Cellular repair of oxidatively induced DNA base lesions is defective in prostate cancer cell lines, PC-3 and DU-145

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Mutagenic oxidative DNA base damage increases with age in prostatic tissue. Various factors may influence this increase including: increased production of reactive oxygen species, increased susceptibility to oxidative stress, alterations in detoxifying enzyme levels or defects in DNA repair. Using liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry, we show increased levels of oxidative DNA base lesions, 8-hydroxyguanine (8-oxoG), 8-hydroxyadenine (8-oxoA) and 5-hydroxy-cytosine (5OHHC) over the baseline in PC-3 and DU-145 prostate cancer cells following exposure to ionizing radiation and a repair period. Nuclear extracts from PC-3 and DU-145 prostate cancer cell lines are defective in the incision of 8-oxoG, 5OHHC and thymine glycol (TG) relative to the non-malignant prostate cell line. Consistent with reduced expression of OGG1 2a, incision of 8-oxoG is reduced in PC-3 and DU-145 mitochondrial extracts. We also show a correlation between severely defective incision of TG and 5OHHC and reduced levels of NTH1 in PC-3 mitochondria. The antioxidant enzymes, glutathione peroxidase (GPx), catalase and superoxide dismutases (SOD1, SOD2), have altered expression patterns in these cancer cell lines. Genetic analysis of the OGG1 gene reveals that both PC-3 and DU-145 cell lines harbor polymorphisms associated with a higher susceptibility to certain cancers. These data suggest that the malignant phenotype in PC-3 and DU-145 cell lines may be associated with defects in base excision repair and alterations in expression of antioxidant enzymes.

Introduction

Prostate cancer, is the most prevalent form of cancer among American men and is the second leading cause of their cancer mortality (1). In the US, there will be 220 900 new cancer cases in 2003 making prostate cancer one of the fastest growing cancers in terms of incidence among American men (2). While certain factors including dietary, genetic, lifestyle and environmental are associated with prostate cancer risk, the molecular mechanisms underlying the etiology of the disease are largely unknown (3).

Several genes associated with heritable forms of prostate cancer have been identified and somatic alterations in these genes are presumed to set the stage for the development and/or progression of the disease. To this end, it has been shown that hypermethylation of the p-class glutathione S-transferase gene (GSTP1) promoter region inhibits transcription of the gene and is associated with prostate cancer development (4–6). The GSTP1 gene product probably protects genomic DNA in prostate cells from the deleterious effects of genotoxic agents (3,7,8). Environmental carcinogens such as polycyclic aromatic hydrocarbons may play a role in the etiology of prostate cancer since 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine has been shown to induce prostate cancer in rats (9,10).

Reactive oxygen species (ROS), most notably the hydroxyl radicals, generated endogenously by cellular metabolism are known to cause oxidative DNA damage that has been implicated in prostate carcinogenesis (11,12). Research on the development of prostate cancer suggests that symptomatic and asymptomatic chronic and acute inflammation occurs in the prostate over the life span and acts in synergy with other factors to cause injury to prostatic epithelium (13). In response to this injury, cellular proliferation occurs followed by oxidative stress related to the ongoing inflammatory process that may in turn result in high rates of oxidative damage to DNA (14). Furthermore, Bostwick et al. have reported low levels of SOD1, SOD2 and catalase in prostate intraepithelial neoplasia and prostate cancer relative to benign prostate epithelium thereby implicating oxidative DNA damage in prostate carcinogenesis (15). There is also a significant increase in the proportion of mutagenic oxidatively induced DNA base lesions, 8-hydroxyadenine (8-oxoA), and 8-hydroxyguanine (8-oxoG) in malignant prostatic tissue as well as an increase in the levels of these lesions in benign prostatic tissue with aging (12,16,17). The existence of OGG1 genetic polymorphisms in prostate cancer patients further supports the notion that defective DNA repair may be associated with prostate cancer risk (18). Taken together these data suggest that reactive oxygen species and oxidative DNA damage may play a critical role in the development of prostate cancer.

Oxidative DNA damage has been shown to be higher in the mitochondrial than in the nuclear genome due to the higher metabolic rate in the mitochondria relative to the nucleus (19). DNA lesions caused by ROS are numerous and include: DNA strand breaks, apurinic/pyrimidinic (AP) sites, modified DNA bases and DNA-protein cross-links (20). Thymine glycol (TG), 8-oxoG and other DNA base lesions may lead to deleterious biological consequences. TG is a cytotoxic lesion that blocks both DNA replication (21) and transcription (22), causing cell death. On the other hand, 8-oxoG is a pre-mutagenic

Abbreviations: AP, apurinic/pyrimidinic; BER, base excision repair; GC/MS, gas chromatography/mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; MMR, mismatch repair; TG, 5OHHC, 5-hydroxy-cytosine; 8-oxoA, 8-hydroxyadenine; 8-oxoG, 8-hydroxyguanine; ROS, reactive oxygen species; TG, thymine glycol.
lesion that results in GC to TA transversions (23), whereas, 8-oxoA causes both AT to GC transition and AT to CG transversion mutations (24). Indeed, spontaneous transversion mutations have been observed in proto-oncogenes and the tumor suppressor gene, p53 (25,26), a commonly mutated gene in cancer that has been shown to play a role in DNA repair (for review see ref. 27). Oxidatively induced mutations in the mtDNA can lead to cellular dysfunction and have been implicated in degenerative diseases, cancer and aging (28). This damage must be repaired in order to maintain proper genetic integrity.

