Lack of Colocalization of HBxAg and Insulin like Growth Factor II in the Livers of Patients with Chronic Hepatitis B, Cirrhosis and Hepatocellular Carcinoma

To evaluate the possibility that HBxAg is related to an enhanced expression of IGF-II, immunohistochemical staining was performed for distribution and colocalization of HBxAg and IGF-II in liver tissues from 40 chronic active hepatitis (CAH-B), 51 cirrhosis and 46 hepatocellular carcinoma (HCC) patients using polyclonal rabbit anti HBxAg raised against full length-recombinant HBxAg and monoclonal mouse anti IGF-II. HBxAg in CAH-B, cirrhosis and HCC tissues was detected in 95%, 39% and 17%, whereas IGF-II in the same tissues was seen in 0%, 92% and 100%, respectively. There was a gradual decrease in the prevalence of HBxAg expression in cirrhosis and HCC, as compared to CAH-B tissues. All of the cirrhosis and HCC samples with positive staining for HBxAg expressed IGF-II. However, 55% of cirrhosis and 100% of HCC samples without HBxAg staining also expressed IGF-II. Moreover, colocalization at neighboring sections, even in both HBxAg and IGF-II positive samples, was not regularly observed. It is concluded that HBxAg expression in CAH-B may play a role in the pathogenesis of CAH-B. Although HBxAg may be related to the expression of IGF-II in some cirrhotic and HCC tissues, IGF-II expression in a large majority of these cases may be related to other factor(s) than HBxAg.
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Key Words : *Hepatitis B antigens; Hepatitis, Chronic active; Carcinoma, Hepatocellular; Liver cirrhosis; Insulin-like growth factor II*

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INTRODUCTION

Hepatitis B virus (HBV) is a causative agent of acute and chronic hepatitis in humans, and its chronic infection is strongly associated with the development of hepatocellular carcinoma (HCC) (1). This pathogenetic pathway frequently involves the appearance of cirrhosis which could progress and develop to HCC (2). Although it has been known that liver injuries in chronic HBV infection are immune mediated (3, 4), that cytotoxic T lymphocytes are involved in the removal of infected hepatocytes (5), the specific mechanism of the HBV-induced hepatocarcinogenesis has not yet been clearly defined. Insertional mutagenesis of cellular genes by integration of HBV-DNA may sometimes be a factor in hepatocarcinogenesis (6), but consistent integration sites of host genes involved in growth control have not been found.

Recently it has been shown that hepatitis B x antigen (HBxAg) is capable of transactivating a variety of viruses (7) and host promoters (8), suggesting that HBxAg in HBV-infected hepatocytes may be involved in HBV-associated hepatocarcinogenesis. This observation seems to be supported by the recent findings that HBxAg is detectable in most liver tissue samples taken from patients with chronic hepatitis-B (9), cirrhosis (9) and HCC (10, 11). We previously reported that insulin-like growth factor II (IGF-II) was detectable in most liver tissue samples from patients with HBV-associated cirrhosis and HCC (12), suggesting that IGF-II may play a role in hepatocyte proliferation in cirrhotic nodules and in tumor cell proliferation in HCC tissues. Recently it has also been reported that IGF-II has an angiogenic effect in experimental animals (13).

Hence, this study was carried out to test whether HBxAg expression is closely associated with an enhanced expression of IGF-II in liver tissues of patients with HBV-associated chronic active hepatitis B (CAH-B), cirrhosis and HCC, by means of immunohistochemical staining.

MATERIALS AND METHODS

Patients

One hundred and thirty seven patients were studied

Table 1. Clinical characteristics in patients with chronic active hepatitis B, cirrhosis and hepatocellular carcinoma

Parameters	Findings		
	CAH-B	LC	HCC
No.	40	51	46
Gender (M:F)	26:14	38:13	38:8
Age (mean, range)	33.4	41.7	50.9
Seropositivity for :			
HBsAg	40/40 (100%)	50/51 (98%)	43/46 (93%)
HBeAg	37/40 (93%)	41/51 (80%)	31/46 (67%)
anti HBc	40/40 (100%)	51/51 (100%)	46/46 (100%)
anti HBe	0	10/51 (20%)	15/46 (31%)
anti HCV	0	0	0
AST (IU/L)	118.7±31.5	84.6±46.4	130.0±136.6
ALT (IU/L)	136.5±42.8	87.9±50.9	105.0±87.7

CAH-B : chronic active hepatitis-B, LC : liver cirrhosis, HCC : hepatocellular carcinoma

