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Expressions of ASC and caspase-1 but not AIM2 are disrupted in chronic HBV infected patients

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PEER REVIEW

Peer reviewer

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Comments

This paper is an interesting research on expression of the AIM2 inflammasome in CHB infection. The results show that there are significant differences in expression of ASC and caspase-1 between CHB patients and controls. These findings suggest that there is a link between CHB infection and AIM2 inflammasome function.
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ABSTRACT

Objective: To determine the mRNA levels of absent in melanoma 2 (AIM2), apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) and caspase-1 in the peripheral blood mononuclear cells of chronic hepatitis B virus (CHB) infected patients.
Methods: This study was conducted on 60 CHB patients and 60 healthy controls and the mRNA levels of AIM2, ASC and caspase-1 were examined in parallel with beta-actin (as housekeeping gene) using real-time polymerase chain reaction technique.
Results: Our results indicated that expression of ASC and caspase-1 but not AIM2 were significantly decreased in peripheral blood mononuclear cells isolated from CHB patients compared to healthy controls.
Conclusions: According to our results, it is likely that CHB patients are unable to firmly express ASC and caspase-1 genes and in turn properly activate IL1β and IL-18 subsequent to hepatitis B virus infection. Thus, these results propose a mechanism which almost partially may describe a reasonable fact that why the infection is sustained in the CHB patients.

KEYWORDS

Chronic HBV infection, AIM2, ASC, Caspase-1, Real-time PCR

1. Introduction

Chronic hepatitis B virus (CHB) infected patients exhibit a clinically active form of hepatitis B in which hepatitis B virus (HBV) is not completely removed either from liver hepatocytes or serum of patients, resulting in an intermediate symptomatic liver disease[1,2]. Several research teams have proposed that CHB could be considered as one of the main etiological features involved in pathogenesis of hepatocellular carcinoma and cirrhosis[3,4]. The predominant mechanism(s) responsible for the development of CHB and other manifestations associated with HBV infection to initiate these pathologies has yet to be identified. Researchers believe paramount importance of multifactorial parameters, such as immunological, genetical and epigenetical characteristics,

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of hosts are responsible for the differences between individuals with resistant HBV strains and those who overcome the disease by clearing the virus[2,5-7]. Absent in melanoma 2 (AIM2) is amongst the main intracellular receptors that recognize microbial dsDNA, including viruses, to induce apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) and caspase-1 activation[8]. Activated caspase-1 in turn cleaves pro-IL1β and IL-18, the two inflammatory cytokines, to yield their activated form[9]. The cleaved IL-1β and IL-18 induce expression of several inflammatory molecules including selectins and integrins[10]. In addition to the above concerns, these inflammatory cytokines also rule some immune-associated reactions such as neutrophils infiltration, fever, *etc*[10]. Therefore, insufficient expressions of AIM2, ASC and caspase-1 may lead to inappropriate immune responses against viral infection. Due to the fact that CHB infected patients fail to completely eradicate intracellular HBV from the hepatocytes, it seems that these patients are functionally defective in their immune systems. According to the important role played by AIM2, ASC and caspase-1 in the processes of dsDNA recognition along with induction of immune responses against dsDNA genomic viruses, it may be speculated that these molecules are crucially important in pathogenesis of CHB. Therefore, the main aim of this study was to investigate the mRNA levels of AIM2, ASC and caspase-1 in the peripheral blood mononuclear cells (PBMCs) of CHB patients.

2. Materials and methods

2.1. Subjects

Peripheral blood samples were collected from 60 CHB infected patients and 60 healthy controls in Rafsanjan and Yazd (Located in southeastern and central parts of Iran) in 5.5 mL tubes with and without anti-coagulant. CHB infected patients with co-infected hepatitis C virus and HIV were excluded from the study. The diagnosis of CHB was performed based on the Guide of Prevention and Treatment in Viral Hepatitis[11] by an expert internal medicine specialist. All of patients were enrolled in the study based on assessment of their previous clinical records and controls were selected with the same age, sex and socio-economic status. Evaluation of socio-economic conditions was measured regarding the levels of education and the monthly income of participants. The samples without anti-coagulant were centrifuged at 3 500 r/min for 4 min, and to achieve qualitative specimens, the serums were separated within 8 h after collection. The serum samples were stored at -20 ℃ for a maximum of 2 months or at -70 ℃ when longer storage periods were required for analysis. The anti-coagulant treated samples were also immediately subjected to RNA extraction (to obtain either more quantitative or qualitative RNA samples) following entrance to the laboratory. This study was approved by the ethical committee of the Rafsanjan University of Medical Sciences and written informed consent was obtained from all of participants prior to sample collection.

