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Plasma Levels of Interleukin-10 (IL-10) and transforming growth factor β (TGF-β) in Hepatitis B virus (HBV) Infected Egyptians

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RJAB

Abstract

Hepatitis B virus infection (HBV) is a non-cytopathic virus; therefore, liver damage leading to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) is considered to be the direct result of the immune response to viral infection. Cytokines play critical roles in the pathogenesis of HBV. The balance between Th1/Th2 has a great impact on the outcome of HBV infection. Interleukein (IL-10) and transforming growth factor (TGF-β1) are important regulators of the immune response due to their anti-inflammatory action on the immune system. Given the importance of these cytokines in the complicated immune response against HBV, it is advantageous to assess their levels in the plasma of HBV patients and comparing them with normal healthy controls. Measuring IL-10 and TGF-β1 levels were achieved by enzyme-linked immunororbent assay (ELISA) in 118 Egyptian HBV patients and 119 normal healthy controls. Our results showed a significant ($p<0.001$) sharp reduction in the level of IL-10 in comparison to controls. Hepatitis B was significantly correlated with reduction in plasma IL-10 level ($r= -0.864$; $p<0.001$). On the other hand there, results presented here indicated that there is a significantly elevated serum level of TGF- β1 ($p<0.001$) compared with that of controls. A direct correlation was reported between TGF-β1 and HBV infection ($r= 0.157$, $p<0.05$). Accordingly, high levels of TGF-β1 may be a mechanism by which immune response against HBV is suppressed. In conclusion, HBV might be associated with reduction in IL-10 coincides with elevation in TGF-β1. Our results give a shed of light about the importance in balance between different cytokines in pathogenesis of HBV and its role in suppressing the immune system during viral infection.

Key Words: IL-10, TGF-β, hepatitis B, Egyptians

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1. Introduction

However, there is available highly effective vaccine, Hepatitis B virus infection (HBV) remains a global dilemma and it is still a significant etiology of chronic HBV, cirrhosis and hepatocellular carcinoma (HCC), especially in Asian and African countries (**Dienstag, 2008; Sonneveld *et al*., 2010**). More than 350 million people globally, representing 5% of world population, are being affected by HBV infection with a mortality rate over 1.2 million deaths per year (**Liang et al., 2009; Kew, 2010**). Both viral and host genetic factors are involved in the viral pathogenesis of liver disease (**Miyazoe *et al*., 2002**). HBV infection may persist and progress to chronic hepatitis, fibrosis and HCC (**Fattovich, 2003**). The risk of HBV persistence is related mainly to two major factors, the age at which infection is contracted and the immune status of the host since chronic hepatitis B occurs in less than 5% of adults and more than 90% of infants (**Vildózola and Salinas, 2009**). Thus, host immune response to HBV is believed to be the major determinant of the clinical course of HBV infection. In self-limiting acute hepatitis, there is strong T cell response against multiple viral epitopes, while this response is weak or absent in chronically infected patients (**Chisari and Ferrari, 1995**).

Cytokines are small glycoproteins which are produced by the immune cells whereas they are involved in both inflammatory and anti-inflammatory responses during the course of infection (**Arababadi *et al*., 2010**). Cytokines play an important role in defense since they directly inhibit viral replication and indirectly determine the predominant pattern of the host immune response (**Koziel, 1999; Takakura *et al*., 2007**). Therefore, the interplay of different cytokines determines the fate of HBV infection. There are two major types of T-helper cells which are the prevailing cytokine-producing cells, which are Th1 and Th2. Both types have distinct functions, and they secrete a wide variety of cytokines. Th1 cells mainly produce interferon-γ (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and enhance cellular immune responses, in contrary, Th2 cells secrete IL-4, IL-5, and IL-10 and promote humoral immune reactions. Preferential activation of these T cell subsets in many infectious diseases is thought to determine the outcome of disease. It has been established that HBV patients with a Th1 polarization are capable of being ultimately recovered (**Penna *et al*., 1997; Miyazoe *et al*., 2002**). On the other hand, Th2 polarized HBV patients may have a persistent chronic infection (**Xing *et al*., 2001**).

