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Role of hemodialysis and hepatitis C virus infection in circulating Th1 and Th2 cytokines in patients with chronic renal disease

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Abstract

The present study was performed to evaluate the possible role of Th-1cytokine (IFN-γ, TNF-α) and Th-2cytokine (IL-10) in immunopathogenesis of hepatitis C in hemodialysis patients. The sandwich ELISA assay was performed for the detecting the serum levels of these cytokines in 23 hemodialysis HCV positive (HD/HCV+) patients, 20 HD hemodialysis HCV negative (HD/HCV-) patients in comparison with apparently healthy control and non-uremic chronic HCV infected patients. Where, the HCV-positive hemodialysis patients had a blunted TNF-α response (P<0.01) and failed to increase the stimulated IFN-γ (P<0.01) compared with chronic HCV infected patients without renal disease. On the contrary, IL-10 stimulation was higher in HCV-positive hemodialysis patients (P<0.01). The results showed that the disturbed cytokine response appeared to focus in the Th1 because the stimulation of Th2 cytokines (IL-10) was not impaired.

Key words: HCV, Hemodialysis, INF-**γ**, TNF-**α**, IL-10.

Introduction

Hepatitis C virus (HCV) is blood-borne pathogen that appears to be endemic in most parts of the world. It is estimated by the World Health Organization that there are 170 million HCV-infected persons worldwide (Al Dhahry *et.al.*, 2003; Senevirathna *et.al.*, 2008). The populations most affected by HCV are patients that undergo multiple blood transfusions, individuals who are intravenous and inhalant drug users, hemophiliacs, and hemodialysis (HD) patients (Alavian *et.al.,* 2011). Immune mediated mechanisms are believed to play an important phathogenetic role in HCV infection. Several cytokine induced by viral infection play directly or indirectly roles in antiviral defense. Liver disease progression from chronic liver disease to hepatocellular carcinoma due to HCV infection is associated with an imbalance between Th1 and Th2 cytokines.

Martin *et.al.*, (2000) have hypothesized that HD procedure may introduce changes in the pattern of cytokines; and within the group of HD patients, HCV infection may modify the cytokine profile in the infected versus the non-infected individuals. Uremic patients have an impaired cell-mediated immunity and phagocytic activity, which accounts for their susceptibility to infection, malignancies and vaccination resistance.

This condition may disrupt the cytokine network by depressing Th1 response, enabling Th2 type mediators to prevail (Burra *et..al.*, 2008). Th1 cells are involved in the innate cell-mediated immunity and produce several proinflammatory cytokines, notably TNF-α, IL-12 and INF-γ. Although Th2 cells are involved in humoral immunity and produce cytokines, such as IL-4, IL-5, and IL-10 they also produce IL-6 and thus also have a role in the systemic inflammatory response (Davenport and Tipping, 2003; Li *et.al.*, 2011). The combination of an impaired immune response coupled with persistent immune stimulation may have a role in the low-grade systemic inflammation and altered cytokine balance that characterizes the uremic state (Stevinkel *et.al.,* 2005). To analyse these hypotheses, we have studied serum levels of cytokines from the Th-1 phenotypes (IFN-γ & TNF-α) and Th-2 (IL-10).

Materials and Methods

This study included 23 hemodialysis HCV positive (HD/HCV+) patients (16 males and 7 females, mean age 44.35±15.06), 20 HD hemodialysis HCV negative (HD/HCV-) patients (9 males and 11 females, mean age 43.9±15.60). Sampling lasted from May to October 2010.

All patients were dialyzed 2 or 3 times per week and each HD treatment took three to four hours. Dialyzer membranes were disposable and single use. The clinical diagnosis was obtained from patient records and interview and ethical approval for use of all specimens was obtained. Exclusion criteria include history of receiving antiviral and/or interferon therapy for HCV (+) subjects, uncontrolled elevated blood pressure, diabetes mellitus, concomitant chronic hepatitis B or other well known liver diseases such as metabolic or autoimmune disorders and various infectious states of the liver, cryglobulinemia, human immune deficiency virus (HIV) infection, chronic respiratory insufficiency, rheumatoid arthritis, cirrhosis, or malignant tumor.

