Yonago Acta medica 2001;44:7–16

Immunohistochemical Study of Dendritic Cells and Kupffer Cells in Griseofulvin-Induced Protoporphyric Mice

Ryota Kashiwagi, Toshiyuki Kaidoh and Takao Inoué

Second Department of Anatomy, Faculty of Medicine, Tottori University, Yonago 683-0826 Japan

To determine whether protoporphyric liver injury affects hepatic dendritic cells (DC) and Kupffer cells, we examined liver tissues of griseofulvin-induced protoporphyric mice using histological and immunohistochemical methods. After 1 week of griseofulvin feeding, the protoporphyric liver showed prominent hepatomegaly and a gradual increase in histopathological changes such as hepatocellular hypertrophy, focal necrosis and brown pigment deposits. After 4 weeks of treatment, marked ductular reaction was observed in the liver tissue. Immunohistochemical analyses indicated that the density of NLDC-145-positive hepatic DC gradually decreased during griseofulvin feeding. However, the index of the number of DC in the whole liver appeared to fall sharply after 6 weeks. In contrast, the density of F4/80-positive Kupffer cells gradually increased during griseofulvin feeding. In the spleen and lung, no significant differences were noted in the distribution of NLDC-145-positive DC between experimental and control mice. These results suggested that griseofulvin-induced protoporphyria leads to a specific decrease in the density of hepatic DC due to hepatomegaly until 4 weeks of treatment and is substantial after 6 weeks. This substantial decrease of hepatic DC might have been induced by some alterations in protoporphyric liver injury including ductular reaction at a later stage in this experiment. Since hepatic DC were reduced in number, they seemed to have no significant relation to the progression of griseofulvin-induced protoporphyric liver injury. However, the decrease of hepatic DC might affect the cellular immune response in protoporphyria.

Key words: dendritic cell; griseofulvin; immunohistochemistry; Kupffer cell; protoporphyria

Although human porphyrias are often associated with the development of liver injury, they are relatively uncommon, preventing adequate investigation in patients. Griseofulvin, an antifungal drug, is known to inhibit hepatic ferrochelatase activity (Lochhead et al., 1967) and disturb porphyrin metabolism in the mouse (De Matteis and Rimington, 1963). This experimental mouse model shows liver injury with accumulation of brown protoporphyrin crystals, resembling human erythropoietic protoporphyria. This mouse model has therefore been used to study the pathogenesis of protoporphyric liver injury (Matilla and Molland, 1974; Gschnait et al., 1975; Denk et al., 1979; Kawahara et al., 1989; Woltsche et al., 1991; Cadrin et al., 1995). However, these studies have focused on changes in hepatocytes or Kupffer cells, so little is known about changes in dendritic cells (DC) in the liver.

DC are professional antigen-presenting cells, which contribute to the initiation of T cell-mediated immune responses. DC are intimately related to the liver in both normal and pathological conditions. Recently, DC have been shown to migrate from the blood to the lymph via the hepatic sinusoids (Matsuno et al., 1996; Kudo et al., 1997). It appears that this is the major migration pathway for DC from the blood, and Kupffer cells play an important role

Abbreviations: BSA, bovine serum albumin; DC, dendritic cells; PBS, phosphate-buffered saline

in this migration (Matsuno et al., 1996; Suda et al., 1996; Kudo et al., 1997). In addition, DC have been suggested to infiltrate into the liver tissues and then play a pivotal role in the development of chronic inflammatory liver diseases such as primary biliary cirrhosis, chronic hepatitis type B and type C, and autoimmune hepatitis (van den Oord et al., 1990; Kaji et al., 1997; Shinomiya et al., 1998; Tanimoto et al., 1999). However, there have been no attempts to quantify changes in hepatic DC or Kupffer cells in protoporphyric liver injury. Therefore, we examined changes in the numbers of these cells in griseofulvin-induced protoporphyric mice using immunohistochemistry to determine whether protoporphyric liver injury affects these cells and to speculate on the pathological role of these cells, particularly hepatic DC, in protoporphyric liver injury.

