Expression Of Fas/Apo-1(Cd95) In Patients With Hepatocellular Carcinoma

Abdelgwad T*, El-Sayed IH, Al-Rabat A*, Badra G*** and Abdelhafeez N***

* Clinical pathology Department, Faculty of Medicine, Benha University, **Molecular Biology Department, Institute of Genetic Engineering and Biotechnology, Minofiya University, ***Internal Medicine Department, National Institute of Liver, Minofyia University.

E-mail: ibrahimelsayed@yahoo.com

ABSTRACT: Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor and one of the most common cancers worldwide. Apoptosis and the Fas system are believed to be involved in the process of hepatocellular carcinogenesis. Fas-expressing cytotoxic T lymphocytes (CTLs) are important antitumor effector cells. Peripheral blood lymphocytes were isolated from 16 patients with HCC and 17 patients with liver cirrhosis and Fas expression was analyzed by flow cytometry. The results were compared with normal control volunteers. The results showed that the Fas mean was significantly higher in patients with HCC compared with liver cirrhosis patients and normal controls (52.09±17.7% vs. 16.68±10.21 vs. 14.19±7.25%, respectively). In conclusion, the elevated blood levels of Fas have been observed in patients with hepatocellular cancer, indicating a significant role in liver cancer tumorigenesis. [Report and Opinion 2010;2(9):62-67]. (ISSN: 1553-9873).

KEYWORDS: Fas, Hepatocellular carcinoma (HCC), flow cytometry, liver cirrhosis

INTRODCTION

Hepatocellular carcinoma (HCC) comprises nearly 6% of all incident cancer cases worldwide, with the overwhelming majority occurring in the developing world. One of the least curable malignancies, HCC is the third most frequent cause of cancer mortality among men worldwide (Parkin et al., 2005). Hepatocellular carcinoma is the second most frequent cause of cancer incidence and mortality among men in Egypt (Freedman et al., 2006). Hospital-based studies from Egypt have reported an increase in the relative frequency of all liver-related cancers in Egypt (>95% as HCC), from approximately 4.0% in 1993 to7.3% in 2003 (Hassan et al., 2001; Strickland et al, 2002; El-Zayadi et al., 2005). The Middle East Cancer Consortium recently reported that the incidence rate among males was seven times greater than the next highest rate (among Israeli Jews) and more than three times that reported in the United States Surveillance Epidemiology and End Results (SEER) summary (Freedman et al., 2006).

Apoptosis is widely accepted as a prominent tumor-suppression mechanism. Bcl-2 family has emerged as a dominant regulator of apoptosis in cancer cells. The mitochondrial- mediated pathway of apoptosis is regulated by the Bcl-2 family of anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) and pro-apoptotic proteins (Bax, Bad, and Bak). Bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with Bax/Bak (Oltvai et al., 1993). Defects in apoptosis signaling contribute to tumorigenesis and chemotherapy resistance of HCC cells. In HCC, there is an imbalance between the pro- and anti-apoptotic of Bcl-2 family members. The expression of anti-apoptotic Bcl-xL and Mcl-1 is increased in HCC, whereas the expression of pro-apoptotic Bid and Bak protein is decreased (Ma et al., 2009)

Fas (CD95/Apo-1) and Fas ligand (FasL, CD178) system contribute to programmed cell death (PCD). The death signal of PCD occurs when FasL binds to the Fas receptor on cell surfaces, activating caspase 8 and eventually caspase-3. Fas consist of two isoforms, membrane anchored (mFas) and soluble (sFas), whereas mFas induces apoptosis and sFas functions as an inhibitor. The membrane isoform (mFas) is a 45-kDa cell surface protein containing a single transmembrane region and induces apoptosis in normal or tumor cells, whereas the soluble isoform (sFas) lacks the transmembrane

domain because of alternative splicing of the transcript and is thought to block Fas-mediated apoptosis by binding and subsequent inactivation of FasL (Oltvai et al., 1993). In the current study, we investigated CD95 flow cytometrically using specific monoclonal antibody in peripheral blood obtained from two patients groups. The first group with hepatocellular carcinoma and the other group with liver cirrhosis.

MATERIALS AND METHODS

Patients

The current study included 52 patients divided into three groups. The first group comprised of 16 patients with hepatocellular carcinoma, the second group included 17 patients with liver cirrhosis, and the last group was normal control composed of 19 volunteers. All cases were recruited from National Institute of Liver (NLI), Minufiya University. All the required investigations were done for confirmation the final diagnosis. The laboratory investigation included CD95, AFP, ALT, AST, bilirubin, GGT, prothrombin, alkaline phosphatase, total protein, and albumin.

