Involvement of Fas receptors (CD95) and ligands (CD95L) in CD4+ T-cells and, CD8+ T-cells depletion and hepatic cytolysis in patients with Chronic Viral Hepatitis B

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Abstract

Background: Chronic Viral Hepatitis B is characterized by a progressive destruction of hepatocytes and T-cells depletion. The mechanisms of the CD95-CD95L signaling pathway during this chronic disease and the cirrhotic process remains unclear. Objective: Our objective was to evaluate the involvement of the CD95-CD95L receptor-ligands in T-cells depletion and hepatic cytolysis in patients with chronic Viral Hepatitis B.

Methods: A cross-sectional study was conducted from September to December 2018, at the Yaoundé General Hospital, Cameroon. Four milliliters of whole blood were collected and analyzed. The CD95, CD95L levels and CD4+ T-cells, CD8+ T-cells counts were performed by Enzyme-linked immunosorbent assay and Flow cytometry, respectively. The data were analyzed using EpiInfo 7.0 and GraphPad PRISM 5.0, with the significant threshold set at $p \leq 0.05$ and a 95% confidence interval.

Results: Of the 130 patients, 36 (27.7%) were cirrhotic and 94 (72.3%) were non-cirrhotic. The plasma level of CD95 and CD95L were significantly elevated in cirrhotic patients, compared with non-cirrhotic patients ($p < 0.001$ and $p = 0.001$, respectively). The CD4/CD8 ratios were lower in cirrhotic patients, compared to non-cirrhotic patients ($p < 0.001$). There were statistically significant correlations between CD95 and CD4+ T-cells, between CD95 and CD8+ T-cells, between CD95 and the CD4/CD8 ratio, between CD95 and fibrosis scores, and between CD95L and fibrosis score.

Conclusion: CD95-CD95L could be involved in T-cells depletion and hepatic cytolysis during the pathogenesis of chronic Viral Hepatitis B, and could potentially be used as biomarkers for immunological and hepatic monitoring in patients with chronic Viral Hepatitis B.

Introduction

The Hepatitis B Virus (HBV) is the leading cause of chronic liver disease and remains a major global health burden. Chronic Viral Hepatitis B (VHB) infection is an inflammatory disease of the liver, caused by HBV, whose surface antigen has persisted in the host for at least six months [1]. It is characterized by the progressive onset of fibrotic hepatic lesions, whose cause remains unclear. The CD4+ and CD8+ T-cells, which play an indispensable role in the resolution of HBV infection [2], are significantly lower in chronic VHB, but the mechanisms involved remains unclear [3].

The severity of liver lesions caused by infection varies according to the immune status of the patient [2]. An appropriate immune response leads to the necrosis of infected hepatocytes and the elimination of the virus [1]. An excessive immune response induces massive hepatocyte destruction; the immunotolerance is marked by an abundant, but asymptomatic viral multiplication without hepatocyte involvement. In chronic carriers of HBV, the immune response exists, but it is insufficient to eliminate the virus [3].

The persistence of HBV in hepatocytes leads to repeated attempts to eliminate them by T-cells [2]. This is mediated by several immune mechanisms, including apoptosis, which is a physiological process of cell death, implementing an actual cell lysis program. The triggering of this "programmed death" is done through the activation of specialized signaling pathways, namely the CD95-CD95L pathway [3].

CD95 belongs to the Tumor Necrosis Factor-Receptor/Nerve Growth Factor-Receptor (TNF-R/NGF-R) family. Members of this family are characterized by the presence of cysteine-rich domains in their extra cytoplasmic portion (3 for CD95). The "death receptor" subfamily is distinguished at the intracytoplasmic portion that contains a domain of about 80 amino acids called the "death domain" [3].

