The Role of Humic Substances in Drinking Water in Kashin-Beck Disease in China

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We conducted in vitro and in vivo assays in a selenium-deficient system to determine if organic matter (mainly fulvic acid; FA) is involved in a free radical mechanism of action for Kashin-Beck disease. Cartilage cell culture experiments indicated that the oxygen or hydroxy functional groups in FA may interfere with the cell membrane and result in enhancement of lipid peroxidation. Experiments with rats demonstrated that toxicity from FA was reduced when the hydroxy group was blocked. Induction of lipid peroxidation by FA in liver and blood of rats was similar to that exhibited by acetyl phenyl hydradrene. FA accumulated in bone and cartilage, where selenium rarely concentrates. In addition, selenium supplementation in rats' drinking water inhibited the generation of oxygen-free radicals in bone. We hypothesized that FA in drinking water is an etiological factor of Kashin-Beck disease and that the mechanism of action involves the oxygen and hydroxy groups in FA for the generation of free radicals. Selenium was confirmed to be a preventive factor for Kashin-Beck disease. Key words: free radical, fulvic acid, Kashin-Beck disease, humic substances, selenium. Environ Health Perspect 107:293–296 (1999). [Online 11 March 1999] http://ehpnet1.niehs.nih.gov/docs/1999/107p293-296penganabstract.html

Humic substances (HSs) are usually found in soil, water, sediment, coal, and peat in the environment. Humic substances include humic acid and a soluble fraction, fulvic acid (FA), which consists of a mixture of complex macromolecules that have polymeric phenolic structures with the ability to chelate with metals and inorganic substances. Reduction, oxidation, and microbial degradation of HSs produce resorcinol, other phenolic and phe-no- nolic carbonyl compounds, and ortho and meta phthalic acids. Because HSs play an important role in binding and fate of inorganic and organic compounds in the natural environment, there have been many studies on the effect of HSs on behavior of inorganic and organic pollutants (1). In recent decades, it was found that HSs, mainly FA, are related to human health. Lu et al. (2) found humic acid in the well water of blackfoot disease endemic areas in Taiwan and conducted systematic in vitro and in vivo research. Lu (3) also reviewed the results of the studies on HSs and their effects on animals and assumed that a close relationship existed between HSs and the cause of blackfoot disease. In addition, because the degradants of HSs were potent inhibitors of thyroid peroxidase, HSs were implicated as environmental goitrogens (4). Epidemiological studies in a blackfoot disease endemic area in Taiwan revealed increased prevalence of goiter, hepatoma, bladder cancer, vascular disease, and diabetes mellitus among the residents; therefore, it was hypothesized that humic acid–metal complexes were possible etiological factors of goiter, hepatoma, bladder cancer, vascular disease, and diabetes mellitus, and free radicals were the common causative factor (5). The close correlation of these diseases with humic acid was surprising and deserves further study.

In 1987, we reported that there was a close relationship between FA in drinking water and the cause of Kashin-Beck disease (KBD) (6). The etiological factors could also be considered as the basis of free radical mechanism. KBD, which may lead to skeletal deformation and dwarfism, is an endemic osteoarthropathy with a high prevalence in several areas of China. Allander (7) reviewed the history and development of KBD and discussed various factors attributed to the disease. Recently, these factors have been summarized as follows: 1) low levels of selenium in nutrition (8); 2) high levels of humic acids (e.g., fulvic acid) in drinking water (9); and 3) high concentrations of mycotoxins from the mold Fusarium on rotten grain (10).

We attributed the occurrence of KBD to low selenium content and higher concentration of FA in water and to mycotoxin in maize. After about 15 years of epidemiologic investigation and laboratory studies and comprehensive research on the roles and relationship of FA, Se, and mycotoxin, a series of important results has been gained: FA may injure cartilage cell, which is the target site of KBD, and selenium deficiency may contribute to the damage from FA (9); selenium could inhibit the toxicity of FA and mycotoxin from Fusarium (11); in animal tests, Yang et al. (12) demonstrated that with low Se and high FA concentrations, FA could cause the impaired conversion of pro-PN-collagen II to collagen II and the structural alteration of collagen II. One efficient method for the prevention of KBD is the improvement of drinking water quality (13). It has been concluded from previous research (6) that not only selenium but also FA in drinking water (and mycotoxin in grain) has a direct relationship with KBD, and the etiology of KBD was not the only composed factor.