Sampling was performed after informed consent was obtained from each patient included in the study to use the samples and clinical data for research purposes after being informed about the nature of the study. The study protocol conforms to the most recent ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by the National Liver Institute (NLI) human research committee.

CD95 Analysis

Detection of FAS (CD95) was performed using monoclonal antibody (Dako, Denemark). This test depends on the ability of a monoclonal antibody to bind to the surface of the cells expressing CD95 lymphocytes and measured by flow cytometry. For each sample two tubes were prepared one for test and the other as control. Ten microlitres of Dako FITC –conjugated antibody of the same isotype as control and 10 µl of Dako FITC–conjugated monoclonal mouse anti-human FAS (CD95) (Dako, Denemark), 100 µl of blood was added to each tube, vortexed and incubated in the dark for 30 min. Two ml of freshly prepared reagent (ammonium chloride 8.02g, sodium bicarbonate 0.82 g, disodium EDTA 0.37 g and distilled water up to 100 ml). The mix was centrifuged at 3000 rpm for five minutes, supernatant was removed and 0.5 ml PBS to each tube was added. The samples were analyzed by the flow cytometer (Coulter EPICS – XL Coulter Electronics, Hialeah, FL, USA)

Statistical analysis

Quantitative data were expressed as mean ± standard deviation. Comparing of two groups was analyzed by Mann-Whitney *U* test, while Kurskal-Wallis test was performed to compare more than two groups and *P* values < 0.05 were considered statistically significant. Data were tabulated and analyzed using the PASW 18 statistical package (SPSS Inc., Chicago, IL).

RESULTS

The study population was classified into three groups. Group one included 16 patients with HCC, 14 males and two females with a mean age of 58.37± 11.3 years. Group two included 17 patients with cirrhosis, 12 females and 5 males with a mean age 52.88 ± 8.13 years. Group three included 19 normal healthy adults as controls, including 15 males and 4 females with a mean age of 53.05± 11.11. There was no significant difference between the three groups regarding the age and sex (Table 1).

The results indicated that liver function tests (AST, ALT, ALP, GGT, Albumin, Total protein, Bilirubin and Prothrombin Time), in addition to alfa fetoprotein are different significantly among the studied group (Table 2). The mean ± SD of α-fetoprotein (normal value up to 11 ng/ml) was 5.55 ± 2.26 ng/ml in healthy controls, 40.41 ± 29.69 ng/ml in liver cirrhotics and 1123.3±934.1 ng/ml in HCC patients (P <0.001).

The mean ± SD of CD95 was 14.19 ± 7.25 vs. 16.68 ±10.21 vs. 52.09±17.7 in control group, liver cirrhotics, and HCC respectively and the difference was statistically significant (P < 0.001)(Table 2). The mean ± SD of CD95 was 50.75±17.85 in male HCC patients versus 61.50 ± 19.08 in female patients (P > 0.05). On the other hand, the mean ± SD of CD95 was 39.19 ± 15.32 in single focal lesion versus 65.0 ± 7.61 in multiple focal lesions (P <0.01). The statistical analysis of data indicated that liver function tests including AST, ALT, ALP, GGT, albumin, total protein, bilirubin, and prothrombin time, in addition to alfa fetoprotein, tumor size are non-statistically correlated with CD95 in HCC group (P > 0.05 for each). Moreover, age and sex were correlated insignificantly with CD95 in HCC group as well (P > 0.05 for each).

Table (1): Age and gender of studied groups

	Normal controls		Cirrhosis n = 17		HCC n = 16		P
Age (yrs):							
Mean ± SD	53.05±11.11		52.88±8.13		58.37±11.3		> 0.05
Gender:	No	%	No	%	No.	%	
Male	15	79	12	70.6	14	87.5	> 0.05
Female	4	21	5	29.4	2	12.5	
Total	19	100	17	100	16	100	