(Table 1). The patients were grouped according to the histological evaluation of liver tissues obtained by needle biopsy performed for diagnostic purposes or from tissues resected during surgery. All patients were seropositive for HBV markers but negative for anti-HCV and HCV RNA. Three groups of tissue samples were analyzed. Group 1 included 40 liver samples obtained from patients with CAH-B. The patients (26 men and 14 women, ranging in age from 18 to 43 years : mean age, 33.4), had a elevated serum levels of transaminase activity for at least 6 months and a diagnosis of CAH-B on liver biopsy in which 17 samples were mild degree of CAH and 23 were moderate degrees of CAH. Group 2 consisted of 51 samples from patients with cirrhosis. All these cases (38 men and 13 women, ranging in age from 37 to 51 years : mean age, 41.7) had compensatory cirrhosis as evaluated by endoscopic, ultrasonographic examination, and technetium-99m-sulfur colloid scans of the liver. Forty one of these patients were seropositive for HBeAg. All patients were diagnosed by needle biopsy. Group 3 included 46 samples from patients with HCC. These patients (38 men and 8 women, ranging in age from 31 to 57 years : mean age, 50.9) had a clinical history of HBV-related chronic liver diseases for at least 10 years. Increased levels in serum of alpha fetoprotein (AFP) over 500 ng/ml were observed in 27 of 46 HCC patients. Twenty-nine HCC samples were obtained from liver tissues resected during surgery and another seventeen samples were from needle biopsy specimens. Both tumorous and nontumorous liver samples were available in 15 of 29 resected HCC samples. All seventeen specimens taken from needle biopsy were tumor tissues. Thirty one of these patients were seropositive for HBeAg. In 31 of 46 cases with HCC, tumors had developed on a background of established cirrhosis. HCC tissues were graded histopathologically, according to the criteria of Edmondson and Steiner (14).

Viral Markers in Serum

Serum HBsAg and HBeAg were tested by radioimmunoassay (RIA) (Ausria II, Abbott Laboratories, IL, USA). Antibody to hepatitis B core antigen (anti HBc) and antibody to HBeAg (anti HBe) were also determined by RIA (Corab, Abbott Laboratories). Anti HCV was tested by using the Abbott HCV EIA test system (Abbott Co.) following the manufacturer's instructions. HCV-RNA in peripheral blood was detected by nested reverse transcriptase-polymerase chain reaction (nested RT-PCR) (15).

Immunohistochemical staining

For the detection of HBxAg and IGF-II, formalin- fixed, paraffin-embedded liver tissue sections were stained by using the method described by Park et al. (12) and Stross et al. (16). Briefly, paraffin blocks were cut at 4 μm, deparaffinized with xylene-histoclear solution (1:3, National Diagnostics, NJ, USA) and dehydrated. After treatment with 3% hydrogen peroxide and blocking reagent, tissues were incubated with primary antibodies, polyclonal rabbit anti HBx IgG or monoclonal mouse anti IGF-II (Upstate Biotechnology Inc., New York, USA). Polyclonal rabbit anti-HBx IgG was prepared by immunizing rabbits against the recombinant DNA-generated X protein, glutathion S transferase (GST)-fused HBx protein, including 154 amino acids and purified using protein A and GST affinity column chromatography. The specificity of polyclonal anti-HBx was checked by western blot analysis using the GST-fused HBx protein (17).

In order to remove the non-specific binding activity to human hepatocytes, anti-HBx and anti IGF-II were preincubated with normal liver tissue before immunohistochemical staining. After washing with immunoassay buffer (Dako corp, CA, USA), each section was treated with biotinylated secondary antibody (Dako corp) and avidin-biotin-peroxidase (Dako corp), sequentially. 3-Amino-9-ethyl-carbazole (Dako corp) was used as a chromogen to each section and the sections were counterstained with hematoxylin and mounted with crystal mount (Biomeda corp, Foster city, USA). Primary antibodies were diluted with antibody diluting buffer (Biomeda corp.) after several tests for optimization.

Controls

Specificity controls for polyclonal rabbit anti-HBx IgG

and monoclonal mouse anti-IGF-II IgG were carried out by staining the tissue sections with the following primary antibodies. 1) normal rabbit serum, 2) normal mouse IgG, 3) anti-HBx IgG preincubated with purified GST- fused HBx protein, 4) anti IGF-II preincubated with excess recombinant human IGF-II (R and D system, Mineapolis, MN, USA), and 5) ten human fetal liver samples, from 21 weeks to 30 weeks of gestation. The liver samples were kindly provided by Dr C.W. Kim (Dept Pathology, Seoul National University Hospital, Seoul, Korea) and used as a positive control for anti IGF-II.

Criteria for immunohistochemical reactivity

The percentage of stained cells was estimated on a scale of 1-4, in which 1 denoted positive staining in less than 25% of cells; 2, positive staining in 25-50%; 3, positive staining in 51-75%; 4, positive staining in 76-100%. The types of staining were classified as follows : 1) cytoplasmic, 2) nuclear, and 3) membranous. The locations of positively stained cells were classified as periportal and lobular.