CD95 (Fas/APO-1) is the receptor of CD95L (Fas ligand, FasL). The induction of apoptosis by CD95 follows its oligomerization by an agonist monoclonal antibody or by its natural ligand CD95L. The latter belongs to the superfamily of TNF/NGF. The CD95 molecule is expressed in many tissues (liver, heart, hematopoietic tissue) [3]. However, a complete lack of expression of the CD95 protein in humans has no direct consequences on the lymphoid system [4]. CD95 is expressed on the surface of the membrane of activated

T-lymphocytes or B-lymphocytes. After a second activation, the T-lymphocyte expresses CD95L and kills the activated lymphocytes (death in "trans"), including itself (death in "cis"), which is called "Activation-Induced Cell Death" (AICD) [5]. The CD95-CD95L interaction therefore represents a preferred route of control of the immune response, and in particular of the regulation of autoimmune proliferations. CD95-mediated T-cells apoptosis has been known for a long time as a mechanism for contraction of T-cells responses for the prevention of immunopathogenesis and the maintenance of immune tolerance, and this is described as an immune checkpoint mechanism [5]. Three mechanisms maintain peripheral T-cells homeostasis: T-cells anergy, suppression by regulatory T-cells and AICD [6]. The population of effector T-cells is controlled by AICD, which is initiated by repeated stimulation of T-Cell Receptor (TCR) via apoptosis mediated by CD95 [7].

Furthermore, the interaction between CD95 and CD95L induces AICD [6]. Activated T-cells, which express CD95 and CD95L, are destroyed either by suicide or by mutual interaction [4]. Peripheral tolerance is maintained by the system that disintegrates activated T-cells [6].

Over activated effector T-cells are dangerous for the immune system and must therefore be removed from the peripheral circulation. Thus, AICD induced by apoptosis mediated by CD95 therefore plays a very important role within the peripheral immune system [6].

The mechanisms of the CD95-CD95L apoptotic signaling pathway during chronic VHB remains to be elucidated. Our objective was to evaluate the involvement of CD95-CD95L receptor-ligands in T-cells depletion and hepatic cytolysis in patients with chronic VHB.

Methods

Ethical considerations

Ethical approval to conduct the study was obtained from the Institutional Ethics and Research Committee for Human Health (N°2019/0803/CEIRSH/ESS/MIM). Written and verbal informed consent was given by all participants. The study was conducted according to the ethical principles guidelines and guidelines of the international Declaration of Helsinki 2013. All procedures were standard and presented minimal risk to participants.

Study design

We performed a cross-sectional study from September to December 2018 on patients with chronic Viral Hepatitis B (VHB) infection. These patients were received for consultation at the Hepato-gastroenterology Department of the Yaoundé General Hospital, Cameroon. The recruitment was consecutive and non-probabilistic. The results of the biological analysis were returned to the patients and incorporated into their medical records.

Study population

From the fibrotest examination, patients were divided based on their METAVIR fibrosis score into cirrhotic and non-cirrhotic HBV infected patients. Patients with liver disease or other etiologies were excluded. Patients with hepatocellular carcinoma, those receiving or treated with antiviral therapy were excluded.

There were no gender restrictions in patient recruitment, but patients recruited had to be between 18 and 60 years old. Patients with both HBV infection and a history of autoimmunity, drug-dependence or co-infection with other viruses, including HCV, VHD, HIV, HTLV-1, were excluded from the study.

The selection criteria for the control participants were: not having a medical history of HBV, HCV, HDV, HTLV-1, HIV infection, autoimmunity and not having consumed alcohol. Social characteristics (age and sex) and clinical information (cirrhosis statute, levels of CD95 and CD95L, values of CD4+ T-cells, CD8+ T-cells and the CD4/CD8 were collected for each participant using a standard questionnaire.

Sample collection and analysis site

Four milliliters (4 mL) of whole blood was collected under standard conditions, into Ethylene Di-amine Tetra Acetate Acid (EDTA) anticoagulant tubes (Greiner Bio-One International GmbH,

Kremsmünster, Austria), and transported at room temperature to the Immuno-virology Laboratory of the Center for the Study and Control of Communicable Diseases (CSCCD) of the Faculty of Medicine and Biomedical Sciences of the University of Yaoundé 1 (FMBS/UY1). These samples were analyzed within 24 hours for CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratio. Plasma were obtained by centrifugation of whole blood at a speed of 5000 revolutions per minute (rpm) for 5 minutes with an electric centrifuge, and aliquoted in cryovials. Plasma samples were frozen at 20°C, for the subsequent determination of CD95 and CD95L levels. METAVIR scores of fibrosis (Fibrosis score) were collected from patients' medical records.

