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ARTICLE

Significance of Serum Vascular Endothelial Growth Factor in Chronic Liver Disease and Hepatocellular Carcinoma: An Exploratory Study.

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Abstract

Background and Objectives: Vascular endothelial growth factor (VEGF) is the most potent directly acting angiogenic growth factor that plays an important role in inducing tumor-associated angiogenesis. We evaluated the clinical significance of the circulating VEGF in patients with hepatocelluar carcinoma (HCC) and chronic liver disease (CLD). **Patients and Methods:** The study included 65 patients [20 with chronic viral hepatitis (CVH), 20 with liver cirrhosis (LC) and 25 with HCC] and 15 age- and gender- matched healthy subjects as controls. For each studied subject, detection of hepatitis viral markers, and assessment of liver function tests, α-fetoprotein (AFP) and VEGF were performed. **Results:** There was a significantly higher VEGF level in the sera of HCC patients as compared to other groups ($p< 0.001$). Serum VEGF level was significantly associated with portal vein tumor thrombosis ($p< 0.01$), but was not related to tumor size. There was no significant difference between the serum VEGF levels in either LC or CVH groups when compared to the controls. Moreover, no significant difference was detected between the different Child-Pugh classes among LC patients. Furthermore, no correlation was found between the level of serum VEGF and AFP, serum albumin, aminotransferases or prothrombin time (PT) in all the studied groups. **Conclusions:** Serum VEGF may be useful as a tumor marker for diagnosis of HCC and as a prognostic marker for tumor invasion. Large studies with greater numbers of patients and controls is required to support our conclusion.

Key words: Chronic liver disease, hepatocellular carcinoma (HCC), tumor marker, vascular endothelial growth factor (VEGF)

Abbreviations: AFP: α-fetoprotein, CLD: Chronic liver disease, CVH: Chronic viral hepatitis, CT: computed tomography, ECM: extracellular matrix, HCC: Hepatocelluar carcinoma, LC: liver cirrhosis, PT:

Prothrombin time, VEGF: Vascular endothelial growth factor

Introduction

Vascular endothelial growth factor (VEGF) is a 34-46 kD glycoprotein with a potent angiogenic activity (1). VEGF has five molecular isoforms generated by alternative splicing of VEGF mRNA. They are 206, 189, 165, 145, and 121 amino acid residues. $VEGF_{121}$ and $VEGF_{165}$ are both secreted into the circulation, but $VEGF_{165}$ is the predominant isoform secreted by most tumors including HCC (2). The other isoforms do not enter the circulation in a significant amount because they are either bound to the extracellular matrix (ECM) ($VEGF_{145}$) or not secreted ($VEGF_{189}$ and $VEGF_{206}$) (3). VEGF mediates the secretion and activation of enzymes involved in degrading the ECM. Acting on endothelial cells, VEGF induces expression of plasminogen activator and its inhibitor, urokinase receptor, matrix metalloproteinase, interstitial collagenase and gelatinase A. VEGF also decreases the levels of tissue inhibitors of metalloproteinases 1 and 2 (4). Furthermore, it acts as a survival factor for endothelial cells through inhibition of apoptosis and is essential for mobilization of bone marrow-derived endothelial cell precursors in the promotion of vascularization (5). VEGF production is upregulated by several substances, including oxygen, steroid hormone, reactive oxygen metabolites and protein kinase C agonists (6). VEGF is significantly expressed by sinusoidal endothelial cells and hepatocytes, whereas modest and inconstant expression has been reported for Kupffer cells (7). VEGF has been shown to induce the proliferation of hepatic sinusoidal cells through increasing expression of its receptors; fms-like tyrosine kinase (FLt) and fetal liver kinase (FLK-1) after experimental partial hepatectomy (8).