Table (2): The biochemical and the investigated criteria of different groups

Liver Function Test	Control group (n=19)	Cirrhosis (n=17)	HCC (n=16)	P
AST (U/L)	19.6±2.7	54.11±44.68	89.23±65.62	<0.001
ALT (U//L)	17.55±2.24	47.31±39.02	75.76±75.92	<0.01
ALP (U/L)	27.20±4.47	129.0±60.60	122.73±46.30	<0.001
GGT (U/L)	23.55±3.15	70.60±58.4	154.2±159.5	<0.01
T. prot. (g/dL)	7.35±0.34	6.02±1.12	6.55±0.68	<0.001
Alb. (g/dL)	3.91±0.19	2.45±0.68	2.73±1.03	<0.001
T. Bil. (mg/dL)	0.66±0.11	5.64±8.48	3.58±3.35	<0.05
D. Bil. (mg/dL)	0.36±0.08	3.39±5.99	2.10±2.67	0.051
PT (%)	96.3±3.51	56.7±23.2	64.5±18.4	<0.001
AFP (ng/ml)	5.55 ± 2.26	40.41 ± 29.69	1123.3 ± 934.1	<0.001
CD95 (Positive Cells, %)	14.19±7.25	16.68±10.21	52.09±17.7	<0.001

DISCUSSION

Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor and one of the most common cancers worldwide. HCC is a primary malignancy of hepatocyte origin. About 80% of people with hepatocellular carcinomas have cirrhosis. Chronic infection with the hepatitis B virus and hepatitis C virus also increases the risk of developing hepatocellular carcinoma. As apoptotic cell death plays an important role in development and homeostasis of multicellular organisms, the failure of cells to undergo apoptosis might be involved in the pathogenesis of a variety of human diseases, including autoimmune diseases, viral infection, and malignancies Thompson, 1995. Regulation of normal cell growth and turnover is balanced between cell proliferation, cell differentiation and apoptosis. A disruption of this balance is thought to be an important event leading to carcinogenesis .One of the effector molecules in apoptosis is Fas antigen. Fas/Apo-1(CD95) is a type1 transmembrane glycoprotein that signals sensitive cells to die by apoptosis upon ligation with anti-Fas/Apo-1 monoclonal or the natural ligand for Fas (Suda et al., 1993). Fas-Fas ligand (FasL) interactions play an important role in cytotoxic T lymphocyte (CTL)-induced cell death. Engagement of Fas antigen (CD95, APO-1) by its ligand, FasL (both members of the tumor necrosis factor [TNF] superfamily), triggers a cascade of cell events leading to apoptosis and cell death, and it is believed that this interaction plays an essential role in maintaining homeostasis of the lymphocyte population (Suda et al., 1997; Suda and Nagata, 1997). It has been reported that FasL expression by tumors results in the survival of tumor cells by inducing apoptosis of Fas-expressing tumor-infiltrating lymphocytes (TILs) (Bennett et al., 1998). The down-regulation of Fas expression by tumor cells also has been reported, resulting in their relative resistance to Fas-mediated apoptosis. These tumor escape immune mechanisms have been well documented in patients with hepatocellular carcinoma (HCC) Ito et al., 1998).

CTLs are important antitumor effector cells. Despite this, tumor cells can escape antitumor immunity by various adaptive mechanisms. This includes the reduction or loss of immunogenic peptide presented by MHC antigens, which are recognized by CTLs (Luo et al., 1997). CTLs are more susceptible to tumor-induced apoptosis, possibly through engagement by tumor-expressed FasL. The purpose of this study is to evaluate the Fas expression in the blood lymphocytes of patients with hepatocellular carcinoma compared with patients with liver cirrhosis (LC), and healthy controls. We found that CD95 levels in HCC patients are highly significant in multiple focal lesions. In HCC, the levels of sFas correlated with the multiplicity of tumor focus rather than the size of a solitary tumor. It is not clear whether sFas levels are dependent on the number of tumor foci or the total mass volume (Jodo et al., 1998). Most interestingly, in all sFas-positive HCC patients who had undergone surgical removal of each solitary tumor, the levels of sFas rapidly decreased by 78.8% (mean of percentage reduction; range 50.6–93.8%) one week post-operation, and all these patients became sFas-negative. These findings strongly suggest that sFas may be produced by or closely linked with the tumor cells. The lack of correlation between sFas levels and tumor size, however, may not support the production of sFas by the tumor itself. One possibility to explain this conflicting result is that HCC cells, especially those inside the tumor, may lose the capacity to produce sFas due to an insufficient blood supply as the tumor grows. Another possibility is that sFas may derive from infiltrating lymphocytes or unidentified cells which are highly activated by HCC. In either possibility, sFas levels may be tightly related to tumor multicentricity rather than to the size of a unifocal tumor (Jodo et al., 1998).

