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Health Effects of Toxic Organic Substances from Coal: Toward "Panendemic" Nephropathy

INTRODUCTION

Coal contains myriad organic compounds, some known to be toxic and others that are potentially toxic. Toxic organic compounds found in coal of particular interest include: *i)* condensed aromatic structures (e.g., polycyclic aromatic hydrocarbons), which can act as mutagens, cancer promoters, and endocrine disrupters; *ii)* aromatic amines, which have probable nephrotoxic activity; and *iii)* heterocyclic compounds, which may be carcinogenic and nephrotoxic. Toxic organic compounds can be leached from coal into water supplies, and long-term human exposure to these compounds may lead to disease occurrence, including cancer and renal disease. Despite these potential hazards, little is known about the impact and toxicity of organic substances derived from coal in water supplies. One example of a disease hypothesized to be linked to coal-derived toxic organic compounds in water supplies is Balkan endemic nephropathy (BEN).

In this paper, we summarize results from our studies linking BEN to the leaching of toxic organic compounds from low rank (lignite) Pliocene coal deposits into water supplies (well and spring water) of the rural villages where the disease occurs. We also introduce the idea of panendemic nephropathy (PEN) for BEN-like diseases that are linked to coal-derived toxic organic compounds in water supplies, but that occur outside the Balkans. Preliminary results supporting the PEN hypothesis are presented, with results from proposed PEN areas in Wyoming (WY) and Louisiana (LA). Results of toxicological studies of the effects of organic compounds isolated from water supplies in BEN and PEN areas on human cell cultures are also discussed. China, India, Turkey, and Portugal represent other areas where BEN-like diseases may occur, as a result of the presence of extensive low rank coal deposits and rural populations using untreated water in contact with coal in these nations.

BALKAN ENDEMIC NEPHROPATHY

Balkan endemic nephropathy (BEN) is a kidney disease of unknown origin, geographically confined to rural villages located along tributaries of the lower Danube River in Bosnia, Bulgaria, Croatia, Romania, and Serbia (1, 2). Villages affected by BEN occur in clusters, and the location of BEN clusters has not changed greatly since the disease was first described in the 1950s (3). Cases of BEN are still being diagnosed (4), and estimates are that 25 000 people currently have BEN (5).

Balkan endemic nephropathy is a tubular interstitial nephropathy leading to end-stage renal failure, with dialysis the typical therapeutic approach. Clinically, BEN most closely resembles analgesic nephropathy. At least 40% of patients diagnosed with BEN also develop renal/pelvic cancer (RPC). Renal/pelvic cancer is rare in the general population, and its high rate of occurrence with BEN is a significant feature of the disease, possibly linked to etiology (6, 7).

Perhaps the biggest challenge in the study of BEN is understanding its etiology (2). Many of the features of BEN suggest an environmental link (1, 2), but genetic factors may also be important (8, 9). Balkan endemic nephropathy etiology has been studied by researchers from a variety of disciplines, and many factors (microorganisms, metals, radioactivity, trace element imbalances in soil, chromosomal aberrations, mycotoxins, plant toxins, etc.) have been proposed as etiological agents for BEN (1, 2). Despite these efforts, the etiology of BEN is still unknown (1, 10).

PLIOCENE LIGNITE AND BEN ETIOLOGY

Studies by the US Geological Survey in the early 1990s revealed an apparent close association between BEN endemic areas and the occurrence of Pliocene lignite coal deposits in the former Yugoslavia (11, 12). The link between BEN and Pliocene lignite deposits has more recently been documented in Romania (13, 14). There is also evidence of new BEN areas in Kosovo and in eastern Serbia near the Bulgarian border that are associated with Pliocene lignite deposits. Several hyperendemic BEN villages in Serbia are located directly on top of the extensive Kolubara Pliocene lignite. There are no known Pliocene lignite deposits associated with the small BEN area near Vratza, Bulgaria, but there may be small, undocumented coal deposits, or possibly coaly sediments or shales present.

