Original Article

Studies on Mutual Relationship between Anti-HBx and sFas, IL-10 or IL-12 in Sera of Cases with Chronic Hepatitis B Infection

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Objective To study the mutual relationship between anti-HBx and IL-10, IL-12 or soluble Fas (sFas) in sera of patients with chronic HBV infection and to explore the importance of anti-HBx detection as well as its role in the development of chronic HBV infection.

Methods Total of 90 cases with chronic HBV infection were randomly selected, including 10 of asymptomatic carriers (ASC), 28 of chronic hepatitis B (CHB), 26 of liver cirrhosis (LC) and 26 patients of hepatocellular carcinoma (HCC). Their clinical data and blood samples were collected, and serum was prepared and stored at -73°C. Anti-HBx was detected with an indirect ELISA established in our earlier research, and levels of IL-10, IL-12 and Fas were determined with commercial double-antibody sandwich ELISA kits. The mutual relationship between anti-HBx and IL-10, IL-12 or sFas in serum were analyzed with the software SPSS 20.0.

Results All levels of IL-10, IL-12 and sFas in peripheral blood showed a rising trend with development of chronic HBV infection. The levels of IL-10 in ASC, CHB, LC and HCC groups were 13.93 ± 14.40 ng/L, 39.38 ± 20.77 ng/L, 69.06 ± 46.37 ng/L and 62.82 ± 23.42 ng/L, respectively, levels of IL-12 in the 4 groups were 15.64 ± 23.04 ng/L, 68.50 ± 23.14 ng/L, 76.83 ± 12.82 ng/L and 83.74 ± 24.88 ng/L, respectively, and levels of sFas were 58.17 ± 77.42 ng/L, 179.88 ± 104.36 ng/L, 249.22 ± 107.80 ng/L and 252.98 ± 87.65 ng/L, respectively. Twenty-seven out of 90 patients showed a positive result for anti-HBx detection, including 1 in ASC, 4 in CHB, 12 in LC and 10 in HCC group. The levels of IL-10, IL-12 and sFas were higher in anti-HBx positive group than in negative group. Statistical analysis demonstrated significant differences of IL-10 and IL-12 between the two groups ($P < 0.05$), but the differences of sFas had no statistical significance ($P = 0.094$).

Conclusions Anti-HBx antibody is not protective, and is closely related to IL-10, IL-12 and sFas. It may be an important serum indicator for aggravation from chronic hepatitis B to liver cirrhosis or hepatocellular carcinoma in patients with chronic HBV infection.

Key words: Hepatitis B virus; Chronic infection; Anti-HBx; sFas; IL-10; IL-12

Chronic hepatitis B (CHB) is an infectious disease which has serious effects on human health. There are more than 300 million patients infected with hepatitis B virus all over the world.¹ Different patients are at different clinical states, including asymptomatic carriers (ASC), chronic hepatitis B (CHB), HBV-related cirrhosis (LC) and

hepatitis B-related hepatocellular carcinoma (HCC). So far, why different patients have different prognosis remains indistinct. As it is known to all, the incidence of CHB is caused by the interaction of HBV antigens system and the immune system. Many studies have shown that the X protein of hepatitis B virus (HBV) was the unique protein encoded by the genome of HBV, which has a variety of regulatory functions. The X protein also known as HBx protein may affect the pathogenesis of HBV by a variety of mechanisms and transactivation of other genes. Furthermore, it has a close relationship with integration of viral genes and gene mutations in its host cells.²⁻⁴ In recent years,

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more researches on the relationship between HBV X gene and liver cancer have been carried out. Immune status of the host at different stages of chronic HBV infection and various cytokines in serum of CHB case have become the research hotspot. However, there are fewer studies focusing on anti-HBx and the relationship between anti-HBx and the various cytokines in the host body. Therefore, anti-HBx, soluble Fas protein (sFas), interleukin-10 (IL-10) and interleukin-12 (IL-12) in sera of 90 patients at different clinical stages of chronic HBV infection were detected and compared in this study.

MATERIALS AND METHODS

Data collection

Total of 90 cases with chronic HBV infection examined in outpatient or inpatient departments of our hospital from September, 2011 to February, 2012 were randomly selected, who all came from Shandong Province. The diagnosis of the 90 cases was in accordance with the criterion in the 2010 edition of "the Guideline for Prevention and Management of Chronic Hepatitis B".⁵ Patients with hepatitis caused by other viruses, drugs or alcohol and autoimmune hepatitis were excluded. There were 10 cases of asymptomatic carriers, 28 cases of chronic hepatitis, 26 cases of liver cirrhosis and 26 cases of hepatocellular carcinoma. The male/female ratio of the 4 groups were 7/3, 23/5, 22/4 and 23/3, respectively, and mean age of the 4 groups were 32.00 ± 7.87 , 31.82 ± 10.44 , 45.14 ± 8.52 and 52.32 ± 7.59 years, respectively.

