Relation Of Smoking And Serum Vascular Endothelial Growth Factor (VEGF) To Hepatic Fibrosis In Chronic Hepatitis C Patients

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Background: Liver fibrosis is an important pathological event in chronic hepatitis (CHC) patients that eventually progresses to liver cirrhosis. Host factors can affect the progression of liver fibrogenesis in CHC patients. Pathologic angiogenesis is linked to necroinflammation and fibrosis in CHC patients. Vascular endothelial growth factor (VEGF) is a major pro-angiogenic factor, trigerred by hypoxia as in smokers, to stimulate angiogenesis and perpetuate hepatic inflammation and fibrosis. ***Aim of this study***: was to assess serum VEGF in smoker and non-smoker CHC patients in relation to liver inflammation and fibrosis. ***Patients and methods***: We determined serum VEGF level by competitive enzyme immunoassay method (ng/L) in 60 CHC patients and 30 healthy controls. The studied 60 CHC patients were divided into: group I (30 smoker CHC patients) and group II (30 non-smoker CHC patients). 30 healthy controls were included in group III. Complete blood count (CBC), liver biochemical profile including: serum ALT, AST, bilirubin, albumin and prothrombin time were determined for all groups. Serological diagnosis by determination of HCV-antibodies was done in group I and group II in addition to determination of viral load by quantitative polymerase chain reaction (PCR). Histopathological diagnosis according to histological activity index (HAI) was done in CHC patients who were naïve to antiviral therapy. ***Results***: We found significantly increased serum VEGF level in CHC patients compared to controls and in group I compared to group II (403±96.5 *vs* 320.5±102.8 *vs* 49.5±23.31, F= 18.4, P<0.01). Serum VEGF was significantly correlated to fibrosis stage in group I (r= 0.64, P<0.01) and group II (r= 0.38, P< 0.05). Serum VEGF was significantly correlated to grade of inflammation in group I (r= 0.72, P<0.01) and group II (r= 0.42, P<0.05). Significant fibrosis (F ≥S2) was present in higher percentage of smoker than non-smoker CHC patients (χ^2= 14.8, P<0.01) and serum VEGF was correlated with number of cigarettes/day in group I (r= 0.61, P<0.01). ***Conclusion***: We concluded that serum VEGF stimulated by smoking and possibly other hypoxic condition, is involved in pathological angiogenesis which is linked to fibrosis progression in CHC patients. Smoking seems to be a prognostic factor in CHC patients with impact on fibrosis progression and response to antiviral therapy.

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Introduction:

Hepatitis C virus (HCV) infection is an important public health problem. It is a major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. It is a major indication of liver transplantation world-wide ***(Berenguer, 2001)***.

In more than 80% of infected person HCV infection can induce persistent hepatic injury which leads to disease progression from periportal inflammation to chronic hepatitis with bridging fibrosis, to frank cirrhosis ***(Kelher and Afdhal, 2005)***.

Risk and natural history of fibrosis associated with HCV infection can run a remarkably variable course, from decades of viremia with little fibrosis, to rapidly progressive disease that leads to cirrhosis in less than 20 years. It is host, rather than virus factors that correlate with fibrosis progression in chronic hepatitis C patients ***(Benhamou et al., 2001)***.

Risk factors for rapid progression of fibrosis in HCV patients include: old age at time of infection (more than 40 years), concurrent liver disease due to HBV infection or alcohol intake (>50gm/day), male gender, increased body mass index associated with hepatic steatosis, HIV infection or immunosuppression after liver transplantation and iron overload ***(Angelucci et al., 2002)***.

Recent data suggest that cigarette smoking may be independently related to increased inflammatory activity and hepatic fibrogenesis ***(Pessione et al., 2003)***. This effect of smoking may be due to nicotine induced oxidative damage or enhancing angiogenesis due to hypoxia ***(Mikko et al., 2009)***.

Smoking is a prognostic factor in chronic hepatitis C patients is still controversial ***(De Luca et al., 2009)***.

Aim of the study is to evaluate the role of VEGF in hepatic fibrosis in CHC patients, both smokers and non-smokers.