The observation that BEN areas are in close proximity to Pliocene lignite deposits led to the hypothesis (Pliocene lignite hypothesis) that these coal deposits may be linked to the etiology of BEN. Many wells from BEN villages were observed to have brownish-colored water, suggesting the presence of organic matter (15). It is hypothesized that organic compounds from Pliocene lignite located in the hills surrounding BEN villages are leached into groundwater and transported to water supplies (wells or springs) located in the villages below. People who use the well/spring water are then exposed to the coal-derived toxic organic compounds. The relatively low concentrations of toxic organic compounds present (individual compounds typically $<1\ \mu g\ L^{-1}$, and various compound classes 10–100 $\mu g\ L^{-1}$) would favor slow development of the disease over 10 to 30 y or more. The frequent association of BEN with RPC may indicate that the coal-derived toxic organic compounds may elicit both nephrotoxic and carcinogenic effects in susceptible individuals (16, 17).

GEOCHEMICAL STUDIES TESTING THE PLIOCENE LIGNITE HYPOTHESIS

The first test of the Pliocene lignite hypothesis of BEN etiology involved the examination of water from BEN villages and control sites. If the hypothesis is valid, then the chemistry of water supplies in BEN villages must be different from control sites in the quantity and/or composition of the organic compounds present.

Sampling trips were conducted at least twice yearly (spring and summer) from 2000 to 2006 to collect water samples from BEN areas and control sites. Targeted BEN areas included: *i)* the Drobeta Turnu Severin area in southwestern Romania; *ii)* the Resita area in western Romania; *iii)* the area west of Nis, Serbia; *iv)* the Kolubara area south of Belgrade, Serbia; and *v)* the Vratza area of northwestern Bulgaria (17, 18). Several types of control sites were examined: *i)* villages near BEN areas, but with no incidence of BEN ever reported, *ii)* municipal water supplies from cities or towns near BEN areas, and *iii)* water from rural villages well outside of the BEN areas. Both well and spring water samples were collected from BEN and control villages. Details on sampling sites and sampling protocols for water collection are provided elsewhere (13, 14, 17, 18).

Initial work focused on solvent-extractable organic compounds present in water samples. Water samples were liquid/liquid extracted with dichloromethane and organic compounds in the extracts analyzed by gas chromatography-mass spectrometry (GC-MS), (13, 14, 17, 18). Results clearly demonstrated that well and spring water samples from BEN villages had greater numbers and concentrations of dichloromethane-extractable organic compounds compared with control sites. Organic compound classes identified in the water from BEN villages included biphenyls, aromatic amines, terpenes, and N-, S-, and O-containing heterocyclic compounds. These compounds were not observed in water from control sites. All of these compound classes are present in low rank coal, and many compounds in these classes are known or suspected of being nephrotoxic or carcinogenic. Seasonal variation in the number and concentrations of organic compounds present in water samples from the BEN villages was also observed, with greater amounts present during the higher rainfall spring season. These results are consistent with the Pliocene lignite hypothesis for BEN.

In addition to examining dichloromethane-extractable organic compounds in water supplies from BEN villages and control sites, we also examined high molecular weight (HMW) organic matter in these water supplies beginning in 2004. High molecular weight organic matter was isolated from water supplies using tangential flow ultrafiltration (TFU), with a 7500 Da nominal molecular weight cutoff membrane cell. The TFU unit operates somewhat like an artificial kidney, continuously concentrating the higher molecular weight organic material and allowing lower molecular weight substances to pass through with the water. In initial work using TFU, 10–30 L of well/spring water was concentrated to 120 ml. Results of TFU experiments showed that wells/springs from BEN villages produced highly colored, organic matter-rich TFU concentrates, while the same volume of water from wells/springs at control sites produced only slightly colored, organic matter–poor TFU concentrates. Thus, there appears to be a much larger amount of HMW organic matter in wells/springs from BEN villages than in water supplies from control sites. This result also supports the Pliocene lignite hypothesis for BEN. The chemical composition of the HMW organic matter in well/spring water from BEN villages is currently under investigation, and it is not known whether any of the material can cause kidney nephropathy or RPC. To address the issue of toxicity, the TFU concentrates isolated from BEN and control sites were used in toxicology studies on human cell cultures, as described later.

