Aristolochic acid mutagenesis: molecular clues to the aetiology of Balkan endemic nephropathy-associated urothelial cancer

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Balkan endemic nephropathy (BEN) is found in certain rural areas of the Balkans and affects at least 25 000 inhabitants. Of the many hypotheses on BEN, the Aristolochia hypothesis has recently gained ground substantiated by the investigations on aristolochic acid nephropathy (AAN). On both clinical and morphological grounds, AAN is very similar to BEN. That exposure to aristolochic acid (AA) of individuals living in endemic areas through consumption of bread made with flour contaminated with seeds of Aristolochia clematitis is responsible for BEN is an old hypothesis, but one which is fully consistent with the unique epidemiologic features of BEN. Here, we propose an approach to investigate AA-induced mutagenesis in BEN that can provide molecular clues to the aetiology of its associated urothelial cancer. The molecular mechanism of AA-induced carcinogenesis demonstrates a strong association between DNA adduct formation, mutation pattern and tumour development. A clear link between urothelial tumours, p53 mutations and AA exposure should emerge as more tumour DNA from BEN patients from different endemic areas becomes available for mutation analysis. We predict that the observed p53 mutation spectrum will be dominated by AT → TA transversion mutations as has already been demonstrated in the human p53 gene of immortalized cells after exposure to AAI and urothelial tumours from BEN patients in Croatia. Moreover, the demonstration of AA-specific DNA adducts in renal tissue of a number of BEN patients and individuals living in areas endemic for BEN in Croatia provides new evidence that chronic exposure to AA is a risk factor for BEN and its associated cancer.

Introduction

Balkan endemic nephropathy (BEN), a chronic renal interstitial fibrosis with slow progression to end-stage renal disease and urothelial malignancy, is found in certain rural areas of Bulgaria, Bosnia, Croatia, Romania and Serbia along the Danube river basin (1,2). At least 25 000 individuals suffer from BEN or are suspected of having the disease, whereas the total number of people at risk in these countries may exceed 100 000. Although first described 50 years ago, the aetiology of BEN remains unclear and is a matter of debate (1,2). In recent years, evidence has accumulated that BEN is an environmental disease (3). Of several hypotheses that have been proposed to explain the environmental cause of this disease, the major three are (i) the mycotoxin hypothesis, which postulates that BEN is caused by the fungal mycotoxin ochratoxin A (OTA) found in contaminated foodstuffs (4,5), (ii) the Pliocene lignite hypothesis, which proposes that BEN is caused by long-term exposure to polycyclic aromatic hydrocarbons (PAHs) and other toxic organic compounds leaching into well drinking water from low-rank coals (1) and (iii) the aristolochic acid (AA) hypothesis, which postulates that BEN is caused by chronic dietary intoxication with seeds of Aristolochia clematitis (6–10).

Chinese herbs nephropathy (CHN), first reported in a group of young patients with end-stage renal disease in Belgium in 1993, is a rapidly progressive renal interstitial fibrosis associated with a high risk of urothelial cancer (11–13). The observed nephropathy has been traced to the ingestion of herbal medicinal remedies that have included Aristolochia species containing AA and is now called aristo-

AA: an old herbal drug known since antiquity

AAs are found primarily in various species of the genus Aristolochia (e.g. A.clematitis, Aristolochia fangchi and Aristolochia manshurien-

AAn and urothelial cancer

The outbreak of so-called CHN in Belgium in 1993 was associated with the ingestion of Chinese herbal remedies prescribed by a single
Aristolochia species offered
naturally occurring nephropathy in which the unequivocal role of AA has been proven that it has been proposed to designate this novel nephropathy in which the unequivocal role of AA has been

Carcinogenic mechanism of AA in rodents. AA is a strong carcinogen in rodents (19). In rats treated orally with 0.1, 1 and 10 mg AA/kg body wt/day for 3 months, a high incidence of tumours was observed (25, 85 and 100%, respectively). Main targets for tumour formation were forestomach, kidney and urinary tract, with 72, 28 and 17% of the animals treated with 10 mg AA/kg body wt having tumours of these organs, respectively (31). DNA adduct formation was investigated in rats by 32P-post-labelling and the structures of the major AA–DNA adducts were identified as 7-(deoxyadenosin-N6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N2-yl)aristolactam I and 7-(deoxyadenosin-N6-yl)aristolactam II (32). AA-induced mutagenicity was investigated at the same doses in the transgenic Big Blue rat model (31,33).

