Morphometric Study of Hepatocytes Containing Hepatitis B Surface Antigen

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The development of hepatocellular carcinoma (HCC) is probably related to infection with hepatitis B virus (HBV). Hepatocytes in livers of patients with HCC have been reported to show putative preneoplastic changes such as hyperplasia, dysplasia, or adenomatous regeneration. To determine quantitatively whether these morphologic changes are associated with HBV-infected cells, the authors performed morphometry of hepatitis B surface antigen (HB_sAg)-positive hepatocytes in the nontumorous portion of 10 livers with HCC and in 10 livers without HCC. The diameter of nuclei and cytoplasm of HB_sAg-positive hepatocytes was measured after demonstration of HB_sAg by the peroxidase–antiperoxidase method. As controls, HB_sAg-negative hepatocytes in the same liver sections were measured as well as hepatocytes of 20 age-matched HB_sAg-negative patients with normal liver or alcoholic cirrhosis. HB_sAg-positive hepatocytes exhibited significantly larger nuclei and a higher nucleocytoplasmic ratio than control hepatocytes. In addition, HB_sAg-positive cells were often arranged in foci that consisted of two cell populations: hypertrophic (enlarged nuclei and nucleocytoplasmic ratio) and hyperplastic (two-cell-thick plates of small cells with a high nucleocytoplasmic ratio). While precancerous cells have been difficult to identify, these morphologic changes are frequently associated with the development of malignant neoplasia. (Am J Pathol 1984, 114:217–221)

MULTIPLE LINES of evidence suggest that the development of hepatocellular carcinoma (HCC) is related to infection with hepatitis B virus (HBV).[1,2] Morphologic and immunomorphologic observations have contributed significantly to the evidence in support of this hypothesis. Considerable uncertainty exists concerning the premalignant lesions both in man and experimental animals.[3,4] Farber[3] considers the hyperplastic nodule or similar lesion as one site of origin of cancer in the multistep model of hepatocarcinogenesis; in contrast, Sell and Leffert[4] call attention to multiple cellular lineages in the development of experimental hepatocellular carcinoma. In man, many organs display consistently several types of putative precancerous lesions, such as atypical hyperplasia, dysplasia, and carcinoma *in situ*.[3] Hepatocytes in livers of patients with HCC have been reported to show presumed preneoplastic changes such as hyperplasia, dysplasia,[5] and adenomatous regeneration.[6] Anthony[7] and others[8,9] demonstrated a statistically significant association between hepatocellular dysplasia and HBV infection, while Cohen et al[10] failed to demonstrate such a relation in South Africa. Many investigators detected HBV markers both in HCC tissue and in the surrounding nontumorous liver (reviewed by Gerber and Thung[11]). The present study attempts to determine quantitatively whether hypertrophy, hyperplasia, and dysplasia are associated with HBV-infected hepatocytes.

Materials and Methods

Morphometry was performed on hepatocytes of 10 hepatitis B surface antigen (HB_sAg)-positive cases