VEGF expression increases significantly during the course of liver fibrosis development in experimental studies and that VEGF participated in sinusoidal capillarization in the liver (9). In addition to hepatocytes, activated hepatic stellate cells, which play an important role in liver fibrogenesis, have been shown to increase VEGF expression during activation (10). Establishment of neovasculature to support HCC growth involves many cell types as liver sinusoidal cells. Kuppfer cells, hepatic stellate cells and circulating endothelial progenitors (11). HCC is one of the most common cancers worldwide and it usually develops in chronically damaged liver (12). CVH, LC and HCC are common in Egypt (13). The aim of this study was to explore the clinical significance of circulating VEGF levels in CVH, LC and HCC.

Patients and Methods

Study population

The study included 40 patients with CLD, 25 patients with HCC and 15 healthy age- and gender- matched controls. Patients were selected from Menoufiya University Hospital. Cases with chronic inflammatory diseases, hematological malignancy or cancers of any other organ were excluded from the study. An informed consent was obtained from all subjects before enrollment in the study. All patients were subjected to thorough history taking, complete clinical examination, abdominal ultrasonography, computed tomography (CT) scan and liver biopsy. The studied patients were classified into 3 groups according to the results of abdominal ultrasonography, liver biopsy and alpha fetoprotein (AFP), as follows. CVH group: included 20 patients with chronic viral hepatitis B or C. Diagnosis was performed on the basis of clinical and laboratory findings (14) and cirrhosis was excluded by ultrasonography. LC group: included 20 patients with LC, who were selected on the basis of clinical and laboratory findings (14) and cirrhotic ultrasonography findings (shrunken liver, coarse echo pattern, attenuated hepatic vein and fine regular surface). HCC group: included 25 patients with HCC, who were diagnosed by ultrasonography, alpha fetoprotein level, CT scan and liver biopsy.

Sampling

Venous blood (7 ml) was obtained from each subject by aseptic technique. Each blood sample was distributed as follows: 1.8 ml was delivered in a graduated vacutainer plastic tube containing 0.2 ml trisodium citrate for prothrombin time (PT). 5.2 ml of blood were delivered in a vacutainer plain test tube. Blood was left for a sufficient time to clot, serum was then separated after centrifugation at 3000 rpm for 10 minutes, then liver function tests were done immediately and the rest of the serum was divided into three aliquots and stored at -80ºC to be tested for viral hepatitis markers, AFP and VEGF.

Analytical Methods

Liver function tests including serum total bilirubin, direct bilirubin, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALP), gamma glutamyl transferase (γ GT) and alkaline phosphatase (ALP) were determined using Synchron CX5 autoanalyzer (Beckman instrumentation, CA, USA). Hepatitis B Surface Antigen

(HBsAg) was detected by a sandwich EIA using Dia. Sorin s.r.l kit (1340 Saluggia,Vercelli, Italy) (15). Hepatitis C Virus Antibody (Anti-HCV) was detected by EIA, using Murex anti-HCV 4th generation kit (Murex VK 47188, England.) (16). Qualitative detection of HCV-RNA by PCR was performed by a direct DNA probe test that utilizes nucleic acid amplification and hybridization using amplicor kit (Roche Diagnostics, Branchburg, USA.). The test is based on reverse transcription of the target RNA to generate a complementary DNA (cDNA) and amplification using primers KY78 and KY80 that define a 244 nucleotide sequence within the highly conserved 5' untranslated region (17). The amplicons were chemically denatured to form single strands and were detected in a microwell plate containing a specific oligonucleotide probe KY150. An avidin-horseradish peroxidase conjugate was added to the plate. The avidin binds to the biotin-labelled amplicons captured by the plate-bound probe KY150. A substrate (TMB + hydrogen peroxide) was added and then the reaction was stopped and the optical density was measured at 450 nm in an automated microwell plate reader (Dynatech MR 700). The results were compared to the supplied cut off value. Serum AFP was measured by Cobas Core (Roche Diagnostics, Instrument center CH-6343. Rotkreuz, Schweiz). The method used was a two-step, solid phase EIA based on the sandwich principle (18). Serum VEGF was measured by competitive enzyme immunoassay (EIA) using the Accucyte Human VEGF kit (CYTIMMUNE Sciences, Inc., College Park Maryland 20740. USA.) (19).

