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• BRIEF REPORTS •

Existence and significance of hepatitis B virus DNA in kidneys of IgA nephropathy

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Abstract

AIM: To investigate the existence and significance of hepatitis B virus (HBV) DNA in the pathogenesis of IgA nephropathy (IgAN).

METHODS: Fifty cases of IgAN with HBV antigenaemia and/or hepatitis B virus antigens (HBAg, or HBsAg, HBcAg) detected by immunohistochemistry in renal tissues were enrolled in our study. The distribution and localization of HBV DNA were observed using *in situ* hybridization. Southern blot analysis was performed to reveal the state of renal HBV DNA.

RESULTS: Among the 50 patients with IgAN, HBs antigenemia was detected in 17 patients (34%). HBAg in renal tissues was detected in 48 patients (96%), the positive rate of HBAg, HBsAg, and HBcAg was 82% (41/50), 58% (29/50), and 42% (21/50) in glomeruli, respectively; and was 94% (47/50), 56% (28/50) and 78% (39/50) in tubular epithelia, respectively. Positive HBV DNA was detected in 72% (36/50) and 82% (41/50) cases in tubular epithelia and glomeruli respectively by *in situ* hybridization, and the positive signals were localized in the nuclei of tubular epithelial cells and glomerular mesangial cells as well as infiltrated interstitial lymphocytes. Moreover, 68% (34/50) cases were proved to be HBV DNA positive by Southern blot analysis, and all were the integrated form.

CONCLUSION: HBV infection might play an important role in occurrence and progress of IgAN. In addition to humoral immune damages mediated by HBAg-HBAb immune complex, renal tissues of some IgAN are directly infected with HBV and express HBAg *in situ*, and the cellular mechanism mediated by HBV originating from renal cells *in situ* may also be involved in the pathogenesis of IgAN.

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Key words: Hepatitis B virus; DNA; IgA nephropathy

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INTRODUCTION

IgA nephropathy is one of the most common glomerular diseases worldwide. Its prevalence varies considerably among and within countries, and its pathogenetic mechanisms still remain largely uncertain[1-3]. 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 by some other studies[5-9]. In 1996 Zhang *et al*[5] investigated HBV DNA in renal biopsies from fifty patients with many kinds of glomerulonephritis using *in situ* hybridization, and found that 72% (36/50) cases were HBV DNA positive detected by *in situ* hybridization, and 82% (17/23) cases were proved to be HBV DNA positive in Southern blot analysis. Moreover, among the five patients with IgAN, four and two patients were HBV DNA positive using *in situ* hybridization and Southern blot analysis, respectively. Wang *et al*[6,9] reported the integrated form of positive HBV DNA in renal biopsies from one of two patients with IgAN in 1996 by using Southern blot analysis, 12 of 20 patients with IgAN by using *in situ* hybridization and 8 of 10 patients with IgAN were the integrated form of positive HBV DNA using Southern blot analysis in 2003. Since China is an endemic area of hepatitis B virus (HBV) infection, and has an incidence of 32% IgA nephropathy in primary glomerulonephritis, according to a clinical analysis of 1001 cases by Li *et al*[10]. Hence, the relationship between IgA nephropathy and HBV infection is attracting increasing attention. In order to clarify the role of HBV DNA in the pathogenesis of IgA nephropathy, we took advantages of the sensitivity and specificity of molecular techniques using both *in situ* hybridization and Southern blot analysis to detect HBV DNA in kidney tissues from patients with IgA nephropathy.

MATERIALS AND METHODS

Patients

Fifty patients with IgA nephropathy who were admitted to our hospital with positive serum HBV infectious markers and/or HBAg detected in renal biopsy by immunohistochemistry were enrolled in this study. Of the 50 patients in this study, 27 were males and 23 were females, aged 18-66 years (average 34.6 years). Their clinical data were complete and pathological diagnoses were confirmed by light microscopy and immunofluorescence examination. The criteria for selection of patients included no history of jaundice or liver disease, blood transfusion, or intravenous drug addiction; normal liver functions; absence of cryoglobulinemia; and no clinical and laboratory evidence of secondary renal lesions such as lupus nephritis and *Henoch-Schonlein* purpura glomerulonephritis. None had liver biopsies.

