Tissue Expression of TNF α and VEGF in Chronic Liver Disease and Hepatocellular Carcinoma

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Abstract

Aim and Background: The molecular mechanisms that lead to cancer in chronic inflammation and the role of angiogenesis in inflammation-associated cancer remain poorly understood.

We measured levels of messenger RNA (mRNA) transcripts, mature protein for TNF-a and VEGF and serum TNF-a level to assess the possible implication of TNF-a-induced angiogenesis in providing a molecular link between inflammation and the development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC).

Patients and Methods: Ninety patients were enrolled: 30 cases of CHC without cirrhosis, 28 cases of CHC with liver cirrhosis, and 32 cases of HCC and hepatitis C virus infection. Ten wedge liver biopsies, taken during laparoscopic cholecystectomy, were served as normal controls. Serum TNF-a levels were measured using ELISA technique; TNF-a and VEGF proteins were detected in hepatic tissue by indirect Immunohistochemical technique; In Situ Hybridization was performed for measurement of mRNA for TNF-a and VEGF.

Results: The highest hepatic expression of TNF-a was noticed in LC specimens compared to non cirrhotic CHC and HCC. Hepatic expression of VEGF and serum level of TNF-a revealed significant increase with the progression of the disease and in cases with higher grades of inflammation or stages of fibrosis. Expression of mRNA of both TNF-a and VEGF shows increasing expression with positive correlation to progression of viral hepatitis to cirrhosis with more positivity in cases that developed HCC.

Conclusions: VEGF signaling could be one of the molecular signaling pathways involved in TNF-a induced angiogenesis which might pose an important link between inflammation and fibrosis in CHC and HCC development and progression. Moreover, serum inflammatory biomarkers can be used to monitor the disease progression.

Key Words: TNF α – VEGF expression – HCC – Chronic hepatitis

Introduction

HEPATOCELLULAR carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide, and each year, approximately 750,000 new cases are diagnosed 111. Early detection of patients with HCC gives better prognosis as HCC tends to grow slowly and stay confined to the liver [2]. The ongoing goals are to identify diagnostic precursor lesions of HCC and to better elucidate the numerous pathophysiological mechanisms of the underlying disease processes that contribute to hepatocarcinogenesis and tumor progression [3].

Increasing evidence support the hypothesis that Chronic and persistent inflammation contributes to cancer development and even predisposes to carcinogenesis. An unresolved inflammation due to any failure in the precise control of the immune response can continue to perturb the cellular microenvironment, thereby leading to alterations in cancer-related genes and posttranslational modification in crucial cellular proteins [41. In addition, there are data indicating that leukocyte infiltration can promote tumor phenotypes, such as angiogenesis, growth and invasion. These inflammatory cells probably can influence cancer promotion by secreting cytokines, growth factors, chemokines and proteases, which stimulate proliferation and invasiveness of cancer cells [5]. Among pro—inflammatory gene products involved in such interactions are tumor necrosis factor alpha (TNF-a). Induced by a wide range of pathogenic stimuli, TNF-a plays a crucial role in the initiation and amplification of inflammatory reactions, with actions directed towards both tissue destruction and recovery from damage. Hence, when dysregulated and secreted in the circulation, TNF-a can mediate a wide variety of diseases, including cancer [6].
Angiogenesis is also a complex multistep process of growth and remodeling involving degradation of the extracellular matrix, cell migration and proliferation, and tube formation [7]. Under normal conditions, this process requires a balance between pro-angiogenic factors and anti-angiogenic factors. Angiogenesis also requires the activation of many receptors by their cognate ligand. Vascular endothelial growth factor (VEGF) is one of these ligands known to play the most important role in angiogenesis [8].

Angiogenesis is essential for tumor growth and metastasis, controlling tumor-associated angiogenesis is a promising tactic in limiting cancer progression [9].

