DOI : 10.35124/bca.2019.19.2.2891

Biochem. Cell. Arch. Vol. 19, No. 2, pp. 2891-2895, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

KINETIC STUDY OF GRANZYME B IN PATIENTS WITH CHRONIC HEPATITIS TYPE B

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(Received 27 October 2018, Revised 14 December 2018, Accepted 6 January 2019)

ABSTRACT : Chronic hepatitis B (CHB) virus infection is affecting more than 350 million people worldwide ,and can predispose for serious complications like liver cirrhosis and hepatocarcinoma. The development of CHB from active stage is the direct result of debilitated immune system which mainly depends on cytotoxic T- lymphocyte (CTL) and natural killer (NK) cells for controlling viral replication. These cells eliminate their targets through the secretion of two proteins : perforin and granzymes (Gzms). Thus, this study aimed to evaluate the activity and concentration of GzmB in patients with CHB. A total of 60 patients (30 newly diagnosed and 30 treated) were recruited for this case-control study. Other 30 age- and gender-matched apparently healthy subjected (negative for HBsAg) were involved as controls. Granzyme B kinetics and concentration were measured using ready commercial kits. Treated patients had significantly higher values of Km (8.57±0.796 X 10^{-5} M), than either untreated patients (7.17±0.69 X 10^{-5} M) or controls (7.24±0.82 X 10^{-5} M), while treated patients showed significantly lower values for Kcat and Kcat/Km (3.77±0.39 s^{-1} and 4.55±0.89 x 10^{4} M^{-1} s^{-1} respectively) than either untreated patients (4.37±0.31 s^{-1} and 6.18±0.98 x 10^{4} M^{-1} s^{-1} respectively) or controls (4.34±0.37 s^{-1} and 6.187±1.167 x 10^{4} M^{-1} s^{-1} respectively). Mean GzmB concentration in control was 104.4±26.37 Pg/mL which significantly higher than that of untreated patients (44.3±8.73 Pg/mL which did not differ significantly from treated patients (42.33±9.09 Pg/mL). These data indicate the reduction in GzmB concentration in CHB which may reflect a defect in the production of this enzyme that predispose un individual to the chronic infection.

Key words : Granzyme B, hepatitis B, kinetics.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem that causes inflammation of the liver. It affects over 350 million people worldwide and over 1 million die each year from this infection. The virus causes either temporary (acute) hepatitis or chronic hepatitis which goes for a long period of time that may develop in the liver fibrosis, cirrhosis and carcinoma [1].

In response to the infection, innate and adaptive immunity have involved in different tasks to control infections. Through recognition of viral nucleic acids, viral proteins or tissue-damage, innate immunity is triggered during the early phases of viral infections [2]. The activation of innate immunity is also necessary for the efficient recruitment of the adaptive immune system, which acts through maturation and expansion of B-cell to produce antibodies specific to Hepatitis B antigen and T-cell clones that specifically recognize and kill infected hepatocytes [3]. Genetic defects in any arm of immune system could increase the susceptibility of an individual to HBV infection [4].

Cytotoxic T lymphocytes and NK cells eliminate virus-infected cells principally by releasing the contents of cytotoxic granules into the immune synapse formed with their target cell. The granule mediators of target cell are serine proteases, known as granzymes, which induce programmed cell death, after they are delivered into the target cell cytoplasm by the pore-forming protein perforin. Granzyme B is the most important one that induces target cell apoptosis in a mitochondria-dependent manner [5,6].

Granzymes should work at maximum speed with lower Km and should be free of the inhibitors in order for successful initiated apoptosis [2]. In hepatitis B patients, 5% do not respond to treatment in which infected cells do not undergo apoptosis. Some of the possibilities for what is going on with those patients might be either defects in

GzmB kinetics or either the presence of inhibitors that might interfere with the enzymes function [7].

This study aimed to evaluate the activity and concentration of GzmB in a sample of Iraqi patients with CHB.

MATERIAL AND METHODS

This is a case control study conducted in Al Imamain Al-Kadhumain Medical City (Gastroenterology and Hepatology department), Gastroenterology and Hepatology Hospital/ Baghdad during the period from January to August, 2018. This study included 60 patients with CHB (30 newly diagnosed and 30 treated or longstanding patients). Other 30 age- and gender- matched apparently healthy subjects were recruited to represent a control group. Venous blood sample (5 mL) was collected taken from subject. Sera were separated after blood clotting and centrifugation, and divided into small aliquots for analysis. Granzyme B concentration was measured using ready commercial kit that has been designed for the quantitative measurement of GzmB in Human serum (Abcam/ USA). Granzyme B kinetic was measured by using Ac-IEPD-pNA (chromogenic GzmB substrate) produce by (Kamiya Biomedical/ USA) following the manufacturer's instructions. Data of patients and control were obtained through direct interview or from patients' records. The study protocol was approved by the Ethical Committee of College of medicine/ AL-Nahrain University. Consent from explaining the study was obtained from each subject.