Statistical analysis

Data were analyzed using statistical package SPSS version 10.0. Kruskal-Wallis, Mann-Whitney (U), Spearman's rank correlation coefficient (r) Chi-square (X^2) and ANOVA (F) tests were done at 5% level of significance (20).

Results

Table (1) shows comparison between different studied groups regarding age, gender, viral markers, and liver function tests. The highest levels of serum bilirubin, AST, ALT, ALP and γ-GT were found among HCC patients. On the other hand, the lowest levels of total protein and albumin were detected among LC patients. Table (2) shows that there was a significantly higher serum level of AFP among HCC ($p<0.001$) and LC ($p<0.01$) patients compared with CVH and control groups. Table (2) shows that there was a significantly ($p< 0.001$) higher level of serum VEGF in sera of patients with HCC compared to that of other groups. However, no significant difference was detected among LC, CVH and control groups. There was no significant relation between serum level of VEGF and size of the tumor. On the other hand, there was a significant ($p<0.01$) association with portal vein tumor thrombosis as shown in table (3). There was no significant relation between VEGF and stage of cirrhosis as determined by Child- Pugh classification of LC (table 3). Moreover, there was no significant correlation between the levels of VEGF and AFP, albumin, aminotransferases or PT among all the studied groups, suggesting that VEGF reflects neither the hepatic synthetic functions nor the inflammatory activity (table 4).

Discussion

In this study, there was a significant increase in the serum level of VEGF in HCC patients compared with LC, CVH and control groups. The preoperative serum VEGF was reported to be higher among patients with HCC (1). These findings indicate that VEGF may play a significant role in angiogenesis, growth and development of HCC (2). An *et al* (22) reported that VEGF was expressed in HCC cells and hepatocytes and on vascular endothelial cells. It was found that VEGF was about 7 times more commonly detected in carcinoma areas compared to non-carcinoma areas, suggesting that it is a very important angiogenesis factor for HCC growth. It was reported that the expression of VEGF was upregulated by HCC tumor and that there was a positive correlation between the serum VEGF level and tumor VEGF expression evaluated by immunohistochemical staining, suggesting that the serum VEGF level, at least in part, reflects the tumor VEGF expression (23). In addition, overexpression of VEGF mRNA and protein in HCC compared with LC and normal liver was reported (1).

Hypoxia was suggested as a central stimulus of angiogenesis and liver carcinogenesis through upregulation of VEGF gene expression by at least two distinct molecular mechanisms: activation of VEGF gene transcription and an increase in VEGF mRNA stability (24). The expression of VEGF is also potentiated by activation of oncogenes such as ras, or inactivation of tumor suppressor genes, such as P53, by cytokines, such as transforming growth factor beta (TGF-β) and nitric oxide, and by upregulation of nuclear factor κB/hypoxia-inducible factor (25).

There was no significant difference between patients with small and large HCC tumors according to Milan criteria

Table 1. General characteristics and liver function tests of the different studied groups.

The studied parameter	HCC (No = 25)	LC (No = 20)	CVH (No = 20)	Controls (No = 15)	Test of significance	p value
Age (years) mean ± SD	53.0 ± 5.6	48.8 ± 6.5 (*)	47.9±3.2(*)	47.3±3.8(*)	F= 3.54	<0.05
Gender: male/female	7/6	8/6	6/7	8/7	X^2= 0.34	>0.05
HB_sAg: -ve/+ve	8/5	7/7	8/5	15/0	X^2= 9.79	< 0.05
HCV: -ve/+ve	0/13	4/10	0/13	15/0	X^2= 42.36	<0.001
AST(IU/L)	(a,b,c) 168±152	(c) 95±79	44±21	17±9	F= 8.35	<0.001
ALT (IU/L)	(a,b,c) 78±39	(c) 50±42	34±16	17±4	F= 10.8	<0.001
Total bilirubin (mg/dl)	(a,b,c) 14.2±12.6	3.3±3.3	0.76±0.31	0.62±0.23	F= 13.6	<0.001
Direct bilirubin (mg/dl)	(a,b,c) 11.7±11.1	2.3±2.8	0.20±0.15	0.19±0.23	F= 12.9	<0.001
Total protein (gm/dl)	(b,c) 6.4±1.0	(b,c) 6.1±0.7	7.3±0.5	7.3±0.47	F= 11.4	<0.001
Albumin (gm/dl)	(b,c) 2.4±0.53	(b,c) 2.2±0.5	4.2±0.43	4.4±0.4	F= 83.1	<0.001
Alkaline phosphatase (IU/L)	(a,b,c) 265±267	97±39	74±22	57±12	F= 7.1	<0.01
γ GT (IU/L)	(a,b,c) 235±196	59±29	61±33	21±13	F= 12.9	<0.001
Prothrombin Time %	(b,c) 48.7±20.0	(b,c) 49.9±15.2	80.8±8.8	99.3±1.1	F= 50.1	<0.001

