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• VIRAL HEPATITIS •

Role of hepatitis B virus infection in pathogenesis of IgA nephropathy

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Abstract

AIM: To investigate the role of hepatitis B virus (HBV) in the pathogenesis of IgA nephropathy (IgAN).

METHODS: HBV antigens (HBAg, or HBsAg, HBcAg, and HBeAg) in renal tissues with IgAN were detected by immunohistochemical technique. The distribution and localization of HBV DNA were observed by using *in situ* hybridization. Southern blot analysis was performed to reveal the state of renal HBV DNA.

RESULTS: Among 100 patients with IgAN, HBs antigenemia was detected in 18 patients (18.00 %). HBAg in renal tissues was detected in 31 patients (31.00 %), the positive rate of HBAg, HBsAg and HBcAg was 64.52 % (20/31), 32.26 % (10/31), 32.26 % (10/31), respectively in glomeruli. HBcAg was also found in tubular epithelia and interstitia, which was 45.16 % (14/31) and 6.45 % (2/31), respectively. Five out of six cases with positive HBV DNA by *in situ* hybridization were proved to be HBV DNA positive by Southern blot analysis, and all were of the integrated form. Eight specimens were demonstrated to be HBV DNA positive by *in situ* hybridization, which was localized in the nuclei of tubular epithelial cells and glomerular mesangial cells as well as in infiltrated interstitial lymphocytes.

CONCLUSION: There is a relationship between HBV infection and IgAN. In addition to the humoral immune damage mediated by HBAg-HBAb immune complex, the cellular mechanism mediated by HBV originating from renal cells *in situ* may be also involved in the pathogenesis of IgAN.

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INTRODUCTION

The association between chronic hepatitis B virus (HBV) infection and glomerular diseases was first described in 1971[1], and various morphological patterns including membranous nephropathy, membranoproliferative glomerulonephritis (MN), mesangial proliferative glomerulonephritis (MPGN), minimal change nephropathy, and IgA nephropathy have been reported since then[1-28]. IgA nephropathy is considered the most common glomerular disease worldwide. Its prevalence varies considerably among and within countries, yet the pathogenetic mechanisms still remain largely uncertain[29,30]. Coexistence of mesangial proliferative glomerulonephritis with predominant mesangial IgA deposits and persistent hepatitis B virus surface antigenemia was first reported in five patients by Nagy *et al*[4], and later in some other reports[13,15,17,25,27], but the number of IgA nephropathy was fewer. Since China is an endemic area of hepatitis B virus (HBV) infection, and there is an incidence of 32 % IgA nephropathy in primary glomerulonephritis, according to a clinical analysis of 1001 cases by Li *et al*[31]. The relationship between IgA nephropathy and HBV infection is attracting increasing attention. In order to clarify the possible role of HBV infection in the pathogenesis of IgA nephropathy, we detected the serum HBV marker, HBV antigens (HBsAg, HBcAg, HBeAg) in renal tissues by immunohistochemistry technique, and HBV DNA in renal tissues by *in situ* hybridization and Southern bolt analysis.

MATERIALS AND METHODS

Patients

One hundred patients with IgA nephropathy who were admitted to our hospital during the period from 1982 to 1993 were included in the study. Their clinical data were complete and pathological diagnoses were confirmed by light microscopy and immunofluorescence examination (fresh renal tissue was used for immunofluorescence). The criteria for patients selection were no prior history of jaundice or liver disease, no previous history of blood transfusion, normal liver functions, no history of intravenous drug addiction, absence of cryoglobulinemia, and no clinical and laboratory evidences of secondary renal lesions such as lupus nephritis, *Henoch-Schonlein* purpura glomerulonephritis. All the patients received the following laboratory tests of urinalysis, serum creatinine and blood urea concentration, proteinuria, creatinine clearance at varying intervals during the study period. None had liver biopsies. Five patients without any HBV infection markers in serum or renal tissue were used as control. The following serial investigations were performed.

