Potential Biomarker for Early Risk Assessment of Prostate Cancer

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BACKGROUND. Catechol estrogen quinones (CEQ) derived from 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) react with DNA to form depurinating—N7Gua and—N3Ade adducts. This damage leads to mutations that can initiate breast and prostate cancer. To determine whether this damage occurs in humans, urine samples from men with prostate cancer and benign urological conditions, and healthy controls were analyzed. The objective was determining whether any of the cancer patients had formed the depurinating 4-OHE1(E2)-1-N3Ade adducts.

METHODS. The adducts were extracted from samples by using affinity columns equipped with a monoclonal antibody developed for detecting 4-OHE1(E2)-1-N3Ade adducts. Eluted extracts were separated by capillary electrophoresis with field-amplified sample stacking and/or ultraperformance liquid chromatography. Absorption/luminescence spectroscopies and mass spectrometry were used to identify the adducts.

RESULTS. 4-OHE1-1-N3Ade was detected at higher levels in samples from subjects with prostate cancer (n = 7) and benign urological conditions (n = 4) compared to healthy males (n = 5).

CONCLUSION. This is the first demonstration that CEQ-derived DNA adducts are present in urine samples from subjects with prostate cancer. Prostate 66: 1565–1571, 2006.

KEY WORDS: estrogens; DNA adducts; risk assessment; biomarkers; prostate cancer

INTRODUCTION

The natural estrogens, estrone (E1), and estradiol (E2), are metabolized at the 2- or 4-position with the formation of catechol estrogens, which, in turn, are metabolically oxidized into catechol estrogen quinones (CEQ). The latter have been implicated in the etiology of human breast cancer by various research studies [1–5]. The reaction of the CEQ, in particular CE-3,4-Q, with DNA forms the depurinating 4-OHE1(E2)-1-N3Ade and 4-OHE1(E2)-1-N7Gua adducts [1,5,6]. These two adducts constitute >99% of the total adducts formed. The CE-2,3-Q form only small amounts of the depurinating 2-OHE1(E2)-6-N3Ade adducts [7]. These adducts generate apurinic sites that may lead to cancer-initiating mutations [8–11], which transform cells [12,13], thereby initiating cancer.
Exposure to estrogens is a well-established risk factor for breast cancer [14]. The possible role of estrogens in prostate cancer is thus far less well established than in breast cancer. Limited evidence exists of an association between estrogens and risk of prostate cancer [15]. One important piece of evidence is the higher level of circulating estrogens observed in African–American men, who have a twofold higher risk of prostate cancer, compared to European–American men [16]. More direct evidence in support of the role of estrogens in prostate carcinogenesis comes from experiments using Noble rats treated with testosterone plus E2 [17,18]. This combined treatment induces ductal adenocarcinoma of the prostate in 100% of the rats [17], whereas treatment with only testosterone causes prostate cancer in only 40% of the rats. Treatment with 5α-dihydrotestosterone, which unlike testosterone cannot be converted to E2, results in only a 4% incidence of prostate cancer. These carcinomas have been suggested to arise from estrogen-induced initiation and testosterone-produced promotion of the prostate tissue [15].

In our earlier work, CE-3,4-Q-derived DNA adducts were identified in tissue extracts from breast cancer patients [19]. In this case, samples were analyzed by CE interfaced with room-temperature absorption and low-temperature (laser-excited) phosphorescence spectroscopies. The level of the 4-OHE1-1-N3Ade in the breast tissue extracted from a patient with breast carcinoma (8.40 ± 0.05 pmol/g of tissue) was larger by a factor of about 30 than the level in the breast tissue sample from a woman without breast cancer (0.25 ± 0.05 pmol/g of tissue) [19]. Although more breast tissue samples from women with and without breast cancer need to be studied, these results suggested that the -N3Ade adducts could serve as biomarkers to predict the risk of breast cancer.