In the current study, it was found in the present results, no significant relation between CD95 and gender, liver function tests and alpha fetoproteins. Moreover, it was found that no significant relation between CD95 and the age in studied HCC. Others found that Fas antigen positivity was not linked with other parameters such as age and sex (Zandieh et la., 2003; Capri et al., 2006). The expression of CD95 increases until age 74 years, whereas in the oldest old it tends to decrease again. The trend of CD95 expression seems to be related to the change of expression of CD95 on CD4+ lymphocytes, because the CD8+/CD95+ population rose steadily throughout the entire age range (Potestio et al., 1999). Significant decline in the number of CD95+ cells, whose expression is known to be linked with activation, may be implicated as a mechanism by which cells that have reached a stage of replicative senescence remain in the peripheral T cell pool. Anti-CD3-mediated activation of cells from both groups revealed much lower proliferative responses from the older group, supporting the idea that there is an age-associated increase in the number of cells that have reached their replicative limit. These cells may not be lost from the peripheral pool because they fail to express CD95.(Age-related changes in the absolute

number of CD95 positive cells in T cell subsets in the blood (Aspinall et al., 1998).

In the present study, it was found that there is a statistically highly significant elevation of Fas expression in blood lymphocytes of patients with HCC, compared with liver cirrhosis. These finding strongly suggest that Fas may be produced by or closely linked with the tumor cells, or derived from infiltrating lymphocytes which are highly activated by HCC, so that the escape from the immune surveillance may play an important role in tumorigenesis. It was found that, serum levels of sFas and sFasL in both HCC and cirrhotic patients were significantly higher than those of normal controls but there was no significant difference between cirrhosis and HCC patients (Wang et al., 2003). In another study, the results showed that positive frequency of serum sFas levels was 100% in HCC patients, 98% in LC patients and 3% in healthy controls. Serum sFas levels were higher in HCC than in LC and healthy ones. Serum sFas levels in HCC patients were significantly higher than those in normal controls. Down-regulation of Fas expression, up-regulation of FasL expression in hepatocytes and elevation of sFas level in serum might contribute to tumor escape from immune surveillance of the body (Sacco et al., 2000). The median percentage of Fas expression by CD3 positive T cells was significantly higher in patients with HCC compared with normal controls and this was attributable solely to Fas expression by CD4 positive PBLs. In contrast, Fas expression was significantly higher in all the subtypes of TLs (CD3 positive, CD4 positive, CD8 positive, NK cells, and natural T cells) compared with controls (Yuen et al., 2001). It was found that there is a significant elevation of serum levels of sFas in patients with HCC, compared with LC and healthy donors, in all sFas-positive HCC. About 40% of patients with HCC showed no significant elevation of serum sFas levels. There is recent evidence that some tumor cells, including HCC, do express functional FasL which can counterattack Fas-positive lymphocytes since human FasL is rapidly cleaved off by matrix metalloproteinase, increased levels of FasL may interfere with sFas detection in the assay system (Jodo et al., 1998). Compared with LC, apoptotic indices (AI) in HCC tissues were significantly reduced ($P < 0.001$), expression of Fas was decreased ($P < 0.05$), and that of FasL was increased ($P < 0.05$).

Serum sFas levels in HCC patients were significantly higher than those in normal controls. Down-regulation of Fas expression, up-regulation of FasL expression in hepatocytes and elevation of sFas level in serum might contribute to tumor escape from immune surveillance of the body. Apoptosis and the Fas system are significantly involved in the process of liver cirrhosis converting into hepatocellular carcinoma (Peng et al., 2001). In conclusion, the elevated blood levels of Fas have been observed in patients with hepatocellular cancer, indicating a significant role in liver cancer tumorigenesis.

Correspondence to:

Dr. Ibrahim El-Sayed

Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Minufiya University, Sadat City, P.O 22857-79,Minufiya,Egypt. E-mail: ibrahimelsayed@yahoo.com

REFERENCES

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. Cancer Journal for Clinicians 2005; 55: 74-108.

[2] Freedman LS, Edwards BK, Ries LAG, Young JL, eds. Cancer Incidence in Four Member Countries (Cyprus, Egypt, Israel, and Jordan) of the Middle East Cancer Consortium (MECC) Compared with US SEER. National Cancer Institute. NIH Pub. No. 06-5873. Bethesda, MD, 2006.

[3] El- Zayadi AR, Badran HM, Barakat EM, Attia Mel-D, Shawky S, et al. Hepatocellular carcinoma in Egypt: A single center study over a decade. World Journal of Gastroenterology 2005; 11: 5193-5198.

