APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 8, 223–228 (1994)

Peripheral Vasculopathy in Rats Induced by Humic Acids

Fung-Jou Lu,* Tien-Shang Huang,* Yung-Sung Lin,† Victor Fei Pang† and Shih Yuh Lin‡

* Departments of Biochemistry and Medicine, College of Medicine, National Taiwan University, Jen-Ai Road, Section 1, Taipei, Taiwan, † Department of Veterinary Medicine, College of Agriculture, National Taiwan University, 142 Chou-San Road, Taipei, Taiwan, and ‡ Taiwan Provincial Research Institute for Animal Health, 376 Chung-Cheng Road, Tansui, Taiwan, Republic of China

Adult male Wistar rats averaging 380 g in weight were injected with single doses (100 mg kg^{-1} body weight) of humic acids intraperitoneally. 'Black tails' were noticed 12 days later. In severely affected regions, thrombosis with mild to moderate acute necrotizing vasculitis was seen in most of the blood vessels located in the dermis, subcutaneous fat and muscle. These blood vessels were markedly dilated. Frequently, colonies of a short-rod bacterium of varying sizes were observed in necrotic tissues. There was mild to moderate edema and minimal accumulation of mast cells and macrophages in the dermis. Acute fat necrosis with fibrin deposition was also seen in the subcutaneous fat. Based on the pathological findings, we diagnosed that the tails of Wistar rats which had been injected with humic acids had severe multifocal thrombosis and mild to moderate, multifocal, acute necrotizing vasculitis with bacterial colonization, mild dermatitis, and fat necrosis. The relevance of these observations to the discussions on arsenic as an etiological factor of human Blackfoot disease is examined.

Keywords: Humic acids, blackfoot disease, thrombosis, vasculopathy, gangrene

INTRODUCTION

'Blackfoot disease' is a folk term for an endemic peripheral vascular disease prevalent along the south-west coast of Taiwan. The initial symptom of this disease is usually an insidious onset of numbness or coldness at one or more of the extremities. It usually progresses, resulting in an area of ulceration with subsequent gangrenous changes giving, as the name suggests, the characteristic black discoloration of dry gangrene, especially in the feet. The clinical symptoms, signs and course are similar to those of Buerger's disease.[1] Pathologically, the patients have vascular changes compatible with either thromboangitis obliterans or arteriosclerosis obliterans.[2] The first case was reported in 1954,[3] and more than 1800 cases were reported by 1985.[4] Arsenic and the fluorescence compounds[5,6] have generally been thought to be two possible etiological factors found in the well water that the inhibitants drank.

Fluorescence of the drinking well water of the Blackfoot-disease endemic area was first noticed in 1975.[7] Under ultraviolet light irradiation, the well water emitted a bluish-green fluorescence. Crystallized fluorescent compounds isolated from the well water were stable to heat and acid–base, and contained —COOH, —OH, and C═O as the main functional groups. Furthermore, there were metal organic complexes containing inorganic metals such as arsenic, iron, manganese, lead, cadmium, zinc and nickel, and these were also identified as humic substances.[8]

Previous investigations in this labortory[9] demonstrated that daily intraperitoneal injection of 5 mg of well-water fluorescent compounds per 20 g of body weight in Balb/c mice resulted in crippling, phlegmasia, ulceration, necrosis and gangrene of the extremities in half of the mice after 14–32 days; the tail of one of these mice developed gangrene. Fluorescent compounds were conjectured to be a possible etiological factor of Blackfoot disease.[9]

The purpose of the present study was to investigate the effect of commercial humic acids on rat organs and the premise that well water humic acids might be a cause of Blackfoot disease.

CCC 0268–2605/94/030223–06

Received 17 December 1993
Accepted 31 January 1994

MATERIALS AND METHODS

Preparation of humic acids

Humic acids are allomelanins resulting from the decomposition of organic matter, especially dead plants; they are usually found in soil, coal and peat. Humic acids consist of a mixture of complex macromolecules having polymeric phenolic structures with the ability to chelate metals and inorganic and organic substances.[10–16]

Humic acids (sodium salt; tech. grade) were obtained from Aldrich Chemical Co., Milwaukee, WI, USA, and were further purified by acid precipitation with 0.1 M HCl, pH 2.0 and passage through a Sephadex G-25 column; they were eluted with distilled water and collected at the first peak. The purified humic acids were diluted with distilled water, pH 7.0–7.5, sterilized through a 0.45 μm Minipore filter and preserved at 4 °C.