To determine whether this type of DNA damage occurs in men, urine samples from subjects with prostate cancer, benign tumors, benign prostate hyperplasia, and a urological condition, as well as healthy males, were analyzed in a blind study. A primary objective was determining whether any of the subjects had formed 4-OHE1(E2)-1-N3Ade (Fig. 1), one of the major adducts formed by CE-3,4-Q. We showed that CE-3,4-Q-derived DNA adducts are present in human urine samples, and that their identification can be accomplished by a combination of techniques.

**METHODS AND MATERIALS**

**Study Population and Samples**

Single spot urine samples were obtained from two sources: (1) Healthy controls: samples were obtained from men who had not been diagnosed with prostate cancer (Table I). (2) Urology clinic: samples were obtained from patients being seen at the urology clinic of Johns Hopkins Bayview Medical Center (Table I). Of these, two had benign urological conditions (erectile dysfunction or benign prostatic hyperplasia), two were undergoing prostate biopsy, and six were meeting with a surgeon to discuss surgery for newly diagnosed prostate cancer. Both of the patients undergoing prostate biopsy had urine collected prior to the biopsy; in both cases the biopsy was negative for prostate cancer. Urine samples were collected in a sterile container placed on dry ice and shipped untreated to the laboratory of Dr. Jankowiak by overnight mail.

**Caution**

CEQ are hazardous chemicals and should be handled carefully in accordance with NIH guidelines.

**Chemicals and CEQ-DNA Adduct Standards**

4-OHE1 and 4-OHE2 were synthesized according to Dwivedy et al. [20]. The 4-OHE1- and 4-OHE2-derived DNA adduct standards were synthesized as previously described [6,21]. Structural analysis of the above standards was accomplished via NMR and mass spectrometry (MS) [6,21]. Ultra-pure grade glycerol was obtained from Spectrum Chemical (Gardena, CA). The purity of standards for CEQ-derived DNA adduct standards, originally separated by HPLC, was verified in our laboratory by capillary electrophoresis (CE) and low-temperature luminescence spectroscopy. CEQ-derived DNA adducts, which are heat- and oxygen-sensitive, were kept for longer-term storage at −80°C under an inert atmosphere (N2 or Ar). Samples were
dissolved in methanol:buffer (80:20), with the following buffer content: 0.1 M ammonium acetate and 1 mg/L ascorbic acid in nanopure water, pH 4.5. Tris[hydroxymethyl] aminomethane was purchased from Fisher Scientific (Fairlawn, NJ). Phosphoric acid and polyoxyethylene 8 cetyl ether (C16E8) were obtained from Sigma-Aldrich (St. Louis, MO).

**Monoclonal Antibodies (MAb)**

Ovalbumin (OA) and keyhole limpet hemocyanin (KLH) were purchased from Pierce Biotechnology, Inc., Rockford, IL. Delbecco’s Modified Eagle medium and horse serum were purchased from Mediatech, Inc., Herndon, VA, and Valley Biomedical, Inc., Winchester, VA, respectively. \(\text{N}-(9\text{-Fluorenyl})\) methoxycarbonyl multiple antigenic peptides (Fmoc MAP) resin was purchased from Applied Biosystems, Foster City, CA. Well-established methods [22] were used to generate an immune response in mice. The 4-OHE\textsubscript{1}-1-N3Ade linker (synthesized similarly to the 4-OHE\textsubscript{1}-2-NAcCys-16z,\(\beta\)-MCC linker [23, unpublished results]) was conjugated to KLH and used in an immunization protocol with 25 \(\mu\)g of antigen/mouse/injection using Freund’s incomplete adjuvant. Serum titers were established using 4-OHE\textsubscript{1}-1-N3Ade conjugated to OA. Mouse spleen cells were fused with an equal number of SP2/O cells (40 million of each) and plated in 16 \times 96-well microtiter plates. When hybridoma wells started to turn yellow, the plates were screened using an ELISA assay. Five hundred nanograms of OA-4-OHE\textsubscript{1}-1-N3Ade linker in binding buffer (100 mM NaHCO\textsubscript{3}, pH 9.3) was used to coat each well of a Nunc Maxisorb plate. Hybridoma cell lines were screened using 4-OHE\textsubscript{1}-1-N3Ade conjugated to OA. Since there is no immunological cross reactivity between KLH and OA, positive hybridoma cell lines secreting antibody against 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade could be rapidly identified using OA-4-OHE\textsubscript{1}-1-N3Ade.

