Role of Environmental Toxins in Endemic (Balkan) Nephropathy

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Endemic (Balkan) nephropathy is a chronic tubulointerstitial nephritis affecting residents of rural villages located in valleys near tributaries of the Danube River in Bosnia, Bulgaria, Croatia, Romania, and Serbia.1 Although endemic nephropathy was first described in the literature in the late 1950s, anecdotal reports and church records suggest that this disease was present in these countries many decades before.2

The significant epidemiologic features of endemic nephropathy include its focal occurrence in certain farming villages, with unaffected villages located in close proximity; a familial but not inherited pattern of disease, frequently affecting members of the same household; occurrence only in individuals who are older than 18 yr; occurrence in <10% of households in endemic villages; and a strong association with upper urinary tract transitional cell (urothelial) cancer.3 These epidemiologic findings suggest the involvement of an environmental toxin in both the nephropathy and urothelial cancer associated with endemic nephropathy. A variety of environmental factors have been explored during the past 50 yr, including heavy metals, polyaromatic hydrocarbons, viruses, and trace elements,4 with ochratoxin A being the most recent focus of research.5 In this article, we describe exciting new advances in endemic nephropathy research, presented at an international symposium held in Zagreb, Croatia, in October 2006 (abstracts of symposium presentations and posters may be accessed at http://www.endemic-nephropathy.mef.hr/). Happily, this “tale of two toxins” sheds new light on the cause of this devastating renal disease.6

**ABSTRACT**

An international symposium, held in Zagreb, Croatia, in October 2006, brought together basic scientists and clinical investigators engaged in research on endemic (Balkan) nephropathy, a chronic renal tubulointerstitial disease of previously unknown cause that often is accompanied by upper urinary tract urothelial cancer. Although this disease is endemic in rural areas of Bosnia, Bulgaria, Croatia, Romania, and Serbia, a similar clinical entity occurs throughout Europe, Asia, and North America. Recent advances in the understanding of endemic nephropathy now favor the causative role of aristolochic acid over the ubiquitous mycotoxin known as ochratoxin A. Specifically, aristolactam-DNA adducts have been found in renal tissues and urothelial cancers of affected patients. A “signature” p53 mutation in the upper urothelial cancer associated with this disease provides evidence of long-term exposure to aristolochic acid. In addition, the renal pathophysiology and histopathology observed in endemic nephropathy most closely resemble the entity known as aristolochic acid nephropathy. Public health authorities in countries harboring this disease are encouraged to reduce the potential for dietary exposure to Aristolochia clematitis.

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**OCHRATOXIN A AND ENDEMIC NEPHROPATHY**

The ubiquitous presence of ochratoxin A in a variety of common foodstuffs, including cereal grains, was recognized more than three decades ago. Shortly thereafter, ochratoxin A was shown to be a powerful rodent carcinogen,7 and concerns arose regarding its safety in humans. The original hypothesis that ochratoxin A might be involved in the cause of endemic nephropathy was based on similarities between the histopathology of endemic nephropathy and mycotoxin-induced porcine nephropathy.8 Subsequently, residents of endemic villages were shown to have high blood concentrations of ochratoxin A, as do certain otherwise healthy individuals in countries throughout the world.9

In a lecture and several posters, Castegnaro and Pfohl-Leskowicz (Auzeville-Tolosane, France) presented...
data in support of their hypothesis that ochratoxin A is involved in the pathogenesis of endemic nephropathy and upper urothelial cancer10 (reviewed by Pfohl-Leszkowicz and Manderville5). Central to this notion is their interpretation of the 32P-postlabeling analyses of DNA obtained from tissues of rodents and pigs treated with ochratoxin A5 and from urinary tract tumors of patients residing in endemic villages in Bulgaria.11 These labeling studies generate multiple radioactive "spots," two of which seem to co-migrate chromatographically with C8-deoxyguanosine adducts prepared by photolabeling of ochratoxin A. The conclusion from these experiments—namely, that ochratoxin A undergoes enzymatic biotransformation to produce the same reactive species as observed in the photolabeling reaction, forming covalent adducts with DNA5,12—conflicts sharply with data from the laboratories of Dekant13 and Turesky,14,15 who, despite using a variety of ultrasensitive analytical methods, failed to detect ochratoxin A-DNA adducts in cells or in rodents treated with high dosages of ochratoxin A (level of detection <3 adducts per 10^9 nucleotides). At this meeting, Mally (Würzburg, Germany) reported that so-called ochratoxin A-DNA adducts isolated from cells treated with tritium-labeled ochratoxin A do not contain part of the ochratoxin A molecule.