Analysis of inorganic substances (metals, anions, nutrients, etc.) in water samples from BEN and control sites was also conducted. Earlier studies found no evidence for involvement of inorganic substances in water supplies in the etiology of BEN (1, 2). Our results concur with these previous studies. We found concentrations of most inorganic substances in water supplies well below US Environmental Protection Agency criteria for drinking water, and no significant differences between concentrations in water supplies from BEN and control sites were observed (17). Only nitrate concentrations (up to 225 mg L^{-1}) exceeded drinking water standards, but nitrate concentrations were equally high at BEN and control sites. Nitrate, therefore, cannot account for the characteristic geographic restriction of BEN. Other workers have also observed ubiquitous high nitrate concentrations in water from rural villages in the Balkans (19), probably from the use of manure and other fertilizers in agriculture. Nitrate could be a cofactor in the etiology of BEN, but because of its ubiquitous occurrence in both BEN and control villages it is unlikely to be the primary etiological factor.

THE PLIOCENE LIGNITES IN BEN AREAS

Studies of the organic and inorganic geochemistry of the Pliocene lignites from BEN areas were carried out to determine whether these coals had characteristics consistent with the Pliocene lignite hypothesis. Laboratory leaching of Pliocene lignites by aqueous solution was conducted to examine the amount and nature of organic compounds extracted from these coals. Fresh coal samples were collected from several coal mines in the BEN areas of Romania and Serbia for these studies. Coal samples from non-BEN areas of these countries were also collected for comparison.

Results show that Pliocene lignites from the BEN areas are less thermally altered than most other lignites but have no unusually high concentrations of inorganic elements (16). Solid-state ^{13}C nuclear magnetic resonance spectra of BEN area Pliocene lignites show the presence of abundant methoxyl and phenolic functionality, and even the presence of residual cellulose, indicators of a low degree of thermal alteration (20, 21). Elemental organic analysis indicated a high degree of heteroatom (N, O, S) content compared with other lignites (16). These structural features of the Pliocene lignites favor aqueous solubility through hydrogen bonding of organic compounds in the immature coal structure with water.

Laboratory leaching experiments were conducted on the BEN area Pliocene lignites, and other coal samples of comparable and higher rank, using water (extraction at room temperature and 80°C) and a polar solvent (methanol). In these experiments, BEN area Pliocene lignites exhibited the highest yield of extractable organic compounds. Compounds identified in the leachates included many of the compounds identified in water supplies from BEN villages, including cycloalkanes/alkenes, steranic structures, monoaromatic and polyaromatic terpanes, polycyclic aromatic hydrocarbons, aromatic amines, and N-, S-, and O-containing heterocyclic compounds. Many of these compounds have attached oxygen-based functional groups (hydroxy-, phenol-, keto-, methoxy-), and some of them contain heterocyclic nitrogen or amino groups, structural features that could make them nephrotoxic and carcinogenic. Very few of these compounds are encountered in leachates of lignite and higher rank coal from non-BEN areas (17).

PANENDEMIC NEPHROPATHY (PEN)

The hypothesis linking low rank coal (Pliocene lignites) to BEN is supported by both field and laboratory tests conducted to date. The hypothesis accounts for many of the important features of BEN, including its characteristic geographic distribution, its occurrence only in rural villages (untreated water supply), and its long incubation period (low levels of toxic contaminants). Since low rank coal is found worldwide, however, this hypothesis raises the question of whether diseases similar to BEN might exist outside of the Balkans. We use the term PEN to refer to BEN-like diseases occurring outside the traditional BEN area. Characteristic features of PEN would include high incidence of unexplained kidney disease (including end-stage renal disease and RPC) in a population using untreated water, and water supplies hydrologically connected to low rank coal deposits,

