Clonal Growth of Hepatitis B Virus-integrated Hepatocytes in Cirrhotic Liver Nodules

Hideaki Yasui, Okio Hino, Keiko Ohtake, Rikuo Machinami, and Tomoyuki Kitagawa

Departments of Pathology [H. Y., K. O., T. K.] and Experimental Pathology [O. H.] of the Cancer Institute, Tokyo, 1-37-1, Kami-ikebukuro, Toshima-ku, Tokyo 170, and the Department of Pathology [H. Y., R. M.], Faculty of Medicine, University of Tokyo School of Medicine, 7-3-1, Hongo, Bunkyo-ku Tokyo 113, Japan

ABSTRACT

A total of 83 cirrhotic nodules (pseudolobules) individually collected from 11 cirrhotic livers of hepatitis B virus carrier patient were analyzed for the frequency and mode of hepatitis B virus integration as well as histological features. Southern blot analysis disclosed discrete bands at higher molecular weight region in 26 of 83 nodules (31.3%), indicating a clonal growth of hepatocytes with viral integration. Considerable variation (0–75%) existed in the positive rates for discrete bands in nodules among livers. Molecular cloning revealed the sequence flanking an integrated viral sequence to be host DNA and thus confirmed true integration.

Histological analysis, however, did not reveal any neoplastic-appearing foci of growth within nodules, despite the fact that the detection sensitivity would predict clones of more than 10^6 cells to give rise to clonal integration patterns on Southern blot analysis. The question of whether clonal expansion of hepatocytes reflects any viral integration-associated growth advantage and/or a preneoplastic condition awaits future studies.

INTRODUCTION

HCCs developing in chronic hepatitis B virus (HBV) carriers frequently show HBV DNA integration (1–4). Since carcinomas are the result of clonal cell proliferation (5), HBV integration would occur before or at the initial stage of neoplastic proliferation. Liver cirrhosis is frequently encountered under HCC and although the cirrhotic nodules have commonly been considered as polyclonal regenerative lesions, recent analyses of HBV DNA integration in hepatocytes have questioned this conclusion. Thus, there have been several studies demonstrating clonal integration patterns of HBV DNA in noncancerous liver tissues (4, 6–10). In all but one of these studies, however, noncancerous tissue were only collected in an arbitrary manner, mainly by needle biopsy, and the histological features of nodules with viral integration were not detailed.

In the present study cirrhotic nodules were therefore examined individually in terms of both HBV DNA integration and histological character. Since the simple demonstration of a discrete band in the high molecular weight region by Southern blot analysis would not completely rule out the possible existence of extrachromosomal viral DNA in the complex form of oligomers (11) which might present pseudopositive signals, we performed molecular cloning for a discrete HBV DNA band to confirm the real integration of viral DNA into host DNA. This paper documents the results.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBs-Ag, hepatitis B virus surface antigen; HBe-Ag, hepatitis B virus e antigen.

MATERIALS AND METHODS

Cirrhotic nodules were obtained from 11 cirrhotic livers of serum HBs-Ag-positive Japanese patients, 9 with HCC, 7 at autopsy, and 4 at surgery. Relatively large cirrhotic nodules measuring 5–10 mm in diameter were selected and sampled from the cut surfaces, counterpart sections being fixed in 10% formalin for routine histological examination.

Serological Examination. Serum HBs-Ag was measured by reverse passive hemagglutination, anti-HBs by passive hemagglutination, and HBe-Ag and anti-HBe by enzyme immunoassay.

DNA Preparation and Southern Blot Analysis. Total cellular DNA was extracted from individual cirrhotic nodules and HCC nodules. Southern blot analysis was performed according to the method previously reported (3). In brief, purified DNA (10 µg) was digested or undigested with a restriction enzyme HindIII, which does not cleave HBV DNA. Each digested and undigested DNA sample was electrophoresed on a 1% agarose gel, transferred onto nitrocellulose filters, and hybridized with a cloned 32P-labeled HBV DNA probe covering the entire virus genome. The possibility of bacterial contamination, especially in autopsy materials (3), was ruled out by hybridizing the filters with 32P-labeled pBR322 DNA.

Histological Study. Hematoxylin and eosin-stained histological specimens were prepared by using another paired surface of the cirrhotic nodules sampled. The histological features were studied blind by two pathologists independently, that is without information of HBV integration. At the same time the absence of metastatic HCC cells was confirmed.