Dose-dependent increases in mutant frequency (MF) and AA–DNA adduct formation measured by 32P-post-labelling were observed for liver (non-target organ) and kidney (target organ) (Figure 2A and B). MF was at least 2-fold higher in kidney compared with liver as were AA–DNA adduct levels, suggesting higher genotoxicity in the target organ for tumour development. Here, we show that there is a strong correlation between DNA adduct levels and MF both in liver (r = 0.995, P < 0.001) and kidney (r = 0.999, P < 0.001) (Figure 2C), indicating that mutagenic effects of AA are clearly associated with the formation of AA–DNA adducts. Carcinogenic effects were also observed in mice. Oral treatment with 5 mg AA/kg body wt/day for 3 weeks resulted in tumour formation in the forestomach, lungs and kidneys (34). In Muta2TMMouse treated with 15 mg AA/kg body wt once a week for 4 weeks, high MFs were found in the target organs (forestomach and organs of the urogenital tract), whereas only small increases in MFs were seen in non-target organs (e.g., glandular stomach and liver) (Figure 3) (35). Although DNA adduct formation has not been investigated in this study, organ-specific AA-induced
and bladder (35). Translesional bypass of adenine adducts of AA (dA-AAI and 7-(deoxyadenosin-β-N-yl)aristolactam II) points to a mutagenic potential resulting from dAMP incorporation opposite the adduct by DNA polymerase (36), suggesting that AT → TA transversion mutations would be the mutagenic consequence. AT → TA transversions are typical mutations observed in H-ras in tumours of rodents treated with AA and correspond with DNA adduct formation at adenine residues (37–39). This mutation occurs exclusively at the first adenine of codon 61 (CAA) in all forestomach and ear duct tumours of rats treated with AAI (37). This selectivity of AAI for mutations at adenine residues is consistent with the extensive formation of dA-AAI adducts in the target organ and their long-term persistence in forestomach DNA (22,40). Collectively, these data may indicate the probable molecular mechanism whereby AA induces tumours in rodents.

On the other hand, specific DNA damage due to AA in urothelial cells and cell-specific alterations at the transcription level of proteins might impair physiological processes (41,42). This may not only be of primary importance to explain the strong nephrotoxicity of AA but also indicates a potential mechanism on the seemingly tissue specificity of AA-induced oncogenesis. Changes in gene expression were examined in liver and kidney of rats treated with 10 mg AA/kg body wt five times per week for 3 months (43,44). Many more genes had altered expression levels in the target kidney than in the non-target liver following AA treatment. Significant alterations of biological processes related to defense response, apoptosis and immune response, as well as organic acid metabolism were found in kidney but not in liver (43).

Fig. 2. Total DNA adduct levels (RAL, relative adduct labelling) and cII MF in liver (non-target) and kidney (target) tissue of Big Blue rats treated with different doses of AA (adapted from ref. 31). (A) Dose dependence of MF. (B) Dose dependence of DNA adduct formation. (C) Correlation of MF with DNA adduct formation. Correlation coefficients were determined by linear regression using Statistical Analysis System software version 6.12.

