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EXPRESSION OF FAS PROTEIN (CD95) AND FAS LIGAND IN LIVER TISSUE OF PATIENTS WITH HCV-INDUCED CHRONIC LIVER DISEASE AND ITS CORRELATION WITH THE DISEASE PROGRESSION

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Abstract

This study evaluated hepatic expression of both Fas and Fas ligand (FasL) in patients with hepatitis c virus (HCV)-induced chronic liver disease and its correlation with the histopathological activity and laboratory parameters as an early predictor of advancement of the disease.

The selected patients were (39) males and (21) females, their ages ranged from (20-67years) with a mean of 43.5±4.5 years, as well as (10) subjects (normal individuals) serving as a control group. They were (7) males and (3) females, their age ranged from (26-53 years) with a mean of 39.5±7.3 years. Patients were grouped as (1) Chronic hepatitis (CH) group including (30) patients with chronic viral hepatitis C. (2) Liver cirrhosis (LC) group including (30) patients with post hepatitis C cirrhosis. Liver biopsy was done for all subjects using an automated 18-gauge true cut needle. Sections were stained with Haematoxylin and Eosin for histopathological diagnosis and with Maisson and Trichrome for assessment of fibrosis. Unstained paraffin sections from each case were subjected for immuno-histochemical procedures using indirect immunoflourescence technique for detection of apoptotic hepatic and lymphocytic cells using monoclonal antibodies. Semiquantitative analysis of the pattern and distribution of the Fas antigen and Fas Ligand as indicators for hepatic apoptosis was studied and assessed.

Key words: Egypt Fas; Fas ligand; apoptosis; HCV; chronic liver disease.

Introduction

For every cell, there is a time to live and a time to die. Prompt removal of unwanted cells, such as senescent, damaged, genetically mutated, or virus infected cells, is crucial for the maintenance of liver health. This process is naturally achieved through a highly regulated programmed form of the cell death called apoptosis (Guicciardi and Gores, 2005). Fas is a (45KD) glycosylated cell-surface protein, ubiquitously expressed in various tissues, in particular in thymus, liver, heart, kidney, and in activated mature lymphocytes or virus-trans-formed lymphocytes (Guicciardi and Gores, 2010). In HCV

infection, Fas expression is up-regulated in the liver cells in line with the severity of liver inflammation. The Fas/FasL system is indeed the pathway most commonly used by immunocytes to kill virally infected cells (Yoneyama *et al*, 2002). When the HCV specific T cells migrate into hepatocytes and recognize the viral antigen via the T cell receptor, they become activated and express Fas ligand that transduce the apoptotic death signals to Fas-bearing hepatocytes resulting in their destruction. Thus, the Fas system plays an important role in liver cell injury by HCV infection (Feig *et al*, 2007). FasL (CD95L) is a type n trans-membrane protein with homotrimeric structure, mainly expressed on the cell surface of activated T cell. In the liver, the interaction between FasL-positive cytotoxic T lymphocytes and target cells, such as virus-infected cells or cancer cells, which usually overexpress Fas, represents a powerful tool to eliminate potentially toxic cells (Guicciardi and Gores, 2005). Kupffer cells, liver-specific phagocytes, can also express FasL and induce hepatocyte apoptosis (Canbay *et al*, 2003). Therapeutic approaches aimed to modulate Fas-mediated apoptosis may ultimately be effective in reducing liver damage in several human liver diseases. Preliminary studies on animal models of liver injury have already generated promising data regarding the feasibility and effectiveness of genetic inhibition of Fas as a possible therapy to prevent fulminant liver failure (Guicciardi and Gores, 2010). This study aimed to assess the expression of Fas and FasL in fine needle liver biopsy samples of patients with chronic HCV and post hepatitis C cirrhosis compared with normal individuals (controls), to correlate their expression with the different pathological grading and staging of chronic viral hepatitis and liver cirrhosis and to study their clinical significance as markers of apoptosis for possible prediction of progression of the disease in chronic viral hepatitis and liver cirrhosis.

Patients, Materials and Methods

The present study was conducted on 70 selected individuals, sixty of them had chronic liver disease (CLD) (chronic hepatitis C), referred to The Tropical Medicine Department, El-Hussein and El- Sayed-Galal University Hospitals, as well as (10) subjects (normal individuals) serving as a controls. The selected patients were (39) males and (21) females, their ages ranged from (20 to 67 years) with a mean of 43.5±4.5 years, as well as (10) subjects (normal individuals) serving as a control group. They were (7) males and (3) females, their age ranged from (26 to 53 years) with a mean of 39.5±7.3 years.

