IL-18 promoter -137G/C polymorphism correlates with chronic hepatitis B and affects the expression of interleukins

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Summary. – The relationship between the interleukin (IL)-18 promoter -137G/C polymorphism and plasma levels of IL-18, IL-12, IL-4, and IFN-γ in chronic hepatitis B (CHB) patients and healthy subjects was investigated. The polymorphism was genotyped by a ligase detection reaction-PCR (LDR-PCR), while the cytokines were assayed by ELISA. Compared with healthy subjects, CHB patients exhibited an increased frequency of the G allele, GG genotype and increased IL-4 levels, but decreased levels of IL-18, IL-12, and IFN-γ. A positive correlation for IL-18 ~ IL-12 ~ IFN-γ and a negative correlation for IL-18 ~ IL-4 were found. We conclude that the IL-18 promoter -137G polymorphisms correlated with CHB infection and influenced the expression of IL-18. The studied interleukins represent an immunomodulatory network that plays important roles in host immune responses to CHB infection.

Keywords: chronic hepatitis B; polymorphism; interleukins; interferon gamma; cytokines

Introduction

Hepatitis B is an infectious inflammatory illness of the liver caused by the hepatitis B virus (HBV) that affects humans. China is among the highly endemic countries with approximately 8% of the population being chronically infected with the virus (Maddrey, 2000). During the HBV infection, the host immune response causes both hepatocellular damage and viral clearance. Th1 cells are characterized by their capability of producing Th1 cytokines, interferon γ (IFN-γ), IL-2, and tumor necrosis factor α (TNF-α), whereas Th2 cells are able to synthesize the Th2 cytokines IL-4 and IL-10 (Mosmann *et al*., 1996). The communication network between Th1 and Th2 cytokines is complex. Recent serological studies suggest that the Th1-Th2 balance may contribute to the induction and/or maintenance of persistent HBV infection (Maruyama *et al*., 1993).

IL-18 is a pleiotropic proinflammatory cytokine that has immunomodulatory effects on both the innate and acquired immune systems (Dinarello, 1999). It promotes development of Th1 lymphocyte response by induction of gamma-interferon production in synergy with IL-12 (Yoshimoto *et al*., 1998). IL-18 expression and effector function has been described in the complex pathogenesis of allergic inflammation (Kodama *et al*., 2000), in the development of chronic graft-versus-host disease and autoimmune disease (Bossù *et al*., 2000; Okamoto *et al*., 2000), in various hematological disorders (Takubo *et al*., 2000), rheumatoid arthritis (Leung *et al*., 2000), human sepsis (Grobmyer *et al*., 2000), and in human immunodeficiency virus 1 (HIV-1) infection (Torre *et al*., 2000). Polymorphisms in the gene promoter probably lead to different levels of cytokine expression and let some individuals have unique immune responses. Genetic associations between single nucleotide polymorphisms (SNPs)

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in the IL-18 promoter G/C or C/C genotype at −137 and spontaneous hepatitis C virus clearance have been reported in African-American injection drug users (An *et al.*, 2008). In order to investigate the relationship of the IL-18 promoter -137G/C polymorphism to the expression of cytokines, we determined the abovementioned polymorphism and plasma levels of IL-18, IL-12, IL-4, and IFN-γ in CHB patients and healthy subjects. We found that (i) IL-18 promoter -137G/C polymorphism correlated with CHB and influenced the expression of IL-18, (ii) IL-18 and other interleukins construct immunomodulatory network, which plays important role in host immune responses against CHB.

Materials and Methods

Subjects. A total of 276 cases of CHB patients (167 males, 109 females) aged 34–56 years were recruited in Xiangyang central hospital from December 2006 to December 2010. The diagnosis of all the patients was confirmed according to the criteria for CHB (Lok *et al.*, 2001), and the patients did not have other viral hepatitis. 254 healthy subjects (138 males, 116 females) aged 32–54 years were randomly selected in Xiangyang area, China, during the same period, with negative antibodies specific for viral antigens HBsAg, HBe, and HBc, and with no history of HBV vaccination. They did not have any abnormalities based on physical examination, chest radiography, electrocardiogram, urinanalysis and routine laboratory blood testing. Liver, renal, endocrine and cardiovascular disorders were excluded. All study subjects were unrelated Chinese Han people in Xiangyang region, P. R. China. Each participant was recruited with signed informed consent. The ethics committee of the Xiangyang central hospital approved the study.