Determination of CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratio

We used the whole blood collected for the determination of CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratio. This was performed by flow cytometry, using the BD FACSCount system, (Becton Dickenson, Fluorescent Activated Cells Sorting, Biosource, San Jose, California, USA). Results were obtained automatically for the absolute CD4+ T-cells, CD8+ T-cells, including the CD4/CD8 ratio. This was done according to the manufacturer's recommendations.

Determination of CD95 and CD95L plasma level

The determination of the levels of CD95 and CD95L were performed using plasma, by the sandwich Enzyme-linked immunosorbent assay (ELISA) technique (Quantikine®, R&D Systems, Abingdon, Barton Lane, United Kingdom), following the manufacturer's instructions. Optical densities were measured at 450 nm using an ELISA reader (Sunrise Tecan, Tecan GmbH, Grödig/Salzburg, Austria), and all samples were assayed in duplicate. The CD95 and CD95L levels in the samples were determined by extrapolating the results from a standard curve.

Statistical analysis

Data from this study were recorded in the Microsoft Office Excel 2016 software, and statistical analysis was performed using EpiInfo® 7.0 software (Division of Public Health Surveillance and Informatics Epidemiology Program Office, MS K74 Centers for Disease Control and Prevention (CDC) Atlanta Georgia, USA) and Graph Pad PRISM 5.0 software package (Graph Pad Software Inc., La Jolla, California, USA). Comparisons between CD4+ T-cells, CD8+ T-cells, CD4/CD8 ratio, CD95 and CD95L between different groups, were performed using the non-parametric test of Mann-Whitney and Kruskal-Wallis. The correlations between CD95, CD95L, CD4+ T-cells, CD8+ T-cells, CD4/CD8 ratio and the fibrosis score were established using the Spearman's correlation coefficient (r). All values of $p \leq 0.05$ were considered statistically significant, for a confidence interval of 95%.

Results

Of the 130 patients enrolled, 36 (27.7%) were cirrhotic and 94 (72.3%) were non-cirrhotic. Among the cirrhotic patients, 23 (64%)

were male and 13 (36%) were female, with an average age of 38 ± 16 years (Table 1). Among non-cirrhotic patients, 52 (55.3%) were male and 42 (44.7%) were female, with an average age of 31.54 ± 9 years (Table 1).

The level of CD95 in cirrhotic patients ranged from 0.6 to 6.5 pg/mL, with a median of 5.3 pg/mL. The level of CD95 in non-cirrhotic patients ranged from 0.7 to 6.3 pg/mL, with a median of 3.2 pg/mL. The difference between these 2 groups was statistically significant, with a p-value <0.001 (Figure 1A). The level of CD95L in cirrhotic patients ranged from 1.8 to 5.9 pg/mL, with a median of 4.2 pg/mL. The level of CD95L in non-cirrhotic patients ranged from 1.3 to 5.3 pg/mL, with a median of 3.6 pg/mL. The difference between these 2 groups was statistically significant, with a p value =0.001 (Figure 1B).

The CD4+ T-cell values in cirrhotic patients ranged from 608 to 1024 cells/µL, with a median of 784.5 cells/µL. The CD4+ T-cell values in non-cirrhotic patients ranged from 688 to 1116 cells/µL, with a median of 901.3 cells/µL. The difference between these 2 groups was statistically significant, with a p-value=0.003 (Figure 2A).

The CD8+ T-cell values in cirrhotic patients ranged from 508 to 1003 cells/µL, with a median of 774.5 cells/µL. The CD8+ T-cell values in non-cirrhotic patients ranged from 585 to 1013 cells/µL, with a median of 786 cells/µL. The difference between these 2 groups was not statistically significant, with a p value of 0.417 (Figure 2B).

