Detection of Carcinogen-DNA Adducts in Exfoliated Urothelial Cells of Cigarette Smokers: Association with Smoking, Hemoglobin Adducts, and Urinary Mutagenicity

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Abstract

The presence of covalent modifications in DNA obtained from exfoliated urothelial cells of smokers and nonsmokers was determined using 32P postlabeling methods. Urine and blood samples were procured from 73 persons. Cells were removed from the urine by filtration. DNA was isolated using an enzyme-solvent extraction method and then coprecipitated with glycogen. Sufficient DNA to detect 1 carcinogen-DNA adduct/10^8 normal nucleotides was obtained from 40 of the 73 samples. DNA was hydrolyzed to 3'-phosphodeoxyribonucleotides and then 32P postlabeled under conditions of excess [32P]ATP. Carcinogen-DNA adducts were resolved using anion-exchange thin-layer chromatography and visualized by autoradiography; film exposures lasted as long as 7 days. Twelve different carcinogen-DNA adducts and a diagonal zone of radioactivity could be found, but no sample contained all adducts. At least four adducts appeared to be cigarette smoking related. These adducts were from 2 to 20 times higher in the smokers than the nonsmokers. Two carcinogen-DNA adducts were qualitatively very similar to adducts described earlier in a study of human bladder biopsies. One of these corresponded to N-(deoxyguanosin-8-yl)-4-aminobiphenyl. Adducts were correlated significantly with the levels of 4-aminobiphenyl hemoglobin adducts and number of cigarettes smoked. In addition, levels of the putative N-(deoxyguanosin-8-yl)-4-aminobiphenyl adduct and a measure of total adducts were correlated with the mutagenic activity of the individual's urine.

These data suggest that noninvasive, biological monitoring techniques can be applied to the study of carcinogen-DNA adducts in humans at high risk for bladder cancer.

Introduction

Various epidemiological studies have indicated that 40–85% of the bladder cancer incidence in the general population is attributable to cigarette smoking (1, 2). Cigarette smoke is a complex mixture containing carcinogenic compounds in the vapor phase and bound to particulates. Carcinogens in cigarette smoke include polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines (1, 3). The tobacco curing process apparently has a significant impact upon the quantity of carcinogens in the resultant products. Black (air-cured) tobacco contains a higher concentration of carcinogens than does blond (flue-cured) tobacco (3). And the incidence of bladder cancer is significantly higher in smokers of black tobacco than it is in persons who smoke blond tobacco (4–7).

Identification of specific chemical processes integral to carcinogenesis has led to the development of biomarkers of internal dose and effect (8). DNA is the critical target for many chemical carcinogens. Carcinogen-DNA adducts, the covalent reaction products of electrophilic chemical carcinogens with nucleophilic sites on DNA, represent the initial damage to the genetic material caused by these agents (9). Carcinogen-DNA adducts have been associated with mutation and chromosome damage in vitro and in vivo (10–12). Investigations of the relationship between carcinogen-DNA adduct levels and smoking status in target organs have shown that adduct levels are significantly higher in lungs, urinary bladders, and oral mucosa of the smoking population (13–15). In contrast, studies involving a surrogate tissue (e.g., blood lymphocytes) to monitor tobacco smoke exposure have been negative generally in human and in animal models (16, 17). Because many compounds are organotropic in their carcinogenicity and because some surrogate tissues do not seem to reflect the events occurring in the target organ, the development of noninvasive techniques to monitor carcinogen-DNA adducts in internal organs is a priority. Recently, we have described a method using 32P postlabeling to detect 4-aminobiphenyl-DNA adducts in DNA from exfoliated urothelial cells obtained from the urine of treated dogs (18). The current report describes the application of these noninvasive methods to monitor carcinogen-DNA adducts in the human urinary bladder, a target organ for tobacco-induced cancers.
Detection of Carcinogen-DNA Adducts in Urothelial Cells of Cigarette Smokers