Cells utilize base excision repair (BER) pathway as the primary means of minimizing the deleterious effects associated with oxidative DNA damage (reviewed in ref. 29). In addition to DNA repair, cells express antioxidants that detoxify ROS produced during aerobic respiration. The genome of cancer cells is more prone to oxidative damage due to the high rate of metabolism associated with increased cellular proliferation (30). This high metabolic rate in cancer cells may result in increased production of ROS that could significantly increase oxidative DNA damage and may be accompanied by alterations in antioxidant levels. It is plausible that increased oxidative DNA damage coupled with alteration in antioxidant levels in cancer cells may result in insufficient DNA repair. Indeed, this is the case in a few cancer cell lines studied showing defective BER in breast cancer cell lines (31,32). Recently, defects in DNA mismatch repair (MMR), the pathway that removes errors arising during DNA replication, have been reported in prostate cancer (33-36). Although, levels of oxidative DNA damage have been shown to be increased in cancerous cells or tissues relative to non-cancerous cells or tissues (reviewed in ref. 30), the relationship between elevated oxidative DNA damage and DNA repair in carcinogenesis is still poorly understood.

To our knowledge, there have been no reports addressing the repair of oxidatively induced DNA base lesions in prostate cancer cells. Therefore, we hypothesize that oxidative DNA repair may be defective in prostate tissue as a result of uncontrolled oxidative stress and increased oxidative DNA damage. We used two well-studied prostate cancer cell lines, PC-3, DU-145 and a non-malignant prostate cell line, RWPE-1 to address our hypothesis.

Materials and methods

Cells and culture conditions

One non-malignant prostate cell line (RWPE-1) and two prostate cancer cell lines (PC-3, DU-145) were used in this study. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The RWPE-1 cell line was established by immortalizing non-neoplastic adult human prostatic epithelial cells from a white male donor with human papillomavirus 18 (37). The RWPE-1 cells were cultured in keratinocyte serum-free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 ng/ml bovine pituitary extract (Life Technologies, Rockville, MD). The PC-3 cancer cell line was grown in Ham’s F-12K medium (ATCC) supplemented with 10% FBS (Life Technologies). The DU-145 cells were cultured in Eagle’s Minimum Essential Medium (ATCC) supplemented with 10% FBS. All cells were grown at 37°C and 5% CO2.

Comet assay (single cell gel electrophoresis)

Prostate cell lines, RWPE-1, PC-3 and DU-145 cells were seeded onto multi-well plates 1 day before experiment and cultured in their primary media in humidified incubator at 37°C with 5% CO2. Basal levels of DNA damage were assessed by comet assay in untreated cells.

The comet assay was performed under alkaline conditions following the procedure of Singh et al. (38) with some modifications. Cells were suspended in 0.75% low melting point agarose (Invitrogen, Carlsbad, CA) in PBS and spread on full frosted microscope slides (A. Daiger and Company, Wheeling, IL) pre-coated with 0.5% normal agarose (Invitrogen). Cells were then lysed for 1 h at 4°C in lysis buffer (10 mM Tris-HCL, pH 10, 2.5 mM NaCl, 100 mM EDTA, 1% Triton X-100). The treatment of double-stranded DNA was performed in situ in the presence of bacterial DNA glycosylase Fpg (New England Biolabs, Beverly, MA) or endonuclease III (Sigma-Aldrich, St Louis, MO) in Fpg/Endo III buffer (40 mM HEPES, pH 8.0, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA) (39) for 2 h at 37°C. The unwinding step was performed for 40 min at 4°C in unwinding/electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13). Electrophoresis was performed at ambient temperature of 8°C for 30 min in unwinding/electrophoresis buffer at electric field strength 0.7 V/cm (330 mA). The slides were then neutralized with a neutralizing buffer (0.4 M Tris-HCL, pH 7.5), rinsed with distilled water, air dried, stained with 2 μg/ml ethidium bromide and covered with cover slips.

Comets were scored under an Eclipse E-400 fluorescence microscope (Nikon, Japan) attached to a Pulsix video camera (Kinetic Imaging, Liverpool, UK) connected to the image analysis system Komet version 4.0 (Kinetic Imaging). Fifty images were randomly selected for each sample and the Olive tail moments were measured. The Olive tail moment is positively correlated with the level of DNA damage in a cell (40). The mean tail moment in a particular sample was taken as an index of DNA lesions in the sample.

Measurement of 8-oxoG, 8-oxoA and 5-hydroxycytosine (5OHC) in genomic DNA

Liquid chromatography/mass spectrometry (LC/MS) with isotope-dilution technique was used to quantify the nucleoside forms of 8-oxoG and 8-oxoA, respectively, following enzymatic hydrolysis of genomic DNA isolated from primary prostate cells. The level of 5OHC was measured by liquid chromatography/mass spectrometry (GC/MS) with isotope-dilution technique. After irradiation at the indicated doses in a Gammarcell 40 Exactor 137Cs γ-ray radiation source (Nordion, Ontario, Canada), the cells were incubated in the complete medium at 37°C for 30 min. Cells were harvested and DNA was isolated by the salt extraction procedure (41). Measurements of 8-oxoG, 8-oxoA and 5OHC were performed as described (20,42-44).