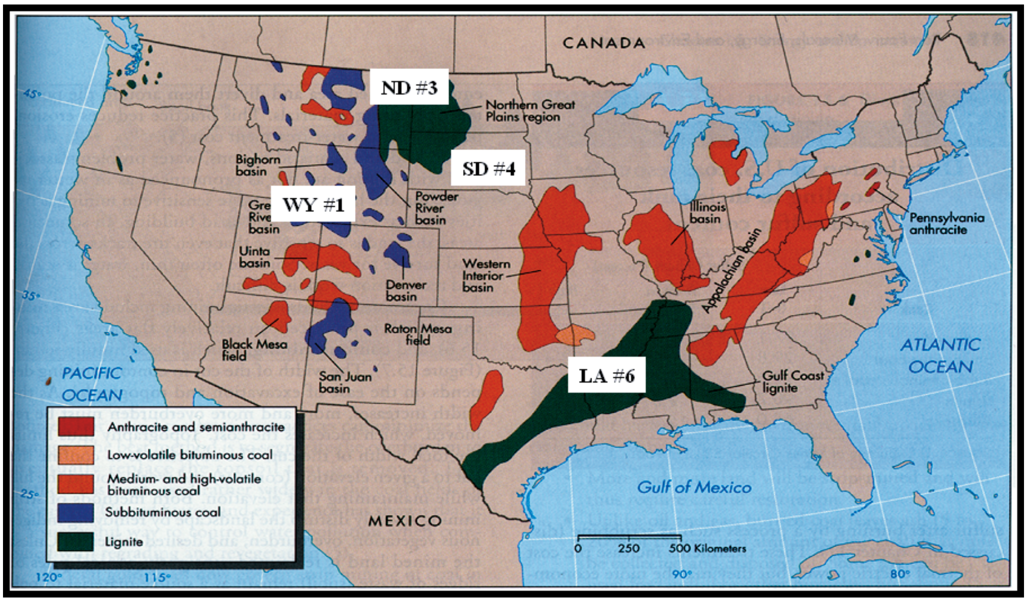

Figure 1. Coal map of the United States showing states with the highest incidence and number of deaths from renal/pelvic cancers (similar to the uroepithelial cancers associated with BEN). All of these states have large rural populations that use well water, and extensive low rank coal deposits (similar to the Pliocene lignites linked to BEN).

Statistics from the National Institutes of Health, USA (22) show that Wyoming (WY), North and South Dakota (ND, SD), and Louisiana (LA) rank among the top 10 states in the United States in incidence and deaths from RPC. Interestingly, all of these states have extensive low rank coal deposits (Fig. 1), and large rural populations using well water. In semi-arid WY, ND, and SD, coal seams are often viewed as good aquifers for water supply. In LA, the highest rates of RPC are in the northwestern part of the state, corresponding to the zone where the lignite deposits are found. A high incidence of RPC is a characteristic feature of BEN, as mentioned earlier. Our emphasis on RPC rather than kidney disease reflects the greater difficulty in separating BEN-like nephropathy from other types of nephropathy in the statistics.

Water supply wells from northwestern LA and from the Powder River Basin (PRB), WY, and coproduced water from coalbed natural gas wells in the PRB were examined for the presence of coal-derived toxic organic compounds. The coproduced water, although not used as a water supply by people, is in direct contact with coal seams, providing information on what types of organic compounds may be leached from coal under natural conditions. Dichloromethane-extractable, and HMW organics (TFU isolation) were examined using the sampling and analytical methods described earlier for the BEN work. Produced water from the PRB contained abundant dichloromethane-extractable and TFU organic matter. Compounds identified in the PRB produced water included polycyclic aromatic hydrocarbons, aromatic amines, and heterocyclic compounds, similar to compounds observed in water extracts of coal, and from BEN area water supplies. Similar results were observed for water supply wells in coal aquifers in the PRB (23). In northwestern LA, water supply wells in the coal area with high rates of RPC had higher numbers and concentrations of organic compounds compared with control sites, and a statistically significant association between the incidence of RPC and the number of different organic compounds in well water and their concentration was determined (24, 25). These results support the concept of PEN.