Patients and Methods:

Patients:

Sixty chronic hepatitis C patients, serologically and histopathologically diagnosed, were included in this study. They were enrolled from patients attending Ain Shams University Hospitals and they were naïve to antiviral therapy. They were categorized into 2 groups:

Group 1: Included 30 patients of CHC who are cigarette smokers. The duration of smoking ranged from 8 years to 35 years and number of cigarettes/day ranged from 15cigarettes/day to 50cigarettes/day.

Group II: Included 30 patients of CHC who are non-smokers. All patients in both groups were males.

In addition to the two patient groups, 30 healthy control males were included as a control group (group III).

This is a randomized case-control study.

Exclusion criteria:

- Females were excluded from patients and control groups (to avoid the effect of gender on hepatic fibrosis).
- Patients with history of alcohol intake or active alcohol consumption at time of the study were excluded.
- Patients who are HBS Ag positive were excluded.
- Patients with previous antiviral therapy for CHC were excluded.

Methods:

For all patients in group I and group II and healthy controls in group III, the following was done:

1. Full clinical assessment by detailed history and physical examination.
2. Complete blood count (CBC).
3. Laboratory biochemical liver profile including: serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum albumin, total serum bilirubin and prothrombin time.
4. Serological diagnosis by enzyme immunoassay (EIA) to detect HCV-antibodies (second generation) in the 2 patient groups. All patients were HBsAg negative. All healthy control person in group III were negative for B and C virus infection.
5. Determination of HCV-RNA by polymerase chain reaction (PCR) (quantitative test) to determine viral load.
6. Real time abdominal ultrasonography (U/S) to comment on liver parenchymal echopattern, portal vein diameter (maximum 1.2cm), splenic vein diameter (maximum 0.6cm), and splenic length (maximum 12cm).
7. Ultrasound guided percutaneous liver biopsy and histopathological examination to assess necro -inflammatory activity and fibrosis stage according to ***Ishak et al. (1995)*** in the two patient groups only. This was done as a step in their evaluation before antiviral therapy.
8. Serum VEGF was determined by competitive enzyme immunoassay method (Accucyte human VEGF).

   Principle: Antirabbit antibodies are allowed to compete for VEGF present in sample (or standard) and coated kit wells. After washing, biotinylated VEGF conjugate attached to the well is visualized using streptavidine alkaline phosphatase conjugate. The higher the optical density (OD), the lower the

VEGF concentration in the sample. VEGF level was expressed in ng/L (range in normal control 15-220ng/L).

9. Statistical analysis of the collected data was done using SPSS computer program (statistical package of social science) version 12. Data were expressed as mean and standard deviation (Mean ±SD). Comparative statistics for parametric data in the 3 groups was done using ANOVA tests (one way analysis of variance). Comparison between different groups for qualitative data was done using Chi-square test (χ^2) and correlation statistics was done using Spearmen correlation coefficient (r) test.

   P-value >0.05 was considered statistically non significant, P-value <0.05 was considered significant and P-value <0.01 was considered highly significant.

Results:

Sixty CHC patients and thirty healthy control persons were included in this study.

Age and sex distribution: Patients in group I and group II and healthy control in group III were comparable regarding age (52.24±12.30 *vs* 50.81±11.21 *vs* 51.15±13.25; F= 0.16, P >0.05). All CHC patients and control persons were males.

Average duration of smoking was 21.2±9.85 years and average number of cigarettes/day was (31.8±11.8).

Comparison of the different haematolgic and biochemical laboratory data in the 3 groups showed significantly lower platelet count in group I compared to group II and group III while Hb% and WBCs were non significantly different in the 3 groups. Serum ALT and AST levels were significantly higher in group I and group II compared to group III reflecting the necroinflammation due to HCV infection.

Ultrasound parameters were compared in the 3 groups where splenic vein diameter, splenic length and portal vein diameter were significantly higher in group I and group II compared to group III (Table II).

The patient groups I and II showed fibrofatty pattern (bright liver) in all patients (100%) while in group III (control group) only 3 patients showed mild bright echopattern of the liver (10%) which was highly significant difference between the 3 groups (χ^2= 24.21, P<0.01).