The CD4/CD8 ratio in cirrhotic patients ranged from 1.0 to 1.2, with a median of 1.0. The CD4/CD8 ratio in non-cirrhotic patients ranged from 1.0 to 1.4 with a median of 1.2. The difference between these 2 groups was statistically significant, with a p-value <0.001 (Figure 2C).

There was a statistically significant correlation between CD95 and CD4+ T-cells (r=0.042, p=0.016), between CD95 and CD8+ T-cells (r=-0.029, p=0.016), between CD95 and the CD4/CD8 ratio (r=0.123, p=0.01), and between CD95 and fibrosis score (r=0.021, p=0.003) (Table 2).

We also found a statistically significant correlation between CD95L and the fibrosis score (r = 0.099, p = 0.003) (Table 2).

There were statistically significant correlations between the fibrosis score and the CD4+ T-cells (r =-0.280, p<0.001), and between the fibrosis score and the CD4/CD8 ratio (r=-0.455, p<0.001) (Table 3).
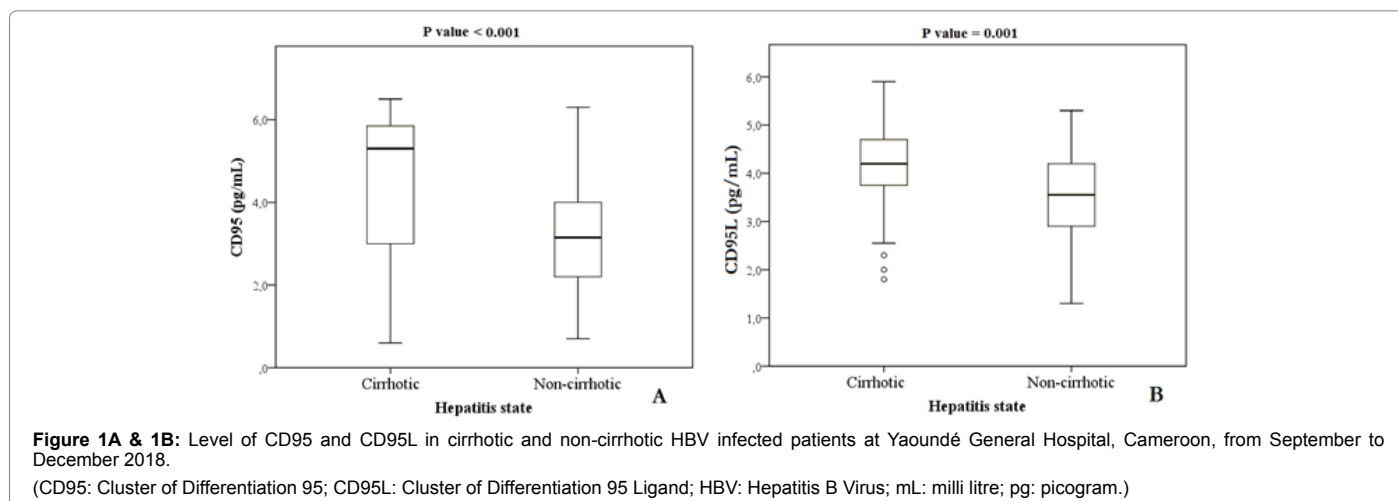
Discussion

