ORIGINAL ARTICLE

CIRCULATING VASCULAR ENDOTHELIAL GROWTH FACTOR AND NITRIC OXIDE IN PATIENTS WITH LIVER CIRRHOSIS: A POSSIBLE ASSOCIATION WITH LIVER FUNCTION IMPAIRMENT

Mohamed Ahmed Abdelmoaty, Ahmed Mohamed Bogdady*, Mervat Mohamed Attia* and Nayel Abdelhamed Zaky*

Departments of Biochemistry and *Internal Medicine, Sohag Faculty of Medicine, Sohag University

ABSTRACT

Hemodynamic disorders of liver cirrhosis complicated with portal hypertension are associated with an increased angiogenesis in animal model of portal hypertension and cirrhosis which were linked to increased expression of vascular endothelial growth factor (VEGF) and nitric oxide (NO). The aim of study was to evaluate the serum concentration of VEGF and total nitric oxide (NO) in liver cirrhosis and the possible association with the degree of liver insufficiency. VEGF and NO were measured in serum of 64 patients with liver cirrhosis by ELISA and spectrophotometry respectively. The significant increase of serum VEGF was observed in liver cirrhosis compared to healthy individuals as well as serum NO (106.1 ± 66.7 vs. 41.5 ± 6 pg/mL, $P < 0.05$; 113.5 ± 65.8 vs. 20.8 ± 3.8 μ mol/l, $P < 0.001$, respectively). Serum VEGF and NO showed significant associations with biochemical indices of liver function and with Child-pugh score where they were increased respectively to the degree of liver insufficiency. A significant association of raised serum NO in early stage of portal hypertension reflect its benefit in early expect of portal hypertension but, high serum VEGF in late stage may reflect its prognostic value in liver cirrhosis.

KEY WORDS

Liver cirrhosis, Nitric oxide, Portal hypertension, Vascular Endothelial Growth Factor.

INTRODUCTION

Cirrhotic patients have some characteristic changes such as low systemic vascular resistance and hypotension lead to secondary disturbances in renal and hepatic blood flow and ascites (1). The exact mechanisms of these hemodynamic disorders have not yet been clearly elucidated (2). Different experimental studies suggested the role of angiogenesis in the pathogenesis of portal hypertension secondary to liver cirrhosis. Sieber et al (3) reported an increased angiogenesis in the abdominal cavity of portal hypertensive rats. Also, Geerts et al (4) provided an increased angiogenesis in the mesenteric

microvasculature in animal model of portal hypertension with cirrhosis. Moreover, they showed high levels of Vascular Endothelial Growth factor (VEGF) and endothelial nitric oxide in mesentery suggested their contribution in portal hypertension secondary to liver cirrhosis.

VEGF is a secreted, 46 kDa dimeric protein, active as a direct and specific mitogen for vascular endothelial cells thus a well known mediator of angiogenesis in physiological and pathological conditions (5). VEGF production is known to be regulated by various substances, including oxygen, steroid hormones, reactive oxygen metabolites and protein kinase C agonists (6). An upregulation of VEGF in cirrhotic livers was previously demonstrated in animal models (7) as well as in human clinical studies (8, 9) and linked with ongoing angiogenesis and portal hypertension. Moreover, it was suggested that VEGF may contribute to liver fibrosis development by inducing the proliferation of hepatic stellate cells and sinusoidal endothelial cells (10). The usefulness of

Address for Correspondence :

Dr. Mohamed Ahmed Abdelmoaty

Sohag Faculty of Medicine,

Sohag University, Egypt

Tel: +2-0126388153

E-mail: abdelmoaty5@yahoo.com

serum VEGF measurement was validated as a prognostic marker in hepatocellular carcinoma patients (11).

Nitric oxide (NO) which is a short-lived radical synthesized from L- arginine by nitric oxide synthase (NOS). It has been proposed to play a crucial role in these hyperdynamic circulatory disorders which characterized by vasodilation and increased cardiac output, are associated with cirrhosis (12). NO, discovered as an endothelium-derived-relaxing factor (EDRF) is implicated in certain physiopathological processes such as smooth muscle relaxation, cardiac contractility, platelet clotting, neuronal mediation, human immune defence and septic shock (13-17). NO production has a relationship to renal function and hyperdynamic circulation in patients with liver cirrhosis (18).