Fibrosis stage was compared in the 2 patient groups (Table III), where significant fibrosis (fibrosis ≥S2) and fibrosis <S2 was compared in the 2 groups and showed highly significant difference between the 2 groups (χ^2= 14.8, P <0.01). More advanced fibrosis is expected to occur in CHC patients who are smokers than non-smokers.

Grade of necroinflammation in both patient groups was compared (Table IV) and group I (smokers) showed highly significant increase in moderate and severe grades of inflammation compared to group II (χ^2= 12.8, P<0.01).

Serum VEGF level was compared in the 3 groups (Table V) showing highly significant increase of serum VEGF from group I through group III (F= 18.4, P<0.01) and further com-parison between group I and group II showed highly significant VEGF level in group I (LSD, P <0.01) and highly significant difference between group II and group III (LSD P<0.01).

This shows the important role of VEGF in the inflammatory process in CHC patients and its possible impact on progression of liver fibrosis. Correlation between serum VEGF and fibrosis stage in group I and group II showed significant correlation in both groups (Table VI). This may support the role of VEGF and enhanced angiogenesis in liver fibrogenesis.

Correlation between serum VEGF and grade of inflammation showed significant correlation in group II and highly significant correlation in group I (Table VII). This supports the relation between inflammation, angiogenesis and fibrogenesis and points to the possible risk of smoking in relation to fibrosis progression. Correlation between serum VEGF and number of cigarettes/day showed positive highly significant correlation (Fig. I). This shows the possible substantial effect of smoking being a trigger of angiogenesis and possible fibrosis in CHC patients.

Table I: Comparison of the 3 groups regarding the different laboratory data (ANOVA test)

Parameters	Group I (n= 30)	Group II (n= 30)	Group II (n= 30)	F	P
Hb (gm/dL)	13.4±3.9	12.7±4.8	12.1±4.2	0.21	>0.05
WBCs (10^9/L)	6.6±1.4	6.1±2.1	5.8±2.2	0.43	>0.05
Platelet count (10^9/L)	151.4±38.3	161.2±48.51	181.8±65.72	4.21	<0.05
ALT (IU/L)	82.3±19.8	75.4±21.4	31.5±9.8	7.2	<0.01
AST (IU/L)	102.8±28.4	92.4±23.5	39.2±12.7	8.3	<0.01
Albumin (gm/dL)	3.8±1.82	3.9±1.68	4.2±1.32	0.34	>0.05
Total bilirubin (mg/dL)	1.2±0.61	1.4±0.52	0.9±0.36	0.29	>0.05
P.T (sec)	13.4±4.2	12.7±3.8	14.1±3.3	0.18	>0.05

P>0.05= Non significant; P<0.05= Significant; P<0.01= Highly significant.

Table II: Comparison of ultrasound parameters in the 3 groups (ANOVA test)

Parameters	Group I (n= 30)	Group II (n= 30)	Group II (n= 30)	F	P
Splenic vein diameter (cm)	0.93±0.31	0.74±0.28	0.62±0.32	1.2	<0.05
Splenic length (cm)	13.4±3.81	12.7±4.3	11.8±3.13	1.4	<0.05
Portal vein diameter (cm)	1.3±0.72	1.2±0.61	0.9±0.52	0.9	<0.05

Table III: Comparison of fibrosis stage in group I (smokers) and group II (non-smokers)

Fibrosis stage	Group I (n=30)	Group II (n=30)	χ^2	P
Fibrosis S>2/6	N= 18 (60%)	N= 9 (30%)	14.8	<0.01
Fibrosis S<2/6	N= 12 (40%)	N= 21 (70%)		

Table IV: Comparison of inflammatory grade in group I and group II

Grade of inflammation	Group I (n=30)	Group II (n=30)	χ^2	P
Grade >6/18	N= 21 (70%)	N= 16 (53%)	12.8	<0.01
Grade <6/18	N= 9 (30%)	N= 14 (47%)		

Table V: Comparison of serum VEGF level in the 3 groups (ANOVA test)

Parameters	Group I (n= 30)	Group II (n= 30)	Group II (n= 30)	F	P
VEGF (ng/L)	403±96.5	320.5±102.8	49.6±23.31	18.4	<0.01

LSD: Group I-Group II P<0.01
Group I-Group III P<0.001
Group II-Group III P<0.001

LSD: Least significant difference.