PCR. Fasting venous blood was collected from an antecubital vein in 10 ml Na-EDTA. Total DNA was extracted from peripheral blood leukocytes using standard techniques and frozen at -20°C. The sequences of the PCR primers used are listed in Table 1. PCR amplifications were carried out in a volume of 50 μl and included 10 mmol/l Tris-HCl buffer pH 8.3 containing 50 mmol/l KCl, 2.0 mmol/l MgCl$_2$, 200 μmol/l dNTPs, 300 nmol/l each of forward and reverse primers, 1.5 U of Amplitaq Gold DNA polymerase and between 50 and 100 ng of total DNA. The polymerase was activated by heating to 95°C for 15 min, and amplification was achieved by thermal cycling for 35 or 40 cycles at 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min, and 72°C for 7 min for a final extension. The polymerase was inactivated by addition of 2 μl of proteinase K to each reaction, incubating at 56°C for 20 min and then 95°C to denature the proteinase. Four microliters of the PCR product were analyzed alongside appropriate negative and positive controls on a 2% agarose gel to verify the presence of the expected amplification product.

LDR-PCR. The sequences of the LDR-PCR probes used are listed in Table 1. LDR-PCR reactions were carried out in a 20 μl mixture containing 20 mmol/l Tris-HCl pH 7.6, 10 mmol/l MgCl$_2$, 100 mmol/l KCl, 10 mmol/l DTT, 1 mmol/l EDTA, 1 mmol/l NAD+, 12.5 nmol/l

of the oligonucleotides, 3 μl of PCR product from each sample, and 0.1 mmol/l Thermus thermophilus (Tth) DNA ligase. LDR-PCR reactions were thermally cycled for 30 cycles of 30 sec at 94°C and 2 min at 60°C. Reactions were stopped by adding 0.5 μl of 0.5 mmol/l EDTA. Aliquots of 2.5 μl of the reaction products were mixed with an equal volume of loading buffer (80% formamide, 10 mmol/l EDTA, and 1.2% Blue Dextran). The mixture was subjected to PAGE in denaturing conditions.

Sandwich ELISA. Sandwich ELISA was performed to measure the concentrations of the plasma IL-18, IL-12, IFN-γ, and IL-4, using the human IL-18, IL-12, and IL-4 ELISA kit (Bender Medsystems Inc., Burlingame, Calif., USA), and the human IFN-γ ELISA kit (R&D Systems Europe, Ltd., Abingdon, UK). All the biochemical measurements were carried out using standard methods.

Statistical analysis. Data are expressed as means ± SD for normally distributed variables. Qualitative data are presented as numbers (percentages). All calculations were performed using Statistical Package for Social Science (SPSS) software (version 13.0). A difference with P ≤0.05 was considered significant.

Results

IL-18 promoter -137G polymorphisms correlate with CHB infection

The -137G/C polymorphism of IL-18 was genotyped by the LDR-PCR. Genotype and allele frequencies for IL-18 polymorphisms are summarized in Table 2. The genotype distribution was in line with the Hardy-Weinberg equilibrium. As shown in Table 2, there were GG, GC, and CC genotypes at the position -137. Of the 276 CHB patients studied, 221 had the GG type (80.1%), 51 had the GC type (18.5%) and 4 had the CC type (1.4%). 168 of the 254 healthy subjects were type GG (66.1%), 80 were GC (31.5%) and 6 were CC (2.4%). There was a significant difference in the genotype distribution and in the allele frequency between the CHB patients and the healthy subjects. Expressed in genotypes, the GG at the position -137 was present at a significantly higher frequency in the CHB patients compared to those in the controls. The allele G at -137 had a significantly higher frequency in the CHB patients than in the control group ($\chi^2 = 7.94$, P = 0.003). We conclude that IL-18 promoter -137G polymorphisms correlate with CHB infection.

