

## Enzyme-altered Liver Cell Foci in Woodchucks Infected with Woodchuck Hepatitis Virus

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The histochemical characteristics of liver cell foci in woodchucks were investigated. The foci appeared to be distributed throughout the liver and were observed only in the woodchuck hepatitis virus (WHV)-positive animals, including all 19 woodchucks with hepatocellular carcinoma (HCC), and 7 without HCC. No foci appeared in 11 WHV-negative animals. Histochemical studies revealed that liver cell foci and carcinoma cells were characterized by positive gamma-glutamyl transpeptidase (GGT) enzymatic reactions and decreased glucose-6-phosphatase enzyme activity compared to non-neoplastic liver. Furthermore, serum GGT was significantly elevated in almost all of the animals which had larger carcinomas. Ultrastructural findings of foci showed some resemblance to carcinoma cells, being characterized by abundant free ribosomes within the cytoplasm and undeveloped endoplasmic reticulum. These results suggest that the liver cell foci are potential precursors of HCC in WHV-infected animals, and that serum GGT may be a useful marker for indicating the development of carcinoma.

Key words: Woodchuck — Woodchuck hepatitis virus — Liver cell foci — Glucose-6-phosphatase — Gamma-glutamyl transpeptidase

The woodchuck hepatitis virus (WHV) belongs to the hepadna virus group of viruses. Persistent infection with hepatitis B virus (HBV) or with WHV is associated with chronic liver disease and hepatocellular carcinoma (HCC) in the natural hosts.<sup>1,2</sup> The woodchuck infected with WHV, therefore, represents a useful animal model for the study of the pathogenesis of chronic HBV infection in humans.

The histologic study of hepatic lesions in woodchucks infected with WHV reveals some resemblance to human hepatitis B and some characteristic differences.<sup>3,4</sup> In humans, progression of chronic hepatitis to cirrhosis occurs frequently prior to or concurrently with development of hepatic tumors, but in woodchucks, cirrhosis characteristically is absent. In contrast to observations in humans chronically infected with HBV, we have found that woodchucks develop liver cell foci.<sup>5</sup> These foci are similar to those associated with chemically induced hepatomas of rats, and mice, and are considered to be putative preneoplastic lesions during chem-

ical hepatocarcinogenesis.<sup>6,7</sup> This study was designed to investigate the enzyme-histochemical changes found in HCC and to determine the histochemical nature of liver cell foci in woodchucks.

### MATERIALS AND METHODS

**Animals** Thirty imported WHV-positive woodchucks trapped in Pennsylvania, USA, and estimated to be 2 to 5 years old, and were used in this study. The animals were maintained in our breeding colony and fed tap water and a rabbit diet made by special order into blocks (Funabashi Farms, Chiba). Blood samples were obtained under general anesthesia with a combination of xylazine (5 mg/kg) and ketamine (25 mg/kg). Open liver biopsies were performed under general anesthesia induced with a combination of xylazine and ketamine and maintained with halothane.

**Light Microscopic Examination** All liver biopsies and autopsy specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections stained with hematoxylin and eosin (HE) were examined by light microscopy.