Statistical Analyses

Statistical package for social sciences (SPSS) was used for data analysis. Continuous variable were expressed as mean ± SD, while categorical variables were expressed as frequency and percentage. All continuous data was subjected to normality test. Data with normal distribution was examined by ANOVA (analysis of variance), while those with abnormal distribution were tested with Kruskal Wallis test to evaluate the differences among groups. Chi square test was used to test the categorical data. A P-value of < 0.05 was considered significant.

RESULTS

Demographic and Laboratory Data of the Study Population

Two demographic characteristics were considered in this study: age and gender, both of which did not differ significantly between the three groups (Table 1). All patients with hepatitis (treated and untreated) were positive for HBsAg and HBcAb-IgG, while none positive for HBcAb-IgM. Two-third of untreated patients (66.67%) was positive for HBeAb compared to 40% in treated patients. For HBeAb, the reverse was true in that one-third of untreated patients were positive for these antibodies compared to up to 70% of treated patients who had these antibodies. Interestingly HBsAb was positive in 0%, 63.33% and 30% in untreated patients, treated patients and control respectively.

Table 1 : Demographic and laboratory characteristics of the study population.

Variables	Un-treated Patients	Treated Patients	Controls	P- value
Age	42.5±7.06[a]	46.23±16.5[b]	40.17±12.65[b]	0.241
Gender				
Male	20(66.67%)	16(53.33%)	17(56.67%)	0.55
Female	10(33.33%)	14(46.67%)	13(43.33%)	
HBsAg				
Positive	30(100%)	30(100%)	0(0%)	<0.001
Negative	0(0%)	0(0%)	30(100%)	
HBeAg				
Positive	20(66.67%)	12(40%)	0(0%)	<0.001
Negative	10(33.33%)	18(60%)	30(100%)	
HBeAb				
Positive	10(33.33%)	21(70%)	0(0%)	<0.001
Negative	20(66.67%)	9(30%)	30(100%)	
HBsAb				
Positive	0(0%)	19(63.33%)	9(30%)	<0.001
Negative	30(100%)	11(36.67%)	21(70%)	
HBcAb-IgM				
Positive	0(0%)	0(0%)	0(0%)	<0.001
Negative	30(100%)	30(100%)	30(100%)	
HBcAb-IgG				
Positive	30(100%)	30(100%)	0(0%)	<0.001
Negative	0(0%)	0(0%)	30(100%)	

Table 2 : Granzyme B kinetics and concentration.

Variables	Un-treated Patients	Treated Patients	Controls	P-value
Km (10^{-5} M)	7.17±0.69a	8.57±0.796b	7.24±0.82a	<0.001
Kcat (s^{-1})	4.37±0.31a	3.77±0.39b	4.34±0.37a	<0.001
Kcat/Km (104M-1s-1)	6.18±0.989a	4.55±0.896b	6.187±1.167a	<0.001
Concentration Pg/mL	44.3±8.73A	42.33±9.09A	104.4±26.37b	<0.001