We sought to assess the implications of CD95-CD95L receptor-ligands in T-cells depletion and hepatic cytolysis, in patients with chronic VHB. We found that the plasma levels of CD95 and CD95L were significantly elevated in cirrhotic patients, compared to non-cirrhotic patients. The CD4/CD8 ratios were lower in cirrhotic patients, compared to non-cirrhotic patients. There were statistically significant correlations between CD95 and CD4+ T-cells, between CD95 and CD8+ T-cells, between CD95 and the CD4/CD8 ratio, between CD95 and fibrosis scores and between CD95L and fibrosis score.
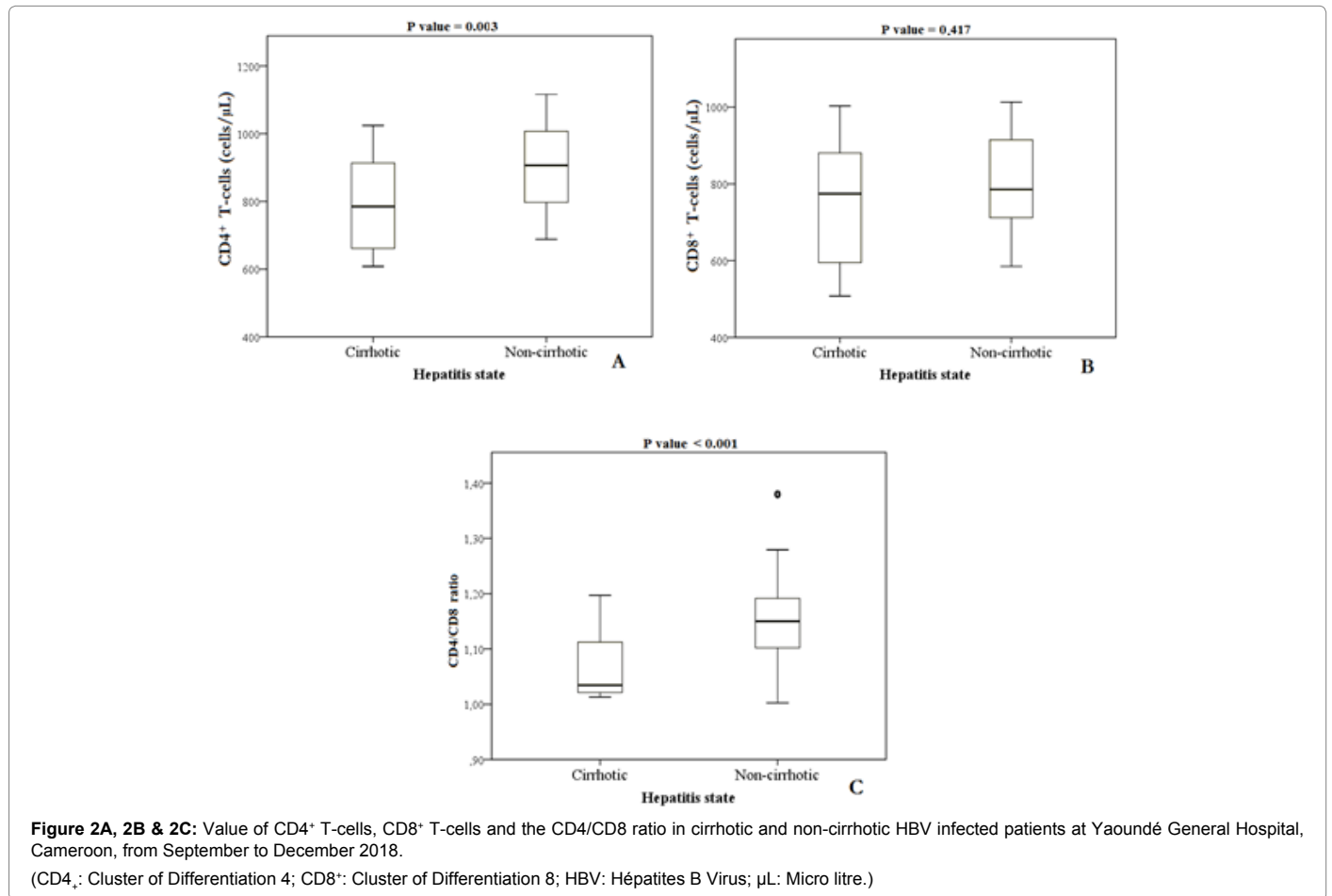
The concentrations of CD95 and CD95L were higher in cirrhotic patients than in non-cirrhotic patients, with a statistically significant difference between the two groups. These results corroborate those of Peter et al., who had observed a low constitutive expression of CD95 in non-cirrhotic patients with chronic VHB, compared to patients with cirrhosis related to HBV [8]. The results obtained in this study are also in accordance with literature that CD95 is overexpressed during HBV infection [9]. This expression of CD95 would be increased in response to a primary stimulus, and would make hepatocytes more susceptible to stimulation by CD95L. This hypothesis is supported by the observation that induction of CD95 expression occurs following chronic lymphatic histiocytic inflammation in different epithelial cells [6]. These results suggest that liver destruction in HBV infected patients may primarily involve the destruction of hepatocytes by T-cells using the CD95-CD95L receptor-ligand system.