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without HCC and in the nontumorous portion of 10 HB_sAg-positive cases with HCC. The specimens were obtained from the United States, Korea, and China by needle or wedge biopsy (17 cases) and at autopsy (3 cases). Histologically, 2 cases showed chronic persistent hepatitis, 7 cases chronic active hepatitis, 10 cases cirrhosis, and 1 case diffuse nodular transformation of liver. Because of an age-related increase in polyploidization of human hepatocytes,[12,13] the patients were matched according to age and the method of specimen procurement. There were 10 cases of normal liver and 10 cases of alcoholic cirrhosis. The average age was 44.3 years in the HCC group, 43.3 in the HB_sAg-positive group without HCC, 47.4 in the normal group, and 49.6 in the alcoholic cirrhosis group. There were 1 to 2 females in each group. Formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin-eosin and for HB_sAg by the peroxidase-antiperoxidase (PAP) method. Briefly, sections were deparaffinized and digested in 0.1% trypsin, followed by incubation in 3% H_2O_2 and subsequently in 10% egg albumin to block endogenous peroxidase activity and to reduce nonspecific staining. Then the sections were incubated with goat antibody to HB_sAg, followed by rabbit antibody to goat γ-globulin and goat antibody to rabbit γ-globulin. This was followed by reaction with rabbit PAP complex and demonstration of the reaction product with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2. The sections were counterstained with hematoxylin or Wilder's reticulin. The specificity of the demonstration of HB_sAg was proven by absorption of the primary antibody with HB_sAg, replacement of the primary antibody with normal goat serum, omission of the secondary and tertiary antibodies, testing for endogenous peroxidase activity, and staining of known positive and negative control sections. In addition, 9 HB_sAg-positive cases were stained for HB_cAg, 10 for α-antitrypsin, and 11 for α-fetoprotein by the PAP method as described previously.[14] Morphometry was performed by measuring the largest diameter of 60 HB_sAg-positive hepatocytes and their nuclei with a horizontal eyepiece micrometer graticule calibrated by means of a stage micrometer. The HB_sAg-containing hepatocytes were often arranged in groups of either large or small cells. Therefore, in 10 cases, 60 HB_sAg-positive small hepatocytes, 60 HB_sAg-positive large hepatocytes and 60 HB_sAg-negative hepatocytes were also measured. In the HB_sAg negative control specimens (10 normal livers and 10 alcoholic cirrhosis), 60 randomly selected hepatocytes were measured. Binucleated and fat-containing hepatocytes were excluded. The data were entered in the City University of New York's IBM 370 computer for analysis. Statistical calculations were done with the use of the BMDP[15] software package. Analysis of variance was used to test the hypothesis that there would be no differences among the group means. Multiple comparisons were tested with Fisher's protected least significant differ-

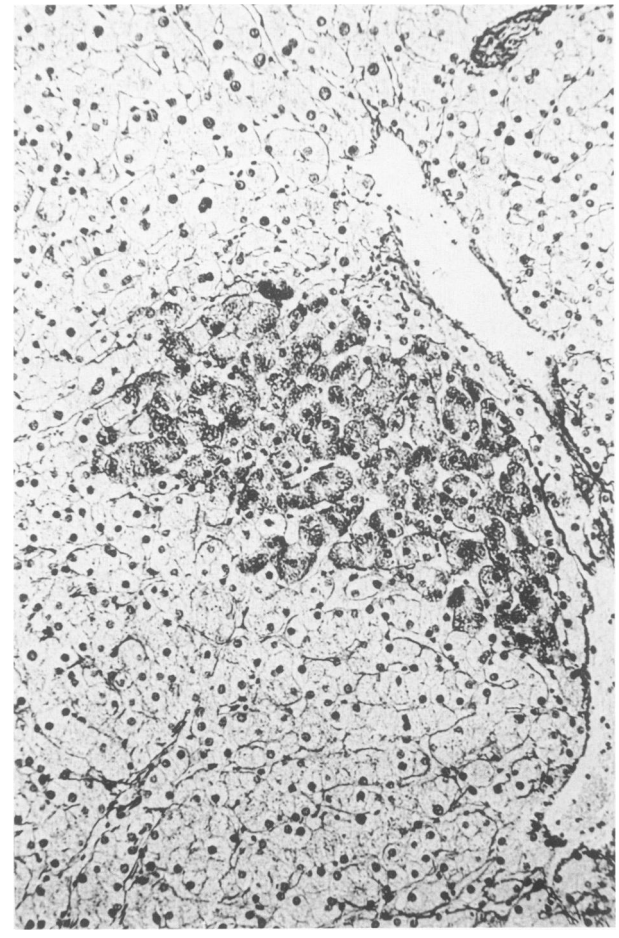

Figure 1 – Large group of HB_sAg-positive hepatocytes with slight compression of HB_sAg-negative cells and reticulin above. (PAP method for HB_sAg followed by reticulin stain, × 100).