Animal treatment

Fifteen adult male Wistar rats averaging 380 g in weight were purchased from the Animal Center of the Medical College of the National Taiwan University. They were fed with Rodent Chow (5001; Ralston Purina Company, USA) and placed in separate cages at 20 °C. Water and food were freely accessible. The experimental group of ten rats was injected intraperitoneally with 1.33 cm^3 of a 30 mg cm^{-3} solution so as to result in 100 mg kg^{-1} body weight of the humic acids prepared as described above. The control group of five rats was injected intraperitoneally with the same amount of distilled water. Rats were examined daily and sacrificed after the development of gangrene in their tails. The tails were fixed in 10% buffered formalin for more than 24 h, and decalcified in D-Calcifier (Lerner Lab, USA) for 12 h. They were then embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin (H&E). Light-microscopic examination was performed thereafter.

RESULTS

The rats tolerated intraperitoneal injection of humic acids, except that rats which had been injected with humic acids ate less and lost some weight (approx. 20 g) compared with the control. Their activity appeared the same as the controls. On the tenth day after a single intraperitoneal injection of humic acids, the tails of two of the ten experimental rats turned red, gradually extended proximally, and then turned black, ulcerated, and became gangrenous (Fig. 1). The gangrenous tail finally amputated spontaneously. The inside section of the tail also showed a dark appearance similar to that of the surface. The scales and hairs of the tail were grossly intact, but the temperature of the tail had decreased.

Microscopic examination of the affected tails revealed thrombosis. The intima of larger arteries was damaged, and adhered with platelet plugs, and thrombi (Fig. 2). The inner vessel wall was connected with clotted fibrin and distorted. Several kinds of blood cells collected in the area of the red thrombus (Fig. 2). The dead endothelial cells of vessels were detached from the vascular wall. Some of veins also showed thrombus formation and were swollen.

The necrotic tissues of rat tail were stained red with H.E. stain, and blood cells were trapped in the area of the thrombus. Various degrees of leucocyte and mononuclear cell infiltration were found in the skin and between muscle bundles and fibers. The regular saw-shaped appearance of the intima of arterioles disappeared. Some of the necrotized regions of tail were colonized with bacteria, most of which appeared around the hypodermis and dermis (Fig. 3). The active bacterial clumps stained blue with H.E. stain. There was mild or moderate edema and minimal accumulation of mast cells and macrophages in the dermis. Acute fat necrosis with fibrin deposition was seen in the subcutaneous fat.

DISCUSSION

Blackfoot disease is an endemic peripheral vascular disease found among the inhabitants along the south-west coast of Taiwan who have used the artesian well water for drinking for a long time. This well water contains high concentrations of arsenic and humic acids. The clinical onset is usually insidious; it may also be quite sudden and usually begins with numbness (75.1%), or coldness (5.70%) in one or more extremities, usually the feet. Intermittent claudication is also a common initial symptom; less common initial symptoms are cyanosis of the feet (15.3%), a burning sensation of the soles (15.0%), pale feet