Negative correlation between IL-18 promoter -137G polymorphisms and IL-18 plasma levels

We studied the correlation between the plasma IL-18 protein levels and the genotypes of IL-18 polymorphism at the -137 position, and we obtained the following data (Table 3). The plasma IL-18 protein concentration levels

Table 1. The primers and probes used in PCR and LDR-PCR

	Sequence (5'-3')	PCR product (bp)
Primers for PCR		
	TCAGAAAGAGGTACAGGTTTTGG	155
	CCTTCAAACAGGCTCTTTCC	
Probes for LDR-PCR		
	P-TTTTCTTCCGTAAAAGTTGGGGCT CTTTTTTTTTTTTTTTTTTTTTTT-FAM	
	TTTTTTTTTTTTTTTTTTTTTTGTAA TATCACTATTTTCATGAAATG	94
	TTTTTTTTTTTTTTTTTTTTTTTTGT AATATCACTATTTTCATGAAATC	96

Table 2. IL-18 promoter -137G/C polymorphism in CHB patients and healthy subjects

Polymorphism	Healthy subjects	CHB patients	χ^2	P
Genotypes				
GG	168 (66.1)	221 (80.1)	8.49	0.002
GC	80 (31.5)	51 (18.5)	7.78	0.005
CC	6 (2.4)	4 (1.4)	0.56	0.483
Alleles				
G	416 (81.9)	493 (89.3)	7.94	0.003
C	92 (18.1)	59 (10.7)		

Table 3. Negative correlation between the IL-18 promoter -137GG genotype and IL-18 plasma levels in CHB patients and healthy subjects

	Genotype	n	IL-18	P
Healthy subjects	GG	168	73.1±25.5	
	CG/CC	86	94.7±29.6	<0.001
CHB patients	GG	221	36.6±17.2	
	CG/CC	55	54.4±21.5	<0.001

IL-18 levels are given in pg/ml.

Table 4. Plasma levels of interleukins in CHB patients and healthy subjects

	Healthy subjects	CHB patients	P
IL-18	84.5±28.2	53.6±20.3	<0.001
IL-12	110.3±38.4	72.1±32.8	<0.001
IFN-γ	243.7±67.6	182.4±54.2	<0.001
IL-4	33.7±17.4	61.5±20.7	<0.001

Interleukin levels are given in pg/ml.

in GG homozygotes are significantly lower than in carrier of the C allele in CHB patients group or healthy subjects, respectively. As shown in Table 3, IL-18 plasma levels in CHB patients carrying the GG genotype are significantly lower than in healthy subjects with the GG genotype at the position -137.

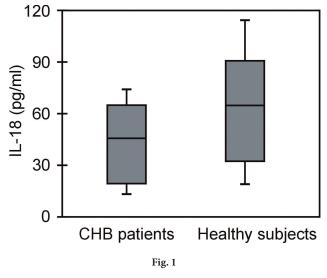*Levels of plasma cytokines in CHB patients and healthy subjects*

The levels of Th1 cytokines IL-18, IL-12, IFN-γ, and Th2 cytokine IL-4 were analyzed in CHB patients and healthy subjects (Table 4, Fig. 1). As shown in Table 4, the concentrations of IL-18, IL-12, and IFN-γ were significantly lower in the CHB patients than in healthy subjects, but IL-4 was opposite. These data indicate that the secretion of Th1 cytokines in CHB patients differs significantly from healthy subjects.

Discussion

In this study, we demonstrated that, compared to the healthy subjects, CHB patients present a characteristic reduced production and flat secretory pattern of Th1 cytokines, preceding an increased and/or prolonged secretion of the Th2 cytokines. These findings further support the concept that CHB is associated with an imbalance between the up-regulated Th2 and the down-regulated Th1 arms of the immune system. In immunopathogenesis of CHB, the HBV-induced liver injury and viral clearance are mediated by the host immune response, the peripheral cytotoxic T-lymphocyte response is usually weak or undetectable and narrow in focus, and a significant role seems to be played by a disturbed secretory function of Th1 cells. An activated humoral response develops, however, which is characterized by the production of IL-4, IL-5, and IL-10 that are secreted by the Th2 cells. This response promotes antibody production rather than viral clearance (Guidotti *et al.*, 2000). In contrast to previous reports, where cytokine levels in CHB patients were sampled separately (Kimura *et al.*, 2002), we examined Th1 cytokines IL-18, IL-12, and IFN-γ, and the Th2 cytokine IL-4. This enabled us to provide a more comprehensive analysis of the Th1/Th2 imbalance in CHB patients. IL-18 is a unique cytokine that enhances innate immunity and both Th1- and Th2-driven immune responses (Nakanishi *et al.*, 2001). During the HBV infection, the protein HBx induces IL-18 expression in liver, which may be associated with hepatic injury by amplifying FasL expression (Lee *et al.*, 2002). IL-18 can induce the peripheral blood mononuclear cells (PBMCs) from CHB patients to produce high levels of IFN-γ, and increase their ability to kill the cells infected

