

T cells Are Depleted in HCV-Induced Hepatocellular Carcinoma Patients: Possible Role of Apoptosis and p53

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Egypt has possibly the highest Hepatitis C Virus (HCV) prevalence worldwide. A high proportion of HCV infections become chronic and lead to liver cirrhosis and hepatocellular carcinoma (HCC). The cellular and molecular mechanisms behind HCV infection complication are not completely understood although apoptosis has been implicated in this process. Using flow cytometry, we examined whether T lymphocyte; isolated from patients with HCV and HCV-associated HCC (HCV-HCC); are predestined *in vivo* to undergo spontaneous apoptosis. Also, the role of p53; a key protein in apoptotic process; in the development of HCC was examined. Our data showed that T cells were severely depleted in HCV-HCC patients and its spontaneous apoptosis was higher in patient groups as compared to normal controls. In addition, p53 expression in liver tissue (determined by ELISA) was higher in the HCC patient groups as compared to normal controls and correlated well with the HCC grade. In conclusion, HCV infection induces peripheral T cell apoptosis, depletion and subsequently immune-suppression and this may lead to persistence of infection. Also, p53 is implicated in the poor prognosis of HCV-HCC and could be used as a predictive marker to assess the prognosis of HCC patients.

Hepatitis C Virus (HCV) causes more than 90% of parenterally transmitted non-A, non-B hepatitis (Poynard, 1997; Seeff, 1997). The infection leads to chronic hepatitis in about 85% of HCV-infected patients (Hoofnagle, 1997), and slowly progresses to fibrosis, cirrhosis and ultimately to hepatocellular carcinoma (HCC) (Di Bisceglie, 1997; Purcell, 1997). Egypt has possibly the highest HCV prevalence in the world (Frank et al., 2000). Although there is no firm data available, death rates from HCC in Egypt appear to be increasing over the last decade and it is now well-documented that HCV is a leading cause of liver cancer and cirrhosis. There are reports suggesting that apoptosis or programmed cell death plays an important role in HCV progression although it is still unclear which cellular and molecular mechanisms participate in the process (Bantel

et al., 2000; Nakamoto and Kaneko, 2003; Patel et al., 1998).

In HCV infection, apoptosis was considered by different investigators as a host defense mechanism against infection and tumorigenesis. In this regard, HCV core protein may have a regulatory function in modulating apoptosis by either enhancing it through Fas, tumor necrosis factor (TNF) and lymphotoxin or inhibiting it through C-myc, cisplatin, or TNF (Patel et al., 1999). T lymphocytes appear to be major effectors for protection against cancer and many infectious diseases (Snyder et al., 2003). Regarding HCV, it has been suggested that enhanced T-cell apoptosis in cases of HCV infection may lead to down-regulation of their cellular immune response, thus contributing to the persistence of the virus (Toubi et al., 2001). The involvement of the host immune response

in disease pathogenesis suggests that the cytolytic pathway associated with cytotoxic T lymphocyte (CTL), or the cytokines released by the CD4⁺/CD8⁺ T cells may be involved in inducing apoptosis of the infected hepatocytes (Lau et al., 1998). Besides the Fas-Fas ligand (FASL) pathway, the activation of the immune mediated apoptotic pathways in chronic HCV is corroborated by the fact that the transcripts for perforin/granzyme B are elevated in patients with HCV-related cirrhosis compared to normal liver, or other non inflammatory causes of liver cirrhosis (Lau et al., 1998). Recently, it was found that apoptosis of activated CD4⁺ and CD8⁺ T cells was enhanced by co-culture with hepatocytes expressing HCV structural proteins, a mechanism that is modulated by FasL induction (Iken et al., 2006). The authors concluded that the increased apoptosis of activated T cells induced by HCV structural proteins could amplify the ability of the liver to down-modulate T cell responses, leading to attenuation of anti-viral responses and facilitating viral persistence. In HCC, there is always a mutant or inactive p53 gene; a tumor suppressor gene (Endo et al., 2000), which leads to the abnormal growth of cells and ultimately carcinogenesis (Cheng et al., 2003). The wild-type p53 protein (wt-p53) has a short half-life and is expressed in very low amounts under normal conditions (Koskinas et al., 2005). Exposure of liver cells to a variety of stress factors, results either in an increased rate of synthesis and stability of wt-p53, or mainly in the production of a mutated protein with a longer half-life (Tabor, 1997). Mutations of p53 are common in human HCC, vary considerably geographically, ranging from 10 to 60% in incidence and have been associated with histological grade, size of tumor, age and gender of the patients (Koskinas et al., 2005).

