Aristolochic acid and the etiology of endemic (Balkan) nephropathy


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Endemic (Balkan) nephropathy (EN), a devastating renal disease affecting men and women living in rural areas of Bosnia, Bulgaria, Croatia, Romania, and Serbia, is characterized by its insidious onset, invariable progression to chronic renal failure and a strong association with transitional cell (urothelial) carcinoma of the upper urinary tract. Significant epidemiologic features of EN include its focal occurrence in certain villages and a familial, but not inherited, pattern of disease. Our experiments test the hypothesis that chronic dietary poisoning by aristolochic acid is responsible for EN and its associated urothelial cancer. Using 32P-postlabeling/PAGE and authentic standards, we identified dA-aristolactam (AL) and dG-AL DNA adducts in the renal cortex of patients with EN but not in patients with other chronic renal diseases. In addition, urothelial cancer tissue was obtained from residents of endemic villages with upper urinary tract malignancies. The AmpliChip p53 microarray was then used to sequence exons 2–11 of the p53 gene where we identified 19 base substitutions. Mutations at A:T pairs accounted for 89% of all p53 mutations, with 78% of these being A:T → T:A transversions. Our experimental results, namely, that (i) DNA adducts derived from aristolochic acid (AA) are present in renal tissues of patients with documented EN, (ii) these adducts can be detected in transitional cell cancers, and (iii) A:T → T:A transversions dominate the p53 mutation spectrum in the upper urinary tract malignancies found in this population lead to the conclusion that dietary exposure to AA is a significant risk factor for EN and its attendant transitional cell cancer.

environmental mutagen | p53 mutation | urothelial cancer | DNA adduct


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Abbreviations: AA, aristolochic acid (mixture of AA-I and AA-II); AAN, aristolochic acid nephropathy; AL, aristolactam; dA-AL-I, 7-(deoxyadenosin-N6-yl) aristolactam-I; dA-AL-II, 7-(deoxyadenosin-N6-yl) aristolactam-II; dG-AL, 7-(deoxyguanosin-N7-yl) aristolactam-II; dNs, nucleotides/nucleosides; EN, endemic (Balkan) nephropathy.

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for dA-AL-I:

had not been exposed to Aristolochia in these tissues was determined by quantitative 32P-postlabeling/PAGE analysis (29). Samples in lanes 1–3 and 4–6 were excised from the right and left kidneys, respectively. Lanes 1 and 4 are from the renal cortex; lanes 2 and 5, from the renal medulla; and lanes 3 and 6, from the renal pelvis. Oligonucleotides containing dA-AL-I and dG-AL-I (1 adduct per 10^6 dNs), digested in parallel, were used as standards.

Results

Identification and Quantitation of AL-DNA Adducts in Human Tissues. Prophylactic bilateral nephrectomy was performed, with informed consent, on an American woman who developed end-stage renal failure after usage of an herbal remedy containing Aristolochia. AA-derived DNA adducts were detected in the renal cortex, medulla, and pelvis of this patient. Oligonucleotides containing dA-AL-I and dG-AL-I (1 adduct per 10^6 dNs), digested in parallel, were used as standards.

Fig. 1. Detection of AL-DNA adducts in renal tissues of a patient with AA. DNA (20 μg) was extracted from the renal cortex, medulla, and pelvis of an American woman who developed end-stage renal failure after treatment with an herbal remedy containing Aristolochia. The level of AL-DNA adducts in these tissues was determined by quantitative 32P-postlabeling/PAGE analysis (29). Samples in lanes 1–3 and 4–6 were excised from the right and left kidneys, respectively. Lanes 1 and 4 are from the renal cortex; lanes 2 and 5, from the renal medulla; and lanes 3 and 6, from the renal pelvis. Oligonucleotides containing dA-AL-I and dG-AL-I (1 adduct per 10^6 dNs), digested in parallel, were used as standards.