with the virus (Kimura *et al.*, 2002; Sun *et al.*, 2003). IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell-stimulating factor and can stimulate the growth and function of T cells (Hsieh *et al.*, 1993). IFN-γ is the primary cytokine, which defines Th1 cells. It causes more naive T cells to differentiate into Th1 cells, thus representing a positive feedback loop, while suppressing Th2 cell differentiation. IL-4 is a cytokine that induces differentiation of naive T cells into Th2 cells. Our results suggest an inhibited function of Th1, as reflected by a reduced release of IFN-γ, IL-18, and IL-12, but Th2-dominated immune responses (such as the expression of IL-4) increased. Because the anti-HBV activity of Th1 lymphocytes is strongly induced by IL-12+IL-18 and may contribute to viral clearance in HBV infection, the ability of immune cells to kill cells infected by virus decreased and HBV could not be eliminated (Szkaradkiewicz *et al.*, 2005). These data enhance the importance of a comprehensive cytokine analysis and suggest that IL-18 plays an important role in the development of Th imbalance by modulating the local cytokine network.

The outcome of an HBV infection is mainly influenced by the virus, immune response and genetic diversity (Thursz *et al.*, 2001; Chu *et al.*, 2002). Many studies strongly support the role of host genetic components in determining the outcome of HBV infection (Ben-Ari *et al.*, 2003; Frodsham, 2005; Ramezani *et al.*, 2008). Diverse polymorphic variants of cytokine genes may induce high or low levels of protein production (Arimitsu *et al.*, 2006). Genetic susceptibility to the HBV infection is considered to be determined at different functional levels, such as cytokine production, antigen presentation and receptor recognition, and the SNPs of cytokine genes involved in the immune response after HBV infection show the importance of the genetic susceptibility, which may highlight the genetic background of HBV infection (Cheong *et al.*, 2010). It was found that SNPs in the promoter of IL-18 gene at the position -137G/C (RS187238) can influence the expression of IL-18 and potentially also of the IFN-gamma (Giedraitis *et al.*, 2001).

In this study, we have evaluated the associations between CHB patients and healthy individuals at the IL-18 gene position -137. A significantly higher frequency of G allele or GG-genotype was observed in CHB patients compared to healthy individuals, indicating that the GG homozygotes had a higher occurrence rate for CHB when compared to the C allele carriers. It has been found that the -137 G/C polymorphism was in the functional promoter region. Our data shown that the IL-18 promoter -137G polymorphisms correlate with the production of IL-18, affect the expression of IL-18 and potentially also of IFN-γ, which is consistent with an earlier report (Arimitsu *et al.*, 2006). We found that IL-18 protein concentration

Fig. 1

IL-18 levels in CHB patients and healthy subjects

levels in GG homozygotes were significant lower than in C allele carriers in CHB patients group or control group, respectively. Therefore, the people with allele C at the position -137 in the promoter of the IL-18 gene may be protected against chronic HBV infection (Zhang *et al.*, 2005).

In summary, the findings of this study provides further evidence that immune response and genetic diversity are important in the pathogenesis of HBV infection. Our results indicate that the IL-18 promoter -137G/C polymorphism correlates with chronic hepatitis B and affects the expression of interleukins. IL-18 and other interleukins construct immunomodulatory network, which plays an important role in host immune responses against HBV. However, the real roles of IL-18 gene promoter polymorphisms in the pathogenesis of developing chronic hepatitis B should be further investigated by large population-based studies.

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