Table 1 – Morphometric Data for Hepatocytes

	Cytoplasm (μ)	Nucleus (μ)	N/C ratio
HB_sAg + cases with HCC			
HB_sAg + hepatocytes	19.94	7.99	0.40
HB_sAg – hepatocytes	19.63	7.45	0.38
HB_sAg + cases without HCC			
HB_sAg + hepatocytes	21.04	7.98	0.38
Normal liver			
HB_sAg – hepatocytes	21.60	7.06	0.33
Alcoholic cirrhosis			
HB_sAg – hepatocytes	20.10	6.96	0.35

ence (LSD) procedure.[16] The significance level for all statistical tests was 0.05.

Results

As shown on Table 1, the nuclear/cytoplasmic (N/C) ratio of HB_sAg-positive hepatocytes in livers with or without HCC (0.40 and 0.38, respectively) was significantly higher than that of hepatocytes in normal livers (0.33) or livers affected by alcoholic cirrhosis (0.35). This was related to significant enlargement of nuclei of HB_sAg-positive hepatocytes (7.99 μ and 7.98 μ, respectively) in comparison with hepatocytic nuclei of HB_sAg-negative cases (7.06 μ and 6.96 μ, respectively). No other significant differences were observed between the nuclear diameters of HB_sAg-negative hepatocytes in HB_sAg-positive cases with HCC, normal livers, or alcoholic cirrhosis and between the cytoplasmic diameters in all cases.

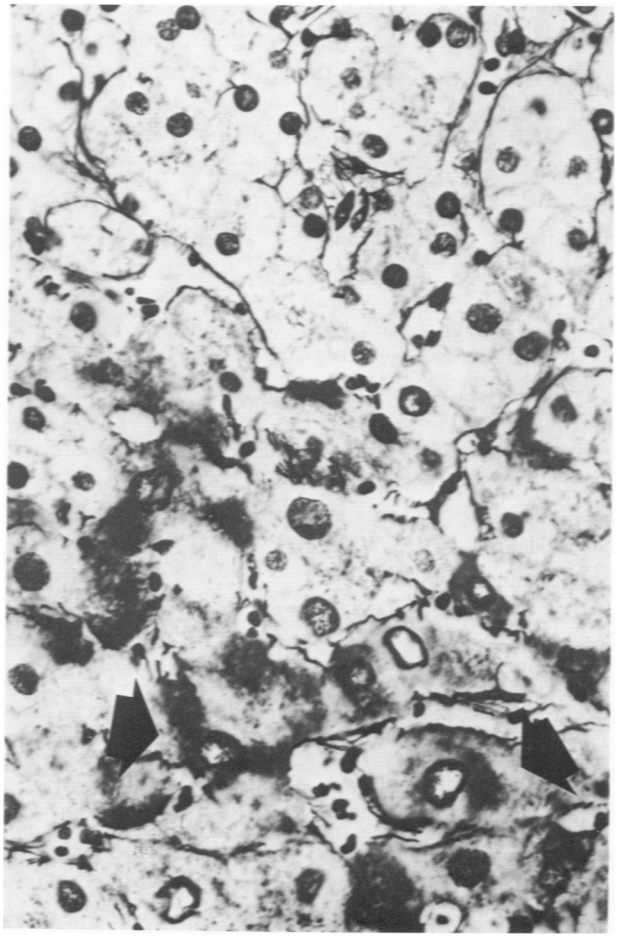

Figure 2—Group of large HB_sAg-positive hepatocytes (dark reaction product in cytoplasm) with large hyperchromatic nuclei (*arrows*). Compare with HB_sAg-negative hepatocytes above. (PAP method for HB_sAg followed by reticulin stain, ×250)