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Fig. 2. Liquid chromatography electrospray ionization/multistage mass spectrometry (LC-ESI/MS/MS) analysis of dA-AL adducts in renal tissue. AL-DNA adducts were detected in all EN patient samples by the 32P-postlabeling/PAGE assay, with levels ranging from 0.8 to 5.9 adducts per 10^7 dNs for dA-AL and 0.2–6.2 adducts per 10^7 dNs for dG-AL (Fig. 3A). dA-AL and dG-AL adducts were not detected in the renal cortex of five patients with upper urinary tract transitional cell cancers who resided in a nonendemic region of Croatia (Fig. 3B) or in five patients with common forms of chronic renal disease (data not shown). Using 10 μg of DNA, the limit of detection in the 32P-postlabeling/PAGE assay is 3 × 10^9 adducts per dNs (29).

AL-DNA Adducts in Renal Tissues of Patients with EN. Criteria established by the World Health Organization (30) were used to establish the clinical diagnosis of EN. In most cases, the clinical diagnosis was confirmed by the unique renal histopathology; in some, the pattern of interstitial fibrosis characteristic of EN was obscured by changes associated with end-stage renal disease. Formalin-fixed renal tissues embedded in paraffin blocks were obtained from four patients who met the diagnostic criteria. Histopathologic examination of these tissues, performed independently by three renal pathologists, revealed the typical EN pattern of dense interstitial fibrosis with minimal inflammation and relative sparing of glomeruli (31). AL-DNA adducts were detected in all EN patient samples by the 32P-postlabeling/PAGE assay, with levels ranging from 0.8 to 5.9 adducts per 10^7 dNs for dA-AL and 0.2–6.2 adducts per 10^7 dNs for dG-AL (Fig. 3A). dA-AL and dG-AL adducts were not detected in the renal cortex of five patients with upper urinary tract transitional cell cancers who resided in a nonendemic region of Croatia (Fig. 3B) or in five patients with common forms of chronic renal disease (data not shown). Using 10 μg of DNA, the limit of detection in the 32P-postlabeling/PAGE assay is 3 × 10^9 adducts per dNs (29).
Histopathologic analysis; eight of the nine remaining patients had tumors localized in the renal pelvis and/or ureter. The one patient whose bladder cancer was analyzed (no. 9) also had cancer of the renal pelvis. In two patients, insufficient renal tissue was available for histopathologic examination. DNA (10^6, patient 4. (8) Five non-EN patients with upper urothelial tract malignancies. After unilateral nephroureterectomy, tumor tissues were fixed in buffered formalin, stained with H&E and Mallory’s trichrome and subjected to histopathologic examination. DNA adduct levels, determined after 32P-postlabeling PAGE, were 0.7–1.6 adducts per 10^8 dNs for the dA-AL adduct (Fig. 4). AL-DNA Adducts in Upper Urinary Tract Transitional Cell Cancers. Urothelial and renal cortical tissues were obtained from three long-term residents of endemic villages who had upper urinary tract malignancies. After unilateral nephroureterectomy, tumor tissue was frozen in liquid nitrogen for DNA adduct analysis. All tissues were fixed in buffered formalin, stained with H&E and Mallory’s trichrome and subjected to histopathologic examination. DNA adduct levels, determined after 32P-postlabeling PAGE, were 0.7–1.6 adducts per 10^8 dNs for the dA-AL adduct and 0.3–0.5 adducts per 10^8 dNs for the dG-AL adduct (Fig. 4).

p53 Mutations in Transitional Cell Cancers. The seven women and four men in this study who resided in endemic villages for a minimum of 15 years. With two exceptions, all were born before 1934. Histopathologic examination revealed all tumors to be transitional cell carcinomas. Ten of the 11 cancers analyzed were fixed in formalin and Mallory’s trichrome and subjected to histopathologic analysis. DNA adduct levels, determined after 32P-postlabeling PAGE, were 0.7–1.6 adducts per 10^8 dNs for the dA-AL adduct and 0.3–0.5 adducts per 10^8 dNs for the dG-AL adduct (Fig. 4).

