Hepatitis B x Antigen in Hepatitis B Virus Carrier Patients with Liver Cancer

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ABSTRACT

Formalin-fixed, paraffin-embedded specimens from 110 cases of primary hepatocellular carcinoma were stained for hepatitis B x antigen (HBxAg), hepatitis B surface antigen (HBsAg), and hepatitis B core antigen (HBcAg). Eighty-four percent of these patients were HBxAg positive in their tumor cells. Among the 110 cases studied, 80 had adjacent nontumorous tissue in the same block, and 65 of these nontumorous liver tissues were positive for HBxAg (81%). HBsAg was positive in 19% of cases within tumor tissue and 61% in surrounding nontumorous tissue. HBcAg was positive in 11% of cases within tumor tissue and 26% in surrounding nontumorous tissue. These findings show that HBxAg is a common marker in the liver of patients with hepatitis B virus (HBV)-associated primary hepatocellular carcinoma and that it is closely associated with tumor cells in these individuals. In addition, the finding of HBxAg in the absence of detectable HBsAg and HBcAg in the liver tissues of many HBsAg carriers suggests that HBxAg could be expressed independent of HBV replication and implies that the synthesis of this antigen may be directed from integrated HBV DNA templates. The finding of HBxAg in the nucleus of hepatocytes from primary hepatocellular carcinoma patients with dysplasia, combined with the known trans-activating properties of HBxAg, implies that HBxAg plays one or more important roles in hepatocarcinogenesis. The finding of HBxAg in bile duct epithelium and cholangiocarcinoma tissues is compatible with the hypothesis that HBV may contribute to this other primary tumor type in the liver. Together, these results further implicate HBxAg in the pathogenesis of primary liver cancers.

INTRODUCTION

It is well established that the HBV chronic carrier state and the presence of progressive liver disease are risk factors which play central roles in the development of PHC (1-5). HBV infection may result in chronic hepatitis, followed by liver cirrhosis (2, 3, 5, 6). Most PHC occurs in individuals with cirrhosis (80%) or CAH (10-15%) (3). PHC is closely associated with cirrhosis, particularly macronodular cirrhosis, which is thought to play an important role in pathogenesis. However, the contribution of HBV to the pathogenesis of PHC remains to be elucidated (1, 5-8).

The prevalence, distribution, and possible significance of HBsAg and HBcAg in liver tissues from patients with chronic liver disease and PHC have been well documented (9-12). However, few studies focused on the characteristics of HBxAg in chronic infection have been reported. Evidence that HBxAg with trans-activating properties could be produced from a viral template integrated into the host genome during chronic infection (13), that HBxAg could trans-activate oncogenes such as c-myc (14), and that HBxAg could "transform" non-tumorigenic cell lines into lines capable of growing as tumors in nude mice (14-16) support the hypothesis that HBxAg may play an important role in the pathogenesis of HBV-associated chronic liver disease, including PHC. Previous results from this laboratory, documenting the presence of HBxAg in dysplastic hepatocytes, combined with the predominantly nuclear distribution of HBxAg in patients with cirrhosis and dysplasia (17), also support this hypothesis. Hence, this study was carried out to test whether HBxAg was closely associated with tumor cells in patients with PHC.

MATERIALS AND METHODS

One hundred ten cases of surgically resected specimens from HBV carriers with PHC were obtained from the Pathology Laboratory of the First Teaching Hospital at the Fourth Military Medical University, Xi'an, People's Republic of China. For comparative studies, additional HBV-infected patients with chronic liver diseases but no evidence of PHC were also used. The characteristics of these latter populations of patients have been described earlier (17). Tissue staining for HBsAg, HBcAg, and HBxAg was carried out by a standard immunoperoxidase procedure (17) using polyclonal anti-HBx raised from a synthetic peptide antigen (spanning HBxAg residues 100-114) (18) or from a recombinant DNA-derived full length HBxAg polypeptide made from an expression vector in Escherichia coli (19). The recombinant HBxAg and corresponding antibody were kindly provided by Dr. X. Ma, Institute of Basic Medical Sciences, AMMS, Beijing, People's Republic of China.