RESULTS

Colocalization of HBxAg and IGF-II

In order to disclose the relationship between the expression of HBxAg and IGF-II, we stained the neighboring sections of 40 CAH, 51 cirrhotic and 46 HCC tissue samples for HBxAg and IGF-II, separately (Table 2). As shown in Fig. 1, thirty-eight (38/40, 95%) of 40 CAH-B tissue samples were positively stained for HBxAg in hepatocytes (Fig. 2A), lymphocytes (Fig. 2B) and bile duct cells (Fig. 2C) in the portal area, whereas none of the CAH-B samples expressed IGF-II. In 51 cirrhotic samples, HBxAg was found in 20 cases (20/51, 39%). Among these positively stained samples, IGF-II-positive hepatocytes were seen in all cases (20/20, 100%). However, colocalization of both HBxAg (Fig. 2D) and IGF-II (Fig. 2E) at neighboring sections was not regularly observed. In 31 cirrhotic tissues without HBxAg expression (31/51, 61%), IGF-II was also seen in 17 cases (17/31, 55%).

Table 2. Expression of IGF-II in patients with CAH-B, cirrhosis and HCC according to the expression of HBxAg

Groups studied	Expression of IGF-II, number(%)	
	HBxAg-positive	HBxAg-negative
CAH (n=40)	0/38(0%)	0/2(0%)
Cirrhosis (n=51)	20/20(100%)	17/31(55%)
HCC (n=46)	8/8(100%)	38/38(100%)

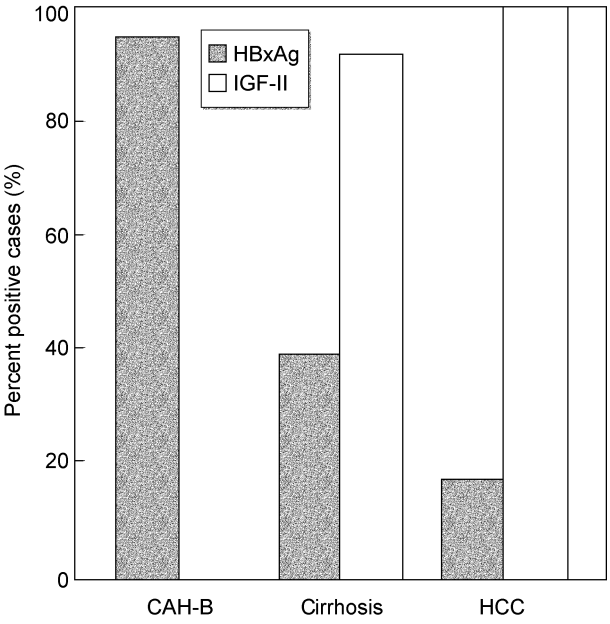

Fig. 1. The percent positivity of HBxAg and IGF-II staining in chronic active hepatitis-B, cirrhosis and hepatocellular carcinoma.

Among 46 HCC tissues, HBxAg-positive tumor cells were seen in 8 cases (8/46, 17%). Among these cases, IGF-II-containing hepatocytes were found in all cases (8/8, 100%), but colocalization of both HBxAg (Fig. 2F) and IGF-II (Fig. 2G) at neighboring sections in a tissue sample was not regularly observed as seen in cirrhotic tissues. However, in 38 HCC samples without HBxAg expression (38/46, 83%), IGF-II-positive tumor cells were found in all cases (38/38, 100%).

Distribution and pattern of HBxAg

In CAH-B samples with positive staining for HBxAg, HBxAg was seen in periportal hepatocytes in 36 samples (36/38, 95%) and 2 (2/38, 5%) in lobular hepatocytes. HBxAg staining in hepatocytes was found both in cytoplasm and nucleus, but no membrane staining was observed. The proportion of positive cells varied widely from 1% to 30%. No relationship between the distribution pattern of HBxAg and the degree of liver injuries was observed. HBxAg staining in lymphocytes and bile ducts in portal areas was seen in most cases with CAH-B. Kupffer cells were also positively stained for HBxAg.

Of 51 cirrhotic samples, 37 (73%) were nodular cirrhosis and 14 (27%) were early cirrhosis. HBxAg was

