Effect of Cigarette Smoking on Urinary 3,N\textsuperscript{4}-Ethenocytosine Levels Measured by Gas Chromatography/Mass Spectrometry

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Etheno DNA adducts are DNA damages derived from exogenous carcinogens as well as endogenous lipid peroxidation and oxidative stress. Elevated levels of etheno DNA adducts were found in cancer-prone tissues and blood samples, suggesting that these promutagenic lesions correlate with risk of cancers. We previously reported the detection of 3,N\textsuperscript{4}-ethenocytosine (εCyt) in the urine samples of two smokers using the isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS) assay (Chen et al., 2001, Chem. Res. Toxicol. 14, 1612–1619). Since smokers are found to have elevated levels of lipid peroxidation and oxidative stress, we examined the association between urinary εCyt levels with cigarette smoking. Among the 23 samples analyzed, the average concentration of urinary εCyt in smokers was significantly higher than that of nonsmokers, 2.65 ± 4.0 versus 0.61 ± 0.90 ng/kg/g creatinine (p = 0.03). Albeit the number of subjects is limited, the results indicate that the measurement of εCyt in human urine may provide a useful non-invasive biomarker for oxidative DNA damage and cancer chemoprevention studies.

Key Words: biomarker; cigarette smoking; 3,N\textsuperscript{4}-ethenocytosine; gas chromatography; mass spectrometry; urine

Etheno DNA adducts, including 1,N\textsuperscript{6}-ethenoadenine (εAde), 3,N\textsuperscript{4}-ethenocytosine (εCyt), and N\textsuperscript{2},3-ethenoguanine (N\textsuperscript{2},3-εGua), have been known to derive from exogenous environmental chemicals, such as ethyl carbamate (urethane), occupational carcinogen vinyl chloride, and their metabolites (Fernando et al., 1996; Guengerich, 1992). There has been growing interest in the formation of etheno adducts from endogenous lipid peroxidation end products (Chung et al., 1996; Nair et al., 1999). Trans-4-hydroxy-2-nonenal is one of the major α,β-unsaturated aldehydic products of lipid peroxidation (Wu and Lin, 1995). 2,3-Epoxy-4-hydroxynonalal, the possible metabolite of trans-4-hydroxy-2-nonenal, might be an important contributor to the endogenous formation of etheno DNA adducts (Chen and Chung, 1995; Chen et al., 1998a, 1998b). Etheno adducts are also formed in nitric oxide–induced lipid peroxidation and are thus associated with DNA damage due to chronic infections and inflammation (Nair et al., 1998b). A recent in vitro study demonstrated that the decomposition of lipid hydroperoxides by ascorbate might be another possible mechanism for in vivo etheno adducts formation (Lee et al., 2001).

Elevated levels of εAde and εCyt were found in cancer-prone tissues, such as in the livers of patients with the genetic metal storage disorders Wilson’s disease and primary hemochromatosis, compared to normal individuals (Nair et al., 1998a). An epidemiological study showed that the levels of εAde and εCyt in the white blood cells of female volunteers on a high omega-6 polyunsaturated fatty acid diet were much higher than the control subjects (Nair et al., 1997). Increased levels of εAde and εCyt were found in colonic polyps compared to unaffected colon tissue in familial adenomatous polyposis patients (Schmid et al., 2000). Since lipid peroxidation is implicated in tumorigenesis, it has been postulated that DNA damage caused by these aldehydic products plays an imperative role in the initiation and progression of carcinogenesis (Bartsch et al., 1999; Chung et al., 1993, 1996; Nair et al., 1999; Schmid et al., 2000). Etheno adducts block DNA polymerases, cause misincorporations, and produce various degrees of mutations in bacteria and mammalian cells (Barbin 2000). Although εCyt induces low mutation frequency in bacteria (Basu et al., 1993; Moriya et al., 1994; Pandya et al., 1996), it is highly mutagenic in mammalian cells (Moriya et al., 1994; Pandya et al., 1996).