Serologic tests for HBV markers

Tests for HBV antigens and antibodies were performed before renal biopsy and regularly thereafter. Double antibody sandwich ELISA was used for detecting HBsAg and HBeAg, while double antigen ELISA was used for detecting anti-HBs and antibody competitive ELISA for detecting anti-HBe and anti-HBc. The kits of the test reagents were purchased from Shanghai Medical Laboratory.

Immunohistochemistry

The biopsy tissue was cut into three to four pieces. One piece

was fixed in 95 % ethanol and processed for 4 μm thick paraffin sections, which were stained by hematoxylin and eosin (HE) and periodic acid silver methanamine (PASM). The second piece was embedded in OCT compound (Miles Inc., Elkhart Inc., USA), and cut into 5 μm thick sections for detecting IgG, IgA, IgM and C_3 with direct immunofluorescence. The relevant antibodies were labelled with fluorescein (FITC) (Dako Corporation, Santa Barbara, CA, USA). The third piece was prefixed with 0.25 % glutaldehyde and postfixed with 1 % osmium and cut into ultrathin sections by conventional methods for electron microscopic observation. The fourth part was freshly preserved at -70 ℃ for Southern blot analysis.

Detection of HBVAg in renal tissue

The immunohistochemistry method was used mainly to detect the distribution of immunoglobulin, HBsAg, HBcAg, and HBeAg. The 4 μm thick tissue sections were digested with 0.05 % trypsin for 15 min at 37 ℃ to expose the epitopes of HBeAg, HBsAg and HbcAg. The ABC (Avidin-Biotin-Peroxidase) complex method kit for examining HBeAg and rabbit-anti-HBcAg in peroxidase-antiperoxidase (PAP) complex kit were purchased from Dako Company (Dakopatts, Denmark, U.S.A.). The PAP kit for HBcAg, and horseradish peroxidase-labelled goat anti-human IgG, IgA, IgM for immunofluorescence examination, and other antibodies were prepared by the Department of Pathology, Shanghai Medical University. The first antibodies for HBV antigens were goat anti-HBs, mouse anti-HBe and rabbit anti-HBcAg, respectively. The specificity of staining for HBV antigens was determined by blocking and absorption procedures as previously described by Lai *et al*[2]. Cross reactivities of anti-HBV antigen (s) with each other and with immunoglobulins, complements, and fibrinogen, normal and sclerosed glomerular tissues from HBsAg-negative controls were not found. Normal sheep and rabbit sera were used as negative control.

In situ hybridization

Paraffin fixed 4 μm thick sections were used for *in situ* hybridization. The digoxigenin labelled full-length HBV DNA probe prepared from an HBV plasmid clone of pBR322 and labelled with digoxigenin by random labelling and detection kit[32] were provided by Beijing Hepatitis Research Institute. The main procedures of *in situ* hybridization were as follows: (1) Paraffin fixed sections were dewaxed by conventional methods and digested with proteinase K (Sigma,0.5 mg/ml in Tris-HCL,0.01 mol/L pH 7.2) at 37 ℃ for 15 min and washed in 0.2 % glycine/0.01 % phosphate buffered saline (PBS) at pH 7.4. (2) The sections were fixed in 4 % paraformaldehyde at room temperature for 10 min. (3) The sections were then treated with 0.2 % acetic anhydride/triethanolamine (0.1 mol/L pH 8.0) at RT for 10 min. (4) The sections were treated with prehybridized medium (5×standard saline citrate (SSC)/0.1 % N-lauroylsarcosine/0.02 % sodium dodecylsulfate (SDS)/0.1 % blocking solution of BK) at room temperature for 15 min (5) Digoxigenin labelled HBV DNA probe was added (400 ng/mL), denatured at 100 ℃ for 10 min, hybridized at 60 ℃ for 16-20 h and washed in 2×SSC, 0.2×SSC, 0.1×SSC and 0.1 % Tween 20/buffer 1 (according to BK) sequentially, (6) Then it was pretreated with 5 % normal sheep serum/3 % bovine serum albumin (BSA) at room temperature for 20 min. (7) Sheep anti-digoxigenin labelled with alkaline phosphatase was added (BK) at 37 ℃ for 2 h, then washed in 0.1 % Tween 20/buffer III (according to BK). (8) it was then visualized by nitroblue tetrazolium salt(NBC)/5-bromo-4-chloro-3 indolyphosphate koluidium salt (BCIP;BK).