The molecular mechanisms that lead to cancer in chronic inflammation and the role of angiogenesis in inflammation-associated cancer remain poorly understood. In this study, we measured levels of messenger RNA (mRNA) transcripts and mature protein for TNF-a and VEGF to assess the possible implication of TNF-a-induced angiogenesis in providing a molecular link between inflammation and the development of HCC in patients with CHC. In addition, we evaluated the possible use of the serum level of circulating TNF-a as an early predictor of HCC development and tumor progression.

**Material and Methods**

A total 90 patients of chronic hepatitis C (CHC) patients (54 males and 36 females, age range between 24-66ys with a mean of 48.32±7.65) admitted to the Hepatology and Gastroenterology Department at Theodor Bilharz Research Institute (TBRI) and to the Endemic Medicine and Hepatology Department and Surgical Departments at Kasr Al Aini Hospital, Cairo University from 2010 — 2012, were enrolled in this study. They included 30 cases of CHC without liver cirrhosis, 28 cases of CHC with liver cirrhosis (LC) who had undergone ultrasound guided liver biopsy and 32 cases of HCC from whom tumor specimens were taken by ultrasound guided liver biopsy and surgical specimens from partial hepatectomies at Kasr Al Aini Hospital. Patients presented with schistosomiasis, other chronic viral diseases, nonalcoholic steatohepatitis, biliary disorders or other malignancies were excluded. Ten control wedge liver biopsies were taken from age- and sex-matched individuals subjected to laparoscopic cholesystectomy (6 males and 4 females, age ranges between 37-48ys with a mean of 42.21±4.54). Written informed consent was obtained from all participants and the study was approved by TBRI local ethical committee.

**Laboratory investigations:**

Complete hemogram using automated hemogram, liver function tests were carried out, circulating anti-HCV antibodies were detected by ELISA technique and HCV-RNA in patients’ sera was detected by real-time polymerase chain reaction using the Amplicor test.

**Abdominal ultrasound was done to all patients included in the study:**

**Measurements of serum TNF-alpha levels:**

Two ml of blood were withdrawn from each subject of the study into plain tube and centrifuged shortly after clot formation. All samples were stored at —70°C in aliquots and used for analysis of measurement of serum TNF-a using the Quantikine Mouse TNF-a Immunoassay (R & D Systems) according to the manufacturer’s recommended protocol.

**Histopathological study:**

Four micrometer thick sections were cut from formalin-fixed paraffin-embedded tissue blocks of core liver biopsies were stained with hematoxylin & eosin and Masson trichrome stains for proper evaluation of tissue fibrosis according to a histological METAVIR [10] scoring system. Using two separate scores, one for necro inflammatory grade (A for activity) A1: Minimal activity, A2: Moderate activity, A3: Severe activity and another for the stage of fibrosis (F) which scores fibrosis from F0-F4. A score of F 1 to F2 signifies significant fibrosis, while a score of F3 and F4 signifies advances fibrosis.

**Immunohistochemical study:**

Immunohistochemical reaction was performed using an avidin biotin complex (ABC) immunoperoxidase technique IIII. The sections were dewaxed in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in 0.3% hydrogen peroxide in methanol for 20min. Antigen retrieval was performed by microwaving the sections in citrate buffer (PH 6.0) for 15min at 700W. Sections were incubated overnight at 4°C with the anti-human primary antibodies against TNF-a and VEGF (purchased from Santa Cruz Biotechnology Inc., Santa Cruz, USA). Monoclonal antibodies were diluted at 1:100 and 1:150 respectively in BPS. Next day, after thorough washing in PBS, the sections were incubated with streptavidin-biotin-peroxidase preformed complex and using a peroxidase/DAB (3, 3’-diaminobenzidine) enzymatic reaction. Staining is completed by 5-10 minutes results in a brown-colored precipitate at
the antigen site. The cell nuclei were counterstained with Mayer's hematoxylin. The cover slips were mounted using Dpx. Positive and negative control slides for each marker were included within each session. As a negative control, a liver tissue section was processed in the above sequences but the omission of the primary antibody and PBS was replaced.

