Aristolochic Acid Nephropathy: Harbinger of a Global Iatrogenic Disease

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This review constitutes an overview of our investigations of aristolochic acid nephropathy, a chronic kidney disease associated with carcinomas of the upper urinary tract. Our studies began by confirming the hypothesis that chronic dietary poisoning by aristolochic acid was responsible for endemic (Balkan) nephropathy. A unique TP53 mutational signature in urothelial tumors and the presence of aristolactam-DNA adducts in the renal cortex, defined in the course of this research, proved to be robust biomarkers of exposure to this potent nephrotoxin and human carcinogen. Armed with this information, we used molecular epidemiologic approaches and novel mechanistic information to establish the causative role of aristolochic acid in upper urinary tract carcinoma in Taiwan, where one-third of the population had been prescribed herbal remedies containing Aristolochia, and the recorded incidence of upper urinary tract cancers is the highest in the world. As traditional Chinese medicine is practiced similarly in Taiwan and China, it is likely that upper urinary tract carcinomas and their attendant aristolochic acid nephropathy are prevalent in China and other Asian countries where Aristolochia herbs have been used for centuries in the treatment and prevention of disease, creating a potential public health problem of considerable magnitude. Environ. Mol. Mutagen. 54:1–7, 2013. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

First recognized in the late 1950’s, Balkan endemic nephropathy (EN) is a chronic progressive renal disease affecting residents of rural farming villages located near tributaries of the Danube River (Fig. 1). Unique epidemiologic features of this disease include its focal occurrence within certain villages; a familial, but not inherited, pattern of disease; occurrence in adults but not children; and a close association with carcinomas of the upper urinary tract (UUC) [Djukanović and Radovanović, 2003]. These relatively rare

2011 EMS AWARD

The Environmental Mutagen Society conferred this award on Arthur P. Grollman in recognition of his fundamental studies of mechanisms of mutagenesis and DNA repair and his public health investigations linking environmental mutagens to human disease. Dr. Grollman and his collaborators have made landmark contributions to our understanding of the molecular mechanisms by which DNA repair proteins process DNA damage caused by reactive oxygen species. Dr. Grollman also was honored for his work on aristolochic acid-induced cancer and nephropathy. These multidisciplinary studies established that Balkan endemic nephropathy, Chinese herbs nephropathy, aristolochic acid nephropathy, and their associated carcinomas of the upper urinary tract are caused by ingestion of aristolochic acid, a component of Aristolochia herbs. This research led to the characterization of signature TP53 mutations as well as the mechanisms of mutagenesis and repair for this environmental carcinogen.

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urothelial (transitional cell) cancers develop eventually in \(~50\%\) of all patients with EN [Petronic, 2000].

Over the past 50 years, investigations of the etiology of EN have focused on the role of environmental agents, including heavy metals, trace elements, organic chemicals, and, especially, ochratoxin A (OTA) [reviewed in Voice et al., 2006]. While high levels of OTA are sometimes present in the blood and urine of patients with EN or UUC, similar levels are found in individuals from countries where endemic nephropathy does not exist [Clark and Snedeker, 2006]. In fact, there is no solid epidemiologic or experimental evidence supporting the association of this ubiquitous mycotoxin with EN [Grollman and Jelakovic, 2007].

In 1969, Ivic proposed an etiologic mechanism for chronic Aristolochia poisoning in EN in which seeds from these plants, which grow abundantly in local wheat fields, co-mingle with wheat grain during the harvesting process [Ivic, 1969]. Ivic speculated that human exposure to a toxic component of Aristolochia might occur through ingestion of bread prepared from flour derived from contaminated grain, and demonstrated in rodent models that Aristolochia seeds induced nephropathy and sarcomas of the skin. Remarkably, this astute hypothesis never was pursued. As a result, the etiology of EN remained an enigma for more than 50 years [Batuman, 2006; Bamias and Boletis, 2008].