Molecular Cloning. The total cellular DNA was extracted from cirrhotic nodule A in Case 8 and a restriction endonuclease fragment containing integrated HBV DNA was identified by Southern blot analysis, using a cloned 32P-labeled HBV DNA probe (Fig. 6). HindIII-digested DNA was size fractionated and a specific sized fraction was ligated into the HindIII site of Charomid 9-36 (12) (provided by Dr. Saito, NIH, Tokyo, Japan) and packaged in vitro by using a gene transfer kit (Gigapack II Gold, Stratagene, Inc.). The recombinant Charomid library was screened with a purified 32P-labeled HBV DNA probe by colony hybridization (13), and a positive clone was designated C-8.

RESULTS

The clinical and serological features of the 11 patients are summarized in Table 1. Serologically, 8 cases were HBe-Ag positive and anti-HBe negative, 2 cases were HBe-Ag negative and anti-HBe positive, and 1 case was HBe-Ag and anti-HBe negative.

A representative autoradiograph of the results of Southern blot analysis is shown in Fig. 1. 6 cirrhotic nodules (Lanes a to f) collected from Case 11 being analyzed. Undigested DNAs formed smears in the high molecular weight region. After digestion with HindIII, discrete bands representing clonal integration patterns were observed for 4 nodules (Lanes a, b, d, and f). These bands were various in size and were all larger than that of HBV DNA (3.2 kilobases). In Lanes c and e, weak smears were demonstrated in the high molecular weight region for the undigested DNA, but no discrete bands were produced after digestion with HindIII, indicating random integration of HBV DNA. Since DNAs derived from livers free from HBV...
infection did not present such smears under our hybridization and washing conditions, the possibility of looking at nonspecific cross-hybridization from background noise could be ruled out. In Lane a, in both HindIII and undigested cases, a diffuse smear was also observed below the HBV DNA size, indicating the presence of free HBV DNA.

As summarized in Table 2, 26 of 83 cirrhotic nodules (31.3%) showed discrete bands after HindIII digestion. Single discrete bands were found in 20 nodules and 2 were evident in 6 nodules, but no cases of 3 or more were apparent. The incidence of spontaneous integration showed discrete bands after HindIII digestion. Single discrete bands ranging from none of 10 nodules (0%) in Case 5 to 6 of 8 nodules (75.0%) in Case 4. In the 9 HCC-complicated cases, the sizes of discrete bands from HCC nodules differed from the sizes of any bands from cirrhotic nodules (data not shown).

Representative histological pictures of nodules are presented in Figs. 2 and 3. Histopathological examination failed to reveal any differences between cirrhotic nodules positive for discrete bands of HBV DNA and negative ones. In both types, portal triads were often observed and hepatocytes were arranged to form cords one or two cells thick, and in some areas several cells thick. In none of the nodules could intranodular foci be distinguished from surrounding cells, in terms of either cytological or structural features. Cellularity and nucleus/cytoplasm ratio of the component cells were also essentially identical between HBV integration-positive and -negative nodules. Occasionally various degrees of so-called liver cell dysplasia (14) and/or focal fatty metamorphosis were observed in both types of nodules.

The cloned DNA from the cirrhotic nodule A in Case 8 (C-8) had a length of 7.4 kilobases, equal to the size of the genomic DNA observed in Southern blot analysis. As shown in Fig. 4, restriction enzyme mapping disclosed that the viral DNA retained the S and part of the X region of HBV DNA. Restriction enzyme mapping did not reveal any major rearrangement. A restriction endonuclease fragment (HindIII-Xhol fragment) containing unique cellular DNA was isolated from the flanking putative cellular sequence of C-8. Southern blot analysis with the use of the DNA fragment as a probe, after digestion by HindIII or EcoRI, generated the same discrete bands sized 4.6 and 5.2 kilobases, respectively, in cirrhotic nodules A, B, and HCC of Case 8 (Fig. 5). In cirrhotic nodule A, additional bands sized 7.4 and 8.5 kilobases were observed. These two bands were exactly the same as observed when hybridized with HBV DNA probe (Fig. 6). This result thus indicated that the flanking sequence originated from the host DNA and that therefore true HBV DNA integration into the host chromosome had occurred.

**DISCUSSION**

The present investigation revealed clonal integration patterns of HBV DNA in cirrhotic nodules at a relatively high (31.3% on average) incidence but with considerable variability (0 to 75%) between patient cases. We also confirmed, in one of the samples, that the clonal integration pattern actually reflects true viral integration in host DNA and that we were therefore looking at clonal expansion of the cells. Conventional histological observation, however, failed to reveal any neoplastic-appearing hepatocytic growth foci in clonal integration-positive cirrhotic nodules.