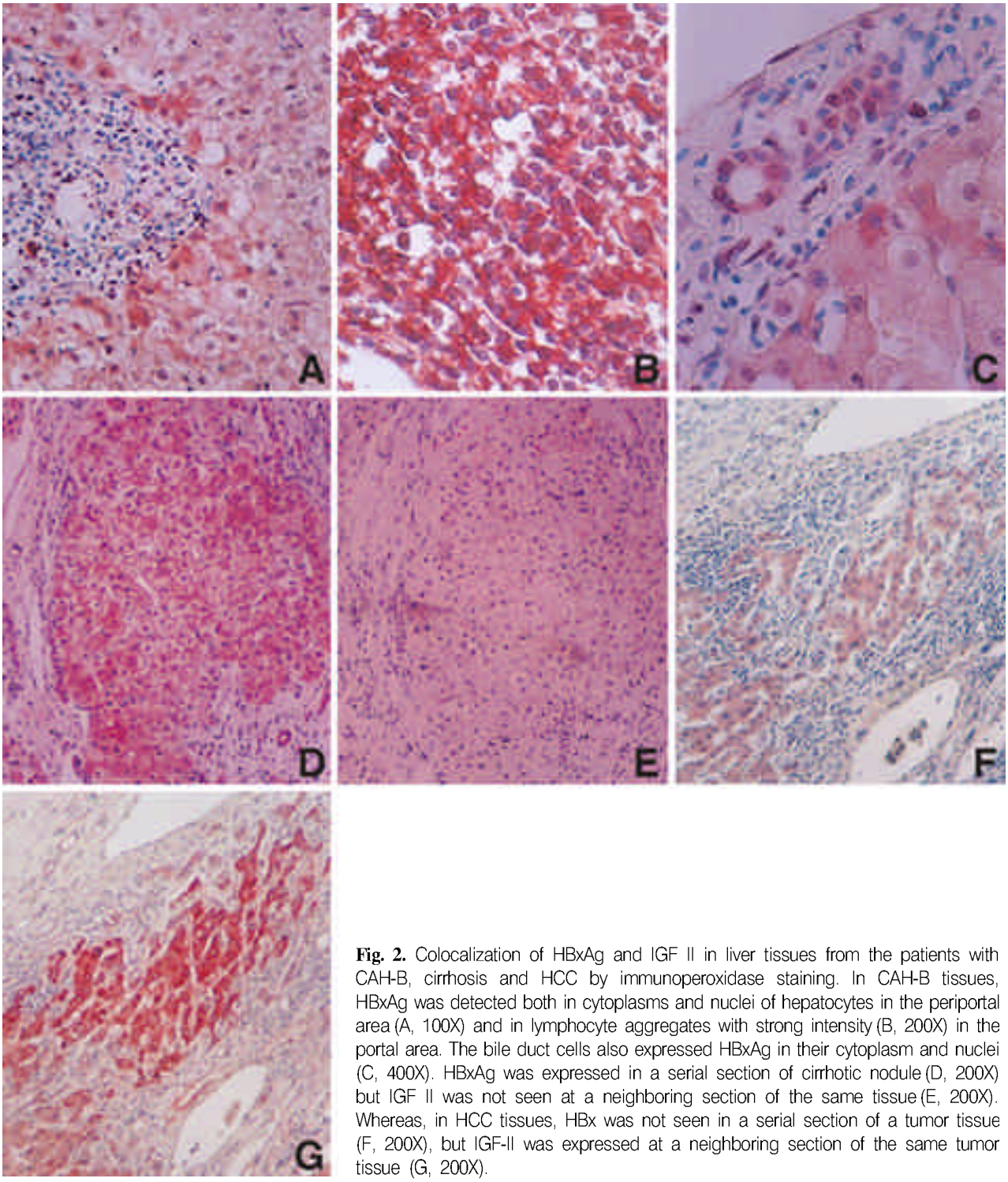

Fig. 2. Colocalization of HBxAg and IGF II in liver tissues from the patients with CAH-B, cirrhosis and HCC by immunoperoxidase staining. In CAH-B tissues, HBxAg was detected both in cytoplasms and nuclei of hepatocytes in the periportal area (A, 100X) and in lymphocyte aggregates with strong intensity (B, 200X) in the portal area. The bile duct cells also expressed HBxAg in their cytoplasm and nuclei (C, 400X). HBxAg was expressed in a serial section of cirrhotic nodule (D, 200X) but IGF II was not seen at a neighboring section of the same tissue (E, 200X). Whereas, in HCC tissues, HBx was not seen in a serial section of a tumor tissue (F, 200X), but IGF-II was expressed at a neighboring section of the same tumor tissue (G, 200X).

seen throughout the cytoplasm of hepatocytes in all of the positive cases, but nuclear and perinuclear staining were also observed. There were several distribution patterns of HBxAg in liver tissues. In the sparse distribution type, only occasional positively stained cells were observed in some areas of the tissue section. HBxAg-containing cells in most cases, however, were evenly distributed within the cirrhotic nodules (Fig. 3A), but focal distribution limited to a part of the nodules was also observed. In the localized type, a few positively stained cells as groups were distributed in some areas of a section. In the diffuse type, many positively stained