Our interest in unraveling the mystery of this environmental disease was inspired by reports of a chronic renal disease that developed in a group of otherwise healthy Belgian women who had ingested Chinese herbs as part of a slimming regimen [Vanherweghem et al., 1993]. Over time, approximately 100 of these women developed chronic renal insufficiency, with many requiring dialysis or transplantation. In certain cases, where the diseased kidneys had been removed, urothelial atypia and carcinomas were found in the upper urinary tract [Cosyns et al., 1999; Nortier et al., 2000]. Cosyns first called attention to the unique renal histopathology of so-called Chinese herbs nephropathy (CHN) with its striking similarity to EN [Cosyns et al., 1994].

Toxicologists have long been aware of the nephrotoxic properties of Aristolochia sp. [Grollman et al., 2009] and, in fact, more than a century ago, Pohl administered extracts prepared from seeds and roots of A. clematitis, to rabbits, revealing its nephrotoxic effects. In addition, veterinary literature from Yugoslavia revealed that horses that ingested hay mixed with A. clematitis developed chronic renal failure [Martinic, 1958]. Toxicity notwithstanding, Aristolochia sp. have been used worldwide for centuries as herbal remedies.

Aristolochia sp. contain a family of structurally related nitrophenanthrene carboxylic acids, principally aristolochic acid I (AA-I), and aristolochic acid II (AA-II). In cells, AA-I and AA-II are subjected to enzymatic nitroreduction, generating a reactive intermediate that binds to the exocyclic amino groups of dA and dG to form aristolactam (AL)-DNA adducts (Fig. 2) [Pfau et al., 1990]. AL-dA adducts persist for years in the renal cortex [Nortier et al., 2000], serving as robust biomarkers of exposure to AA. Schmeiser and his associates conducted pioneering studies on the molecular mechanism of AA-induced carcinogenesis [reviewed in Arlt et al., 2002]. Later, methods developed in the course of this research...

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**Fig. 1.** Map depicting discrete pockets of endemic nephropathy and associated upper urinary tract carcinoma in rural farming villages in Croatia, Bosnia-Herzegovina, Serbia, Bulgaria, and Romania.

**Fig. 2.** Metabolic activation and DNA adduct formation by aristolochic acid I and aristolochic acid II. NR = nitroreductase.
were applied to the identification of AL-DNA adducts in tissues of Belgian women with CHN [Nortier et al., 2012].

Our studies of EN began with a pilot epidemiologic study, led by a Stony Brook medical student who was fluent in Serbo-Croatian [Hranjec et al., 2005]. Working in an endemic region of Croatia, our research team confirmed earlier reports [Martinčić, 1958; Ivić, 1969] that Aristolochia clematitis could be found in cultivated fields, where it was considered to be a harmless weed. Moreover, as Ivić had observed, traditional methods used by local farmers for harvesting and milling of wheat allowed seeds of A. clematitis to contaminate the grain used to prepare home-made bread, a dietary staple among local farmers. Finally, conversations with EN patients disabused any notion that A. clematitis had been used in the endemic region as an herbal remedy.

Thus, in 2005, we developed our guiding hypothesis that dietary ingestion of AA, in conjunction with individual genetic sensitivity, accounts for all epidemiologic, clinical and pathophysiologic features of EN and UUC. This hypothesis has an important corollary; namely, Balkan endemic nephropathy, Chinese herbs nephropathy, and aristolochic acid nephropathy are the same disease [Grollman et al., 2009].

Our hypothesis entailed several predictions. First, chronic dietary exposure to AA should result in the accumulation of AL-DNA adducts in target tissues; in this case, the renal cortex and upper urinary tract. Second, translesion synthesis past AL-DNA adducts should lead to mutations in the TP53 tumor suppressor gene. A series of investigations were undertaken to test these assumptions and the results of this research are summarized below.

ARISTOLACTAM-DNA ADDUCTS AND EN

The presence of AL-DNA adducts in target tissues provides a tangible link between dietary exposure to AA and its nephrotoxic and carcinogenic effects. We identified the predominant dA-AL-1 adduct using 32P-postlabelling/polycrylamide gel electrophoresis, by demonstrating its comigration with authentic synthetic standards [Dong et al., 2006]. In addition, we subjected renal cortical DNA to multistage tandem mass spectrometric analysis, providing unequivocal chemical identification of dA-AL adducts in the tissues of patients from endemic regions [Grollman et al., 2007; Jelaković et al., 2012].