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**Table 2 Occurrence of discrete bands in cirrhotic nodules**

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<th>No. of nodules with discrete bands</th>
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<td>6</td>
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**Table 1 Clinical and serological status of patients**

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<th>Age (yr)</th>
<th>Sex</th>
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<th>Serum Anti-HBs</th>
<th>Serum HBeAg</th>
<th>Serum Anti-HBe</th>
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**Fig. 1.** Representative autoradiograph illustrating the results of Southern blot analysis of different cirrhotic nodules (Lanes a to f) in Case 11. Molecular weight markers are shown bilaterally. kb, kilobases.

**Fig. 2.** Histological appearance of representative cirrhotic nodules at low magnification. The largest one measuring 10 mm in diameter was clonal integration pattern positive. Note presence of portal triads and absence of any neoplastic-appearing focus within the nodules. H & E, × 6.
CLONAL GROWTH OF HBV-INTEGRATED HEPATOCYTES

The connection between the clonal integration rate in cirrhotic nodules and the presence or absence of HCC. Nor were unique gross features evident in cirrhotic livers with high clonal integration rates in comparison with others.

Our investigation is the first in which the histological features of individual nodules were precisely studied in connection with findings for HBV integration. The minimum quantity of HBV DNA required for detection by Southern blot analysis is about 0.1 to 1.0 pg, which roughly corresponds to $10^5$ to $10^6$ HBV DNA copies. Therefore, assuming that each cell contains one copy of HBV DNA integrated in the same host DNA site, at least $10^5$ to $10^6$ clonally expanded cells should exist in order to give rise to discrete bands on Southern blotting. Such numbers of hepatocytes, assuming focal proliferation, should form a focus measuring at least a few mm in diameter.

In experimental hepatocarcinogenesis in rodents, putative precancerous lesions designated as altered cell foci are well known to be comprised of clonally expanded hepatocytes (15, 16). These lesions have distinct enzyme histochemical features which make them clearly discernible from surrounding tissue (17). Even in hematoxylin and eosin preparations, most of them, including those less than 1 mm in size, are recognizable on the basis of cytoplasmic features (clear, basophilic, or eosinophilic) or nuclear irregularities with prominent nucleoli (17, 18). In the human liver, the adenomatous hyperplasias which have been assumed to be precancerous lesions (19) are usually grossly identifiable lesions larger than cirrhotic nodules and microscopically clearly distinguishable from surrounding tissue by their structural and cellular atypia, even when only of low

Previously, several investigators have reported clonal integration patterns of HBV in noncancerous portions of chronically diseased liver (4, 6–10). Most of them analyzed samples arbitrarily obtained by needle biopsy and the positive rates for clonal integration were recorded by case: 5 of 11 (4), 6 of 6 (7), and 4 of 18 (9). Aoki and Robinson (10), on the other hand, examined clonal integration rates of HBV in 3 cirrhotic livers by collecting and analyzing individual nodules. They detected clonal integration patterns in 5 of 20, 1 of 57, and 0 of 52 of the nodules, respectively, after digestion with HindIII and EcoRI. Our results document particularly clearly the remarkable differences between individual cases. The underlying reasons for the differences remain unclear and there was no apparent con-

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Fig. 3. Histological appearance of cirrhotic nodules showing hepatocytes arranged in cords, several cells in thickness, and with fatty change (a), or with clear cell change (b). H & E, × 50 and × 40, respectively.

Fig. 4. Restriction endonuclease map of the integrated HBV DNA sequence in cloned DNA (C-8). The arrows show the locations of the two HBV gene regions. S, S gene; X, X gene. ■, integrated HBV DNA; □, flanking cellular DNA. kb, kilobase.

Fig. 5. Hybridization patterns of two different cirrhotic nodules A and B, and one hepatocellular carcinoma using the flanking cellular DNA probe in Case 8. H, HindIII digested; E, EcoRI digested; LC, liver cirrhosis; HCC, hepatocellular carcinoma. Molecular weight markers are shown on the right. kb, kilobases.
Clonal integration-positive and -negative nodules. In all these cases, it is important to note that weight markers are shown on the right, kb, kilobases.

Concerning the third possibility, evidence for viral integration-related growth advantage has been demonstrated (24-28). Integration or not, would clearly be of importance for both initiation and/or multistep progression of carcinogenesis. The present investigation thus calls for more attention to the existence of clonally expanding hepatocyte populations in cirrhotic livers and clarification of their nature.

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REFERENCES


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