2.2. Detection of serological HBV markers

All of the samples were screened for hepatitis B virus surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) using enzyme-linked immuno sorbent assay (Behring, Germany) according to the manufacturer's guidelines.

2.3. HBV-DNA extraction and real-time polymerase chain reaction (PCR) conditions

The viral DNA was purified from 200 μL of plasma from CHB patients using a commercial kit (Cinnaclon, Iran) according to manufacturer's guidelines. The HBV-DNA amplification was also undertaken using a commercial kit (Primer Design Company, UK) following the manufacturer's instructions.

2.4. RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was extracted from PBMCs using RNX solution from Cinnaclon Company (Iran). The extracted RNA quality was determined by electrophoresis on the ethidium bromide pretreated agarose gel and measured by absorption on 260/280 nm by spectrophotometer following by cDNA synthesize using a cDNA synthesis kit (Parstous, Iran) with both oligo and random hexamer primers (Aryatous, Iran). The reverse transcription step was performed with the following program: 70 ℃ for 10 min (without reverse transcription enzymes), -20 ℃ for 1 min (cooling), 42 ℃ for 60 min (added reverse transcription enzymes) and 95 ℃ for 10 min (reverse transcription enzymes inactivation). Quantitative real-time PCR was performed by addition of SYBR green master mix (Parstous, Iran), 200 ng of produced cDNA, and 2 pg/μL of appropriate primers (Table 1).

Table 1
Primer sequences of evaluated genes.

Target gene	Primer sequences
AIM2	F: 5'-CAG GAG GAG AAG GAG AAA GTT G-3'
	R: 5'-GTG CAG CAC GTT GCT TTG-3'
ASC	F: 5'- AAC CCA AGC AAG ATG CG-3'
	R: 5'-TTA GGG CCT GGA GGA GCA AG-3'
Caspase 1	F: 5'-GCT TTC TGC TCT TCC ACA CC-3'
	R: 5'-CAT CTG GCT GCT CAA ATG AA-3'
β-Actin	F: 5'- GGC ACC CAG CAC AAT GAA G -3'
	R: 5'- CCG ATC CAC ACG GAG TAC TTG -3'

Following program was set on the Bio-Rad CFX96 system (Bio-Rad Company, USA): one cycle of 95 ℃ for 15 min, 40

cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. Real-time PCR were carried out in triplicate and the β-actin as housekeeping gene was used for normalization of amplification signal of target genes. The relative amounts of PCR product were determined using the $2^{-\triangle\triangle Ct}$ formula. The dissociation stages, melting curves and quantitative analyses of the data were performed using CFX manager software version 1.1.308.111 (Bio-Rad, USA).

N.b.: PCR products were also electrophoresed on 1% gel containing 0.5 mg/mL ethidium bromide to check the size of PCR product.

2.5. Data analysis and statistical methods

The parametric statistical analyses were performed using the *t*-test and ANOVA under SPSS software version 18. *P* value less than 0.05 was considered significant.

3. Results

3.1. Detection of HBsAg and HBeAg

Our results demonstrated that all of patients were HBsAg positive, and only two patients were positive for HBeAg.

3.2. Quantification of HBV-DNA copy numbers

Our results showed that all of patients had detectable HBV-DNA. Among the patients, 40 had HBV DNA levels less than 20 000 copies/mL, 12 patients between 20 000 to 400 000 copies/mL, and 11 patients were higher than 1 000 000 copies/mL.

3.3. Expression of target genes

Our results showed that expression of AIM2 in the PBMCs of CHB patients was decreased 1.35-fold compared with healthy controls. Statistical analysis of data revealed that the difference was not significant (P=0.458) (Figure 1). The results also demonstrated that expression of ASC and caspase-1 was reduced 2.63- and 3.33-fold in the PBMCs of CHB patients compared with healthy controls. Statistical analysis showed that the differences between groups regarding ASC (P<0.001) and caspase-1 (P=0.04) were significant (Figure 1). Our results also demonstrated that expression levels of AIM2 (P=0.961) and caspase-1 (P=0.417) were not changed in CHB patients with different viral load, while the mRNA levels of ASC was significantly decreased (P=0.019) in CHB patients with HBV-DNA viral load less than 20 000 and higher than 1 000 000 copy copies/mL in comparison to patients suffering from viral load between 20 000 to 400 000 copies/mL (Figure 2).

