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Full Length Research Paper

Association of interleukin 27 expression and p28 gene polymorphism with chronic hepatitis B virus infection

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Interleukin 27 (IL-27) is a newly discovered cytokine encoded by 2 genes, EBI3 and p28. We investigated IL-27 serum levels and p28 gene -964 A>G polymorphism in patients with chronic HBV infection and in healthy individuals, with the aims to define the correlation between IL-27 expression and chronic hepatitis B development. 168 patients, 99 recovered individuals and 152 healthy individuals were enrolled in this study. IL-27 level was measured by dual antibody sandwich-ELISA, and p28 polymorphism was determined by PCR restriction fragment length polymorphism. IL-27 serum levels were significantly higher in patients than in healthy controls (P < 0.001). Among -964 A/G polymorphisms, A/A genotype was more closely associated with patients than healthy individuals (P = 0.013). There were no differences between recovered individuals and healthy individuals in both IL-27 levels and p28 polymorphism. IL-27 levels and p28 polymorphism are associated with the development of chronic hepatitis B.

Key words: Interleukin 27, chronic hepatitis B, IL-27 p28 -964 A>G polymorphism, cytokine, single nucleotide polymorphism.

INTRODUCTION

It is estimated that over 400 million people globally are hepatitis B virus (HBV) carriers and a large proportion of these infected individuals do not clear the virus, which may progress to persistent infection with or without liver disease (Akbar et al., 2007). Although an efficient cellular cytotoxic T-lymphocyte (CTL) immune response is a major contributor to HBV elimination, virus specific T cell appears to target a very few infected hepatocytes when chronicity develops (Bertoletti and Gehring, 2006). Numerous studies have shown that cytokines are of critical importance for modulating the intensity and duration of the host immune responses against HBV infections. Certain inflammatory cytokines, such as IFN-gamma, TNF-alpha (Togashi et al., 2000), and IL-2 (Guidotti et al., 1994) released by activated lympho/ mononuclear cells, are capable of restraining HBV gene expression and replication and play important roles in the clearance of viruses from the remaining infected cells by noncytolytic mechanisms. In contrast, IL-10 is a potent immunosuppressive cytokine that contributes importantly to immune tolerance toward HBV infections (Blackburn and Wherry, 2007). Thus, the host immunity might exert effects in the disease progression of hepatitis through the dynamic interaction of different cytokine expression profiles with HBV.

Interleukin 27 (IL-27), a novel member of the IL-12 cytokine family, is a heterodimeric molecule that consists of IL-12 p40-related protein encoded by the Epstein-Barr virus-induced gene 3 (EBI3) and IL-12 p35-related polypeptide encoded by the p28 gene. IL-27 is produced and released early by antigen-presenting cells in response to various inflammatory stimuli. It promotes rapid clonal expansion and differentiation of naive CD4+ T cells and plays an essential role in a link between innate and adaptive immunities. The cellular effects of IL-27 are mediated through a heterodimeric receptor complex that includes WSX-1 (specific for IL-27 and also named as T cell cytokine receptor) and gp130 (ubiquitously expressed by a variety of immune and non-immune cells) (Cordoba-Rodriguez and Frucht, 2003; Hunter, 2005; Hunter et al., 2004; Kastelein et al., 2007; Pflanz et al., 2002). As a close structural and functional homologue of IL-12, IL-27 is also involved in the host defense against the infections of intracellular pathogens (Fakruddin et al., 2007; Matsui

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Table 1. Characteristics of individuals investigated.

Characteristics	Patients with chronic hepatitis B	Recovered individuals	Healthy individuals
Number	168	99	152
Age	56.3 ± 10.4	47.1 ± 9.7	48.6 ± 13.5
Male/Female	110/58	60/39	95/57
ALT(IU/L)	286.2 ± 253.8*	30.1 ± 19.4	28.6 ± 16.7
AST(IU/ L)	264.7 ± 239.3*	25.7 ± 13.6	21.6 ± 10.5
Bilirubin (mg/dL)	2.42 ± 1.63*	1.03 ± 0.21	1.09 ± 0.17

ALT, alkanine aminotransferase; AST, aspartate aminotransferase; *P < 0.05 (Patients with chronic hepatitis B *versus* healthy individuals).