*(a) significantly different from LC, (b) different from CVH, (c) significantly different from, * significance of difference from HCC. ANOVA (F) and Chi-square (X^2) tests were all done at 5% level of significance.*

(21). Similar results were previously reported by some investigators who demonstrated an association between high VEGF expression in HCC and portal vein tumor thrombosis but no difference between large and small HCC tumors (26). On the other hand, Zhao *et al* (1) and Kamel *et al* (13) reported that there was a positive correlation between serum level of VEGF and tumor size. Moreover, other investigators have reported higher VEGF expression in small and well-differentiated HCC and suggested that VEGF plays its most important role in a relatively early stage of angiogenesis (24,26). Angiogenesis plays an important role in the development and progression of HCC and other tumors (9).The relation between tumor size and VEGF expression might be complex and dynamic because HCC has different vascular growth patterns (9,27).

Among our HCC patients, there was a significant increase in serum level of VEGF in patients with portal vein tumor thrombosis compared to patients without this complication. It was shown that the plasma level of VEGF was correlated with clinicopathologic parameters and poor overall survival (28,29). Some investigators (23) reported that in HCC, a high serum VEGF level was significantly correlated with absence of tumor capsule, presence of intrahepatic metastasis, presence of microscopic venous invasion, and advanced stage. The association between increased serum level of VEGF and portal vein tumor thrombosis may be attributed to the fact that VEGF- transduced cells had a marked increase in their invasive activity (26). More expression of VEGF in HCC was more evident with vascular and capsular invasion (30). VEGF may increase the permeability of microvessels, causing a significant vascular leakiness. An increase in tumor vessel permeability could

Table 2. Serum level of AFP and VEGF in the different study groups

Study parameter	HCC No=25	LC No= 20	CVH No=20	Controls No=15	Kruskal-Wallis	p value
Serum AFP (ng/ml):						
Mean	*(a,b,c)* 12017	*(b)* 32	10	3	32.5	<0.001
Standard Deviation	13070	19	6	1		
Minimum	1234	12	4	2		
Maximum	42433	63	26	4		
Serum VEGF (ng/ml):						
Mean	*(a,b,c)* 1.4	0.4	0.3	0.4	23.5	< 0.001
Standard Deviation	0.8	0.2	0.2	0.2		
Minimum	0.4	0.0	0.0	0.1		
Maximum	3.2	0.8	0.7	0.7		

(a) More significant than LC (b) More significant than CVH (c) Much more significant than controls.
The serum level of VEGF was significantly (p< 0.001) higher in patients with HCC compared to the other groups.

Table 3. Serum levels of VEGF in relation to portal vein tumor thrombosis and size of the tumor in HCC patients and to Child-Pugh class in LC patients.

Study parameter	VEGF (ng / ml)		Mann-Whitney U-test	p-value
	Mean ± SD	Range		
Size of the tumor (cm) < 5 (n=11) ≥ 5 (n=14)	 1.4 ± 0.9 1.4 ± 0.6	 0.4 – 3.2 0.6 – 2.1	0.3	> 0.05
Portal vein tumor thrombosis Positive (n = 9) Negative (n=16)	 2.3 ± 0.6 1.1 ± 0.4	 1.9 – 3.2 0.4 – 1.7	2.8	<0.01
Child-Pugh class in LC patients Child class B (n=8) Child class C (n=12)	 0.5±0.3 0.3±0.2	 0.2-0.8 0.0-0.6	1.2	>0.05

Table 4. Correlation of serum level of VEGF to liver function tests and AFP among the different groups of patients.