Five cases without any HBV infectious markers in both serum or renal tissue were used as controls.

Serologic tests for HBV

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 test reagent kits were purchased from Shanghai Medical Laboratory.

Immunohistochemistry

The biopsy tissue was divided into three to four pieces. One piece was fixed in 950 mL/L ethanol and cut into 3 μm thick sections and stained with hematoxylin and eosin (HE) and periodic acid silver methanamine (PASM). The second piece was embedded in ornithine carbamoyltransferase (OCT) compound (Miles Inc. Elkhart, In, USA), 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 2.5 g/L glutaldehyde and postfixed with 10 g/L osmium and cut into ultrathin sections with conventional methods for electron microscopic observation. The fourth piece was freshly preserved at -70 ℃ for Southern blot analysis.

Detection of HBVAg in renal tissue

Immunohistochemical methods were used to detect the distribution of immunoglobulin, HbsAg and HBcAg. The 4 μm thick sections were digested with 0.5 g/L trypsin for 15 min at 37 ℃ to expose the epitopes of HBsAg and HBcAg. Rabbit anti-HBcAg and peroxidase-antiperoxidase (PAP) complex were purchased from Dako Company (Dakopatts, Denmark, USA.). PAP kit for HBcAg, horseradish peroxidase-labelled goat anti-human IgG, IgA and 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 and rabbit anti-HBcAg. The specificity of staining for HBV antigens was checked by blocking and absorption procedures as previously described by Lai *et al*[11] and Zhang *et al*[12]. No cross reactivities of anti-HBV antigen (s) with each other and with immunoglobulins, complements, fibrinogen, normal and sclerosed glomerular tissues from HBsAg-negative controls were found. Primary antibodies were replaced by normal sheep and rabbit sera as negative controls.

In situ hybridization

Paraffin-embedded 4 μm thick sections were used for *in situ* hybridization. The digoxigenin labeled full-length HBV DNA probe was prepared from a HBV plasmid clone of pBR322 and labeled with digoxigenin by random labelling using a detection kit[13] (Beijing Hepatitis Research Institute). The main procedures of *in situ* hybridization were the same as reported previously[5-9].

Southern blot analysis

Fresh specimens preserved at -70 ℃ were processed for the detection of renal HBV DNA state by Southern blot analysis. The ^{32}P(a)-dCTP- labelled HBV DNA probe and the procedures used were the same as reported previously[5-9].

Statistical analysis

To analyze the seropositivity of HBV marker or the presence of HBAg and HBV DNA in renal biopsies in IgAN patients, Fisher's exact test or chi-sequare test was used as appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Serologic findings

Seventeen patients (34%) were found to be HBsAg positive in their sera, serum HBeAg was detected in 3 patients (6%), anti-HBe was positive in 10 patients (20%), HBcAb was positive in 26 patients (52%), and HBsAb was positive in 10 patients (20%).

Detection of HBAg in renal tissue

HBAg (HBsAg and/or HBcAg) in renal tissues was detected in 48 patients (96%) by immunohistochemistry. The distribution of positive HBAg on glomeruli was found in 41 cases (82%), including HBsAg in 58% (29/50) cases and HBcAg in 42% (21/50) cases. The distribution of HBAg on glomeruli was found either on capillary loops or in mesangial region or both. The expression of HBAg in tubular epithelial cells was detected in 47 cases (94%). HBsAg and HBcAg in tubular epithelial cells were also found in 56% (28/50) and 78% (39/50) cases. The positive rate of tubular HBcAg (78%) was higher than that of tubular HBsAg (56%), glomerular HBsAg (58%) and HBcAg (42%). Both HBsAg and HBcAg were located in cytoplasm of tubular epithelial cells. Occasionally, HBcAg could be visualized in the nuclei of tubular cells.