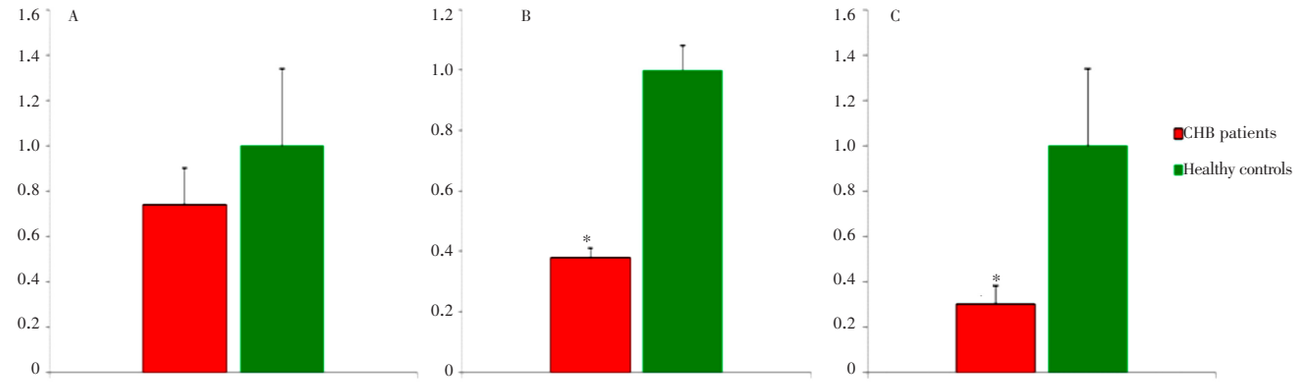

Figure 1. mRNA levels of AIM2, ASC, and caspase-1 in PBMCs of CHB patients and healthy controls.
A: AIM2, B: ASC, C: caspase-1. Results are shown as mean±SE. *: Significant differences between CHB patients and healthy controls.

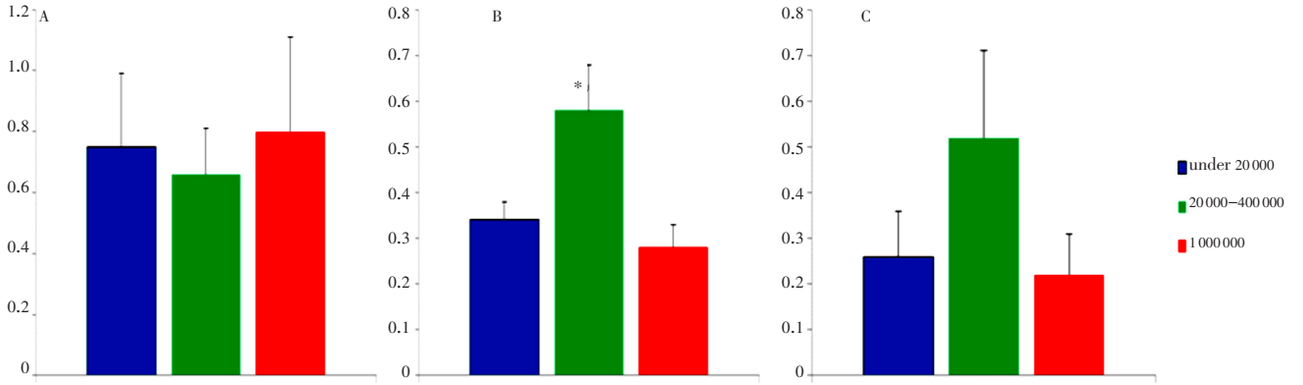

Figure 2. mRNA levels of AIM2, ASC, and caspase-1 in PBMCs of CHB patients with different HBV-DNA viral load.
A: AIM2, B: ASC, C: caspase-1. Results are shown as mean±SE. *: Significant differences in ASC molecule expression between CHB patients with less than 20 000 HBV-DNA copies/mL compared to patients with viral load between 20 000 to 400 000 (P=0.03) and higher than 1 000 000 (P=0.02) copies/mL.