Methods

Informed consent was obtained from a group of 73 healthy male blood donors between the ages of 45 and 64 in Turin, Italy. Blood and urine samples were obtained from each individual, who also completed a questionnaire detailing his ethnic background and smoking, dietary, and occupational history. Urine samples were collected from the individuals for 24 h. Samples were assigned a code number, which was broken only after the analyses were complete. This group of 73 was part of a population of 97; 24-h urine samples were not collected from the remaining 24 persons. Aliquots of the urine samples were removed for determination of urinary mutagenicity in Salmonella typhimurium strain TA 98 in the presence of liver 5-9 from Arochlor-treated rats at the International Agency for Research on Cancer as described earlier (19, 20). Exfoliated urothelial cells were isolated by vacuum filtration using 500-ml stainless steel funnels and 5-μm filters (Type SM; Millipore). Filters were coded and then frozen and shipped on dry ice. Cells were washed off the filters with a buffer consisting of 0.25 M sucrose, 1.8 mM calcium chloride, 25 mM potassium chloride, and 50 mM Tris-HCl, pH 7.5. Cells were washed several times by resuspension in the same buffer followed by centrifugation at 1800 rpm in a refrigerated centrifuge. Cells were then brought to 20% glycerol and frozen at −80°C.

A 20-ml blood sample was also collected. The RBC were washed with isotonic saline, frozen, and shipped to the Massachusetts Institute of Technology for amino-biphenyl-hemoglobin analysis, as described in an earlier report (21, 22).

DNA Isolation. DNA isolation of the thawed cell pellets was as described earlier for exfoliated urothelial cells obtained from treated dogs (18). In brief, the washed cells were homogenized in 1% sodium dodecyl sulfate, treated sequentially with both RNase A and RNase T<sub>1</sub>, and then proteinase K. Hydrolysates were extracted with phenol, then phenol-chloroform, and finally chloroform-isomyl alcohol (23). At this point, 0.1 volume of 4.5 M LiCl and 30 μg of glycogen were added. DNA was coprecipitated with the glycogen by the addition of 2 volumes of ice-cold ethanol, followed by cooling to −80°C for 30 min. Samples were centrifuged at 7000 x g in a refrigerated centrifuge and washed twice in ice-cold 70% ethanol, and the DNA was dissolved in a small volume (∼40 μl) of 1.5 mM NaCl-0.1 mM sodium citrate. The amount of DNA obtained from these cells was often below that which could be detected by UV spectrophotometry without exhausting the valuable sample. The <sup>3</sup>P postlabeling analysis was then used to quantify the amount of DNA.

<sup>31</sup>P Postlabeling. <sup>14</sup>P postlabeling of the exfoliated urothelial cells was performed essentially as described earlier (18). Because the concentration of DNA in the samples was not known, 4 μl of each were analyzed. In order to quantify accurately the amount of DNA in each sample, <sup>31</sup>P postlabeling was done under conditions of ATP excess. Since our earlier studies indicated that no more than about 125 pmol of DNA would be present in 4 μl of a given sample, the necessary excess of ATP was provided by the addition of 132 pmol of [<sup>31</sup>P]ATP (∼3000 Ci/mmol, 400 μCi) to each sample. The excess [<sup>31</sup>P]ATP was then destroyed by incubation with apyrase. An aliquot of each sample was removed for normal nucleotide analysis, and the remainder was then applied to the polyethyleneimine sheets for chromatography essentially as described earlier, except that 0.65 M sodium phosphate buffer (pH 6.0) was found to be the appropriate D1 solvent (14, 24, 25). Autoradiography was performed by exposing Kodak XAR-5 film to the chromatograms in cassettes containing Kodak Regular intensifying screens at −80°C for as long as 120 h. When individual adducts could be visualized, they were excised and counted. Adducts often appeared within a diffuse diagonal zone of radioactivity. When an adduct could be distinguished within the zone, it was excised and counted separately. The radioactivity in the diagonal radioactive zones was also determined. The total adducts in the diagonal radioactive zone were determined by summing the activity in the zone and the adducts that appeared to be part of the zone (Adducts 3, 4, and 9). Carcinogen-DNA adduct levels were calculated by determining the RAL, which was defined as

\[
\frac{cpm_{adducts}}{cpm_{normal nucleotides}} \times 10^n
\]

The amount of DNA in each sample was estimated by determining the proportion of the total <sup>31</sup>P incorporated into normal nucleotides and multiplying that value by the molar amount of [<sup>31</sup>P]ATP added per sample.

In earlier studies we found that different populations of carcinogen-DNA adducts were intensified by nuclease P<sub>1</sub> and butanol extraction (14). The methods employed in this study should be sensitive to both populations of carcinogen-DNA adducts because there is excess [<sup>31</sup>P] ATP.