DNA for these studies was obtained from the renal cortex and tumor tissue of patients from Croatia, Serbia, and Bosnia who had undergone nephroureterectomy for UUC [Jelaković et al., 2012]. dA-AL adducts, with levels ranging between 0.2 and 19 adducts per 10^8 nucleotides, were detected in the renal cortex of 70% of the endemic cases (Table I). dA-AL adducts also were detected, albeit at lower levels, in DNA obtained from urothelial cancer tissue [Grollman et al., 2007]. In contrast, dA-AL adducts were not found in the renal cortex of UUC patients residing in non-endemic areas, such as the cities of Belgrade and Zagreb.

An important advance in the ability to analyze DNA adducts was achieved when we demonstrated that UPLC-ESI/MS^n, a highly sensitive, specific and robust analytical method, could replace the 32P-postlabeling techniques used for biomonitoring of DNA adducts in human tissues. For 25 years, 32P-postlabeling has served as an ultra-sensitive but non-specific method for quantifying DNA adducts in molecular epidemiology studies. Now, quantitative mass spectroscopy can be used for this purpose while, at the same time, corroborating the chemical identity of the lesion under study [Yun et al., 2012].

MUTATIONAL SIGNATURE OF AA IN TP53

Translesion DNA synthesis past AL-dA adducts generates predominantly A→T transversions, as established by studies of AA-induced mutagenesis of the H-ras gene in rats [Schmeiser et al., 1990], in human TP53 knock-in mouse cells treated with AA [Nedelko et al., 2009], and in site-specific mutagenesis experiments in which plasmid DNA containing a single AL-dA adduct was allowed to replicate in mouse cells [Attaluri et al., 2010]. This mutational specificity was confirmed by our analysis of TP53 mutations in UUC, drawn from a population with an established dietary exposure to AA (Fig. 3). Moreover, these studies revealed a pattern of mutations unique to this environmental mutagen [Grollman et al., 2007; Moriya et al., 2011]. Thus, the mutational spectrum of TP53 in this cohort is dominated by A:T→T:A transversions (68% of the total), a class of mutations found in only 5.3% of all human cancers, 4.8% of all urothelial (transitional cell) carcinomas and 1.4% of all UUC (renal pelvis and ureter). A→T mutational hotspots were observed at codons 131 and 179 and at the splice acceptor splice site for intron 6. Mutations at these sites have not previously been associ-
results of the foregoing experiments proved important in several respects. First, we validated the biomarkers used in this molecular epidemiologic study. Second, in fulfilling the several predictions made in undertaking this research, we provided compelling evidence that AA was the etiologic agent responsible for EN and UUC [de Jonge and Vanherweghem, 2008]. Finally, as a result of these investigations, EN joined CHN in being properly referred to as aristolochic acid nephropathy (AAN) [De Broe, 2012].

**ARISTOLOCHIA HERBAL REMEDIES AND UUC**

*Aristolochia* herbs have been used for medicinal purposes throughout Europe for more than 2500 years [Dawson, 1927]. The earliest mention of *Aristolochia* in China dates to the fifth century; references to various species of this herb appear in every important treatise of traditional Chinese medicine [Zhu, 2002]. Surprisingly, descriptions of the therapeutic use of *Aristolochia* rarely mention any intrinsic toxicity. For this reason, we and others [Debelle et al., 2008; Grollman et al., 2009] raised a provocative and potentially disturbing question: Given the widespread use of *Aristolochia* sp. in traditional herbal remedies, could aristolochic acid be responsible for a previously unrecognized global disease?

To address this issue, we adopted the molecular epidemiologic approach we had used in our Balkan study, with AL-DNA adducts in the renal cortex serving as a robust and sensitive biomarker of exposure, together with *TP53* mutational spectra, as a specific biomarker of exposure and carcinogenic effects. As a locale for our study, we selected Taiwan, the country with the highest recorded incidence of UUC and one where extensive use of *Aristolochia* herbal remedies has been documented by the systematic analysis of prescriptions in a national database. These analyses show that approximately one-third of the population of Taiwan have been exposed to herbs containing, or likely to contain, AA [Hsieh et al., 2008]. Moreover, a linear dose-response relationship has been established between the consumption of herbal remedies containing AA and the risk of developing UUC [Lai et al., 2010].