Preparation of mitochondrial extracts

Mitochondrial extracts were prepared as described in refs 31,45. Briefly, 1–4 × 1010 cells were homogenized in 1× MSHE buffer (10 mM HEPES buffer, pH 7.4, 0.21 M mannitol, 0.07 M sucrose, 0.15 mM spermine, 0.75 mM spermidine, 1 mM EGTA, 1 mM EDTA, 5 mM DDT, and protease inhibitors with the shown final concentrations: 1 mM phenylmethylsulfonylfluoride (PMSF), 2 μM benzamide, 1 μg/ml chymostatin A, 1 μM E64, 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml bestatin). Mitochondria and nuclei were separated by centrifugation at 500 g for 7 min at 4°C and the supernatant containing mitochondria was transferred to a fresh tube. The nuclear pellet was stored at –80°C and later used for preparation of nuclear extract. The homogenate was spun at 10 000 g for 7 min at 4°C. The mitochondrial pellet was resuspended in 1 ml of 1× MSHE buffer and further purified by gradient ultracentrifugation (Beckman XL-90) through 50% percoll in 1× MSHE buffer at 50 000 g for 1 h at 4°C. The band containing mitochondria was removed and washed by centrifugation in 50 mM KCl/lx MSHE buffer (1500 g, 10 min, 4°C). The mitochondria were lysed in buffer containing 20 mM HEPES, pH 7.4, 5% glycerol, 1 mM EDTA, 5 mM DTT, protease inhibitors, 0.05% Triton X-100. Total protein content was quantified using Bradford assay (Bio-Rad, Hercules, CA) and the extracts were aliquoted and stored at –80°C.

Cytochrome c oxidase assay

The viability of mitochondria was assessed colorimetrically using Cytochrome c Oxidase Assay Kit (Sigma-Aldrich). The reactions were performed using 25 μl of mitochondrial extracts in 1.1 ml of buffer containing 520 mM KCl, 45 mM sucrose, 23 μg/ml α-dodecyl-β-D-maltoside, 123 μg/ml reduced cytochrome c, 9.5 mM Tris-HCl, pH 7.0. The cytochrome c oxidase activity was calculated from the initial rate of cytochrome c oxidation.

Preparation of nuclear extracts

The nuclear extracts were prepared from the nuclear pellet obtained during mitochondrial extract preparation. The nuclear pellet was re-suspended in 2 vol of buffer A (20 mM HEPES buffer, pH 7.9, 25% glycerol, 0.2 mM EDTA, protease inhibitors, 5 mM DTT). The nuclear membranes were disrupted by sonication (Ultrasonic Processor GE 130, Sonics and Materials, Newtown, CT) in ice water for three consecutive cycles performed at settings of 2 and 6 W/60 s and finally at 10 W/40 s. The three sonication cycles were performed for 5 s each and were characterized by 1 s pause intervals. After sonication, the samples were left on ice for 30 min and then spun two times at 25 000 g, for 30 min, at 4°C (Beckman XL-90). The supernatant was aliquoted and the total protein concentration determined by Bradford assay (Bio-Rad).
Preparation of whole cell extracts

Whole cell extracts were prepared by the method described by Manley et al. (46) with some modifications. Briefly, 5–20 × 10^6 cells were harvested. Cell pellet was re-suspended in four packed-cell volumes of hypotonic lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, protease inhibitors) and subjected to 35 strokes of homogenization in a glass-teflon homogenizer. Four packed-cell volumes of sucrose–glycerol buffer (50 mM Tris–HCl, pH 7.5, 25% sucrose, 50% glycerol, vol/vol, 10 mM MgCl₂ and 2 mM DTT) were then added and the mixture was stirred gently. To this suspension, one packed-cell volume of saturated ammonium sulfate (neutralized with NaOH to pH 7) was added dropwise and stirring continued for 30 min. The extract was ultracentrifuged at 84 000 g for 3 h at 4°C (Beckman XL-90) and the supernatant precipitated by addition of finely ground ammonium sulfate (30.33 g/ml of suspension). After dissolving the salt, 1 M NaOH was added (10 μl/g of ammonium sulfate added) and the mixture was stirred for another 30 min. The extract was spun at 31 400 g for 20 min, at 4°C. The dry pellet was re-suspended in dialysis buffer (20 mM HEPES, pH 7.4, 5% glycerol, 1 mM EDTA, 5 mM DTT, protease inhibitors), and dialyzed (Slide-a-Lyzer 10,000 MWCO, Pierce, Rockford, IL) overnight against dialysis buffer. The sample was centrifuged at 12 000 g for 10 min at 4°C and the supernatant aliquoted.

The total protein amount was determined by Bradford assay (Bio-Rad) and the protein aliquots stored at −80°C.