This paper reviews the free radical mechanism of damage generated by FA using cell and animal tests. FA in drinking water may accumulate on cartilage and bone. FA, as an exogenous free radical carrier, induced the lipid peroxidative process through oxy and hydroxyl functional groups under oxygend conditions.

Materials and Methods

Fulvic acid. Drinking water from the KBD region was acidified with HCl and subsequently passed over a GDX-102 resin (Tianjin 2nd Chemical Factory, Tianjin, China). The water soluble FA was absorbed on the resin and eluted with a solvent containing ethanol/ammonia (1:2). FA purification has been previously described (14).

Cartilage cell culture. Cells were obtained by dissociating the limb cartilage from 12-day-old white Leghorn embryonic chicks. The culture system used in the study has been previously described (15). Cells were incubated over 2 days at 37°C, and the media was replaced at 48-hr intervals. The cultured cells were divided at random into five treatment groups. One treatment group received standard media (control); the other groups received media supplemented with FA (50 ppm), FA + seleno-methionine, FA + seleno-cysteine, or FA + selenite. The concentration of selenium in compounds was 0.1 ppm. After another 5 days of incubation, we assayed lipid peroxidation (LPO) by the method of Fletcher et al. (15).

Tests for model compounds were carried out as described above, except that FA

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was replaced with various model compounds.

**Animal tests.** In Test 1, the -OH groups in FA were blocked by the method of acetylation of Schnitzer and Skinner (16). Experimental rats with the same diet were divided into three groups: rats were given 1) FA from the KBD region (Tianshui), 2) OH-blocked FA from Tianshui, or 3) FA from the KBD region plus 0.1 ppm Se(IV) added to drinking water at a dose of 100 μg/g body weight. At 72 hr after injection, blood was drawn from both tail and liver and examined for the LPO content.

In Test 2, four groups (six male rats in each group) of Kunming rats fed with standard diet were injected with test solution into the abdominal cavity as follows: 1) control group (physiological saline); 2) acetyl phenyl hydrazine (APH; 200 mg/kg); 3) FA from the KBD region (50 mg/kg/day); 4) FA from the KBD region (100 mg/kg/day); or 5) FA from wheat (p-FA; 50 mg/kg). This experiment lasted 10 days. Blood and liver were collected and the malonaldehyde (MDA) content was determined using the methods of Uchiyama and MiHara (17) and Stocks et al. (18), respectively.

In Test 3, The FA was tritium labeled using the catalytic isotope exchange method. The labeling was carried out at the Institute of Atomic Energy Research, Chinese Academy of Sciences. To each Wistar rat fed with normal diet, 1.5 ml of solution containing 19 mg FA and 20 μCi 3H-FA was orally administered every other day, for a total of 10 treatments. After administration, rats were sacrificed at 10, 20, 30, and 40 days (10 rats per time point). The sacrificed rats were immediately dissected to obtain the organs, bone, and joint cartilage, which were wiped dry with filter paper and weighed. The radioactivity was measured for different organs using an LS 6000 TA Scintillation Counter (Beckman, Los Angeles, CA) after digestion.

For Test 4, juvenile Wistar rats were separated into three groups of eight (equal numbers of male and female). Rats in the KBD group were fed corn and wheat (1:4) from the KBD region and received water containing 200 ppm FA extracted from soil by the edge of wells (14). The FA was added to the KBD region served as the control group. The third group was fed the same diet as the KBD group but received Se(IV) (0.1 ppm) in water. After feeding for 5 weeks, all the rats were decellularized and counted bone samples.

**Results and Discussion**

**Influence of FA and its model compounds on LPO.** Because the pathophysiological process of KBD occurs mainly in articular cartilage, cartilage cells are the target cells of KBD. Cultured cells were assayed for LPO by the fluorescent method (9). The results are summarized in Figure 1, which indicates the significant enhancement of LPO in the FA group as compared with the control group. FA seems to generate reactive oxygen radicals that may directly interfere with cell membranes and result in lipid peroxidation. However, Se-containing inorganic or organic compounds reduced LPO that was enhanced by FA.