The subjects in our study [Chen et al., 2012] comprised 151 patients with UUC and 25 patients with renal cell...
carcinoma (RCC). Among patients with UUC, 60% of women and 50% of men with TP53 mutations displayed the signature A:T→T:A transversions. Remarkably, despite significant differences in the dose, frequency and timing of exposure to AA, the overall distribution and positions of A:T→T:A transversions in TP53 of UUC patients from Taiwan are almost identical to those observed in residents of the EN regions in Bosnia, Croatia and Serbia (Fig. 3B).

In both cohorts, these singular mutations appear almost exclusively on the nontranscribed strand, with hotspots at various 5’AG acceptor splice sites. Direct evidence of exposure to AA was established by the presence of dA-AL adducts in the renal cortex of 60% of all cases of UUC and of 84% of patients with A:T→T:A mutations in TP53 (Table I). AL adducts also were detected in 60% of patients with RCC, reflecting the widespread exposure to AA in Taiwan. However, in this control group, A→T mutations were not present in TP53, as AL-DNA adducts do not drive the neoplastic process in RCC.

We conclude from this study that AA, an established human carcinogen [National Toxicology Program, 2011], and an intrinsic component of all Aristolochia herbal remedies, contributes significantly to the high incidence of UUC in Taiwan. This finding has implications for public health internationally [Chen et al., 2012].

GLOBAL PUBLIC HEALTH CONSIDERATIONS

The traditional practice of Chinese herbal medicine in Taiwan mirrors that in mainland China. In the past, large amounts of Aristolochia herbs have been produced annually in China [Hu et al., 2004], and several Aristolochia sp. are still listed in the 2005 Chinese Pharmacopoeia. Importantly, the nephrotoxic effects of AA are irreversible and its carcinogenic effects may not become manifest until 30 or more years after exposure. Thus, UUC and its attendant AAN are likely to be prevalent in China and other Asian countries where Aristolochia herbs have been used for the treatment and prevention of disease. Indeed, it is estimated that in China 100 million people may be at risk [Hu et al., 2004].

PERSPECTIVES

The discoveries described in this review (Fig. 4) were made in a relatively short period of time. We credit our
success to adopting a paradigm embodied in the seminal research of Wogan, Groopman, and Kensler on the environmental toxicology of aflatoxin [Kensler et al., 2011]. Their work illustrates the power of combining mechanistic information with molecular epidemiologic approaches in establishing causative linkages between exposure to an environmental mutagen and increased disease risk.

Accordingly, while pursuing the etiology of EN, we also investigated the molecular toxicology of AA. These parallel, interdisciplinary studies included the development of robust methods for quantifying AL-DNA adducts [Dong et al., 2006; Yun et al., 2012], chemical synthesis of AA-derived analogs [Attaluri et al., 2010], elucidation of mechanisms of AA transport in the proximal tubule [Dickman et al., 2011], demarcation of pathways involved in the excision of AL-DNA adducts by nucleotide excision repair [Sidorenko et al., 2012], identification of genes and enzymes responsible for detoxifying AA [Rosenquist et al., 2010; Shibutani et al., 2010], and documentation, via site-specific techniques, of the mutational specificity and frequency of dA-AL and dG-AL adducts in mammalian cells [Attaluri et al., 2010]. These diverse mechanistic investigations played a crucial role in the design and execution of our translational research and in the interpretation of our results.

Our prediction of the global nature of chronic renal disease and upper urothelial carcinoma attributable to AA sets the stage for research on prevention and early diagnosis, which will be required to deal with this devastating disease. AA is unusual among environmental carcinogens in its persistence in target tissues and in harboring a distinctive mutational signature, properties that should facilitate the development of biomarkers for the identification and screening of populations at risk.

Finally, significant questions remain to be addressed, thereby clarifying and extending on our research. Among the most important of such initiatives are the identification of genes governing human susceptibility to AA and AA-induced UUC, as well as the elucidation of molecular and cellular mechanisms by which AA exerts its profound nephrotoxic effects. Answers to these central questions should help to illuminate the pathogenic processes that contribute to these and other forms of human disease.

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