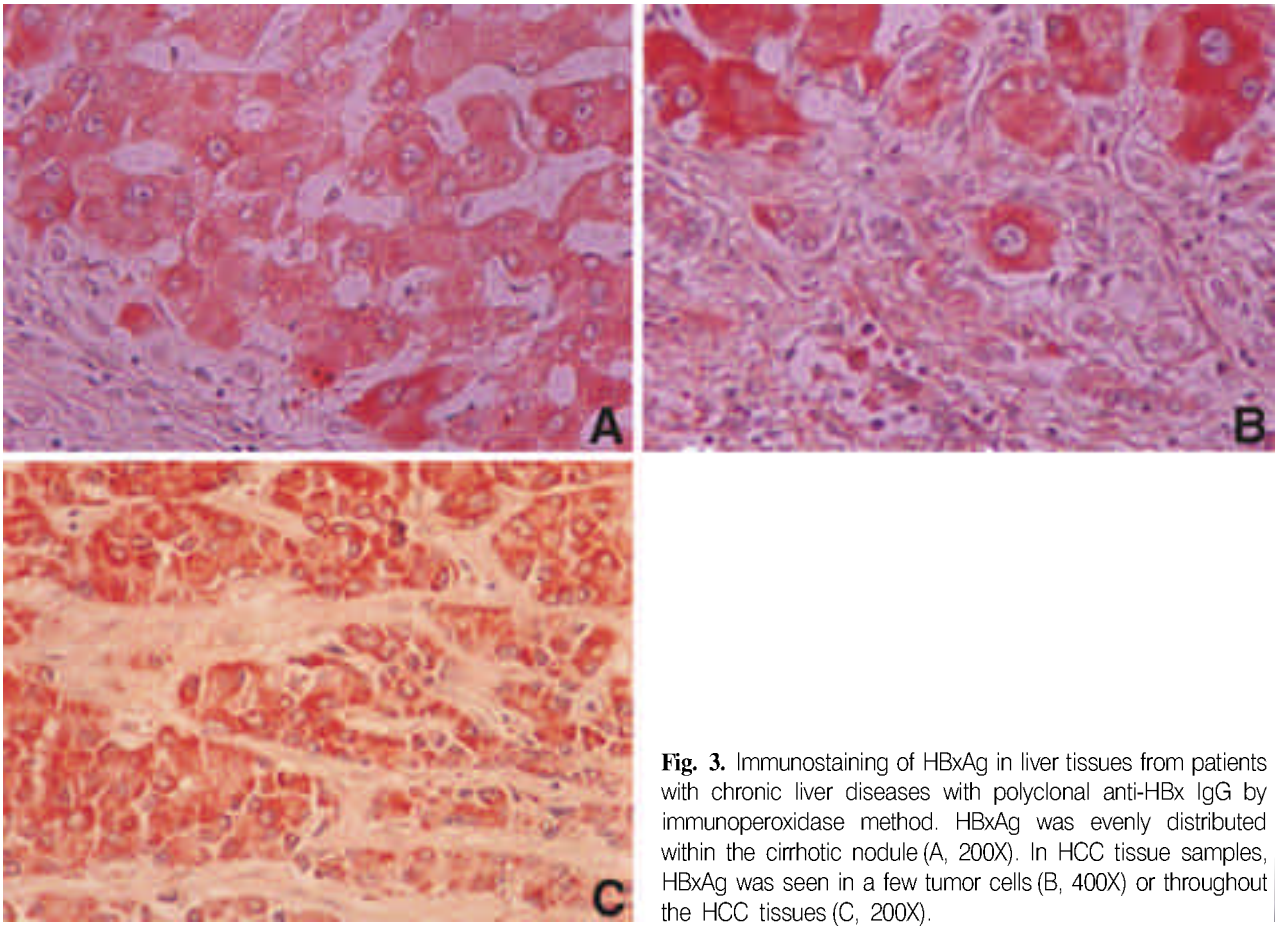

Fig. 3. Immunostaining of HBxAg in liver tissues from patients with chronic liver diseases with polyclonal anti-HBx IgG by immunoperoxidase method. HBxAg was evenly distributed within the cirrhotic nodule (A, 200X). In HCC tissue samples, HBxAg was seen in a few tumor cells (B, 400X) or throughout the HCC tissues (C, 200X).

cells were diffusely distributed throughout the section. In many cases, the distribution of HBxAg-positive cells was perivascular and along fibrous septa of the cirrhotic nodules. HBxAg was also present in the lymphoid cells and bile ducts in portal areas.

HBxAg was seen in 8 (17%) of 46 HCC samples within tumor cells (Fig. 1). HBxAg was also detected in the surrounding nontumorous tissues in 7 (47%) of 15 samples in which this tissue was available. When the frequency and intensity of HBxAg staining were compared in tumorous and nontumorous tissues, both frequency and intensity of HBx staining were greater in nontumorous tissues compared to tumor tissues. The percentage of HBxAg positive tumor cells varied among the different tumor samples. HBxAg was localized mainly in the cytoplasm of tumor cells, and less frequently in the nuclei. No membrane staining was seen. Both localized (Fig. 3B) and diffuse staining (Fig. 3C) were observed in tumor sections. Localized staining was the dominant pattern in the large majority of tumor samples while diffuse staining was present in only 10% of the tissue samples. The pattern of HBxAg staining was not related to the histologic type or grade of HCC.

Distribution and pattern of IGF-II

CAH-B tissue (Fig. 4A) did not express IGF-II. Among 51 cirrhotic samples, IGF-II was expressed in 47 samples (92%) and all of the 46 HCC samples (100%) tested (Fig. 1). IGF-II in cirrhotic livers was seen in the cytoplasm of the hepatocytes and was evenly distributed in regenerating nodules (Fig. 4B). IGF-II was expressed throughout the tumor tissue in positive cases (Fig. 4C), but in most cases, focal expression limited to a part at the growing edge of the tumor tissue was also observed. IGF-II positivity was mainly localized in cytoplasm, the expression of IGF-II was not related to the histologic type or grade of HCC.