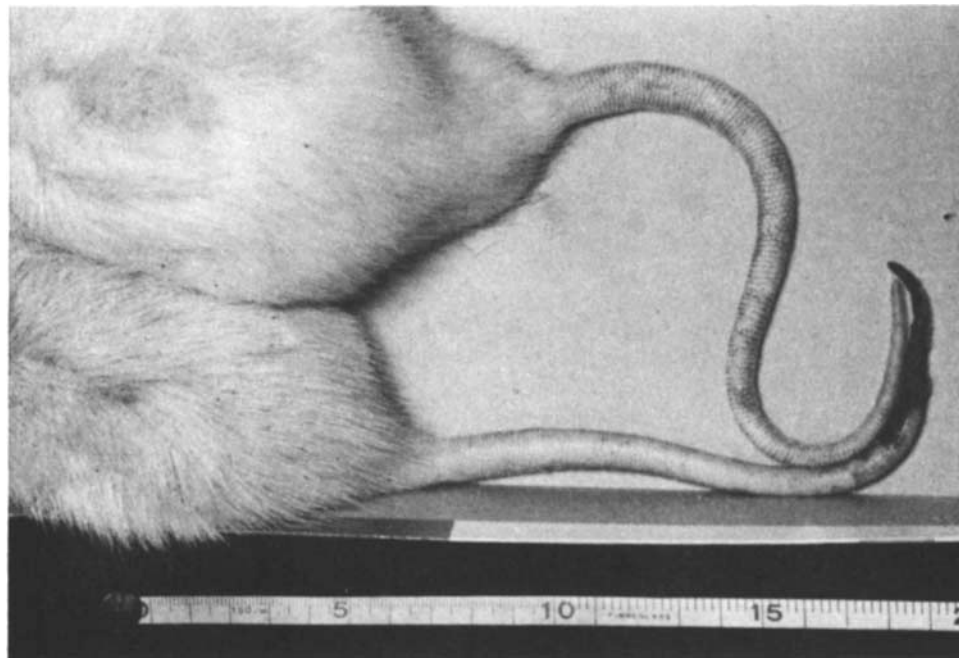

Figure 1 Rat tail on the 20th day after intraperitoneal injection of humic acid, showing ulceration and gangrene. The upper rat was a control. The scale is in centimeters.

(14.9%), weakness of extremities (10.2%), and itching in the sole (5.3%).[17] After a period varying from several days to two years but usually within six months, gangrene develops which gives the affected limbs the characteristic black discoloration.[1] Nearly 70% of the victims eventually underwent surgical amputation or the diseased parts amputated spontaneously.

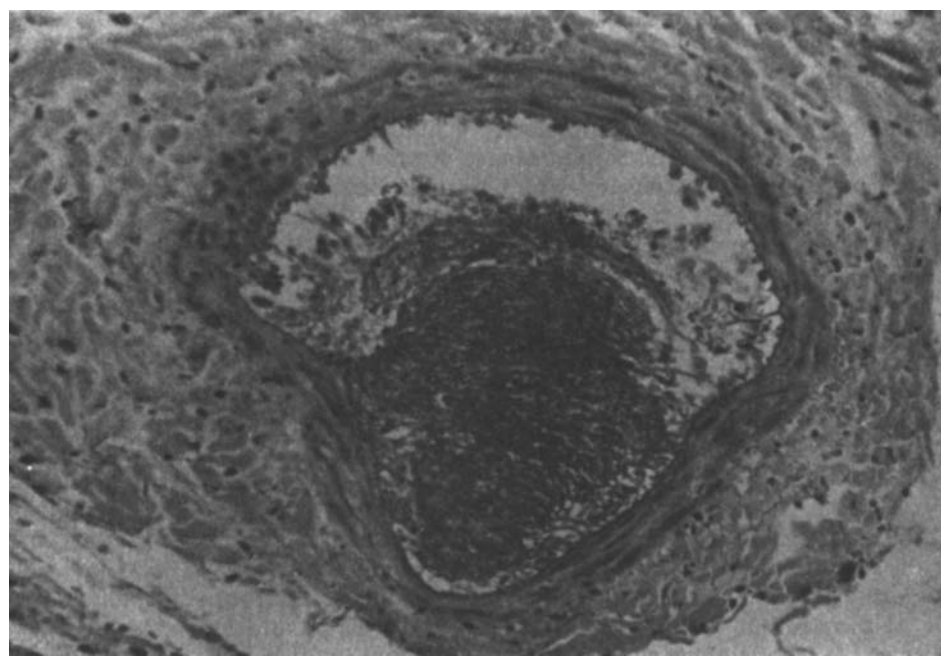

Figure 2 Thrombus formation in a distorted arteriole (H.E. stain × 200).