In spite of these important roles, the relationship between the pathological

significance of p53 mutation and prognosis in HCC has not been fully established. In summary, virus-host interactions may determine viral persistence, extent and severity of liver inflammation and possibly viral hepatocarcinogenesis (Kountouras et al., 2003). In this study, we determined whether peripheral blood mononuclear cells (PBMCs), especially T lymphocytes, of patients with HCV and HCV-associated HCC (HCV-HCC) are predestined *in vivo* to undergo spontaneous apoptosis. Also, we discussed the possible role of p53 in the development of HCC.

Materials and Methods

Patients

After informed consent, 15 patients with HCV- induced HCC (10 males and 5 females; 8 grade I and 7 grade II) and 10 chronic HCV patients (8 males and 2 females), older than 21 years, negative for HBV, schistosomiasis and HIV were recruited from Kasr El-Aini hospital in the period from 2003 to 2004. No patients had undertaken interferon therapy. All patients underwent a complete routine clinical assessment including liver functions. Ten control samples for liver biopsies were obtained from healthy liver transplant donors (liver transplant volunteers undergoing liver biopsies as routine investigations before the operation) in the same period and hospital. Ten healthy blood donors/laboratory personnel (4 males and 6 females) were individuals selected from the staff at VACSERA, Egypt.

Extraction of RNA and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from plasma of both control and patient groups with trizol reagent (Life Technology, Gaithersburg, MD) and chloroform (Sigma Chemical Co., St. Louis, MO, USA) as recommended by the manufacturer (Invitrogen, USA). RNA pellets were reconstituted in 20µl diethyl pyrocarbonate (DEPC)-treated water and treated with RNase-free DNase treated water by moving the pipette up and down several times. For cDNA synthesis, reverse transcription (RT) of total RNA was performed with Molony Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, USA). All reactions were carried out in an MJ Research PTC-100 thermal cycler (Watertown, MA) in a mixture containing: DEPC-treated water, KCl buffer (5x), random primers

(Promega, USA), dNTP_s (10mM) (Promega, USA) and RNase inhibitor (25U/ml) (Amersham). To generate complementary DNA (cDNA), master mix was added to the total extracted RNA and incubated at 37 °C for 45 min (for reverse transcription) and for 5 min at 95°C for inactivation of the enzyme.

A portion of cDNA (10 or 20µl according to reaction volume) was used to amplify HCV-specific sequences using nested PCR. The cDNA was amplified by first PCR using T_{aq} DNA polymerase (5 U/ml) (Amersham Pharmacia, Uppsala, Sweden) and a mixture containing: DEPC water, KCl buffer (10x), MgCl₂ (25 mM) (Promega, USA), the first primer, CH₁ (Biomedica, Austria), second primer CH₂ (50 pmol/µl) (Biomedica, Austria). The sequences of the primers were as follows: CH₁ (5'-GGTGACGGTCTACGAG ACCTC-3'), and CH₂ (5'-AACTACTGTCTTACGC AGAA- 3'). Then samples were added and the thermal cycler was programmed (initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 2 min). A total of 35 cycles were performed. The amplification was ended with a long extension time at 72 °C for 10 min. For nested PCR, 3 µl of the 1st reaction was removed and added to 47 µl of master mix (using the same concentration of reagents as in the 1st round of PCR except for the primers which were replaced with CH₃ and CH₄ with the same concentrations). The sequences of these primers were as follows: CH₃ (5'-GCGACCAACACTACTCGGCT-3') and CH₄ (5'-ATGGCGTTAGTATGAGTG-3'). After that, the thermal cycler was programmed (initial denaturation at 95°C for 4 min, denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72 °C for 1 min) and the reaction was started. A total of 25 cycles were performed. The amplification was ended with a long extension time at 72 °C for 10 min. The products of RT-PCR were electrophoresed through a 1.5 % agarose-Tris-EDTA buffer gel (Sigma, MO).

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples (10ml) were collected from patients and controls into sterile vacuotainer tubes containing heparin (Becton Dickinson, NJ, USA). Heparinized venous blood samples were diluted in equal amounts of phosphate-buffered saline (PBS, pH=7.2) at room temperature and separated by density gradient centrifugation over Ficoll-Hypaque (Sigma, MO) at 2000 rpm. Plasma samples were collected, divided into 200µl aliquots and kept frozen at -70°C until used for p53 ELISA. The mononuclear cell layer was separated and following two washings in PBS, PBMCs were suspended in RPMI 1640 medium (Invitrogen, USA) supplemented with Penicillin G

(200U/ml), Streptomycin sulfate (100µg/ml), L-glutamine (2mM) and 10 % heat-inactivated pooled human AB serum (Hyclone, USA). The cell count and viability were then determined using trypan blue (Invitrogen, USA) exclusion technique. All the steps of PBMCs separation were performed in a biosafety cabinet using aseptic technique. All cell suspensions were adjusted to 1x10⁶ cell/ml.