IL-10, a T regulatory cytokine, has a promising immunosuppressive and anti-inflammatory role (**Spits and Malafyt, 1992; Moore *et al*., 2001**). IL-10 is produced by various cells including T cells, monocytes/macrophages, Kupffer cells, hepatocytes, and hepatic stellate cells (**Grove *et al*., 2000**). It exerts its immuno-suppressive via the inhibition of macrophage dependent antigen presentation, T-cell proliferation, and down-regulation of Th1 cytokines such as IFN-γ, IL-2 and TNF-α (**Fiorentino *et al*., 1989; Fiorentino *et al*., 1991a; Redpath *et al*., 2001; Pestka *et al*., 2004; Wu *et al*., 2010**). Additionally, IL-10 exerts its potent anti-inflammatory effects via down-regulating the synthesis of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-12, IL-18, TNF-α) and chemokines by monocytes/macrophages and Kupffer cells and

RJAB

up-regulates the synthesis of IL-1 receptor (IL-1R) antagonist. Neutrophil chemotaxis and chemokine expression are also down-regulated. IL-10 has also been reported to play a vital role in the regulation of immune responses in HBV infection (**Payvandi *et al*., 1998; Powell *et al.*, 2000**). IL-10 has also anti-fibrogenic effects in the liver (**Tsukamoto, 1998**) through inhibition of collagen gene transcription and increased collagenase expression by hepatic stellate cells (**Louis *et al*., 1998**).

TGF-β1 is a critical anti-inflammatory cytokine which is produced by from a variety of liver cell populations including HSCs, hepatocytes, and LSECs in addition to platelets and infiltrating mononuclear cells **(Bedossa *et al*., 1995; Gressner, 1995)** during the homeostasis and tissue remodeling (**Regateiro *et al*.,2011**). TGF-β1is the chief fibrogenesis inducing factor in the liver as its over-expression leads to hepatic fibrosis (**Sanderson *et al*., 1995; George *et al*., 1999; Kanzler *et al*., 1999; Qi *et al*., 1999**). Moreover, TGF-β has potential impact on the immune response since it has immunosuppressive effects like its inhibitory effect on T-cells proliferation via IL-2 down-regulation (**Kehrl *et al*., 1986**). Moreover, it inhibits B cell proliferation, induces apoptosis of immature or resting B cells, and blocks B cell activation and isotype class switching **(Li *et al*., 2006)**. Chronic HBV patients are incapable of eradicating the infection from their hepatocytes in which some light was shed the possible role TGF-β1 up-regulation in suppression of the immune response in the liver hepatocytes. Thus, it is plausible to hypothesize that the down-regulation or up-regulation may lead to inappropriate immune responses against HBV. Therefore, we have tailored the following study to assess the plasma levels of IL-10 and TGF-β1 in Egyptian HBV patients.

2. Materials and Methods

2.1. Patients and controls

One hundred and eighteen patients with HBV infection recruited from the National Liver Institute, Menofia University, Egypt were enrolled in this study. The males over numbered the females (98 men and 20 women) with mean age of 34.4±10.69 years (range: 68-22). One hundred and nineteen healthy controls with no history of previous liver disease, normal liver function tests, and negative HBV and HCV serology were involved in the study. Patients with HCV or other viral infections or any liver diseases were excluded from the study. All investigations were performed in accordance with the Menofia University, Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethics committee approved the study protocol and informed consents were got from all subjects.

2.2. Virological assessment

Hepatitis B surface antigen (HBsAg) was tested using commercially available kits (Sorin Biomedica, Milan, Italy) and confirmation of the presence of HBV-DNA in HBV-positive patients was tested by a standard polymerase reaction (Roche Diagnostics Corp., Indianapolis, IN). HCV antibodies were tested by using enzyme-linked immunosorbent assay (ELISA) (Murex Biotech Ltd., Dartford, U.K.) All

patients were positive for HBsAg, HBV-DNA, and negative for HCV antibodies. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) (bioMérieux S.A, Marcy l'Etoile, France), direct and indirect bilirubin (Roche Diagnostics Corp., Indianapolis, IN), and albumin (Human Gesellschaft Fur Biochemica Und DiagnosticaMbh,Wiesbaden, Germany), were all measured according to their respective kits' manufacturers' instructions.

