RESEARCH ARTICLE

Programmed Cell Death Protein 1-overexpressed CD8+ T Lymphocytes Play a Role in Increasing Chronic Hepatitis B Disease Progression

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Abstract

BACKGROUND: T lymphocyte activation depends on the balance of co-stimulatory and co-inhibitory signals determined by Cluster of Diffrentiation (CD)28 and Programmed Cell Death Protein 1 (PD-1) expression. Alteration in CD28 and PD-1 expression might affect the progression of chronic hepatitis B (CHB). Current study was conducted to evaluate the correlations of the CD28 and PD-1 expressions of T lymphocytes and CHB progression.

METHODS: Subjects were recruited, selected and divided into 3 groups, inactive CHB, active CHB and CHB with End-Stage Liver Disease (ESLD). HBeAg was determined by using Enzyme-Linked Fluorescence Assay while HBV-DNA was carried out by the RT-PCR method. Numbers of T lymphocytes expressing CD3, CD4, CD8, CD45, CD28 and PD-1 molecules were determined by flowcytometry.

RESULTS: There was no significant difference in the expression of CD28 by CD4+ and CD8+ T lymphocytes of inactive CHB, active CHB and CHB with ESLD subjects. There was also no significant difference in the expression of PD-1 in CD4+ lymphocytes of inactive CHB, active CHB and ESLD subjects. In contrast there was a significant increase in the expression of PD-1 in CD8+ T lymphocytes of ESLD subjects.

CONCLUSION: CD28 expression among CHB subjects was within normal range and not related to disease progression, but PD-1 expression of CD8+ T lymphocyte was increased along with disease progression, especially in CHB subjects with ESLD. This suggests that PD-1-overexpressed CD8+ T lymphocyte play a role in increasing CHB disease progression.

KEYWORDS: chronic hepatitis B, CD28, PD-1, T lymphocyte, disease progression

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Introduction

Hepatitis B Virus (HBV) infection has become a global threat to human health. Globally, 2 billion people are infected by HBV and 350-450 million of the cases become chronic hepatitis B (CHB). Approximately 15-40% of CHB patient become cirrhosis and hepatocellular carcinoma (HCC). Moreover, there are approximately 200.000 death resulting from cirrhosis and 300.000 from HCC per annum. Indonesia is one of the most endemic areas of HBV and has the third-highest prevalence of HBV infection in the world with a moderate prevalence, in which 7.1% of the population is infected with hepatitis B. The prevalence of hepatitis B surface antigen (HBsAg) is 37-76% in patients with liver cirrhosis and 37-68% in hepatocellular carcinoma (HCC) patients.(1) This condition causes HBV infection to have a significant contribution in the health system.(2)

In CHB patients, there is a change in cellular immune response particularly in T lymphocyte function. The dysfunctions of T lymphocyte were characterized as impaired proliferation, differentiation, decreased antiviral cytokine secretion and disruption of effector T lymphocyte activity which causes persistent viremia.(3-6) The attachment of the HBV antigen presented by the Human Leucocyte Antigen (HLA) molecule to the T cell receptor (TCR)/Cluster of Differentiation (CD)3, causes the T lymphocyte to issue a primary signal (first signal) which resultantly initiate the activation of T lymphocyte.(7,8) T lymphocyte activation is also regulated by a second signal determined by the balance between positive (stimulation) and negative signals (inhibition). This balance is maintained by the interaction between co-stimulatory and co-inhibitory molecules on the surface of T lymphocytes with the ligands on the surface of Antigen Presenting Cell (APCs). These stimulatory and inhibitory signals produced by APCs and integrated by T lymphocytes to determine the final outcome of T lymphocyte activation.(9,10)

CD28 and Programmed Cell Death Protein (PD)-1 molecules are the main co-stimulator and co-inhibitor molecules on the surface of T cells. Expression of CD28 was decreased in CD4$^+$ and CD8$^+$ T lymphocytes of CHB patients. This decreased expression affects T lymphocyte activation which play a central role in the host cellular immune response to HBV infection.(10,11) Meanwhile, expression of Programmed Cell Death Protein 1 (PD-1) was increased on the surface of T lymphocytes of CHB patients. HBV DNA and Hepatitis B e antigen (HBeAg) were the cause of the increased PD-1 expression. High PD-1 expression is an indicator of dysfunction T lymphocyte.(6)

Changes in co-stimulatory and co-inhibitor expressions, each of which contributes to the functional activity and proliferation of T lymphocytes, ultimately affect viral persistence and progression of CHB.(12) Due to the high prevalence level of HBV infection in Indonesia, this study was conducted to investigate the correlation between the expression of co-stimulator (CD28) and co-inhibitor (PD-1) with the disease progression of CHB patients in Indonesia. To the best of our knowledge, this study was the first to evaluate the correlation of CD28 and PD1 expressions with the progression of CHB.