**Evaluation of TNF alpha expression by IHC:**

The scoring of TNF alpha in liver tissue were evaluated by the semi quantitative technique, relating the score of 0 to 4 points to the fraction of stained cells (score 0, 0% cells; 1, less than 5% cells; 2, 5-20% cells; 3, 20-40%; and 4, more than 40% positive cells) according to Kasprzak et al., [12]. Liver sections were examined by Zeiss light microscopy at X400 power for both markers; the number of positively stained cells with the highest expression was semi-quantitatively recorded within ten successive fields and counted/section and the final value represents the mean. Zero percentage was given to unstained sections.

**Evaluation of VEGF expression by IHC:**

All brown-stained endothelial cell or endothelial cell cluster, which was clearly separate from connective tissue elements, was considered a micro vessel. Cell clusters were counted as one micro vessel. Stained sections were observed at 100x magnification to identify the areas with the highest number of positive cells. Counts were performed in five regions at 400x magnification. The immunohistochemical results for VEGF are classified as follows: <5% was considered negative for VEGF, 5-25% was considered weak positive, 26-50% was considered mild positive, 51-75% was considered moderate positive, >75% was considered intense positive [13].

**In situ hybridization:**

In situ hybridization (ISH) for mRNA VEGF and mRNA TNF-a.

Paraffin sections were deparaffinized by incubation in xylene followed by dehydration and washing in diethylypyrocarbonate-treated water (DEPC-H2O) for 1min. The sections were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20min, washed in 3X PBS for 5min and rehydrated. The sections were then treated with 0.2M HC1 at room temperature (RT) for 20min and washed in DEPC-H2O for 5min. For proteolysis the sections were incubated with pronase (0.125mg pronase, Sigma, in 100m1 PBS) at RT for 10min and the reaction was stopped with 0.1M glycine DEPC-H2O. The sections were washed twice in PBS, 1X for 30sec, fixed again in PFA and washed in 1X PBS for 3min. Acetylation was performed in 0.25-0.5% acetic anhydride in 0.1M triethanolamine for 10min after which the sections were washed in 1X PBS, and air dried. Then the sections were treated for 2 hrs at 42°C with the pre-hybridization mixture (10mM dithiothreitol (DTT, Sigma), 10mM Tris-HCl, 5mM dinitrtrimidineta acetic acid (EDTA), 0.3M NaCl, 0.02% NaCl, 0.02% (w/v), 0.75mg/ml yeast tRNA, 50% deionized formamide, dextran sulphate, 12.5% (w/v), 1.5% (w/v) salmon sperm DNA, Ficoll, 0.02% (w/v) polyvinylpyroldone, 0.02mg/ml bovine serum albumin (BSA, Sigma) and 6.5% (v/v) DEPC-H2O.

In hybridization approximately lug VEGF-digoxigenin in 30111 hybridization solution and lug TNF-a digoxigenin in 30111 hybridization solution (as pre-hybridization solution but without DEPC-H2O) were first denatured by heating at 95°C for 10min and placed on ice for 5min. Hybridization was carried out at 42°C overnight. A human VEGF-cDNA probe and TNF-a-cDNA was kindly provided by Bruno Voss, BGFA, Germany.

The post-hybridization washes were in formamide washing solution (twice for 20min containing 50% deionized formamide, 1% 2-mercaptoethanol, 10% of 20X standard saline citrate (SSC) (3M NaCl, 3mM Na citrate) and 39% DW) at 47°C; 20min in 2X SSC at 58°C; 3min in 1X PBS at RT; 30min in 0.5% H2O2 in PBS at RT and 5min in PBS at RT. The slides were blocked by 30min incubation with blocking buffer (1% BSA, 0.2% skimmed milk in PBS). Anti-digoxigenin horseradish peroxidase Fab fragment (1:100 (v/v) Boehringer Mannheim) in blocking buffer was applied for 30min at RT. Slides were washed 3 times for 5min in PBS at RT. The reaction signal was amplified by the use of tyramide signal amplification (TSA) kit (New Life Science Products) in which TSA fluorescein 1:60 was applied in the dark for 5min at RT and washed 3 times for 5min with PBS in the dark. Counterstaining was done with propidium iodide (1:10,000) in PBS and the slides were washed for 10min with PBS. Sections were rehydrated and kept moistened with glycerol.