Control groups were age and sex matched: (1) chronic hepatitis C patients without known renal disease (*n*=20) (14 males and 6 females, mean age 44.55±15.81 years) were randomly recruited from the gastroenterology outpatient clinic. In patients with indication for antiviral therapy the sera were collected before the start of antiviral therapy; and (2) healthy volunteers (*n*=20) (11 males and 9 females, mean age 42.95±16.07 years) were used as normal control. No individual had serological markers of hepatitis B or human immunodeficiency viruses infections. Samples of blood (5 ml) were obtained by vein puncture using disposable latex gloves and syringes immediately before Hemodialysis sessions. Sera were separated from whole blood, for this purpose the blood samples were allowed to clot in the room temperature for 20 minutes and then centrifuged at 2,000 rpm for 10 minutes. All samples were divided into three aliquots then immediately frozen and stored at (-20ºC) for serological assays to prevent cross contamination and unnecessary thawing and freezing.

Sandwich ELISA-kits for detection of INF-γ (MABTECH, Sweden, product code: 3420-1A-6), TNF-α (MABTECH, Sweden, product code: 3510-1A-6) and IL-10 (MABTECH, Sweden, product code: 3430-1A-6).

The limit of detection for INF-γ, TNF-α and IL-10 were 2 pg/ml, 8 pg/ml, and 0.5 pg/ml, respectively. To produce a standard curve was done by using a duplicate sample of each standard and the mean of them was used. The procedure done in a two days, as following: In the first day: the high protein binding ELISA plate was coated with mAb (2μg/ml diluted in PBS, pH=7.4), by adding 100μl/well and incubated overnight at (4°C). In the next day: the plate was Emptied and washed twice with PBS. Then, uncoated sites were blocked with 200μl/well of blocking buffer for one hour at room temperature; incubation was carried out in a shaker incubator. The plate was emptied and washed five times with washing buffer. Then 100μl/well of each patient and control's samples and each diluted standard were added to each well and the plate then incubated for 2 hours at room temperature. After that, the excess of non-reacted sera were removed through a five times wash with washing buffer, while the reacted sera were detected by adding to each well 100μl/well biotenlated mAb (specific to IL-10, INF-γ, or TNF-α Ab) at 1μg/ml concentration in incubation buffer and the plate was incubated for 1 hour at room temperature, then the excess of non reacted sera were removed through five times wash buffer. The reacted sera were detected by adding to each well 100μl/well 1/1000 streptavidin-ALP in incubation buffer, incubated for 1 hour at room temperature and washing five times with washing buffer. While, the reacted reagent were detected by adding 100μl/well freshly prepared substrate solution (P-Nitrophenyl-phosphate solution) and the mixture allowed to react in a dark place for 30 minutes at room temperature. Then, the absorbency was measured at 450 nm for each sample using an automated microtiter plate reader.

Statistical significance was measured using the statistical package for social studies (SPSS) program for windows software package release 15. All data are expressed as means ± S.D., Student's t-test was used to evaluate the mean differences between two groups, while ANOVA was used to compare means of more than two independent groups. Correlations between variables were estimated using Pearson's correlation coefficient. The level of significant in all cases was set at a two-tailed (P<0.05).

Results and Discussion

In the present study, the solid sandwich ELISA

assay was performed for the detecting the serum levels of Th1 and Th2 cytokines in 23 haemodialysis HCV positive (HD/HCV+) patients (16 males and 7 females, mean age 44.35±15.06), 20 HD haemodialysis HCV negative (HD/HCV-) patients (9 males and 11 females, mean age 43.9±15.60), 20 non-uremic patients with chronic hepatitis (C/HCV+) (14 males and 6 females, mean age 44.55±15.81) as patients control, and 20 apparently healthy volunteers (H/HCV-) (11 males and 9 females, mean age 42.95±16.07) as normal control. There was no significant difference in age and sex (P>0.05) between patients group and control (Table 1).

The comparison of cytokines level in the HD patients and controls detected that the serum TNF-α levels in HD patients was higher than their levels in the healthy control group 45.60±17.86 vs. 13.7±3.79 pg/ml, P<0.001), (Table 2). TNF-α is the main mediator of the acute inflammatory responses to microbial infections and may contribute to the innate immunity in stimulating the adaptive immune responses (Najafizadeh *et.al.*, 2007). Furthermore, chronic uremia is considered a pro-inflammatory state associated with high cardiovascular morbidity and mortality. As well as, the present study results also showed that the level of IL-10 in the serum of hemodialysis patients were significantly higher than apparently healthy control group (89.77± 57.30 vs. 18.2±6.49 pg/ml, P<0.001).