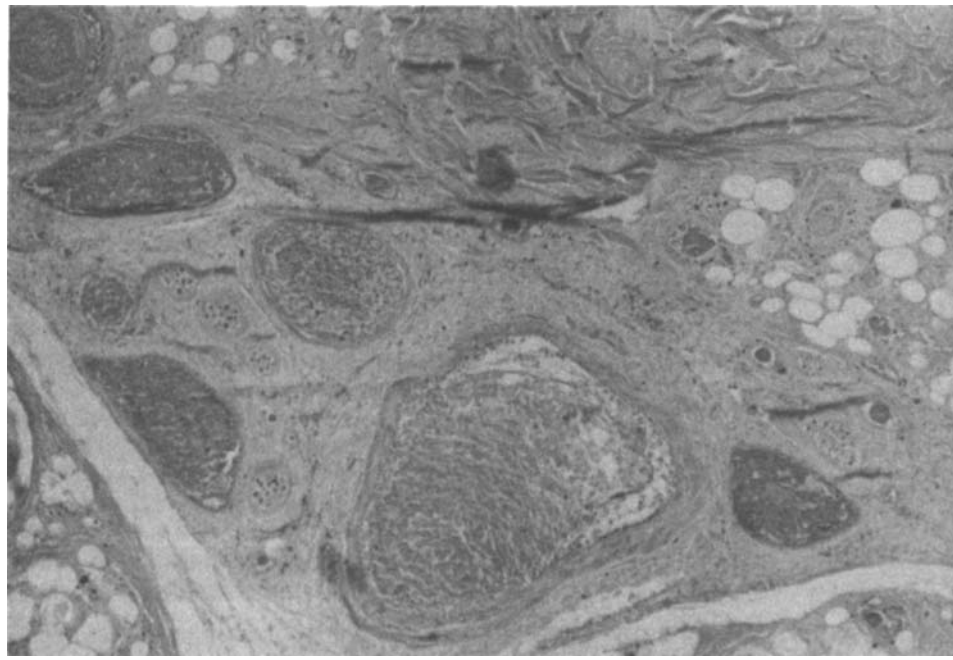

Figure 3 Bacterial clumps in the subcutaneous fat region stained blue with H.E. (H.E. stain × 100).

The histopathology of Blackfoot disease can be divided into two distinctly different groups: one, the thromboangitis obliterans group; and the other, the arteriosclerosis obliterans group. In the thromboangitis obliterans group, the basic initial change is fibrinoid degeneration of the intimal connective tissue of either arteries or veins, with or without inflammatory cell reaction. An intimal collar fibrosis, usually described as intimal proliferation, was noted in large vessels. Thrombotic occlusion may ensue in small vessels. Sometimes, the fibrinoid degeneration was associated with a marked inflammatory reaction. Such vascular changes are seen involving small arteries in skeletal muscles, but are never seen in ordinary thromboangitis obliterans. In the arteriosclerosis obliterans group, the occluding lesions are variable—from a red or mixed thrombus to complete occlusion. Marked intima sclerosis has been found. The most common intimal change is fibrous thickening. Atheroma formation and calcification can be found, and necrosis in deeper layers sometimes occurs. Periarterial fibrosis is not uncommon in this group, though not as marked as in the thromboangiitis obliterans group.[17]

In our previous experiments, we induced black discoloration of the tails of rats and mice with humic substances.[9,18] In one experiment, 200 mg (100 g^{-1}) body weight of humic substances (dissolved in 1 cm^3 of distilled water) obtained from artesian well water in the Blackfoot disease endemic area was injected intraperitoneally into ten male Sprague Dawley rats daily. On the 13th day, the tail of one of the ten rats became gangrenous with necrosis of skin, vascular congestion and thrombosis of arterioles.[18] In another experiment, 16 male Balb/c mice were injected intraperitoneally with a smaller dose of the same humic substances [5 mg (20 g body weight)$^{-1}$] daily.[9] During an interval of 14–32 days, eight mice developed crippling, phlegmasia, ulceration, necrosis and gangrene in extremities. The whole tail of one of the eight mice blackened. All the mice survived in the experiment.[9]