2.3. Measurement of plasma IL-10 and TGF-β1 levels

Blood was collected by withdrawal of 5 ml venous blood from each individual involved in this study into sterile vacutainer tubes containing $EDTA.K_3$ (Tri-potassium Ethylene diamine tetra acetic acid), then the tubes were centrifuged at 1500 rpm for 10 minutes. Plasma was separated, aliquoted and stored at-80º. Total concentrations of TGF-β1 plasma levels were measured in HBV patients and normal controls by sandwich enzyme linked immunosorbent assay (ELISA) (DuoSet® ELISA Development Systems, R&D System, Inc., Minneapolis, USA) according to manufacturer's instructions. IL-10 was measured as previously described by **Talaat *et al.* (2007)** with slight modifications.
Briefly, microtiter plates were coated with anti-cytokine monoclonal antibody for 1 hr at 37°C followed by overnight incubation at 4°C. On the next day, coated plates were washed and blocked with blocking buffer for 2.5 h at 37°C to saturate non-specific binding sites. Plasma specimens were added in in duplicate wells. Plates were incubated for 2 h and subsequently washed with washing buffer. Polyclonal detecting antibody was added and incubated for additional 2 h. After washing excess antibodies, horseradish peroxidase (HRP)-conjugated goat anti-human IgG was used prior to color development with 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide substrate solution The reaction was stopped by 50 µl/well of 1M HCl stopping buffer. The absorbance of each well was measured at 450nm using a microplate reader (SunriseTM, Tecan Group Ltd. Männedorf/Switzerland) and plotted against a standard curve with standard levels expressed as pg/ml. Each plasma sample was analyzed in duplicates. The ELISA reader-controlling software (Softmax) readily processes the digital data of raw absorbencies value into a standard curve from which cytokine concentrations of unknown samples can be derived directly and expressed as pg/ml.

RJAB

2.4. Statistical analysis

The statistical analyses were performed by SPSS statistical package version 19 (SPSS, IBM Corporation, USA). Comparisons were made using independent *t* test and results were presented as Mean ± SD. Correlation between variables was determined using Spearman's correlation test. In all of the tests the level of significance was set at $P < 0.05$.

3. Results

All samples were examined for the biochemical and virological parameters. The demographic and biochemical characteristics are presented in **Table (1)**. The HBV patients were positive for HBsAg and HBV-DNA whereas the level of HBV-DNA is

statistically significant ($p<0.05$). They were all negative for HCV antibody and HCV-RNA. As expected, level of liver enzymes showed significant increase in the patients group ($p<0.001$) compared with normal subjects.

Table (1): Demographic and biochemical characteristics of HBV patients and healthy controls

Parameter	HBV group (N=119)	Control group (N=118)	P
Age (Mean± SD)	44.92±11.76	32.11±14.89	NS
Gender (Male ♂: Female ♀)	53/12 (81.5 / 18.5%)	14/36 (28% / 72%)	p<0.001
<u>Liver function tests</u>			
AST (IU/L)	42.01±2.75	20.96±0.53	p<0.001
ALT (IU/L)	44.12±4.13	18.91±0.46	p<0.001
Albumin (g/L)	3.42±0.09	4.35±0.07	p<0.001
Total Bilirubin (mg/dl)	1.09±0.07	0.66±0.01	p<0.001
Direct Bilirubin (mg/dl)	0.31±0.06	0.12±0.01	p<0.001
<u>Kidney function tests</u>			
Creatinine (mg/dl)	1.11±0.04	0.89±0.01	p<0.001
Urea (mg/dl)	33.4±1.73	28.93±0.645	p<0.001
HBV DNA (IU/L)	1641329±958497	-	-

* All data are presented as mean ± SE. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST)

A positive association was recorded between the level of ALT ($r=0.4541$; $p<0.001$) and AST ($r=0.489$; $p<0.001$). In addition, the significant reduction of Albumin ($p<0.001$) which was negatively correlated with the ($r=-0.541$; $p<0.001$). Furthermore, levels of both total and direct bilirubin were statistically significant ($p<0.001$) in HBV patients in relation to normal controls with positive correlation with the disease ($r= 0.518$, $p>0.001$; $r= 0.247$, $p<0.001$ for total and direct bilirubin; respectively. Concerning the kidney function, levels of both urea and creatinine were significantly elevated ($p<0.001$).