Methods

Subjects Recruitment and Selection
Forty-five CHB subjects were included in this study. Fifteen subjects were not receiving antiviral treatment, while 30 subjects were receiving antiviral treatment at the Internal Medicine Department of Arifin Ahmad General Hospital Pekanbaru, Riau, Indonesia. Based on the clinical and laboratory records, the subjects were divided into three groups: inactive CHB (n=15), active CHB (n=15) and CHB with End-Stage Liver Disease (ESLD), including liver cirrhosis and hepatocellular carcinoma (HCC) (n=15). The criteria for CHB were previously described (13,14) based on clinical history, laboratory and imaging examinations. All CHB subjects were HBsAg positive, anti-HCV negative and anti-HIV negative. CHB subjects with other causes of chronic liver abnormalities were excluded from this study. Each subject signed an informed consent. The research protocol was approved by the Ethics Committee of Faculty of Medicine, Universitas Riau, Indonesia (514/UN.19.1.1.8/ UEPKK2017).

Preparation of Samples
Peripheral blood of each subject was collected in a tube containing EDTA. The serum was separated, stored at 2-8oC and later used for HBsAg, HBeAg, ALT, CD28, PD-1, and HBV-DNA examinations.

HBeAg Examinations
HBeAg was determined by using Enzyme-Linked Fluorescence Assay kit (Vidas, Biomereux, Marcy-l'Étoile, France). All procedures were performed according to the standardized protocol. All reagents were ready-to-use and pre-dispensed in the sealed reagent strips. All steps were performed automatically by the instrument. After dilution, the samples were cycled in and out of the Solid Phase Receptacle (SPR). Biotin-conjugated monoclonal anti-HBe antibody bounds were detected by incubation with alkaline phosphatase-conjugated streptavidin. The fluorescence of was measured at 450 nm.

ALT Examination
Serum ALT levels were measured by Cobas Integra 250 Autoanalyzer (Roche Diagnostic). All procedures were performed according to the standardized protocol. Briefly, ALT catalyzed the reaction between L-alanine and 2-oxoglutarate. The pyruvate formed was reduced by nicotinamide adenine dinucleotide (NAD) hydrogen in a reaction catalyzed by lactate dehydrogenase to form L-lactate and NAD$^+$.

HBV-DNA Examination
DNA was extracted with magnetic glass particle and amplified with Real-Time Polymerase Chain Reaction on COBAS AmpliPrep Cobas Taqman Analyzer HBV version

2.0 (Roche Molecular System, Munich, Germany). The detection sensitivity was 2.00 x 10^1 viral genome IU/mL (1 IU = 5.82 copies).

Flowcytometric analysis

Numbers of $CD28^+$ T lymphocytes was carried out using following antibodies: PE-conjugated anti-human CD28 (Cat.555729, BD Biosciences, California, CA, USA), FITC-conjugated anti-human CD4 (Cat. 555346, BD Biosciences), PerCP-Cy-5.5-conjugated anti-human CD8 (Cat. 341051, BD Biosciences), Alexa Fluor 647-conjugated CD279 (PD-1) (Cat. 560838, BD Biosciences) and BD Multitest CD3/CD8/CD45/CD4 (Cat. 340499, BD Biosciences). The examinations were carried out using FACS Canto II flow cytometer (BD Biosciences) with FACS Diva 8.0.1 software (BD Biosciences) at Prodia Clinical Laboratory, Jakarta.

Statistical analysis

Statistical analysis was carried out with SPSS. for Windows version 18 (SPSS Inc, Chicago, IL, USA). Analysis of Variance (ANOVA) test or Kruskal Wallis test was performed based on the results of Shapiro-Wilk test of normality. The p-values<0.05 were considered statistically significant.