Oligonucleotide sequences and 5′-end labeling

The oligonucleotides used had either, 8-oxoG, TG, 5OH or AP sites at specific positions. The oligonucleotides were prepared by 5′-ATACGACATTACCCGGAGG(8-oxoG)1TCGGCC-

GAATGGATCTAAC-3′, 5′-AAAGAAGGGAGAAGCTTATT-3′, 5′-AAAGAAGGGAGAAGCTTATT-3′, 5′-AAAGAAGGGAGAAGCTTATT-3′, GCAGACTTATCGTGACATTTACGCATTAATT-3′. Approximately, 100 ng of the single-stranded oligonucleotides were incubated at 37°C for 1 h with 30 μCi of [γ-32P]ATP in the presence of T4 polyadenyli-

tide kinase. The oligonucleotides were annealed to 4-fold excess of their complementary oligonucleotides by heating to 90°C for 5 min and then slow cooling to room temperature. Complementary oligonucleotides contained cyto-

sine opposite 8-oxoG, adenine opposite TG, guanine opposite 5OH and AP sites. The efficiency of the annealing reaction was ascertained by native 20% polyacrylamide gel electrophoresis. The duplex oligonucleotides were purified by passage through G-25 spin columns (Amersham Biosciences, Piscataway, NJ).

Incision assay

Duplex oligonucleotides were incubated at 37°C with either nuclear or mito-

dochondrial extracts. This assay was performed using either varying amounts of extract at a constant time or a fixed amount of extract at increasing time. The incubation buffer contained: 20 mM HEPES buffer, pH 7.4, 5% glycerol, 0.1 mM NaCl, 5 mM EDTA, 5 mM DTT, 100 μg/ml BSA, 25 μg/ml poly(dl-DC). Oligonucleotides containing normal bases in place of the damaged bases were used as controls for specific activities. All reactions were stopped by incuba-

tion with 0.2% SDS and 0.1 mg/ml proteinase K at 56°C for 30 min. Proteins were removed by phenol–chloroform extraction. The reaction products were sep-

arated on 20% polyacrylamide gel containing 7 M urea at 15 W for ~80 min. The radiolabeled wet gels were placed in PhosphorImager cassettes and the gel images analyzed using ImageQuant version 5.1 (Amersham Biosciences).

Western blot analysis

Nuclear, mitochondrial and whole cell extracts (15–100 μg) were denatured by heating at 95°C for 3 min, loaded on a 6 or 12% Tris–glycine gel (Invitrogen). Purified proteins, OGG1 and catalase (Sigma-Aldrich) were used as size markers. The proteins were separated by electrophoresis in 1× Tris–glycine SDS buffer and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked for 1 h in 5% dry milk in PBS-Tween (0.2%) and then probed with the following primary antibodies: OGG1 1a, OGG1 2a, NHEJ-1, XLF, HDR1, SOD2, glutathione peroxidase (Novus Biologicals, Littleton, CO), SOD1 and catalase (Calbiochem, La Jolla, CA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary anti-

body (anti-rabbit, anti-mouse or anti-goat, depending on the origin of the primary antibodies) (Vector Laboratories, Burlingame, CA). The membranes were developed using enhanced chemiluminescence plus western detection kit (Amersham Biosciences). The membranes were stripped at 50°C for 30 min in stripping buffer (62.5 mM Tris–HCl, pH 7.4, 100 mM β-mercaptoethanol, 2% SDS) and re-probed with β-actin antibody (Santa Cruz Biotechnology), and after washing, were incubated with anti-goat horseradish peroxidase-

conjugated secondary antibody (Vector Laboratories). The bands were quan-

tified using ImageQuant software version 5.1 (Amersham Biosciences).

Sequencing of OGG1 gene

Genomic DNA was isolated from prostate cells using FlexiGene DNA kit (Qiagen, Valencia, CA) and OGG1 gene sequence was determined (Seqwright, Houston, TX). The sequence of all exons including a 200 bp promoter adjacent to exon 1 and introns in the proximity of splicing sites were analyzed.

Statistical analysis of data

Results are given as arithmetic means ± SEM calculated from at least three independent experiments. Differences between means were tested for signific-

cance using Student’s t-test. More than two means were statistically analyzed using one-way ANOVA. The Holm-Sidak and Dunnett’s methods of multiple comparisons with control groups were applied as post-hoc tests. A P value <0.05 was taken to be statistically significant.

Results

Analysis of the levels of oxidative DNA damage in prostate cancer cells by comet assay, LC/MS and GC/MS

In order to address the hypothesis that the prostate cancer cell lines PC-3 and DU-145 may be overwhelmed by DNA damage, we examined baseline and induced oxidative DNA damage levels using comet assay, LC/MS and GC/MS. The alkaline comet assay showed that there were comparable base-

line levels of SSB, DSB and AP sites in all three cell lines (Figure 1A, I). There were also comparable levels of Endo III-

sensitive sites (TG and 5OH) based on the alkaline comet assay (Figure 1A, III). Furthermore, our results showed that there was a significantly lower level of Fpg-sensitive sites (8-oxoG) in the prostate cancer cell lines relative to the control cell line, RWPE-1 (Figure 1A, II). The baseline levels of 8-oxoG and 5OH were significantly lower in the cancer cell lines than in the control as measured by LC/MS and GC/MS, respectively (Figure 1B, II and III). Baseline levels of 8-oxoA were the same for all cell lines (Figure 1B, I). Analysis of the levels of DNA base lesions after γ-irradiation and a 30-min repair period showed that the levels of 8-oxoA and 8-oxoG in PC-3 cells were higher than the baseline levels (compare Figure 1C, I with B, I and C, II with B, II, clear bars) as well as a significantly higher level of 5OH over the baseline in both cancer cell lines (compare Figure 1C, III with B, III, clear and gray bars). There were no changes in the levels of DNA base lesions in the control cell line, RWPE-1 (compare Figure 1C, I–III with B, I–III). These observations suggest that repair of these lesions is defective in prostate cancer cell lines in agreement with the incision data.