Scoring for VEGF mRNA, TNF-a mRNA: (+) = normal or weak expression, (+++) = moderate expression , (+++) = over expression.

**Statistical analysis:**

Statistical evaluation of results was done using SPSS computer program (Version 12 Windows). Results were expressed as mean±standard deviation (SD) or number (%). Comparisons between the mean values of different parameters in the different
groups were performed using one-way analysis of variance (ANOVA) with post hoc test using the least significant difference. Correlation between parameters was performed using Spearman’s rank correlation coefficient (r). p-value<0.05 was considered significant and <0.01 was considered highly significant.

Results

TNF-a protein was observed in the cytoplasm of hepatocytes and inflammatory cells. The pattern of VEGF immunoreactivity in hepatocytes and endothelial cells lining blood vessels were that of diffuse cytoplasmic. In the current study, normal liver specimens showed no detectable TNF-a protein expression and faint VEGF protein expression. Both antigens were significantly increased in the diseased groups (p<0.01) compared to the control specimens. The highest expression of TNF-a was noticed in LC specimens compared to non cirrhotic CHC and HCC (p<0.01). Expression of VEGF revealed significant increase with the progression of the disease (p<0.01). Serum level of TNF-a showed significant increase in all the diseased groups compared to the control (p<0.01). Significant increase was also observed with progression of the disease (p<0.01) (Tables 1-3).

In CHC patients, cases with higher grades of inflammation or stages of fibrosis showed significant increase in serum level of TNF-a and expression of both TNF-a and VEGF compared to those with lower scores (Tables 4,5).

Expression of TNF alpha mRNA shows increasing expression in all diseased specimens compared to controls (p<0.01). A significant increase (p<0.05) was also noticed in LC & HCC groups compared to non cirrhotic CHC. Moreover, expression of VEGF mRNA shows increasing expression with positive correlation to progression of viral hepatitis to cirrhosis with more positive expression with cases that developed HCC (Tables 6,7).

Table (1): Tissue TNF-a and VEGF and serum TNF-a expression among the studied groups.

<table>
<thead>
<tr>
<th>Items</th>
<th>Controls (n=10)</th>
<th>CHC without cirrhosis (n=30)</th>
<th>Cirrhosis (n=28)</th>
<th>HCC (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue TNF-a, within 10 successive microscopic fields (x400) / section (mean percentage) +ve cells ±S.D</td>
<td>0.0±0.0</td>
<td>24.8±14.8a</td>
<td>51.28±15.99abc</td>
<td>36.93±18.83abd</td>
</tr>
<tr>
<td>Tissue VEGF, within 10 successive microscopic fields (x400) / section (mean percentage) +ve cells ±S.D</td>
<td>1.3±0.5</td>
<td>14.03±5.46a</td>
<td>25.42±13.48abc</td>
<td>66.56±11.7abd</td>
</tr>
<tr>
<td>Serum TNF-a pg/mL</td>
<td>10.2±2.65</td>
<td>34.5±15.33a</td>
<td>61.67±27.37abc</td>
<td>108.28+43.37abd</td>
</tr>
</tbody>
</table>

Abbreviations: CHC: Chronic hepatitis C. HCC: Hepatocellular carcinoma.

Table (2): Tissue expression of TNF-a immunostaining in liver tissue of different studied cases.

<table>
<thead>
<tr>
<th>Histopathologic diagnosis (n)</th>
<th>Positive Cases</th>
<th>Range %</th>
<th>Intensity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>5-20%</td>
<td>20-40%</td>
</tr>
<tr>
<td>Controls (10)</td>
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<td>0</td>
</tr>
<tr>
<td>CHC without cirrhosis (30)</td>
<td>12</td>
<td>40a</td>
<td>0</td>
</tr>
<tr>
<td>CHC with cirrhosis (28)</td>
<td>23</td>
<td>82.1ab</td>
<td>0</td>
</tr>
<tr>
<td>HCC (32)</td>
<td>22</td>
<td>68.7abc</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CHC: Chronic hepatitis C. HCC: Hepatocellular carcinoma. a p<0.01 significant difference relative to control group. b p<0.01 significant difference relative to CHC without cirrhosis. c p<0.01 significant difference relative to CHC with cirrhosis.