Materials and Methods

Twenty-nine male ICR mice, 5 weeks old and weighing about 30 g, were fed a powdered diet containing 3.5% griseofulvin (Sigma Chemical Co., St. Louis, MO) and water ad libitum. They were analyzed at 1 (n = 5), 2 (n = 5), 3 (n = 5), 4 (n = 5), 6 (n = 5) and 8 (n = 4) weeks after the commencement of griseofulvin feeding. Eight male ICR mice, 5 (n = 5) and 13 (n = 3) weeks old on a powdered diet without griseofulvin, served as controls. The 13-week-old control mice were the same age as the mice fed griseofulvin for 8 weeks. Animals were weighed and killed under diethyl ether anesthesia. The liver, spleen and lung were removed and the liver was weighed. Small pieces of these specimens were processed for histological and immunohistochemical studies.

Liver tissues were fixed in 10% formalin and processed using standard histological methods to yield paraffin sections 4 μm thick stained with hematoxylin and eosin.

Specimens of the liver, spleen and lung were snap-frozen in OCT medium with liquid nitrogen. Cryostat sections 4 μm thick were fixed in acetone for 10 min and single or double immunostaining was carried out. The single immunostaining method was as follows. After blocking nonspecific binding with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4) for 10 min and then with avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA), liver sections were incubated with rat anti-mouse macrophage monoclonal antibody F4/80 (diluted 1:50; Serotec, Oxford, United Kingdom) and those of the spleen and lung were incubated with rat anti-mouse dendritic cell monoclonal antibody NLDC-145 (diluted 1:25; Serotec) for 60 min (Austyn and Gordon, 1981; Witmer-Pack et al., 1995). Bound monoclonal antibody was detected with biotinylated rabbit anti-rat IgG (diluted 1:200 for F4/80, 1:1000 for NLDC-145; Vector) for 30 min. The sections were then treated with avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP Kit; Vector) for 30 min and labeled cells were colored red with alkaline phosphatase substrate kit I (Vector Red; Vector). Sections were counterstained with hematoxylin and mounted in Aquatex (Merck, Darmstadt, Germany). Since NLDC-145 recognizes not only DC but also proliferating bile ductules, we performed double immunostaining of the liver using anti-keratin antibody as follows to distinguish these structures. Endogenous peroxidase activity was eliminated by treatment with 1% H_2O_2 and 0.1% NaN_3 for 10 min. After blocking nonspecific binding with 1% BSA in PBS for 10 min, the sections were incubated with rabbit polyclonal anti-keratin, Wide Spectrum Screening (diluted 1:600; Dako, Carpinteria, CA) for 30 min to label the bile ductules (Shiojiri, 1994). The reaction of the antibody was detected using goat anti-rabbit immunoglobulin conjugated to peroxidase labeled-dextran polymer (Dako EnVision+; Dako) for 30 min and labeled cells were colored brown with 3,3'-diaminobenzidine tetrachloride (Dojin Chemical, Kumamoto, Japan). The sections were then treated with 5% normal rabbit serum in PBS for 20 min and with avidin-biotin blocking kit. Then, they were reacted with NLDC-145 and processed by the indirect immunoalkaline phosphatase method as described above. On double immunostaining, hepatic DC (red) could be distinguished from bile ductules (brown).

The number of NLDC-145-positive DC was quantified by counting cells per unit area on the sections in a blinded fashion, and the density of these cells was determined. Since the protoporphyric liver showed prominent hepatomegaly, we considered that hypertrophy of hepatocytes decreased the density of NLDC-145-positive DC. Therefore, we calculated the index of the number of DC in the whole liver as follows. We multiplied cell density (/mm^2) by liver weight (g) in each mouse and calculated the mean value of the products in each stage. Then the ratio of the value to that of the 5-week-old controls was evaluated. On the other hand, to quantify the density of Kupffer cells, we counted the number of F4/80-positive cells only in the sinusoidal area and excluded macrophages in the portal area in randomly selected fields. Since Kupffer cells were counted only in the sinusoidal area, we could not estimate the changes in Kupffer cell number in the whole liver. In these analyses, only the labeled cells where the nucleus could be identified were counted.