Examination of *in sit*u hybridization was retrospectively performed within several days by the same person. The specificity of *in situ* hybridization was confirmed by the negative controls. For example, the specific HBV DNA-digoxigenin probe was omitted, pBR328 DNA- digoxigenin probe was used (BK) instead of the HBV DNA- digoxigenin probe, DNase (Sigma, Chemical Co., St Louis, MO, USA 100 ug/mL) was digested.

Southern blot analysis

The fresh specimens preserved at -70 ℃ were processed for the detection of renal HBV DNA by Southern blot analysis. The ^{32}P(a)-dCTP labelled HBV DNA probe and the procedures used were the same as reported previously[18-27].

RESULTS

Serologic findings

Of the 100 patients in this study, 52 were males and 48 were females, (ranging 18-62 years, average 32.8 years). Eighteen patients were found to be HBsAg positive in their sera. Serum HBeAg was detected in four patients. Positive anti-HBe, HBcAb and anti-HBs were found in 7, 13, and 1 patients, respectively.

Detection of HBVAg in renal tissue

Detectable rate of HBAg (HBsAg, HBcAg and HBeAg) in glomeruli and renal tubules by immunohistochemistry was 31 % (31/100). The glomerular distribution of positive HBAg in 31 cases was as follows. Twenty cases were HBAg positive in the glomeruli, including HBsAg in 10 (32.26 %), and HBcAg in 10 (32.26 %). In addition, HBcAg was deposited in tubular epithelial cells (14 cases, 45.16 %) and renal interstitia (2 cases, 6.45 %). Three out of 20 cases were found to be HBeAg positive in glomerular cells (15 %). The distribution of HBAg in glomeruli varied either in capillary loops or in mesangial region or both. Both HBsAg and HBcAg were located in the cytoplasm of tubular epithelium. Occasionally HBcAg could be visualized in the nuclei of tubular cells.

Serologic and clinical findings

Among the 31 patients with positive HBVAg in renal tissues, eight were found to be HBsAg positive in their sera. Serum HBeAg was detected in one patient, HBeAb was positive in three patients, HBcAb was positive in seven patients. Clinical findings showed gross hematuria in 9 cases, microscopic hematuria in 5 cases, proteinuria in 6 cases, nephrotic syndrome in 5 cases, chronic renal failure in 2 cases and hypertension in 4 cases.

Detection of HBV DNA in renal tissue by in situ hybridization

HBV DNA was detected in eight out of the 31 specimens of renal tissues by *in situ* hybridization. Among the eight patients, four were found to be HBsAg positive in their sera. HBeAg was detected in one patient, HBeAb was positive in two patients, HBcAb was positive in four patients and HBsAb was positive in one patient. HBVAg was positive in the renal tissue, HBcAg was positive in glomerular cells and tubular cells in 7 and 3 cases respectively. HBsAg was positive in 1 and 4 cases, respectively. Eight cases were demonstrated to be HBV DNA positive in tubular epithelia by *in situ* hybridization, the positive rate was 100 % (Figure 1). The amount of HBV DNA positive cells varied in different cases. The positive signal of hybridization was localized within the nucleus. HBV DNA was detected in glomeruli in 6 out of the 8 specimens, the positive rate was 75 % (Figure 2).

Detection of HBV DNA in renal tissue by Southern blot

Five out of the 6 specimens with positive HBV DNA in glomeruli were found to be HBV DNA positive by Southern

blot analysis, the positive rate was 83.33 %. All of these positive specimens were identified to be the integrated form of HBV DNA , and none was identified as non-replicating free form of HBV DNA with a single band of signals at 3.2 Kb in length. The integrated HBV DNA by Southern blot analysis showed a high molecular weight single band before digestion, and revealed irregular multiple bands after ECoRI restrictive enzyme treatment.