Detection of HBV DNA in renal tissue by in situ hybridization

Forty-six of the 50 cases (92%) were HBV DNA positive in renal tissues by *in situ* hybridization. Seventeen of them were found to be HBsAg positive in their sera, HBeAg was detected in 3 patients, HBeAb was positive in 10 patients, HBcAb was positive in 25 patients and HBsAb was positive in 10 patients. All these 46 patients were HBVAg positive in the renal tissue, while HBcAg was positive in glomerular cells and tubular cells in 21 and 39 cases, respectively and HBsAg was positive in 29 and 28 cases, respectively. Of these 50 cases, 41 (82%) were HBV DNA positive in tubular epithelial cells. HBV DNA was detected in glomeruli in 72% cases (36/50). The positive signals of hybridization were localized within the nuclei of tubular epithelial cells and glomerular mesangial cells as well as infiltrated interstitial lymphocytes.

Detection of HBV DNA in renal tissue by Southern blot

Of the 50 cases, 34 (68%) were HBV DNA positive by Southern blot analysis, all these positive specimens were the integrated form of HBV DNA , and none was a non-replicating free form of HBV DNA with a single signal band of 3.2 kb. The integrated state of HBV DNA by Southern blot analysis showed a high molecular weight single band before digestion and revealed irregular multiple bands after *E. cori* restrictive enzyme treatment.

Relationship among HBV infectious markers in sera, and HBAg and HBV DNA in renal tissues

The relationship among HBV infectious markers in sera, and HBAg and HBV DNA in renal tissues of the 50 cases with IgAN are shown in Table 1. Neither HBAg nor HBV DNA was found in the renal tissues from the five control cases of mesangioproliferative glomerulonephritis.

DISCUSSION

The association between chronic hepatitis B virus infection, characterized by persistent hepatitis B surface antigenemia and glomerular disease was first described in 1971[14].

Table 1 Relationship among HBV infectious markers in sera, and HBAg and HBV DNA in renal tissue in 50 cases with IgA nephropathy

Case	Age (yr)	Sex	Course	Serum HBV markers					Renal HBAg				Renal HBV DNA		
				HBAg		HBAb			T		G		ISH		Southern bolt
				HBsAg	HBeAg	HBsAb	HBeAb	HBcAb	HBsAg	HBcAg	HBsAg	HBcAg	T	G	
1	30	F	4 yr	+	-	-	-	+	-	+	+	+	+	+	+
2	32	M	8 mo	+	-	-	-	-	-	+	+	-	+	+	+
3	35	M	2 yr	-	-	-	-	+	-	+	+	-	+	+	+
4	38	M	13 yr	+	-	-	+	+	-	+	-	+	+	+	+
5	35	F	5 yr	-	-	+	-	-	+	-	-	-	+	-	-
6	36	F	7 mo	+	-	-	-	+	+	-	+	+	+	+	+
7	32	M	2 mo	-	-	-	+	-	-	+	-	-	+	-	-
8	26	M	6 yr	-	-	-	-	+	-	+	+	-	+	+	+
9	22	M	5 mo	+	-	-	-	-	+	-	-	+	+	+	+
10	47	F	4 yr	+	-	-	-	+	-	+	+	-	+	+	+
11	24	M	6 yr	-	-	-	+	+	-	+	-	-	+	+	-
12	43	M	9 mo	-	-	-	-	+	+	-	-	+	+	+	+
13	26	F	1 yr	-	+	-	-	-	+	+	+	-	+	+	+
14	28	M	2 yr	-	-	+	-	+	-	+	-	+	+	-	+
15	45	M	3 mo	-	-	-	-	+	-	+	-	+	-	+	-
16	38	F	2 mo	-	-	+	-	-	+	+	+	-	+	-	+
17	53	F	4 yr	+	-	-	-	+	+	+	+	+	+	+	+
18	59	M	1 yr	-	-	-	+	+	+	+	+	-	+	+	+
19	64	M	11 mo	-	-	+	-	-	+	-	+	-	-	+	-
20	58	F	7 mo	-	-	+	-	-	-	+	-	+	-	+	+
21	66	M	2 yr	-	+	-	-	-	+	+	+	+	+	+	+
22	21	F	4 mo	-	-	-	-	-	+	-	+	-	-	+	-
23	30	M	1 yr	-	-	-	+	+	+	+	+	-	+	+	+
24	29	F	4 mo	-	-	-	-	+	-	-	-	-	-	-	-
25	43	F	3 y	+	-	-	-	-	+	+	+	-	+	+	+
26	36	M	1 mo	-	-	+	-	+	-	+	+	-	+	+	+
27	27	M	2 mo	-	-	-	-	-	-	+	-	-	+	-	-
28	53	F	2 yr	-	-	-	-	+	+	+	+	+	+	+	+
29	49	F	5 mo	-	-	+	-	-	-	-	+	-	-	-	-
30	16	M	3 mo	-	-	-	-	+	+	+	+	-	+	+	+
31	28	F	6 mo	-	-	-	+	+	-	+	+	-	+	-	+
32	33	F	4 mo	+	-	-	-	-	+	+	+	-	+	+	+
33	41	M	7 mo	+	-	-	-	-	+	+	+	+	+	+	+
34	20	F	1 mo	-	-	-	-	+	-	+	-	+	+	-	-
35	18	F	2 mo	-	-	-	-	-	-	+	-	-	-	-	-
36	31	M	6 mo	+	-	-	-	-	+	+	+	+	+	+	+
37	29	M	1 yr	-	-	-	+	+	+	+	+	-	+	+	+
38	42	F	3 mo	-	-	+	-	-	+	-	-	-	+	-	-
39	21	F	2 mo	-	-	-	-	+	-	-	-	-	-	-	-
40	36	M	1 yr	+	-	-	-	-	+	+	+	-	+	+	+
41	60	F	2 yr	+	-	-	-	-	-	+	+	-	+	+	+
42	25	M	3 mo	-	-	-	-	+	-	+	-	+	+	-	-
43	19	M	2 mo	-	-	+	-	-	+	-	-	-	+	-	-
44	56	F	7 mo	+	-	-	-	-	+	+	+	+	+	+	+
45	22	M	3 mo	-	-	-	+	+	+	+	+	-	+	+	-
46	43	F	2 yr	+	-	-	-	+	+	+	-	+	+	+	+
47	39	M	6 mo	-	-		+	-	+	+	-	+	-	+	+
48	25	M	7 mo	+	+	-	-	-	+	+	+	+	+	+	+
49	36	F	8 mo	-	-	+	+	+	-	+	-	+	+	+	+
50	32	M	3 yr	+	-	-	-	+	+	+	-	+	+	+	+
Total				17	3	10	10	26	28	39	29	21	41	36	34