Vasodilatation was induced by intravenous injection of VEGF in rats (19). Such effect appeared to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived nitric oxide, as assessed by the requirement for an intact endothelium and the prevention of the effects by N-methyl-arginine (20). Accordingly, VEGF has no direct effect on contractility or rate in isolated rat heart in vitro (19). These hemodynamic effects of angiogenic factors such as VEGF, aFGF and bFGF may be induced by nitric oxide-mediated vasodilatation and hypotension (21). The aim of present study was to evaluate the serum concentration of VEGF and total nitric oxide (nitrate and nitrite) and their possible association with liver function impairment in liver cirrhosis individuals.

MATERIALS AND METHODS

Sixty four patients with cirrhosis (mean age 50 ± 3 years, with a median age of 51, minimum 41, maximum 57 years, 44 males, and 20 females) and 16 healthy volunteers (mean age 48 ± 8 years, with a median age of 47, minimum 42, maximum 58 years, 10 males and 6 females) were included in this study. 28 (43.8%) cirrhotic patients were associated with Hepatitis C virus (HCV) antibody positive, but 22 (34.4%) of these were associated with positive Hepatitis Bs antigen (HBsAg). Patients selected in this current study admitted in the Internal Medicine Department, Sohag faculty of Medicine hospital. Ethics Committee approval was obtained for the current study and the volunteers gave their oral consent to participate in the study after being informed of the purpose and steps of the trial. Liver cirrhosis was diagnosed on the basis of clinical signs, ultrasound imaging, CT and endoscopic finding of oesophageal varices. The severity of the disease was evaluated according to the classification of Child–Pugh (22, Table 1). Patients with congestive heart failure, renal failure,

Table 1: The Child–Pugh score

Points	1	2	3
Encephalopathy	None	Minimal	Advanced (coma)
Ascites	Absent	Controlled	Refractory
Bilirubin (mmol/l)	<34	34–51	> 51
Albumin (g/l)	>35	28–35	<28
Prothrombin conc.	>50%	40–50%	<40%

lung disease, malignancy, hepatic encephalopathy or gastrointestinal bleeding were excluded. Diuretics, antibiotic therapy or nitrate-containing medications were stopped for 2 weeks before the study. All patients and control subjects received the same dietary protein intake and fasted for at least 10 h before serum samples were collected.

Ascites, encephalopathy, prothrombin concentration, bilirubin, aminotransferase and albumin concentrations were evaluated in all cirrhotic patients to divide them into three groups according to Child–Pugh score (Table 2).

Table 2: Groups selected in cirrhotic patients and control

Groups	Child-Pugh Score	h	Mean age (range) y.	Sex(M/F)
Group 1	A (5-6)	18	48 (41-56)	13/5
Group 2	B (7-9)	21	50 (44-56)	16/5
Group 3	C (10-15)	25	50 (43-57)	15/10
Control group	-	16	48 (42-58)	10/6

VEGF concentration was measured in serum of all patients and control groups by using RayBiotech Human VEGF ELISA (Enzyme-Linked Immunosorbent Assay kit; Cat. No ELH-VEGF-001) with minimal detectable dose of 3.5 pg/ mL. The colour change from blue to yellow after adding stop solution and the intensity of colour change was measured at 450 nm. According to Van Bezooijen et al, NO was assayed by spectrophotometric method. Sample deproteinization by zinc sulphate followed by reduction of nitrate to nitrite by activated cadmium with aminochloride and sodium borate. Griess reagent (sulphanilamide, naphthylethylene diamine dihydrochloride and orthophosphoric acid) was added to the reduced clear serum supernatant. Measurement was done at OD_{550nm} and the total NO in the sample was calculated from the standard curve (22).

Statistical analysis: Values were expressed as mean and standard deviation (SD). The significance of differences was calculated by unpaired Student's t-test. Differences with *P*

values < 0.05 were considered significant by using SPSS software (release 10.0).