Table VI: Correlation of VEGF and stage of fibrosis in group I and group II

Fibrosis stage	Group I (VEGF)	Group II (VEGF)
FS >2/6	392±132.56	289±95.61
FS <2/6	256±108.62	214±89.35
R	0.64	0.38
P	P <0.01	P <0.05

Table VII: Correlation of VEGF and grade of necroinflammation in group I and group II

Grade of inflammation	Group I (VEGF)	Group II (VEGF)
Grade >6/18	401±96.36	278±93.52
Grade <6/18	328±78.32	226±81.72
r	0.72	0.42
P	<0.01	<0.05

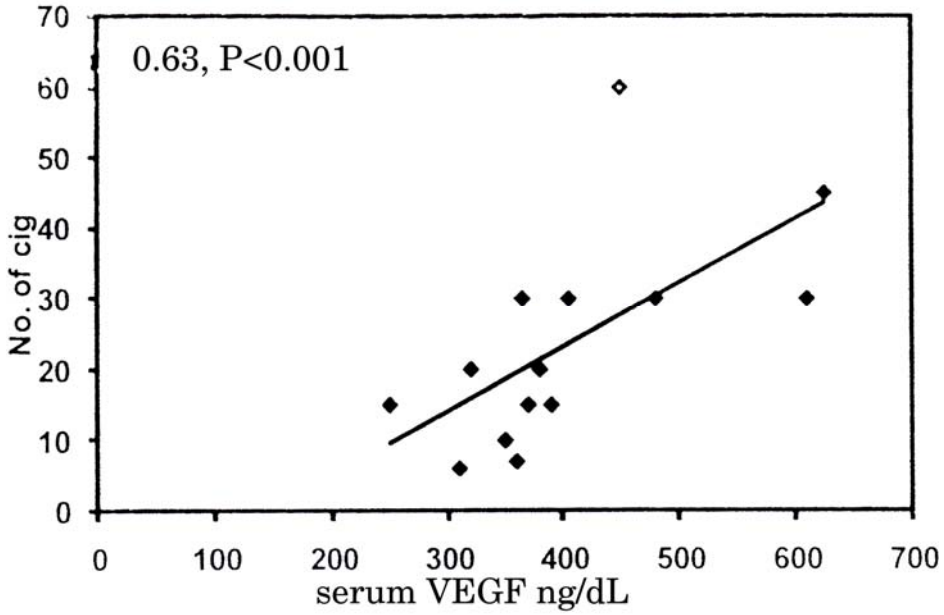

Fig. (I): Correlation of VEGF and number of cigarettes/day in group I (smokers).

Discussion:

Hepatic fibrosis is a well-recognized sequalae of chronic hepatitis C (CHC), yet the severity and rate of firbosis progression vary among patients.

Host factors that affect fibrosis progression include age, age at infection, duration of infection, sex, presence of steatosis, body mass index, iron overload and concurrent liver morbidity as HIV or HBV infection or alcohol intake ***(Feld and Liang, 2006)***.

Recent data suggest that cigarette smoking is related independently to increased fibrogenesis in CHC patients and this was attributed to stimulation of hepatic angiogenesis through upregulation of pro-angiogenic factors as VEGF ***(Roy et al., 2006)***.

In this study, we determined the serum VEGF level in a cohort of Egyptian CHC patients, both smokers and non smokers, in comparison with age and sex matched healthy controls. We found highly significantly increased serum VEGF level in smokers than non-smokers CHC patients and in all patients compared to healthy controls. This agreed with previous reports of ***Mikko et al. (2009)***, and ***Deluca et al. (2009)*** who reported that hypoxia induced by smoking in CHC patients trigerred VEGF release from T-cells and other cells and resulted in enhanced fibrogenesis.