Ochratoxin A is nephrotoxic in rabbits, mice, and pigs; cytotoxic to cultured renal cells; and carcinogenic in rodents.5,9 Oxidative DNA damage may account for some or all of these harmful effects.16 Evidence supporting an oxidative mechanism was presented by Fuchs and Peraica (Zagreb, Croatia), who reported that levels of malondialdehyde and protein carbonyl were increased in renal tissue of male rats treated with low dosages (5 ng/kg) of ochratoxin A. Marin-Kuan and Schilter (Lausanne, Switzerland) used gene expression profiling to demonstrate ochratoxin A–induced disruption of pathways regulated by the transcription factor Nrf2, thereby reducing cellular defenses against oxidative stress. They proposed that a network of interacting epigenetic mechanisms, including protein synthesis inhibition, oxidative stress, and the activation of specific cell signaling pathways, may be responsible for renal cell tumors in rats treated with ochratoxin A.17

In discussions of presentations and posters related to ochratoxin A, it became clear that ochratoxin A has never been documented as a cause of human renal disease or human cancer; ochratoxin A is detected in the blood of individuals throughout Europe, whereas endemic nephropathy is limited to isolated villages in the endemic region; chemically characterized ochratoxin A-DNA adducts do not form under physiologic conditions in rodents or cultured cells treated with high dosages of ochratoxin A; and the so-called "ochratoxin A-DNA adducts" described in 32P-postlabeling studies of cells treated with ochratoxin A do not contain this molecule. Therefore, it seems highly unlikely that ochratoxin A is a direct-acting genotoxic carcinogen or that it plays a significant role in the cause of endemic nephropathy or upper urothelial cancer. A similar conclusion was reached by a European Union Committee on Food Safety, whose report stated "there is no convincing evidence from human epidemiology to confirm the association between ochratoxin A exposure and the prevalence of endemic nephropathy or urothelial cancer."18

ARISTOLOCHIC ACID AND ENDEMIC NEPHROPATHY

Jelaković (Zagreb) drew attention to the well-documented but largely overlooked reports from Croatia of nephrotoxicity in horses that ingested hay contaminated with Aristolochia clematitis,19,20 a plant containing aristolochic acid. Examination of renal tissue from these animals revealed histopathologic changes very similar to those associated with endemic nephropathy. The prevalence of A. clematitis in crop fields in Croatia was noted in these early accounts, providing credence to an intriguing hypothesis formulated in 1967 by Ivić.21 Observing that seeds of A. clematitis commingled with wheat grain during the annual harvest, Ivić suggested that a toxic constituent of this plant might be inadvertently introduced into home-baked bread, a dietary staple of farm families in the endemic region. Remarkably, during the next 35 yr, no attempt was made to confirm or follow up Ivić’s prescient observations despite frequent references to his hypothesis in literature reviews.

This critical oversight was rectified in 2004, when Hranjec et al.22 conducted a case-controlled molecular epidemiologic study involving residents of several endemic villages in Croatia; residents of a nearby disease-free village served as control subjects. DNA was obtained from all participants for genotyping studies. The results of this investigation excluded the possibility that this population’s exposure to aristolochic acid might relate to the medicinal use of herbal remedies and strongly supports Ivić’s proposal that dietary exposure to seeds of A. clematitis is an important risk factor for endemic nephropathy.