[4] Strickland GT, Elhefni H, Salman T, Waked I, Abdel-Hamid M, *et al*. Role of hepatitis C infection in chronic liver disease in Egypt. American Journal of Tropical Medicine and Hygiene 2002; 67: 436-442

[5] Hassan MM, Zaghloul AS, El-Serag HB, Soliman O, Patt YZ, et al. The role of hepatitis C in hepatocellular carcinoma: A case control study among Egyptian patients. Journal of Clinical Gastroenterology 2001; 33: 123-126.

[6] Oltvai ZN, Milliman CL, Korsmeyer K. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74: 609-619.

[7] Ma S, Chen GG, Lai PBS. Bcl-2 family members in hepatocellular carcinoma (HCC): mechanisms and therapeutic potentials. In Chen GG and Lai PBS (eds); Apoptosis in carcinogenesis and chemotherapy, apoptosis in cancer. 2009, Pages 219-235, Springer.

[8] Marsik C, Halama T, Cardona F, Wlassits W, Mayr F, Pleiner J, Jilma B. Regulation of Fas (Apo-1, CD95) and Fas ligand expression in leukocytes during systemic inflammation in humans. Shock 2003; 20 : 493-496.
[9] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; 267:1456 62.
[10] Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993; 75(6):1169-78.
[11] Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. J Exp Med. 1997; 186:2045-50.
[12] Suda T, Nagata S. Why do defects in the Fas-Fas ligand system cause autoimmunity? J Allergy Clin Immunol 1997; 100:S97-101.
[13] Bennett MW, O'Connell J, O'Sullivan GC, Brady C, Roche D, Collins JK, Shanahan F. The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. J Immunol. 1998; 160:5669-75.
[14] Ito Y, Takeda T, Umeshita K, Sakon M, Wakasa K, Matsuura N, Monden M. Fas antigen expression in hepatocellular carcinoma tissues. Oncol Rep 1998; 5(1): 41-4.
[15] Luo KX, Zhu YF, Zhang LX, He HT, Wang XS, Zhang L. *In situ* investigation of Fas/FasL expression in chronic hepatitis B infection and related liver diseases. J Viral Hepatol 1997; 4: 303-7.
[16] Jodo S, Kobayashi S, Nakajima Y, Matsunaga T, Nakayama N, Ogura N, Kayagaki N, Okumura K, Koike T. Elevated serum levels of soluble Fas/APO-1 (CD95) in patients with hepatocellular carcinoma. Clin Exp Immunol. 1998; 112:166-71.
[17] Zandieh T, Fard AS, Aghaipur M. Expression of Fas antigen (CD95) on human leukemic cells and assessment of apoptosis on Fas+ and Fas-samples by flowcytometry method. Iranian J of Allergy, Asthma and Immunology 2003; 2: 203-207.
[18] Capri M, Salvioli S, Altilia S, Sevini F, Remondini D, Mesirca P, Bersani F, Monti D, Franceschi C. Age-dependent effects of *in vitro* radiofrequency exposure (mobile phone) on CD95+ T helper human lymphocytes. Ann N Y Acad Sci. 2006; 1067:493-9.
[19] Potestio M, Pawelec G, Di Lorenzo G, Candore G, D'Anna C, Gervasi F, Lio D, Tranchida G, Caruso C, Romano GC. Age-related changes in the expression of CD95 (APO1/FAS) on blood lymphocytes. Exp Gerontol. 1999; 34:659-73.
[20] Aspinall R, Carroll J, Jiang S. Age-related changes in the absolute number of CD95 positive cells in T cell subsets in the blood. Exp Gerontol. 1998; 33:581-91.
[21] Wang XZ, Chen XC, Chen YX, Zhang LJ, Li D, Chen FL, Chen ZX, Chen HY, Tao QM. Overexpression of HBxAg in hepatocellular carcinoma and its relationship withFas/FasL system. World J Gastroenterol. 2003; 9:2671-5.
[22] Sacco R, Leuci D, Tortorella C, Fiore G, Marinosci F, Schiraldi O, Antonaci S.Transforming growth factor beta1 and soluble Fas serum levels in hepatocellularcarcinoma. Cytokine. 2000; 12:811-4.
[23] Yuen MF, Hughes RD, Heneghan MA, Langley PG, Norris S. Expression of Fas antigen (CD95) in peripheral blood lymphocytes and in liver-infiltrating, cytotoxic lymphocytes in patients with hepatocellular carcinoma. Cancer. 2001; 92:2136-41.
[24] Peng Z, Tang H, Ling Y, Han G. Apoptosis and Fas system are significantly involved in the process of liver cirrhosis converting into hepatocellular carcinoma. J Tongji Med Univ. 2001; 21:126-9, 148.

1/9/2010