TOXICOLOGY STUDIES: BEN AND PEN

Toxicology studies were conducted to further test and validate the hypothesis linking toxic coal-derived organic compounds in drinking water supplies to BEN and PEN. Toxicology studies conducted to date have been limited to *in vitro* experiments conducted on several types of human cells: *i)* mesenchymal stem cells (MSCs) at different passages obtained from the bone marrow of patients (from non-BEN areas) with hematologic disorders, able to proliferate spontaneously for several months without immortalization, and sensitive to radiation and xenobiotic agents; *ii)* an erythroleukemia cell line (K562), with greater resistance to xenobiotic agents compared with MSCs; and *iii)* a commercial human kidney cell line (HK-2). Cells were exposed to two types of organic compounds: *i)* water extracts of Pliocene lignite from BEN areas and low rank coal from hypothesized PEN areas in the United States (WY and LA) and *ii)* TFU organic concentrates from water in wells and springs from BEN areas and control sites in the Balkans, and from water collected from wells penetrating the coal in WY and LA. Toxicity testing was conducted in standard well plates for periods of 24 to 120 h (26, 27). Exposure levels to organic toxins were controlled by the amount of material added to each well plate and by the duration of the test. Both positive (cells with no addition) and negative (cells with triton-X or dexamethasone solution added) controls were used. Cell viability and proliferation was assessed using a standard MTT assay (28, 29), and visual changes in cell morphology were noted.

Results from these preliminary experiments showed two principal effects. For the HK-2 and K562 cell experiments, lower levels of exposure to organic compounds from coal extracts and TFU concentrates resulted in increased proliferation of the cells relative to controls, while higher levels of exposure resulted in higher rates of cell necrosis relative to controls. Exposure effects (cell proliferation at low exposure and cell necrosis at higher exposure) were most pronounced for the Pliocene lignite extracts and for TFU concentrates from BEN villages in Romania and Serbia. A somewhat lower degree of cell proliferation and necrosis was observed for water samples from coal deposits in the PRB and northwestern LA. The more sensitive MSCs had higher rates of cell necrosis at all levels of exposure to the organic compounds, relative to controls. Cells exposed to the organic compounds also showed morphological changes relative to controls (Fig. 2), including the presence of large vacuoles in the cells.

CONCLUSIONS

The hypothesis implicating coal-derived toxic organic compounds in the etiology of BEN has been tested, and results support the hypothesis. This hypothesis accounts for both the