In parallel studies, Shibutani (Stony Brook, NY) and colleagues23,24 used ultrasensitive, quantitative 32P-postlabeling methods, in conjunction with HPLC and mass spectroscopic techniques, to quantify dA-aristolactam (dA-AL) and dG-aristolactam (dG-AL) adducts in the renal cortex of patients with endemic nephropathy and in urothelial cancer tissues of residents of endemic villages. In addition, A:T→T:A mutations were shown to dominate the p53 mutational spectra of urothelial cancers removed at surgery from patients residing in the endemic region24 (Figure 1A). This “signature” mutation, rarely observed in transitional cell cancers25 (Figure 1B), is consistent with the mutational spectra induced by aristolochic acid-I in the Hras gene of rats,26 transgenic rodent models,27 the p53 gene in a ureteral tumor from a patient with documented aristolochic acid nephropathy,28 site-specific mutagenesis studies by Yang and Moriya (Stony Brook) in which a single dA-AL adduct is transfected into NER-deficient human cells, and nine of 11 mouse cell lines carrying the human p53 gene.29,30 Indeed, the predominance of
A:T→T:A transversions in the p53 mutational spectra now may be regarded as a signature mutation for human exposure to aristolochic acid.24

The foregoing collaborative research, involving investigators at Stony Brook University, University of Zagreb, Institute Rudjer Bošković, and Croatian Center for Endemic Nephropathy (Croatia), summarized in a lecture by Grollman (Stony Brook), provides strong support for the hypothesis that long-term dietary poisoning with aristolochic acid is involved in the cause of endemic nephropathy and endemic urothelial cancer. This breakthrough in identifying an important environmental risk factor for endemic nephropathy prompted the Fogarty Center, the National Institute of Environmental Health Sciences, and the Croatian Ministries of Science and Health to join forces in supporting a new interdisciplinary translational research program centered on the genetic epidemiology and molecular toxicology of aristolochic acid.

Slade (Zagreb) discussed the application of p53 mutational data to upper urothelial cancer. Arlt et al.26 (Sutton, Great Britian) reviewed the mutational specificity of aristolochic acid, including studies conducted in the Hollstein laboratory (Heidelberg, Germany), in which the human p53 gene was introduced into mouse fibroblasts (Hupki cells) and then treated with aristolochic acid.29,30 As noted, the mutational spectra observed in vitro (A:T→T:A) is similar to that reported in the Ha-ras gene of rodents treated with aristolochic acid.26 Stiborová et al.31 (Prague, Czech Republic) described studies in which she and her colleagues used human hepatic and renal microsomes and, in some cases, purified enzymes to establish potential roles for Cyp1A1, Cyp 1A2, NADPH:CYP reductase, prostaglandin H synthase, NQO1, and xanthine oxidase in catalyzing the formation of AL-DNA adducts.

Dickman (Stony Brook) used the isolated perfused rat kidney model to measure the renal clearance of several aristolochic acids, and isolated purified rat proximal tubules to study aristolochic acid-I metabolism and transport. She reported that the renal excretion of aristolochic acid-I involves net secretion of aristolochic acid-1a, a metabolite formed by demethylation of the toxin in the proximal tubule. Importantly, this reaction, catalyzed by human CYP IA1/2 (Einhoff, West Hanover, NJ), abolishes the nephrotoxic properties of aristolochic acid-I. Additionally, several novel aristolochic acid metabolites were identified and subsequently characterized by mass spectroscopy (Figure 2).