et al., 2004). Lines of evidence support the participation of IL-12 in HBV clearance since this cytokine enhances Th1 activity and negatively regulates HBV gene expression resulting in elimination of HBV viral antigens and effective treatment of chronic hepatitis B (CHB) (Rossol et al., 1997). However, for unknown reasons, little information is available on the association between IL-27 expression and HBV infection. Variations of cytokine expression and regulation among individuals are common and are dominated mainly by the host factors (e.g., gender, age, racial background and especially the gene polymorphisms). Polymorphisms in several cytokine genes, such as IFN-gamma (Qi et al., 2005; Yu et al., 2006), TNF-alpha (Du et al., 2006; Hohler et al., 1998), IL-10 (Cheong et al., 2006), IL-18 (Hirankarn et al., 2007; Zhang et al., 2005), and IL-1B (Migita et al., 2007), are considered to be correlated with the severity of liver disease in patients with HBV infection. In human IL-27 p28 gene, there is an important A versus G transition at position -964 in the promoter region, which was reported to be associated with susceptibility to asthma (Chae et al., 2007), providing the first available evidence of the association of IL-27 genetic variant with the development of chronic disease.

We conducted pilot studies to examine the differential serum levels of IL-27 in a cohort of chronic HBV infected Chinese patients with well-defined clinical status as well as in healthy individuals and evaluate the possible association of the -964 A>G polymorphism of IL-27 p28 gene with susceptibility to chronic hepatitis B infection, to allow an assessment of the correlation between IL-27 expression and the development of CHB.

MATERIALS AND METHODS

Patients and controls

One hundred and 68 patients with chronic hepatitis B infection were recruited from Zhongnan hospital of Wuhan university in this study from December 2007 through July 2008 (Table 1). The diagnosis of chronic hepatitis B was confirmed by the serological examination of HBsAg and the persistence of abnormal aminotransferase activity for more than 6 months. In addition, there were detectable anti-HBc IgG and HBV DNA but no anti-HBs in these patients, and 39 of them (23%) were serum hepatitis B early antigen (HBeAg) positive. All patients were heterosexuals and did not receive any anti-viral or immunosuppressive treatment before entry into this study.

The control group included 99 healthy individuals who recovered from HBV infection (HBsAg negative, anti-HBs positive and anti-HBc IgG positive) and 152 healthy volunteers (HBsAg negative, anti-HBs negative and anti-HBc IgG negative) who were matched for gender, age, ethnicity and residence to the chronic hepatitis B patients. All study subjects were seronegative for markers of HCV, HDV and HIV and had no other types of liver damage such as autoimmune hepatitis, alcoholic liver disease and Wilson's disease.

All the research protocols were reviewed and approved by the ethics committee of Wuhan university and informed consents were obtained from all participating individuals.

Detection of the levels of IL-27 in sera

Blood samples were acquired from all subjects and collected in pyrogen-free glass tubes and centrifugated at 1000 rpm for 10 min. Sera were removed and kept in pyrogen-free plastic tubes at -70℃ until analysis. The levels of IL-27 in sera were measured by dual antibody sandwich-ELISA according to the protocol provided by the manufacturer (R&D Systems, Inc., USA). Antibody AF2526 was used as a capture reagent and biotinylated antibody BAF2526 was used for detection. Standard curve using known amounts of recombinant human IL-27 (2526-IL) was generated. Each sample was tested in duplicate and the concentrations of IL-27 were calculated based on the standard curve. The minimum detectable dose of IL-27 was 0.1 pg/ml. This dual antibody sandwich-ELISA was also tested for the specificity, which showed no cross-reactivity with other IL-12 related cytokines (rmIL-27, rhIL-12 and rhIL-12 p40).

Genotyping the -964 A>G polymorphism of IL-27 p28 gene promoter

DNA was extracted from blood samples using phenol-chloroform following standard procedures. IL-27 p28 -964 genotypes were determined by PCR restriction fragment length polymorphism (PCR-RFLP). The primers designed for the IL-27 p28 -964 A>G promoter region were: 5'-GGCTGTGCTGGAAGGGAGAC-3' (forward) and 5'-ATATCTGGGACCAGGGTTAGG-3' (reverse). A 25 µl of PCR reaction mixture contained 0.2 µg of DNA, 5 pmol/l of each primer, 5 mmol/l of dNTPs, 1 U of Taq polymerase and PCR buffer with 1.5 mmol/l MgCl2. PCR conditions were one cycle of 94℃ for 5 min, followed by 35 cycles of 94℃ for 1 min, 56℃ for 45 s, 72℃ for 1 min and a final extension step at 72℃ for 10 min. 15 µl PCR products were subjected to digestion with XhoI restriction enzyme, which yielded DNA fragments of 468 bp for A/A genotype, 468/347/121 bp for A/G, and 347/121 bp for G/G. The digested PCR products were detected on 2% agarose gel electrophoresis and visualized under UV light with ethidium bromide stain.