Results

Characteristics of 45 CHB subjects were shown in Table 1. The subjects were distributed equally, 15 inactive CHB, 15 active CHB and 15 ESLD subjects. Most of the subjects were male in the age of 41-60 years old. There were 5 (33.33%) $HBeAg^+$ inactive CHB subjects, 11 (73.33%) $HBeAg^+$ active CHB subjects and 11 (73.33%) $HBeAg^+$ ESLD subjects. Highest levels of ALT and HBV DNA were observed in ESLD subjects.

Table 1. Subject characteristics (n=45).

Gender	n	%
Male	28	62.22
Female	17	37.78
Age (year)	**n**	**%**
18-40	18	40
41-60	23	51.11
>60	4	8.89
ALT (IU/mL)	**n**	**Mean±SD**
Inactive CHB	15	23±4.10
Active CHB	15	41±4.22
ESLD	15	61±21.20
HBV DNA (IU/mL)	**n**	**Mean±SD**
Inactive CHB	15	<20 (UDL)
Active CHB	15	$1.82 \times 10^5 \pm 5.92 \times 10^5$
ESLD	15	$1.45 \times 10^6 \pm 5.55 \times 10^6$

ALT: alanine aminotransferase; UDL: Under Detection Limit.

Lymphocytes and Lymphocyte Subsets of Inactive CHB, Active CHB and ESLD Subjects

As shown in Table 2, total lymphocyte count, T lymphocyte count, T lymphocyte percentage, $CD4^+$ T lymphocyte count, $CD4^+$ T lymphocyte percentage and $CD8^+$ T lymphocyte count of inactive CHB, active CHB and ESLD subjects were in the normal ranges with no significant differences. However, $CD8^+$ T lymphocyte percentages of ESLD subjects were significantly the highest. Meanwhile CD4/CD8 ratios of ESLD subjects were significantly the lowest.

$CD28^+$, $CD4^+$ and $CD8^+$ T Lymphocytes of Inactive CHB, Active CHB and ESLD Subjects

$CD28^+CD4^+$ T lymphocytes numbers and percentages of ESLD subjects were the lowest, while $CD28^+CD8^+$ T

Table 2. Averages of lymphocytes and lymphocyte subsets of inactive CHB, active CHB and ESLD subjects.

Lymphocyte	Inactive CHB	Active CHB	ESLD	p-value
Total lymphocyte (cell/μL)	2004.27	1913.20	1944.87	#0.826
T Lymphocyte (cell/μL)	1180.20	1232.47	1320.60	$0.828
T Lymphocyte (%)	60.09	63.13	68.00	$0.116
$CD4^+$ T Lymphocyte (cell/μL)	659.67	724.33	602.73	#0.655
$CD4^+$ T Lymphocyte (%)	33.02	37.55	30.83	$0.100
$CD8^+$ T Lymphocyte (cell/μL)	476.87	488.60	686.40	$0.191
$CD8^+$ T Lymphocyte (%)	23.51	24.26	35.51	#,*0.009
CD4/CD8 ratio	1.91	1.74	0.99	#,*0.006

#Kruskal Wallis Test, $ANOVA Test, *$p$<0.05.

Table 3. Averages of CD28+, CD4+ and CD8+ T lymphocytes of inactive CHB, active CHB and ESLD subjects.

T Lymphocyte	Inactive CHB	Active CHB	ESLD	*p*- value
CD28+ CD4+ (cell/μL)	650.47	678.53	596.40	#0.785
CD28+CD4+ in CD4+ (%)	98.87	94.34	93.13	#0.475
CD28+ CD8+ (cell/μL)	412.27	421.33	655.40	$0.079
CD28+ CD8+ in CD8+ (%)	92.99	85.45	93.62	#0.192

#Kruskal Wallis Test, $ANOVA Test

lymphocytes numbers and percentages of ESLD subjects were the highest. However, all CD28+CD4+ and CD28+CD8+ T lymphocytes numbers and percentages of inactive CHB, active CHB and ESLD subjects were within the normal ranges with no significant differences (Table 3).

PD-1+, CD4+ and CD8+ T Lymphocytes of Inactive CHB, Active CHB and ESLD Subjects

There was no significant difference in the expression of PD-1 in CD4+ lymphocytes of inactive CHB, active CHB and ESLD subjects. However, there was a significant increase in the expression of PD-1 in CD8+ T lymphocytes of ESLD subjects (Table 4).