Carcinogenic mechanism of AA in humans. Human cytosolic enzymes [e.g. NAD(P)H:quinone oxidoreductase (NQO1)] and microsomal enzymes (e.g. CYP1A1 and CYP1A2) activate AA by simple nitroreduction leading to DNA-binding species (45,46). Among the activating metabolizing enzymes is also prostaglandin H synthase that is highly expressed in urothelial tissue. Interestingly, it was reported that to date only 3–5% of the patients treated with the slimming regimen in Belgium have suffered from nephropathy (9,13,47). One possible explanation for the differential responses of patients may be individual differences in the activities of the enzymes catalysing biotransformation (activation and/or detoxification) of AA. The most abundant DNA adduct detected by 32P-post-labelling in urothelial tissue is dA-AAI (12,21,22,24–27). However, AA–DNA adducts are also found in various tissues outside the urinary tract (24,26,48), indicating that additional factors may be critical for the high incidence of urothelial tumours. Long-term persistence of the dA-AAI adduct in various organs (including kidney) in rats is in line with its detection in Belgian AAN patients almost 10 years after the patients stopped taking the herbal slimming regimen (12), thus demonstrating that AA–DNA adducts are not only suitable biomarkers of exposure to AA but also markers of cancer risk. In Belgian AAN patients, the risk to develop urothelial tumours was related to the cumulated intake of Aristolochia fangchi (12,23).

In AAN, urothelial atypia were associated with the over-expression of the p53 protein (13), suggesting that p53 is mutated in AAN-associated cancer (49). More than 50% of all human tumours contain a mutation in p53 (50). Interestingly, in one AAN patient from the UK available for analysis, a characteristic AT → TA transversion mutation was found in p53 (exon 5; codon 139 AAG) in urothelial tumour cells (48). It is noteworthy that the mutated base adenine has the same neighbour-base pairs in codon 138/139 (GCC AAG) of p53 as in codon 61 (CAA) of H-ras, suggesting a sequence-specific mechanism during mutation induction. Other mutations in p53 were also found in a papillary transitional cell carcinoma resected from the bladder of one Belgian AAN patient (see below) (9). To examine AA-induced mutation spectra in the human p53 gene in laboratory animals, a human p53 knock-in (Hupki) mouse has been constructed (51,52). When the Hupki p53 of immortalized cells derived from primary Hupki embryonic fibroblasts exposed to AAI were sequenced, specific AT → TA

mutagenesis correlates with the sites of tumour development following exposure to AA.

The AA-induced mutation pattern in the kidney in the Muta™ Mouse and Big Blue assays was similar (Figure 4A and B) (31,33,35). In addition, in Big Blue rats the overall pattern of mutations induced by AA in liver was similar to that in kidney (31,33). While the mutation spectrum of controls was dominated by GC → AT transitions, AT → TA transversions were the predominant AA-induced mutation type (Figure 4A and B). Similarly, in AA-treated Muta™ Mouse, AT → TA mutations were also predominant in target organ forestomach DNA adduct formation. Correlation coefficients were determined by linear regression using Statistical Analysis System software version 6.12.
transversion mutations in p53 were observed (Figure 4C) (53,54). Interestingly, in one cell line a characteristic AT → TA transversion was found at the first adenine of codon 139 (AAG) identical to the mutation found in the urothelial tumour cells of the UK AAN patient (48,54). These data may indicate a probable molecular mechanism whereby AA causes urothelial cancer.

Mutational specificity of a carcinogen often serves as indirect evidence for tumour initiation caused by interaction of the carcinogen with these specific DNA sequences (55–57). In the same AAN patient from the UK, a GC → AT transition mutation (exon 7; codon 245) was also found in p53 in a breast tumour and a liver metastasis, indicating that the mutation most probably arose before metastasis (48). However, given that GC → AT transitions are not the typical mutations induced by AA (usually AT → TA transversions), it was concluded that it is not likely that the p53 mutation in the breast and liver tumours was induced by AA.