Specificity controls were carried out as described (17). Fifteen uninfected human liver samples and several tissue types from other organs (spleen, lymph node, muscle, nerve, and gallbladder), were tested with immune sera. Preimmune and normal rabbit sera were tested with positive liver sections. The HBxAg synthetic peptide used to raise immune sera was tested for blocking in tissues staining positive with the anti-HBx reagent. Recombinant DNA-derived HBxAg polypeptide (19) was used to block the reaction of antibodies raised against this recombinant polypeptide with positive tissues (kindly supplied by Dr. X. Ma). Liver powder made from uninfected human liver tissues (20) was used to absorb the primary antibodies prior to staining. Anti-HBx reagents were tested by Western blotting with E. coli lysate from bacteria expressing HBxAg polypeptide compared to a similar lysate from untransfected host cells. Hep G2.2.15 cells making HBV particles were tested with anti-HBx reagents as were virus-negative Hep G2 cells. Cell supernatants and extracts were also analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western blotting (18). Additional control tissues included liver containing unrelated metastatic cancers.

Among the 110 carriers studied, there were three different tumor types. One hundred two patients had PHC, 3 had HChC, and another 5 had ChC. Eighty of these PHC patients had nontumorous liver in the same histological sections. The diagnosis of ChC and HChC was confirmed by Dr. Zachary Goodman, Hepatic Pathology Branch, Armed Forces Institute of Pathology. PHC was divided into three grades of differentiation, based upon morphological criteria in each case, according to the guidelines suggested by the WHO (21) and others (22). Grade I represented differentiated PHC, grade II moderately differentiated PHC, and grade III undifferentiated PHC.

The definitions and grades of CAH and liver cell dysplasia used in these studies have been outlined earlier (17).
RESULTS

Frequency of HBxAg in Tumor and Surrounding Nontumor Tissues from PHC Patients. Among 110 HBsAg carrier cases with liver tumors, 102 had PHC, 3 had HChC, and 5 had ChC. Eighty of these patients had surrounding nontumorous tissue on the same tissue section; cirrhosis was present in 54 cases and CAH was present in the remaining 26 (Table 1). Dysplasia was present in 50 of 54 cases (93%) with liver cirrhosis and in 12 of 26 cases (46%) with CAH. HBxAg was detected in 92 of 110 patients with liver cancer (84%) within tumor cells using peptide antibodies, as described (17) (Table 1). Eighty-five of these were seen in tumor cells of PHC, 3 in HChC, and 4 in ChC. Representative results for PHC patients are presented in Fig. 1. In addition to patients with PHC, 7 of 8 HBsAg carriers with HChC or ChC were also HBxAg positive within tumor cells (Table 2; Fig. 2), suggesting that HBxAg may be a prevalent marker in these tumor types as well as in PHC. HBxAg was also detected in the nonneoplastic tissues in 70 of the 80 patients (88%) with these tissues available. Among these 70 HBxAg-positive cases, 64 had PHC, 2 had HChC, and 4 had ChC. HBxAg was detectable in 45 of 54 PHC patients with cirrhosis (83%). Forty-three of these cirrhotic patients also had dysplasia. Among the remaining 20 PHC patients with nontumorous tissue, all had CAH, 10 had dysplasia, and 19 were HBxAg positive (Table 1). HBxAg was also present in 2 ChC patients with dysplasia (Table 2). As in earlier work (17), more than 70% of patients with liver cell dysplasia were HBxAg positive. These results show that HBxAg is often present in nontumorous liver tissues adjacent to tumors. Among patients with liver tumors, there were a handful who were also HBxAg positive in bile duct epithelium (Fig. 2). In all cases, the staining in bile ducts was generally weaker than in hepatocytes. Combined with the above results demonstrating the presence of HBxAg in HChC and ChC sections, these results are consistent with an association between HBV and bile duct associated and/or derived cells.