The density of hepatic DC and Kupffer cells was analyzed using the Mann-Whitney *U* test. A P value < 0.05 was taken to indicate statistical significance.

Results

Griseofulvin-induced protoporphyric mice showed retardation of growth. Mean body weight of these mice at 8 weeks was about 80% of that of the 13-week-old controls (Fig. 1). These mice showed prominent hepatomegaly, with the liver being dark brown in color. The livers of the mice fed griseofulvin for 8 weeks were about 3.6-fold as heavy as those of the 13-week-old controls (Fig. 1).

The intensity of histopathological changes in the liver gradually increased over the experimental period (Fig. 2). Hepatocytes showed nuclear and cytoplasmic hypertrophy, and vacuolated cytoplasm. Focal necrosis in the parenchyma occurred at all stages. Brown pigment deposits appeared 1 week after the commencement of griseofulvin feeding. They accumulated in hepatocytes, Kupffer cells, portal macrophages, bile ducts and bile ductules. Plugs of the brown pigment frequently obstructed the bile ducts and ductules. Proliferation of bile ductules was obvious at 3 weeks and became marked after 4 weeks. This was accompanied by a variable number of connective tissue elements and infiltration of neutrophils and mononuclear cells as reported previously (Weston-Hurst and Paget, 1963; Gschnait et al., 1975). These changes, the proliferation of bile ductules accompanied by inflammatory infiltration and connective tissue elements, are similar to so-called “ductular reaction”. After 4 weeks of griseofulvin feeding, this ductular reaction became a marked pathological change in the injured liver. It extended from the portal tracts toward neighboring portal tracts and penetrated the parenchyma between the liver cell plates.

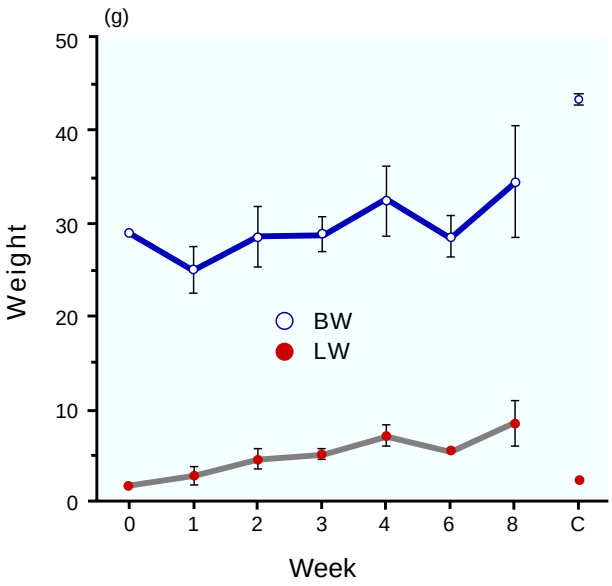

Fig. 1. Body weight (BW) and liver weight (LW) in control and griseofulvin-treated mice. Data are expressed as mean ± SD. Bars represent SD. C, 13-week-old control mice, identical in age to the mice treated with griseofulvin for 8 weeks.