Shibutani et al.,32 using a mouse model of aristolochic acid nephropathy, discovered that one of the aristolochic acids found in Aristolochia species of A. clematitis (aristolochic acid-I) is nephrotoxic, whereas the other major constituent (aristolochic acid-II) is not; however, both compounds form covalent adducts with DNA. Rosenquist (Stony Brook) used inbred strains of mice to map quantitative trait loci contributing to aristolochic acid nephropathy in the mouse. Zavadil (New York, NY) used microarray techniques to explore the mechanisms of toxicity of aristolochic acid-I and aristolochic acid-II, validating his gene expression studies by a proteomic analysis in collaboration with Josic (Providence, RI). This powerful approach was used to establish key genetic regulatory and signaling pathways involved in the pathophysiology of aristolochic acid nephropathy (Figure 3). This information could lead to the identification of potential targets for the treatment of renal epithelial injury and interstitial fibrosis.

ENDEMIC (BALKAN) NEPHROPATHY = CHINESE HERBAL NEPHROPATHY = ARISTOLOCHIC ACID NEPHROPATHY

In the lecture “Kidney Disease among the Romans,” Scarborough (Madison, WI) reported that herbal recipes used in Roman-Greco times contained Aristolochia in amounts estimated to be highly nephrotoxic. Indeed, Aristolochia species have been used widely for more than 2000 years in Chinese and Ayurvedic medicine as well as in Europe, Latin America, and the United States.33 Remarkably, the potent toxicity of Aristolochia in humans was not fully appreciated until the early 1990s, when a cluster of renal failure cases was identified in Brussels. Subsequently, additional cases of so-called Chinese herb nephropathy were reported worldwide.34 Importantly, all species of Aristolochia contain aristolochic acid.35

Nortier (Brussels, Belgium) provided a clinical and epidemiologic update to
Figure 2. Metabolism of aristolochic acids in the rat. Aristolochic acid-I is metabolized via two major pathways.43 Demethylation to aristolochic acid-Ia generates a non-nephrotoxic product that is subject to phase II biotransformation to form glucuronide or sulfate conjugates. Alternatively, reactions catalyzed by cellular nitroreductases (NR) generate reactive intermediates that form covalent adducts with DNA and protein. Further reduction ultimately yields the non-nephrotoxic aristolactams. AL, aristolactam; UGT, UDP-glucuronyltransferase; SULT, sulfttransferase; CYP, cytochrome P450.

As noted by Stefanović (Niš, Serbia) and Jelaković, differing diagnostic criteria used in various countries harboring endemic nephropathy represents a problem when comparing epidemiologic data for research purposes. Furthermore, the lack of sensitive and specific biomarkers makes it difficult to determine accurately the prevalence of this disease. Recent data are available only for a few endemic foci. Bukvić (Lazarevac, Serbia) reported that the prevalence of endemic nephropathy in Lazarevac, Serbia, has been unchanged for the past 20 yr. Miletić-Medved (Slavonski Brod, Croatia) observed the same prevalence in 2005 as in 1980 to 1995 for the focus under study in Croatia. In addition, Dimitrov (Sofia, Bulgaria) used patient registry analyses of endemic nephropathy, which tend to underreport this disease, recording a decreasing prevalence of endemic nephropathy in Bulgaria. Imamović (Tužla, Bosnia) reported that the prevalence of endemic nephropathy in a Bosnian focus was essentially the same as in 1977. In all endemic areas, the median age at which patients initially receive a diagnosis of endemic nephropathy has increased significantly during the past 40 yr.

A large clinical research study led by Jelaković and Miletić-Medved and supported by the Croatian Ministries of Science and Health serves as a model for future epidemiologic studies of endemic nephropathy. This field study, including a detailed questionnaire developed for a case-controlled pilot study,22 involved more than 1000 individuals residing in endemic and “control” villages. Blood and urine samples were obtained, with informed consent, for this nested-case-double-control, molecular epidemiologic study. DNA was secured for high-throughput genotyping studies, following encouraging
results from a pilot study (Chen; Stony Brook) in which several candidate genes that seem to confer susceptibility to endemic nephropathy were identified.