Specificity of anti HBx IgG and anti IGF-II IgG

The specificity of polyclonal rabbit anti HBx IgG and monoclonal mouse anti IGF-II IgG was determined in several ways. To remove the non-specific binding activity of anti HBx to normal liver tissue, the anti HBx was preincubated with excess amounts of normal liver tissue taken from a person who had received an abdominal

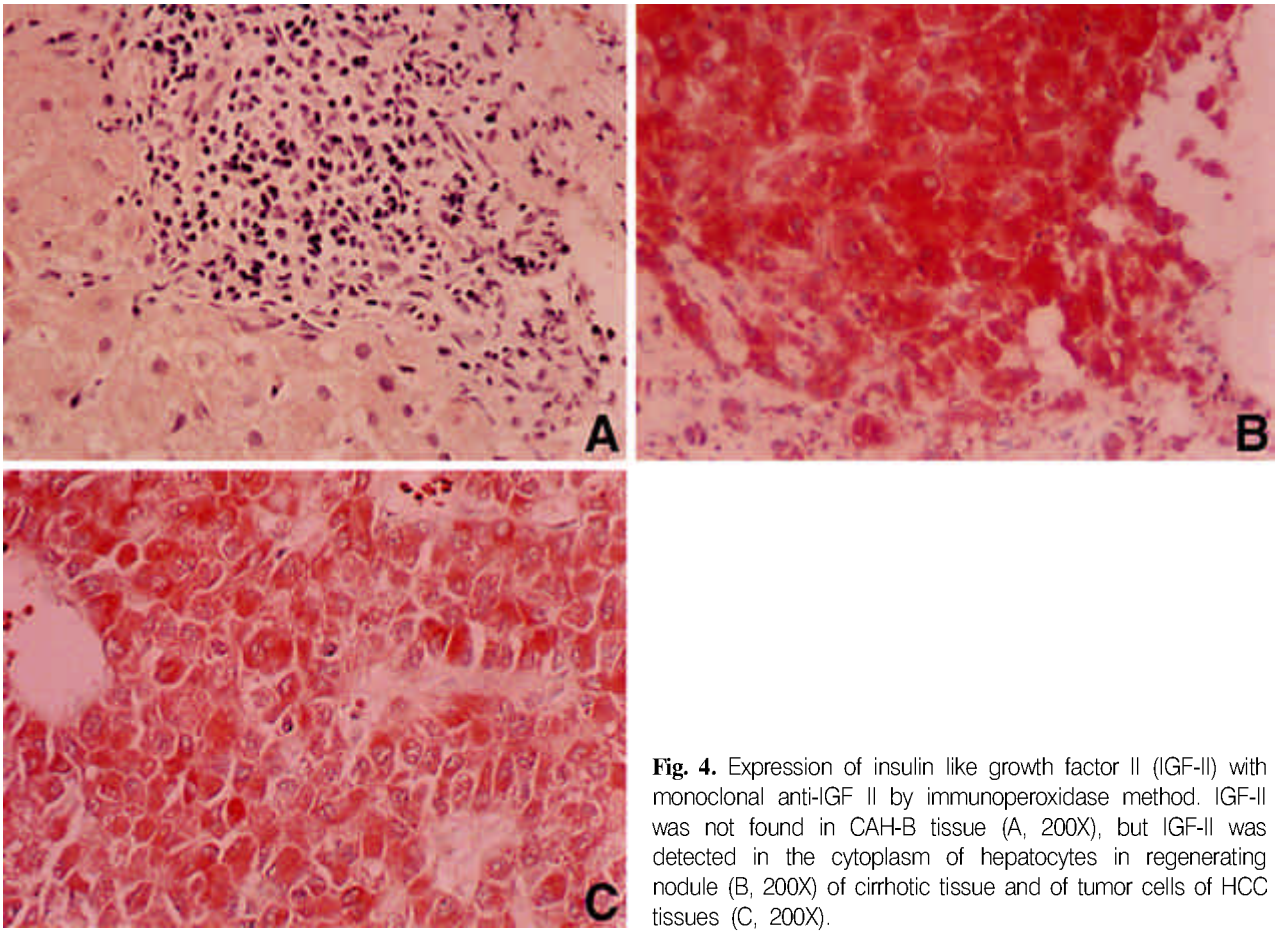

Fig. 4. Expression of insulin like growth factor II (IGF-II) with monoclonal anti-IGF II by immunoperoxidase method. IGF-II was not found in CAH-B tissue (A, 200X), but IGF-II was detected in the cytoplasm of hepatocytes in regenerating nodule (B, 200X) of cirrhotic tissue and of tumor cells of HCC tissues (C, 200X).

surgery and was seronegative for all HBV and HCV markers, before all immunohistochemical staining procedures. Although this preincubated anti HBx showed a clear negative staining to normal liver tissue (Fig. 5A). HBxAg was not blocked in all positive samples, suggesting that the staining is not due to one or more cellular proteins. In addition, the positive staining in a sample (Fig. 5B) was blocked by incubation of anti HBx with excess amount of purified GST-fused HBxAg, an immunogen used to raise anti HBx in this study (Fig. 5C). Liver section with positive staining for HBxAg became negative when anti HBx was replaced with normal rabbit IgG (data not shown).

