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Assessment the process of apoptosis by the measurement of Fas and Fas ligand levels in some Iraqi Hepatitis B patients

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Abstract

This study was aimed to evaluation the process of apoptosis through the estimation of Fas and Fas ligand levels in some Iraqi Hepatitis B patients. A total of 103 Iraqi patients infected with hepatitis B were involved in this study, these patients attended the Gastroenterology Department of Baghdad Teaching Hospital during the period from mid-June 2012 to April 2013. Their ages ranged between (20 -60) year. These patients included 66 males and 37 females, in addition to forty apparently healthy individuals with age range from (20- 60) year were studied as control group. This group included 26 males and 14 females. The ratio between male to female among patients group was 1.78:1 whereas 1.85:1 among control group. The results of this study recorded that the mean age among patients group was 44.16±13.97 year and more than half of patients (58.3%) are located within third and fourth decade whereas the mean age among control group was 35.1±17.1 year, moreover the more than half of control group (62.5 %) are located within second and third decade. Furthermore the results of this study proved that in hepatitis B patients the levels of Fas and Fas ligand were significantly elevated ($p<0.001$) as compared to control group. In patients group the Fas levels was 251.5±72.2ng/ml and Fas ligand level was 65.7±24.8ng/ml in comparison to control group was 136.01±37.68ng/ml and 31.2±7.8ng/ml respectively. In conclusion, our findings prove the influence of apoptotic factors (Fas and FasL) on hepatitis B patients.

Keywords: Apoptosis, Fas, Fas ligand, Hepatitis B.

Introduction

Fas and FasL are “death domain”, are proteins which regulate the process of apoptosis in patients of chronic inflammatory diseases such as viral hepatitis (Bantel and Osthoff, 2003). Fas (CD95/APO-1) is a cell-surface membrane member of the tumor necrosis factor (TNF) receptor super family and mediate programmed cell death, or ‘apoptosis’, upon engagement by its ligand, FasL. Fas is widely expressed in numerous different cell types throughout the body, whereas FasL expression appears to be more restricted (Connell, 2001; Nagata, 1997). Following activation, different cell types within the immune system express FasL, including T and B cells. FasL is also expressed in cells within areas of ‘immune privilege’, including the eye and reproductive organs. FasL-induced apoptosis plays both regulatory and effector functions in the immune system and appears to contribute to inflammatory process (Nagata, 1997). Hepatitis B virus (HBV) infection with its associated sequelae is a disease of major public health importance worldwide. Globally, it is estimated that about 320–350 million individuals are chronic carriers of hepatitis B virus (HBV) and about 1.5 million people die annually from HBV-related causes (Wright, 2006). It is based on this high prevalence, and the various sequelae of HBV infection, especially liver cirrhosis and primary liver cell carcinoma (PLCC) that makes HBV infection to continue to remain a public health concern (Wurie *et al.,* 2005). At least seven different types of viruses are referred to as hepatitis viruses and they generally cannot be distinguished clinically, these are hepatitis A virus (HAV) the etiologic form of infectious hepatitis; hepatitis B virus (HBV) which is associated with (serum hepatitis); hepatitis C virus (HCV); hepatitis D virus (HDV); hepatitis E virus (HEV); and hepatitis G virus (HGV) (Wright, 2006). Transmission methods of HBV are; sexually by persons with more than one sex partner, persons diagnosed with a sexually transmitted disease, homosexual men, injecting drug users, household contacts of infected persons, infants born to infected mothers, infants or children of immigrants from areas with high HBV rates, health care and

public safety workers who are exposed to blood, and hemophiliacs, and haemodialysis patients (CDC, 2005; Liang, 2009). There is significant evidence that Fas and FasL play a significant role in the pathogenesis of a wide spectrum of liver and gastrointestinal diseases (Lapenski *et al.,* 2004). Death receptors, especially Fas, are widely expressed in all liver cell types, likely in response to the evolutionary pressure to eliminate hepato-tropic viruses, the Fas/FasL system is indeed the pathway most commonly used by immunocytes to kill virally infected cells. Because of this high level of death receptor expression in hepatocytes, apoptosis in the liver occurs mainly via the extrinsic pathway (Krammer, 2000). The contribution of Fas and Fas ligand to some disorders is suggested by the finding of elevated Fas levels in serum of patients with conditions such as myocarditis, liver disease, hypertension and rheumatoid arthritis these findings suggested that Fas have a pro-inflammatory role which may be inhibited by inhibition of lymphocyte apoptosis (Hohlbaum *et al.,* 2000; Natori *et al.,* 2001). Abnormalities in apoptosis, particularly affecting the soluble Fas / Fas ligand are implicated in the pathogenesis of hepatitis B viral infection (Terradillos *et al.,* 2002), therefore the aim of this study was undertaken to evaluate the process of apoptosis by measurement of Fas and FasL levels in some Iraqi hepatitis B patients.