Apoptosis assay using annexin V staining and flow cytometry

For positive control, 10⁶ cells were killed by heating at 56 °C for 45 minutes and mixed with equal volume of alive cells. We used unstained cells as a negative control staining. Of the freshly isolated PBMCs (test and control samples), 1x10⁶ cells were placed into 12x75 polystyrene tubes and washed with wash buffer (PBS containing 2 % heat-inactivated human AB serum) and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 100µl of wash buffer containing 2 µl of annexin-V-fluorescein, 2 µl of Propidium iodide (PI; Roche Diagnostics, Mannheim, Germany); 3 µl allophycocyanin (APC)-labeled monoclonal antibodies against human CD3, 3 µl of peridinin chlorophyll protein (PerCP)-labeled monoclonal antibodies against human CD8. Both CD3 and CD8 monoclonal antibodies were from Beckton Dickinson (New Jersey, USA). The cell suspension and the reagents were incubated for 15 min at 25 °C in a dark place. The cells were then washed twice with 2 ml of wash buffer. The cell pellet was resuspended in 500 µl wash buffer and analyzed immediately on a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA). One hundred thousand events were acquired and data analysis was performed using the FlowJo software (Tree Stars Inc., San Carlos, CA).

Assessment of p53 using ELISA

Fresh tissue samples (biopsy specimens) were collected under supervision of clinicians and were divided into two parts. One part was used for histological studies by fixing it immediately in tissue preservative (40 % formalin). This part was cut and stained for inspection of proper histopathological diagnosis. Diagnosis and grading of HCC was based on clinical and laboratory findings and histopathological features of the liver as defined by the Edmondson-Steiner grading system (Edmondson and Steiner, 1954). The other part was cryogenically stored in liquid nitrogen and used to test for p53 protein using p53 pan ELISA kit (Roche Diagnostics, Mannheim, Germany). Liver extracts were prepared according to the manufacturer's instructions. Extracts were prepared by detergent lysis using low salt RIPA buffer (20 mmole/L Tris-HCl, pH= 7.5, 0.5 mmole/L EDTA, 1 % Nonidet P 40, 0.5 % sodium

deoxycholate, 0.05 % sodium dodecyl sulfate, 1 mmole/L phenylmethylsulphonylfluoride, 1 µg/ml aprotinin (Sigma), 2 µg/ml leupeptin (Sigma)). Liver biopsy sample tubes were placed on ice and 4 volumes of RIPA detergent lysis buffer was added per gram of liver tissue. Homogenization was carried out by manual homogenizer, centrifugation at 1500 rpm for 10 min at 4°C and the resultant supernatants were collected. These supernatants were used to determine the liver content of p53 using ELISA. Individual plasma samples were obtained as described previously and were used to determine plasma p53 content.

The protein content of the liver homogenates and plasma was determined by a colorimetric assay using the dye binding protein assay method of Bradford (1976) using commercially available BIO-RAD kit according to the manufacturer's instructions (BIO-RAD laboratories, USA). The level of expression of p53 in the liver homogenates and plasma samples were measured quantitatively using one-step immunoassay with the p53 pan ELISA kit (Roche Diagnostics, Mannheim, Germany). The method was conducted according to the manufacturer's guidelines. Briefly, 100µl of standards (provided with the kit) and unknown supernatant of liver homogenate and plasma samples were added in triplicates into anti-p53 pre-coated microtiter plate, followed by the addition of 100 µl of anti-p53-peroxidase. The microtiter plate was tightly covered with adhesive cover foil and incubated for 2 h at 25 °C on an orbital shaker. After 2 h, anti-p53-peroxidase was removed by suction and the wells were rinsed 5X with 300 µl of wash buffer (1 min each) at room temperature. After washing, 200 µl of the substrate solution were added and incubated on a shaker for 20 min at RT until color development was sufficient for photometric detection. 50 µl of the stopping solution were added to each well after shaking for approximately 1 min on a microtiter plate shaker. Absorbance was measured at 450 nm using the UV-max ELISA plate reader (Molecular device UV max-Kinetic microplate reader). The ELISA reader-controlling software (Softmax) readily processes the digital data of raw absorbance value into a standard curve from which p53 concentration of the unknown samples were directly derived.

Statistical analysis

Data were expressed for each group of patients and controls as mean level ± the standard error of the mean (SE). Analysis of variance (ANOVA) and paired Student t-test (two tailed) were used to compare the sample data with cut off value. All statistics were carried out using statistical analysis system (SAS) program version 6.12 (SAS, 2000). Differences were

considered statistically significant when p is < 0.05 and highly significant when p is < 0.01.

Results

Detection of HCV-RNA in patients' sera

Amplifying and detecting the RNA using RT-PCR assay can demonstrate the presence of RNA viruses. All patient groups, including HCC patients, were enrolled for detection of HCV-RNA by RT-PCR assay as described in Materials and Methods. In Figure (1), a representative example of RT-PCR data shows that some HCC patients have detectable HCV-RNA levels in serum (positive HCV-RNA) suggesting that these patients were having HCV-associated HCC. Negative HCV-RNA patients were excluded from the study.