Conversely, IFN- γ level was significantly lower in hemodialysis patients γ (4.07±2.00 pg/ml) than those of the normal controls (10.00±1.65 pg/ml, P<0.001). Similar results reported by Borazan *et.al.*, (2004) who found that serum TNF-α and IL-10 levels in the CAPD and HD patients were higher than those in the control group. The two typical cell factors (IFN-γ /IL-10) detected in this study may represent Th1/Th2. In hemodialysis patients, it seems that Th2 function overwhelms Th1. One potential mechanism is the bad biocompatibility of the dialysis membrane and the endotoxin mixed in dialysate, varieties of active peptides are released, and then the humoral immunity system is stimulated to respond to these antigens. This process needs many Th2 cells, which can produce profuse IL-10. As indicated above, IL-10 can decrease the number of Th1 cells and the accompanying secretion of IFN-γ (Li *et.al*., 2011).

Also the serum cytokines levels were determined in patients with chronic HCV infection in comparison with apparently healthy control group. The results showed that serum levels of INF-γ and TNF-α were significantly higher in chronic HCV-infected patients than control group (P<0.001). These results in accordance with that reported by (Soube *et.al.*, 2001; Najafizadeh *et.al.*, 2007). In contrast, the serum level of IFN-γ was significantly reduces in HCV infected patients (Ebeid and El-Bakry, 2009; R-Viso *et.al.*, 2010). Also our results showed that IL-10 was significantly higher in chronic HCV patients than healthy control group (P<0.05) (Table-2). Similarly results reported by others (Abayli *et.al.*, 2003; Zekri *et.al.*, 2005; R-Viso *et.al.*, 2010).

Furthermore, we found that the elevated levels of Th1 cytokines are greater than Th2 cytokine in non-uremic chronic HCV patients. Thus, our study indicates that enhanced Th1 responses are present during chronic HCV infection, which may partly be responsible for the liver injury. Moreover there was a significant reverse correlation between the hemodialysis period and the levels of INF-γ (r = -0.304, P<0.05). In contrast, a significant positive correlation has been seen between hemodialysis period and the levels of IL-10 (r = +0.418, P< 0.05), (Table-3). If a change in IFN-γ and IL-10 levels in hemodialysis patients is induced by hemodialysis, it is understandable that this kind of change may be time dependent. Surprisingly, there was no significant correlation between the level of TNF-α and length of the hemodialysis period in our current data (r = +0.191, P>0.05), one presumed reason is that our sample size was not very large and this caused a statistical distortion.

The comparison between HD patients and C/HCV patients showed that IL-10 serum concentrations were significantly higher in HD patients (89.77±57.30 pg/ml; P < 0.001) than in non-uremic chronic HCV patients (53.55 ± 14.17 pg/ml), as shown in table (4). In contrast, our results also showed that HCV-positive hemodialysis patients had a blunted TNF-α response and failed to increase the stimulated IFN-γ production compared with chronic HCV patients without renal disease (TNF-α: 49.26±2.19 vs. 64.05±18.32 pg/ml, P<0.01; INF-γ: 3.57±2.19 vs. 53.75±9.53 pg/ml, P<0.01), as shown in tables (4 and 5). Similar results reported by others (Martin *et.al*., 2000; Horoz *et.al.*, 2006; Burra *et.al.*, 2008).

HCV infection promotes a predominant Th2-like (IL-10) over a Th1 (IFN-γ) type response in HD. Sobue *et al.*(2001) found that both CD4+ and CD8+ cells, interferon IFN-γ-producing cell populations increased, while there was no difference in interleukin IL-10 production, indicating a shift to a Th1 cytokine profile with the progression of liver disease. In contrast, Abayli *et al.* (2003) suggested the involvement of Th2 cytokines in the pathogenesis of chronic hepatitis C virus liver disease. However, HD/HCV-positive patients showed lower TNF-α production (49.26±2.19 vs. 64.05 ±18.32 pg/ml, *P*<0.01) but more IL-10 secretion (105.39±55.16 vs. 53.55±14.17 pg/ml, *P*<0.01) compared with chronic hepatitis C (C/HCV) patients. Finally, the HD/HCV-positive individuals had a marked decreased of IFN-γ compared with the C/HCV patients (3.57±2.19 vs. 53.75±9.53 pg/ml, *P*<0.01) (Data not shown). These characteristics may account for the finding that the manifestations of HCV-associated disease are milder in HD patients compared with chronic HCV patients in whom Th1 responses are predominant (Martin *et al*., 2000).