Many of the above sections were also stained with antibodies raised against full-length HBxAg polypeptide made from a recombinant DNA expression vector in E. coli (19). The results were very similar to those obtained with the X peptide antibodies above, with the exception that the intensity of staining was sometimes lower using antibodies against the recombinant protein compared to the peptide antisera (data not shown). Specificity was demonstrated by showing that staining was absent from HBxAg-positive sections in which preimmune rabbit serum was used in place of anti-HBx as primary antibody (Fig. 1) and by showing that staining could be completely blocked by preincubation of the antisera with the immunizing antigen (HBxAg synthetic peptide or recombinant DNA-generated HBxAg) prior to assay (Fig. 2). Controls outlined in "Materials and Methods" have also been carried out with these antisera (17) which confirm the specificity of antigen staining. These results, then, support the conclusion that HBxAg is present in both tumor and nontumorous liver cells from chronic carriers.

Cellular and Tissue Distribution of HBxAg. The percentage of HBxAg positive tumor cells varied among the different liver cancer patients. Among the 80 patients with paired tumors and nontumorous samples for evaluation, HBxAg was undetectable in tumor cells from 13 cases, was present in less than 30% of tumor cells in 29 cases, and was detectable in more than 30% of tumor cells in the remaining cases (Table 3). In most cases, the intensity of HBxAg staining increased with the percentage of HBxAg-positive cells in liver sections, so that the patients with the largest percentage of HBxAg-positive tumor cells often had the most intense staining in those cells (usually moderate (+) to dark (+++) staining). Similar analysis of surrounding nontumorous tissues in these patients showed 4 cases without detectable HBxAg. In these cases, both the tumorous and nontumorous tissues were negative for HBsAg and HBCAg. HBxAg was present in less than 30% of nontumorous cells from an additional 7 patients and was present in more than 30% of nontumorous liver cells from the remaining individuals (Table 3). Again, the intensity of staining correlated with the percentage of HBxAg-positive cells in these patients. When the frequency (percentage of HBxAg-positive cells) and intensity of HBxAg staining were compared in tumorous and nontumorous tissues among patients with CAH, cirrhosis, and/or dysplasia, both frequency and intensity of HBxAg staining was greater in nontumorous compared to tumor tissue. Hence, the proportion of patients with intense HBxAg staining was greater in the nontumorous compared to tumorous cells.

When the frequency and intensity of HBxAg staining were examined in patients without PHC and compared to the results of this study (Ref. 17; Table 4), it was observed that a higher proportion of PHC-negative patients with CAH or cirrhosis were also HBxAg positive in more than 30% of their hepatocytes compared to patients with chronic persistent hepatitis and in tumor cells among patients with differentiated (grades I and II) or undifferentiated (grade III) PHC. An analogous observation was made when the parameter of HBxAg staining intensity was evaluated (Table 4). For example, among patients with chronic persistent hepatitis and PHC, the intensity of HBxAg staining in tissue was negative to mild (− to +). Combined with the above finding that more than 30% of liver cells examined in sections from most patients with CAH or cirrhosis were positive for HBxAg and the staining in these cases was moderate to dark (++ to +++), these results suggest that the number of cells expressing HBxAg and the intensity of staining increases in carriers with progressive liver disease preceding the appearance of PHC.

The subcellular localization of HBxAg in the cells of tumorous and nontumorous tissues is summarized in Table 3. HBxAg was localized mainly in the cytoplasm of the liver and tumor cells, and less frequently, on the cell membrane and/or in the nucleus. Both membranous and nuclear HBxAg were observed in tumorous as well as nontumorous cells. A striking feature is the relatively high frequency of HBxAg in the nuclei of hepatocytes in 18 of 50 cirrhotic patients with dysplasia (36%). Nuclear HBxAg was also observed in more than 50% of cirrhotic patients with dysplasia who had no evidence of PHC (Ref. 17; Table 4). In contrast, PHC patients with cirrhosis but...
no dysplasia did not have evidence of nuclear HBxAg, while more than 40% of PHC-negative patients with cirrhosis had evidence of HBxAg (Tables 3 and 4). While these results are consistent with the conclusion that the patterns of nuclear HBxAg are different among patients with CAH or cirrhosis, depending upon whether they also had PHC, the differences may be due to the fact that there are only 4 cirrhotic patients with PHC compared to 33 without PHC (17) and that comparison of such small numbers is not enough to illustrate the true frequencies. Hence, the finding of nuclear HBxAg in many patients with dysplasia is consistent with the conclusion that nuclear HBxAg plays an important role in pathogenesis of chronic infection leading up to PHC.

