Proinflammatory Cytokines and Its Correlation with Liver Injury

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ABSTRACT

Background: *A persistent infection of hepatitis B virus (HBV) can cause liver cirrhosis and hepatocarcinoma even though the virus itself is non-cytopathic and does not cause cell injury. It has been asserted that liver injury in chronic HBV infection is attributed to the host immune system responding to HBV infection. Cytokines have a critical role in mediating immune responses to viral infection. This study aimed to determine the correlation between the levels of serum interferon gamma (IFN-γ), interleukin-2 (IL-2), interleukin-17 (IL-17), and tumour necrosis alpha (TNF- α) with the progression of liver fibrosis that was determined through aspartate aminotransferase/AST to platelet ratio index (APRI) score and FibroScan in patients with chronic HBV infection.*

Method: *Blood samples were collected from patients with chronic hepatitis B and the levels of serum IFN-γ, IL-2, IL-17, and TNF-α were measured by using enzyme-linked immunosorbent assay (ELISA). The correlation between each cytokine levels and APRI Score and FibroScan were analyzed by using the Spearman correlation test with a p value of < 0.05 is considered as statistically significant.*

Results: *A total of 47 samples were collected from patients with chronic hepatitis B (n = 38), chronic hepatitis B with liver cirrhosis (n = 6), and chronic hepatitis B with hepatocellular carcinoma (n = 3). No significant correlation between cytokines and the progression of liver fibrosis was found.*

Conclusion: *There was no significant correlation between the levels of serum IFN-γ, IL-2, IL-17, and TNF-α with liver fibrosis, which may indicate that those cytokines are not directly involved in liver fibrosis.*

Keywords: *Hepatitis B virus (HBV), Hepatitis B, interferon gamma (IFN-γ), interleukin-2 (IL-2), interleukin-17 (IL-17), tumour necrosis factor alpha (TNF-α)*

ABSTRAK

Latar belakang: *Infeksi virus hepatitis B (HBV) yang persisten dapat menyebabkan sirosis hepar dan hepatokarsinoma walaupun HBV sendiri merupakan virus non-sitopatik dan tidak menyebabkan kerusakan sel. Diketahui bahwa kerusakan hepar pada infeksi HBV kronis disebabkan oleh respon sistem imun terhadap infeksi HBV. Sitokin memiliki peran penting dalam memediasi respon imun terhadap infeksi virus. Penelitian ini bertujuan untuk menentukan korelasi antara kadar interferon gamma (IFN-γ), interleukin-2 (IL-2), interleukin-17 (IL-17), dan tumour necrosis alpha (TNF-α) dalam serum dengan progresivitas fibrosis hepar yang ditentukan berdasarkan nilai aspartate aminotransferase/AST to platelet ratio index (APRI) dan FibroScan.*

Metode: *Sampel darah diambil dari pasien dengan hepatitis B kronis dan kadar IFN-γ, IL-2, IL-17, dan TNF-α dalam serum diukur menggunakan enzyme-linked immunosorbent assay (ELISA). Korelasi antara kadar sitokin yang diperiksa dengan nilai aspartate aminotransferase/AST to platelet ratio index (APRI) dan FibroScan dianalisis menggunakan uji korelasi Spearman dengan nilai p < 0,05 dianggap sifgnifikan secara statistik.*

Hasil: *Total 47 sampel telah dikumpulkan dari pasien dengan hepatitis B kronis (n = 38), hepatitis B kronis dengan sirosis hepar (n = 6), dan hepatitis kronis dengan hepatokarsinoma selular (n = 3). Tidak ditemukan korelasi yang signifikan antara sitokin dan progresivitas fibrosis hepar.*

Simpulan: *Tidak didapatkan korelasi yang bermakna antara kadar IFN-γ, IL-2, IL-17, dam TNF-α dalam serum dengan progresivitas fibrosis hepar, dapat mengindikasikan bahwa sitokin-sitokin tersebut tidak terlibat langsung dalam fibrosis hepar.*

Kata kunci: *Hepatitis B virus (HBV), Hepatitis B, interferon gamma (IFN-γ), interleukin-2 (IL-2), interleukin-17 (IL-17), tumour necrosis factor alpha (TNF-α)*