Because FA consists of a mixture of poorly defined organic compounds with a wide variety of reactive groups, it is difficult to define which one plays the main role. Humic substances in drinking water from soil surrounding wells are a mixture of degradation products from bodies of animal and plant remains that were decomposed by microorganisms. To simulate the toxic effect of FA molecules, model compounds of the predecessors and decomposition products of FA were used in the cartilage cell culture system. The results are shown in Figure 2. Based on the morphology of cells and the degree of LPO, the model compounds were grouped and followed an order of quinonic-polyphenolic = FA > phenolic and organic acid-xanthonic functional groups. This is an indication that the toxic effect of FA could be due to the oxy or hydroxy functional groups in the structure. The quinone and semiquinone structures evolve into free radicals when pH and E(H) (oxidative and reductive potential) of the system changed, and the oxy-free radicals can induce the production of both hydroxyl-free radicals and peroxides (19). The elimination of peroxides depend heavily on the activity of peroxidase and other antioxidants in biological media.

The influence of FA with a blocked OH group on LPO content in the blood and liver of rats (animal test 1). If the source of toxic effect of FA is the hydroxyl group, the action would be reduced when the hydroxyl group is blocked. However, the hydroxyl groups were blocked by acetylation of FA, as compared with the FA group. The injurious effect of active oxygen came mainly from hydroxyl free radicals. Wang et al. (20) compared the effects of organic acid reagents such as ferulic acid, P-cumaric acid, α-valeric acid, benzoic acid, 3,4-dihydroxybenzoic acid, etc., on cartilage cells and found that the injurious degree of cells was closely related to hydroxyl groups contained in organic acid. P-Cumaric acid and

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**Figure 1.** Stimulation of lipid peroxidation (LPO) by fulvic acid (FA) and protection from different selenium (Se) compounds in a cartilage cell culture system. See "Materials and Methods" for details.

**Figure 2.** Influence of fulvic acid (FA) and model compounds on the lipid peroxidation (LPO) of cultured cartilage cells. Abbreviations: Q, average of 2-hydroxy-1,4-quinone and 3-hydroxy-1,6-quinone; P, average of 1,4-diphenol, 1,3-diphenol, and 1,3,5-triphenol; FA, FA from the Kashin-Beck disease region; N, α-naphthol; SA, salicylic acid; BA, benzoic acid; X, xanthene; MP, average of 4-methyl-phenol and 3,4-dimethyl-phenol.
3,4-dihydroxybenzoic acid have a greater effect than other acids (20).

Influence of FA on LPO in rats compared to LPO induced by APH (animal test 2). Acetyl phenyl hydrazine is a lipid peroxidation inducer. Comparison of FA from the KBD region with APH should confirm whether FA can induce LPO. The LPO content, expressed by MDA in the samples, is shown in Table 2. The role of FA was similar to that of APH, indicating a similar mechanism for both.

Table 2 shows that FA from the KBD region significantly enhanced MDA in liver and erythrocytes, which increased with the higher FA dose. FA induced LPO, as did APH. However, FA isolated from peat did not induce MDA. FA in water or soil generated reactive oxygen and membrane LPO, but FA from peat did not. We reported earlier (14) that FA in water and soil were smaller molecules and had more reactive groups than FA from peat. The reason for this needs further study.

Accumulation of FA in bone and cartilage (animal test 3). The free radical theory, as related to human health, has attracted much attention in recent years. With regard to KBD, it is not known if the FA attacks the bone and then causes a series of pathological changes. Our objective is to confirm whether FA is a causative factor or a toxicant that may directly or indirectly reach the target tissues in KBD.

The distribution of 3H-labeled FA in different organs of rats is shown in Figure 3. FA was distributed in all organs of the rats, and the incorporation was not specific. The distribution of radioactivity in different organs follows the order of kidney-liver-cartilage-bone-muscle-lung-brain. The main metabolic organs for FA in rats are liver and kidney, where the radioactivity is high and the turnover time is shorter than that in other organs. Because the metabolism of FA is relatively slow, accumulation is reflected in bone and cartilage. Xu (21) found, by in vivo experiments, that there was very little selenium distributed in bone. This suggested that the target tissue of KBD could receive higher pathogenic doses of FA but have lower protective factor (Se). This is consistent with the environment, where FA is higher and selenium is lower. This may explain how FA generates oxidative damage in bone.