Among PHC cases, membranous HBxAg was detected in 10% of patients with cirrhosis and dysplasia, but in none of the individuals with CAH (Table 3). In contrast, 30–40% of the patients with chronic hepatitis and cirrhosis but no PHC were positive for HBxAg on their hepatocyte membranes (Table 4). Hence, there is an apparent difference in the proportion of patients with membranous HBxAg from those with and without PHC. These findings are also consistent with the conclusion that membranous HBxAg may play a prominent role in the process leading up to PHC (7).

Both localized and diffuse staining was observed for HBxAg in PHC cells (Fig. 1; Table 3). Localized staining was the dominant pattern in the large majority of patients while diffuse staining was present in only 10–20% of PHC tissues. Sparse staining was rare in PHC tissue, as it was in nontumorous liver samples (17). Further, the tissue distribution of HBxAg staining did not correlate with HBsAg or HBeAg, nor was it associated with any type of chronic liver disease. Similar patterns of HBxAg were observed in tumorous and nontumorous tissues from patients with HChC and ChC.

The patterns of HBxAg staining were also examined in differentiated PHC (grades I and II) and compared to the patterns in undifferentiated tumors (grade III) (Table 4). Seventy-five % of patients with differentiated PHC were HBxAg positive in more than 30% of their cells, which was similar to...
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that observed in the livers of chronic HBV carriers without PHC. In contrast, only 40% of patients with undifferentiated PHC were HBxAg positive in more than 30% of their tumor cells. When the intensity of HBxAg staining is considered, moderate and intense staining was observed in most patients independent of the grade of PHC. Similar intensity profiles were also observed among HBV carriers without evidence of PHC (Table 4). The cellular staining patterns for HBxAg showed a dominant cytoplasmic distribution in nearly all patients, independent of the grade of PHC, and in patients without PHC. Membranous HBxAg was detected in 17% of patients with differentiated PHC but was 30–40% in patients with undifferentiated PHC and without PHC (Table 4). Membranous HBxAg was lowest among PHC patients with adjacent nontumorous tissue. Nuclear staining was lower in the tumor cells of PHC patients (24% for grades I and II; 19% for grade III) than in patients with cirrhosis (50%) or in cirrhotic patients with dysplasia (54%). Finally, the distribution of HBxAg staining in tissue was mostly localized and, to a lesser extent, diffuse in patients from all categories of patients (Table 4).

Patterns of HBsAg and HBcAg Expression in Patients with Liver Cancer in Relation to HBxAg. Fifteen % of patients with liver cancer were HBsAg positive in tumor cells. Seventeen of

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Diagnosis in nontumorous tissue</th>
<th>Serum HBsAg status</th>
<th>HBV antigens in liver sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>HBcAg HBxAg HBsAg HBcAg HBxAg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHC CAH</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CHC CAH</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>CHC CAH</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* These patients have dysplasia in nontumorous liver tissue.