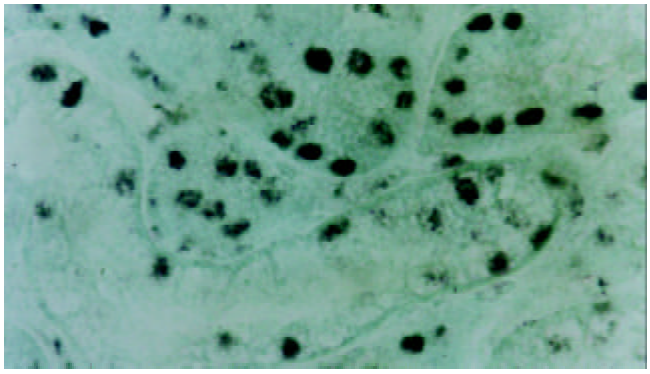

Figure 1 HBV DNA signals detected in nuclei of tubular cells by *in situ* hybridization. X200

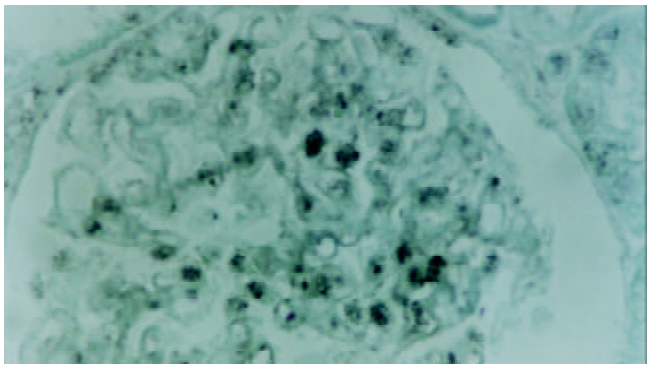

Figure 2 HBV DNA signals detected in nuclei of glomerular mesangial cells by *in situ* hybridization. X400

Relationship of positive HBV infectious markers in sera and HBAg and HBV DNA in renal tissues

The appearance of HBV infection markers in sera and HBAg and HBV DNA in renal tissues of the 8 cases of IgAN is shown in Table 1.

Neither HBAg nor HBV DNA was found in the sera or renal tissues in the five control cases of IgA nephropathy.

DISCUSSION

IgA nephropathy has been confirmed to be an immune complex-mediated glomerulonephritis defined morphologically by mesangial deposition of IgA, but the etiology is still uncertain[29,30]. The etiologic role of HBV antigenemia and HBV antigen deposition in IgA nephropathy remains speculative. It most likely involved mesangial and subendothelial trapping of circulating immune complexes (CICs), and the observations that preformed CICs primarily resulted in mesangial and subendothelial deposits supported this mechanism[29,30]. The possible role of HBV antigens was highly suspected especially in endemic areas of HBV infection such as in Southeastern Asia[13,27]. Many efforts have been contributed to this field[13,15,17,25,27], yet the data are scattered and incomplete because of the difficulty in obtaining tissue specimens, complicated clinical settings, and less specific and sensitive detection techniques. Lai *et al*[13] reported HBs antigenemia was detected in 21 out of 122 patients (17.2 %), which was significantly higher than the prevalence of HBsAg carriers in the general population ($P<0.01$). Humoral immune responses triggered by HBAg-HBAb immune complexes were traditionally regarded as the mechanism of tissue injury resulting in HBV-glomerulonephritis (GN). HBsAg, HBcAg and HBeAg with immunoglobulins and complement deposits in the glomeruli of HBV-GN have been demonstrated in many investigations[1-28]. It remains perplexing why some chronic HBsAg carriers develop IgAN whereas some develop membranous glomerulonephritis (MGN) or mesangiocapillary glomerulonephritis (MCGN). This could well be related to HBV antigens as well as the size and charge properties of HBV antigens and their antibodies. The nephrogenicity of the three different kinds of HBAg was therefore suggested. Takekosh[5], Ito[6], Hirose[8], and Zhang[15] emphasized that the molecular weight of HBeAg was the smallest (1×10^5), enabling it to pass through the glomerular basement membrane and to result in the formation of subepithelial dense deposits by microscopy. This is the well known morphologic characteristics of MGN. Local formation of antigen and antibody complexes has been well established to induce diffuse subepithelial immune deposits and proteinuria characteristic of HBV-MGN. Therefore, HBeAg is considered to be nephrogenic for MGN[5-8,15]. In contrast, the large size of HBsAg[2-4,7,9,13,15,25,27] and anionic nature of IgAN[27,29,30] favor the mesangial localization of HBsAg-anti-HBs complexes in HBV-IgAN. However, high frequencies of HBsAg (3×10^6) and HBcAg (8×10^6) were also detected in the capillary loops of glomeruli of MGN by other investigators[1,6,17,20,25,34].