Table (3): Tissue expression of VEGF immunostaining in liver tissue of different studied cases.

<table>
<thead>
<tr>
<th>Histopathologic diagnosis (n)</th>
<th>Positive Cases</th>
<th>Range %</th>
<th>Intensity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>5-25%</td>
<td>26-50%</td>
</tr>
<tr>
<td>Controls (10)</td>
<td>2</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>CHC without cirrhosis (30)</td>
<td>10</td>
<td>33.3a</td>
<td>3</td>
</tr>
<tr>
<td>CHC with cirrhosis (28)</td>
<td>16</td>
<td>55abc</td>
<td>9</td>
</tr>
<tr>
<td>HCC (32)</td>
<td>24</td>
<td>75abc</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations: CHC: Chronic hepatitis C. HCC: Hepatocellular carcinoma. a p<0.01 significant difference relative to control group. b p<0.01 significant difference relative to CHC without cirrhosis. c p<0.01 significant difference relative to CHC with cirrhosis.
Table (4): Tissue TNF-α and VEGF and serum TNF-α expression according to METAVIR activity scoring system in CHC with or without cirrhosis groups.

<table>
<thead>
<tr>
<th>Items</th>
<th>Al (n=25)</th>
<th>A2 (n=23)</th>
<th>A3 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue TNF-α, within 10 successive microscopic fields (x400)/section (mean percentage) +ve cells ±S.D</td>
<td>18.42±15.91</td>
<td>32.84±20.11a</td>
<td>59.91±17.41ab</td>
</tr>
<tr>
<td>Tissue VEGF, within 10 successive microscopic fields (x400)/section (mean percentage) +ve cells ±S.D</td>
<td>10.39±4.0</td>
<td>18.46±2.14</td>
<td>28.66±3.89ab</td>
</tr>
<tr>
<td>Serum TNF-α pg/ml.</td>
<td>15.84±3.31</td>
<td>38.3±6.58a</td>
<td>73.58±8.32ab</td>
</tr>
</tbody>
</table>

a p<0.05 Statistically significant from Al group.
b p<0.05 Statistically significant from A2 group.

c p<0.05 Statistically significant from A3 group.

Table (5): Tissue TNF-α and VEGF and serum TNF-α expression according to METAVIR fibrosis scoring system in CHC with or without cirrhosis groups.

<table>
<thead>
<tr>
<th>Items</th>
<th>F1 (n=18)</th>
<th>F2 (n=12)</th>
<th>F3 (n=20)</th>
<th>F4 (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue TNF-α, within 10 successive microscopic fields (x400)/section (mean percentage) +ve cells ±S.D</td>
<td>14.33±7.03</td>
<td>24.96±14.45a</td>
<td>30.28±5.16a</td>
<td>50.28±1 5.99abc</td>
</tr>
<tr>
<td>Tissue VEGF, within 10 successive microscopic fields (x400)/section (mean percentage) +ve cells ±S.D</td>
<td>13.47±9.43</td>
<td>16.66±5.77</td>
<td>27.42+3.48ab</td>
<td>48.42+6.06abc</td>
</tr>
<tr>
<td>Serum TNF-α pg/ml.</td>
<td>12.55±3.12</td>
<td>24.0±5.29a</td>
<td>61.67+2.37ab</td>
<td>82.67+7.10abc</td>
</tr>
</tbody>
</table>

a p<0.01 Statistically significant from F1 group.
b p<0.01 Statistically significant from F2 group.
c p<0.05 Statistically significant from F3 group.

d p<0.05 Statistically significant from F4 group.

Table (6): Tissue expression of TNF-α mRNA in liver tissue of different studied cases.