Study Groups	**Serum VEGF**									
	AST	ALT	Total bilirubin	Direct bilirubin	Total Protein	Albumin	Alkaline phosphatase	GGT	PT	AFP
HCC (n=25)	0.28	0.29	0.27	0.28	-0.03	-0.47	0.31	0.21	-0.30	0.20
LC (n=20)	0.23	0.34	-0.20	-0.16	0.04	0.30	0.16	-0.18	-0.11	-0.06
CVH (n=20)	0.04	0.10	0.21	0.01	0.38	0.48	-0.29	-0.15	0.02	-0.42

Spearman's rank correlation coefficient test was done at 5% level of significance. There was no significant correlation between serum levels of VEGF and liver function tests or AFP

increase the chance of tumor cell entry into the circulation, and newly formed vessels or capillaries may have leaky and weak basement membranes through which tumor cells could penetrate more easily than those of mature vessels, thus accelerating the hematogenous metastasis (31). Others postulated that platelets adhering to circulating tumor cells may be activated to release VEGF at points of adhesion to endothelium, leading to hyperpermeability and extravasation of tumor cells (23).

In the present study, the serum level of VEGF among patients with CVH or LC was not significantly different compared to that of the controls. Moreover, there was no significant association between the serum level of VEGF and stage of LC or biochemical indices of hepatocyte injury and/or function. Despite absence of correlation between the levels of VEGF and liver function tests, our findings showed that the levels of AST, ALT, bilirubin, alkaline phosphatase and γ GT were significantly higher in HCC patients compared to those with LC. A marked increase was found in the levels of bilirubin (mainly the direct bilirubin), alkaline phosphatase and γ GT among HCC patients, a finding which may be attributed to cholestasis or to presence of tumor (32). However, Desideri and Ferri (7) reported that serum VEGF levels decrease according to Child–Pugh classification in LC patients, and to severity of histologic findings in patients with CVH. Meanwhile, Shi *et al* (33) reported that VEGF has a tendency to increase in CVH and to decrease in LC both in tissue expression and circulating levels. On the other hand, it was reported that hepatic VEGF expression significantly increased during LC (10,33). An increased VEGF level in both HCC and LC was reported by Gadelhak *et al* (34). A positive correlation was found between serum level of VEGF and grade of the disease in patients with CVH (35).

Angiogenesis has a role in progression of various liver diseases (9). It was shown that neovascularization significantly increased during the development of liver fibrosis in both human and animal experimental studies (36). Furthermore, a semisynthetic analogue of fumagillin, TNP-470, which possesses antiangiogenic activity, suppressed experimental liver fibrosis development (37). These results suggest that angiogenesis plays an important role in the development of liver fibrosis (24). VEGF is responsible for LC-associated angiogenesis in which intrahepatic shunts and capillarization of sinusoids are well-established characteristics of LC (6,7). In addition to its angiogenesis activity, VEGF may induce extravasation of plasma proteins and increase mRNA levels of connective tissue growth factor leading to increased ECM production (38). VEGF also stimulates proliferation of both activated hepatic stellate cells and sinusoidal endothelial cells (10). Hepatic stellate cells are the primary ECM producing cells but sinusoidal endothelial cells also produce ECM components, such as type IV collagen and several proteoglycans (39). Therefore, VEGF was suggested to be involved in pathogenesis of CLD in addition to development of HCC (40).

In conclusion, VEGF was higher among HCC patients as compared to those with CVH or LC, and there was an association with portal vein tumor thrombosis in HCC, suggesting that it can be used as a useful biological marker of tumor invasiveness and a prognostic factor in HCC. However, further study is needed on a large scale in patients with HCC and follow up after tumor resection.

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