RESULTS

The significant increase of serum VEGF and total NO was observed in liver cirrhosis compared to healthy individuals (106.1 ± 66.7 vs. 41.5 ± 6 pg/ml, $P < 0.05$; 113.5 ± 65.8 vs. 20.8 ± 3.8 $\mu\text{mol/l}$ $P < 0.001$, respectively). Differences in serum VEGF and NO did not change significantly in patients with post-hepatitis cirrhosis than those with non post- hepatitis form. Serum VEGF showed significant associations with biochemical indices of liver function (prothrombin time, albumins, bilirubin concentration) and also, clinical signs (ascites and encephalopathy) (Table 3), but not with markers of liver injury as aminotransferases (serum aspartate transaminase and alanine transaminase).

Interestingly, among clinical parameters of liver dysfunction only ascites revealed significant association with serum VEGF and NO. The elevation in serum VEGF in individuals with ascites was more than 2-folds compared to those without (149.2 ± 63.7 vs. 52.7 ± 15.4 pg/ml, respectively $P < 0.05$). Also, the elevation in serum NO in cirrhotic patients with ascites was more than 3-folds compared to those without (161.7 ± 27.4 vs. 47.4 ± 7.1 $\mu\text{mol/l}$, respectively $P < 0.05$). Furthermore, the highest level of serum VEGF was observed in patients with refractory ascites compared to diuretic response ascites (178.8 ± 24.2 vs. 70.7 ± 16.1 pg/ml, respectively $P < 0.001$) but, no significant change in NO was found between refractory and response ascites.

Serum VEGF and NO levels in all cirrhotic patients were increased, respectively, to the degree of liver insufficiency (Table 3 and Figure 1). It was demonstrated through a

Fig 1 (A)

Fig 1 (B)

Fig 1: Serum VEGF (A) and nitric oxide (B) concentrations in liver cirrhosis patients with different score of Child-Pugh and control group

significant positive correlation with Child-Pugh score: $r = 0.38$, $P < 0.001$ for VEGF and $r = 0.77$, $P < 0.001$ for NO. The most prominent increase of serum VEGF was observed in patients

Table 3: Serum VEGF and Nitric oxide levels in different groups selected

	Serum VEGF		Serum Nitric oxide		Serum aspartate transaminase		Serum alanine transaminase	
	(pg/ml)		($\mu\text{mol/l}$)		(U/L)		(U/L)	
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
Group 1	52.28 ± 9.5	39-69	26.39 ± 8.3	16-49	$172 \pm 41.7^{\S}$	22- 241	$142 \pm 36.1^{\S}$	14- 206
Group 2	72.95 ± 15.3	37-99	$157.76 \pm 28.7^{??}$	121-211	$183 \pm 35.7^{?}$	45-264	$152 \pm 29.7^{?}$	24-228
Group 3	$193.12 \pm 24.2^{\# + o}$	155-244	$165.2 \pm 26.6^{\# +}$	134-233	$179 \pm 54.4^{\#}$	47-294	$147 \pm 44.5^{\#}$	37-241
Control group	41.5 ± 6	33-51	20.81 ± 3.8	15-28	18 ± 7.9	5-34	14 ± 5.7	6-18

$\S$ Significant change ($P < 0.05$) between control group and group 1; $?$ Significant change ($P < 0.05$) between control group and group 2; $\#$ Significant change ($P < 0.05$) between control group and group 3; $?$ Significant change ($P < 0.05$) between group 1 and group 2; $+$ Significant change ($P < 0.05$) between group 1 and group 3; o Significant change ($P < 0.05$) between group 2 and group 3

with group 3 liver cirrhosis (Child- Pugh class C, Table 3), where patients serum VEGF concentrations in group 1 and 2 were not significant changed comparing to healthy individuals. Also, there was no significant change between group 1 and 2 (Child- Pugh class A and B respectively) of liver cirrhosis.

DISCUSSION

Vascular endothelial growth factor (VEGF) is a well-known angiogenic mediator and has a role in vascular permeability (5). High serum VEGF was observed in cirrhotic livers of bile duct ligated rats (7, 24). In human, high Serum VEGF measurement was validated in patients with hepatocellular carcinoma and its level associated with poor response to treatment as well as shorter survival (11). In these study, we detected its relation to liver cirrhosis and its degree according to Child-Pugh score (22). There are conflicting results concerning the serum VEGF in liver cirrhosis. Many published studies report its increase (25, 26). We observed increase over two folds of VEGF in liver cirrhosis compared to healthy controls. The highest concentrations of VEGF were observed in patients with advanced stages of liver cirrhosis, which was reflected by a positive correlation with Child-Pugh score specially score C. These suggest that VEGF might be involved in cirrhosis associated angiogenesis as well as portal hypertension. Correspondingly, there was no difference in serum VEGF between various etiologies of disease. Interestingly, among clinical parameters of liver dysfunction only the presence and intensity ascites revealed significant association with serum VEGF.