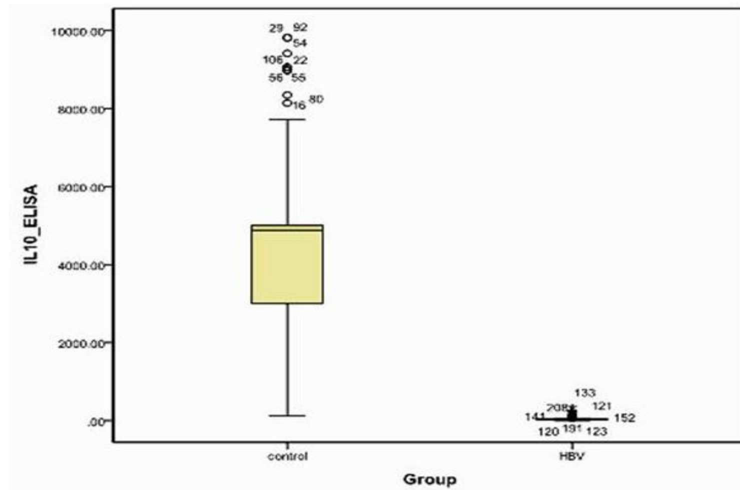

Figure (1): Serum levels of IL-10 in HBV patients in comparison with normal controls. Data are presented as box plots with lines inside the box representing medians, boxes representing the 25th and 75th percentiles and the lines outside the boxes indicating the 10th and 90th percentiles

As regards IL-10, mean plasma concentrations was significantly lower ($p<0.001$) in HBV patients than normal controls (40.0±4.98 versus 4398±209 pg/ml) (**Fig 1**). Hepatitis B was significantly correlated with reduction in plasma IL-10 level ($r= -0.864$; $p<0.001$).

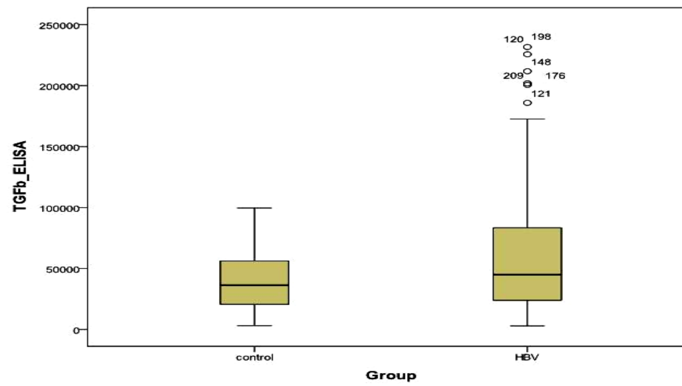

Figure (2): Serum levels of TGF-β1 in HBV patients in comparison with normal controls. Data are presented as box plots with lines inside the box representing medians, boxes representing the 25th and 75th percentiles and the lines outside the boxes indicating the 10th and 90th percentiles

On the other hand, mean serum concentrations of TGF-β1 were significantly increased in HBV patients (60881±4955) as compared to normal cases (39210±2443 pg/ml) (**Fig 2**). A direct correlation was reported between TGF-β1 and HBV infection ($r= 0.157$, $p<0.05$)

4. Discussion

HBV is a non-cytopathic, hepatotropic virus. Cytokines network plays important role during immune response against viral infection (**Arababadi *et al.*, 2012**). During HBV infection CD4+T cells , including Th1 and Th2, recognize the antigens on viral nucleocapsid which are presented by MHC class II (Major histocompatibility complex) on the surface of APCs, as consequence, CD4+T cells proliferate and release cytokines which induce antiviral humoral and cell-mediated response. Th1 cells produce IFN-γ, IL-2 and TNF-α which activate cell-mediated effector response such as T cell proliferation and up-regulation of MHC molecules on hepatocytes. On the other hand, Th2 produce IL-4, IL-5, IL-6 and IL-10 which play a vital role in B cell development and induction of humoral response. Th1 and Th2 counteract each other whereas IL-10 inhibits Th1. Therefore, Th1/Th2 cytokines balance is believed to be a factor determining the infection outcome since some studies have shown that low levels of IFN-γ and TNF-α have been in correlation with chronic HBV infection (**Ferrari *et al.*, 2003; Buke *et al.*, 2004; Akpolat *et al.*, 2005**). Therefore, the pathogenesis of HBV infection is highly affected by imbalance of Th1/Th2 cytokines (**Penna *et al.*, 1997**). The identification of biomarkers with potential diagnostic or prognostic significance for chronic hepatitis is considered a priority of clinical hepatology.