According to the history taking, the physical examination, laboratory investigations, imaging techniques and histopathological examination, the patients were grouped as: G1-CH including (30) patients with chronic viral hepatitis C. G2-LC including (30) patients with post hepatitis C cirrhosis. G3-Control included (10) subjects with normal liver function tests and negative viral seromarkers.

The patients were included according to the following inclusion criteria: Patients with the chronic HCV infection proved clinically, biochemically, serologically and histopathologically. Post hepatitis C liver cirrhosis. The diagnosis was based upon clinical, biochemical, serological and ultrasonographic findings. Prothrombin concentration >60% and platelets count >60000/cmm to allow liver biopsy performance.

All the studied subjects were subjected to: 1- Complete clinical assessment. 2- Laboratory evaluation including: A- Routine tests: The complete urine and stools analysis to exclude *Schistosoma* ova, complete blood picture, and erythrocyte sedimentation rate. B- Biochemical tests: Liver and renal function tests. C- Hepatitis sero-markers: For HBV (HBsAg, anti HBc and anti HBs) and for HCV (anti-HCV) using ELISA technique according to Abbott laboratories. D- HCV RNA tested by PCR nested quantitative by IU/ml. E - Alpha fetoprotein after El Far *et al*. (2006)

Imaging procedures: A- Conventional Abdominal Ultrasonography by using Toshiba SSA-340A machine with a 3.5MHZ curved convex probe. B- Ultrasound guided liver biopsy.

HistopathoIogical examination: All patients were subjected to specific tests where unstained paraffin sections from each case were cut on slides treated with 3-amino propyl-triethoxy saline (TESPA) and subjected for immunohistochemical procedures using indirect immunoflourescence technique for detection of apoptotic hepatic and lymphocytic cells using monoclonal antibodies (Bauwens *et al*, 2011).

The semiquantitative analysis of the pattern and distribution of the Fas antigen and Fas Ligand as indicators for hepatic apoptosis was studied and assessed. Immunohistochemical scoring of Fas and Fas L: The expression of Fas and Fas L was measured in 5 successive high power fields (X400). It was expressed as brown coloration on hepatocytes cell surface accompanied by cytoplasmic involvement. The degree of Fas and Fas L expression within the hepatic lobules was evaluated semi-quantitatively according to the percentage of positively stained cells.

The intensity of staining was determined for each case as mild, moderate and intense (over expression) and the extent of immunolabeling of each case was scored as: a- 0 cell (no cell stained), b- Negative (1-<5 %), c- Focally positive (5-89%) and d-Diffusely positive (≥90%) according to Lee *et al.* (2003). Statistical analysis: The SPSS software was used for data management and analysis. Quantitative data were presented as mean ± SD. Qualitative data were presented as frequencies and percentages. For comparison of 3 group's means, Mann-Whitney U test was used to study the relationship between two variables Spearman's correlation coefficient was calculated. All tests were two tailed and considered statistically significant when ($p < 0.05$).

Results

The results are shown in tables (1, 2, 3, 4, 5, 6, 7 & 8).

Table 1: Fas and Fas Ligand expression in studied groups

Item	Fas Mean± SD	Fas Ligand Mean± SD	P value
Control	1.8±2.3	0±0	P<0.001
CH	32±15.7*	33.4±14.9*	
LC	61.4±14.7*,^	49.8±18.2*,^	

*P value <0.001 relative to control group (VHS), ^P value 0.001 relative to CH group (VHS), VHS: very high significant.

