring. The slides were finally stained with ethidium bromide (2 ng/mL) and the comets scored under a Zeiss fluorescence microscope coupled to KOMET 5.0 software (Kinetic Imaging, Durham, NC). The alkaline comet assay detects alkali-labile sites in the DNA, which are a global assessment of DNA single-strand breaks, DNA double-strand breaks, and DNA base damage. In brief, cells were suspended in 0.5% low melting-point agarose and spread on glass microscope slides precoated with 1% normal agarose. After immersion in lysis solution (2.5 mol/L NaCl, 100 mMol/L EDTA, 10 mMol/L Trizma base, 10% DMSO, and 1% Triton X-100) at 4°C for 1 hour to remove cellular proteins, the slides were immersed in electrophoresis buffer [300 mMol/L NaOH, 1 mMol/L EDTA (pH >13)] for unwinding DNA. After a single electrophoresis of 20 to 30 minutes at 300 mA for a total of 20 minutes. Neutralized and dehydrated slides were finally stained with ethidium bromide (2 ng/mL; Sigma-Aldrich) before scoring.

Repair of DNA base damage was scored by treating the DNA with a lesion-specific glycosylase (formamidopyrimidine-DNA glycosylase). This enzyme recognizes oxidative damage as specific DNA base modifications including 8-oxo-7,8-dihydro-2′-deoxyguanosine, 7-methyl-guanine, 5-OH- cytosine, and 5-OH-uracil. Cells were suspended in 0.5% low-melting point agarose and spread on glass microscope slides precoated with 1% normal agarose. Slides were immersed in lysis solution (2.5 mol/L NaCl, 100 mMol/L EDTA, 10 mMol/L Trizma base, 10% DMSO, and 1% Triton X-100) at 4°C for a minimum period of 1 hour and then equilibrated with enzyme reaction buffer (HEPES 9.5g, KCl 7.5 g, and EDTA 0.5 mol/L). Bovine serum albumin fraction V was then added [0.2 per mL (pH 8.0)] for 10 minutes on ice. The slides were finally treated with formamidopyrimidine-DNA glycosylase enzyme (obtained from Dr. Karel Angelis, Institute of Experimental Botany, Praha, Czech Republic; 1:100 dilution in enzyme reaction buffer) at 37°C for 30 minutes in a moist box. The enzyme treatment was terminated by dipping the slides in denaturing solution [0.3 mol/L NaOH and 5 mMol/L EDTA (pH 12.1)] for 20 minutes, followed by neutralization in 1× Tris-buffet-boric acid-EDTA for 5 minutes and, finally, electrophoresis. The ratio of DNA migration between the enzyme treated and buffer control slides gives an
estimate of the formamidopirimidine-DNA glycosylase sensitive sites in the sample.

For all of the comet assays, the comet parameter, “Olive Tail Moment” (i.e., % DNA x distance of center of gravity of DNA) was used as the indicator of DNA damage (19). One hundred consecutive cells were scored at random from the middle of each slide for two to three independent experiments and the final result expressed as the (mean of the median Olive Tail Moment values) ± SEM of the medians. For graphical purposes in the formamidopirimidine-DNA glycosylase assay, the difference between the Olive Tail Moment for formamidopirimidine-DNA glycosylase-treated slides versus the Olive Tail Moment of same population treated with buffer alone were plotted as the final end point of formamidopirimidine-DNA glycosylase-sensitive sites. Statistical differences in Olive Tail Moment values were determined using non-parametric Mann-Whitney analyses.

Chromosomal Aberration Assays. The frequency of spontaneous, ionizing radiation-induced (4 Gy) or mitomycin C-induced (40 ng/ml for 24 hours) chromosomal aberrations were determined in exponentially growing cell cultures of PrSC, LNCaP, DU-145, and PC-3. The cells were harvested by trypsinization 24 hours after treatment and incubation with 1 µg of Colcemid/ml for 2 hours to collect metaphase spreads for analysis. The cells were fixed, after treatment with hypotonic solution (0.6% sodium citrate), in EtOH-glacial acetic acid (3:1). Air-dried preparations were made and slides were stained 4’, 6-diamidino-2-phenylindole/Vectashield antifade mixture (Vector Laboratories, Burlingame, CA). For chromosomal aberrations, 25 mitotic cells were analyzed for each treatment per cell line.