Table 1. Human p53 mutations observed in tumors of EN patients

<table>
<thead>
<tr>
<th>Patient, no.</th>
<th>Codon/position</th>
<th>Type of mutation</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>274-3</td>
<td>GTT → GTG</td>
<td>V → V</td>
</tr>
<tr>
<td>2*</td>
<td>251-1</td>
<td>ATC → TTC</td>
<td>I → F</td>
</tr>
<tr>
<td>3</td>
<td>179-2</td>
<td>CAT → CTT</td>
<td>H → L</td>
</tr>
<tr>
<td>4*</td>
<td>241-1</td>
<td>TCC → ACC</td>
<td>S → T</td>
</tr>
<tr>
<td>5</td>
<td>158-2</td>
<td>CGC → CAC</td>
<td>R → H</td>
</tr>
<tr>
<td>6</td>
<td>280-3</td>
<td>AGA → AGT</td>
<td>R → S</td>
</tr>
<tr>
<td>7</td>
<td>274-3</td>
<td>GTT → GTG</td>
<td>V → V</td>
</tr>
<tr>
<td>8</td>
<td>291-1</td>
<td>AAG → TAG</td>
<td>K → Stop</td>
</tr>
<tr>
<td>9</td>
<td>131-1</td>
<td>AAC → TAC</td>
<td>N → Y</td>
</tr>
<tr>
<td>10</td>
<td>280-1</td>
<td>AGA → TAG</td>
<td>K → Stop</td>
</tr>
<tr>
<td>11</td>
<td>144-2</td>
<td>CAG → CTG</td>
<td>Q → L</td>
</tr>
<tr>
<td>12</td>
<td>209-1</td>
<td>AGA → TAG</td>
<td>R → Stop</td>
</tr>
<tr>
<td>13</td>
<td>280-3</td>
<td>AGA → AGT</td>
<td>R → S</td>
</tr>
<tr>
<td>14</td>
<td>130-2</td>
<td>CTC → CAC</td>
<td>L → H</td>
</tr>
<tr>
<td>15</td>
<td>139-1</td>
<td>AAG → TAG</td>
<td>K → Stop</td>
</tr>
<tr>
<td>16</td>
<td>158-1</td>
<td>CGC → GGC</td>
<td>R → G</td>
</tr>
<tr>
<td>17</td>
<td>176-3</td>
<td>TGC → TGG</td>
<td>C → W</td>
</tr>
<tr>
<td>18</td>
<td>193-2</td>
<td>CAT → CTT</td>
<td>H → L</td>
</tr>
<tr>
<td>19</td>
<td>203-3</td>
<td>GTG → GTC</td>
<td>V → V</td>
</tr>
<tr>
<td>20</td>
<td>209-1</td>
<td>AGA → TAG</td>
<td>R → Stop</td>
</tr>
<tr>
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<td>280-3</td>
<td>AGA → AGT</td>
<td>R → S</td>
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<td>GAA → GTA</td>
<td>G → V</td>
</tr>
<tr>
<td>24</td>
<td>313-1</td>
<td>AGC → TGC</td>
<td>S → C</td>
</tr>
</tbody>
</table>

Colors designating patient and codon/position correspond to those in Fig. 6. *Only exons 5–8 were sequenced in these patients.

Fig. 4. Identification of AL-DNA adducts in urothelial cancer tissues of patients residing in endemic villages. DNA (10 μg) extracted from upper urinary tract cancer tissues of patients residing in endemic villages of Croatia was used for quantitative analysis of AL-DNA adducts.

Fig. 3. Detection of AL-DNA adducts in the renal cortex. (A) DNA (20 μg) was extracted from seven formalin-fixed, paraffin-embedded renal cortical tissues obtained from four Croatian patients who met the diagnostic criteria for EN and used to quantify AL-DNA adducts by 32P-postlabeling/PAGE techniques. Lanes 1 and 2, patient 1; lane 3, patient 2; lanes 4 and 7, patient 3; lanes 5 and 6, patient 4. (8) Five non-EN patients with upper urothelial tract malignancies.