Interleukin-10 is anti-inflammatory Th2 cytokine. It mainly inhibits Th1 cytokines as IFN-γ and cytokines produced by macrophages such as TNF-α, IL-1, IL-12 and chemokines. Previous studies have reported the inhibitory effect of IFN-γ and TNF-α on HBV replication through non-cytolytic pathways (**Guidotti *et al.*, 1994; Guidotti *et al.*, 1996; Guidotti and Chisari, 2001**) but it was found that IL-10 acts against their actions (**Fiorentino *et al.*, 1989; Fiorentino *et al.*, 1991a; Mosmann, 1994**). Therefore, IL-10 may compromise the host immune response to acute viral infection (**Moore *et al.*, 1993; Cacciarelli *et al.*, 1996; Koziel, 1999**). Assessment of the plasma IL-10 levels showed a significant sharp reduction in HBV patients than in the healthy controls which is consistent with the previous study which reported the reduction of IL-10 mRNA expression in liver patients than in controls (**Napoli *et al.*, 1996**).). In agreement with our data, a significant decrease of IL-10 in non-responders HBV infected patients was previously observed **(Velu *et al.*, 2008).** On contrary to our results, **Bozkaya *et al.* (2000)** and **Wu *et al.* (2010)** reported that patients who contracted chronic HBV infection had serum IL-10 levels higher than the healthy controls. Furthermore, other studies reported that IL-10 levels didn't show difference between the patients and healthy controls in other disease (**Cookson *et al.*, 1999)**

TGF-β1, as an anti-inflammatory cytokine, plays key roles in the regulation and suppression of immune responses (**Regateiro *et al.*, 2011**). Therefore, any significant alteration in the expression of TGF-β1 may lead to inappropriate immune responses

against viral infections. Our results demonstrated that the expression of TGF-β1 significantly increased in HBV patients in comparison with healthy controls. Our results are in agreement of the previous studies of **Khorramdelazad *et al*., (2012).** They found an elevation in the TGF-β1 level as a sign of the suppression exerted on the immune system. **Khorramdelazad *et al.* (2012)** results revealed that the serum levels of TGF-β were significantly increased in chronic HBV patients in compare to healthy controls. Many studies conducted before to show the effect of TGF-β1 on the liver and its diseases (**Divella *et al*., 2012; El-Tayeh *et al*., 2012; Guo *et al*., 2008**) in which all of them demonstrated the elevated level of TGF-β1. A conspicuous point to state is that elevation in TGF-β1 in HBV patients, more than healthy controls can be justified as the immune response is complex system in addition to the oddness of immune response during HBV infection (**Mondelli *et al*., 2010**). Moreover, some researches have demonstrated the strong relationship between the immune response and the normal functioning of the hepatocytes (**McMahon, 2009; Bertoletti *et al*., 2010**). Viral factors may also be culpable of altering the TGF-β1 Level as in a study of **Li *et al*., (2010)** revealed that HBcAg induces TGF-β1 production in HBV patients.

5. Conclusion

In conclusion, cytokines may be useful marker in the monitoring of disease progression and might be an inseparable part of assessment of chronic hepatopathies. TGF-β1 may have a role in the immune suppression in HBV patients according to our results. Therefore, conducting a comprehensive study on a large size sample will be a confirmatory to unravel what are the underlying relationships between TGF-β1 and hepatocytes as one side and TGF-β1 and the viral factors as the other side. Analysis of further cytokines participating in HBV is essential in further studies.

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RJAB

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RJAB

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