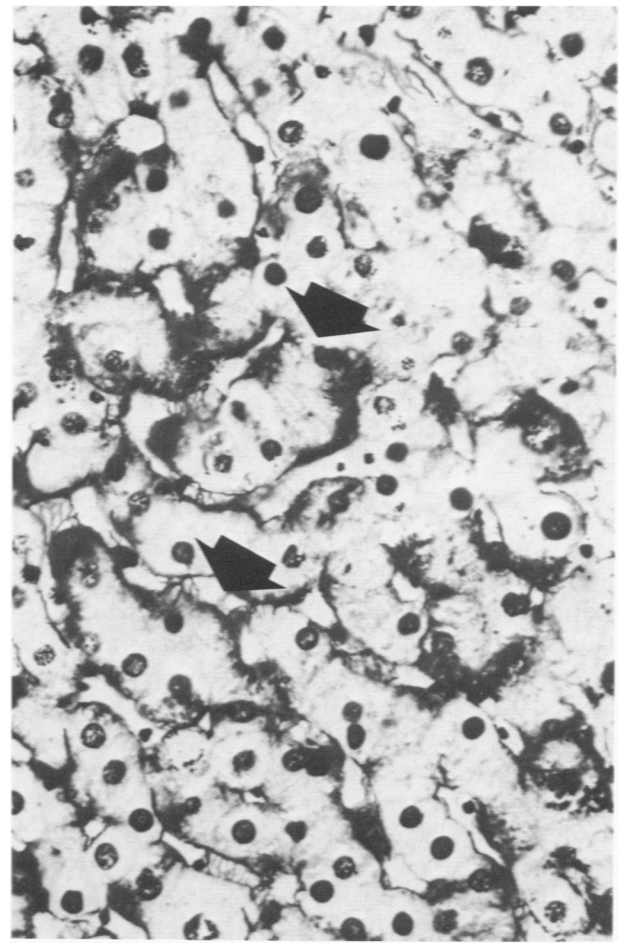

Figure 3—Group of small HB_sAg-positive hepatocytes arranged in two-cell-thick plates (*arrows*). (PAP method for HB_sAg followed by reticulin stain, ×250)

The HB_sAg-positive hepatocytes in livers of patients with or without HCC were often arranged in groups (Figure 1). In some foci, HB_sAg positive hepatocytes were large with large pleomorphic hyperchromatic nuclei and prominent nucleoli (Figure 2). In other groups, HB_sAg-positive hepatocytes appeared to be smaller and were arranged in two-cell-thick plates (Figure 3). Morphometry of these two types of HB_sAg-positive cells, selected by eye, revealed that the mean diameter of nucleus and cytoplasm of large HB_sAg-positive hepatocytes was significantly larger than that of small HB_sAg-positive hepatocytes (Table 2). The N/C ratio of the small HB_sAg-positive hepatocytes was significantly higher than that of large HB_sAg-positive hepatocytes.

HB_cAg was detected in scattered hepatocyte nuclei of 1 of 6 cases with HCC and 2 of 3 cases without HCC examined. $Alpha_1$-antitrypsin was found in hepatocyte cytoplasm of 5 of 8 cases with HCC and

Table 2—Morphometric Data for Two Populations of HB_sAg-Positive Hepatocytes

	Cytoplasm (μ)	Nucleus (μ)	N/C ratio
Large HB_sAg + hepatocytes	23.19	9.03	0.39
Small HB_sAg + hepatocytes	16.58	7.04	0.43

both cases without HCC tested, and α-fetoprotein was found in 3 of 8 cases with HCC and 1 of 3 cases without HCC. Comparison with parallel sections after reaction with antibody to HB_sAg did not reveal preferential expression of these antigens in HB_sAg-positive hepatocytes.

Discussion

The liver represents an ideal tissue for morphometric studies.[17] The size of normal human hepatocytes and their nuclei has been measured by various morphometric techniques.[12,13,18-20] From these studies the mean nuclear and cytoplasmic diameter can be calculated to range from 6.5 to 7.3 and 17.8 to 23.4 μ, respectively. These variations may be due in part to an age-related increase of polyploidy of human hepatocytes.[12,13] The values for the mean nuclear and cytoplasmic diameter of normal hepatocytes recorded here (7.06 and 21.6 μ, respectively) fell well within this range. HB_sAg-positive hepatocytes in patients with or without HCC exhibited a striking enlargement of their nuclei to a diameter of 7.99 and 7.98μ, respectively. Although HB_sAg-containing ground-glass hepatocytes showed marked proliferation of smooth endoplasmic reticulum,[21,22] the diameter of the entire cell remained within the normal range. Therefore, the N/C ratio of HB_sAg-positive hepatocytes increased significantly, a characteristic of dysplastic and anaplastic cells. The enlargement of nuclei of HBV-infected hepatocytes may be a morphologic expression of integration of the HBV genome into the cellular DNA of patients with chronic HBV infection. Molecular hybridization analysis demonstrated integrated HBV DNA sequences in both HCC and nontumorous portion of HB_sAg-positive liver tissue.[23-26]