INTRODUCTION

Hepatitis B is a liver disease caused by hepatitis B virus (HBV). HBV infection still remains as global health issue. There are 257 million people that had been infected by HBV and caused more than a million deaths per year, mostly caused by cirrhosis and hepatocarcinoma (HCC).[1] HBV is known to be a non-cytopathic virus and does not damage the infected liver cells; i.e., hepatocytes. The damage of liver cells during HBV infection is mainly caused by specific immune responses against infected hepatocytes in order to eliminate the virus. A persistent HBV infection can cause chronic liver disease and liver injury, leading to liver cirrhosis, hepatocarcinoma and liver failure. T Cells play a crucial role in the course of HBV infection. An adequate T-cell response is essential for spontaneous resolution from HBV acute infection and necessarily important to prevent persistent infection. T-cells' responses are controlled by other substances such as cytokines.[2,3]

Cytokines are produced by immune cells, mainly by T-cells. T helper 1 (Th1)-associated cytokines such as interferon gamma (IFN-γ) and interleukin-2 (IL-2) contribute to cellular immune responses to viral infection. On the other hand, T helper 2 (Th2)-associated cytokines such as IL-4, IL-6, and IL-10 are involved in humoral responses. Another group of cytokines such as IL-17, IL-22, and IL-23 is associated with Thelper17 (Th17) and they mediate inflammation. Apart from T cells, macrophages also produce a cytokine called the tumour necrosis factor (TNF) family which play a critical role in inflammation and the acute phase of infection. During HBV infection, all of those cytokines are involved in various pathways. For example, the IFN family enhances the activities of macrophages, natural killer (NK) cells, and $CD8^+$ T cells to eliminate the virus and inhibits the replication of HBV whilst the TNF family mediates liver inflammation and cell injury. Another example is IL-2 enhances the activities of cytotoxic T cells (CTL) and NK cells aiming at HBV clearance whereas IL-17 inhibits the replication of HBV.[4]

Chronic infection of HBV can develop into liver fibrosis, liver cirrhosis, and hepatocellular carcinoma.[5-9] Among 15-20% patients with active viral replication during chronic infection of HBV, cirrhosis will develop within 5 years, and the incidence of hepatocellular carcinoma increased 70-90% in patients with cirrhosis.[6] Liver fibrosis is an important link in the progression of chronic viral hepatitis to cirrhosis.[10] Before experiencing liver cirrhosis and HCC, patients with chronic hepatitis B will experience liver fibrosis which can be observed using CT scan, MRI, or FibroScan. The accumulation of fibrous tissue changes the normal structure of liver and lead to the loss of liver function.[11-13] Cirrhosis is a final stage of chronic liver disease, resulting from a continuous process of inflammation, destruction, and regeneration of liver parenchyma, representing a form of diffuse fibrosis and regenerative nodules. Liver cirrhosis itself is a risk factor for hepatocellular carcinoma.[14]

Aspartate transaminase (AST) and alanine transaminase (ALT) are transaminase enzymes found mainly in liver and other organs, such as muscle, heart, pancreas, kidneys, and red blood cells. Increased AST and ALT levels may indicate liver injury. A damage in liver cell can cause immediate release of AST and ALT into the bloodstream.[15,16] As we know, HBV infection can cause liver cells damage because the excessive response of immune system responding to

the virus infection. Elevated ALT levels are generally more specific for liver damage, whereas elevated AST levels can be caused by other extrahepatic organs, such as muscle disorders.[17] Previous study has stated that increased AST/ALT ratio is associated with poor outcome in patients with hepatocarcinoma.[18] However, increased AST and ALT levels does not necessarily occur in every patients with HBV infection, and because AST and ALT can be found in organs other than liver, AST and ALT are less specific for liver injury or liver fibrosis.[16,17,19]

It has been well known that liver biopsy is a golden standard for diagnosing liver tissue injury as this method is highly sensitive and specific. However, liver biopsy is an invasive procedure that can only be performed by specialized health workers. Besides, the procedure is quite expensive and is often not readily available in many health centres in developing countries. Therefore, a new diagnostic procedure that is cost-effective, less-invasive, and generalist is needed to help the clinician ascertain an accurate diagnosis in HBV infection. Previous studies have already validated the utility of AST to platelet ratio index (APRI) as a non-invasive predictor of liver fibrosis with 30% sensitivity and 93% specificity.[20-23] WHO recommend two cut-off levels to define cirrhosis using APRI. A lower cut-off points, 0.5, which has high sensitivity to rule out the presence of fibrosis, and higher cut-off value, 1.5 which has high specificity used to diagnose fibrosis or indicates a high probability of having cirrhosis.[24] APRI is an index for assessing liver fibrosis and cirrhosis that is easy to perform, cost-effective, only require simple serum and haematology test, and does not require specialized training.