Incision of oxidatively induced DNA base lesions in oligonucleotides by nuclear extracts of RWPE-1, PC-3 and DU-145

The increased oxidative DNA damage in PC-3 and DU-145 cells warranted examination of the DNA repair capacity in the two prostate cancer cell lines. We examined the ability of nuclear extracts to incise the DNA lesions, 8-oxoG, TG, 5OH and AP sites. Concentration-dependent studies showed that the incision of 8-oxoG, TG and 5OH was reduced in the two cancer cell lines relative to the non-malignant cell line RWPE-1 (Figures 2A–C, 3A–C and 4A–C). Time course analysis of incision activity revealed that both PC-3 and DU-145 had reduced activity relative to the control cell line. There was a 6-fold reduction in incision of TG and 5OH by PC-3 extracts, while the incision of 8-oxoG was reduced 2-3-fold compared with the non-malignant control. The incision of TG, 5OH and 8-oxoG by DU-145 extracts was reduced 2-3-fold when compared with RWPE-1. These results clearly show that the ability of the nuclear extracts of the two studied prostate
cancer cell lines to repair oxidative DNA damage is defective. Conversely, the incision of AP sites in the prostate cancer cell lines was indistinguishable from the non-malignant control cell line, RWPE-1 (Figures 3D and 4D). The wild-type incision of AP sites by the cancer cell lines is not surprising since this lesion is repaired by the abundant AP endonucleases and the lyase activity of certain DNA glycosylases.

Incision of DNA base lesions in oligonucleotides by mitochondrial extracts of RWPE-1, PC-3 and DU-145

Since the level of oxidative DNA damage is higher in the mitochondria than in the nuclei (19), we determined whether mitochondrial extracts from prostate cancer cell lines are proficient in removal of 8-oxoG, TG and 5OHC. Incision of all three lesions by PC-3 mitochondrial extracts was reduced 2–3-fold relative to RWPE-1 (Figures 5A–C and 6A–C). The incision of 8-oxoG by DU-145 mitochondrial extracts was reduced 2–3-fold when compared with the control (Figures 5A and 6A). Interestingly, the incision of TG and 5OHC by DU-145 mitochondrial extracts was increased 2–3-fold over the non-malignant control (Figures 5B and C and 6B and C). These results show that the ability of mitochondrial extracts from both prostate cancer cell lines to repair 8-oxoG is defective, mirroring the result seen for the nuclear extracts.

Fig. 1. Analysis of oxidative DNA damage using comet assay, LC/MS and GC/MS in prostate cells. Comet assay: we analyzed baseline oxidative DNA damage in growing cells using alkaline comet assay. In this assay, SSB, DSB and AP sites were analyzed. Additional oxidative DNA damage was analyzed as either Fpg (8-oxoG) or endonuclease III (TG, 5OHC)-sensitive sites. The results are shown in (A, I–III). LC/MS and GC/MS analyses: actively growing cells were exposed to 5 Gy of γ-irradiation and incubated for 30 min at 37°C to allow DNA repair to take place. Genomic DNA was isolated and analyzed for 8-oxoA, 8-oxoG as their nucleosides by LC/MS and for 5OHC by GC/MS. (B, I–III) Unchallenged cells; (C, I–III) challenged cells. The bars are shown as: black for RWPE-1, clear for PC-3 and gray for DU-145. All the values in this study for both the comet assay, LC/MS and GC/MS were obtained from at least three independent experiments and expressed as mean ± SEM. The data presented in (A) and (B) for prostate cancer cell lines were compared to RWPE-1 and the asterisks denote statistically significant differences, P < 0.05. The asterisks shown in (C) denotes statistically significant differences (P < 0.05) between data in (C) and (B). The bars in (C) have a dividing line to indicate the position of the baseline damage.
Fig. 2. Representative gels showing incision of TG by nuclear extracts from prostate cell lines. A 5'-end-labeled duplex TG-containing oligonucleotide (0.2 ng) was incubated with increasing amounts of nuclear extracts with the indicated amounts in micrograms for 2 h. Control reactions were performed using oligonucleotide containing undamaged base (TG-C). An oligonucleotide sizing marker is shown on the left of each gel. (A) Represents the results of RWPE-1. (B and C) represent the results of PC-3 and DU-145, respectively. The positions of the expected products and remaining substrate are shown by side arrows.

Fig. 3. Analysis of the removal of oxidatively induced lesions by nuclear extracts from prostate cell lines. A 5'-end-labeled duplex oligonucleotide containing a single lesion was incubated at 37°C with increasing amounts of extract for 2 h. The reaction products were separated by electrophoresis through 20% denaturing (7 M urea) polyacrylamide gels. The reactions were stopped and then analyzed by PhosphorImager. (A) The incision of 8-oxoG, (B) the incision of TG, (C) the incision of 5OHC and (D) AP sites. The symbols used are: circles for RWPE-1, triangles for PC-3 and squares for DU-145. The results were obtained from three independent experiments and presented as mean ± SEM.
However, the repair of TG and 5OHC is up-regulated in the mitochondrial extracts of DU-145 in contrast to the results for the nuclear extracts. Similar to the nuclear extract results, the incision of TG and 5OHC was defective in PC-3 cells.