Statistical analysis

Data were analyzed with SPSS11.5 software (SPSS Inc., Chicago,

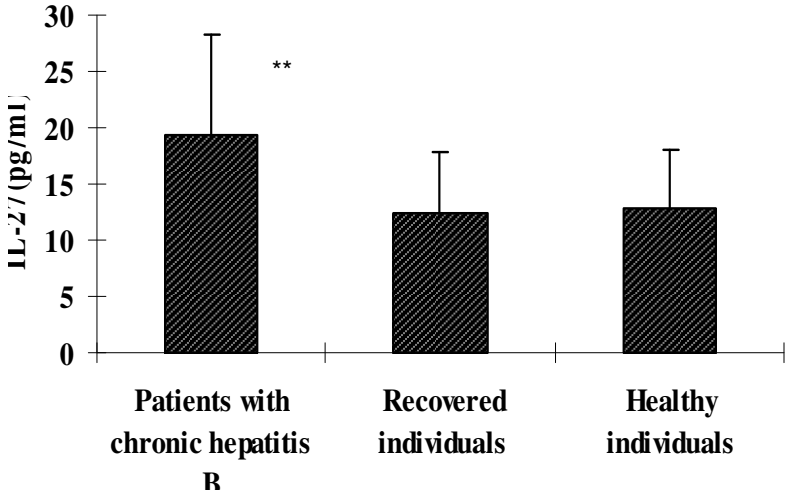

Figure 1. Serum IL-27 levels in the investigated groups, **P < 0.001 (Patients with chronic hepatitis B *versus* healthy individuals).

IL, USA). Serum IL-27 levels of different groups were compared using the student's t-test and analysis of variance (ANOVA). The chi-square test was used to determine Hardy-Weinberg equilibrium and compare the genotype and allelic frequency distributions of polymorphism in the chronic hepatitis B patient and control groups. The odds ratio (OR) was calculated by means of logistic regression and the confidence interval (CI) was calculated at the 95% level. All P values were 2-tailed and P values < 0.05 were considered to be statistically significant.

RESULTS

It has been reported that IL-27 can act as an inhibitor of HIV-1 replication in CD4+ T cells and macrophages and also as a potent adjuvant for epitope- specific CTL induction against HCV infection (Fakruddin et al., 2007; Matsui et al., 2004). In this study, we first investigated the differential levels of IL-27 in sera between patients with chronic HBV infection and healthy individuals. Results from enzyme immunoassay showed that the level of IL-27 in the sera of patients with chronic HBV infection was 19.4 ± 8.7 pg/ml and in healthy individuals was 12.9 ± 5.2 pg/ml, implicating IL-27 serum level in patients was significantly higher than that in healthy individuals (P < 0.001) (Figure 1), which was consistent with our previous data already published (Zhu et al., 2008). In addition, we noticed that the level of IL-27 in the sera of individuals recovered from hepatitis B virus infection was 12.3 ± 5.6 pg/ml, suggesting there was no significant difference between this group and the healthy control group (Figure 1).

To investigate the correlation between the polymerphism of IL-27 and the susceptibility to chronic hepatitis B, the -964 genotypes of IL-27 p28 gene were determined by the approach of PCR-RFLP and compared by the chi-square test in all the patients and controls. The distribution of genotype frequencies was found to be in Hardy-Weinberg equilibrium. Results showed that the distribution rates of -964 AA, AG and GG genotypes of IL-27 p28 gene were 56.2, 32.1 and 11.7% in patients with chronic hepatitis B, 40.4, 39.4 and 20.2% in individuals recovered from HBV infection and 42.1, 38.5 and 19.4% in healthy controls, respectively (Table 2). These results suggested that the genotype frequency of AA in patients with chronic hepatitis B was significantly higher than that in healthy controls (OR = 1.75; 95% CI: 1.12 - 2.72; P = 0.013). However, the genotype frequency of AA in individuals recovered from HBV infection was similar to that in healthy individuals. We also noticed that the allelic frequency of A and G was 72.0 and 28.0% in patients with hepatitis B, 60.1 and 39.9% in individuals recovered from HBV infection and 61.5 and 38.5% in healthy individuals, respectively (Table 2). These results demonstrated that the allelic frequency of A was significantly higher in patients with chronic hepatitis B compared to that in healthy individual group (OR=1.61; 95% CI: 1.16 - 2.25; P = 0.005). Also, the allelic frequency in individuals recovered from HBV infection was similar to that in healthy individuals.