4. Discussion

Initiation of immune responses against viral infections starts via cytokine production. The IL-1β and IL-18 are the main cytokines produced as the first lines of immune responses against viral infections[12]. These cytokines are expressed as inactive pro-cytokines and are activated subsequent to cleavage by caspase-1[13]. Although our results demonstrated that mRNA expression of AIM2 did not differ, ASC and caspase-1 mRNA levels were significantly decreased in the CHB patients when compared to healthy controls. Therefore, based on our results, it can presumably be concluded that CHB patients are unable to express adequate levels of ASC and caspase-1. Thus, they probably are incapable to activate IL-1β and IL-18 due to lack of favorable amount of ASC and caspase-1 to response against HBV infection and in turn completely eradicate the infection. Interestingly, there are two additional viral sensors (NLRP3 and RIG-I) that use ASC and caspase-1 pathway to produce activated IL-1b and IL-18[14]. Therefore, it seems that the NLRP3 and RIG-I pathways are also defected in the CHB patients. Due to the fact that IL-1β and IL-18 showed the ability to inhibit HBV replication[15], it seems that HBV suppresses ASC and caspase-1 expression via unknown mechanism(s) to overcome of immune system inhibitory effects on its replication. Interestingly, the current results revealed that the decreased expression of ASC is associated with high HBV-DNA copy numbers (higher than 1 000 000 copies/mL), hence, it seems that HBV-DNA can suppress ASC expression. Moreover, Manigold *et al.* reported that hepatitis B core antigen (HBcAg) can causes induced IL-18 production by induction of caspase-1[16]. Because the findings of Manigold *et al.* are based on *in vitro* examinations of the effect of HBcAg taking together with our *in vivo* model, it seems that chronic HBV replication inhibits the effect of HBcAg *in vivo*. Additionally, HBcAg can also stimulate IL-10, an anti-inflammatory cytokine, production by immune cells of CHB patients[17]. IL-10 has antagonistic effects with ASC and caspase-1[18], hence, it seems that HBcAg can suppress expression of ASC and caspase-1 possibly in an IL-10 dependent pathway *in vivo*. The caspase-1 activation not only is required for activation of IL-1β and IL-18 but also is necessary for maximal production of other pro-inflammatory cytokines[19]. For instance, active caspase-1 has been shown to facilitate secretion of IL-1α, tumor necrosis factor and IL-6 through cleavage of the toll-like receptor adapter protein TIRAP (Toll/interleukin-1 receptor domain containing adapter protein) and consequently enhance TIRAP signaling[20]. Therefore, it seems that down-regulation of caspase-1 in CHB patients may attenuate immune responses due to deficit in pro-inflammatory cytokines production. Interestingly, pyroptosis (the phenomenon of caspase-1 dependent cell death) is regulated by caspase-1[21], hence, one of the mechanisms responsible for elevated risk for hepatocellular carcinoma in the CHB patients may be related to reduction of hepatocyte-derived caspase-1. In order to confirm this hypothesis, more accurate studies using the liver biopsy of CHB patients are needed. To the best of our knowledge, this is the unique and first report regarding expression of AIM2, ASC and caspase-1 in CHB patients, but some other studies demonstrated that AIM2 and its corresponding molecular signaling is essential for activation of IL-1β and IL-18 during infection with vaccinia virus[22], cytomegalovirus[22] and *Listeria monocytogenes*[23]. Collectively, based on our results and other reports, it can be concluded that AIM2 downstream signaling molecules (ASC and caspase-1) expressions are defective in the CHB patients. However, we can not propose the exact mechanism underlying down-regulation of these signaling molecules in CHB patients, but these findings may open an avenue for the investigators of this field to focus on designing therapeutic reagents to overcome this defect in CHB patients. Furthermore, results of current study suggest a model for future therapy which up-regulation of these molecules is of paramount importance. This may be based on cytokine activation via these molecules and subsequently eradication of HBV from CHB patients.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Chronic HBV infection is an important global health problem affecting about 350 million people worldwide and is associated with cirrhosis and hepatocellular carcinoma. AIM2 is an important intracellular receptor recognizing viral dsDNA and activates ASC and caspase-1. This process lead to expression of inflammatory molecules and appropriate immune responses against viruses.

Research frontiers

In this study, mRNA expression of AIM2, ASC, and caspase-1 has been evaluated by real-time PCR technique in both 60 chronic HBV infected patients and 60 healthy controls. Therefore, the report compares mRNA expression levels in CHB patients with controls in southeast Iran.

Related reports

This is the first study investigating expression of AIM2, ASC and caspase-1 in CHB patients. However, there are some studies about AIM2 inflammasome that is essential for host defense against cytosolic bacteria and DNA viruses. AIM2 is essential for inflammasome activation in response to *Francisella tularensis*, vaccinia virus, mouse cytomegalovirus, and *Listeria monocytogenes* (Rathinam *et al.* 2010).

Innovations & breakthroughs

This is the first report evaluating mRNA levels of AIM2, ASC, and caspase-1 in chronic hepatitis B patients. AIM2 has a crucial role in the activation of caspase-1, the protease responsible for the processing of pro-inflammatory cytokines IL-1β and IL-18.

Applications

The results demonstrate that ASC and caspase-1 mRNA levels were significantly decreased in CHB patients. Therefore, deficient expression of ASC and caspase-1 in CHB patients could lead to an inappropriate immune response against HBV. The results of this study may be helpful in treating chronic HBV infection.

Peer review

This paper is an interesting research on expression of the AIM2 inflammasome in CHB infection. The results show that there are significant differences in expression of ASC and caspase-1 between CHB patients and controls. These findings suggest that there is a link between CHB infection and AIM2 inflammasome function.

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