Affinity columns were developed and used to purify MAb against 4-OHE\textsubscript{1}-1-N3Ade, by passing 3 mL of medium from the selected hybridoma over the column. Then the columns were washed with 50 mL of PBS and antibody eluted with 100 mM acetic acid, pH 2.5. MAbs were concentrated by centrifugal filters (Millipore Corporation, Bedford, MA) using an Amicon Ultra 100,000 molecular weight cut. This allowed separating solutes from low MW compounds. The eluted antibody was isotyped using a kit specific for mouse IgG antibody, confirming that the antibody was of mouse origin and not from the horse serum used in growing the cells. This purified MAb (assigned as 15G8) was immobilized on an agarose bead column (Aminolink kit, Pierce Inc.) and used to detect 4-OHE\textsubscript{1}-1-N3Ade in PBS buffer that was spiked with various concentrations of -N3Ade adduct (data not shown). We have demonstrated that the 15G8 MAb binds with high affinity to 4-OHE\textsubscript{1}-1-N3Ade adducts \((K_a = 10^8/M)\), highly discriminating against a large spectrum of closely related CEQ-derived metabolites [unpublished results]. It has

<table>
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\(\textsuperscript{a}\)NA, data not available; TUNA, trans-urethral needle ablation (a minimally invasive treatment for BPH).
\(\textsuperscript{b}\)Not measured.
been used here for detecting 4-OHE$_1$(E$_2$)-1-N3Ade adducts in urine samples. The immunoaffinity columns were used to capture and preconcentrate the hapten of interest from the urine samples.

**Capillary Electrophoresis**

The analysis of urine extracts was done using a P/ACE MDQ CE system (Beckman/Coulter, Fullerton, CA) with a photodiode array (PDA) detector for simultaneous detection of electropherograms and UV absorption spectra of separated analytes. A bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with 21 cm effective length and 31.2 cm total length (75 μm I.D. and 360 μm O.D.) was used. The running buffer was 0.5% C$_{16}$E$_8$ in 0.25 mM Tris-phosphate (pH 3.5). Before injection, the solvent in the sample was evaporated under vacuum; the sample residue was then diluted with the same volume of 75 μM H$_3$PO$_4$ solution. The same extract was also separated with an ISCO (Lincoln, NE) model 3140 Electropherograph System, and re-analyzed by low-temperature luminescence spectroscopy. The CEQ-derived DNA adduct standards, and the extracts from the immunoaffinity columns were analyzed with field-amplified sample stacking (FASS) conditions. FASS [24,25] was used for analyte preconcentration. To achieve reproducible and accurate stacking results, a water plug was injected into the capillary before the sample (at 0.2 psi for 12 sec) followed by the electrokinetic injection of urine extract sample at +10 kV for 30 sec. The applied electric field for separation was 480 V/cm, and the running temperature was 25°C. The absorption detection was set at the PDA mode to obtain the electropherograms at different UV wavelengths and the absorption spectra of the separated analytes. After each run, the capillary was rinsed with 0.1 M NaOH for 2 min, and running buffer for 5 min. Electropherograms were obtained in the absorbance mode. CE-separated DNA adducts were identified based on the characteristic migration times and corresponding absorption spectra. Various detection wavelengths for the CE electropherograms were utilized (e.g., 214, 260, and 276 nm).