p53 Mutations in Transitional Cell Cancers. The seven women and four men in this study who resided in endemic villages for a minimum of 15 years. With two exceptions, all were born before 1934. Histopathologic examination revealed all tumors to be transitional cell carcinomas. Ten of the 11 cancers analyzed were fixed in formalin and Mallory’s trichrome and subjected to histopathologic analysis. DNA adduct levels, determined after 32P-postlabeling PAGE, were 0.7–1.6 adducts per 10^8 dNs for the dA-AL adduct and 0.3–0.5 adducts per 10^8 dNs for the dG-AL adduct (Fig. 4).
respectively. Colored arrows represent mutations in the same patient. Single mutations are represented by a black arrow. Numbers corresponding to amino acid residues are shown below the bar.}

**Discussion**

The principal results of these studies include the (i) identification of DNA adducts derived from AA in the renal cortex and medulla of patients with documented EN, (ii) detection of these adducts in transitional cell cancers of long-term residents of endemic villages in Croatia, and (iii) demonstration that A:T → T:A transversions, a mutational signature for exposure to AA, dominate the p53 mutational spectrum in these malignancies. Taken together, these data suggest that AA is the environmental agent responsible for EN and its attendant transitional cell cancer.

Identification of DNA adducts formed by AAs in renal tissues represents prima facie evidence of exposure to an established nephrotoxin/carcinogen. Toward that end, we used a highly sensitive 32P-postlabeling/PAGE method (29) to quantify AL-DNA adducts and validated this approach by performing this analysis on renal tissue from a patient with documented exposure to AA. Remarkably, dA-AL adducts were detected 3 years after the patient terminated her exposure. In this individual, liquid chromatography electrospray ionization/multistage mass spectrometry (LC-ESI/MS/MS) also was used to confirm the chemical identities of the dA-AL-I and dA-AL-II adducts. To our knowledge, this is the first study to employ MS3 product ion spectra to identify environmental carcinogen DNA adducts in humans. With this technique, dA-AL adducts can be detected at <0.2 fmol on column. With the incorporation of stable, isotopically labeled adducts as internal standards, we expect to quantify dA-AL adducts at levels approaching several adducts per 10^6 dNs, when 100 µg of DNA is used for analysis. This level of sensitivity approaches the detection limit of the 32P-postlabeling methods, while simultaneously providing quantitative measurements and spectral data to confirm details of adduct structure.

The 32P-postlabeling/PAGE method was applied to the analysis of AL-DNA adducts in target tissues of patients with clinically and histopathologically confirmed EN. In these individuals, dA-AL- and dG-AL-DNA adducts were identified by demonstrating comigration of putative adducts with authentic standards. AL-DNA adducts were detected in the renal cortex of patients with EN but not that of patients with other forms of chronic renal disease. In addition, dA-AL and dG-AL adducts were detected, albeit at significantly lower levels, in transitional cell cancers of residents of EN villages. The presence of these adducts in target tissues is consistent with their postulated role in the initiation of urothelial cancer. In an earlier study, Arlt et al. (33) used 32P-postlabeling methods to detect dA-AL-I, dA-AL-II, dG-AL-I, and putative OTA-DNA adducts in kidney tissues of two patients from an endemic region of Croatia. One patient was diagnosed with upper urothelial tract malignancy, and the other reportedly suffered from ureteral stenosis. The lack of clinical, epidemiologic, and histopathologic data in this study prevents classification of either patient as suffering from EN.

AL-DNA adducts serve as biomarkers of exposure and give rise to a specific pattern of mutations in the p53 gene. The significance of these mutational spectra is best appreciated by comparing the data in Table 1 with that reported for sporadic transitional cell carcinomas in the October 2006 edition (R11) of the largest international p53 mutational database (www.p53.iarc.fr) (32). In that database, A:T → T:A mutations occur infrequently in transitional cell carcinomas of the renal pelvis (0%), ureter (5.0%), and bladder (4.8%) and, at somewhat higher frequency (14.2%), in other (unspecified) urinary tract organs (Fig. 5B). In contrast, the frequency of A:T → T:A mutations in transitional cell carcinomas in patients with EN or suspected EN is 78% (Fig. 5A). Nine of the 19 mutations observed in our study (seven sites) are unique among the 696 transitional cell carcinomas listed in the IARC database.