Discussion

Based on current results, there was no any significant difference in lymphocyte counts and subsets of inactive CHB, active CHB, as well as ESLD groups, suggesting that lymphocyte counts and subsets were not correlated with the progression of CHB. The total lymphocyte averages of inactive CHB, active CHB, and ESLD subjects were in the normal range (1000-3000/μL).

Similarly, the CD4+ T lymphocyte and CD8+ T lymphocyte averages of inactive CHB, active CHB, and ESLD subjects were in the normal range of 64-1721/μL and 135-852/μL respectively. In addition, CD4/CD8 ratios

of inactive CHB, active CHB, and ESLD subjects were categorized in the normal range.(15) There was another report showing that there was no difference in lymphocyte counts of peripheral blood and liver tissue of 21 active and 13 inactive CHB subjects. However, when the lymphocyte counts of CHB subjects were compared with the ones of HCV, there was a significant increase in the lymphocyte count, CD4+ and CD8+ T lymphocytes of HCV subjects compared with the ones of CHB subjects. This suggested that there was an adequate cellular immune response of HCV subjects to determine the effectiveness of virus clearance and the degree of liver cell damage compared to CHB subjects.(16)

One of the factors responsible for T lymphocyte dysfunction is the signal imbalance between the co-stimulator and co-inhibitor molecules. This occurs due to the decrease in the number of T lymphocytes expressing the main CD28 co-stimulator molecule and an increase in the number of T lymphocytes expressing the PD1 main co-inhibitory molecule on its surface. Changes in the expression of co-stimulator and co-inhibitor factors in T lymphocytes with CHB have been widely reported in previous studies.(17,18) In this study, more than 90% of CD4+ and CD8+ T lymphocytes of CHB subjects expressed the CD28 co-stimulatory molecule. There was not any significant difference of CD28 expression in inactive CHB, active CHB, as well as ESLD groups. The results showed that viral persistence, chronicity of disease and

Table 4. Averages of PD-1+, CD4+ and CD8+ T Lymphocytes of Inactive CHB, Active CHB and ESLD subjects.

T Lymphocyte	Inactive CHB	Active CHB	ESLD	*p*- value
PD-1+ CD4+ (cell/μL)	209.07	334.93	342.40	$0.142
PD-1+ CD4+ in CD4+ (%)	34.96	46.64	52.95	#0.019
PD-1+ CD8+ (cell/μL)	99.53	145.27	309.67	$,*0.001
PD-1+ CD8+ in CD8+ (%)	22.88	32.80	42.47	#,*0.004

#Kruskal Wallis Test, $ANOVA Test, *p<0.05.

progression due to T cell dysfunction were not affected by the expression of CD28, however, it might be caused by other factors such as viral or environmental factors. The result of this study showed no impaired expression of CD28 molecules in CHB subjects, hence, the impairment in T lymphocytes function in CHB subjects was not caused by decreased CD28 expression. When the expression of the CD28 was normal, there should be other probable factors responsible for T lymphocyte dysfunction and immune system failure which resulted in viral persistence and disease progression.

There was a significant increase in the expression of PD-1 molecule on CD8^{+} T lymphocyte in relation to disease progression. However, there was no correlation between the expression of PD-1 in CD4^{+} T lymphocyte in relation to disease progression. Other study reported that an increase in the expression of PD-1 inhibitory molecules in CD8^{+} T lymphocyte in CHB subjects.(19) The PD-1 is an inhibitory molecule that inhibits T cell activation and play an important role in the regulation of the immune system and self-tolerance. Increased expression of PD-1 molecules on the T lymphocyte surface inhibit T lymphocyte activation and constitute one of the mechanisms responsible for the failure of the immune response in eliminating viruses and clean malignant cells in CHB subjects with liver cirrhosis or HCC.(19-21)

Conclusion

CD28 expression among CHB subjects was within normal range and not related to disease progression, but PD-1 expression of CD8^{+} T lymphocyte was increased along with disease progression, especially in CHB subjects with ESLD. This suggests that PD-1-overexpressed CD8^{+} T lymphocyte play a role in increasing CHB disease progression.

Authors Contribution

F, EN, and NZ were involved in concepting and planning the study/research. F performed the data acquisition/collection. F, EN, and NZ conducted the data analysis and also interpreted the results. F and FS prepared, drafted the manuscript and designed the presented tables and figures. All authors took parts in giving critical revision of the manuscript.

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