In selected positive liver section for IGF-II (Fig. 5D), the specific binding activity of anti IGF-II was blocked when anti IGF-II was preincubated with excess amount of recombinant human IGF-II (Fig. 5E). The positively stained tissue became negative when anti IGF-II was replaced with mouse IgG. For a positive control for IGF-II, fetal liver sections were used (Fig. 5F).

DISCUSSION

The frequency and pattern of HBxAg expression were determined and compared to IGF-II expression in liver tissues from 137 patients with chronic active hepatitis-B, cirrhosis and HCC.

In the present study, it is noteworthy that none of 38 CAH-B samples with positive staining for HBxAg expressed IGF-II, indicating that HBxAg observed in CAH-B tissues does not enhance the expression of IGF-II. Although all of the cirrhotic and HCC tissue samples with positive staining for HBxAg expressed IGF-II, 55% of cirrhotic and 100% of HCC tissue samples without HBxAg staining also expressed IGF-II. Moreover, colocalization of both HBxAg and IGF-II at neighboring sections, even in both HBxAg and IGF-II positive samples, was not regularly observed. These findings suggest that, although HBxAg may be related to an enhanced expression of IGF-II in some cirrhotic and HCC samples, IGF-II expression in a large majority of these tissues may be enhanced by other factor(s) than HBxAg. Indeed, the current results were further sup-