Etheno adducts can be repaired by separate base excision repair enzymes, and their repair efficiencies varies. Among human DNA glycosylases capable of repairing the etheno adducts, a mammalian m’A-DNA glycosylase can excise εAde and N\textsuperscript{2},3-εGua but not εCyt (Matijasevic et al., 1992). Human mismatch-specific thymine-DNA glycosylase (Hang et al., 1998; Saparbaev and Laval, 1998) and a DNA glycosylase from human He La cells (Hang et al., 1996) have been shown to possess high εCyt glycosylase activity. Excised adducts are excreted into biological fluids, such as urine. Among etheno adducts, εAde was detected in the urine of rats exposed to chloroethylene oxide and in untreated rats (Holt et al., 1998; Yen et al., 1998) and 1,N\textsuperscript{2}-εGua and N\textsuperscript{2},3-εGua were found in

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healthy human urine (Gonzalez-Reche et al., 2002). Our recent finding that eCyt is present in human urine (Chen et al., 2001) suggests that it might be at least partially due to the activity of human eCyt glycosylases (Hang et al., 1996, 1998; Saparbaev and Laval, 1998). Urinary etheno deoxyribonucleoside adduct, 1,6-etheno-2'-deoxyadenosine, was detected in Japanese women, and its level is associated with salt-induced inflammation and lipid peroxidation (Hanaoka et al., 2002).

Since levels of etheno adducts appear to increase with oxidative stress and cancer development (Barbin, 2000; Bartsch et al., 1999; Chung et al., 1993, 1996; Nair et al., 1997, 1999; Schmid et al., 2000), etheno adducts might be valid biomarkers for cancer-risk assessment (Barbin, 2000; Bartsch et al., 1999; Chung et al., 1993, 1996; Nair et al., 1999). Although cigarette smoking did not increase the levels of eAde and eCyt in pancreatic DNA (Kadlubar et al., 1998), the correlation in urinary adduct levels was not studied, mainly due to the lack of an appropriate analytical method. 32P-Postlabelling may be the most sensitive technique to analyze adducts in DNA, but it cannot be applied to analyze excited bases in biological fluids. In this study, we investigate the relationship between urinary eCyt with the cigarette smoking status of 23 subjects not occupationally exposed to industrial chemicals using the stable isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS) method developed in our laboratory (Chen et al., 2001).

MATERIALS AND METHODS

Materials. 3,4-etheno-2'-Cytosine was purchased from Sigma Chemical Co. (St. Louis, MO). Pentafluorobenzyl bromide (PFB-Br), diisopropylethylamine, anhydrous methanol, and anhydrous phosphorous pentoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bond Elut C18-OH and Si solid-phase extraction (SPE) columns (500 mg, 3 ml) were obtained from Varian (Harbor City, CA). The isotope standard [13C4,15N3]PFB-Cyt was synthesized as described in Chen et al. (2001).

Urine pretreatment. Urine samples collected over a 24-h period were stored at −80°C in a freezer. The samples were defrosted in an ice-water bath and centrifuged at 15,000 × g for 10 min at 4°C and the precipitate was discarded.

Adduct enrichment by C18-OH SPE column. Before the samples were used, each new batch of SPE columns was tested for consistency in their elution pattern with 1.0 μg of standard eCyt. After elution with the conditions described below, the fractions were collected every 3 ml. These fractions were evaporated and analyzed by reversed-phase high performance liquid chromatography (HPLC) with photodiode array detection as described in Chen et al. (2001).

Assay procedures. Typically, a urine sample (0.1 ml) was enriched with a C18-OH SPE column, followed by derivatization with PFB-Br, and postderivatization clean-up by an SPE column before GC/NICI/MS analysis. The analysis was performed using a Hewlett Packard 6890 GC with a 5973 MSD mass selective detector with a negative ion chemical ionization (NICI) source (Agilent Technologies, Palo Alto, CA). Selective ion monitoring (SIM) at m/z 134 and 141 was used to monitor and quantify PFB-eCyt and [13C4,15N3]PFB-eCyt for their respective [M − 181]− fragment ions using the conditions described in Chen et al. (2001).

Assay calibration. For the eCyt assay, [13C4,15N3]eCyt (1.0 ng) was added to sample vials containing various amounts (0, 0.25, 0.5, 1.0, 5.0, 10, and 50 pg) of eCyt. Each sample was processed through the C18-OH SPE column, pentafluorobenzylation, Si SPE purification, and GC/NICI/MS analysis. Quantification of eCyt was based on intrapolation of the ratio of the peak area of PFB-eCyt versus [13C4,15N3]PFB-Cyt on the calibration curve obtained from at least duplicated experiments.