The impact of the - 964 A>G single nucleotide polymerphism (SNP) of IL-27 p28 gene promoter on the expression level of IL-27 in the sera of investigated groups was also evaluated (Table 3). These results suggested that there was no significant correlation between the genotype of p28 gene promoter and the expression of IL-27.

DISCUSSION

Upon activation, naive T helper cells can differentiate into either Th1 or Th2 cells, 2 functionally distinct clones classified on the basis of the pattern of cytokines produced. In human, Th1 cells produce IFN-gamma and lymphotoxin, augment cell-mediated immunity (CMI) and help control intracellular pathogens, which are often referred to as Th1- type immune responses (Lucey et al., 1996). Cytokine IL-12 is thought to be the most important factor

Table 2. -964 polymorphism of IL-27 p28 gene promoter detected in the investigated groups.

Polymorphism	Patients with chronic hepatitis B	Recovered individuals	Healthy individuals
Genotype frequencies	n (%)	n (%)	n (%)
AA	94* (56.2)	40 (40.4)	64 (42.1)
AG	54 (32.1)	39 (39.4)	59 (38.5)
GG	20 (11.7)	20 (20.2)	29 (19.4)
Allelic frequencies	n (%)	n (%)	n (%)
A	242# (72.0)	119 (60.1)	187 (61.5)
G	94 (28.0)	79 (39.9)	117 (38.5)

* OR = 1.75; 95% CI: 1.12 - 2.72; P = 0.013 (Patients with chronic hepatitis B versus healthy individuals).
# OR = 1.61; 95% CI: 1.16 - 2.25; P = 0.005 (Patients with chronic hepatitis B versus healthy individuals).

Table 3. IL-27 serum levels in the investigated groups according to the three genotype distributions.

Groups	Genotype		
	AA	AG	GG
	IL-27 (pg/ml)	IL-27 (pg/ml)	IL-27 (pg/ml)
Patients with chronic hepatitis B (n = 168)	18.7 ± 8.4	20.8 ± 9.6	19.1 ± 8.8
Recovered individuals (n = 99)	12.8 ± 5.6	11.7 ± 6.1	12.9 ± 5.8
Healthy individuals (n = 152)	12.6 ± 5.0	13.6 ± 5.7	12.1 ± 5.2

that influences the development of Th1-type immune responses (Trinchieri, 1995). In contrast, Th2 cells secrete cytokines such as IL-4, IL-5 and IL-9, which are involved in humoral immunity, allergic responses and host defense against helminths (Lucey et al., 1996). Previous investigations on IL-27 function revealed that this cytokine promotes the development of naive CD4+ T cells into Th1 cells at an earlier stage and synergizes with IL-12 to trigger the production of signature Th1 cytokine, IFN-gamma, in naive CD4+ T cells and NK cells. Thus, IL-27 is also believed to be necessary for the efficient induction of Th1 responses and critical for the initial host defense against intracellular pathogens.