On the other hand, there was a significant correlation between serum total nitric oxide (NO) level and liver cirrhosis. Patients with a gross ascites had an increased level of C-reactive protein, showing that an inflammatory process occurs in these patients, leading to increase in inflammatory cytokines coupled with an increase in NO production (27, 28). Experimental studies in animal models of cirrhosis and portal hypertension have shown that NO contributes to systemic and splanchnic vasodilation and hypo-responsiveness to vasoconstrictors (29). Few in vivo human studies have examined the relationship between NO and the hyperdynamic circulation. NO participate in systemic hyperdynamic circulatory may be due to stimulation of constitutive NO synthase in patients with cirrhosis (30). Also, anemia, which is a frequent complication in liver cirrhosis, may therefore be a factor in enhanced activity of NO as haemoglobin is a potent inhibitor of NO (31).

Portal hypertension secondary to liver cirrhosis is due to increase intrahepatic vascular resistance and increase blood

flow through the portal venous system. An attention focused on the potential role of increase nitric oxide production in the splanchnic territory is observed in portal hypertension associated with liver cirrhosis (32). Nevertheless, the role of structural changes like angiogenesis has been suggested by experimental studies in the pathophysiology of portal hypertension and cirrhosis. Sieber et al (3) showed an increased angiogenesis in the abdominal cavity of portal hypertensive rats without cirrhosis. An increased VEGF expression was previously shown in vitro in the mesentery of portal hypertensive mice (4). Studies by Fernandez et al (33, 34) showed a decrease of the intestinal neovascularization, splanchnic blood flow and formation of portosystemic collaterals in portal hypertensive rats by the administration of anti-VEGF receptor-2 monoclonal antibodies. Moreover, Chun et al (35) reported that transforming growth factor beta1 (TGF-beta1) can activate macrophages to express angiogenic mediators such as VEGF. Serum TGF-beta1 demonstrated strong correlation with degree of hepatic insufficiency (36).

Geerts et al (4) found in vivo increased angiogenesis in the mesenteric microvasculature of an experimental model of portal hypertension rats with cirrhosis. Authors showed increased expression of VEGF and endothelial cell-nitric oxide synthase in the mesentery of those rats, which was significantly higher compared with the control groups. Authors concluded that portal hypertension with or without cirrhosis could able to increase angiogenesis in the splanchnic microvasculature. More recently Fernandez et al (37) showed in portal vein ligated rats that treatment with rapamycin (VEGF signaling inhibitor) and Gleevec (PDGF signaling inhibitor) reduced splanchnic neovascularization and significantly decreased portal pressure. Furthermore, Iavarone et al. (38) showed a significant VEGF gradient between hepatic vein and peripheral vein serum levels that correlated with the degree of hepatic vein pressure gradient (HVPG) in hepatocellular carcinoma (HCC)-free patients. Interestingly, such association was not found in patients with cirrhosis and HCC, suggesting, that in these patients serum levels of VEGF were mainly influenced by VEGF synthesis by neoplastic liver cells rather than by portal hypertension. Our results support those observations in clinical settings. In fact, highest concentrations of VEGF were observed in patients with refractory acites and correlated with the degree of liver insufficiency. Moreover, recently published data by Saphr et al (39) showed that prolonged administration of octreotide in liver cirrhosis decreased HVPG which was associated with the decrease in circulating VEGF. Our results could also point the usefulness of serum VEGF as potential prognostic factor in liver cirrhosis. The serum VEGF measurement might be helpful in selecting diuretic

resistant or refractory ascites individuals who could benefit from ascites drainage rather than diuretic therapy.

In conclusion, our study suggests that serum VEGF and nitric oxide may reflect the hepatic function impairment in liver cirrhosis and seems to be associated with portal hypertension symptoms. In liver cirrhosis, Serum nitric oxide can be used as diagnostic test of portal hypertension but, serum VEGF is useful as a prognostic factor.

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