Fig. 2. Detection of HBxAg in bile duct cells and primary liver tumors with ductular morphology. A, HBxAg in bile duct epithelial cells from a HBV carrier with chronic hepatitis. B, control of same patient as in A in which peptide antibody was preincubated for 1 h at 37°C with an excess of immunizing synthetic peptide prior to staining. C, HBxAg in tumor cells from a patient with cholangiocarcinoma. Staining was carried out using antibodies raised against recombinant DNA-generated HBxAg. M, 17,000 polypeptide made in E. coli. HBxAg-positive cells are on the lower half. D, control of the same patient as in C, in which primary antibody was preincubated for 1 h at 37°C with an excess of immunizing M, 17,000 HBxAg polypeptide prior to staining. E, staining of a malignant fibrous histiocytoma of the liver with anti-HBx peptide antibodies from a patient without evidence of HBV infection.
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Table 3 Characteristics of HBxAg in tumor and surrounding nontumorous samples from Chinese HBV carriers with liver tumors

<table>
<thead>
<tr>
<th>HBxAg positive</th>
<th>Frequency in cells</th>
<th>Intensity</th>
<th>Distribution in cells</th>
<th>Distribution in tissue</th>
<th>HBsAg positive (no.)</th>
<th>HBcAg positive (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>&lt;30%</td>
<td>30-70%</td>
<td>&gt;70%</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PHC/CAH (n = 22)</td>
<td>Tumorous</td>
<td>17</td>
<td>4</td>
<td>11</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Nontumorous</td>
<td>19</td>
<td>1</td>
<td>15</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>PHC/cirrhosis/ + dysplasia (n = 54)</td>
<td>Tumorous</td>
<td>45</td>
<td>19</td>
<td>22</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Nontumorous</td>
<td>51</td>
<td>5</td>
<td>18</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>HBC (n = 3)</td>
<td>Tumorous</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nontumorous</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ChC (n = 5)</td>
<td>Tumorous</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Nontumorous</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Samples presented here include those from patients in which both tumorous and nontumorous samples were available.
+ The cutoff values for reporting the frequency of HBxAg positive cells were arbitrary.
C, cytoplasmic; D, diffuse; L, localized; M, membranous; N, nuclear; S, scattered.
Numbers in parentheses, number tested in each group.
All patients in these categories had dysplasia.

Table 4 Characteristics of HBxAg in liver samples from Chinese HBV carriers with different types of chronic liver disease

<table>
<thead>
<tr>
<th>HBxAg positive</th>
<th>Frequency in cells</th>
<th>Intensity</th>
<th>Distribution in cells</th>
<th>Distribution in tissue</th>
<th>HBsAg positive (no.)</th>
<th>HBcAg positive (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>&lt;30%</td>
<td>30-70%</td>
<td>&gt;70%</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CPH</td>
<td>28</td>
<td>18</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CAH</td>
<td>82</td>
<td>17</td>
<td>14</td>
<td>40</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>33</td>
<td>30</td>
<td>3</td>
<td>21</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Cirrhosis/ + dysplasia</td>
<td>65</td>
<td>65</td>
<td>10</td>
<td>38</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>PHC I, II*</td>
<td>66</td>
<td>58</td>
<td>14</td>
<td>37</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Nontumorous</td>
<td>48</td>
<td>47</td>
<td>3</td>
<td>23</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>PHC III*</td>
<td>36</td>
<td>27</td>
<td>16</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Nontumorous</td>
<td>26</td>
<td>25</td>
<td>2</td>
<td>12</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

* M, membranous; C, cytoplasmic; N, nuclear; S, scattered; L, localized; D, diffuse; CPH, chronic persistent hepatitis; PHC I, II, (grade I) differentiated and (grade II) moderately differentiated PHC; PHC III, (grade III) undifferentiated PHC.* Includes PHC patients with and without surrounding nontumorous tissue for evaluation.

19 HBsAg positive patients had PHC (3 grade I, 11 grade II, and 3 grade III) while 1 patient had HChC and another patient had ChC (Tables 2–4). HBsAg was more prevalent in tumor cells from patients with differentiated liver cell carcinomas (grades I and II) compared to undifferentiated tumors (grade III). Among the 80 cases where hepatic tissue surrounding tumor nodules was available, 61% of the patients were HBsAg-positive in nontumorous tissue. Among the 47 HBsAg-positive cases with PHC, 34 had liver cirrhosis and 13 had chronic hepatitis. The HBsAg-positive patient with HChC and the other with ChC both had CAH (Table 2). Eight patients who were HBsAg positive in their tumor cells also had dysplasia. HBsAg staining in tumors and nontumorous tissues from the same case was found in 14 cases (1 grade I, 10 grade II, and 3 grade III). The proportion of patients with HBsAg in surrounding nontumorous tissues was similar regardless of the tumor grade. The patterns of HBsAg staining could be described as the diffuse, membranous, and/or inclusion types in both tumorous and nontumorous tissues. The last type was observed to be the most frequent; multiple types of staining were observed in some cases. HBsAg was detected in 2 cases of PHC in the nuclei and nucleoli (data not shown).