**Electron Microscopic Examination** The liver tissue was fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded

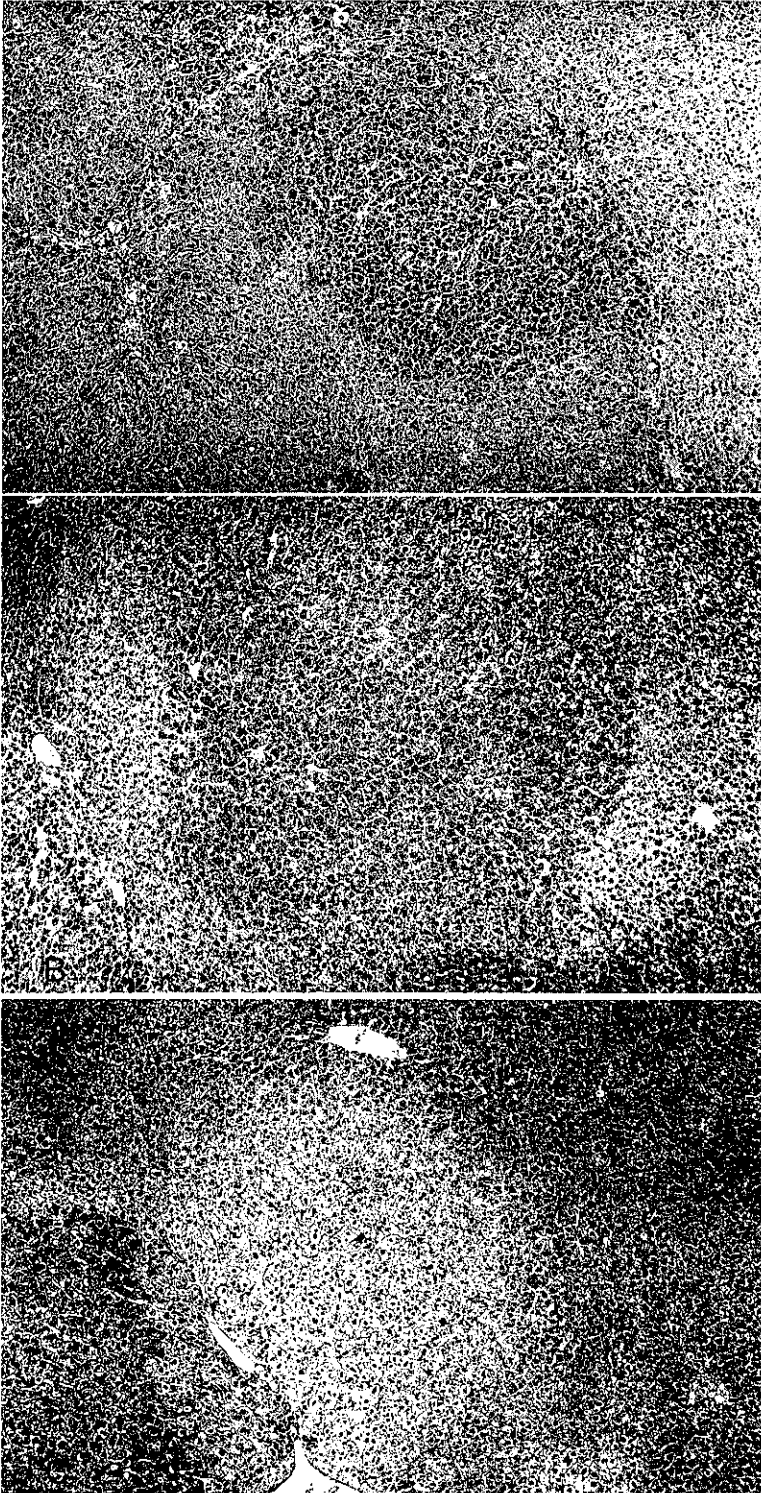


Fig. 1. Liver cell foci comprized of basophilic (A), eosinophilic (B) and clear (C) cells. HE stain,  $\times 40$ .

ethanols, and embedded in epoxy resin. Ultra-thin sections stained with lead citrate and uranyl acetate were examined under an electron microscope.

**Enzyme-histochemical Examination** Serial frozen sections of the liver tissues made in a cryostat were stained for gamma-glutamyl transpeptidase (GGT) by the method described by Rutenburg *et al.*,<sup>8)</sup> and glucose-6-phosphatase (G6P) by the method of Wachstein and Meisel.<sup>9)</sup>

**Assay of Serum GGT** Assay of serum GGT was performed with a commercially available manual colorimetric kit (Marco Pharmaceutical Co., Nagoya).

## RESULTS

**Histopathological Findings** All 30 WHV-positive animals had chronic hepatitis. Twenty-nine of these had chronic persistent hepatitis, which was characterized by infiltration of lymphocytes and plasma cells into portal areas without necrotizing inflammatory changes of hepatocytes and with well-preserved limiting plates. One had chronic active hepatitis. Twenty-five of the 30 WHV-positive animals had liver cell foci. The foci appeared diffusely within the liver (about 1 to 3 foci/cm<sup>2</sup> tissue) and were distinct from the adjacent parenchyma, thus corresponding to real microscopic foci ranging from 1 to 3 mm in diameter. The hepatocytes in these foci were large with basophilic cytoplasm, but rarely with eosinophilic or clear cells, distinct cell borders, large vesicular nuclei, and prominent nucleoli (Fig. 1). Small foci of similar hepatocytes sometimes appeared in the peripheral zone of hepatic lobules. The foci were observed in all 19 WHV-positive animals with HCCs and in 6 WHV-positive animals without HCCs (see the Table I). Grossly, the tumors appeared as multiple firm nodules. Foci of hemorrhage and necrosis were frequent in larger masses. Metastases were not encountered. Histologically, tumors were well-differentiated hepatocellular carcinoma with trabecular patterns and were frequently associated with extramedullary hematopoiesis (Fig. 2). Ultrastructurally, the basophilic hepatocytes in the foci showed abundant free ribosomes in the cytoplasm, undeveloped endoplasmic reticulum and prominent nucleoli, but well-preserved bile canaliculi (Fig. 3). These pathological changes were not seen in WHV-negative animals.