The free radical signal characteristic of rat bone generated by FA (animal test 4). Exogenous factors such as FA taken via drinking water can be accumulated in bone as verified by animal tests described above. The effect of oxy-free radicals generated by FA in bone should be confirmed. We dissolved FA from the KBD region in drinking water of rats. The intensity of the free signal (electron spin resonance, ESR) was found to be correlated with Se content in the diet and selenite-supplemented drinking water, as shown in Figure 4.

The intensity of the ESR signal in bone was highest when rats were fed FA-containing water and a Se-deficient diet. The ESR signal was reduced when rats were given a Se-sufficient diet or selenite in drinking water.

The free radical initiators (mainly superoxide anion, hydroxyl radical, hydrogen peroxide, etc.) oxidize and attack abundant unsaturated fatty acids in cell membranes, causing LPO, and producing lipid peroxide, which reacts directly in the tissue. Related to the injury mechanism, Yang et al. (12) reported that FA could generate reactive oxygen radicals that could indirectly interfere with procollagen processing. Yang et al. (22) analyzed articular cartilage from two patients and found an accumulation of the precursor molecule pro-pN-collagen, which

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**Table 1. The influence of hydroxyl-blocked fulvic acid (FA) and Se supplement on change in glutathione peroxidase (GPx) activity and LPO content in the blood and liver caused by FA in the Kashin-Beck disease (KBD) region**

| Group             | Blood  | Liver  |
|-------------------|--------|--------|
|                   | GPx    | LPO    | GPx    | LPO    |
| KBD region        | 23.23 ± 1.53 | 2.56 ± 0.09 | 723.1 ± 57.9 | 606.8 ± 48.9 |
| Hydroxyl-blocked  | 30.83 ± 1.47 | 2.65 ± 0.28 | 749.1 ± 55.9 | 491.1 ± 62.3 |
| Se supplement     | 30.88 ± 3.57 | 2.60 ± 0.29 | 794.8 ± 47.8 | 513.2 ± 59.7 |

*p<0.05*

**Table 2. Malondialdehyde contents of liver and erythrocytes in rats**

| Group              | Liver (OD) | Erythrocytes (nmol/g Hb) |
|--------------------|------------|--------------------------|
| Control            |            |                         |
| APH (200 mg/kg)    | 0.205 ± 0.075 | 92.70 ± 21.42 (p<0.001) |
| FA (50 mg/kg)      | 0.270 ± 0.075 | 9.20 ± 0.79 (p<0.001)   |
| FA (100 mg/kg)     | 0.354 ± 0.075 | 19.63 ± 3.58 (p<0.001)  |
| p-FA (50 mg/kg)    | 0.190 ± 0.090 | 0.00                     |

*p<0.05*

**Figure 3. Time-dependent radioactivity of 3H-fulvic acid (FA) in different organs of rats.**
was not present in extracts of control fetal cartilage. Furthermore, they developed an animal model of KBD in which Se-deficient mice received FA supplementation for two generations. The results indicated that the growth of the treated mice was slightly retarded, mice had irregular bone formation, and lysine residues in collagen I from bone and cartilage were overmodified. Wang et al. (23) reported that the oxidative damage to type II collagen from pig cartilage was induced by hydroxyl and superoxide anions and by FA from the KBD region; FA acted much like •OH and •O2-. These studies could be linked to the injury effect of oxy-free radicals generated by FA in the initiation and development of KBD.

Conclusion

We have demonstrated the actions of FA from drinking water on "membrane defect" due to peroxidation injury in the onset of KBD. First of all, FA, as well as its degradation products, could result in the damage to cultured cartilage cells and increase the LPO level in cellular matrix. The toxic effect of FA could be mostly due to the oxy or hydroxyl functional groups in the FA structure. This assumption has been verified in an animal test in which the OH-group was blocked. Second, there was an increase in LPO in blood and liver of rats that were fed normal diets and received water containing FA from the KBD region. These results are similar to those of an LPO inducer, APH. In addition, it was found that FA could be incorporated into bone and cartilage of rats, the target tissue of KBD, where the selenium concentration was low. The organic matter in drinking water, mainly fulvic acid, as a exogenous carrier of oxy radical, is one important pathogenic factor of KBD. Hou et al. (13) showed that a change in water sources was an effective method for prevention of KBD occurrence. Taking into account that selenium is a scavenger of free radicals, selenium deficiency apparently is a conditional factor of KBD disease.

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