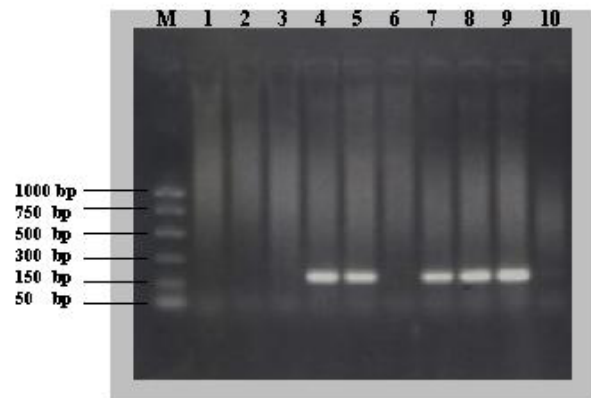


Figure 1. A representative agarose gel showing RT-PCR data of HCV-specific RNA obtained from HCV-positive and negative sera. HCV-specific primers were used to test the sera of patients for HCV-RNA using RT-PCR techniques as described in the Materials and Methods. The amplified genetic material was electrophoresed on a 1.5% agarose gel. The lanes include: lane M, PCR marker; Lanes 1, 2, 3 and 6, for negative samples; Lanes 4, 5, 7 and 8 showing one band at 187 bp for positive samples; Lane 9, positive HCV RNA control sera and the last lane, negative HCV RNA control sera.

Peripheral blood CD3+ T lymphocytes are severely depleted in HCC patients

Figure 2A-C shows a representative example of flow cytometric analysis of CD3 fluorescence intensity as an indication of T cells in the different groups tested using the FlowJo software for analysis. Peripheral

blood T cells were severely depleted in HCC patients compared to chronic HCV patients (Figure 2D). As shown in Figure 2D, the mean percentage of CD3⁺ cells was 58.2 ± 2.6 in control individuals, 43.0 ± 2.0 in chronic HCV-infected patients and 27.6 ± 4.9 in HCC patients. The difference in the mean percentage of CD3⁺ cells was highly significant between the groups tested. In this regard, the mean percentage of CD3⁺ cells was significantly higher in control group than

that in chronic HCV infected patients ($p = 0.0006$) and in HCC patients ($p = 0.0002$). On the other hand, the mean percentage of CD3⁺ cells was significantly higher in chronic HCV infected patients ($p = 0.03$) than in HCC group. These data suggest that peripheral blood T lymphocytes are severely depleted in HCC patients and this process appears to occur in a stepwise fashion during the course of HCV infection.

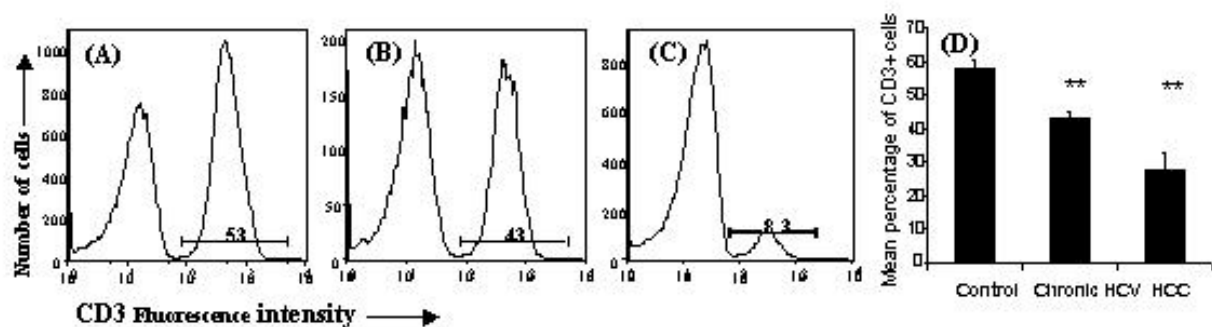


Figure 2. Peripheral blood CD3⁺ T cells are depleted in HCC patients. The percentage of CD3⁺ T cells in normal controls, chronic HCV and HCC patients was determined by Flow cytometry. Peripheral blood mononuclear cells were stained with CD3 and the data were acquired on a FACSCalibur flow cytometer and analyzed using the FlowJo software as described in the Materials and Methods. Representative data from one control (A), one chronic HCV (B) and one HCC patient (C) is shown, respectively. The mean percentage of peripheral blood CD3⁺ T cells in control group (normal, N=10), chronic HCV infected patients (N=10) and HCC patients (N=15) is shown in Panel D. The error bars represent the standard error of the mean and the significance between different groups is shown on the graph. ** Indicates highly significance difference at $p < 0.01$ as compared to control.