It is reasonable to speculate that the first sign we saw in the mice, i.e. crippling, was intermittent claudication equivalent to that observed in man. Edema, a common sign of early Blackfoot disease, also occurred in five (31.3%) of the experimental mice. Ulceration and gangrene, which are both important diagnotic criteria for Blackfoot disease, were observed in five of the experimental mice, too. The most striking characteristic signs of Blackfoot disease—black discoloration (2/16) and spontaneous amputation (1/16) of the affected extremities—were also successfully induced. Both the spreading from one limb to another, and the onset of signs in more

than one limb, in some experimental mice also occurred in Blackfoot disease patients.[1] Sudden onset without any preceding sign, which occurred in one mouse, was also observed in about 13.9% of Blackfoot disease patients.[17] Therefore, both the clinical signs and disease course in mice induced by injecting well-water humic substances were quite similar to those of Blackfoot disease.

The present study showed that a single intraperitoneal injection into rats of commercial humic acids resulted in endothelial damage with platelet plugs and thrombi in multiple peripheral arterioles and venules of gangrenous tails ten days later. Thrombosis of arteries resulted in ischemic gangrene, ulceration, necrosis, black discoloration and finally spontaneous amputation of the tails. The time course of the appearance of the black tail was shorter than in two previous experiments. The bacterial colonization and leucocytge infiltration represented secondary infection of gangrenous tissues rather than disseminated intravascular coagulation, as there was no bleeding tendency nor thromboemboli in other organs and the experimental rats appeared active.

There are similarities and dissimilarities in the pathology of our animal model and endemic Blackfoot disease. Thus in both, arterioles and venules are involved; thrombosis and dry blackened gangrene as well as spontaneous amputation develop; mainly peripheral vessels (limbs, tail or extremities) are involved. One important difference between the animal model and the human disease is that fibrinoid degeneration of intimal connective tissue is lacking in the animal model. Also, cell infiltration is due mainly to mast cells and leucocytes in the animal model, but to lymphocytes and macrophages in the human. Despite these differences, the animal model may be the equivalent of the human disease. The species difference and the rapid course and high dose used in the animal model may alter the pathological picture. It is also possible that humic acids are not the only factors in Blackfoot disease. Metallic or other compounds chelated by artesian humic substances may alter their biotoxicity and produce different tissue reactions.

High arsenic content in the well water was thought for many years to be the causative factor of Blackfoot disease but all the attempts to induce Blackfoot disease in animals with arsenics have failed.[19] Although the cause of Blackfoot disease is unknown, these experiments suggest that humic acids could be etiological factors. Our previous *in vitro* studies have shown that humic acids can cause endothelial damage and stimulate endothelin production,[20] shorten human prothrombin time,[21] and act as potent plasmin inhibitors.[22] We speculate that the duration of exposure, concentration of the humic acids in the vessels and/or predisposing vasculopathy such as arteriosclerosis may contribute to the various tissue reactions seen among Blackfoot disease patients and experimental animals. Thus, as soon as endothelial damage or activation occurs, humic acids facilitate thrombosis and prevent activation of the thrombolytic defense mechanism. In our previous experiments, arteriole thrombosis was seen occasionally in cerebellum, heart and lung (authors' own unpublished data). The predominant involvement of peripheral vessels may be partially explained in that stagnation of blood flow and the acidic medium of peripheral tissues cause humic acids in the blood to precipitate. Unfortunately, there is no method available to quantify humic acids content in tissues. Our hypothesis does not preclude arsenic as a contributing factor. Humic acids are known to chelate various heavy-metal ions to form complexes. Our preliminary studies have shown that metalorganic complexes may enhance biotoxicity.[23] Further studies including experiments to feed water from the Blackfoot area to experimental animals over long time periods will be required to clarify the mechanism of Blackfoot disease.

Acknowledgements This study was supported by grants NSC 82-0412-B002-187 and NSC 82-0412-B002-516 from the National Science Council, Republic of China. The authors are indebted to Miss Su-Whei Lee and Mr Cheng-Chung Lin for performing tissue sections.

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