This study aimed to assess the levels of serum IFN-γ, IL-2, IL-17, and TNF-α in patient with chronic HBV infection and analyze the correlation between those serum cytokines levels and liver fibrosis and/or cirrhosis based on APRI and FibroScan in patients with chronic hepatitis B.

METHOD

This study is an analytic observational study with a cross-sectional approach. Blood samples were collected from patients with chronic hepatitis B who were admitted to Dr Moewardi Hospital, Surakarta, Indonesia from 14 October 2017 to 21 December 2017. The levels of serum IFN-γ, IL-2, IL-17, and TNF-α were measured by using an ELISA kit (Koma Biotech Inc., Korea) according to the standard protocol. Supporting data such as provisional diagnosis, patient's age, FibroScan data, levels of aspartate transaminase, and levels of platelet were retrieved from the patient's medical record. In this study, FibroScan was classified according to METAVIR score, F0 F1, F2, F3, and F4. APRI is calculated by using APRI online calculator (https://www.hepatitisc.uw.edu/page/clinical-calculators/apri) with upper limit of normal AST levels is 35 according to *Pedoman Interpretasi Data Klinik* (clinical data interpretation guidance) by Indonesian Health Ministry in 2011.[25] The data were analyzed by the Spearman correlation test with a p-value of < 0.05 was considered statistically significant. This study is part of a larger research project studying the immunology of hepatitis B and C in Surakarta, Indonesia. The study protocol has been approved by the Human Research Ethics Committee at Dr Moewardi hospital (No. 548/ VIII/ HREC/ 2017).

RESULTS

A total of 47 patients with chronic HBV infection have participated in this study; 38 (81%) were diagnosed with chronic hepatitis B (CHB), 6 (13%) had liver cirrhosis, and the rest (n = 3; 6%) had hepatocellular carcinoma. About one-third (34%) of the samples were collected from early elderly (46-55 years old). 21 (45%) patients had APRI score below lower cut-off points (0.5), and 3 patients (6%) had APRI score above higher cut-off points (1.5) (Table 1).

Only 11 from 47 patients in this study undergo the FibroScan procedure. The limited number of FibroScan samples can be caused by many things, cost, for example. The cost of FibroScan in Indonesia is relatively expensive, so it is not easily accessible to the lower socioeconomic classes who need information about the degree of fibrosis during infection. Liver injury in this study was determined by using APRI and FibroScan (METAVIR score; F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis). A few of the study participants (n = 5; 9%) had severe fibrosis with cirrhosis (Table 1).

The results of ELISA were divided into three groups; the lower quartile, middle quartile, and upper quartile (Table 2). The predominant levels of serum IFN-γ were in the middle quartile group (n = 27; 58), IL-2 in the middle quartile group (n = 37; 79%), IL-17 in the middle quartile group (n = 28; 60%), and TNF-α in the middle quartile group (n = 27; 57%).

Table 1. Characteristics of study participants (n = 47)

Variable	n (%)
Age (years old)	
17-25	1 (2)
26-35	5 (10)
36-45	12 (26)
46-55	16 (34)
56-55	12 (26)
> 65	1 (2)
Provisional diagnosis	
Chronic hepatitis B	38 (81)
Liver cirrhosis	6 (13)
Hepatocellular carcinoma	3 (6)
Serum ALT level	
5-35 U/L	28 (60)
36-70 U/L	7 (15)
>70 U/L	12 (25)
Serum AST level	
5-35 U/L	22 (47)
36-70 U/L	13 (28)
>70 U/L	12 (25)
APRI	
0-0.5	21 (45)
> 0.5-1.0	19 (40)
>1.0-1.5	4 (9)
> 1.5	3 (6)
FibroScan	
F0 F1	2 (4)
F2	1 (2)
F3	3 (6)
F4	5 (9)
NA	36 (79)

ALT: alanine aminotransferase; AST: aspartate aminotransferase; NA: not available

Table 2. The levels of IFN-γ, IL-2, IL-17, and TNF-α in study participants, measured by ELISA