<table>
<thead>
<tr>
<th>Histopathologic diagnosis (n)</th>
<th>Positive Cases</th>
<th>Range %</th>
<th>Intensity%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>5-20%</td>
</tr>
<tr>
<td>Controls (10)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CHC without cirrhosis (30)</td>
<td>15</td>
<td>50a</td>
<td>2</td>
</tr>
<tr>
<td>CHC with cirrhosis (28)</td>
<td>23</td>
<td>82.1ab</td>
<td>0</td>
</tr>
<tr>
<td>HCC (32)</td>
<td>26</td>
<td>81.3ab</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations:  
a p<0.01 Significant difference relative to control group.  
b p<0.05 Significant difference relative to CHC without cirrhosis.

Table (7): Tissue expression of VEGF mRNA in liver tissue of different studied cases.

<table>
<thead>
<tr>
<th>Histopathologic diagnosis (n)</th>
<th>Positive Cases</th>
<th>Range %</th>
<th>Intensity%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>5-25%</td>
</tr>
<tr>
<td>Controls (10)</td>
<td>3</td>
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<td>3</td>
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<tr>
<td>CHC without cirrhosis (30)</td>
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<tr>
<td>CHC with cirrhosis (28)</td>
<td>18</td>
<td>64.2ab</td>
<td>6</td>
</tr>
<tr>
<td>HCC (32)</td>
<td>26</td>
<td>81.2abc</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations:  
a p<0.01 significant difference relative to control group.  
b p<0.01 significant difference relative to CHC without cirrhosis.

c p<0.05 significant difference relative to CHC with cirrhosis.
TNF-α is mainly produced by macrophages in inflammatory tissues and have been implicated in angiogenesis during inflammation, wound repair and growth of tumors [16]. Results of this study showed no detectable TNF-α protein or TNF-α mRNA expression in normal liver specimens. In the CHC and LC specimens, increased expression of TNF-α and its transcripts have been observed. This increased expression was mainly found in hepatocytes and in cells of liver sinusoids (macrophages and endothelial cells). Our data are consistent with the results of previous studies which showed increased TNF-α production in chronic liver disease [17,18]. A role for TNF-α in the pathogenesis of chronic hepatitis C viral infection has been suggested. TNF-α, is an integral part of inflammation in chronic hepatitis C infection [19]. Pro inflammatory TNF-α is produced in response to tissue injury by an overwhelming number of infiltrating TNF-alpha-secreting monocytes and is associated with an increase in cell-cycle progression and oxidative stress through the formation of 8-oxo-deoxyguanosine, an established marker of DNA damage associated with chronic hepatitis in human liver [20]. In addition, hepatitis C virus induces TNF-α expression in human liver and human hepatoma cell lines [21]. Our results revealed significant increase in expression of TNF-α and its transcripts in cases with higher stages of fibrosis and in LC specimens compared to CHC specimens. In liver fibrosis, one of the first events is the activation of resident innate inflammatory cells and the recruitment of additional inflammatory monocytes/macrophages and the liver-resident Kupffer cells [22]. In accordance with our results Wang et al. [23] noticed increased number of TNF-α positive cells in liver tissues from patients with LC compared to chronic hepatitis and suggested that TNF-α may be related to liver fibrosis and might promote liver fibrosis. Data also showed significant increase in the expression of TNF-α and TNF-α mRNA in HCC specimens compared to controls and CHC specimens. In situ hybridization studies revealed no significant difference in TNF-α mRNA between the HCC and LC specimens. Although a high proportion of patients with LC had cells that expressed TNF-α compared to HCC yet the intensity of TNF-α and its transcript was higher in HCC specimens. There is increasing evidence that the inflammatory process is inherently associated with many different cancer types, including HCC [24]. A high level of proinflammatory TNF-α has been associated with carcinogenesis and was detected in HCC patients, especially those with recurrence [25]. In addition, TNF-α level was found to be lower in HCC tumor tissue versus the cirrhotic tissue surrounding the tumor [26].