Materials and Methods

Patients Study Group: A total of (103) Iraqi patients infected with hepatitis B were involved in this study, these patients attended the Gastroenterology Department of Baghdad Teaching Hospital during the period from mid-June 2012 to April 2013. Their ages ranged between (20-60) year. These patients included 66 males and 37 females. They were sequentially selected from cases referred to the hospital at first presentation. They were diagnosis based upon the patient's medical history, physical examination and laboratory test.

Control group: Forty apparently healthy individuals with age range from (20-60) year were studied as control group. This group included 26 males and 14 females. Samples were collected from those individuals only if they were not receiving any medication and did not had a history of a chronic or acute illness.

Specimens collection: From each individual included in this study, 5 ml of blood was drawn by vein puncture using disposable syringes. The blood was placed in plastic disposable tubes, it was left to stand at room temperature (20-25°C) to allow it to clot, then the sera was separated by centrifugation for 5min. and divided into aliquots (250µl) and stored at -20°C till examination. Each aliquot of the serum was used once to avoid thawing and freezing. All sera and reagents were allowed to stand at room temperature before use in the test.

ELISA screening test for HBs Ag: Hepanostika HBsAg Uni-FormII is an ELISA for qualitative determination of HBs Ag subtypes ad and ay in human serum or plasma (Klaus *et al.,* 1996). This test is based on a one step "Sandwich" principle. As to HBs Ag coupled with HRP serves as the conjugate with TMB and peroxide as a substrate. Upon completion of the test, a color develops which directly proportional to the amount of HBs Ag in the sample , the reaction is terminated by addition of sulfuric acid and absorbance is measured at 450nm (Klaus *et al.,* 1996). The detailed procedure was carried out as has been suggested in the leaflet supplied with the test kit (Bio Merieux, France).

ELISA HBs Ag confirmatory test: Hepanostika HBs Ag Uniform II confirmatory reagents are to be used for the confirmation of HBs-Ag in specimens (Rose *et al.,* 2002). HBs Ag confirmatory test requires the use of two solutions: Confirmatory neutralizing antibody (Nab) and confirmatory control, each were pipetted into one of two non-coated wells, then the sample was added to both wells. Following incubation, the mixtures were transferred to HBs Ag Uni-Form II Ab-coated wells containing a conjugate phase. The test continued with the reagent and procedure of the HBs Ag. Sample that contains HBs-Ag showed a reduced color developed in the well neutralized by the confirmatory Nab, the reaction is terminated by addition of sulfuric acid and absorbance is measured at 450nm (Rose *etal.,* 2002). The detailed procedure was carried out as has been suggested in the leaflet supplied with the test kit (Bio Merieux, France).

Determination of human Fas and FasL: Serum levels of Fas and FasL were measured quantitatively in sera of studied groups by enzyme linked immunosorbent assay method using ELISA kits (Bender MedSystems, Austria), measurement as recommended by the manufacturer (Yoon and Gores, 2002; Rust and Gores, 2000). An anti-Fas or anti-FasL monoclonal antibody is adsorbed onto micro wells. Fas or FasL present in sample or standard binds to antibodies adsorbed to the micro wells ; a biotin-conjugate monoclonal anti-Fas or anti-FasL antibody is added and binds to the Fas or FasL captured by the first antibody. Following incubation unbound biotin-conjugate anti-Fas or anti-FasL is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-Fas or anti-FasL. After incubation unbound streptavidin-HRP is removed during the

wash step. And substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of the Fas or FasL present in the sample. The reaction is terminated by addition of sulfuric acid and absorbance is measured at 450nm. A standard curve is prepared by using several dilutions of Fas or FasL and sample concentration determined (Yoon and Gores, 2002; Rust and Gores, 2000). The detailed procedure was carried out as has been suggested in the leaflet supplied with the test kit.

Statistical analysis: The usual statistical methods were used in order to assess and analyze our results and included:

Descriptive statistics: including Mean and Standard deviation (SD).

Inferential-statistics: Data have been analyzed statistically using SPSS program version 20. Analysis of quantitative data was done using t-test and ANOVA (analysis of variance). Acceptable level of significance was considered to be below 0.05.