Immunohistochemistry of Prostate Xenografts. For xenograft studies, 1 × 10^6 of PC-3 or DU-145 cells were injected i.m. into the gastronemius muscle (tail of hind leg) of BALB/c-xu mice. Tumors were mock-irradiated or irradiated at a weight of 0.5 g with 20 Gy using a 250KvP X-ray unit without anesthesia. For irradiation, the mouse was lightly restrained in a lucite holder box with lead shielding, such that only the tumor-bearing hind leg was within the irradiation volume. Tumors were then excised and placed immediately in formalin for subsequent fixation and immunostaining using primary RAD51 antibodies (Ab-1, Oncogene Research Products, San Diego, CA) and secondary horseradish peroxidase antibodies for immunodetection. All of the studies were in ethical compliance with the PMH-UHN Animal Care Committee.

RESULTS

Gene Expression Relating to Atm-p53 DNA Damage Pathways in Prostate Cell Cultures. To initially test the utility of RNase protection assays in quantifying gene expression in our panel of two normal (PrEC-epithelial and PrSC-stromal) and three malignant (LNCaP, PC-3, and DU-145) prostate cell lines, we first quantitated ionizing radiation induction of genes associated with the Atm-p53 DNA damage signaling cascade. We have reported previously the apoptotic and G1-checkpoint responses in these cell cultures (16). All five of the cell cultures undergo minimal and nondifferential apoptosis (<5%) after doses of up to 20 Gy (measured 12–96 hours after ionizing radiation); yet the three malignant cell lines have the highest clonogenic survival (i.e., mean SP2Gy values ranging from 0.40 to 0.55 relative to the values for PrSC and PrEC at 0.25 and 0.10, respectively).

PrSc, PrEC, and LNCaP cells express two wild-type p53 alleles and have an intact G1 cell cycle arrest checkpoint with increased p21WAF protein expression after irradiation. PC-3 cells are devoid of p53 protein expression due to chromosome 17p hemizygosity and a mutation in the remaining allele at codon 138 that results in a premature stop codon at position 169. DU-145 cells express high levels of trans- dominant, mutant (MT) p53 proteins due to missense mutations at codons 223 and 274. Both latter cell lines lack a G1 checkpoint and do not show increased p21WAF protein expression after DNA damage (15). Using the RNase protection assay analyses at 4 hours after 10 Gy, we observed increased expression of p21WAF, Gadd45, Bax, and Bcl-2 genes in the WTp53-expressing cells (i.e., PrSc, PrEc, and LNCaP), whereas similar increases in gene expression were not observed for the null-p53 and MTP53-expressing, PC-3 and DU-145 cells (see Fig. 1A and B).

The RNase protection assay also confirmed a previous observation that the relative up-regulation of p21WAF in PrEC epithelial cells was attenuated in comparison to PrSC stromal cells (16). Neither Atm nor p53 RNA was increased after irradiation, consistent with post-translational modification as the basis for activation of these proteins (6). Furthermore, the level of p53 RNA in PC-3 (null for p53) was at background. These results confirmed previous p21WAF expression data from our laboratory for the same cell lines (16). We also confirmed a dose- and time-dependent (i.e., maximal induction at 4–6 hours after ionizing radiation) induction of both RNA and protein relating to p21WAF in the LNCaP cell line (Fig. 1B inset and data not shown). We conclude that the RNase protection assay is a sensitive indicator of gene expression under the conditions of DNA damage and repair within our panel of prostate cell cultures.

Expression and Functional Assessment of DNA Double-Strand Break and DNA Single-Strand Break Repair Proteins in Prostate Cultures. The initial data relating to the Atmp53 stress response provided confidence in the use of RNase protection assay for quantification of gene expression relating to the nonhomologous (nonhomolo-gous end-joining) and homologous recombination pathways of DNA double-strand break repair. We observed a significant and differential increase (approximately 2–3 fold) in homologous recombination associated genes (i.e., Rad51, Rad54, Xrcc3, and Rad52) in malignant prostate cell cultures at both the RNA and protein levels when compared with PrEC cells (see Fig. 2). Gene expression relating to the MRN complex (Mre11/Rad50/Nbs1-p95) was not differentially expressed. We also found little evidence for increased basal mRNA or protein expression of the nonhomologous end-joining-related Ku70, Ku80, DNA-PKcs, XRCC4, or Lgase IV genes (see Fig. 3A and data not shown). We observed elevated levels of the DNA single-strand break repair protein XRCC1 in malignant cultures (Fig. 3A). In contrast to a previous report (20), homologous recombination and nonhomologous end-joining gene expression at both the RNA and protein levels was invariant after irradiation (Fig. 2A and data not shown). We conclude that the endogenous expression of the homologous recombination-related Rad51, Rad52, Rad54, Xrcc3, and Xrcc1 genes are increased at RNA and/or protein levels in malignant prostate cell lines.