The ratio between CD4⁺ and CD8⁺ T cells is not different in the different groups tested

To determine if spontaneous apoptosis (see below) has a differential effect on T cells subsets in HCC patients and if ratio between CD4⁺ and CD8⁺ T cells is normal, the percentage of these populations were determined in the different groups tested using flow cytometry. PBMCs were stained with APC-labeled monoclonal antibodies against human CD3 and PerCP-labeled monoclonal antibodies against human CD8. The percentage of CD8⁺ T cells was determined directly on the density plot (Figure 3A-C) using the FlowJo software, while the percentage of CD4⁺ T cells was determined as the percentage of CD3⁺/CD8⁻ cells. In the

example shown (Figure 3A), the percentage of CD3⁺ cells in the normal individual was 53 %, and the percentage of CD8⁺ cells was 15 %. So, the percentage of CD4⁺ cells was calculated as 38 %. The mean percentages of CD4⁺ T cells was 37.5 ± 2.2 in the control individuals, 27.9 ± 1.5 in chronic HCV patients and 18.8 ± 3.7 in HCC patient group, respectively (Figure 3D). On the other hand, the mean percentage of CD8⁺ T cells was 19.8 ± 1.0 in the control group, 15.1 ± 0.9 in the chronic HCV patients and 9.5 ± 1.9 in the HCC patient groups, respectively. The difference in the mean percentage of CD4⁺ and CD8⁺ T cells in different groups tested was statistically significant ($p < 0.01$). In this regard, the mean percentage of CD4⁺ T cells in controls was significantly higher than that

in chronic HCV patients ($p = 0.002$) and in HCC patients ($p = 0.001$). In addition, the percentage of CD8⁺ T cells in the control group was significantly higher than that of chronic HCV patients ($p = 0.003$) and that of

HCC patients ($p = 0.0004$). These data show that the ratio of CD4⁺ and CD8⁺ T cells is almost 2:1 in all the groups tested and suggest that the depletion of T cells affects both T cell subsets in a similar manner.

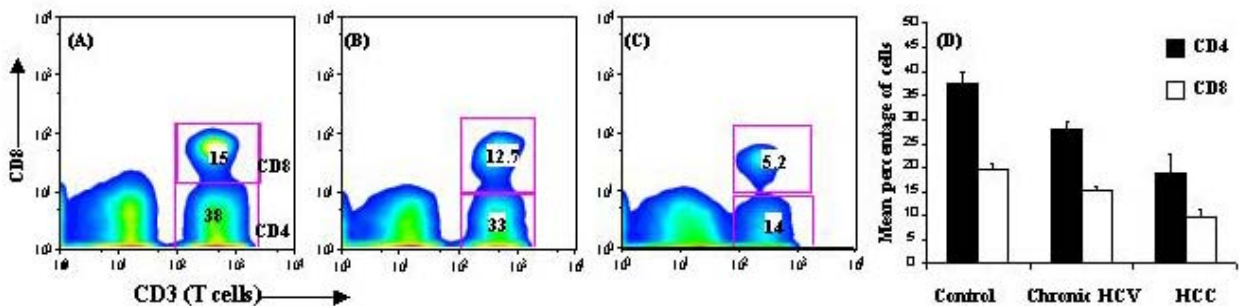


Figure 3. The ratio between CD4⁺ and CD8⁺ T cells is not different in the different groups tested. The percentage of CD4⁺ and CD8⁺ T cell subsets in PBMCs in different groups were determined by flow cytometry as described in the Materials and Methods. The percentage of CD8⁺ T cells was determined directly as shown on the density plots above while the percentage of CD4⁺ T cells was determined as the percentage of CD3⁺/CD8⁻ cells. Panels A-C are representative examples for data from individuals in control (A), chronic HCV (B) and HCC (C) patient groups, respectively. Data are representative of at least three independent experiments. The mean percentage of peripheral blood CD4⁺ and CD8⁺ T cells in control group (N=10), chronic HCV infected patients (N=10) and HCC patients (N=15) is shown in Panel D. As shown on the graph, the ratio between CD4⁺ and CD8⁺ T cells is almost 2:1 in the different groups tested. Error bars represent the standard error of the mean.