Table 2: METAVIR scoring of inflammatory activity of studied patients (70)

	Control (10)		CH (30)		LC (30)		P value
METAVIR Activity	N	%	N	%	N	%	P<0.001
A0 (n=10)	10	100	0	00	0	00	
Al (n=28)	0	00	27	90	1	3.3	
A2 (n=14)	0	00	3	10	11	36.6	
A3 (n=18)	0	00	0	00	18	60	

Table 3: METAVIR scoring of staging of fibrosis of studied patients (70)

	Control (10)		CH (30)		LC (30)		P value
METAVTR Fibrosis	N	%	N	%	N	%	P<0.001
F0 (n=10)	10	100	0	00	0	00	
F1(n=25)	0	00	25	83.3	0	00	
F2 (n=4)	0	00	4	13.3	0	00	
F3 (n=24)	0	00	1	3.3	23	76.7	
F4 (n=7)	0	00	0	00	7	23.3	

Table 4: Fas and Fas ligand expression versus histopathologic inflammation grades

METAVIR Activity	Fas Mean± SD	Fas Ligand Mean± SD
A0 (n=10)	1.8±2.3^,**,^^	0±0^,**,^^
Al (n=28)	33.8±15.8*,^^	35.1±14.2*,^^
A2 (n=14)	27.5±15.4*,^^	25±17.3*,^^
A3 (n=18)	62.6±14.5*,^,**	50.5±18.5*,^,**

*p<0.01 compared to patients with METAVIR (A0)
^p<0.001 compared to patients with METAVIR (Al)
**p<0.01 compared to patients with METAVIR (A2)
^^p<0.001 compared to patients with METAVIR (A3)

Table 5: Fas and Fas Ligand expression versus histopathologic stages of fibrosis

METAVIR Fibrosis	Fas (Mean± SD)	Fas L (Mean± SD)
F0 (n=10)	1.8±2.3^**^^#	0±0^**^^#
Fl (n=25)	32.4±16.2*^^#	35.7±15*^^#
F2 (n=4)	33.8±13.8*^^#	28±10.3*^^#
F3 (n-24)	60 ±16*^**	47.4±20.3*^**
F4 (n=7)	59.3±20*^**	52.4±16.7*^**

*p<0.01 compared to patients with METAVIR (F0)
^ p<0.001compared to patients with METAVIR (Fl)
**p<0.05 compared to patients with METAVIR (F2)
^^p<0.05 compared to patients with METAVIR (F3)
# p<0.05 compared to patients with METAVIR (F4)

Table 6: Fas and Fas Ligand expression versus HCV RNA (PCR)

HCV RNA (P.C.R)	Fas Mean± SD	Fas Ligand Mean± SD
Negative viremia (<100)	2.5±3.3^**^^	3.18±10.6^**^^
Low viremia (100-100,000)	44.3±19.6*^^	36.4±14.6*^^
Moderate viremia (100,000-1000,000)	42.7±20.4*^^	41.7±22.1*^^
High viremia (>1000,000)	63.3±18.7*^**	57.7±15.4*^**

HCV RNA done by PCR= polymerase chain reaction, nested quantitative (IU/ml).
*p<0.001 compared to patients with negative PCR
^p<0.01 compared to patients with low viremia
**p<0.01 compared to patients with moderate viremia
^^p<0.01 compared to patients with high viremia

Table 7: Correlation between Fas, Fas Ligand, steatosis, METAVIR A and METAVIR F in groups

	Fas		Fas Ligand		Steatosis		METAVIR A		METAVIR F	
	r	P	r	p	r	p	r	p	r	p
Fas			0.75	<0.001	0.69	<0.001	0.79	<0.001	0.76	<0.001
Fas Ligand	0.75	<0.001			0.6	<0.001	0.63	<0.001	0.61	<0.001
Steatosis	0.69	<0.001	0.6	<0.001			0.81	<0.001	0.82	<0.001
METAVIR A	0.79	< 0.001	0.63	<0.001	0.81	<0.001			0.69	<0.001
METAVIR F	0.76	<0.001	0.61	<0.001	0.82	<0.001	0.69	<0.001		

Table 8: Correlation between Fas, Fas Ligand, steatosis, METAVIR A and METAVIR F with ALT, AST and HCV RNA (PCR) in groups

	Fas		Fas Ligand		Steatosis		METAVIR A		METAVIR F	
	r	P	r	p	r	p	r	p	r	p
ALT	0.34	<0.01	0.25	<0.05	0.35	<0.01	0.36	<0.01	0.32	<0.01
AST	0.34	<0.01	0.24	<0.05	0.29	<0.05	0.30	<0.05	0.25	<0.05
HCV P.C.R	0.55	<0.001	0.63	<0.001	0.52	<0.001	0.49	<0.001	0.46	<0.001

r. correlation coefficient, p: p value, METAVIR A: METAVIR activity, METAVIR F: METAVIR fibrosis

Discussion

HCV is a major cause of CLD, cirrhosis, and hepatocellular carcinoma worldwide. The infection has a high propensity to chronicity and the majority of HCV carriers have histological evidence of liver inflammation and chronic damage, although with a very wide spectrum of severity and progression rate (Calabrese *et al*, 2000).