Measurement of urinary total antioxidant capacity (TAC). The procedures were modified from the published methods (Kirschbaum, 2001; Miller et al., 1993). Briefly, hydrogen peroxide (final concentration 420 μM) was added to a solution containing urine (10 μl), methemoglobin (0.84 μM), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate (ABTS, 900 μM) in phosphate buffer saline (5-mM potassium phosphate with 70-mM NaCl, pH 7.0) with a total volume of 3.0 ml. The reaction was initiated by the addition of hydrogen peroxide, and the reaction mixture was incubated at 37°C for 10 min before measurement of its absorption at 734 nm on a UV-Vis spectrophotometer (Varian Cary 3E; Varian, Harbor City, CA). The standard curve was constructed from various amounts of uric acid ranging from 0 to 300 μM. Quantification was based on intrapolation of the difference in absorbance, compared with the control experiment without the addition of urine, to the standard curve. The levels of TAC were averaged from the experiments repeated four times.

Statistical analysis. All of the results are reported as the means ± standard deviation (SD). Statistical analysis of two groups was performed by the Mann-Whitney U-test, and p ≤ 0.05 was considered significant. The Spearman rank correlation was used to calculate the correlation coefficient. GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA; www.graphpad.com) was used for these analyses.

RESULTS

GC/NICI/MS Assay

The procedures for analyzing eCyt in the urine sample are described in Figure 1. The isotope standard [13C4,15N3]eCyt was added to 0.1 ml of urine, and a disposable C18-OH SPE column was used for purification and enrichment of the eCyt. After the sample was loaded, it was washed under optimum conditions, eluting with aqueous methanol solution of different methanol contents to obtain the clearest fractions possible. Water was used to remove the very polar components in urine, followed by 10% aqueous methanol in which the eCyt did not elute. The fraction containing eCyt was collected by eluting with 15% aqueous methanol. The nonpolar components of urine were left on the column and discarded. The eCyt-containing fraction was dried and derivatized with the electrophore, PFB-Br. After postderivatization cleanup using a Si SPE column, the sample was analyzed by GC/NICI/MS under SIM mode.

In our previous study (Chen et al., 2001), 1/100 of the processed sample from 3 ml of urine was analyzed by GC/NICI/MS. In the present study, only 0.1 ml of the urine sample was used and 1/10 of the processed sample was injected into the GC/NICI/MS. The levels of eCyt in all of the 23 urine samples analyzed were above the detection limit of the assay. Without using the immunoaffinity chromatography, the recovery of the entire assay was acceptable (ca. 35%). The interday relative standard error of the assay was 5.4% (n = 3), and the intraday relative standard error was 2.0% (n = 4).

The advantageous feature of this assay is the addition of the
stable isotope $^{13}\text{C}_4,^{15}\text{N}_3\text{Cyt}$ as an internal standard for monitoring $\text{Cyt}$ from the beginning of the assay. The stable isotope standard $^{13}\text{C}_4,^{15}\text{N}_3\text{Cyt}$ provides accurate quantification of $\text{Cyt}$ in the complex mixture of the urine sample because, while it has the identical chemical property as $\text{Cyt}$, it can be distinguished from $\text{Cyt}$ by mass spectrometry. The analyte $\text{Cyt}$ was monitored by the mass channel at m/z 134, while the internal standard $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$ was at m/z 141. To make sure that the peak of $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$ at m/z 141 originated from $^{13}\text{C}_4,^{15}\text{N}_3\text{Cyt}$ added to the sample, a urine sample was spiked with a known amount of $\text{Cyt}$ (0.9 ng) and processed through the assay procedures. At the channel of m/z 141, no peaks were present at the retention time of $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$ (19.15 min) (Fig. 2). Thus, the quantification of urinary $\text{Cyt}$ by $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$ was assured to be without interference. The PFB-Ade peak around 20 min at m/z 134 was also observed previously (Chen et al., 2001).

The limit of detection for PFB-$\text{Cyt}$ was reported to be 1.0 fg (3.2 amol) injected on the column, while the limit of quantification in the samples was 1.0 pg (7.4 fmol) of $\text{Cyt}$ due to the background level of the control samples (peak area ratio = 0.005) (Chen et al., 2001). In this study, the peak area ratio of PFB-$\text{Cyt}$ versus $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$ was reduced to 0.002, and subsequently the limit of quantification was improved to 0.25 pg (1.8 fmol) in 0.1 ml of urine, which is equivalent to a concentration detection limit of 18 pM of $\text{Cyt}$ in the sample.