		Clinical status n (%)	
		Cirrhotic	**Non-cirrhotic**
Study population: 130		36 (27.7%)	94 (72.3%)
Sex	Male 75 (57.7%)	23 (64.0%)	52 (55.3%)
	Female 55 (42.3%)	13 (36.0%)	42 (44.7%)
Age (years) Mean ± SD		38.03 ± 16.11	31.54 ± 9.27

Table 1: Social and clinical characteristics of HBV infected patients at Yaoundé General Hospital, Cameroon, from September to December 2018.

HBV: Hepatitis B Virus; n: Effective; SD: Standard Deviation

Figure 1A & 1B: Level of CD95 and CD95L in cirrhotic and non-cirrhotic HBV infected patients at Yaoundé General Hospital, Cameroon, from September to December 2018.

(CD95: Cluster of Differentiation 95; CD95L: Cluster of Differentiation 95 Ligand; HBV: Hepatitis B Virus; mL: milli litre; pg: picogram.)

Figure 2A, 2B & 2C: Value of CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratio in cirrhotic and non-cirrhotic HBV infected patients at Yaoundé General Hospital, Cameroon, from September to December 2018.

(CD4+: Cluster of Differentiation 4; CD8+: Cluster of Differentiation 8; HBV: Hépatites B Virus; µL: Micro litre.)

	CD95 (pg/mL)		CD95L (pg/mL)	
	r	p value	r	p value
CD4+ T-cells (cells/µL)	0.042	0.016	-0.286	0.148
CD8+ T-cells (cells/µL)	-0.029	0.016	-0.155	0.083
CD4/CD8 ratio	0.123	0.01	-0.106	0.1
Fibrosis score	0.021	0.003	0.099	0.003

Table 2: Correlation between CD95, CD95L and CD4+ T-cells, CD8+ T-cells, CD4/CD8 ratio and fibrosis score of HBV infected patients at Yaoundé General Hospital, Cameroon, from September to December 2018.

CD95: Cluster of Differentiation 95; CD95L: Cluster of Differentiation 95 Ligand; HBV: Hepatitis B Virus; µL: Micro litre; mL: Milli litre; pg,: picogram.

	Fibrosis score	
	r	p value
CD4+ T-cells (cells /µL)	-0.28	0.009
CD8+ T-cells (cells /µL)	-0.039	0.426
CD4/CD8 ratio	-0.455	<0.001

Table 3: Correlation between fibrosis score and CD4+ T-cells, CD8+ T-cells, CD4/CD8 ratio of HBV infected patients at Yaoundé General Hospital, Cameroon, from September to December 2018.

HBV: Hepatitis B Virus; µL: Micro liter; r: Spearman's correlation coefficient

Immune system abnormalities are associated with T-cells depletion [10]. We found statistically significant differences in CD4+ T-cells values between cirrhotic and non-cirrhotic patients. Mean values of CD4+ T-cells in cirrhotic patients were lower than those observed in non-cirrhotic patients. In addition, mean values of CD8+ T-cells in cirrhotic patients were higher than in non-cirrhotic patients. These abnormalities can be caused by an abnormal regulation of the activation gene for the antigen 4 of cytotoxic T-cells (CTLA-4), lymphocytes 3 (Lag-3), the immunoglobulin T domain and the mucin 3 domain (TIM- 3), cell death receptor 1 (PD-1), CD244 / 2B4, CD160 and by the T-cells immunoreceptor with Ig and ITIM domains (TIGIT) [11, 12].