The mechanisms leading to liver cell injury, inflammations, and fibrosis in chronic HCV, were not fully understood. Both immune-mediated reactions and more direct cytopathic effects of HCV and of its proteins may be involved. Evidence has been provided that apoptosis of liver cells may play a significant role in the HCV pathogenesis (Bantel *et al*, 2001).

Increased expression of Fas, one of the most important members of the tumor necrosis factor family receptors able to transduce the apoptotic signal to programmed cell death was described in chronic HCV (Calabrese *et al*, 2000).

The hepatic up-regulation of Fas was found to correlate with more severe inflammation and with ongoing HCV infection. Parallel activation of T lymphocytes expressing FasL was detected in liver infiltrating mononuclear cells, allowing transduction of the apoptotic death signal to Fas-bearing hepatocytes and to proinflammatory activated cells that continuously migrate from extrahepatic sites. Both structural and nonstructural HCV proteins have been shown to interact with apoptosis mediators and possibly modulate the active cascade of events leading to programmed cell death (Kim *et al*, 2006).

The immune-mediated apoptosis may play a role in the pathogenesis of chronic HCV and indicate that this type of reaction may occur in the absence of significant alanine transaminase (ALT) elevation (Zekri *et al*, 2007). Thus, assessment of liver cell apoptosis is a valuable indicator in histopathological examination as regard disease progression. In HCV infection, Fas expression is up-regulated in the liver cells in line with the severity of liver inflammation. The Fas/FasL system is indeed the pathway most commonly used by immunocytes to kill virally infected cells (Yoneyama *et al*, 2002).

Concerning the expression of Fas and FasL, there was very low Fas protein expression (1.8±2.3) and no FasL expression in the control group whereas there is a high expression in the CH group (32±15.7). The G3-LC showed the highest expression of Fas and FasL (61±14.7). This denotes that Fas and FasL expression (markers of apoptosis) increase in chronic hepatitis and their level of expression is directly proportional with the disease progression. The mean value of Fas and FasL is statistically very highly significant in CH and LC group relative to controls at p value < 0.001. Also, there was a statistically very highly significant difference between LC group and CH group at p value < 0.001. There was a positive correlation between hepatic expression of Fas and FasL (correlation coefficient $r = 0.75$ and p value <0.001).

The degree of liver cell apoptosis has been shown to be high in chronic HCV patients compared to healthy subject (Kountouras *et al*, 2003). The cellular and molecular processes responsible for the increase in apoptosis remain unclear (Walsh *et al*, 2004). Fas/FasL interaction has been postulated as a major mechanism for HCV-induced hepatocyte apoptosis (Dmitrieva *et al*, 2003).

Fas antigen expression by hepatocytes were significantly higher in patients with moderate and sever grades of necroinflmmatory activity and stages of fibrosis as compared to patients with mild activity and fibrosis and with control group (Zakaria *et al*, 2005).

FasL expression by the activated T cells may initiate the apoptotic death signal in Fas-bearing hepatocytes and in proinflammatory-activated cells that continuously migrate from extrahepatic sites. Enhanced Fas-mediated hepatocyte apoptosis has been well documented in HCV-associated CLD, and the Fas expression increases in parallel to chronic HCV progression, referring to important role of cell death (Jarmay *et al*, 2002).

In the present study, the highest Fas and FasL expression was in METAVIR activity (A3) and the lowest was in METAVIR activity (A0). Fas and FasL expression increased gradually from METAVIR activity (A0) to reach its highest level in METAVIR activity (A3). There is a very high statistically significant difference between Fas and FasL expression with METAVIR activity (A1& A2) and with METAVIR activity (A3) groups in comparison to the control group at p value<0.001. The mean value of Fas and FasL is very highly statistically significant in METAVIR activity (A3) groups relative to METAVIR activity (A1& A2) groups at p value <0.001 (tab. 4).