**Enzyme-histochemical Findings** In normal liver tissue, G6P activity was present in the cytoplasm of all hepatocytes throughout the entire lobule and was especially strong at periportal regions. The staining for GGT activity revealed granular areas of activity in the bile ducts; however, no areas of activity were observed in the hepatocytes and bile canaliculi. In abnormal foci, G6P activity showed variable loss, and there was only weak GGT activity (Fig. 4). Distinct GGT activity could be seen in the cytoplasm of hepatocytes in the foci, but not in the hepatocytes outside the foci (Fig. 5). In tumors, G6P activity was markedly decreased or lost throughout the carcinomatous areas. GGT, on the other hand, stained strongly along the entire cell membrane, bile canaliculi and in the cytoplasm of carcinoma cells in a diffuse, fine and granular pattern.

**Serum GGT** Serum GGT was increased in almost all of the animals which had HCCs. In 9 WHV-positive animals with small tumors (1 to 2 cm<sup>3</sup> in volume), GGT activity ranged from 5–39 mU/ml. Nine animals with larger

Table I. Relationship between Liver Cell Foci and Hepatocellular Carcinoma

WHsAg	Liver cell foci	HCC <sup>a)</sup>	HCC with foci
Positive	25/30(83%)	19/30(63%)	19/19(100%)
Negative	0/11 (0%)	0/11 (0%)	0/0 (0%)

a) Hepatocellular carcinoma.

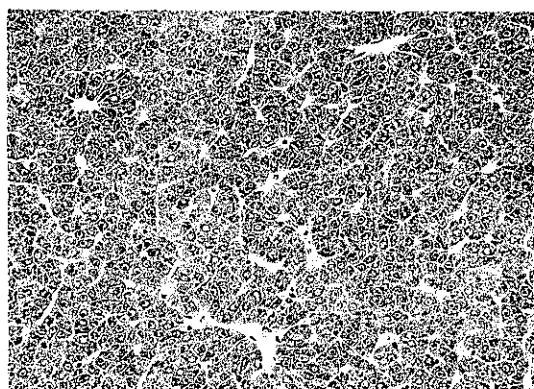


Fig. 2. Well-differentiated hepatocellular carcinoma with trabecular pattern. HE stain,  $\times 100$ .