HB_sAg-positive hepatocytes often formed foci that appeared to consist of two distinct cell populations: large cells with large nuclei and a moderately high N/C ratio, ie, hypertrophic; and small cells with a very high N/C ratio, often arranged in two-cell-thick plates, i.e., hyperplastic. Since submission of this article, Watanabe et al[27] reported in cirrhotic livers with HCC large and small dysplastic hepatocytes, the latter often forming round foci. Morphologic studies suggest that hypertrophy with an increase in the N/C ratio, hyperplasia, and dysplasia are frequently associated with development of malignant neoplasia.[3] The present study of HB_sAg-containing hepatocytes in the nontumorous part of livers with HCC demonstrates quantitatively that these morphologic changes are associated with HBV-infected cells.

In HB_sAg-positive patients without HCC, hyperplasia, hypertrophy, and dysplasia of hepatocytes may indicate preneoplastic changes and may be a useful diagnostic sign. Indeed, in one patient of this series with groups of HB_sAg-positive hepatocytes with a mean nuclear diameter of 8.01 μ and an N/C ratio of 4.02 HCC developed 4 years later. Previous attempts to demonstrate by immunohistochemical techniques preferential expression of phenotypic markers such as α-fetoprotein, carcinoembryonic antigen, or α_1-antitrypsin in dysplastic or HB_sAg-positive hepatocytes were unsuccessful, although these antigens were detected in the majority of livers with HCC.[28,29] Morphologic evidence of hypertrophy and hyperplasia of HB_sAg containing hepatocytes may be a more reliable indicator of preneoplasia than the phenotypic marker antigens currently available. Further studies employing more sophisticated stereologic methods and *in situ* hybridization with sensitive labeled HBV DNA probes are needed to support this suggestion.

References

1. Szmuness W: Hepatocellular carcinoma and the hepatitis B virus: Evidence for a causal association. Prog Med Virol 1978, 24:40-69
2. Gerber MA, Thung SN, Popper H: Viral hepatitis and hepatocellular carcinoma, Clinical and Laboratory Science of Viral Hepatitis. Edited by F Deinhardt, J Deinhardt. New York, Marcel Dekker, 1983, pp 327-341
3. Farber E: Chemical carcinogenesis: A biologic perspective. Am J Pathol 1982, 106:271-296
4. Sell S, Leffert HL: An evaluation of cellular lineages in the pathogenesis of experimental hepatocellular carcinoma. Hepatology 1982, 2:77-86
5. Anthony PP, Vogel CL, Barker LF: Liver cell dysplasia: A premalignant condition. J Clin Pathol 1973, 26:217-223
6. Peters RL: Pathology of hepatocellular carcinoma, Hepatocellular Carcinoma. Edited by K Okuda, RL Peters. New York, London, Sydney, Toronto, John Wiley & Sons, 1976, 107-168
7. Anthony PP: Precursor lesions for liver cancer in humans. Cancer Res 1976, 36:2579-2583
8. Nakashima T, Okuda K, Kojiro M, Jimi A, Yamaguchi R, Sakamoto K, Ikari T: Pathology of hepatocellular carcinoma in Japan. Cancer 1983, 51:863-877
9. Ho JCI, Wu PC, Mak TK: Liver cell dysplasia in association with hepatocellular carcinoma, cirrhosis and hepatitis B surface antigen in Hong Kong. Int J Cancer 1981, 28:571-574
10. Cohen C, Berson SD, Geddes EW: Liver cell dysplasia: Association with hepatocellular carcinoma, cirrhosis