Eleven % of patients with liver tumors were HBCaAg positive in their tumor cells. Among patients with PHC, HBCaAg was observed in the surrounding tissues from 20 cases; 15 with liver cirrhosis (including 14 with dysplasia), and 5 with chronic hepatitis (including 2 with dysplasia). A single patient with ChC was also HBCaAg positive in surrounding nontumorous tissue (Table 3). As with HBsAg, HBCaAg was more prevalent in tumor cells from patients with differentiated liver cell carcinomas compared to undifferentiated tumors (Table 4). HBCaAg-positive cells appeared in both tumorous and nontumorous tissues in 5 PHC patients. HBCaAg was demonstrated in the nuclei and nucleoli, in the cytoplasm, and/or on the cell membrane (data not shown). Cytoplasmic HBCaAg staining was observed in tumor cells, while nuclear HBCaAg staining was dominant in nontumorous tissue. As with HBsAg, the proportion of patients with HBCaAg in surrounding nontumorous tissues was similar regardless of the tumor grade.

Among the 92 HBxAg positive tumors, 23% were associated with HBsAg and/or HBCaAg in tumor cells; 6 of these cases were associated with both antigens, 15 with only HBsAg, and 6 with only HBCaAg (Tables 3 and 4). Among the 72 HBxAg positive cases of nontumorous liver tissue in patients with PHC 48 were also positive for HBsAg and/or HBCaAg. Fifteen of these samples were positive for both HBsAg and HBCaAg, 29 for HBsAg without HBCaAg, and 5 with HBCaAg but no HBsAg. Five of 7 HBxAg-positive cases of HChC and ChC had neither HBsAg nor HBCaAg in tumor tissue (Table 2). Two of the patients in this group who were HBsAg positive in HChC or...
ChC were also HBsAg positive in surrounding liver tissue. One of the HBsAg-positive patients was also HbcAg positive in surrounding liver tissue (Table 2). Hence, HBxAg was expressed in tumor tissue more frequently than HBsAg or HbcAg in this population.

DISCUSSION

The patterns of HBxAg gene expression were determined in liver sections from 110 HBV carrier patients with liver cancer and were compared to liver HBsAg and HbcAg staining in 80 of these patients where tumor and surrounding nontumorous samples were available. HBxAg was found in both tumorous and surrounding nontumorous cells from more than 80% of these patients (Table 1), which is consistent with some observations (23), but not others (24). The differences may be due to the antibody probes used for staining and/or the populations used in the various studies. HBxAg was also found in many tumor tissue samples from HBsAg-positive carriers who had undetectable levels of HBsAg and HbcAg in tumor (Tables 2–4). Again, similar observations have been made in some (23) but not all (24) studies. The presence of HBxAg as the only hepatitis B antigen marker in both tumor and many nontumorous samples implies that the production of HBxAg may occur independent of viral replication. The discordance between HBxAg and HbcAg in these studies is consistent with the conclusion that HBxAg may be generated mainly from one or more integrated HBV DNA templates, which are commonly found in PHC nodules (13, 25, 26) and in some cases of chronic hepatitis (27).