**Luminescence and Absorption Spectroscopy**

Luminescence spectra were obtained using an excitation wavelength of 257 nm with a Lexel 95-SHG-257 CW laser. Emission was dispersed by a Model 218 0.3-m monochromator (McPherson, Acton, MA), equipped with a 300 G/mm grating, providing a resolution of ~1 nm. Spectra were detected with an intensified CCD camera (Princeton Instruments, Trenton, NJ) using gated and non-gated modes of detection. A fast shutter, operated by a Uniblitz driver control (model SD-12 2B), was synchronized with the CCD camera (ICCD-1024 MLDG-E1) and used for time-resolved phosphorescence measurements. Using this setup, time-resolved phosphorescence spectra could be measured in 0.5 sec intervals with a gate width of 0.5 sec. To ensure good glass formation in off-line spectroscopic measurements, glycerol (50% by volume) was added to the samples in buffer just prior to cooling to 77 K in a liquid nitrogen optical cryostat with suprasil optical windows. Samples (ca. 20 μL) were contained in suprasil tubes (2-mm i.d.). All spectra were background corrected.

**UPLC and Mass Spectrometry**

The sample was diluted 1:10 in 50% methanol in water and analyzed twice by ultraperformance liquid chromatography (UPLC). The parent-daughter transition used in analysis was $m/z = 420.1 > m/z = 296.0$, which was analyzed with a Waters Acquity binary solvent manager and Sample Manager Micromass and a Quattro Micro mass spectrometer. The gradient was 80% (H$_2$O, 0.1% formic acid), 20% (acetonitrile, 0.1% formic acid) to 79% (H$_2$O, 0.1% formic acid), 21% (acetonitrile, 0.1% formic acid) in 4 min, to 45% (H$_2$O, 0.1% formic acid), 55% (acetonitrile, 0.1% formic acid) in 6 min. Standard curves were established and quantitation was carried out using QuanLynx v4.0.

**Statistical Methods**

Mean levels of 4-OHE$_1$(E$_2$)-1-N3Ade adduct were compared between patient groups using the Student's $t$-test and analysis of covariance, the latter to permit adjustment for age. Linear regression was used to evaluate the correlation between 4-OHE$_1$(E$_2$)-1-N3Ade adduct levels and prostate specific antigen (PSA). All analyses were performed using SAS version 9.1 software (SAS Institute, Cary, NC).

**RESULTS AND DISCUSSION**

**Detection of 4-OHE$_1$(E$_2$)-1-N3Ade Adducts in Urine Samples**

Urine samples (20 mL each) from 16 subjects were analyzed in blind studies using different detection methods, as described above. Selected characteristics of the subjects are presented in Table I. All specimens were initially separated using affinity column purification, that is, the adducts of interest were extracted from the urine samples using home-built columns equipped with the 15G8 MAb. Eluted extracts from immunoaffinity columns were analyzed by laser-excited low-temperature phosphorescence spectroscopy and UPLC interfaced with tandem mass spectrometry (LC/MS/MS).
In addition, urine samples, after lyophilization and methanol extraction, were pre-concentrated and analytes therein were separated by CE with FASS and detected by absorbance-based electropherograms. A spiking procedure with synthesized DNA adduct-standards and absorption/luminescence spectroscopies were used to identify the biomarkers of interest.

In this initial study, the 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N7Gua adducts was not analyzed for two main reasons. First, development of a MAb to the N7Gua adduct would have delayed the study by 6 months or more. Second, studies on the mutagenicity of 4-OHE\textsubscript{2} and E\textsubscript{2}-3,4-Q reveal only A to G transition mutations \cite{8–11}. These results imply that the instantaneous depurination of N3Ade adducts generates error-prone repair of de-adenylated sites \cite{8}.