Duplicates (at least) of each sample were analyzed independently. Data were accumulated using Lotus 123 software, and statistical analyses were performed using the PC SAS statistical package.

Results

The entire group of 73 samples was <sup>31</sup>P postlabeled. Sufficient DNA for <sup>31</sup>P postlabeling analysis was obtained from 40 of these 73 urine samples. An exfoliated urothelial sample was defined as having insufficient DNA if it contained less than about 35 pmol as determined from the normal nucleotide analysis. Two samples were grossly contaminated with bacteria.

Transportation delays contributed to the losses; approximately 15 samples arrived without dry ice and the DNA in the majority was degraded. As with the earlier study, thawing the samples in the absence of 20% glycerol led to decreased DNA yields (18). The recovery of sufficient DNA from samples thought to be properly prepared was good (about 70%). Another major interfering factor in isolating DNA and in detecting carcinogen-DNA adducts in these samples was the fairly large amount of crystals voided in the urine. Uric acid, triple phosphate (Mg<sub>2</sub>PO<sub>4</sub>·3H<sub>2</sub>O), and oxalate crystals predominated. However, the majority of these could be removed.

The abbreviations used are: RAL, relative adduct labeling; TOTZ, total diagonal zone.
defined above.

Fig. 1. Autoradiogram of normal nucleotides; duplicate lanes for each of five individuals. A 4-μl aliquot of 32P postlabeled and apyrase-treated DNA digests was diluted to 750 μl with 10 mM bicine buffer, pH 9.5, and mixed. Then, 5 μl were spotted at the origin (O). The chromatogram was predeveloped to the origin in distilled water and then developed to the top with 0.17 M sodium phosphate buffer, pH 6.8. The chromatogram was dried and then exposed to Dupont Chronex film for 12 min in a Kodak film cassette with regular intensifying screens.

The 40 samples included 21 from nonsmokers, 18 from current smokers, and one from a pipe smoker, who was excluded from further analysis. These 39 individuals were very similar to the larger population of 97 people from which they were drawn. There were no significant differences for any of the demographic (e.g., age) or analytical variables reported earlier (19, 22). This includes the levels of 4-aminobiphenyl-hemoglobin adducts and urinary mutagens.

A normal nucleotide map for a series of 32P postlabeled human exfoliated urothelial cell DNA samples appears as Fig. 1. There was a wide variation in the quantity of the radiolabeled nucleotides, as would be expected since the number of cells and the amount of DNA in each sample was not known prior to the analysis. An excess of radiolabel is seen in each sample regardless of concentration, indicating that sufficient [32P]ATP was available to label all the nucleotides in the samples.

Fig. 2 depicts autoradiograms from a sample that showed very low adduct levels (Fig. 2A); a sample obtained from a nonsmoker that seemed to contain high levels of adducts (Fig. 2B); a sample from an individual who reported a current smoking history (Fig. 2C). In a previous study of carcinogen-DNA adduct levels in bladder biopsy samples of cigarette smokers, we reported that several carcinogen-DNA adducts were higher in smokers than they were in nonsmokers or former smokers (14). Several of the adducts in the exfoliated urothelial cells displayed chromatographic behavior similar to that of the adducts reported in that study. In particular, Adducts 1, 2, 4, 6, and 9 in the current work were chromatographically similar to Adducts P2, B1 and P1, B4, P6, and B9, respectively, in the earlier report.

The mean levels of several carcinogen-DNA adducts seen in current smokers and nonsmokers are shown in Fig. 3.

As can be seen from the figure, Adducts 1, 2, 4, 9, and 10 and TOTZ adducts are higher on average in smokers than nonsmokers. The fold increase for these adducts in the smokers over nonsmokers is 1.6, 7.0, 1.7, 3.9, 38, and 1.3 times, respectively. However, these differences were not significant at the 0.05 level using the Mann-Whitney statistic. Univariate analysis indicated the carcinogen-DNA adducts in the sample of 39 persons did not follow a normal distribution, making nonparametric statistical analysis appropriate.

The data were analyzed next to determine whether there were correlations between reported cigarette intake, levels of aminobiphenyl-hemoglobin, and urinary mutagenicity. The Spearman (nonparametric) correlation coefficients for the Adducts 1, 2, 4, 9, and 10 and TOTZ with the number of cigarettes smoked, 4-aminobiphenyl-hemoglobin adduct levels, and the levels of mutagens excreted in the urine of smokers are given in Table 1.