The results from this study demonstrate a similar discordance of HBxAg with HBsAg and HbcAg in nontumorous tissues. In contrast, other studies show an apparent concordance of X antigen in liver with classical virus markers, and especially with core antigen (23, 28, 29) suggesting that X antigen expression is related to patterns of virus replication. The latter concept is further supported by the reported findings of X antigen associated with core particles in the liver (30, 31), and with X antigen associated with both hepatitis B e antigen and viral DNA in the serum (18). The differences in interpretation may be due to the differences in the template producing X antigen and the characteristics of the X antigen polypeptides made. During viral replication, studies in this laboratory have shown that X antigen polypeptides are made as X/core fusion components (30, 31), probably from supercoiled viral DNA, which become closely associated with cytoplasmic core particles. Perhaps this is why several studies have shown a close association between X antigen and viral replication (23, 28, 29). Alternatively, many patients with chronic hepatitis, and most with cirrhosis and/or liver cancer are HbcAg negative in the liver and have undetectable levels of virus replication. With little or no replicative forms of virus DNA in the liver, X antigen polypeptide(s) may be made from integrated templates as components unfused to core antigen. Under the latter circumstances, the production of X antigen polypeptides would be expected to occur independently of core particles and virus replication. The latter circumstances appear to be the case in the patients within this study, most of whom have little evidence of virus replication and probably have only integrated HBV DNA.

The presence of HBxAg in the tumor cells from the majority of patients strengthens a close association between chronic HBV infection and the appearance of liver cancer. The fact that HBxAg has also been observed in a human hepatoblastoma cell line producing HBV particles (32), that X region viral DNA has been detected in most liver tumor tissues examined (26), and that functional X polypeptide(s) have been produced from such integrated species (13, 27) not only supports the idea of HBxAg as a common antigen in patients with chronic liver disease, including liver cancer, but also implies that it has one or more roles in the initiation and/or progression of PHC. The finding of several HBxAg-negative tumors, even in patients who were positive for HBxAg in surrounding nontumorous cells, suggests that the continued expression of HBxAg may not be required for the persistence of liver cancer. Observations that liver tumor nodules from some HBV-infected individuals do not have detectable HBV DNA (8) also suggest that tumor persistence is not HBxAg dependent. However, recent reports that HBxAg expression is associated with the transformation of nontumorigenic to tumorigenic cell lines (14–16) and that the expression of HBxAg is associated with the appearance of liver tumors in transgenic mice from one laboratory (33) suggest that HBxAg may participate in one or more critical steps important to the establishment of liver cancer.

The finding of HBxAg in bile duct cells from HBV carriers (Fig. 2) suggests that liver cells other than hepatocytes may be infected with HBV. Infection of bile duct cells may be important because HBxAg staining was also observed in several bile duct cell-derived tumors, namely, HClC and ChC (Fig. 2). These results raise the possibility that HBV may be the etiological agent responsible for these other liver neoplasms in chronic HBV carriers. Further support for this possibility derives from the recent finding that the related duck hepatitis B virus was detected by immunochemical staining in bile duct epithelial cells and that bile duct proliferation is a feature of duck hepatitis B virus infection (34, 35). These observations are consistent with the conclusion that hepadnaviruses may infect bile duct epithelial cells and that such infections could result in proliferation which may be an important step in the pathogenesis of cholangiocarcinoma.

Although the mechanism by which HBxAg contributes to the pathogenesis of liver cancer remains to be determined, the widely documented trans-activating activities of HBxAg (36, 37) may play an important role. As noted previously (17) and here (Tables 3 and 4), the relatively high frequency of HBxAg staining in the nuclei of patients with dysplasia compared to patients with chronic hepatitis and PHC suggests that if alterations in host gene expression take place, these changes are most likely to occur in cirrhotic patients (with dysplasia) just prior to the appearance of liver cancer. If so, then such trans-activation may result in triggering the expression of cellular genes critical for the onset of liver cancer. Alternatively, or in addition, the putative protein kinase activity associated with HBxAg (38) may be important in altering the function of cell cycle-dependent or other growth-regulatory molecules important to hepatocellular homeostasis. These outcomes could occur independent of the continued expression of HBxAg in tumor cells once the appropriate alterations in the behavior of cells have been achieved. Hence, HBxAg may be more important to the onset rather than the persistence of liver cancer.

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