Specimens, reagents and main instrument

All patients were kept stomach empty for more than 8 h, and 5 ml blood was taken from cubital veins, 3 ml of which was sent to the laboratory for detection immediately. Automatic biochemistry analyzer was applied to analyze the liver function indicators such as alanine aminotransferase (ALT) and total bilirubin (TBil). The amount of HBV DNA was detected in quantitative fluorescence PCR assay. Serum was separated from the residual 2 ml blood of each patient and was stored at -73°C . Interleukin-10 (IL-10), interleukin-12 (IL-12) and soluble Fas protein (sFas) detection kits were purchased from TaKaRa Company. The kits were used in double-antibody sandwich enzyme-linked immunosorbent assay. The main instrument was Labsystems microplate reader.

Detection of IL-10, IL-12 and sFas

IL-10, IL-12 and sFas were detected in batches. The serum levels of IL-10, IL-12 and Fas were detected according to the conventional manufacturer's instructions.

Determination of anti-HBx

As there was no available commercial kit for detection of anti-HBx, an indirect ELISA method was set up to detect anti-HBx antibody. A fusion protein of wild-type HBx protein with glutathione S-transferase was obtained with genetic engineering techniques and highly purified in our previous research.⁶ The purified fusion protein, i.e. GST-fused HBx antigen (GST-HBxAg) was diluted to 0.488 ng/ μl (1/1 000). Each well of the microtiter plate was coated with 100 μl sample, and kept at 4°C overnight. Then the microtiter plate was washed four times in PBST, each well was added with 100 μl 1% BSA to block the nonspecific binding sites, then incubated for 2 h at 37°C , and washed for four times in PBST. The sera of cases with chronic hepatitis B virus infection and healthy control were diluted in 1/100, and each well was stuffed with 100 μl of the diluted serum. Each serum sample was set into three wells. The microtiter plate was incubated for 2 h at 37°C , and washed for five times in PBST. After that, goat anti-human IgG labelled with HRP were added into each well, the microtiter plate was incubated for 2 h at 37°C , and washed for five times in PBST once more. Sequentially the chromogenic enzyme substrate solution containing OPD was added in the wells, then A values in 490 nm were detected. A value ≥ 0.23 in serum was set as positive threshold value (threshold value = mean A value of the negative samples + $2 \times$ standard deviation).

Statistical analysis

Data of the general information, clinical diagnosis and IL-10, IL-12, sFas, anti-HBx of the 90 patients were collected. The database was analyzed by SPSS 20.0 statistical software. Normally distributed data were shown in mean $\pm$ SD, the differences among the four groups were compared with variance comparing analysis, the pairwised comparisons between groups were statistically analyzed with LSD method, $P < 0.05$ was considered as statistically significant.

RESULTS

Detection of sFas, IL-10 and IL-12

Results of serum sFas, IL-10 and IL-12 of ASC, CHB, LC and HCC patients were shown in Table 1 and Figure 1. The levels of IL-10, IL-12 and sFas increased gradually with the progression of chronic HBV infection. The *F* values for IL-10, IL-12 and sFas were 11.83, 10.47 and 26.36, respectively. The three indicator of ASC patients showed significant difference among CHB, LC and HCC patients (*P* < 0.001). The levels of IL-10 and sFas of LC and HCC patients were significantly higher than that in CHB patients (*P* < 0.05). The IL-12 level of HCC patients was also significantly higher in CHB patients (*P* = 0.01). However, differences of IL-12 between CHB and LC patients were not statistically significant (*P* = 0.154). Differences of three indicators between LC and HCC patients were not statistically significant (*P* > 0.05).

Detection of anti-HBx

Among the 90 patients with chronic HBV infection, anti-HBx of 27 cases were positive, 1 in ASC group (10%), 4 in CHB group (14.18%), 12 in LC group (46.15%), and 10 in HCC group (38.46%).

Comparison of sFas, IL-10 and IL-12 between anti-HBx positive and negative groups

The levels of IL-10 and IL-12 in anti-HBx positive group were significantly higher than that in anti-HBx negative group (*P* < 0.05), and the level of sFas between the two groups showed no significant differences (*P* = 0.094) (Table 2).