**Urinary $\text{Cyt}$ Levels**

Typical GC/NICI/MS chromatograms of the urine samples of a smoker and a nonsmoker showed exactly the same retention times for the PFB-$\text{Cyt}$ peak monitoring at m/z 134 and the $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$ peak at m/z 141 under the SIM mode (Fig. 3). The identical retention times in both m/z 134 and 141 channels clearly indicated their chemical identity. The peak at 19.77 min in the top left panel in Figure 3 represents 4.3 pg (14 fmol) of PFB-$\text{Cyt}$, and the peak at 19.86 min in the top right panel represents the internal standard $^{13}\text{C}_4,^{15}\text{N}_3\text{PFB-Cyt}$.
panel in Figure 3 represents 0.62 pg (2.0 fmol) of PFB-eCyt. Quantification of urinary eCyt was achieved by intrapolation into the standard curve constructed from various amounts of eCyt, ranging from 0 to 50 pg, in the presence of a fixed amount of $[^{13}C_4,^{15}N_3]$ eCyt. The results correspond to an eCyt concentration of 184 and 27 pg/ml in the smoker’s and the nonsmoker’s urine samples, respectively.

Characteristics of the study population are listed in Table 1. There are no heavy smokers among the smokers’ population, according to the smoking index, which is defined as the number of cigarettes smoked per day times years of smoking. Comparison of urinary eCyt concentrations in smokers and nonsmokers shows a strong association ($p = 0.0019$) with cigarette smoking using the nonparametric Mann-Whitney U-test. Statistically significant correlations ($p = 0.0015–0.0303$) are observed no matter how eCyt levels are justified, either by urinary creatinine concentration, body weight, or both (Table 2). The average concentration of eCyt in smokers was 6-fold higher than that of nonsmokers. The distribution of eCyt concentration (Fig. 4) showed a wide range of variation among smokers (13.5–573 pg/ml).

**TABLE 1**

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<thead>
<tr>
<th>Characteristics of the Study Population</th>
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<td>Smokers, mean ± SD (range)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>43 ± 16 (24–68)</td>
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<tr>
<td>Cigarettes/day</td>
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<tr>
<td>15 ± 6 (5–25)</td>
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<tr>
<td>Smoking years</td>
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<tr>
<td>22 ± 16 (3–50)</td>
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<td>Smoking index*</td>
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<td>331 ± 253 (15–750)</td>
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<td>Nonsmokers, mean ± SD (range)</td>
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*Note. Smokers, $n = 10$; nonsmokers, $n = 13$.

*Smoking index = number of cigarettes smoked per day × years of smoking.

**Urinary TAC**

To provide a convenient measure for the antioxidant defenses of individuals, a modified assay based on quenching the long-lived radical cation of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) was used (Miller et al., 1993; Kirschbaum, 2001). The radical was generated through the peroxidase activity of methemoglobin in the presence of hydrogen peroxide, which can be detected at 734 nm by a spectrophotometer. The TAC measured in the urine sample...
represents the sum of all compounds capable of scavenging the ABTS radical and is standardized against uric acid. In the standard curve, the decrease in absorbance of the sample compared to the control experiment (without sample) was linear with respect to uric acid concentration (0–300 μM). The levels of TAC measured in these 23 urine samples agree with the reported TAC range in human urine (Koracevic et al., 2001). However, no association was found between urinary TAC and Cyt levels. The Spearman rank correlation coefficient (r) of the Cyt level normalized for body weight and creatinine versus TAC was −0.2931 with p = 0.1748 (Fig. 5), and the correlation was thus considered not significant. However, we recently found that 3,N4-etheno-2′-deoxycytidine levels correlated with urinary TAC (unpublished results).

DISCUSSION

Knowledge of how etheno adducts are formed allows one to identify risk factors, such as genetic defects in their repair and/or lifestyle, and to find strategies suppressing the formation of these adducts. Epidemiological studies have established a causal relationship between cigarette smoking and various sites of human cancers (McLaughlin et al., 1995), especially lung cancer (Wynder et al., 1994). Exocyclic etheno DNA adducts are known to arise from endogenous lipid peroxidation and oxidative stress (Chung et al., 1996; Nair et al., 1998b). The effect of diet on etheno adducts formation in tissue DNA has been investigated. The levels of eAde and eCyt in the white blood cells of female volunteers on a high omega-6 polyunsaturated fatty acid diet, who were at a high risk of breast cancer, are much higher than the control subjects, but no difference in adduct levels was observed in men (Nair et al., 1997) or in healthy females (Hagenlocher et al., 2001). Moreover, alcohol consumption led to elevated levels of etheno adducts in the hepatic liver DNA of rats, but it only accounted for a 2-fold increase (Navasumrit et al., 2001).