**Genetic variation of AA-metabolizing enzymes as a risk factor in AAN and/or BEN?**

Variations in AA-activating enzymes such as NQO1 and CYP1A1 and regulatory proteins controlling expression of these enzymes may play a role in cancer susceptibility to AA. The role of genetic polymorphism in several genes relevant for detoxification has already been investigated in BEN patients (2,58,59). BEN patients homozygous for the $\text{NQO1}^2$ allele were at increased risk of developing urothelial malignancy of the upper urinary tract (odds ratio = 13.75, 95% confidence interval 1.17–166.21) (59). Thus, the importance of NQO1 in AA activation could be an explanation for the development of BEN (or AAN) and the high cancer risk of patients suffering from either of these nephropathies. While enzymes catalysing AA activation have already been extensively studied, those participating in its detoxification await further investigation. Preliminary data suggest that cytochrome P450 enzymes can generate AAs, which is considered a detoxification pathway (M.Šiborová, unpublished data). A large-scale investigation in BEN patients on the role of genetic polymorphism in genes of some phase I detoxification enzymes, such as CYP2D6, −3A4 and −3A5 as well as in those of the conjugation enzymes NAT1, NAT2, GSTT1 and GSTM1, revealed a higher risk for BEN (odds ratio = 2.41) in individuals carrying CYP1A1*1 allele G6989 (58). However, whether or not this cytochrome P450 isoform is involved in AA detoxification remains to be determined.

AA mutagenesis: a clue to BEN-associated urothelial cancer? The p53 tumour suppressor protein plays an important role in DNA damage responses, cell cycle control, apoptosis and activation of certain DNA repair systems (60). Disabling p53 function by mutations in the p53 coding sequence leads to the development of tumours. More than 50% of all human tumours contain a mutation in p53 (50). Over 20 000 human tumour mutations in p53 have been registered in the IARC TP53 database (61). In principle, this information can be used to generate hypotheses regarding disease risk factors in a defined population. Three often-cited observations that draw a link between a particular mutation profile and a specific environmental risk factor are (i) the high prevalence of tandem CC → TT mutations in squamous and basal cell carcinoma of skin and exposure to sunlight, (ii) the nearly exclusive occurrence of a codon 249 AGG to AGT hotspot mutation in hepatocellular carcinoma from high-incidence areas where aflatoxin exposure and chronic hepatitis B infection are common and (iii) the high prevalence of p53 GC → TA transversion mutations in lung cancers of tobacco smokers (50).

Figure 5A shows the distribution of mutations along p53 in tumours of the urinary tract (kidney, renal pelvis, ureter and other urinary organs excluding bladder) as found in the IARC TP53 database (http://www-p53.iarc.fr). In the current database (R11 release, October 2006), 137 mutations (with 121 mutations in the coding region) are recorded for these tissues. The mutation spectrum is characterized by

![Fig. 3. MF in the cII gene from various organs of Muta™Mouse treated with AA (adapted from ref. 35).](image)