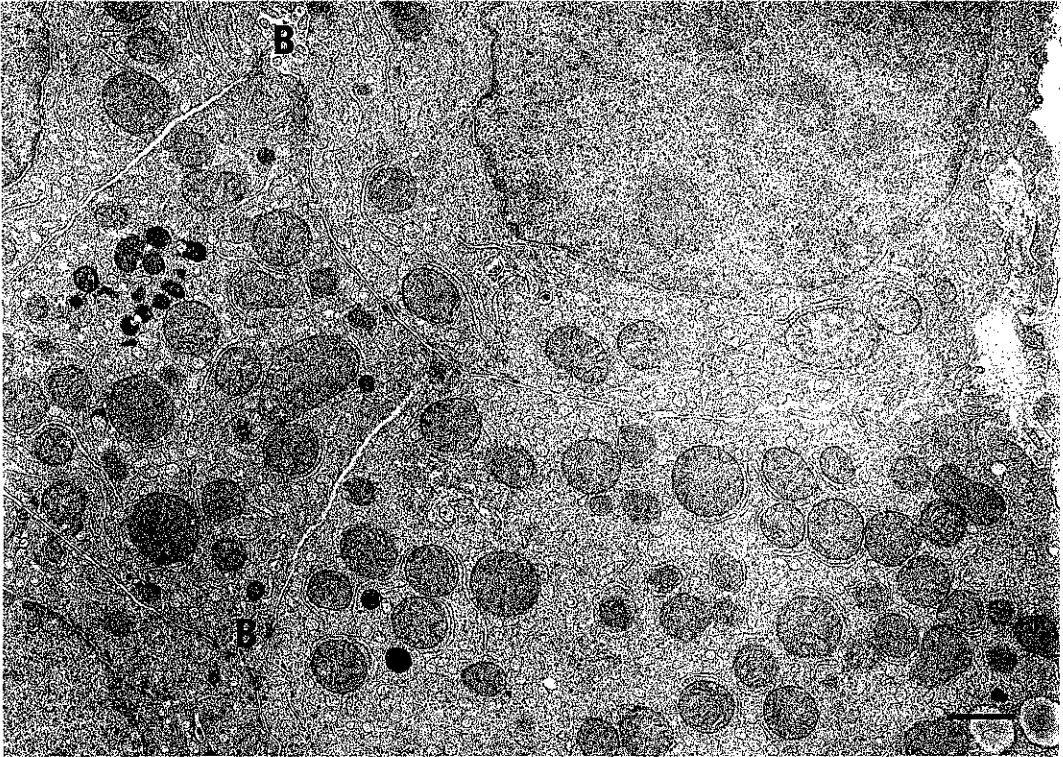


Fig. 3. An electron micrograph showing basophilic hepatocytes in foci containing abundant free ribosomes in the cytoplasm, undeveloped endoplasmic reticulum and prominent nucleoli, but with well-preserved bile canaliculi (B).  $\times 8,360$ . Bar =  $1 \mu\text{m}$ .

tumors (up to about  $100 \text{ cm}^3$  in volume) showed a marked elevation of GGT activity ranging between 100–1679 mU/ml. An additional animal with HCC showed a rapid decrease in serum GGT after surgical removal of carcinomatous tissue. In contrast, the serum GGT activity in animals without HCC was under 9 mU/ml.

#### DISCUSSION

Recently, hepatitis viruses with characteristics similar to HBV have been identified in several different animal species: the woodchuck, Beechey ground squirrel and Pekin duck.<sup>10–12</sup> WHV, originally described by Summers *et al.*, serves as a biological and pathogenetic model for human HBV.<sup>10</sup> The homology between the two viral DNAs was confirmed with the cloned DNAs.<sup>13</sup> Thus, the WHV and HBV are phylogenetically related.

Chronic WHV infection has been associated with a high frequency of HCC (in more than half of the infected animals).<sup>1,2</sup> Furthermore, WHV-DNA hybridization studies of liver tissue demonstrated a strong correlation between the presence of WHV and the development of HCC. Summers has reported that 2 of 5 WHV-positive animals showed integration of WHV-DNA into cellular DNA at the same site in most cells of the tumor.<sup>14</sup> These findings are important in evaluating the behavior of WHV in carcinoma cells and the role of WHV in carcinogenesis. It has been noted that pathological changes seen in animals infected with other hepadnaviruses show important differences from those seen in HBV infections in man. For example, ground squirrels infected with ground squirrel hepatitis virus show little evidence of hepatitis and HCC.<sup>11,15</sup> Woodchucks chronically infected

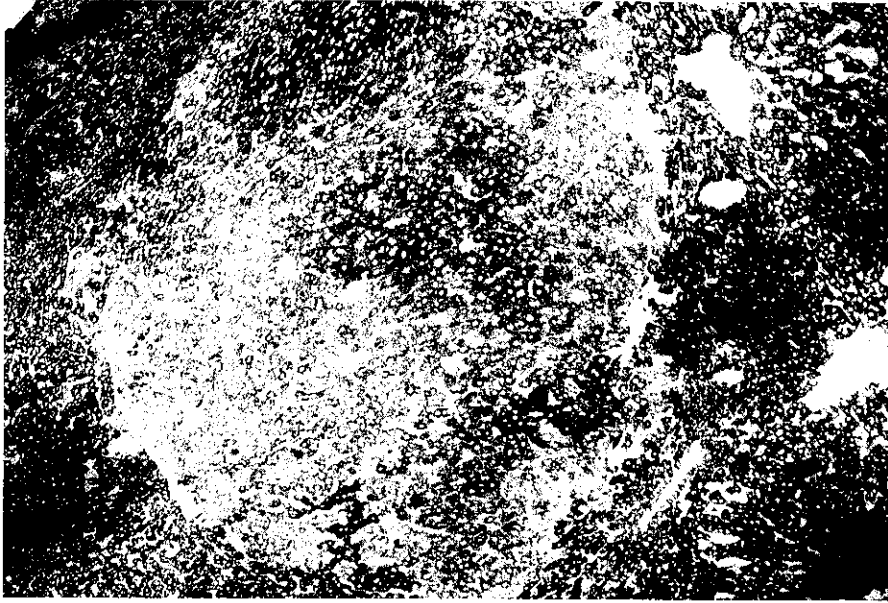


Fig. 4. Glucose-6-phosphatase reaction in liver showing partial depletion of reaction product within foci.  $\times 40$ .