The integrity of mitochondrial extracts was determined by measuring cytochrome c oxidase activity. Cytochrome c oxidase activity in DU-145 extracts was similar to that of the control cell line, whereas, PC-3 mitochondrial extracts were ~2.5 less active than those from RWPE-1 (Table I).
Assessment of expression levels of BER enzymes

We used western blot analysis to determine whether the changes in incision of 8-oxoG, TG and 5OHC in PC-3 and DU-145 cell lines have any correlation with the expression of BER enzymes. The levels of OGG1 1a and NTH1 were comparable with that of the wild-type both in nuclear and whole cell extracts (Figure 7A and C). However, OGG1 2a was reduced in both PC-3 and DU-145 mitochondrial extracts (Figure 7B) while mitochondrial NTH1 was reduced in PC-3 cells and normal in DU-145 cells (Figure 7B). Nuclear and mitochondrial extracts from PC-3 and DU-145 cells expressed wild-type APE1 levels (Figure 7A and B).

Assessment of expression levels of antioxidant enzymes

Levels of antioxidant enzymes may be critical in the cellular response to oxidative stress. In order to determine whether there was a correlation between oxidative DNA damage and the levels of antioxidant enzymes, we examined protein expression of the common antioxidant enzymes. There was a significant increase in SOD1 in both prostate cancer cell lines (Figure 8A and C) and a significant increase in catalase in the PC-3 cell line (Figure 8A and D). We also observed a significant decrease in GPx protein expression in both PC-3 and DU-145 (Figure 8A and E). Analysis of the mitochondrial antioxidant enzyme, SOD2, showed that it was reduced in both cancer cell lines (Figure 8B and F). Our data show that there are altered levels of the detoxifying enzymes studied. These changes in the levels of the antioxidant enzymes may result in altered cellular response to oxidative stress.

Genetic analysis of OGG1 gene

To determine whether the defective incision of 8-oxoG in the prostate cancer cell lines may be associated with the genetic status of the OGG1 gene, we performed sequence analysis of OGG1 gene in RWPE-1, PC-3 and DU-145 cells. Two polymorphic sites were found in the prostate cancer cell lines. One is located in exon 7 at nucleotide 29 resulting in the substitution of guanine for cytosine. This polymorphism results in a change from serine to cysteine at the protein level. This occurs only in the nuclear isoform OGG1 1a. The second polymorphic site is located in intron 4 and as expected does not result in an amino acid change (Table II).

Discussion

We report several critical findings relating to oxidative DNA damage and repair in two well-studied prostate cancer cell lines, PC-3 and DU-145: increased oxidative DNA damage, reduced incision of major oxidatively induced DNA base lesions, altered expression of proteins involved in repair of oxidative DNA damage and detoxification of ROS, and genetic polymorphisms in the OGG1 gene. Genomic DNA analysis using LC/MS and GC/MS revealed significantly higher levels of 5OHC in irradiated cells (Figure 1C, III) relative to the unchallenged cells (Figure 1B, III) in both prostate cancer cell lines. We also observed significantly higher levels of 8-oxoA and 8-oxoG in irradiated PC-3 cell line (Figure 1C, I--II, clear bar) relative to the unchallenged cells (Figure 1B, II, III) in both prostate cancer cell lines. Evidently, 8-oxoA levels were remarkably high in irradiated PC-3 cells (2--3-fold) (compare Figure 1C, I and B, I). These results suggest that the increased oxidative DNA damage in the prostate cancer cells after irradiation may be overwhelming to the DNA repair

<table>
<thead>
<tr>
<th>Cell line</th>
<th>μmol/min/mg protein (mean ± SEM)</th>
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<tbody>
<tr>
<td>RWPE-1</td>
<td>14.7 ± 2.2</td>
</tr>
<tr>
<td>PC-3</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>DU-145</td>
<td>14.1 ± 4.1</td>
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machinery thereby resulting in insufficient repair in PC-3 and DU-145 cells. Higher levels of 8-oxoA and 8-oxoG in benign prostatic hyperplasia, prostatic malignant tissue and tumors from other tissues have been reported (12,16,17,30,47). We must however, emphasize that although this increased damage has been demonstrated in various forms of cancer, the contribution of this damage to the development of cancer is still unclear.

Incision of three major DNA lesions, 8-oxoG, TG and 5OHC by nuclear extracts from both PC-3 and DU-145 was markedly reduced relative to the non-malignant prostate cell line RWPE-1 (Figures 2A--C, 3A--C and 4A--C). These results suggest that the BER pathway that is responsible for repairing the major DNA lesions may be defective in PC-3 and DU-145 prostate cancer cell lines. This reduction in DNA repair activity may be due to a number of factors including: increased oxidative DNA damage, low levels of DNA repair enzymes, mutations in oxidative DNA repair genes, altered levels of antioxidant enzymes and mutations in the tumor suppressor gene, p53. The reduced incision of the oxidative DNA lesions by the nuclear extracts was not due to reduced levels of OGG1 or