Results and Discussion

One hundred and three hepatitis B patients (66 women, 37 men) who age range (20-60 year) were studied. Both sexes can be affected by hepatitis B virus (HBV). By analyzed the distribution results of studied groups according to gender, the results showed that the majority of patients and control groups are males (64.1% and 65.0%) respectively rather than females (35.9% and 35.0%) respectively. The ratio between male to female among patients group was (1.78:1) whereas (1.85:1) among control group (Table 1). This high frequency of infection with HBV among males patients group may be attributed to socio-community nature of Iraqi people which makes men undergone the responsibility of working and eventually are in great contact with the pathogens rather than the women as well as the sex differences among patients group could be explained on the basis that males may have a greater chance to come in contact with risk factors of HBV than females, or alcohol intake being common in males, which may enhance the liver damage caused by HBV infection (Manolakopoulos *et al*., 2003). Most studies denoted to the prevalence of HBV was among men more than women (Ahmed, 2006; Al-Yassiri and Al-Thwani, 2006; Al-Waysi, 2005; Al-Saaedi, 2001; Al-Hilli, 2000).

The distribution of studied groups according to age are shown in (Table 2), the results of this study recorded that the age ranged between (20-60 year) (mean of 44.16±13.97) among patients group and more than half of patients (58.3 %) are located within third and fourth decade (30-49 year) whereas the age ranged between 20-60 year (mean of 35.1±17.1) among control group, moreover the more than half of control group (62.5%) are located within second and third decade (20-39 year) (Table 2). In fact, age at infection seems to be the most influencing factor in prognosis. the results of this study indicated that the mean age for patients group was 44.16 year, these results coincide with the previous studies done in Iraq as Ahmed (2006) and Al-waysi (2005) who establish that 45 year was the mean age for hepatitis B patients, also on other hand Dienstag (2005) registered that the mean age for control and patient groups was 37 year and 42.6 year respectively.

Table (1): Distribution of the studied groups according to gender.

Sex	Patients		Control	
	No.	%	No.	%
Female	37	35.9	14	35
Male	66	64.1	26	65
Total	103	100	40	100
Male / Female ratio	1.78:1		1.85:1	

Table (2): Distribution of the studied groups according to age.

Age groups (year)	Patients		Control	
	No.	%	No.	%
(20-29)	22	21.4	11	27.5
(30-39)	36	35.0	14	35
(40-49)	24	23.3	6	15
(50-60)	21	20.4	9	22.5
Total	103	100.0	40	100.0
Mean	44.16 ± 13.97 year		35.1 ± 17.1 year	

The results of this study proved that in sera samples of hepatitis B patients the levels of Fas and Fas ligand were significantly elevated ($p<0.001$) as compared control group (Table 3 and 4). In patients group the Fas levels (mean ± D) was 251.5±72.2 ng/ml and Fas ligand level (mean ± SD) was 65.7±24.8ng/ml in comparison to control group Fas levels 136.01±37.68ng/ml and Fas ligand 31.2±7.8 ng/ml as shown in Tables (3 and 4). This study showed a remarkable increment in the serum concentrations of Fas and FasL in hepatitis B group as compared to control group, these results are similar to the results obtained by (Yoon and Gores, 2002; Rust and Gores, 2000; Yin and Ding, 2003) reported that the serum concentrations of Fas and FasL in patients with HBV infection were significantly elevated.

Table (3): The comparison between hepatitis B patients and control group regarding to apoptosis parameters (Fas and Fas ligand).

Study group	Factors	No.	Mean	Std. Deviation	Std. Error Mean
Patients	Fas	103	251.5	72.2	7.118
	Fas Ligand	103	65.7	24.8	2.4317
Control	Fas	40	136.01	37.68	5.951
	Fas Ligand	40	31.2	7.8	1.230

Table (4): T-test and P-value for patients group and control group according to Fas and Fas ligand.

Factors	T	P-Value	C.s
Fas \Patients - Fas \Control	10.088	P<0.001	HS
Fas ligand\ Patients - Fas ligand\ Control	9.970	P<0.001	HS

Apoptosis is one of the factors prolonging inflammation in chronic hepatitis B. Impaired apoptosis activity or its hyperactivity may induce unfavourable course of HBV, Fas and FasL are proteins that regulate activity of apoptosis. Fas is dominantly localized on liver cells. Activation of Fas is an after-effect of fusion with FasL lymphocyte receptors. These particles are recognized defining coefficient state of stimulation apoptosis. The concentration of Fas/FasL in serum patients with chronic hepatitis B and C can define the success of therapy (Wang *et al.,* 2003; Zhao *et al.,* 2000). In this study showed that increase concentration of Fas and Fas ligand in patients group in comparison to control group. The possible explanation of this fact is that HBV might stimulate expression of FasL on lymphocytes. The complex FasL and Fas could activate apoptosis and cytotoxic T lymphocytes. Activation of inflammation processes in the liver could influence FasL activity, associated with Fas and FasL complex formation, therefore in patients with hepatitis B, insufficient apoptosis activity could cause chronic inflammation, as well as, the HBV-mediated immune response might be closely associated with Fas-triggered hepatocyte apoptosis. In conclusion, our findings confirm the influence of apoptotic factors (Fas and FasL) on hepatitis B patients (i.e. inflammation activity of HBV is associated with apoptosis).

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