Description	n (%)
IFN- γ	
Lower quartile (< 0.057 pg/mL)	10 (21)
Middle quartile (0.057 – 0.067 pg/mL)	27 (58)
Upper quartile (> 0.067 pg/mL)	10 (21)
IL-2	
Lower quartile (< 0.25 pg/mL)	3 (6)
Middle quartile (0.25 – 0.265 pg/mL)	37 (79)
Upper quartile (> 0.265 pg/mL)	7 (15)
IL-17	
Lower quartile (< 0.37 pg/mL)	11 (23)
Middle quartile (0.37 - 0.405 pg/mL)	28 (60)
Upper quartile (> 0.405 pg/mL)	8 (17)
TNF-α	
Lower quartile (< 0.079 pg/mL)	12 (26)
Middle quartile (0.079 - 0.107 pg/mL)	27 (57)
Upper quartile (> 0.107 pg/mL)	8 (17)
Total	47 (100)

IFN-γ: interferon gamma, TNF-α: tumour necrosis factor alpha, IL-2: interleukin-2, il-7: interlukin-7

No significant correlation was found between IFN-γ, IL-2, IL-17, and TNF-α serum and liver injury defined by APRI and FibroScan (p > 0.05). Table 3 shows significance levels (*p*-value) among variables tested.

Table 3. Significance levels (p-values) of the correlation among variables

	IFN- γ	IL-2	IL-17	TNF-α
AST to platelet ratio index (APRI)	0.10	0.45	0.38	0.28
FibroScan	0.46	0.90	0.18	0.43

IFN-γ: interferon gamma, TNF-α: tumour necrosis factor alpha, IL-2: interleukin-2, il-7: interlukin-7

DISCUSSION

From 47 samples, only 3 samples with APRI score above higher cut-off value which indicates high probability of having liver cirrhosis, and 5 samples had liver cirrhosis (METAVIR score = F4). It has been known that the features of liver cirrhosis are including regenerative nodules, which hepatocytes continually undergo cell death, and fibrotic tissues as a form in response to chronic inflammation and excessive wound healing response.[26,27] During HBV infection, the host's immune response causes viral clearance and also hepatocyte damage called immune-mediated liver injury, mainly caused by the T-cells response which is induced by other substance such as cytokines.[2,3,28] In healthy humans, a number of cytokines are not produced or detected in only small amounts. Cytokine levels vary in every individual, and the releasement of cytokines may differ based on activation signals, target cells, and physiological factors including stress, fitness, digestion and absorption of food in the body.[29,30]

No significant correlation was found between IFN-γ and APRI (p = 0.10) or FibroScan (p = 0.46) in this study indicates that IFN-γ may not directly involved in liver fibrosis. In contrast with this study, Attallah et al demonstrated a significant correlation between IFN-γ and fibrosis activity and/or cirrhosis.[31] It has been known that IFN-γ elicits cell apoptosis, including hepatocytes and hepatic stellate cells (HSCs) through various pathways.[32,33] IFN-γ is capable to inhibit the proliferation of HSCs, which is considered to be the main fibrotic cells in the liver. HSCs can produce various kinds of extracellular matrix proteins and when activated, they release vitamin A and lipid and then develop a myofibroblastic phenotype. Early activated HSCs were sensitive to IFN-γ so the cell proliferation can be inhibited, whereas intermediately activated HSCs were resistant to IFN-γ. A prolonged HBV infection induces the production of retinol metabolites by HSCs and upregulates the suppressor of cytokine signal transduction 1 (SOCS1) gene, leading to the inhibition of IFN-γ signal transduction, resulting in a reduced sensitivity of HSCs to IFN-γ in advanced fibrosis. Apart from having an anti-tumor function, a chronic presence of IFN-γ can promote cancer

immunoevasion in immunogenic or inflammatory tumor microenvironments. However, the threshold level of IFN-γ exposure to regulate the process of tumorigenesis remain undefined. This may show that therapy using IFN-γ is only effective in early fibrosis knowing the dual side of IFN-γ which is capable as an anti-tumor but also promoting cancer.[13,33,34]