Table II. Analysis OGG1 gene polymorphisms in prostate cell lines

<table>
<thead>
<tr>
<th>Position in OGG1 gene</th>
<th>GeneBankb</th>
<th>RWPE-1</th>
<th>PC-3</th>
<th>DU-145</th>
</tr>
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<tbody>
<tr>
<td>EX5-15 (intron 4)c</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>C/G</td>
</tr>
<tr>
<td>EX7+29 (exon 7)d</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>C/G</td>
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</table>

aSingle polymorphisms in OGG1 gene found in prostate cancer cell lines, PC-3 and DU-145. The polymorphism in exon 7 is associated with presence of cysteine instead of serine in codon 326 in isoform 1a of OGG1 protein (C, cytosine; G, guanine).

bReference sequence from GeneBank, accession number NT_005927.

cEX5-15, position 15 nt before 1st nucleotide in exon 5.

dNucleotide 29 in exon 7.
NTH1 since western blot analysis revealed normal expression levels of these two enzymes (Figure 7A). The expression of the recently characterized DNA repair enzymes, NEIL1 and NEIL2 (48,49) was similar in both prostate cancer cell lines compared with RWPE-1 (data not shown) suggesting that the reduced incision of 5OHC and TG that we observed in both PC-3 and DU-145 cannot be explained by altered NEIL1 and NEIL2 activity. While our data show defects in BER in these cell lines, the mechanism may not be connected with a reduction in expression of BER enzymes, as is the case for MMR in prostate cancer. Human MMR proteins, MSH2 and MLH1 are reduced or absent in prostate cancer cell lines, DU-145, PC-3 and LNCaP (34-36) and in prostate cancer patients relative to controls (33). Furthermore, reduced expression of the MMR proteins have been shown to correlate with decreased MMR activity (34) suggesting a link between prostate cancer and defective MMR in the cells studied. It has been shown that APE1 stimulates the activity of OGG1 and NTH1 (50-52).

Kelley and co-workers showed that APE-1/Ref-1 is increased in prostate cancer tissue (53). We therefore analyzed the levels of APE-1 in PC-3 and DU-145 nuclear and mitochondrial extracts. We found that APE1 levels in both prostate cancer cell lines were comparable with the wild-type level (Figure 7A and B). This suggests that the defective incision of 8-oxoG, TG and 5OHC in PC-3 and DU-145 cells cannot be due to ineffective direct stimulation by APE1. Kelley et al. analyzed APE1 expression in tissues obtained from patients, whereas our studies were performed using cell-free extracts. This difference in approach may explain the differences in APE1 expression levels found in these studies. In addition, APE1 expression in PC-3 and DU-145 correlates with wild-type incision activity of AP site (Figure 3D and 4D). Thus, the reduced incision of DNA lesions that we observed is unlikely to be associated with defective APE1. The efficient repair of AP sites reported here is not surprising since this lesion is repaired by the abundant AP endonucleases and also by the lyase activities of bifunctional DNA glycosylases (reviewed in refs 54,55).

We were able to detect mRNAs of the BER enzymes studied in the three cell lines by RT-PCR analysis. This analysis showed that the mRNA level of the BER enzymes were not altered significantly (data not shown). The reduced incision of DNA lesions may be associated with genetic defects in OGG1 and NTH1, the genes that encode DNA glycosylases responsible for repairing 8-oxoG and TG/5OHC, respectively (56-58). Available evidence in the literature has shown reduced incision of 8-oxoG in extracts from mutant mice carrying one disrupted Ogg1 gene copy whereas, total Ogg1 knockout mice were almost devoid of 8-oxoG incision activity (59,60). Reduced incision of TG has also been reported in Nth1 knockout mice (61).

The increased levels of ROS in the mitochondria associated with the electron transport system and high levels of oxidative damage in the mitochondrial extracts to the nuclear DNA (19) prompted us to examine DNA repair activity in mitochondria from the prostate cancer cell lines. Consistent with reduced expression of OGG1 2a in mitochondrial extracts from PC-3 and DU-145 cell lines (Figure 7B), the incision of 8-oxoG was significantly reduced in the mitochondrial extracts of both prostate cancer cell lines relative to RWPE-1 (Figures 5B and 6A). A similar reduction in 8-oxoG repair has been reported previously in mitochondrial extracts from the breast cancer cell lines, MCF-7 and MDA-MB-468 (31). Furthermore, mitochondrial extracts of PC-3 origin exhibited a 2-3-fold reduction in TG and 5OHC incision activity (Figures 5B and C and 6B and C). Cytochrome c oxidase activity was reduced in PC-3 relative to RWPE-1, suggesting abnormal mitochondrial function in this particular cell line (Table I). It is unlikely that the defective incision of 8-oxoG, TG and 5OHC in PC-3 mitochondria is caused by defective mitochondria since incision of 8-oxoG by DU-145 mitochondrial extracts is defective despite wild-type cytochrome c oxidase activity (Figures 5A and 6A and Table I). Moreover, the levels of mitochondrial oxidative DNA damage repair enzymes, OGG1 2a and NTH1 were reduced in PC-3 mitochondria (Figure 7B), implicating enzymatic activity of the two enzymes in the defective repair mechanism. Interestingly, the incision of both TG and 5OHC was increased in mitochondrial extracts of DU-145 origin relative to RWPE-1 (Figures 5B and C and 6B and C). Backup TG and 5OHC repair activity may account for the increased incision of TG and 5OHC in mitochondria of DU-145 cells. There is evidence of backup TG/5OHC repair activity designated TGG1 in mitochondria (61). Presently, it is not known whether NEIL1 and NEIL2 exist in the mitochondria (62). Altered mitochondrial NTH1 and OGG1 2a functional activity may be due to mutation in the corresponding genes. This interpretation is supported by published data showing loss of 8-oxoG activity in mitochondrial extracts from Ogg1 knockout mice (63). There are a number of papers in the literature showing impaired mitochondrial targeting of OGG1 due to mutation in mitochondrial localization sequence (MLS) in kidney cancer (64,65). It is also feasible for mutations to occur in the MLS of NTH1 and mitochondrial isoforms of OGG1 preventing effective translocation of these enzymes to the mitochondria (66).