Glomerular detection of HBV DNA has been reported in the kidneys of chronic HBsAg carriers with different glomerulonephritis[13,20,35], yet the consistency of these findings

Table 1 Detection results of HBV infection markers in sera and HBAg and HBV DNA in renal tissues in 8 cases of IgA nephropathy

Case	Age	Sex	Course	Serum HBVmarkers					Renal HBAg				Renal HBV DNA		
				HBAg		HBAb			T		G		ISH		Southern blot
				HBsAg	HBeAg	HBsAb	HBeAb	HBcAb	HBsAg	HBcAg	HBsAg	HBcAg	T	G	
1	30	F	4a	+	-	-	-	+	-	+	+	+	+	+	+
2	32	M	8m	+	-	-	-	-	-	+	+	-	+	+	+
3	35	M	2a	-	-	-	-	-	-	+	+	-	+	+	+
4	38	M	13a	+	-	-	+	+	-	+	-	+	+	+	+
5	35	F	5a	-	-	+	-	-	+	-	-	-	+	-	-
6	36	F	7m	+	-	-	-	+	+	-	+	+	+	+	+
7	32	M	2m	-	-	-	+	-	-	+	-	-	+	-	-
8	26	M	6a	-	-	-	-	+	-	+	+	-	+	+	+

F, female. M, male. d, day. m, month. a, year. T, tubules. G, glomeruli. ISH, *in situ* hybridization. +, positive. -, negative.

remains controversial, since some investigators have been unable to detect similar findings in chronic HBV carriers with coexisting membranous nephropathy[36]. This issue is of importance in understanding the pathogenesis of HBV-related glomerulonephritis. Therefore, with the help of *in situ* hybridization (ISH) and Southern blot analysis for HBV DNA, and highly sensitive and specific biological techniques, we found evidence for the presence of viral transcription in glomerular cells and renal tubular epithelia, which supported an etiological role of HBV in some chronic HBsAg carriers who developed coexisting glomerulonephritis[18,19,23,24,26,27]. Thereafter, the question of whether the existence of HBV DNA in renal tissue of glomerulonephritis is a general phenomenon and what role HBV DNA plays in the pathogenesis of renal tissue injury has been raised. In this serial investigation of 8 cases of IgAN, we found that HBV DNA detectable rate by *in situ* hybridization was 100 %. The positive frequencies were relevant to HBV antigenaemia and the detectable renal HBAg by immunohistochemistry. Since the presence of HBcAg in glomeruli might be not only from HBV DNA positive glomerular cells but also from circulation, the detectable rate of HBcAg in glomeruli has a close correlation with serum HBV antigenaemia and HBV DNA both in serum and renal tissue. The presence of HBcAg and HBV DNA in tubular epithelia might indicate HBV replication in epithelial cells, which were consistent with other studies in transgenic mice revealing the expression of viral genome of HBcAg or HBeAg only in epithelial cells[37-39]. In our study, 5 out of 6 patients with positive HBV DNA by *in situ* hybridization were HBV DNA positive by Southern blot analysis, all of them were integrated form. Since the number and molecular weight of the bands of HBV DNA signals of integrated form varied, it was suggested that HBV DNA integration was random. The infected cells with free form of HBV DNA, consisting of full genome of HBV, might theoretically express HBAg. However, if only some fragments of HBV DNA integrated into the chromosomes of the host cells randomly, whether the cells expressed HBAg would depend on whether the integrated part was consisted of certain intact HBAg genomes and their matched promotors as the elements of franking sequence of HBV DNA. Therefore, the kidney might carry dormant HBV DNA after HBV infection[19,23,27] (as found in the liver) or expressed HBAg to trigger immune reaction and result in tissue injury, which might be mediated by HBAg-HBAb immune complexes together with complements. Meanwhile, another possibility arises that HBV infected renal cells with the target HBcAg expression might activate T lymphocytes the relevant lymphokines to result in increased permeability of glomerular epithelial cells and glomerular basement membrane (nephrotic syndrome) or proliferation of glomerular cells (proliferative glomerulonephritis or IgAN).