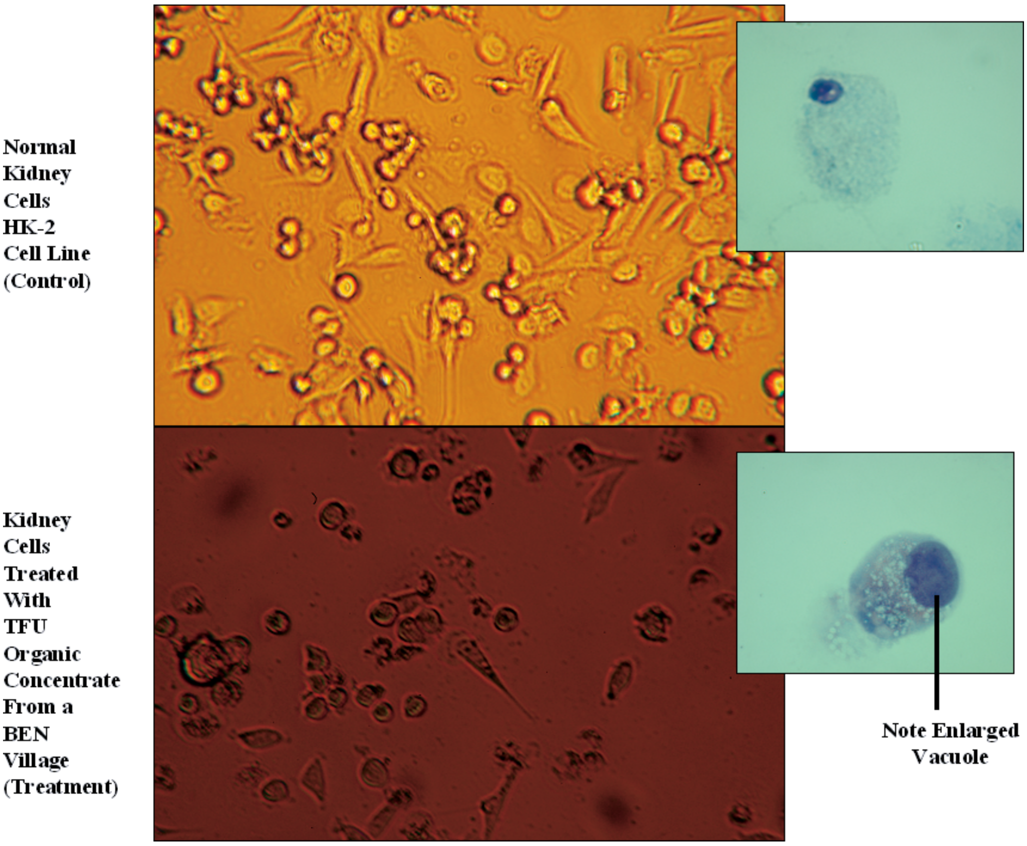

Figure 2. Morphological effects of exposure of human kidney cell line (HK-2) to organic matter from tangential flow ultrafiltration (TFU) concentrate isolated from a water supply well in a BEN village in Serbia. Note morphological difference between the control (top) and the TFU treated cells. Tangential flow ultrafiltration treatment also produced excessive cell proliferation at low dosage, and cell necrosis at high dose.

geographic restriction of BEN, and many of its medical features. Well water from BEN villages contain higher numbers and concentrations of potentially toxic extractable and HMW organic compounds compared with controls. Study results do not support involvement of inorganic substances (except for the possible involvement of nitrate as a cofactor) in water supplies in BEN etiology.

The concept of PEN or BEN-like diseases (especially RPC) that occur outside of the Balkans is supported by preliminary geochemical results from the United States in states with high rates of RPC and low rank coal deposits (LA and WY). Renal disease may also be linked to lignite-containing areas in northern Portugal (Deolinda Flores, Universidade do Porto, Portugal, personal communication).

Preliminary toxicology studies exposing different types of human cell cultures (including kidney cells) to TFU concentrates from BEN wells/springs, and from areas of high RPC in the United States (LA and WY), showed a range of effects, including excessive cell proliferation at lower doses, and cell necrosis at higher doses, relative to controls. These results are consistent with both the cell necrosis associated with BEN, and the occurrence of RPC in BEN and PEN. Tangential flow ultrafiltration concentrates also induced changes in cell morphology, especially the development of large vacuoles within the cells. Additional cell line toxicology studies and animal experiments (rats) with TFU concentrates, and studies of the effects of organic compounds in TFU concentrates on lecithin : cholesterol acyltransferase activity, which is low in BEN patients (29) are currently under way.

Diseases that are like BEN (kidney nephropathy and RPC) linked to toxic organic compounds derived from coal in drinking water supplies may be widespread. This may also include toxic organic compounds in peat water used as water supplies. Ongoing work is exploring the occurrence and etiology of PEN as a global issue in medical geology, focusing on areas where low rank coals and populations using untreated well water occur (e.g. India, Turkey, China, Portugal).

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31. Support for this work was provided by the Energy Resources Program, US Geological Survey, and NATO Science Programme, Collaborative Linkage Grants. Logistical support was provided by the Romanian Ministry of Health, and collaborative assistance from the US Bureau of Land Management, the Ministry of Health, Serbia, F. Margineanu, MD (County Hospital, Drobeta-Turnu Severin, Romania), the Louisiana Geological Survey, and the School of Medicine, Louisiana State University. This paper is a synopsis of a presentation given at the International Symposium on Medical Geology (May 18–19, 2006), Department of Geosciences, Stockholm University, and sponsored by the Marcus Wallenbergs Stiftelse för Internationellt Vetenskapligt Samarbete, the Swedish Committee for UNESCO's Scientific Programmes, the Royal Swedish Academy of Sciences, and the International Medical Geology Association (IMGA). Special thanks to the meeting organizers, Rolf Hallberg (Stockholm University) and Olle Selinus (Swedish Geological Survey).

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