DISCUSSION

Progression of chronic hepatitis B infection is determined by the interaction of HBV antigen systems and the host immune system. IL-10, a potent anti-inflammatory Th2-type cytokine with immuno-

Table 1. Results of sFas, IL-10 and IL-12 in sera of patients in different stages of chronic HBV infection

Groups	Case number	IL-10 (ng/L)	sFas (ng/L)	IL-12 (ng/L)
ASC	10	13.93 ± 14.40	58.17 ± 77.42	15.64 ± 23.04
CHB	28	39.38 ± 20.77	179.88 ± 104.36	68.50 ± 23.14
LC	26	69.06 ± 46.37	249.22 ± 107.80	76.83 ± 12.82
HCC	26	62.82 ± 23.42	252.98 ± 87.65	83.74 ± 24.88

Table 2. Comparisons of levels of sFas, IL-10 and IL-12 between anti-HBx positive group and negative groups

Groups	Case number	sFas (ng/L)	IL-10 (ng/L)	IL-12 (ng/L)
Anti-HBx (-)	63	194.21 ± 111.64	45.98 ± 29.54	64.81 ± 27.57
Anti-HBx (+)	27	238.52 ± 118.33	65.73 ± 43.42	80.23 ± 29.69
<i>P</i> values		0.094	0.014	0.020

Figure 1. Levels of IL-10, IL-12 and sFas in serum of cases suffering from chronic HBV infection increased with disease progression.

Notes: The levels of IL-10, IL-12 and sFas were increased gradually with the progression of chronic HBV infection. The *F* values for IL-10, IL-12 and sFas were 11.83, 10.47 and 26.36, respectively. The three indicator of ASC patients showed significant difference among CHB, LC and HCC patients (*P* < 0.001). The levels of IL-10 and sFas of LC and HCC patients were significantly higher than those in CHB patients (*P* < 0.05).

suppressive activity may alleviate inflammation and protect the body from liver damage, but suppress the body's immunity and cause persistent infection. The level of IL-10 increases when the liver is under inflammation.⁷ IL-12 is a Th1-type cytokine, secreted by B lymphocytes, phagocytic cells and neutrophils under various stimulations, and plays an important role in the clearance of hepatitis B virus. Many studies showed that an imbalance between Th1 and Th2 in the form of dominant Th2 response existed in patients with chronic hepatitis B virus infection.⁸ The results of present study furtherly confirmed the theory. Although IL-10 and IL-12 were both increased with disease progression from asymptomatic carrier to patients with chronic hepatitis B, liver cirrhosis or hepatocellular carcinoma, the increase of IL-10 was more obvious than IL-12. As a result, comparatively higher level of IL-10 and lower level of IL-12 promote persistent infection and disease progression. Apoptosis is a regulatory mechanism which can remove infected, muted and senescent cells, so as to maintain body's physiological balance. The immunocompetent cell combines the apoptotic surface receptor proteins (Fas) of target cells through its ligand (FasL) and therefore initiates apoptosis and eliminates the infected cells.⁹ Serum Fas (sFas), an important soluble molecule with functional activity to regulate apoptosis, secreted by liver tissue, binds to cell membrane FasL, and reduces FasL's immune activity and the ability to cause apoptosis. As a result, the apoptosis of infected cells is reduced. Studies showed that sFas had a close relationship with the increase of TBil and the decline of prothrombin activity and albumin caused by late liver inflammation and damage.⁹ In this study, it is shown that with the disease progression of chronic HBV infection, sFas increased obviously, particularly in cases with cirrhosis or hepatocellular carcinoma, suggesting that sFas might play a very important role in the progression of liver cirrhosis and hepatocellular carcinoma. HBx protein plays an important role in liver tissue damage process in chronic HBV infection, particularly in cirrhosis and hepatocellular carcinoma.¹⁰ HBx protein stimulates the body to produce anti-HBx. However, the significance of anti-HBx and its role in the progress of the disease remain unclear, so does the significance of anti-HBx detection. In recent years, the positive rates of anti-HBx detection in several studies showed significant differences,¹¹⁻¹³ which might be caused by the differences of reagents, detection methods, sources of patient and other factors. The

anti-HBx positive rates for asymptomatic HBV carriers, patients with chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma in present study were 10%, 14.28%, 46.15% and 38.46%, respectively, showing a gradually increasing trend. To some extent, serum IL-10 and IL-12 reflect the humoral and cellular immunity, respectively, while Fas reflects inflammation, necrosis and apoptosis. Researches on the correlation of anti-HBx with IL-10, IL-12 and Fas were conducive to explore the significance of anti-HBx detection and its role in the progression of the disease. This study showed that the levels of IL-10, IL-12 and Fas in sera of anti-HBx positive group were higher than those of anti-HBx negative group, indicating that anti-HBx was the indicator of disease progression, liver inflammation and necrosis, and even cancer. Anti-HBx was not a protective antibody, but the evidence of CHB progression. Detection of anti-HBx in serum may become a serological marker for patients from asymptomatic carrier or chronic hepatitis to liver cirrhosis or hepatocellular carcinoma.

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