Mutations and genetic polymorphisms in DNA repair enzymes have been associated with genetic instability and carcinogenesis (reviewed in refs 67-69). A common OGG1 polymorphism involving Ser326 has been associated with increased risk of lung (69,70) and oropharyngeal cancers (69,71). Our genetic analysis of the OGG1 gene in prostate cancer cell lines PC-3 and DU-145 revealed the presence of two polymorphic changes, one in intron 4 and the other in exon 7 (Table II). Although, the intron 4 polymorphism has been associated with increased incidence of prostate cancer (18), its contribution to the development of prostate cancer is unknown. The polymorphism in exon 7 (Ser326 to Cys326) was found in OGG1 1a but not in the mitochondrial isoform, OGG1 2a. The OGG1 Cys326/Cys326-phenotype has been associated with increased susceptibility to oxidative stress and ROS resulting in elevated oxidative DNA damage and cancer risk (71,72). Incision activity of 8-oxoG by recombinant OGG1 1a proteins with either serine or cysteine at position 326 was indistinguishable (73). It is possible for the observed polymorphisms to alter the functional activity of OGG1 by interfering with potential sites for post-translational modification processes. Such changes including phosphorylation and acetylation have been reported in the literature for OGG1 (74) and other BER proteins (75-79). Although, sequence analysis of the OGG1 gene revealed the presence of Ser326Cys polymorphism in both prostate cancer cell lines, there were no other mutations that would adversely affect the function of OGG1 enzyme.

In examining the DNA repair capacity of these cell lines, it is necessary to consider their p53 status: PC-3 is p53-null and DU-145 has two different mutations in p53 (80,81). The p53 status of the cells may play a role in the defects in BER that we have identified because several groups have shown that p53 is...
involved in BER. There are studies showing that involvement of p53 in BER requires an intact core and C-terminal domain of the protein (82). Zhou et al. (83) reported that p53 protein stimulates BER in vitro and that BER activity is correlated with cellular levels of the protein. There is also evidence that p53 mutant cells have diminished BER capacity (84). If p53 is involved in the defective repair process, its role is probably achieved through protein-protein interactions.

An assumption could be made that increased oxidative DNA damage resulting from ROS would modulate the level of cellular antioxidant enzymes in order to minimize DNA damage. Our studies have revealed that SOD1, the enzyme that converts superoxide anion to hydrogen peroxide, was up-regulated in both PC-3 and DU-145 (Figure 8A and C). However, catalase was over-expressed in only one of the cancer cell line, PC-3 (Figure 8A and D). Furthermore, the level of GPx, an enzyme that converts hydrogen peroxide to water, was reduced in both prostate cancer cell lines (Figure 8A and E). The level of SOD2, the other dismutase that converts superoxide anion to hydrogen peroxide in the mitochondria was reduced in PC-3 and DU-145 mitochondrial extracts relative to RWPE-1 (Figure 8B and F). The uncontrolled expression of antioxidant enzymes in both prostate cancer cell lines may result in altered cellular antioxidant activity. Our results on the expression of antioxidant enzymes in PC-3 and DU-145 are in agreement with those published by Jung et al. (86). Altered expression of SOD1 and SOD2 may result in increased formation of ROS through processes such as Fenton reaction that would give rise to elevated levels of oxidative DNA damage (87, 88). Hydrogen peroxide, one of the damaging agents so produced is usually converted to water by catalase. The level of catalase in PC-3 cells was increased, probably to increase protection from hydrogen peroxide in these cells.

Antioxidants and detoxifying enzymes may influence DNA damage and DNA repair responses. Work by Seo et al. showed that selenium, the essential trace element and constituent of antioxidant enzymes can also induce DNA repair and protect cells from oxidative DNA damage (84). It was found that selenium activates p53 through a redox pathway that requires APE-1/Ref-1 (85). Ongoing prostate cancer clinical trials are testing the hypothesis that selenium compounds can prevent prostate cancer or influence its progression (89-91). Our findings in these prostate cancer cell lines that levels of oxidative DNA damage are persistently elevated after IR damage, antioxidant enzyme levels are altered, and that BER is defective, suggest that selenium compounds may be successful as a preventive therapy for prostate cancer.

We have found a defect in the first step of BER of oxidative DNA base lesions at the nuclear and mitochondrial level in two prostate cancer cell lines. However, our data suggest that the etiologic mechanisms underlying this defect are multifactorial and implicate various genes and proteins involved in the cellular response to DNA damage and repair. Our findings further lend support to the idea that oxidative DNA damage and repair should be considered as critical factors in prostate cancer development; however, this hypothesis must be pursued further using primary benign and malignant tissue.

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References


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