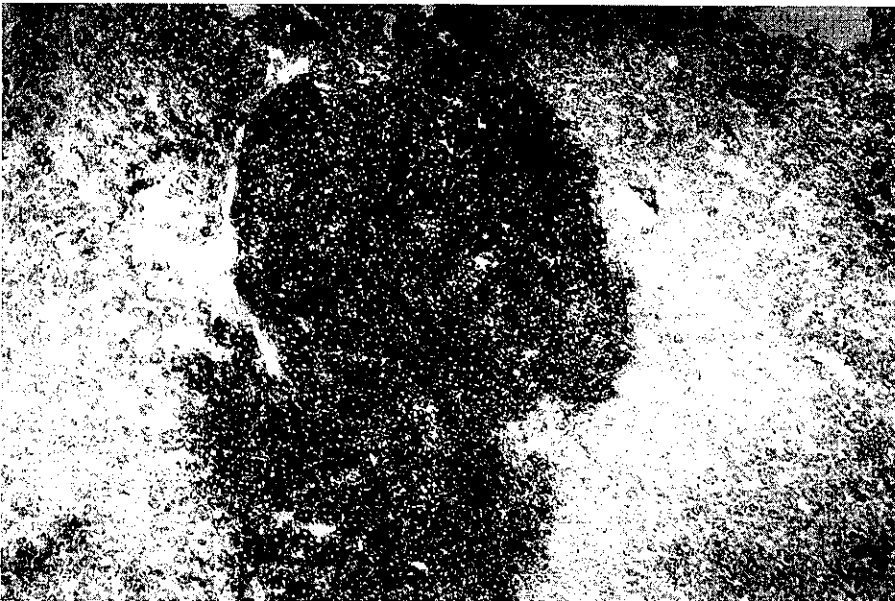


Fig. 5. Gamma-glutamyl transpeptidase reaction in liver showing prominent enzyme activity of the hepatocytes in foci. Counterstained with hematoxylin,  $\times 100$ .

with WHV show signs of mild to moderate hepatitis, but the development of cirrhosis as seen in man has not been found.<sup>3-5)</sup> In the present study, the characteristic presence of liver cell foci in woodchuck was strongly associated with WHV-infection and HCC. These histologic changes, which can be identified during the precancerous period, have features in common with those described for carcinogens such as diethylnitrosamine,<sup>16, 17)</sup> 2-fluorenylacetylamide,<sup>18, 19)</sup> 4-dimethylaminoazobenzene,<sup>20, 21)</sup> N-nitrosomorpholine,<sup>22)</sup> ethionine<sup>23)</sup> and aflatoxin<sup>24-26)</sup> in rodents. Since the term liver cell foci was applied to preneoplastic lesions in the liver during chemical carcinogenesis, the presence of GGT is widely used as a marker of preneoplastic lesions in the liver during experimental studies of chemical carcinogenesis.<sup>27)</sup> By histochemical and biochemical methods, GGT was shown to be present in large amounts in the liver parenchyma of fetal and early neonatal rats, but to be virtually absent in normal adult rat liver.<sup>28)</sup> It has been generally accepted that reacquisition of GGT activity in the liver during hepatocarcinogenesis might be an oncofetal property similar in nature to the expression of alpha-fetoprotein.<sup>29)</sup> Using polarography and colorimetry on rat liver homogenates, Fiala *et al.* demonstrated that GGT is activated by several unrelated hepatocarcinogens in mice and in various rat strains and that it is significantly increased in the hepatocytes during both the precancerous stage and HCC.<sup>30)</sup> The histochemical identification in WHV carrier woodchucks of liver cell foci that are similar to the preneoplastic, GGT-positive foci induced by certain chemical carcinogens suggested that GGT may be a useful marker in further elucidating the early changes occurring during the process of hepatocarcinogenesis in the woodchuck. From these results, liver cell foci in WHV-infected animals were considered as potential precursors of carcinoma and it was speculated that woodchuck HCC may develop multicentrically. Furthermore, serum GGT was also elevated in animals with HCCs, and these results suggested that the serum GGT can be used as a sensitive and specific marker for the development of HCC. Thus, the woodchuck infected with WHV seems a favorable animal model for the study of the pathogenesis of HBV-induced HCC.

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