We next compared the panel of cell cultures for their relative ability to repair DNA double-strand breaks, DNA single-strand breaks, and alkali-labile sites using the Comet assay under neutral or alkaline lysis conditions (Fig. 3B and C). Despite similar amounts of initial DNA damage, the malignant cultures had significantly decreased capacity in repairing ionizing radiation-induced DNA damage. These data suggest that despite high levels of homologous recombination-related and XRCC1 proteins, malignant prostate cells are defective in the rejoining of DNA double-strand breaks and alkali-labile sites (the latter reflecting DNA single-strand breaks and DNA base damage).

Base Excision Repair in Malignant Prostate Cultures. As the alkaline Comet assay also scores abasic sites, we used the formamidopirimidine-DNA glycosylase Comet assay to directly determine whether the malignant cultures had relatively increased spontaneous and ionizing radiation-induced levels of oxidative damage. Treating DNA with formamidopirimidine-DNA glycosylase unmask oxidative damage as 8-oxo-7,8-dihydro-2′-deoxyguanosine, 7-methyl-guanine, 5-OH-cytosine, or 5-OH-uracil DNA lesions (19). The results shown in Fig. 4A are consistent with a decreased capacity for base excision repair of these lesions in the malignant cultures. This defect was not related to decreased levels of the base excision repair-related p53, APE/REF1, or OGG1 proteins. In fact, the malignant
cells lines had increased levels of DNA-polymerase-β and -δ, two key enzymes involved in short-patch and long-patch base excision repair (ref. 21; Fig. 4B and C). We also assessed whether increased expression of homologous recombination-related or base excision repair-related proteins could be secondary to cell cycle bias given that these proteins are optimally expressed in S and G2 phases of the cell cycle (13). In comparing the cell culture doubling times in vitro with mitotic and S-phase biomarkers (phosphorylated-histone 3 and RNase protection assay proteins, respectively), we did not observe any correlation between increased DNA repair protein expression and cell proliferation indices among the five cultures (Fig. 4B).

**Chromosomal Repair and Heterogeneity of RAD51 Protein Expression In vivo.** Homologous recombination and nonhomologous end-joining events can be scored using integrated genetic substrates (22) or characterization of distinct types of chromosomal aberrations after cellular exposure to mitomycin C or ionizing radiation (known to induce DNA cross-links or predominantly DNA strand breaks, respectively). We used the chromosomal damage assay, as it directly compares the capacity for homologous recombination and nonhomologous end-joining in both malignant and normal cells. Normal PrSc and PrEC cells are difficult to transfect with DNA repair plasmid reporter substrates (22). The metaphase spreads in Fig. 5A show representative structural cytogenetic aberrations, which are quantified in Table 1 for four of the five cultures. The protracted doubling time of PrEC cells precluded their assessment in this assay. All three malignant cell lines show an increased incidence of a variety of aberrations associated with aberrant homologous recombination occurring in the S and G2 phases of the cell cycle phases including: chromatid breaks; double minutes; tri-, quad-, and complex-radial chromosomes; abnormal telomeric associations; and centromere fissions. Furthermore, nonhomologous end-joining-associated defects occurring within the G1 cell cycle phase were also observed as increased chromosomal breaks and di- or tricentric chromosomes in malignant cultures. These data support our hypothesis that defects in DNA double-strand break repair.

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**Fig. 1.** Gene expression relating to Atm-p53 signaling in malignant and normal prostate cultures using RNase protection assay analyses. A, representative example of RNase protection assay blot whereby 32P-labeled multiriboprobes were hybridized to the total mRNA derived from mock-irradiated (NIR) or irradiated (IR: 10 Gy-4 hours) asynchronously growing prostate cultures. Specific multiprobes used in this analysis are indicated in the far left margin of the blot aside the corresponding gene band of interest. Also shown are lanes containing probed sequences within HeLa cells (positive control), Yeast (Yst; negative control), and the RNA probes themselves (far right). Gapdh was a housekeeping gene that served as internal control for densitometric quantitation of results. Note IR-induced p21WAF signal in WT-p53 expressing PrSC, PrEC, and LNCaP cell lines consistent with wild-type p53 status and an intact G1-checkpoint in these cells. B, quantitation of Atm/p53-dependent stress response based on RNase protection assay analyses of mRNA in normal and malignant prostate cells. **Top figure** shows basal (i.e., nonirradiated) levels of mRNA expression. Shown are mean gene expression values based on at least three independent experiments; bars, ±1 SEM. **Bottom panel** shows mean values of expression of same genes relative to basal levels at 4 hours after 10 Gy. Significantly increased expression was observed for the p21WAF gene in WT-p53 expressing cells (PrSC, PrEC, and LNCaP), which is both time-dependent (see insert for p21WAF protein expression over 6 hours post-10 Gy in LNCaP cells) and dose dependent from 2 to 10 Gy (data not shown; Mann-Whitney test; \( P < 0.05 \)), Bax, Bcl-2, and Gadd45 mRNA in these cell lines was also significantly increased after irradiation (Mann-Whitney test; \( P < 0.05 \)).
can be observed in malignant cultures and augment our findings using the Comet assay.