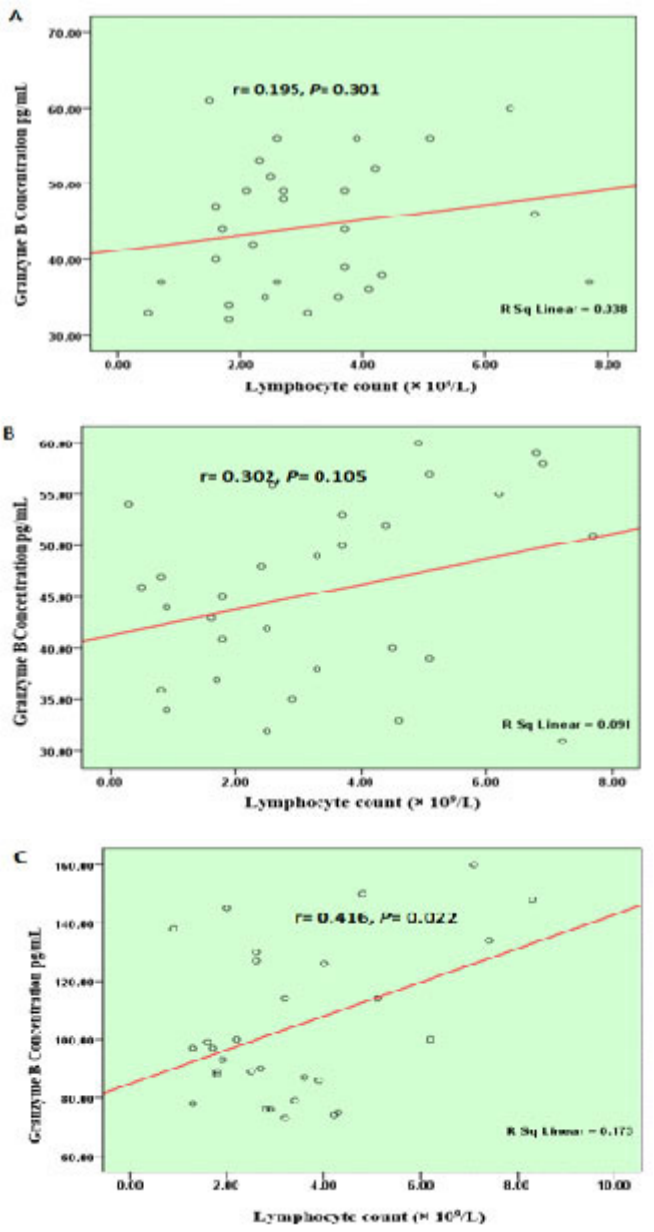
Granzyme B Kinetics and Concentration

Table 2 shows the kinetics and concentration of GzmB in patients and controls. Treated patients had significantly higher values of Km (8.57±0.796 x 10^{-5} M), than either untreated patients (7.17±0.69 x 10^{-5} M) or controls (7.24±0.82 x 10^{-5} M). In contrast, treated patients showed significantly lower values for Kcat and Kcat/Km (3.77±0.39 s^{-1} and 4.55±0.896 x 10^4 M^{-1} s^{-1})respectively) than either untreated patients (4.37±0.31 s^{-1} and 6.18±0.989 x 10^4 M^{-1} s^{-1} respectively) or controls (4.34±0.37 s^{-1} and 6.187±1.167 x 10^4 M^{-1} s^{-1} respectively). Of note, there were no significant differences between untreated patients and controls in these parameters. On the other hand, there was a marked reduction in enzyme concentration in patients compared with controls. Mean GzmB concentration in control was 104.4±26.37 Pg/mL which significantly higher than that of untreated patients (44.3±8.73 Pg/mL which did not differ significantly from treated patients (42.33±9.09 Pg/mL).

Correlation between Granzyme B Concentration and Lymphocyte count

Pearson's correlation and linear regression tests were used to find out the correlation between GzmB concentration and lymphocyte count in each group (Figure 1). Although there was a positive correlation between the two markers in treated and untreated patients, it did not reach the significant level (r = 0.195, P= 0.301 in untreated patients and r = 0.302, *P*= 0.105 in treated patients). In contrast, there was a significant moderate positive correlation between GzmB concentration and lymphocyte count in control group (r = 0.416, *P*= 0.022).

Figure 1 : Correlation between granzyme B concentration and lymphocyte count in untreated patients (A), treated patients (B) and healthy control (C).



DISCUSSION

The current study aimed to investigate the kinetics of ganzyme B in patients with CHB infection (treated and newly diagnosed) in comparison to healthy individuals. No significant differences were found between the three groups regrading age and gender. That is because subjects in control group were intentionally selected to match patients in both age and gender. Otherwise, each of these two factors can influence the incidence of CHB infection. In one study, Khan *et al* [8] screened a total of 4890 Pakistani patients for HBV infection. They reported almost an inverse relationship between infection rate and age. The infection rate rose from 13.39% in teenage to a peak of 34.93% at 21-30 year age class and then began to decline to 23.83% at 31-40 year age class then to 16.13% and 7.09% in people aged 41-50 and > 60 years

respectively. Although the author investigated acute HB infection, it is well-known that chronic infection is derived from acute infection. On the other hand, the same authors reported more than two-time higher HBV infection rate in males than females (the ratio was 2.14:1). In a local cross-sectional study all over Iraqi governorates including 9610 subjects, Tarky *et al* [9] reported that HBV infection (as indicated by the positivity for HBsAg) was higher in older ages (31 years and older) than younger ages, and males were more affected than females. It is reasonable to detect HBsAg in all patients because this antigen is produced in all infection phases and persists until the infection is completely resolved.