![Fig. 4. p53 mutation pattern. (A) Mutation pattern in the kidney cII of Big Blue rat treated with AA (adapted from ref. 33). (B) Mutation pattern in the kidney cII of Big Blue rat treated with AA (adapted from ref. 33). (C) Immortalized HuPi murine embryonic fibroblasts (MEFs). Cell lines are derived from primary cells treated with AAI (N = 12) or not treated (N = 12) (adapted from refs 51–53). (D) Left: mutation pattern of human urinary tract tumours (kidney, renal pelvis, ureter and other urinary organs excluding bladder) as recorded in the IARC TP53 database (R11 release, October 2006), 137 mutations. Morphology inclusion criteria: carcinoma not otherwise specified, papillary carcinoma not otherwise specified, squamous cell carcinoma not otherwise specified, transitional cell carcinoma not otherwise specified, papillary transitional cell carcinoma, carcinoma in situ not otherwise specified and dysplasia not otherwise specified. (D) Right: mutation pattern of human bladder tumours as recorded in the IARC TP53 database (R11 release, October 2006), 898 mutations. Morphology inclusion criteria: carcinoma not otherwise specified, papillary carcinoma not otherwise specified, squamous cell carcinoma not otherwise specified, transitional cell carcinoma not otherwise specified, transitional cell carcinoma in situ, papillary transitional cell carcinoma, carcinoma in situ not otherwise specified, urothelial papilloma not otherwise specified and dysplasia not otherwise specified. (E) Mutation pattern in transitional cell carcinoma from BEN patients in Croatia (N = 11) (adapted from ref. 18).](image)
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mutation hotspots in codons 65, 175, 214, 248, 249, 258, 280 and 285. Some of these codons (175, 248 and 282) contain 5-methylcytosine within a CpG sequence and represent mutation hotspots in many other tumours (50,62). Mutations at these codons may be formed by a methylation–deamination mechanism but enhanced adduct formation at guanines in CpG sites by bulky carcinogens, such as PAHs, may also contribute to mutations at these mutation hotspots (57). Codons 65, 214, 258 and 280 seem more specific for the mutation spectrum of urinary tract tumours. Some of the mutations recorded for these codons are associated with adenine, mutations in codon 65 are even related to AT → TA transversion mutations. However, overall the mutation pattern is dominated by G → A (39%; G → A at CpG 19%) and A → G (20%), followed by G → T (12%), whereas A → T transversion mutations account for only 6% of mutations (Figure 4D). Although it is unclear whether these mutations are formed by endogenous or exogenous sources, some of the tumours have been attributed to smoking (exposure to aromatic amines) or to treatment with analgesic drugs (treatment with phenacetin). In fact, the database also lists the AT → TA transversion mutation found in codon 139 of the AAN patient (48). On the basis of patient data, however, it is likely that all except one of these mutations (as noted above) are not associated with AA exposure. However, in a recent report examining p53 mutations in urothelial tumours of BEN patients in Croatia (N = 11), mutations at A:T pairs accounted for 89% (17/19) of all mutations, with the majority of these (15/17) being AT → TA transversions, representing 78% of all base substitutions detected in the p53 gene (Figure 4E) (18). Interestingly, the mutations in p53 found in two of the cases (AT → TA transversions at codons 209 and 280) were also induced in immortalized cells derived from primary Hupki embryonic fibroblasts exposed to AAI (53,54). In general, we suggest that a clear link between urothelial tumours, p53 mutations and exposure to AA will emerge as more tumour DNA from afflicted persons becomes available for mutation analysis. We predict that this mutation spectrum will be dominated by AT → TA transversion mutations as has already been demonstrated in immortalized cells derived from primary Hupki embryonic fibroblasts after exposure to AAI and in

Fig. 5. p53 mutation spectrum. (A) Mutation spectrum of human urinary tract tumours (kidney, renal pelvis, ureter and other urinary organs excluding bladder) as recorded in the IARC TP53 database (R11 release, October 2006), 121 single-base substitutions. Morphology inclusion criteria: carcinoma not otherwise specified, papillary carcinoma not otherwise specified, squamous cell carcinoma not otherwise specified, transitional cell carcinoma not otherwise specified, papillary transitional cell carcinoma, carcinoma in situ not otherwise specified and dysplasia not otherwise specified. (B) Mutation spectrum of human bladder tumours as recorded in the IARC TP53 database (R11 release, October 2006), 822 single-base substitutions. Morphology inclusion criteria: carcinoma not otherwise specified, papillary carcinoma not otherwise specified, squamous cell carcinoma not otherwise specified, transitional cell carcinoma not otherwise specified, transitional cell carcinoma in situ, papillary transitional cell carcinoma, carcinoma in situ not otherwise specified, urothelial papilloma not otherwise specified and dysplasia not otherwise specified.
urothelial tumours from patients with BEN in Croatia (Figure 4C and E) (18,53,54). With respect to AA as a risk factor for BEN-associated urothelial tumours observed outside Croatia, we would also predict that many of these tumours carry characteristic AT → TA transversion mutations in p53. Those studies in combination with the detection of AA–DNA adducts in BEN patients (18) can provide the molecular clues demonstrating that chronic exposure to AA is a risk factor in areas endemic for BEN and its associated cancer.