Given the observed increase in homologous recombination-related protein expression in vitro, we also compared the expression of similar proteins in vivo within xenografts derived from the same malignant cell lines (e.g., PC-3 and DU-145). Therefore, we immunostained for the RAD51 protein in histologic sections derived from xenografts before and after 20 Gy irradiation in vivo (see Fig. 5B).

Similar to a previous report in pancreatic cancer (23), we observed intratumoral heterogeneity with respect to endogenous RAD51 expression. Both cytoplasmic and nuclear RAD51 expression was observed in PC-3 and DU-145 xenograft histologic sections. However, in contrast to our data in which RAD51 expression in vitro was invariant after DNA damage, nuclear RAD51 expression increased in the PC-3 or DU-145 xenografts irradiated under in vivo conditions. Taken together, our data suggest that RAD51 expression in vivo can be additionally modified by intratumoral biology and physiology with the three-dimensional tumor architecture.

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Fig. 2. Increased expression of the homologous recombination genes, Rad51, Xrc3, Rad52, and Rad54, in malignant prostate cancer cell lines. A. Top panel shows relative basal homologous recombination gene expression based on RNase protection assay analyses in normal and malignant prostate cells. Significant increased mRNA expression of Rad51, Rad54, Rad52, and Xrc3 is noted in the malignant prostate cultures in comparison with normal cell cultures (Mann-Whitney test, P < 0.05). Irradiated (IR) induction of the Rad51, Rad54, Rad52, and Xrc3 genes was observed solely in the PrSC stromal cell line (Mann-Whitney test, P < 0.05), but not the other epithelial cell lines (data not shown; Supplementary Fig. 1A). Bottom panel shows Western blots of selected homologous recombination proteins in which protein expression is invariant before (mock-irradiated, NIR) or at 4 hours after 10 Gy (IR). α-Tubulin is shown as the protein loading control. Increased p21WAF protein expression served as an irradiation control whereby p21WAF protein was elevated in cell lines with wild-type p53 gene status. B. Confirmation of increased basal RAD51, RAD52, RAD54, and XRCC3 protein expression in malignant prostate cells relative to PrEC cells using quantitative densitometry of Western blot analyses (see example blot in Supplementary Fig. 1B). Similar results were obtained among two independent experiments. The dotted line represents the relative expression compared with the PrEC cell line. Homologous recombination-related protein expression was not additionally increased at 4 hours after a 10 Gy (data not shown); bars, ± SEM.

Fig. 3. Expression of nonhomologous end-joining proteins and defects in DNA strand break (DNA double-strand break and DNA single-strand break) repair in malignant prostate cell cultures. A. quantitation of nonhomologous end-joining and DNA single-strand break repair protein expression in malignant prostate cells relative to PrEC cells using quantitative densitometry of Western blot analyses (see example blot in Supplementary Fig. 1C). Similar results were obtained for two independent experiments. The dotted line represents the relative expression compared with the PrEC cell line. The level of XRCC1 protein expression was consistently elevated in all three of the malignant cell lines. Neither nonhomologous end-joining- nor DNA single-strand-break-related protein expression was additionally increased at 4 hours after a 10 Gy dose (data not shown). B, neutral Comet assay of malignant and normal prostate cells before and after 10 or 25 Gy of ionizing radiation. Plotted is the Olive Tail Moment (OTM, i.e., % DNA X distance of center of gravity of DNA) on the Y axis as the indicator of the presence of DNA double-strand breaks for a given time and radiation dose. No significant differences exist between the five cell lines for OTM values at baseline or immediately after irradiation (i.e., time = 0). However, the residual number of DNA double-strand breaks at 24 hours after 25 Gy is greater in the three malignant cultures in comparison with the normal cultures (Mann-Whitney test, P < 0.05). C, alkaline (pH 13.0) Comet assay of malignant and normal prostate cells before and after 6 Gy of ionizing radiation. Similar to B above, the OTM on the Y axis as the indicator of the presence of alkali-labile sites, DNA-double-strand breaks, DNA single-strand breaks, and DNA base damage after irradiation. The residual damage at 24 hours after irradiation in the three malignant cultures is greater than that of the control PrEC cultures (Mann-Whitney test, P < 0.05). For both B and C, 100 consecutive cells were scored from the middle of each slide for two to three independent experiments and the final result expressed as the (mean of the OTM median values); bars, ± SEM.
**DISCUSSION**