**UPPER UROTHELIAL CANCER**

The striking geographic correlation between endemic nephropathy and urothelial cancer, two very rare diseases, points to a common causative agent. Miletic-Medved reported that the mortality associated with urothelial cancer in Croatian endemic nephropathy areas is 55 times higher than in other regions in Croatia. Bukvić reported no significant change in the incidence of urothelial cancer in the past 20 yr in the Lazarevac region. Nikolić (Belgrade) analyzed data from 2110 Serbian patients who had urothelial cancer and had undergone unilateral nephroureterectomy during the past 50 yr. In this cohort, the average age of patients in a given village is inversely proportional to the incidence, suggesting dosage dependence for the environmental toxin responsible for endemic urothelial cancer.

Endemic urothelial cancer has a latency period of 10 to 20 yr and may produce symptoms before renal dysfunction is clinically evident. In the future, then, urothelial cancer may represent a greater public health problem than endemic nephropathy–associated renal failure. In fact, Bašić-Jukić (Zagreb) observed a much higher incidence of urothelial cancer in patients who had endemic nephropathy and underwent renal transplantation compared with patients with other forms of renal failure (22 versus 0.67%), suggesting that bilateral nephrectomy should be performed prophylactically in patients with endemic nephropathy to prevent urothelial cancer.

**EMERGING CONCEPTS AND FUTURE DIRECTIONS**

The cardinal conclusion emerging from the Zagreb meeting is that aristolochic acid, not ochratoxin A, is the principal risk factor for endemic nephropathy and endemic nephropathy–associated urothelial cancer. This critical concept is the key to future basic and translational research agendas and forms a basis for public health programs in endemic regions. Interdisciplinary and translational research focused on aristolochic acid, a powerful nephrotoxin and human carcinogen, are

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**Figure 3.** Gene expression profiling of aristolochic acid nephropathy modeled in the C3H/He mouse. Mice were injected intraperitoneally with aristolochic acid-I or aristolochic acid-II (1.8 mg/kg) daily for 11 d, and renal cortical epithelial mRNA was profiled by microarray analysis. (A) Genes and biological categories common to both aristolochic acid-I and aristolochic acid-II treatment. (B) Genes and biological categories specific for aristolochic acid-I. (C) Genes and biological categories exclusive to the nephrotoxic and fibrogenic effects of aristolochic acid-I. Red and blue colors represent up- and down-modulated mRNA, respectively.
long overdue. Current dietary exposure to aristolochic acid should be evaluated by establishing the prevalence of A. clematitis in wheat fields and by direct measurements of aristolochic acid in the flour used in preparing home-baked bread. The ongoing research program in Croatia constitutes a model for molecular epidemiologic investigations of endemic nephropathy. Toxicogenomic studies can provide unique insights into the genes and gene–environment interactions that govern susceptibility and resistance to this environmental disease. Although banned in many countries, Aristolochia species continue to be used as a component in traditional herbal remedies; as a result, aristolochic acid nephropathy represents a global health problem of considerable magnitude. Investigations of the molecular mechanism(s) of nephrotoxicity and carcinogenicity of aristolochic acid are clearly warranted. Rodent models of aristolochic acid nephropathy may be used to study biotransformation of aristolochic acid and repair of AL-DNA adducts and, importantly, to validate the use of AL-DNA adducts as biomarkers of disease. The murine model will be particularly useful for determining genetic susceptibility to aristolochic acid nephropathy and, by implication, to endemic nephropathy. Microarray, quantitative proteomic, and bioinformatic approaches can be combined with information on the human genome to establish the cellular pathways affected by aristolochic acid-I and aristolochic acid-II, including those involved in aristolochic acid-I–induced interstitial fibrosis.

From the clinical perspective, various diagnostic criteria for endemic nephropathy should be formally reevaluated; a meeting of endemic nephropathy center directors is being convened for this purpose. The detection of AL-DNA adducts in renal tissues of patients with endemic nephropathy/urothelial cancer should be confirmed, using quantitative methods. Most important, public health authorities in the several countries harboring this disease—no longer a medical mystery—should take immediate measures to reduce the potential for dietary exposure of residents to aristolochia.

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DISCLOSURES

None.

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