In Figure 2, the bars in row #1 correspond to the integrated (normalized) area of the absorbance-based CE electropherogram peaks assigned to 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade. Only the samples from the urology patients contained 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade adduct. Among the urology patients, there were no significant differences in mean 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade adduct levels between the patients with prostate cancer (mean = 86.9 pmole/ mg) or the patients with benign urologic conditions (mean = 108.5 pmole/mg), \(P = 0.665\). When the comparison was adjusted for age, there was no significant difference, \(P = 0.385\). Among the eight subjects with recent PSA results, there was no correlation between 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade adduct levels and PSA (\(P = 0.583\)). The adduct level (normalized to creatinine concentration) varied from sample to sample with concentration levels of about 15–240 pmole per mg of creatinine. The identity of the adducts in samples #1–11 in row #1 was confirmed by low-temperature (77 K) luminescence spectroscopy, as shown by the bars in row #2 of Figure 2, which correspond to the integrated (normalized) area of the low temperature phosphorescence spectra obtained for urine samples #1–11 eluted from the immunoaffinity columns. Examples of the phosphorescence spectra obtained for samples #1, 4, and 6 are shown in the right inset of Figure 2; the red spectrum overlapping with the phosphorescence spectrum of sample #6 is that of the standard adduct. In fact, all spectra measured for samples #1–11 revealed emission identical to the phosphorescence spectrum of the 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade adduct standard \cite{19} (data not shown), thus proving again that the analyte eluted from the 15G8-MAb based column corresponds to 4-OHE\textsubscript{1}(E\textsubscript{2})-1-N3Ade. The amount of this adduct in samples #1–11 using low temperature phosphorescence-based calibration curves was about 10–150 pmole per mg of creatinine, depending on the sample. With a detection limit of about 10\textsuperscript{−6} M \cite{19}, 4-OHE\textsubscript{1}-1-N3Ade adducts were not detected in samples #12–16 from healthy control subjects, in agreement with the CE/FASS results. The observed emission intensity was near the background level.
Finally, LC/MS/MS was used for further validation of the above findings. That is, all samples eluted from the immunoaffinity columns were also analyzed by LC/MS/MS. The amounts of the adducts are reported in row #3 of Figure 2. Only samples #1–11 revealed the presence of the 4-OHE$_1$-1-N3Ade adducts. Although similar adduct distribution is observed in all samples using the three different methodologies, the relative adduct concentration observed in elutions from immunoaffinity columns was somewhat smaller than that observed by CE/FASS with absorbance detection. This is not surprising, since recovery from a typical column is 70–80% [19,24]. The UPLC chromatogram obtained for sample #11 is shown in the left inset of Figure 2; the main peak near 2 min in the chromatogram corresponds to the 4-OHE$_1$-1-N3Ade adduct, indicating that the eluent from the immunoaffinity column was relatively pure. The spectrum corresponds to the daughter ions, m/z 135.9 and 296.0, which were obtained from fragmentation of the adduct parent ion, m/z 420.1. Note that 4-OHE$_1$-1-N3Ade (and not 4-OHE$_2$-1-N3Ade) was detected in the urine of all subjects from the urology clinic, but none of the healthy male controls. There were no significant differences between levels from subjects with benign urologic conditions and those with prostate cancer. However, the three highest levels were in a patient with prostate cancer and the two patients with negative biopsies. Thus, it is conceivable that the patients with negative biopsies may harbor undetected prostate cancer, or be at increased risk for prostate cancer. To date, neither man has been diagnosed with prostate cancer at follow-up more than 6 months after the negative biopsy. The sample from the patient with BPH was also among the higher values, indicating that BPH might be related to CEQ-induced DNA damage. However, LC/MS/MS did not reveal the presence of any 4-OHE$_1$-1-N3Ade adduct in samples #12–16 obtained from healthy individuals, in perfect agreement with the low temperature phosphorescence and CE/FASS studies. Urine samples from several other healthy subjects tested by low-temperature phosphorescence (data not shown) also did not have any detectable 4-OHE$_1$-1-N3Ade adduct.

**CONCLUSIONS**

Evidence has been obtained from studies of men with and without prostate cancer that CEQ-derived DNA adducts are formed in humans. We think this is the first demonstration that CEQ-derived DNA adducts are present in urine samples from subjects with prostate cancer. Given the limited sample size, it is difficult to draw conclusions from the lack of significant differences between adduct levels in prostate cancer cases and men with benign urologic conditions. Larger sample sizes are needed to determine whether the presence of depurinating adducts in human urine samples could be used as a risk factor for prostate cancer. Such studies will also allow us to determine the utility of these biomarkers as surrogate endpoints to investigate the hypothesis that metabolically activated endogenous estrogens are involved in initiating prostate cancer.

**ACKNOWLEDGMENTS**

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