Adduct 4 has the strongest relationship with these indices of exposure. This adduct appears qualitatively very similar to butanol Adduct 4 (B4) in the bladder biopsy study, which was characterized as N-(deoxyguanosin-8-yl)-4-aminobiphenyl (14). The average level of Adduct 4 was somewhat higher in black tobacco smokers than in blond tobacco smokers (RAL values of 8.8 and 7.7, respectively), but these levels were not different statistically from those of the nonsmokers (RAL = 4.8). Many samples from smokers and nonsmokers did not contain particular adducts. Adduct 4 was reported positive (nonzero) in 13 of 21 nonsmokers and in 10 of 18 smokers. And, while these proportions are not different statistically, several interesting trends appear. As shown in Table 2, the proportion of samples that were positive for Adduct 4 is increased in black tobacco smokers over blond smokers or nonsmokers.

In addition, when smokers were segregated into three tobacco consumption groups of approximately equal size, light (1–14 cigarettes/day), medium (15–29
Fig. 2. Autoradiograms of 3H postlabeled DNA from several human exfoliated urothelial cell samples. A, autoradiogram from a nonsmoker with low adduct levels; B, autoradiogram from a nonsmoker that was misclassified due to high background activity on the plate; C, autoradiogram from a smoker. Samples were treated and chromatography was performed as described in "Materials and Methods," as well as in earlier references (14).

In addition, Adduct 2 was detected in 57% of the samples of the heaviest smokers (>30 cigarettes/day) but only in about 20% of the samples of nonsmokers or those who smoke fewer than 14 cigarettes/day (Table 3).

The average level of adduct 10 in smokers was 5.4, while the mean level in nonsmokers was 0.14. However, only one of 21 nonsmokers and 4 of 14 smokers were positive for this adduct, making further comparisons tenuous. As is shown in Table 1, adduct 10 was correlated significantly with the amount smoked and levels of 4-amino-biphenyl-hemoglobin adducts.

A smoker's level of total diagonal zone adducts (TOTZ) was correlated significantly with 4-amino-biphenyl-hemoglobin adducts and excretion of urinary mutagens. As mentioned above, the diagonal radioactive zone is the summation of Adducts 3, 4, and 9, and the activity above background associated with the diagonal radioactive zone not incorporated into discrete adducts. This measure is of course correlated with Adduct 4, which is a major constituent.

Discussion
This paper describes the detection of carcinogen-DNA adducts from an internal target organ, obtained by collection of exfoliated urothelial cells from the urine of human volunteers. Several carcinogen-DNA adducts were elevated in cigarette smokers; the increase in particular carcinogen-DNA adducts ranged from 1.6-fold to over 7-fold, which is approximately the range seen in an earlier study of human bladder biopsies. This is also the range of excess risk of bladder cancer in smokers as reported by various epidemiological studies (1, 5, 4, 26). However, these increases were not statistically significant. Nevertheless, further analysis indicated that three adducts and a summary adduct measure were correlated significantly with quantity of tobacco smoked, 4-amino-biphenyl-hemoglobin adducts and/or urinary mutagenicity. Two of these carcinogen-DNA adducts (2 and 4)
were qualitatively similar to adducts observed in bladder biopsy samples of cigarette smokers, as reported earlier.

Adduct 4 was 1.7 times higher in smokers than in nonsmokers and correlated significantly with the amount of tobacco the individual smoked, his level of urinary mutagens, and the level of 4-aminobiphenyl-hemoglobin adducts in the RBC. Adduct 4 levels were somewhat higher in black-tobacco smokers than in those who smoked blond tobacco (RAL values of 8.8 and 7.7, respectively). However, the mean RAL level in black-tobacco smokers was 22.5, which was significantly different from the level in the nonsmoking group at the 0.001 level. Thus, Adduct 2 seems to be a marker of heavy exposure to cigarette smoke; the adduct was found in 18.75% (6 of 32) of the nonsmokers and those who smoked fewer than 15 cigarettes/day, but in 57% (4 of 7) of the heavy smokers. In the bladder biopsy samples, this adduct was detected by both nuclease P1 and butanol extraction enhancement of 32P postlabeling. Since most aromatic amines are detected poorly by nuclease P1, it would seem likely that Adduct 2 is not an aromatic amine. However, Peluso et al. (33) report that DNA adducts caused by exposure to 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine may be detected by both methods (33). In addition, Adduct 2 levels were correlated significantly ($r = 0.51, P \leq 0.03$) with total diagonal zone adducts, again indicating that this DNA adduct may serve as a marker of general, high exposure.