More recently, an increased incidence of bladder tumours has been observed in AAN patients (63). Bladder tumours have also been reported in BEN patients. Figure 5B shows the mutation spectrum of bladder tumours as recorded in the IARC TP53 database. In the current database, 822 single-base substitutions (898 mutations in total) are recorded for bladder. The mutation spectrum is characterized by hotspots in codons 175, 220, 241, 245, 248, 271, 273, 280 and 285. There is some overlap with the codon distribution of mutations as found in tumours of the urinary tract (Figure 5A), and again, the mutation pattern is dominated by G → A (51%; G → A at CpG 19%), followed by G → C (13%), G → T (10%) and A → G (10%), whereas A → T transversion mutations account for only 5% (Figure 4D). Mutations associated with adenine are found in codons 220, 271, 280 and 285, whereas in codon 220 A → G transition is the exclusive mutation type (N = 17), mutations associated with adenine are rare at the other mutation hotspots, respectively. As mentioned above, it is noteworthy that a papillary transitional cell carcinoma from the bladder in one Belgian AAN patient showed an AT → GC transversion and a GC → AT transition mutation in exon 7, codon 230 (ACC) and codon 248 (CGG), respectively (9). However, we predict that a characteristic mutation spectrum (dominated by AT → TA transversions) in relation to AA exposure will emerge in bladder as more tumours from afflicted persons become available for mutation analysis.

A significant amount of research into the aetiology of BEN over the past decades has focused on OTA. Evidence both in favour (5,64-67) and against (68-71) the genotoxicity of OTA in humans and rodents has been published. The literature on short-term in vitro mutagenicity tests on OTA is also inconsistent (71) but there have been no in vivo studies on the mutagenic potency and mutagenic specificity in transgenic rodent mutation assays. IARC has classified OTA as a possible human carcinogen (Group 2B) on the basis of sufficient evidence for mutagenic rodent mutation assays. IARC has classified OTA as a possible human carcinogen (Group 2B) on the basis of sufficient evidence for mutagenic rodent mutation assays. IARC has classified OTA as a possible human carcinogen (Group 2B) on the basis of sufficient evidence for mutagenic rodent mutation assays. IARC has classified OTA as a possible human carcinogen (Group 2B) on the basis of sufficient evidence for mutagenic rodent mutation assays. 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IARC has classified OTA as a possible As indicated earlier, other factors in the pathogenesis of BEN cannot be ruled out.

Conclusions

Epidemiological evidence gathered during the last decades strongly suggests that BEN is an environmentally induced disease. Of the many hypotheses put forward to disclose the causative agents of BEN, the Aristolochia hypothesis has recently gained ground. That dietary intake of AA may be responsible for BEN and its associated urothelial cancer is a theory that was first proposed in 1969 by Ivic and is fully consistent with the unique epidemiologic features of BEN. Experimental evidence such as the detection of AA–DNA adducts in BEN patients and the identification of AA-specific mutation spectra in tumours of BEN patients would establish a molecular link between AAN and BEN. However, the role of other factors in the pathogenesis of BEN cannot be ruled out.

Funding

Association for International Cancer Research; Waltraud-Lewenz PhD student grant (to J.v.B.).

Acknowledgements

The authors gratefully acknowledge the contribution of Drs Nan Mei and Tao Chen, National Center for Toxicological Research, USA Food and Drug Administration, for providing raw data of the Big Blue mutation assay. V.M.A. and D.H.P. are members of the European Environmental Cancer Risk, Nutrition and Individual Susceptibility Network of Excellence. The authors thank Dr A. Grollman, University at Stony Brook, NY, for advance access to his publication subsequent to the online publication of our article.

Conflict of Interest Statement: None declared.

References


The text provided is a list of references, with a mix of journal citations and book citations, which are related to the study of aristolochic acid and its association with cancer. The references cover a range of studies from 1988 to 2004, and they discuss topics such as the carcinogenic effects of aristolochic acid, its detection in tissues, and the role of p53 mutations in cancer development. The references are cited in a natural language style, and they include a variety of sources such as peer-reviewed journals, books, and conference proceedings.

Received March 1, 2007; revised March 29, 2007; accepted April 2, 2007