Spontaneous apoptosis of peripheral blood T lymphocytes

Apoptotic and necrotic T cells were measured among healthy control subject and patient groups by staining PBMCs with annexin V and PI using flow cytometry. Staining with annexin V only is a marker for apoptotic cells (Ann+/PI-), while staining with annexin V and PI (Ann+/PI+) is an indicator for necrotic cells, i.e. double positive (DP). Staining with APC-labeled monoclonal antibodies against human CD3 was used as a marker for T cells. Figure (4A-C) shows a representative flow cytometric analysis for staining with annexin V and PI for CD3-gated cells. Spontaneous apoptotic cells stained with annexin V (lower right quadrants) and were 0.6 % in normal, 1.2 % in chronic HCV infected patients and 3.6 % in HCC patients, respectively (Figure 4A-C). Necrotic cells stained with annexin V and PI and is represented by DP cells (upper right quadrants). These necrotic cells were 0.07 % in normal group, 0.2 % in chronic HCV infected patients and 1.4 % in HCC patients, respectively (Figure 4A-C). In Figure

4D, the mean level of spontaneous apoptosis of CD3⁺ T cells was 1.5 ± 0.52 in control group, 2.0 ± 0.73 in chronic HCV patients and 2.9 ± 0.61 in HCC patients, respectively. The difference in the value of spontaneous apoptosis of peripheral blood CD3⁺ T cells was statistically not significant in the different groups tested ($p > 0.05$). The mean level of necrosis of CD3⁺ cells was 0.18 ± 0.04 in controls, 0.59 ± 0.08 in chronic HCV patients and 0.66 ± 0.1 in HCC patients, respectively. The difference in level of the necrosis of CD3⁺ cells was highly significant in the different groups ($p < 0.01$). Necrosis of CD3⁺ cells in chronic HCV patients and HCC patients was significantly higher than that in control group ($p = 0.0006$, $p = 0.002$, respectively). On the other hand, necrosis of CD3⁺ T cells in HCC patients was not significantly different in comparison with chronic HCV patients ($p = 0.8$). These data suggest that spontaneous apoptosis; although statistically non significant; is increased in HCV and HCC patients as compared to the healthy control subjects.

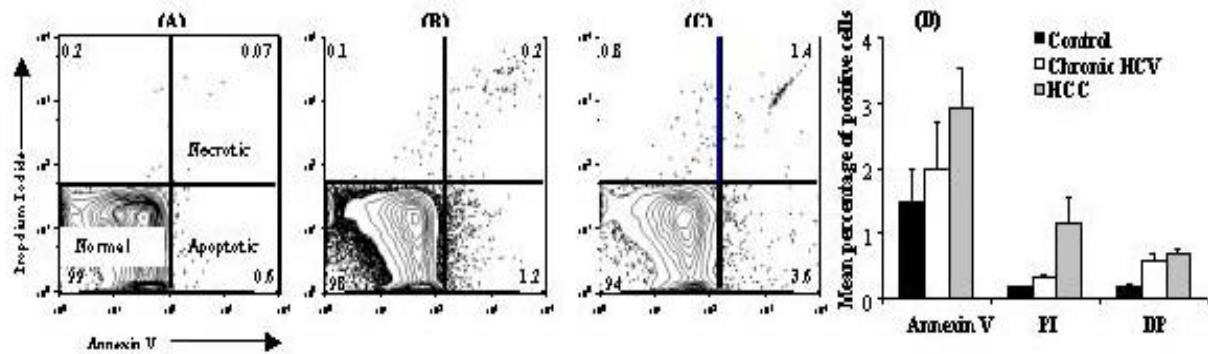


Figure 4. Increased spontaneous apoptosis of peripheral blood T cells in cases of HCC patients. Peripheral blood T cells were stained with annexin V-FITC, PI, APC-CD3 and PerCP-CD8 to examine spontaneous apoptosis in different groups by flow cytometry as described in the Materials and Methods section and a representative contour plot is shown for individuals in control (A), chronic HCV patient (B) and HCC patient (C) groups, respectively gated on CD3 positive cells. Apoptotic cells shown on the graph are annexin V positive and PI negative (Lower right quadrant, Ann+/PI-) while necrotic cells are annexin V positive and PI positive (Upper right quadrant, Ann+/PI+). The mean level of spontaneous apoptosis and necrosis of T cells in control group (N=10), chronic HCV infected patients (N=10) and HCC patients (N=15) is shown in Panel D. PI: propidium iodide, DP: double positive.

Expression of p53 protein

Fifteen liver biopsies from HCC patients, three liver biopsies from chronic HCV patients and 10 liver biopsies from controls (normal liver donors) were collected as described in detail in the Materials and Methods. Plasma samples were also collected from 10 chronic HCV infected patients, 15 HCV-induced HCC and 10 controls. The protein content of both plasma and liver homogenate was determined as described in the Materials and Methods and was taken in consideration before assessment p53 content. It should be noticed that the ELISA kit does not differentiate between wild type and mutant p53. All healthy control subjects had off scale low data for p53 in both liver biopsy and plasma samples. The data also showed that 10 liver biopsies and 10 plasma samples of HCC patients were positive for p53 (10/15, 66.7 %). On the other hand, there were 10

chronic HCV infected patients displaying off scale low levels for plasma p53 (10/10, 100 %) and one chronic HCV infected patient who was positive for liver p53 out of three patients (33.3 %). The p53 content in the liver tissue and plasma samples of HCC patients was correlated with the cancer grade. In Figure (5A), the mean level of p53 (in pg/ μ g protein) in liver homogenate in grade I HCC was 0.04 ± 0.01 , while the level was 0.13 ± 0.03 in grade II. The p53 protein level in the liver was significantly ($p = 0.03$) higher in grade II HCC than that in grade I HCC. On the other hand, the mean plasma level of p53 in pg/ml in grade I HCC was 76.7 ± 7.2 and 108.4 ± 16.0 in grade II, respectively (Figure 5B). The difference in mean plasma p53 protein level was not statistically significant ($p = 0.3$) between grade II and grade I HCC. Our data suggest that p53 is elevated in the sera and liver tissues of HCC patients as compared to chronic HCV patients and healthy controls.