and hepatitis B antigen carrier status. Cancer 1979, 44: 1671–1676
11. Gerber MA, Thung SN: The localization of hepatitis viruses in tissues. Int Rev Exp Pathol 1979, 29:49–76
12. Ranek L, Keiding N, Jensen ST: A morphometric study of normal human liver cell nuclei. Acta Pathol Microbiol Scand [A] 1975, 83:467–476
13. Watanabe T, Tanaka Y: Age-related alterations in the size of human hepatocytes. Virchows Arch [Cell Pathol] 1982, 39:9–20
14. Thung SN, Gerber MA, Sarno E, Popper H: Distribution of five antigens in hepatocellular carcinoma. Lab Invest 1979, 41:101–105
15. BMDP Statistical Software (1981 Edition). Department of Biomathematics, University of California, Los Angeles, University of California Press, 1981
16. Ott L: An introduction to Statistical Methods and Data Analysis. Duxbury Press, 1977, pp 384–388
17. Jagoe R, Sowter C, Dandy S, Slavin G: Morphometric study of liver cell nuclei in hepatomas using an interaction computer technique: 1. Nuclear size and shape. J Clin Pathol 1982, 35:1057–1062
18. Orrego H, Blendis LM, Crossley IR, Medline A, MacDonald A, Ritchie S, Israel Y: Correlation of intrahepatic pressure with collagen in the Disse space and hepatomegaly in humans and in the rat. Gastroenterology 1981, 80:546–556
19. Blendis LM, Orrego H, Crossley IR, Blake JE, Medline A, Israel Y: The role of hepatocyte enlargement in hepatic pressure in cirrhotic and noncirrhotic alcoholic liver disease. Hepatology 1982, 2:539–546
20. Israel Y, Orrego H, Colman GC, Britton RS: Alcohol-induced hepatomegaly: Pathogenesis and role in the production of portal hypertension. Fed Proc 1982, 41: 2472–2477
21. Bianchi L, Zimmerli-Ning M, Gudat F: Viral hepatitis, Pathology of the Liver. Edited by RNM MacSween, PP Anthony, PJ Scheuer. Edinburgh, Churchill Livingstone, 1979, pp 164–191
22. Gerber MA, Hadziyannis S, Vissoulis C, Schaffner F, Paronetto F, Popper H: Electron microscopy and immunoelectron microscopy of cytoplasmic hepatitis B antigen in hepatocytes. Am J Pathol 1974, 75:489–495
23. Brechot C, Pourcel C, Hadchouel M, Dejean A, Louise A, Scotto J, Tiollais P: State of hepatitis B virus DNA in liver diseases. Hepatology 1982, 2:27S–34S
24. Shafritz DA: Hepatitis B Virus DNA molecules in the liver of hepatitis B surface antigen carriers: Mechanistic considerations in the pathogenesis of hepatocellular carcinoma. Hepatology 1982, 2:35S–41S
25. Chen DS, Hoyer BH, Nelson J, Purcell RH, Gerin JL: Detection and properties of hepatitis B viral DNA in liver tissues from patients with hepatocellular carcinoma. Hepatology 1982, 2:42S–46S
26. Kam W, Rall LB, Smuckler EA, Schmid R, Rutter WJ: Hepatitis B viral DNA in liver and serum of asymptomatic carriers. Proc Natl Acad Sci USA 1982, 79:7522–7526
27. Watanabe S, Okita K, Harada T, Kodama T, Numa Y, Takemoto T, Takahashi T: Morphologic studies of the liver cell dysplasia. Cancer 1983, 51:2197–2205
28. Gerber MA, Thung SN, Popper H: Localization of fetal antigens in relation to HBsAG in hepatocytes, Virus and the Liver. Edited by L Bianchi, W Gerok, K Sickinger, GA Stalder. Lancaster, MTP Press Limited, 1980, pp 205–208
29. Sakamoto SI, Kawaharada M, Yachi A, Wada T: Carcinoembryonic Proteins: Chemistry, Biology, Clinical Applications. Edited by FG Lehmann. New York, Elsevier/North Holland Biomedical Press, 1979

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