The comparison between HD/HCV positive & HD/HCV negative patients results showed that IL-10 serum level was higher in HD/HCV positive (105.39±55.16 pg/ml) compared with HD/HCV-negative individuals (71.8±55.67 pg/ml), however it was not reached statistical significant (*P*=0.054) (Table-5). This result in accordance with that reported by Nascimento *et.al.* (2005) who found that the virus provoked an induced Th2 immunosuppression, and both Th1 and Th2 were related to a progressive state of HCV chronicity. Burra *et.al.* (2008) reported that cuprophane membranes reportedly stimulate monocytes to produce high levels of pro-inflammatory cytokines which is followed, within a few hours, by the induction of IL-10. Also, there was no difference regarding INF-γ & TNF-α production between HCV positive and negative groups (*P*>0.05), as shown in table (5). Thus, HCV does not appear to influence cytokine responses in HD patients. The cytokine pattern observed in HD patients is compatible with the hypothesis explaining the relatively benign evolution of HCV-related liver disease in HD patients. Similar results reported by Horoz *et.al.* (2006), who found that inflammatory cytokine responses such as production of TNF-α, IFN-γ by PBMC were abolished in HD patients and this pattern of response similar among hemodialysis patients with or without HCV infection.

Although the precise role of viral, host and/or environmental factors in promoting disease progression have yet to be defined in dialysis subjects, it has been reported that IL-10 concentrations were also related to renal function indices and nutritional markers in patients on long-term HD (Balakrishnan *et.al.*, 2004). Other factors such as age may influence the serum levels of cytokines. A study of Boehmer *et.al.*, (2005) demonstrated that older mice produce more IL-10 than younger mice and less TNF-α following lipopolysaccharide stimulation. In view of negative regulation of Th2 cytokines for immune functions, we consider that enhanced Th2 responses in HD/HCV positive patients may allow the human host to suppress the inflammatory/ immune responses, resulting in reducing the hepatic tissue injury through down-regulation of the inflammatory/immune reaction and leading to inability to eliminate the virus. This is one possible explanation why HCV infection tends to be a chronic condition (Fan *et.al.*, 1998).

Conclusion: The cytokine pattern observed in hemodialysis patients is compatible with the hypothesis explaining the relatively benign evolution of HCV-related liver disease in hemodialysis patients.

Table (1): Demographic features and level of biochemical factors of patients and control groups.

Variable	HD/HCV+ (n=23)	HD/HCV- (n=20)	C/HCV+ (n=20)	H/HCV- (n=20)
Age	44.35 ±15.06	43.9 ±15.60	44.55 ±15.81	42.95 ±16.07
Gender (M/F)	16/7	9/11	14/6	11/9
ALT (IU/L)	13.78 ±15.70	15.85 ±13.46	42.50 ±20.35	22.4 ±1.67
AST (IU/L)	16.78 ±23.96	14.20 ±9.00	39.60 ±18.54	20.8 ±1.36

Table (2): INF-**γ**, TNF-**α** & IL-10 levels in hemodialysis patients, Chronic HCV infected patients and healthy control groups.

Group	INF-**γ** (pg/ml) Mean ± S.D.	TNF-**α** (pg/ml) Mean ± S.D.	IL-10 (pg/ml) Mean ± S.D.
H/control	10.00 ±1.65	13.7 ±3.79	18.2 ±6.49
H.D.	4.07 ±2.00	45.60 ±17.86	89.77 ±57.30
**P*	<0.001	<0.001	<0.001
C/HCV	53.75 ±9.53	64.05 ±18.32	53.55 ±14.17
**P*	<0.001	<0.001	<0.05

**P* value in comparison with apparently healthy control

Table (3): Person correlations between the haemodialysis period & the serum levels of INF-**γ**, TNF-**α** & IL-10.

Correlation	INF-**γ** (pg/ml)	TNF-**α** (pg/ml)	IL-10 (pg/ml)
r	-0.304	+0.191	+0.418
P	<0.05*	>0.05	<0.05*

Table (4): INF-**γ**, TNF-**α** and IL-10 levels in patients with hemodialysis and chronic HCV groups.

Group	INF-**γ** (pg/ml) Mean ± S.D.	TNF-**α** (pg/ml) Mean ± S.D.	IL-10 (pg/ml) Mean ± S.D.
C/HCV	53.75 ±9.53	64.05 ±18.32	53.55 ±14.17
H.D.	4.07 ±2.00	45.60 ±17.86	89.77 ±57.30
P	<0.001	<0.001	<0.001

Table (5): INF-**γ**, TNF-**α** and IL-10 concentration (pg/ml) in HCV positive & negative haemodialysis patients.

Groups	INF-**γ** (pg/ml) Mean ± S.D.	TNF-**α** (pg/ml) Mean ± S.D.	IL-10 (pg/ml) Mean ± S.D.
HD/HCV+	3.57 ±2.19	49.26 ±2.19	105.39 ±55.16
HD/HCV-	4.65 ±1.63	41.40 ±1.63	71.8 ±55.67
P	>0.05	>0.05	>0.05

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