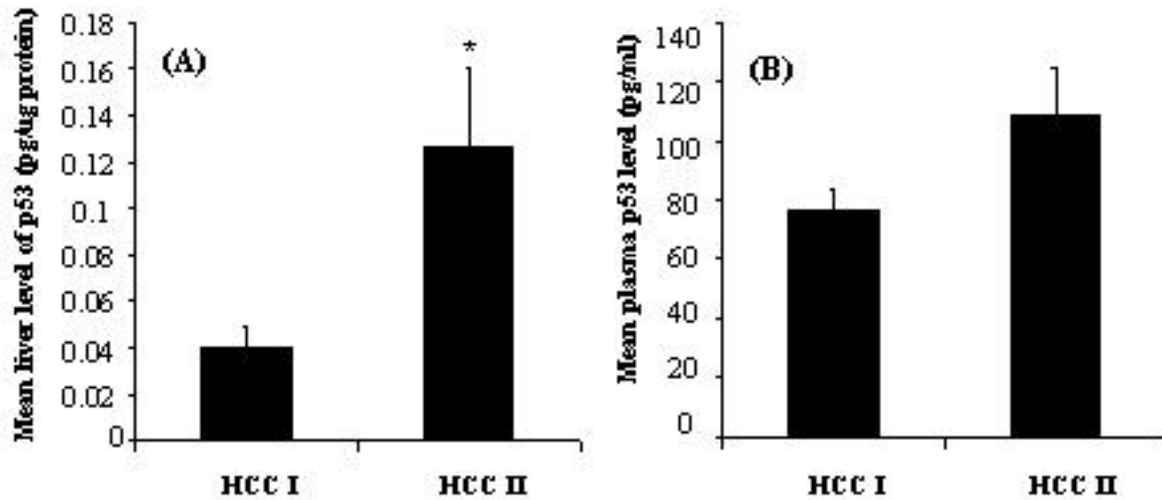


Figure 5. p53 was significantly expressed in liver biopsies from HCC grade II patient but not in plasma. The p53 content of liver tissues and plasma samples was determined by ELISA as described in the Materials and Methods. The mean concentration of p53 (pg/μg protein) in liver tissues of HCC patients grade I and II is shown in panel (A) while that of plasma is shown in panel (B). The error bars represent the standard error of the mean and the significance between different groups is shown on the graph.

Discussion

The objective of this study was to determine whether T lymphocytes from patients with HCV or HCV-associated HCC; were predestined *in vivo* to undergo spontaneous apoptosis *ex vivo*. Our second objective was to examine the role of p53 in hepatocyte and lymphocyte apoptosis and in the development of HCC in HCV-Egyptian patients. It is well established that HCV is efficient in establishing persistent infection, possibly mediated by an impaired immune response to HCV antigens (Soguero et al., 2002). In addition, several studies indicate that HCV core protein is responsible for suppressing T cell responses (Geissler et al., 1998; Kittlesen et al., 2000; Large et al., 1999; Yao et al., 2001). To our knowledge, no study had elucidated the relationship between apoptosis of peripheral blood lymphocytes in HCV genotype 4a infected patients and the severity and progression of the disease. HCV genotype 4a is the main cause of HCV-associated chronic liver disease in Egypt (Angelico et al., 1997; Kamal et al., 2000; Quinti et al., 1997).

In this study, peripheral blood T cells in HCC patients were severely depleted. In addition, T cells were slightly depleted in chronic HCV as compared to normal controls (Figure 2). In this regard, chronic HCV infection and HCC are characterized by low frequencies of CD3+ T-cells in peripheral blood (Soguero et al., 2002). Our data suggest that there is a stepwise depletion of peripheral blood T cells during the course of HCV infection (Figure 2 and data not shown). Further studies are required to test this hypothesis.