T: tubules; G: glomeruli; ISH: *in situ* hybridization; +: positive; -: negative.

Hepatitis B virus associated glomerulonephritis (HBVGN) has been traditionally regarded as a typical immune complex glomerulonephritis mediated by hepatitis B antigen (HBAg) and hepatitis B virus antibody (HBAb) immune complexes deposited on glomeruli[14-18]. Various morphological patterns, including membranous nephropathy, membranoproliferative glomerulonephritis (MN), mesangial proliferative glomerulonephritis (MPGN), minimal change nephropathy, and IgA nephropathy have been reported[18-22,24-30]. IgA nephropathy has been confirmed to be an immune complex-mediated glomerulonephritis, which is morphologically defined by mesangial deposition of IgA[1-3]. The etiologic role of HBV antigenemia and HBV antigen deposition in IgA nephropathy remains speculative[5-9]. It most likely involves mesangial and subendothelial trapping of circulating immune complexes (CICs), and the observation that CICs primarily result in mesangial and subendothelial deposits supports this mechanism[1-3]. The possible role of HBV antigens is highly suspected, especially in endemic areas of HBV infection, such as in Southeastern Asia[5-9,23]. Many efforts have been contributed to this field[12,23,27,31-34], yet there are only scattered and incomplete data because of the difficulty in obtaining tissue specimens, complicated clinical settings, less specific and sensitive detection techniques. Humoral immune responses triggered by HBAg-HBAb immune complexes are 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[4,30-33,35-38]. It remains perplexing why some chronic HBsAg carriers develop IgAN, while others develop membranous glomerulonephritis (MGN) or mesangiocapillary glomerulonephritis (MCGN). This is well related to HBV antigen status as well as the size and charge properties of HBV antigens and their antibodies. The larger size of HBsAg and HBcAg favors the mesangial localization of HbsAg-anti-HBs or /and HBcAg-anti-HBc complexes in HBV-IgAN[11,12,23].