The spectrum of p53 mutations observed in transitional cell cancers of patients with EN is dominated by A:T → T:A transversions (Fig. 5A). This result is consistent with the mutational spectra induced by AA-I (or by a mixture of AA-I and
AA-H) in (i) the H-ras gene of rats (23, 24), (ii) transgenic rodent models (25, 26), (iii) the p53 gene in a urothelial cancer from a patient with AAN (34), (iv) site-specific mutagenesis studies in which a single dA-AL adduct is transfected into NER-deficient human cells (I.-Y. Yang and M.M., unpublished data), and (v) 9 of 11 immortalized mouse cell lines (Hupki cells) carrying the human p53 gene (27, 28). Indeed, the predominance of A:T → T:A transversions in the p53 mutational spectra may properly be regarded as a mutational signature (35) for human exposure to AA.

Confidence in our conclusion regarding AA and the etiology of EN rests, in part, on the biomarker data reported here and, in part, on studies of AAN in human populations, in particular, a cluster of cases affecting women in Belgium (36). The clinical and histopathologic features of EN and AAN are remarkably similar (14). Hallmarks of both diseases include proximal renal tubular dysfunction manifested by low molecular weight proteinuria (37, 38) and dense interstitial fibrosis, decreasing in intensity from the outer to the inner cortex while sparing the glomeruli, an infrequent finding in other forms of renal disease (15). Also, in both disorders, urothelial cell atypia, and/or malignant transformation develop with similar frequencies (40, 50%) (7, 39). Interestingly, based on the prevalence of EN in endemic villages (6) and on the mean cumulative exposure to AA in the cluster of women with AAN (40), only ~5% of individuals exposed to AA develop overt manifestations of disease, suggesting that susceptibility to the toxin is markedly influenced by genetic factors.

Exposure to AA potentially could occur either through ingestion of contaminated wheat-based products or through the use of herbal remedies prepared from Aristolochia plants. The latter possibility (33) was excluded by the results of a questionnaire administered in 2005 to 1,081 residents of the endemic region as part of an ongoing epidemiologic investigation of EN. Recently, similar observations were made in our epidemiologic study in the endemic region of Croatia (16).

During the past 50 years, various hypotheses have been proposed to explain the etiology of EN and its associated urothelial cancer (9–11). Considerable attention has been devoted to the hypothesis that a mycotoxin, ochratoxin A (OTA), is the environmental toxin responsible for EN (12, 13, 42). However, solid epidemiologic evidence supporting an association between OTA exposure and the prevalence of EN or upper urothelial cancer is lacking (43). In fact, a search of the world literature failed to uncover any clearly documented human case of OTA-induced nephrotoxicity. Furthermore, studies using highly sensitive methods of DNA adduct detection, including accelerator MS (44), have shown that OTA does not bind to DNA in reactions catalyzed by human and rat enzymes (45). Additionally, although OTA is strongly carcinogenic in male rats, covalent OTA-DNA adducts have not been detected in this species (46). These investigations cast considerable doubt on claims (42) for genotoxicity as a mechanism for the toxic actions of OTA.

In conclusion, our data strongly support the hypothesis that chronic dietary poisoning with AA is an etiologic factor in the development of EN and its associated transitional cell cancer.

Methods

DNA Adduct Analysis. Methods used for the 32P-postlabeling/PAGE analysis of AA-derived DNA adducts were described in ref. 29. The mass spectrometric methods used in this study have been reported (47); experimental details are provided in the legend to Fig. 2 and in the supporting information (SI).

P53 Mutational Analysis. Transitional cell cancer tissues obtained from patients residing in endemic villages were analyzed in compliance with Institutional Review Boards of Stony Brook University and the School of Medicine, University of Zagreb. DNA was extracted from frozen and paraffin-embedded tissues, using a Qiagen (Valencia, CA) kit. Exons 2–11 were amplified and the fragmented DNA amplicons labeled with biotin at their 3′ termini. The mixture was hybridized to oligonucleotides on the AmpliChip p53 microarray and then washed and stained with phycoerythrin, a streptavidin-conjugated fluorescent dye (see SI for details).

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