Cells respond to DNA damages using various repair mechanisms to reverse the modification. Commonly accepted DNA repair mechanisms include base excision repair (BER), nucleotide excision repair (NER), and direct reversal by O-alkyltransferase (Singer and Hang, 1997). Base excision repair initiated by specific DNA glycosylases releases adducts as free bases, which can be excreted into the urine without being metabolized. The level of urinary base adducts appears to depend on the formation rate of DNA lesions and their repair efficacies by DNA glycosylases (Fraga et al., 1990; Loft et al., 1998). Another possible source of urinary base adducts is RNA modification. However, RNA adducts are not recognized the

<table>
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<th>Smokers, mean ± SD (range)</th>
<th>Nonsmokers, mean ± SD (range)</th>
<th>p-value a</th>
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<tr>
<td>eCyt (pg/ml)</td>
<td>152 ± 167 (13.5–573)</td>
<td>24 ± 24 (2.8–96)</td>
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<tr>
<td>eCyt/creatinine (ng/g)</td>
<td>193 ± 279 (12–949)</td>
<td>37 ± 47 (3.6–171)</td>
</tr>
<tr>
<td>eCyt/body weight (pg/ml)</td>
<td>2.1 ± 2.4 (0.16–8.2)</td>
<td>0.39 ± 0.47 (0.05–1.9)</td>
</tr>
<tr>
<td>eCyt/body weight/creatinine (pg/kg)</td>
<td>2.65 ± 4.0 (0.15–13.6)</td>
<td>0.61 ± 0.90 (0.04–3.35)</td>
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Note. Smokers, n = 10; nonsmokers, n = 13.

aThe two-tailed p-values were obtained by comparing adduct levels among smokers versus nonsmokers using the nonparametric Mann-Whitney U-test.

*p < 0.05.

FIG. 4. Distribution of urinary levels of eCyt in smokers and nonsmokers.

FIG. 5. Correlation between the urinary eCyt levels and total antioxidant capacity of 23 human urine samples.
same way as adducts on DNA, according to a report that methylated adducts in the liver RNA of hamsters exposed to the methylating agent were not repaired (Margison et al., 1979). Although the contribution of urinary DNA adducts from the deoxynucleotide pool (Topal and Baker, 1982) cannot be ruled out, the pool size is normally very small and may not be relevant under physiological conditions (Snow and Mitra, 1988). In addition, the profile of adduct formation in the deoxynucleotide pool with the alkylating agent is different from that in DNA (Topal and Baker, 1982). Thus, it appears that neither RNA nor the deoxynucleotide pool contributes significantly to urinary DNA adducts. Correlation of the dose-dependent urinary excretion of the aflatoxin-guanine adduct with adduct formation in the liver demonstrates the link between urinary adduct with aflatoxin-induced hepatocellular neoplasms in experimental animals (Bennett et al., 1981). Furthermore, the parallel decrease in levels of hepatic DNA adducts and urinary aflatoxin-guanine in rats fed a chemopreventive agent suggests that the aflatoxin-guanine adduct in urine can serve as a noninvasive biomarker for assessing the risk of hepatic cancer and the efficacy of chemoprotective agents (Groopman et al., 1992 53). Nonetheless, interpretation of the level of other urinary adducts should be cautioned (Cooke et al., 2002).

The excretion of the repaired DNA adducts in urine represents the average rate of DNA damage in the total body, whereas the level of addeduct bases in tissue DNA is a concentration measurement in specific tissue/cells at the moment of sampling. The human eCyt glycosylases identified (Hang et al., 1996, 1998; Saparbaev and Laval, 1998) are at least partially responsible for the eCyt detected in urine. If the contribution from diet and cell death is realized and quantified, a measurement of urinary DNA adducts can provide valuable noninvasive biomarkers for DNA damage and repair (Cooke et al., 2002). Although the number of our subjects was limited, the correlation between levels of urinary eCyt with cigarette smoking is significant. Our results indicate that urinary eCyt, like 8-oxo-2’-deoxyguanosine (Loft et al., 1992) and 5-(hydroxymethyl)uracil (Bianchini et al., 1998), represents a form of oxidative DNA damage in an individual, and it might be a valuable noninvasive biomarker in epidemiological studies. In addition, the large variation in levels of urinary eCyt observed in this study might be partially due to dietary factors, and it implies that urinary eCyt can potentially be a biomarker for evaluating the effect of chemopreventive agents with more subjects in a more defined study (Bartsch and Nair, 2000; Bartsch et al., 1999).

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REFERENCES