The demonstration of HBV DNA within the glomeruli in patients with glomerulonephritis[33-35] and the identification of both free and integrated HBV DNA in kidneys of chronic HBsAg carrier, may favor the notion that HBV might also infect resident glomerular cells leading to immune complex formation. Detection of HBV DNA has been reported in kidneys of chronic HBsAg carriers with different glomerulonephritis[33-35], yet the consistency of these findings remains controversial, since some investigators were 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. In 1992 Fang *et al*[30] identified the non-replicating free form and the integrated form of HBV DNA in children's membranous glomerulonephritis by Southern blot analysis of renal DNA. Lin[31] investigated HBV DNA by in *situ* hybridization with ^{3}H-dCTP -labeled full-length HBV DNA probes in 20 child cases of membranous glomerulonephritis, and found that the positive rate of HBV DNA was 87.5% within 6 mo after the disease onset, and only 21% in tubular epithelial cells and none in glomeruli, while the course of glomerulonephritis lasted for more than 6 mo. In 1996, Zhang *et al*[5] investigated the HBV DNA in renal biopsies from fifty patients and found that 72% (36/50) cases were HBV DNA positive by using *in situ* hybridization and 73.9% (17/23) cases were HBV DNA positive in Southern blot analysis. Wang *et al*[9] reported that HBV DNA was positive in 100% renal biopsies from patients with IgAN by using *in situ* hybridization and 80% (8/10) patients with IgAN were the integrated form of positive HBV DNA by Southern blot analysis. Thereafter, the questions that whether the existence of HBV DNA in renal tissue of glomerulonephritis patients is a general phenomenon and what role HBV DNA plays in the pathogenesis of renal injury are raised. Therefore, with the help of *in situ* hybridization (ISH) and Southern blot analysis for HBV DNA, and highly sensitive and specific biological techniques, we have found the evidence for the presence of viral transcription in glomerular cells and renal tubular epithelial cells, which supports an etiological role of HBV in some chronic HBsAg carriers who develop coexisting glomerulonephritis. Since the presence of HBcAg in glomeruli might be not only from the HBV DNA positive glomerular cells, but also from the circulation, the detectable rate of HBcAg in glomeruli has a close correlation with serum HBV antigenaemia and the state of HBV DNA both in serum and renal tissue. The presence of HBcAg and HBV DNA in tubular epithelial might indicate HBV replication in epithelial cells[39,40]. Southern blot analysis, could identify the state of HBV DNA[30]. In our study, 34 of 50 cases (68%) were HBV DNA positive by Southern blot analysis, and all were the integrated form. Since the number and molecular weight of the bands of HBV DNA signals of integrated form varied, it is suggested that HBV DNA integration is random. The infected cells with free forms of HBV DNA, consisting of full genome of HBV, may theoretically express HBAg. However, if only some fragments of HBV DNA randomly integrate into chromosomes of the host cells, whether the cells expressing HBAg would depend on the integrated part 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[5,6,28] or express HBAg triggering immune reaction resulting in tissue injury, which might be mediated by HBAg-HBAb immune complexes together with complements. Meanwhile, another possibility is that HBV-infected renal cells with the target HBcAg expression might activate T lymphocytes with relevant lymphokines resulting in increase of the permeability of glomerular epithelial cells and glomerular basement membrane[41].

In conclusion, the presence of HBV DNA in renal tissues of patients with IgAN, especially with coexisting HBV antigenaemia, appears to be a general phenomenon among IgAN patients in Shanghai, China. The host tissue tropism of HBV is not limited to hepatocytes, and active viral transcription is present in glomerular and tubular epithelial cells. Hepatitis B virus might be the etiologic agent for some chronic HBsAg carriers with coexisting IgA nephropathy. The cellular mechanism mediated by HBV originating from renal cells *in situ* may also be involved in the pathogenesis of IgAN. These concepts might enrich our understanding of the pathogenesis of HBV related IgAN both theoretically and clinically.

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