The average ratio of the CD4/CD8 ratios in cirrhotic patients was similar to that found in 2016 by Yang et al [13]. This mean ratio, however, was lower than that found in non-cirrhotic patients. This could reflect a possible increased immune depletion in these cirrhotic patients, compared to non-cirrhotic patients.

We highlighted the existence of a statistically significant positive correlation between CD95 and CD4+ T-cells in chronically infected individuals with HBV. The correlation coefficient showed that in these patients, these two parameters move in the same direction. At the same time, there was a statistically significant, but negative, correlation between CD95 and CD8. This indicated that in both groups of patients these two parameters are reversed. These results could be explained by the presence of CD95 receptors on the surface of activated T-cells, hence their increase / decrease would be associated with the increase / decrease of CD95. The statistically significant positive correlation between CD95 and the CD4/CD8 ratio observed in HBV infected individuals showed that in these individuals, these two parameters move in the same direction. This could be due to the influence of the amount of CD4+ T-cells in the CD4/CD8 ratios. The increase in CD4+ T-cell involving the increase in the plasma concentration of CD95 receptors.

There was no statistically significant correlation between CD95L and CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratio. The values of the correlation coefficients, all negative, showed that in these individuals, the CD95L evolved in the opposite direction to the CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratio, although there is no significant association between them. This could be explained by the maintenance of the peripheral tolerance of T-cells activated by AICD mediated by the interaction between CD95 and CD95L [6]. Indeed, over activated effector T-cells are harmful to the immune system, and must be removed from the periphery.

In addition, there was a statistically significant positive correlation between the METAVIR fibrosis score and the CD95 and CD95L concentrations in these chronically HBV-infected patients. This reflected an association between liver injury and activation of the CD95-CD95L apoptosis pathway. These results corroborate those obtained by Peter et al. [8] in a study of CD95 receptor and ligand involvement in hepatic injury [8], where CD95 receptor expression was very high in hepatocytes in HBV-related cirrhosis [12]. These results are also parallel to those obtained in the case of infections with Hepatitis C virus by Hayashi & Mita [14], where the expression of CD95 was upregulated according to the severity of inflammation of the liver.

Analysis of the statistically significant negative correlation between fibrosis score and CD4+ T-cell concentration in patients chronically infected with HBV show that these two parameters were associated. There was a statistically significant negative correlation between fibrosis score and the CD4/CD8 ratio in patients chronically infected with HBV. These results reflected an association between the specific cellular immune response via the activation of the CD4+ T-cells who are the "coordinators", and the liver lesions that occurred; and therefore suggests that the destruction of hepatocytes in VHB would be induced by T-cells, using CD95-CD95L mediation [12]. There was no statistically significant correlation between fibrosis score and CD8+ T-cells concentration in patients chronically infected with HBV. These results show that expression levels of the constitutive receptor CD95 are functionally sufficient to mediate apoptosis of hepatocytes and T-cells.

Our study had certain limitations; indeed, this study did not evaluate the activation of the CD95-CD95L signaling pathway during the evolution of viral B infection. We therefore propose, through a longitudinal study, to evaluate the activation of the CD95-CD95L signaling during viral progression from hepatocyte cultures infected with HBV; and evaluate the inhibition of CD95 receptors as an immunotherapeutic medium in the context of chronic VHB.

Conclusion

The levels of CD95 and CD95L were higher in cirrhotic patients compared to non-cirrhotic patients. The CD4+ T-cells, CD8+ T-cells and the CD4/CD8 ratios were respectively lower, higher, and lower in cirrhotic patients compared to non-cirrhotic patients. Thus, CD95-CD95L would therefore be involved in T-cells depletion and hepatic cytolysis during the pathogenesis of chronic VHB, and could potentially be used as biomarkers for immunological and hepatic monitoring in patients with chronic VHB.

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