The current study showed higher GzmB Km average level among the treated group (8.57±0.79 x10^{-5} Molar) than both untreated group (7.17±0.69 x10^{-5} Molar) and control group (7.24± 0.82x10^{-5} Molar). This is due to the treatment with Tenofovir that cause increase in the H concentration in the blood which lead to increase in the Km value and decrease in the GzmB affinity to the substrate. An elevated Km means that enzyme needs more substrate molecules to reach a certain reaction rate, while a low Km means the opposite. Moreover, elevation in Km value decreases the affinity of enzyme to substrate as well as dissociation become high, which means enzyme efficiency for cleavage is low [10].

In a previous study, Poe *et al* [11] used Ná-t-butyloxycarbonyl-L-alanyl-L-alanyl-L-asparty (lB oc-Ala-Ala-Asp) biobenzyl ester as a substrate for purified GzmB from human CTL cell line. They found that the purified enzyme has hydrolyzed the substrate with a Km of 0.15±0.06 M which is much less than the values reported in the current study. Of course the nature of the substrate influence the hydrolysis rate beside many other conditions within the experiment.

The enzyme Kcat values reported in the current study among the untreated group was (4.37 ±0.31 s^{-1}), which is less than that of treated (3.77 ±0.39 s^{-1}) and control group (4.34 ± 0.37 s^{-1}), with significant difference. This is can be explained by the increase in the Km value that leads to decrease the affinity of the GzmB with eventual decrease in Kcat value**.** Recently, Andoniou *et al* [12] studied the kinetic of GzmB using three types of substrates (Abz-IEPD SESQK-dnp, Abz-IEPD SGSQK-dnp and Abz-LEYD LGALK-dnp). The Kcat values for the enzyme in these substrates were 1.316±0.09, 5.106±0.27 and 0.035±0.002 second respectively, which indicates that different substrates can give very different Kcat values.

The current study showed that the Kcat/Km ratio among untreated group was (6.81 ±0.98 x 10^4 M^{-1} s^{-1}) and among treated was (4.55±0.89 x 10^4 M^{-1} s^{-1}) which is lower than the mean level of the control group (6.187 ± 1.16 x 10^4 M^{-1} s^{-1}), with significant difference. The decreased in the Kcat/Km ratio among the treated patients may be attributed to tenofovir disoproxil fumarate which affects GzmB catalytic efficiency tremendously by lowering catalytic efficiency of substrate cleavage. In an in vitro study used 17 substrate to investigate the kinetics of GzmB. They reported that kcat/km ratio ranged from 1.3±0.5 × 103 M^{-1} s^{-1} to 2.5±0.8 × 106 M^{-1} s^{-1}, which indicates a wide range of enzyme catalytic efficiency. Casciola *et al* 1999 [13] .

In this study also GzmB level was measured among the untreated group (44.3±8.73 pg/ml) and treated (42.33 ±9.09 pg/ml) was lower than control group (104.4±26.37 pg/ml), with significant difference. These results indicate that GzmB concentration is too low in both treated group and untreated group comparing to the control group.

Several reasons could be hypothesized for this reduction. Among these, the presence of a defect in the intracellular signal cascades generated from the T-cell receptor (TCR) and CD3 proteins especially defect in the proximal TCR signal transduction or distal signaling pathway lead to effects in the production of GzmB, or it may due to defect in the DNA transcription as well as it could come from mutation in *GzmB* gene polymorphism in the promoter region. Another reason why GzmB level is reduced in patients could be the defect in mRNA splicing or translation in the ribosomes as well as defect from the activation and proliferation of NK cells or cytotoxic cells in the immune system [14].

In control group, there was a significant moderate positive correlation between GzmB concentration and lymphocyte count in control group. This is a logical results because the enzyme is predominantly produced by lymphocyte cell. The absence of this correlation in CHB patients (either treated or untreated) may reflects the diminished ability of lymphocyte to produce the required amount of the enzyme, and this supports the above results that indicate a low concentration of GzmB in patients.

To summarize, these data strongly suggest a reduction in GzmB concentration in CHB patients while the activity of the enzyme is almost intact. Further studies should focus on the possible mechanism that may cause this reduction.

CONCLUSIONS

Granzyme B activity is reduced in CHB patients under the treatment with tenofovir disoproxil fumurate , while the enzyme concentration is reduced in treated and untreated patients. This indicates a defect in enzyme secretion from CTL and/or NK cells.

ACKNOWLEDGEMENT

We highly appreciate the efforts of the National Institutes of Health staff, in Bethesda, Maryland, USA especially Senior investigator Dr. Mark Connors MD, Dr. Rodhe in NIH for the scientific advice and information that had provided for me and the Virology Laboratory staff in the Central Public Health Laboratory in Iraq especially, Miss Intesar Jabar and Mr. Ali Abid Al-Khadim for helping me in the collection of the samples.

Conflict of interest

The authors declare no conflict of interest.

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