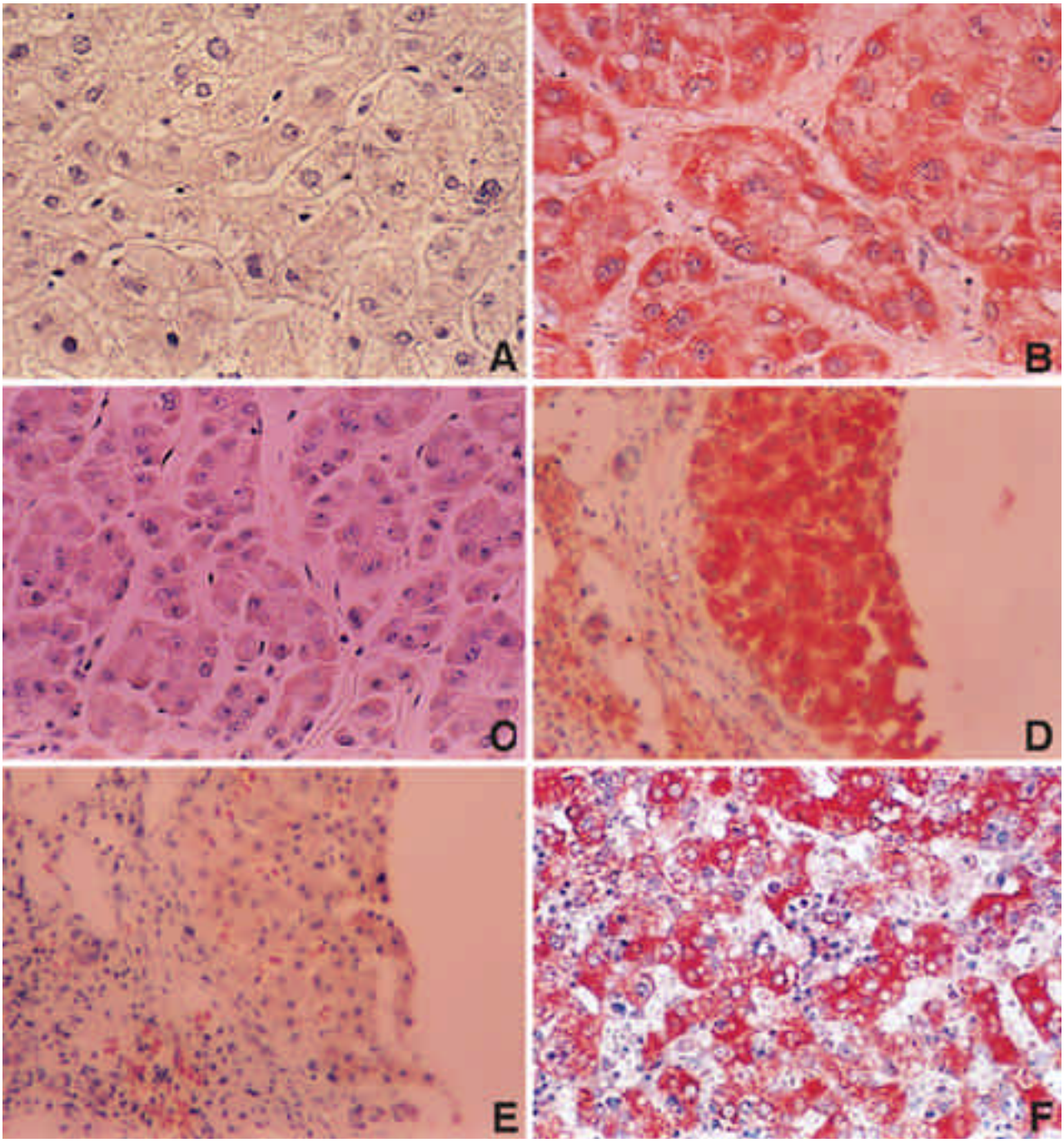

Fig. 5. Determination of the specificity of polyclonal anti-HBxAg and monoclonal anti-IGF II. Normal liver tissue was not stained with anti-HBx preincubated with normal liver tissue (A, 400X). The staining of HBxAg in HCC tissue (B, 400X) disappeared when anti-HBx was preincubated with excess amounts of recombinant HBx protein (C, 200X). A positive staining of IGF-II in cirrhotic tissue (D, 100X) disappeared when anti-IGF-II was preincubated with excess amount of recombinant human IGF-II (E, 100X). A fetal liver tissue was used as a positive control of IGF-II (F, 200X).

ported by the expression of IGF-II in the majority of liver tissues taken from the patients with HCV-associated with cirrhosis and HCC. The simultaneous detection both for HBxAg and IGF-II in chronic liver diseases has also been carried by other workers (18), but with different results. These authors reported that IGF-II was seen not only in HCC (93%) but also in 64% of chronic active hepatitis and 37% of chronic persistent hepatitis, by using polyclonal rabbit anti IGF-II. Clearly, the different results may be due to the use of polyclonal rabbit anti IGF-II

in the detection of IGF-II, whereas in this study, monoclonal anti IGF-II was used.

However, careful attention should be paid to the correct interpretation of the results observed in the present study. In this study, the prevalence of HBxAg expression in cirrhotic (39%) and HCC tissue samples (17%) were considerably lower than that (95%) observed in CAH-B samples, indicating the gradual decrease in the prevalence of HBxAg expression in cirrhotic and HCC tissues, as compared to CAH-B tissues. This finding, obtained by using a polyclonal rabbit anti-HBx IgG raised against recombinant HBx protein including 154 amino acid, is somewhat similar to a finding from Japan (19). The latter authors reported that HBxAg was seen in 64% of the patients with chronic hepatitis and 50% of those with cirrhosis but 2 HCC samples were negative for HBxAg, by using polyclonal rabbit anti-HBx raised against almost the complete HBx protein including 154 amino acids. The present results, however, are not in agreement with the results of other workers (9, 10). These workers observed that 97% of cirrhosis and 84% of HCC samples were positively stained for HBxAg, by using the Chinese liver samples and polyclonal rabbit anti-HBx raised against HBxAg synthetic peptide spanning residues 100 to 114 amino acids (9, 10). Presently, we do not have any definite explanation for these contradictory findings observed in this and the latter work. Although it is worthwhile, in the present study, to perform a comparative study at HBx gene level by in situ hybridization or transcriptional level by northern blot, the current results observed at least in HBx protein level, do not provide supportive evidence for the latter finding. Instead, it should be considered that the nature of antibodies used for immunohistochemical staining may be important because antibodies raised against different portions of HBx protein could affect both the frequency and intensity of HBxAg staining. Therefore, it is conceivable that the problem of these different findings could be settled by using the same kind of anti HBx antibody which was raised against the same portion of HBx protein in a single laboratory.

Taken as a whole, in the present study, we demonstrated that HBxAg in CAH-B, cirrhosis and HCC tissue sample was detected in 95%, 39% and 17% respectively, whereas IGF-II in the same tissues was detected in 0%, 92% and 100%, respectively. Although the highest prevalence of HBxAg expression was observed in the periportal hepatocytes of CAH-B tissues, the relation between HBxAg and severity of liver injury was not observed. Therefore the role of HBxAg in the pathogenesis of ongoing chronic liver injuries requires much more investigation. However, the present study also demonstrated the gradual decrease in the prevalence of HBxAg expression in cirrhosis and HCC tissues, as compared to CAH-B tissues. Moreover, the consistent colocalization of both HBxAg and IGF-II at neighboring sections of cirrhotic and HCC tissues was not regularly observed even in liver samples in which both proteins were positively stained. Although the host immune status against HBxAg and genetic alteration of HBV during progression of chronic active hepatitis B to cirrhosis and HCC should be further investigated, the findings observed in this work do not support the possibility that HBxAg may enhance the expression of IGF-II in HBV-associated cirrhosis and HCC.

In conclusion, the results and clinical implication of the present study indicate that the expression of IGF-II, known as a mitogen for cell proliferation in HCC and an angiogenic factor, in liver tissues from patients with chronic liver injuries may result from other factor(s) than HBxAg itself, although HBxAg may play a role in the pathogenesis of ongoing liver injuries and may be related to the IGF-II expression in some cirrhotic and HCC tissues.

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