This is the first study to report defective DNA double-strand break, DNA single-strand break, and base damage repair in malignant prostate cancer cells and associate these defects with increased chromosomal aberrations and genetic instability. Our data are consistent with homologous recombination-related and base excision repair-related protein dysfunction in malignant cells as a biomarker of a “mutator” phenotype driving genomic instability after cytotoxic insult. These findings are supported by previous data in which LNCaP cells had a decreased ability to repair restriction-enzyme mediated DNA double-strand breaks based on a fluorescence-based plasmid reconstitution assay (24). Additionally, during the preparation of this article, Trzeciak et al. (25) reported that PC-3 and DU-145 cells have defective excision of oxidative lesions with altered levels of superoxide dismutase and glutathione peroxidase supporting our observation of decreased base excision repair in these cell lines. Other reports in the literature within panels of cell lines with varying histopathologic type have reported heterogeneity in DNA polymerase-β activity and elevated PARP expression (26–28).

There have been previous reports of elevated levels of Rad51 mRNA or RAD51 protein expression within human tumor cell lines (29, 30). However, to our knowledge, this is the first direct comparison of DNA double-strand break gene expression within normal and malignant cells from the same histopathologic type linked to a functional assessment of DNA double-strand break repair. Altered stoichiometry of repair proteins or a disconnect between cell cycle checkpoint control and DNA repair may be the basis for the observed discordance between repair protein expression and function (13, 31). Altered homologous recombination-related protein expression might indirectly lead to altered nonhomologous end-joining activity given the interplay between the two pathways during DNA double-strand break repair (32, 33). Maintenance of survival in malignant cells, despite high levels of DNA double-strand breaks and chromosomal aberrations after DNA damage, is probably secondary to loss of potentially deleterious accentric fragments or other chromosomal abnormalities within micronuclei at 48 to 72 hours after irradiation (34, 35).

In isogenic systems, the relationship among RAD51 expression, homologous recombination, and induction of chromosomal aberrations remains controversial (29, 36–38). This may relate to variability in genetic background or the plasmid homologous recombination reporter substrates used for study (22). Transfection studies with forced overexpression of RAD51 have led to observations of both increased and decreased frequencies of homologous recombination, arguing for RAD51 acting as either a promoter or repressor of genetic instability and tumor progression (37, 39). Other transfection studies suggested that RAD51, XRCC3, and XRCC1 protein overexpression leads to increased p21WAF expression and a decreased apoptotic response with resulting radioresistance (23, 40, 41). However, our data do not support such a direct correlation. Our malignant cultures, which overexpress homologous recombination-related proteins have varying G1 checkpoint control and p21WAF expression, and all five of the cultures are resistant to apoptosis (16). The increased DNA repair protein expression observed in the malignant cell lines was independent of p53 status, G1 checkpoint control, androgen responsiveness, clongenic radiation cell survival, cell proliferation, and susceptibility for radiation-induced apoptosis (16, 20, 42).

It had also been hypothesized that RAD51 overexpression might abrogate RAD51-p53 interactions and override the G1 checkpoint leading to aneuploidy and high levels of homologous recombination (37, 43). The decreased levels of MMS- and ionizing radiation-induced homologous recombination-related chromosomal aberrations in the G1 checkpoint-proficient LNCaP cultures, relative to the G1 checkpoint-deficient DU-145 and PC-3 cultures, would support this hypothesis. Additionally, our observed overexpression of the Rad51, Xrcc3, Rad52, and Rad54 genes at both the RNA and protein level suggests that loss of control of homologous recombination expression in malignant cells is operational at the transcriptional level and may be secondary to altered activity of transcription factors in cancer cells. Rad51 gene overexpression can be mediated by Bcr/Abl and STAT5-dependent transcription in addition to inhibition of caspase-3-dependent RAD51 protein expression.
cleavage (44, 45). Similar mechanisms may exist for other homologous recombination-related genes in prostate cancer. Indeed, preliminary data in our laboratory suggests that endogenous BRCA1 and BRCA2 protein expression are also elevated in the three malignant cell lines.5