In this study, we demonstrated that patients with chronic hepatitis B had significant higher serum levels of IL-27 compared to those in healthy controls, indicating that patients with HBsAg positive chronic hepatitis are not immunologically unresponsive and IL-27 production is not impaired during HBV infection. Since IL-27 is important for Th1 promotion and persistent Th1 responses can lead to excessive CMI and uncontrolled tissue damage, it is reasonable to speculate that the incidence of fatal hepatitis in chronic hepatitis B patients may be in part due to the high production of IL-27. However, most chronic HBV infection is in fact characterized by deficient CMI and efficient humoral responses, namely, Th2 predominant responses. It is obvious that the elevated IL-27 expression in chronic hepatitis B patients is not merely a positive signal for them to generate Th1 phenotypes. Indeed, subsequent investigations uncovered that IL-27 in vivo may act primarily as a negative immune regulator by reducing the proliferation and differentiation of a variety of immune cells and limiting the induction of either Th1 or Th2 responses (Batten and Ghilardi, 2007; Villarino and Hunter, 2004). Upon binding to its receptor, IL-27 activates several Jak/STAT signaling pathways to carry on its immunosuppressive functions. But the exact molecular mechanisms that initiate the inhibitory properties of the Jak/STAT signaling remain elusive (Yoshimura et al., 2006). Another important mechanism by which IL-27 functions is the regulation of pro-inflammatory or anti-inflammatory cytokine expression. IL-10, a major immunosuppressive and anti-inflammatory cytokine, has strong correlations with the process of HBV replication and viral persistence in chronic hepatitis B patients. Analyses of the mutual relationship of IL-27 and IL-10 have brought to light the fact that IL-27 up-regulation of IL-10 by certain effector T cells contributes to the immunosuppressive role of IL-27 (Awasthi et al., 2007; Batten et al., 2008; Fitzgerald et al., 2007; Stumhofer et al., 2007). In addition, IL-27 can antagonize potent immune stimulators that are important for T cell proliferation and critical for sustained inflammatory responses. Suppression of IL-2 expression either by IL-27 itself or by IL-27 induction of suppressor of cytokine signaling 3 (SOCS3) is presumed to facilitate the inhibitory effects of IL-27 (Owaki et al., 2006; Villarino et al., 2006). For the above reasons, we postulate that IL-27 may play a major role in promoting Th1 responses during the early acute phase of HBV infection, because the complete clearance of HBV needs a coordinated and quick-responded innate and adaptive cell-mediated immune response. In the situation of chro-

nic HBV infection, due to the extremely long lasting time course of virus-host interaction, the immune responses must be elaborately regulated by key factors including IL-27 to fight the virus and to simultaneously avoid harmful damage to the host. Thus, the increased expression of IL-27 in patients seems to reflect one of the host's self-defensive mechanisms. To this end, the 2 distinct functions of IL-27 are quite impressive.

It has been demonstrated that IL-27 can down-regulate Th2 responses by inhibiting expression of the Th2 factor Gata binding protein 3 (GATA-3) (Yoshimoto et al., 2007), implicating its potential protective role in diseases such as asthma and chronic obstructive pulmonary disease (COPD), which are characterized by airway hyperreactivity and predominant Th2-type immune responses. Indeed, polymorphisms in the IL-27 p28 gene that might influence IL-27 expression were shown to be associated with susceptibility to such diseases (Huang et al., 2008). To our knowledge, this study is the first to report the association of the IL-27 gene promoter polymorphism with susceptibility to chronic HBV infection. A significant difference was found between patients with chronic hepatitis B and healthy individuals in both IL-27 p28 -964 A>G polymorphism genotype and allelic distributions, with the genotype frequency of -964 A/A and the allelic frequency of -964 A increased in chronic hepatitis B group. These results confirmed the clinical significance of p28 polymorphism in the development of chronic diseases.

The statistically significant relationship between IL-27 p28 -964 A>G polymorphism and IL-27 concentrations was not found in this study, however, as it was the only SNP examined, we can not rule out the possibility that genetic variants from other loci of IL-27 may participate in the alteration of transcription activity of IL-27. On the other hand, IL-27 p28 -964 polymorphism and IL-27 serum levels possibly predict the outcomes of infection independently, as is often the case (Wennberg et al., 2002). The elevated IL-27 levels in patients could be aroused by HBV itself because viral proteins have the capacity to activate signal transduction pathways resulting in abnormal expression of cytokines (Mogensen and Paludan, 2001). Actually, our previous data confirmed that transfection of hepatocyte-derived cell lines with HBV protein expression vectors could induce the production of biologically active human IL-27 (Zhu et al., 2008). In summary, chronic hepatitis B infection is a multifactorial process depending on both host and viral parameters. The accumulated information on IL-27 is likely to provide a new prognostic marker for predicting the CHB development. As IL-27 is a pluripotent cytokine with the properties of both exciting and suppressing immune responses, IL-27 must coordinate with host Th1/Th2 cytokines in shaping and fine tuning of the immune responses, which contributes to resistance to and recovery from viral infections. Any imbalance in the Th1/Th2 cytokine microenvironment will to some extent alter the defense mechanism and the capacity of immunity, allowing virus to evade immune surveillance, hence establishing a persistent infection.

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