We did not find any correlation between IL-2 and APRI ($p = 0.45$) or fibroScan ($p = 0.90$). IL-2 stimulates T cell proliferation or increases the number of T cells and NK cells cytotoxicity to destroy infected cells. Because of these functions, IL-2 can strengthen the Th1 immune response and a stronger Th1 immune response can inhibit tumor development.[35] Our study also did not find a significant correlation between the levels of serum IL-17 and APRI ($p = 0.38$) or FibroScan ($p = 0.18$). During the acute phase, HBV-specific T cells cannot produce IL-17 in response to infected hepatocytes thus suggesting that liver inflammation is not directly related to IL-17.[36] However, another study demonstrated the increased IL-17 levels in chronic hepatitis B, liver cirrhosis, and hepatocarcinoma patients.[37] IL-17 regulates anti-apoptosis molecules in hepatocytes thereby increasing hepatocytes survival. IL-17 also maintains a proinflammatory environment by activating the monocytes, and the activated-monocytes promote HSCs activation. The activation of HSCs induces the transformation of HSCs into myofibroblasts and promotes extracellular matrix synthesis therefore promotes liver fibrosis.[36,38] There was a lack of studies that reported the involvement of IL-17 in the development of hepatitis B progression. Most of the previous studies focused on the Th17 role as the main cells that produce IL-17 so that the role of IL-17 in HBV infection needs to be further investigated.

Previous studies showed that the elevated level of serum TNF-α during HBV infection is correlated with hepatic fibrosis and liver tissue injury.[39,40] However, we did not find a significant correlation between TNF-α and liver fibrosis progression estimated by APRI ($p = 0.28$) or FibroScan ($p = 0.43$). TNF-α is produced by macrophages and monocytes and is considered as one of the crucial cytokines for eradicating HBV. TNF-α also plays a crucial role in initiating fibrogenesis and is involved in the initiation, proliferation, angiogenesis, and metastatic of various cancers through binding with specific receptors, such as soluble TNF receptors (sTNFR).[39] The binding of TNF-α with its receptors promotes the production of nuclear factor Kappa B (NF-κB) and subsequently induces the releasement of proinflammatory cytokines and maintains a proinflammatory environment therefore promotes liver fibrosis. In acute and chronic hepatitis B, the increased soluble TNF-α and TNF-α receptors are detected in the patient's liver and serum.[41] A previous study showed that the elevated TNF-α levels in the serum of patients with mild liver inflammation could be used to predict the inflammation degree in the liver even though the liver enzymes were normal.[40]

Prolonged inflammation and liver fibrosis are common in chronic liver infection due to the imbalanced Th1 and Th2 immune responses in the liver. Cytokines play a crucial role in maintaining a patient's immune against HBV infection, but cytokines are also involved in hepatocellular injury especially in patients with chronic infection.[40,42] Factors that may influence the levels of serum cytokines in this study include therapy received by the patients and cytokine polymorphism genes of the samples. Several studies have identified that cytokine polymorphism genes are functionally associated with liver diseases and hepatocellular carcinoma and also contribute to an individual's susceptibility to developing cancer.[35] The levels of serum cytokines vary amongst individuals. The release and effect of cytokines also differ depending on signaling, target cells, and physiological factors such as stress, fitness, and feeding state.[30]

The limitations of this study include the number of samples and the technique of sample processing. Within 2 months of the study period, only 47 samples were collected and the majority of them (81%) were diagnosed as chronic hepatitis B. The short period of sample collection has made the diagnosis-based classification unequal so that correlation analysis between groups of patients with different diagnosis was impossible. In addition, the levels of serum cytokines can be affected by repeated thawing and freezing prior to ELISA measurement. Rapid freezing and slow thawing of serum samples can impair protein stability.[43] Uncertain mechanisms of cytokines in mediating liver injury is probably due to the activity of cytokines themselves that have dual side effects, for example, IFN-γ which has antitumor activities but also promoting cancer immunoevasion.[34] Moreover, there are limited papers on the use of cytokines as potential therapies for hepatitis, possibly because of the high risk of treatment failure or side effects.

CONCLUSION

Study participants had varying levels of cytokines, mostly within the middle quartile group. The intrinsic

factors that contribute to the cytokine levels include polymorphism of genes, signaling mechanism, and individual fitness. Extrinsic factors such as therapy received as well as method of sample processing; i.e., repeated rapid freezing and thawing of serum samples, play important roles in the results of tested cytokines. This study did not find a significant correlation between the levels of serum IFN-γ, IL-2, IL-17, and TNF-α with liver fibrosis and/or cirrhosis. Further research is needed to evaluate non- or minimally invasive method to study the pathogenesis of hepatitis B and determine the degree of liver damage. The use of freshly collected blood samples with minimal repetition of freezing and thawing is recommended for measurement of cytokine levels by using ELISA method.

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AUTHORS'S CONTRIBUTION

TNS was responsible for the conceptualization of study design, seeking research funding, coordinating research. TYP collected blood samples. WR conducted laboratory experiment and analysed the data. TNS and WR contributed equally during manuscript preparation. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

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