Our results implicate DNA repair, and particularly DNA double-strand break repair and homologous recombination, as a potential factor in prostate tumor progression. Importantly, our data may have implications for both prostate cancer diagnosis and therapy. Profiling of DNA repair protein expression may be useful to discover new biomarkers of genetic instability, malignant progression, and aggressive tumor phenotypes (46, 47). We would also hypothesize that prostate intraepithelial neoplasia (PIN) may have altered frequencies of homologous recombination and defective DNA double-strand break repair when compared with nonmalignant epithelium. Unfortunately, the paucity of cell lines for the study of prostatic intraepithelial neoplasia or early stage prostate cancer prevents us from making this direct link in vitro (48).

Prostate tissue arrays will be useful to confirm that our in vitro findings are operational in vivo and that loss of checkpoint control and DNA repair activity is correlated to tumor progression similar to that reported for breast cancer (49). However, the data presented in Fig. 5B suggest that interpretation of tissue arrays may be difficult without added information pertaining to cell cycle phase, oxygenation, nutritional status, or clonal variation, which may affect the intratumoral heterogeneity of protein expression. For example, the relative increase in RAD51 expression after irradiation in vivo may be secondary to the arrest of cells in the G2 phase of the cell cycle, which has higher levels of RAD51 expression (50), or due to microenvironmental factors. Indeed, in a separate study, we have observed that RAD51 expression

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Table 1 Chromosomal aberrations in prostate cell cultures in response to DNA damage

<table>
<thead>
<tr>
<th>Aberration</th>
<th>PRSC Control</th>
<th>MMC Control</th>
<th>IR Control</th>
<th>LNCAP Control</th>
<th>MMC Control</th>
<th>IR Control</th>
<th>DU145 Control</th>
<th>MMC Control</th>
<th>IR Control</th>
<th>PC-3 Control</th>
<th>MMC Control</th>
<th>IR Control</th>
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<tr>
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<td>0</td>
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<td>1</td>
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<tr>
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<td>0</td>
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<td>23</td>
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<td>19</td>
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<td>58</td>
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</table>

NOTE. For each cell culture, the frequency of spontaneous (control), IR-induced (4 Gy), or MMC-induced (40 ng/ml for 24 hours) chromosomal aberrations were determined in exponentially growing cell cultures of PRSC, LNCAP, DU145, and PC-3. The extremely slow doubling time of PrEC cells (48 hours) precluded a similar analysis in this cell type. The cells were harvested by trypsinization 24 hours after treatment and then incubated with 1 μg of Colcemid/ml for 2 hours to collect metaphase spreads for analysis. Twenty-five metaphases were scored for each culture. Both increased chromosome and chromatid types of aberrations are observed in the malignant cells relative to PrEC cells after IR or MMC treatment, consistent with defects in homologous recombination and non-homologous end-joining repair.

Abbreviations: MMC, mitomycin-c; IR, ionizing radiation.

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5 R. Fan, F. Jalali, and R. G. Bristow, unpublished observations.
of intratumoral hypoxia (51). Additional experiments with defined doses and time points at irradiation in vivo using prostate xenographs may clarify these issues and glean more information regarding RAD51 function within solid tumors. We speculate that the use of agents that either augment DNA repair in premalignant prostate epithelium or inhibit the hyper-recombination-genotypic phenotype of malignant cells may be valuable in prostate cancer treatment. For example, the antioxidant selenium has been implicated as a chemoprotective agent for high-risk prostate cancer and has been shown recently to increase DNA repair and chemoprotect normal cells in response to DNA damaging agents (52). Additionally, molecular-targeted agents, which decrease aberrant Rad51 or other DNA repair gene expression, such as STI571-Gleevec (45, 53) or small interfering RNA approaches (54), may be potentially novel strategies to sensitize prostate cancer cells to radiotherapy or chemotherapy (55, 56).

REFERENCES

Defective DNA Strand Break Repair after DNA Damage in Prostate Cancer Cells: Implications for Genetic Instability and Prostate Cancer Progression

Rong Fan, Tirukalikundram S. Kumaravel, Farid Jalali, et al.

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