The levels of total diagonal zone adducts increased with increasing levels of 4-aminobiphenyl-hemoglobin adducts and excreted mutagens. It has been proposed that this zone of carcinogen-DNA adducts consists of overlapping bands of multiple individual adducts; data from experiments involving complex mixtures seem to bear out this hypothesis (34).

This paper details initial efforts to apply noninvasive techniques to monitor carcinogen exposure to internal organs in humans. We have demonstrated that it is possible to obtain sufficient DNA for carcinogen-DNA adduct analysis in the exfoliated urothelial cells and that the levels of adducts are related to exposure and other biomarkers of exposure, namely 4-aminobiphenyl-hemoglobin adducts and urinary mutagenicity. An adduct that was chromatographically similar to $N$-(deoxyguanosin-8-yl)-4-aminobiphenyl was shown to be correlated with levels of 4-aminobiphenyl-hemoglobin adducts. These techniques are relatively simple and, with improvements to decrease background radioactivity on

**Table 1** Spearman correlation coefficients ($r$) and $P$ values for smoking-related adducts in smokers

<table>
<thead>
<tr>
<th>Adduct no.</th>
<th>Cigarettes/day</th>
<th>With hemoglobin adduct levels</th>
<th>With urinary mutagenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.310</td>
<td>0.164</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.52)</td>
<td>(0.34)</td>
</tr>
<tr>
<td>2</td>
<td>0.499</td>
<td>0.410</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.09)</td>
<td>(0.30)</td>
</tr>
<tr>
<td>3</td>
<td>0.050</td>
<td>0.601</td>
<td>0.509</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.01)</td>
<td>(0.03)</td>
</tr>
<tr>
<td>4</td>
<td>0.032</td>
<td>0.010</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>(0.96)</td>
<td>(0.97)</td>
<td>(0.1)</td>
</tr>
<tr>
<td>5</td>
<td>0.502</td>
<td>0.494</td>
<td>0.455</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.04)</td>
<td>(0.058)</td>
</tr>
<tr>
<td>TOTZ</td>
<td>0.337</td>
<td>0.510</td>
<td>0.654</td>
</tr>
<tr>
<td></td>
<td>(0.17)</td>
<td>(0.03)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>

* Correlation coefficients significant at the 0.05 level.

**Table 2** Proportion of samples positive for Adduct 4

<table>
<thead>
<tr>
<th>Exposure classification</th>
<th>Positive Total</th>
<th>Percentage positive</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>13/21</td>
<td>61.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Blond-tobacco smokers</td>
<td>5/11</td>
<td>45.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Black-tobacco smokers</td>
<td>5/7</td>
<td>71.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Number of samples positive for Adduct 4 over all the samples in the group.

**Table 3** Proportion of samples positive for Adduct 2 or 4 in all smokers

<table>
<thead>
<tr>
<th>Cigarettes/day</th>
<th>Adduct 2</th>
<th>Adduct 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Total</td>
<td>% Ratio</td>
<td>Positive Total</td>
</tr>
<tr>
<td>1-14</td>
<td>1/6 17</td>
<td>1.0</td>
</tr>
<tr>
<td>15-29</td>
<td>1/5 20</td>
<td>1.2</td>
</tr>
<tr>
<td>30+</td>
<td>4/7 57</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Number of samples positive for the adduct over all samples in the group. 

**Table 4** Proportion of samples positive for Adduct 2

<table>
<thead>
<tr>
<th>Exposure classification</th>
<th>Positive Total</th>
<th>Percentage positive</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>4/21</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Blond-tobacco smokers</td>
<td>3/11</td>
<td>27.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Black-tobacco smokers</td>
<td>3/7</td>
<td>43.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Number of samples positive for Adduct 2 over all samples in the group. 

* Determined as in Table 2.
the thin-layer plates, should have general application to monitor human exposure to urinary bladder carcinogens.

Acknowledgments

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References


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