On the other hand, in the present study, the highest Fas and FasL expression was in METAVIR fibrosis (F3 & F4) and the lowest was in METAVIR fibrosis (FO). Fas and FasL expression increased gradually from METAVIR fibrosis (FO) to reach its highest level in METAVIR fibrosis (F4). There is a very high statistically significant difference between Fas and FasL expression with METAVIR fibrosis (F1& F2) and with METAVIR fibrosis (F3 & F4) groups in comparison to the control group at p value <0.001. The mean value of Fas and FasL is statistically significant in METAVIR fibrosis (F3 & F4) groups relative to METAVIR fibrosis (F1& F2) groups at p value <0.01 (Fas) and at p value<0.05 (FasL) (table 5).

The present data were similar to that previously reported (Rust and Gores, 2000; Zakaria et al., 2005), and could be attributed to fact that, Fas expression in HCV infection is up regulated in the liver cell in line with the severity of liver inflammation (Nasir *et al*, 2000). Moreover, a positive correlation was found between Fas antigen expression in the studied patients and stage of hepatic fibrosis, indicating that hepatocyte apoptosis through Fas expression is suggestive to be involved in hepatic cell injury in chronic HCV patients (Zakaria *et al,* 2005).

The present study, showed a highly positive correlation of Fas, FasL, steatosis, METAVIR A and METAVIR F with each others at p value <0.001, which indicates that there is a positive correlation between the degree of steatosis, inflammation, fibrosis and the degree of apoptosis represented by Fas, FasL expression (apoptotic markers) (tab. 7).

Fas, FasL showed significant direct correlation with steatosis ($r=0.69$, $p < 0.001$). Steatosis is one of the characteristic histological features of (although not pathognomonic for) chronic HCV infection. Liver steatosis is a frequent finding in the biopsy of patients with HCV infection and in some of them it might be extensive and severe (Alberti, 2003). Liver steatosis may contribute to progression of fibrosis in patients with HCV (Nasir *et al,* 2004).

The present results agreed with Poynard *et al* (2001). Canbay *et al.* (2004) concluded that clearance of apoptosis debris through phagocytosis may directly stimulate fibrogenesis. Zakaria *et al.* (2005) found a significant direct correlation between the degree of steatosis and the severity of histopathological changes and hepatocyte apoptosis

suggesting the latter as a mechanism whereby steatosis contributes to the progression of liver injury in chronic HCV.

In the present study, serum transaminases values correlated significantly with the severity of liver disease and with apoptotic activity represented in areas of Fas antigen expression, with statistical significance (r =0.34, p value <0.01 for Fas & r =0.25, p value 0.05 for FasL) (Tab. 8). This was in agreement with kronenberger et al (2000) and Zakaria et al (2005). However, in another reports, apoptosis did not correlate with transaminase levels and in spite of normal ALT levels, patients showed mild Fas expression. This could be explained by Nakamoto *et al.* (2002) and Kountouras *et al*. (2003) who concluded that T cell apoptosis in patients with chronic HCV is considered to cause a reduction in serum ALT, contributing to HCV persistence in patients with chronic HCV without overt biochemical changes.

The patients with chronic HCV and normal transaminases had significantly lower hepatocyte proliferation rates and show a trend towards lower apoptosis rates compared to patients with elevated transaminases (kronenberger *et al,* 2000).

In the present study the highest Fas and FasL expression was in those with high viremia and the lowest was in those with negative viremia. The pattern of Fas and FasL expression increases with the HCV viral load. There was a positive correlation between hepatic expression of Fas and FasL and level of viremia with a statistically very highly significance (r =0.55 for Fas, r =0.63 for FasL, p value 0.001 for both) (Table 8), indicating that the increased HCV-RNA load is associated with higher rates of hepatocyte apoptosis. The results agreed with Zakaria *et al.* (2005).

Conclusions

The progressive liver injury in chronic HCV infection may be related to an up-regulation of apoptosis through Fas antigen expression that interacts with the anti-Fas antibody (FasL) on cytotoxic T lymphocytes. Liver cell apoptosis (through Fas and FasL expression) in the chronic HCV patients was directly correlated with biochemical, virological and different histopathological grades of inflammatory activity.

The extent of apoptosis (Fas antigen expression) could point to the degree of activity in chronic HCV infection. Hepatic steatosis could be accompanied with a higher grade of necroinflammatory activity and a more sever forms of fibrosis.

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