We suggested that apoptosis plays an important role in the T cell depletion process in cases of HCV infection. Indeed, apoptosis of infected hepatocytes mediated by T lymphocytes especially CTL is thought to play a major role in viral clearance in HCV infection (Calabrese et al., 2000; Lau et al., 1998). So, apoptosis of cancer cells in liver is decreased or may be inhibited because of the less number of T cells due to their spontaneous apoptosis by viral genome encoded proteins (Calabrese et al., 2000; Lau et al., 1998). Consequently, we examined the spontaneous apoptotic process of peripheral

blood T cells in the different groups of patients represented in this study. In favor of all of the above is our finding that spontaneous apoptosis of peripheral T cells from HCV-infected patients was increased compared with that of normal individuals. Our data also revealed a different level of apoptosis of CD3+ T lymphocytes among the patients and control group that was not significant while the necrosis level of CD3+ T lymphocytes in chronic HCV, and HCC patient groups was highly significant ($p = 0.0006$, $p = 0.002$, respectively; Figure 4). Thus, we concluded that the low frequencies of CD3+ T cells in HCC patients might be attributed to apoptosis that occurs gradually during the course of HCV infection. This finding is in agreement with previous reports showing an association between the increased sensitivity of peripheral T cells to apoptosis, a decrease in their number and persistency of HCV infection (Nasir et al., 2000; Taya et al., 2000). It should be noted that the ratio between CD4+ and CD8+ T cells was similar in the studied groups (Figure 3) suggesting that T cell depletion affects both T cell subsets in a similar way.

The p53, tumor suppressor protein, could play an important role in inhibiting hepatocyte apoptosis of HCV-infected patients and subsequently development of HCC. In the present study, we showed that the mean level of p53, detected by ELISA methods, in the liver was significantly higher in grade II HCC than that in grade I HCC, while the mean level of p53 in plasma was not statistically different in both grade II and grade I HCC. It should be pointed out that p53 was not detected in the sera and liver tissues of normal controls. This was also true for sera and tissues collected from patients with chronic HCV infection with only one exception being in one out of three liver biopsies. In this regard, the presence of p53 could be used as an early marker of HCC. However, it was reported that p53 alterations were not an early event in

HCC (Charuruks et al., 2001). This contradiction could be accounted for by a number of factors. These factors may include the small number of subjects examined, different characteristics of the tumors examined, and/or the sensitivity of the ELISA techniques.

It should be noted that the ELISA technique does not differentiate between wild type and mutant p53. Further studies are required to confirm which form of the p53 is present in our samples and to confirm the relationship between plasma p53 status and p53 mutations. However, four issues support our notion that p53 detected by ELISA in our study is of the mutant type. First, the difference in the $t_{1/2}$ of both forms suggests that the ELISA-detected p53 is of the mutant form. Second, because of the short $t_{1/2}$ of wild-type p53 (wt-p53) protein, the p53 protein detected by immunohistochemistry (IHC) is almost mutant-type p53 (mt-p53) as reported recently (Qin et al., 2005). Third, mutation of p53 was found in 41 % of tumor tissues isolated from HCV-infected Egyptians (a total of 17 of 41) suffering from HCC (El-Kafrawy et al., 2005). Fourth, 38.3 % of HCC tissues expressed mt-p53 protein, higher than that in the adjacent non-cancerous and normal liver tissues, indicating that p53 mutation is frequent in HCC. In addition, the level of mt-p53 in HCC correlated with the differentiation of tumor cells (Choi et al., 2001; Itoh et al., 2000; Nakano et al., 2003), that is, with the increasing of mt-p53 level, the differentiation of tumor cells became poorer.

Mutation of the p53 gene has been extensively investigated in HCC associated with HCV in different populations worldwide. A high prevalence of p53 alterations (in 30 %-50 % of HCC patients) was reported in previous studies (Bressac et al., 1991; Hsu et al., 1991). Our study demonstrated that 66.7% of liver biopsies and plasma samples of HCC were positive for p53. The percentage was as high as that reported in previous studies in

which p53 alterations had been studied by complicated molecular techniques (Bressac et al., 1991; Hsu et al., 1991). It has been reported that p53 abnormalities were associated with a poor outcome of HCC (Honda et al., 1998; Mise et al., 1998) and p53 alterations were reported to occur in approximately 30 %–50 % of patients with HCC (Bressac et al., 1991; Hsu et al., 1991). Therefore, the detection of serum p53 positivity could be useful in the planning for HCC therapy. In addition, the relationship between serum p53 positivity and disease severity and survival should be further investigated with longer follow-up periods. In summary, HCV proteins may regulate cellular genes transcriptionally in the liver as tumor suppressor p53 protein may be mutated and non-functionally accumulates in the liver leading to cellular growth and development of liver cancer. Also, studying p53 in patients with HCC, using an ELISA method could be carried out as a biomarker in order to assess the clinical implications and prognosis of HCC patients.

In conclusion, HCV infection may induce immune-suppression by inducing peripheral T cell apoptosis, and depletion and this could lead to persistence of HCV infection. In addition, p53 is implicated in the pathogenesis of HCC in HCV-infected patients and may be used as a biomarker to assess the clinical outcome and prognosis of HCC patients in Egypt.

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