Nicotine is mainly metabolized in the liver and can induce hepatic steatosis, focal and confluent necrosis and immunosuppression ***(De Luca et al., 2009)***.

In CHC patients, hypoxia induced by smoking and by chronic inflammation, leads to expression of hypoxia inducible factors and over-expression of proangiogenic cytokines as VEGF, platelet derived growth factor (PDGF) and angiopoitein-1 (Ang-1) ***(Paternostro et al., 2010)***.

Angiogenesis is a dynamic, hypoxia-stimulated, and growth factor dependent process that leads to new vessel formation from pre-existing vessels. Enhanced hepatic angiogenesis unequivocally is involved in the pathogenesis of chronic liver disease as CHC and is strongly related to necroinflammation and fibrogenesis ***(Novo et al., 2007)***.

In our study, patients with significant fibrosis (F≥S2) and patients with more than mild inflammation (grade ≥6/18) were more among smokers than non-smokers (Table III and Table IV). This reflects enhanced angiogenesis in their livers in parallel to chronic inflammation and fibrogenesis.

Our findings agreed with findings of ***Parola et al. (2008)*** and ***Copple et al. (2009)*** who found that chronic inflammatory response in CHC patients leads to progression of fibrosis towards the end point of

cirrhosis. Several mediators of inflammatory response may stimulate other cells in the hepatic microenvironment to express VEGF and other proangiogenic factors and to sustain angiogenesis. These mediators or cytokines include hepatocyte growth factor (HGF), platelet derived growth factor (PDGF) and nitric oxide (No) ***(Volfré di Bonzo et al., 2009)***.

New vessels formed as a consequence of enhanced angiogenesis help to maintain chronicity of liver injury and perpetuate necroinflammation and fibrosis. The extent of angiogenesis, in addition to maintaining disease progression, may also represent a key limiting factor for fibrosis reversibility particularly in postnecrotic cirrhosis as CHC patients ***(Paternostro et al., 2010)***.

In our study, we found significant positive correlation between serum level of VEGF and fibrosis stage (Table VI) and grade of inflammation (Table VII) in agreement with findings of ***Corpechot et al. (2002)*** and ***Yoshiji et al. (2003)***, who reported that angiogenesis, inflammation and fibrosis occur in parallel in chronic liver diseases as CHC. They also reported that hepatic stellate cells (HSCs) and myofibroblast cells (MFS) in CHC represent strategic cells in this interaction.

HSC/MFs represent hypoxia-sensitive and cyto- and chemokine-modulated cellular crossroad between necro-inflammation, pathological angiogenesis and fibrogenesis. Hepatocytes, under the effect of hypoxia in chronic liver disease, may be a relevant source of vasoactive mediators as VEGF.

This proves the cross-link between hypoxic hepatocytes and surrounding mesenchymal cells (hepatic/mesenchymal interaction) in producing VEGF which has a major role in increasing vascular permeability, triggering neovessel formation and increasing vascular resistance and capacitance to maintain hyperdynamic circulation and portal hypertension in CHC patients ending in cirrhosis ***(Paternostro et al., 2010)***.

We found a significant positive correlation between VEGF level and number of cigarettes day in CHC patients and this again supports the role of hypoxia in smokers and in other hypoxic chronic diseases as chronic obstructive pulmonary disease (COPD) in stimulating VEGF expression and enhanced angiogenesis ***(Mikko et al., 2009)***.

This effect of hypoxia leads to release of VEGH[H.MF from T-cells, HSC/MFS, sinusoidal endothelial cells and hypoxic hepatocytes. This raises, in recent years, the concept that pathological angiogenesis may be used as a potential therapeutic target in CHC and other chronic liver diseases. Antiangiogenic therapy by Sorafenib or Sunitinib or others may be promising to halt the progression of angiogenesis and fibrosis and to delay liver cirrhosis and its complications, in addition to other lines of treatment ***(Shah and Bruix, 2009)***.

We concluded from this study that smoking seems to be a prognostic factor in CHC patients that may affect the rate of fibrosis progression and responsiveness to antiviral therapy. However, this needs further prospective studies on a larger number of patients to evaluate its importance, in addition to other determining factors, in CHC patients.

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