Immune regulation defect was hypothesized to play a key role in the pathogenesis of IgA nephropathy[29]. Immune system disorder seemed to be related with secondary infections such as HBV infection or activation of HBV infection[40-42]. In IgA nephropathy patients, the spectrum of antibodies could be extended from both fixed and circulating endogenous antigens[30]. Studies of T lymphocytes suggested that increased T help (CD4) lymphocytes and decreased T suppresser (CD8) lymphocytes occurred with exacerbation of the disease, but overproduction of IgA was probably the consequence of the involvement of both T and B lymphocytes. This might provide some clue to the elevated serum concentration of IgA in our patients. However, whether clearance impairment of IgA immune complexes in hepatic and splenic phagocytic system or elevated levels of CICs involve in the pathogenesis of IgAN are yet to be determined.

We found that HBAg in renal biopsy specimens was mainly glomerular and renal tubular HBsAg and HBcAg. They are large molecular weight antigens forming CIC after binding to appropriate antibodies. It is believed that HBsAg preferentially deposites in mesangial region and subendothelial cells, while HBeAg as a part of viral nucleoprotein is expected to be capable of inducing membranous nephropathy by preferentially depositing along capillary walls. Since HBV DNA genome was detected in glomerular mesangium of patients with HBV associated glomerulonephritis[25], the possibility of immune complex formation *in situ* couldnot be excluded. It is noteworthy that, although many previous studies described the predominant deposition of HBV in glomeruli similar to ours, HBV DNA (ISH) was confirmed to exist extensively in glomeruli, renal tubules, interstitium and capsule[26]. This phenomenon could be attributed to the sensitive and specific techniques employed. Lai *et al* noticed that HBV DNA was found mainly in cytoplasm of proximal tubular epithelia but not in glomerular cells[24]. Zhang had the similar finding[23]. Whether HBV DNA in renal tubules represents endocytosis of HBV DNA in urinary filtrates or the direct invasion of HBV needs further study. In addition, mesangial IgA deposition was possibly resulted from the structural immunological or physicochemical abnormality in IgA nephropathy[29]. Recently Zhang *et al* examined T cell subsets infiltration in interstitia of HBV-GN by immunohistochemistry with monoclonal antibodies against CD3, CD4 and CD8, and found that there were certain relationships between the expression of HBcAg in tubular epithelial cells and the amount of phenotypes of infiltrated T cells in interstitia[23]. There is a possibility that such a relationship might be involved in the frequent occurrence of tubular atrophy with prominent interstitial inflammation and persistent clinical course in some HBV-GN patients.

Our investigation by both *in situ* hybridization and Southern blot analysis revealed that the presence of HBV DNA in renal tissues of IgAN patients with coexisting HBV antigenaemia appeared to be a general phenomenon among IgAN patients in Shanghai, China. Evidences showed that the renal tissue was infected with HBV, HBAg deposited in glomeruli was not only from circulation but also from infected glomerular cells. However, this study did not provide conclusive evidences for the hypothesis. It is suggested that cellular immune mechanism might be involved in the pathogenesis of HBV-GN in addition to humoral immune injury. These concepts might better our understanding of the pathogenesis of HBV related IgAN both theoretically and clinically.

In conclusion, our findings indicate that the host tissue tropism of HBV is not limited to hepatocytes, and active viral transcription is present in glomerular cells and tubular epithelia. Hepatitis B virus might be the etiologic agent of some chronic HBsAg carriers with coexisting IgA nephropathy. There is a relationship between HBV infection and IgAN. In addition to humoral immune injury mediated by HBAg-HBAb immune complexes, cellular mechanism mediated by HBV originating from renal cells *in situ* might also be involved in the pathogenesis of IgAN.

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