Serum TNF-α level was significantly higher among all patient groups compared to healthy volunteers. Moreover, a highly significant increase in serum TNF-α level was noticed with the disease progression and HCC development and correlate well with its liver immunohistochemical pattern suggesting that peripheral levels could be used as surrogate markers of local TNF expression in CHC patients. Our results are in accordance with Neuman et al. [27] and Neuman et al. [28] who found similar correlation in CHC patients. Serum TNF-α was found to be positively associated with both inflammation and fibrosis in liver biopsies of CHC with and without cirrhosis. Even patients with mild liver inflammation have elevated serum TNF-α levels suggesting that this cytokine could be used as a sensitive predictor of liver inflammation.

Immunohistochemical and In situ hybridization studies revealed that tumor and non tumor sections were more positive for VEGF protein and VEGF mRNA than histologically normal liver. Our study also demonstrated that this increased positivity matched the development of LC. These findings are in accordance with those of Deli et al. and Iavarone et al. [29,30]. Moreover, the highest value of both VEGF mRNA and protein expression was mostly encountered among HCC patients. Similar results were previously encountered and strongly correlated with the degree of vascularization [31,32]. VEGF represents a key mediator of angiogenesis associated with various disorders [33]. Increased expression of VEGF by hepatocytes in the cirrhotic liver has been demonstrated and accompanied by active angiogenesis [34,35]. Our results were in accordance with Amarapurkar et al., [36] who found that VEGF expression was significantly more in stage 3 and 4 compared to stage 1 and 2 fibrosis. One of the sources of VEGF in the fibromuscular stroma may be fibroblasts, since the ability of these cells to produce VEGF was demonstrated earlier [37]. Increased vascularization of the cirrhotic stro-
ma may be either a part of the formation of liver fibrosis and granulation tissue or be a compensatory response to the decreased blood supply and hypoxia in the cirrhotic nodules [32]. Hypoxia also stimulates angiogenesis to support tumor growth in HCC [38]. Results of the present study revealed significant VEGF expression in cases with higher grades of inflammation. This is in accordance with the study in the literature suggesting that capillarization and phenotypic changes within hepatic sinusoids occur with inflammation and liver fibrosis [39]. Moreover, strong correlation between TNF-a and VEGF at both the protein expression level and the mRNA level was detected in all the diseased groups. The data suggested increased expression of VEGF in response to the pro-inflammatory cytokine TNF-a and that TNF-a may mediate its angiogenic effect by up-regulating VEGF. A well known association between inflammation and tumor development has been found in viral hepatitis B and C, alcoholic liver cirrhosis and hepatocarcinoma [40]. VEGF expression in cirrhotic liver was found to be modulated by inflammatory cytokines released from infiltrating inflammatory cells [41,42]. TNF-a has been even reported to mediate macrophage-induced angiogenesis [43]. TNF-a promotes angiogenesis through its ability to synergize VEGF-induced vessel permeability, a prerequisite initial event for plasma exudation and fibrin clot formation, a matrix permissive for angiogenesis [44]. TNF-a is also capable of inducing gene expression of the pro angiogenic molecules VEGF and its receptors (VEGFRs) [45,46]. Indeed, the cellular VEGF mRNA level is potently enhanced in response to TNF-a, probably due to transcriptional activation mediated by the transcription factor SP-1, leading to induction of a paracrine loop for neovascularization under pathological conditions, including cancer [47,48].

In view of all the proceeding data, we can sum up that VEGF signaling could be one of the molecular signaling pathways involved in TNF-induced angiogenesis which might pose an important link between inflammation and fibrosis in CHC and HCC development and progression. Thus, inflammatory biomarkers can be used to monitor the disease progression. Moreover, both inflammatory and angiogenic responses in tumor stroma could be targets for development of anticancer therapeutic drugs and the use of anti-inflammatory drugs as an adjuvant to other therapies, such as anti-angiogenic or cytotoxic agents and may provide highly efficacious therapeutic regimens for the treatment of HCC.

References
Tissue Expression of TNF-α and VEGF in Chronic Liver Disease