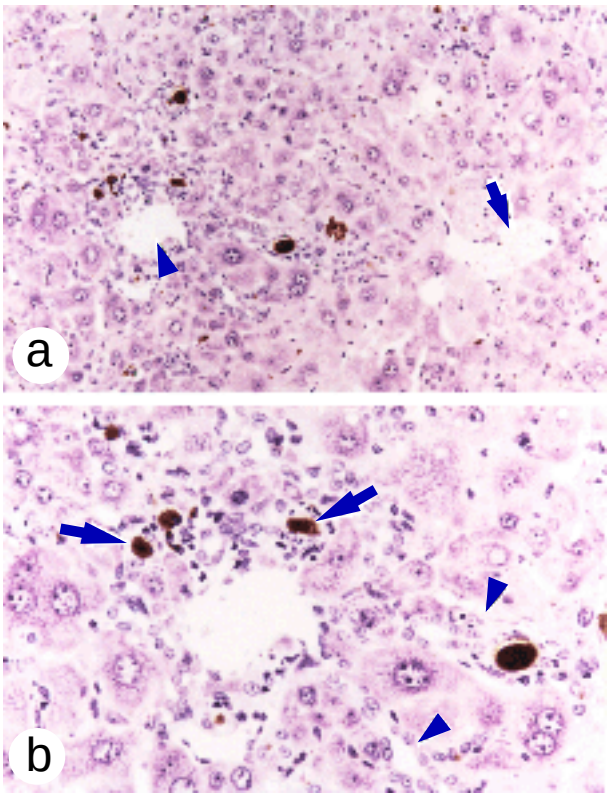

Fig. 2. Light micrographs of the liver of a mouse fed griseofulvin for 8 weeks. Hematoxylin and eosin. **a**: Brown pigment deposits are accumulated in the liver tissue. Central (arrow) and interlobular (arrowhead) veins are visible. × 120. **b**: Hepatocytes show hypertrophy. The proliferation of bile ductules is accompanied by inflammatory infiltrate and connective tissue elements, similarly to the so-called "ductular reaction". It penetrates the parenchyma between the liver cell plates (arrowheads). Note brown pigment deposits in the bile ductules (arrows). × 250.

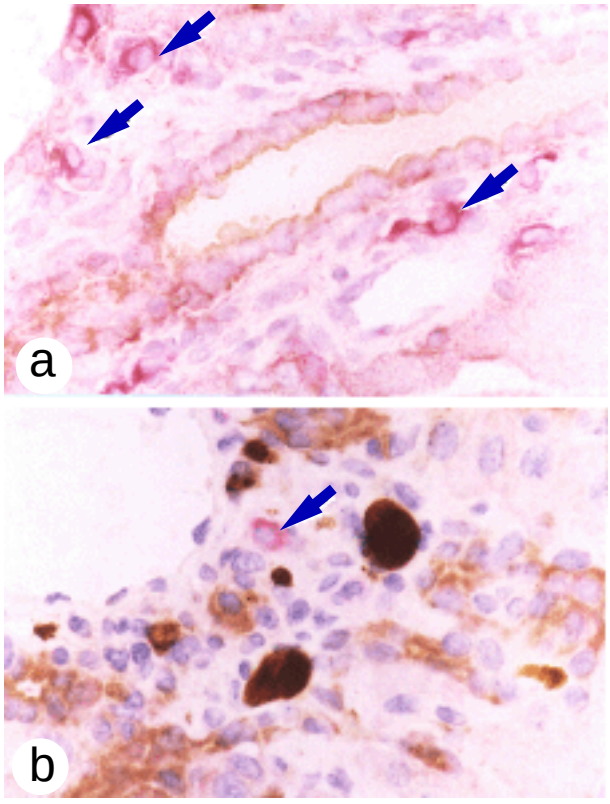

Fig. 3. Double immunostaining of the liver with monoclonal antibody NLDC-145 (red) and polyclonal anti-keratin, Wide Spectrum Screening (brown). **a**: A 5-week-old control mouse. Several NLDC-145-positive dendritic cells (DC) are visible in the portal area (arrows). × 500. **b**: A mouse treated with griseofulvin for 8 weeks. The number of NLDC-145-positive DC (arrow) is obviously decreased. Proliferating bile ductules are colored brown. × 500.