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Role of fibrogenic markers in chronic hepatitis C and associated hepatocellular carcinoma

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Abstract Detection and follow up of fibrogenesis in chronic hepatitis C (CHC) is mandatory for early treatment and risk stratification. The current study included 120 patients with CHC, of whom 30 had liver cirrhosis (LC) and 30 had hepatocellular carcinoma (HCC). 15 wedge liver biopsies, taken during laparoscopic cholecystectomy, were included as normal controls. Cases were subjected to laboratory investigations, serologic markers for viral hepatitis and assessment of circulating levels of hyaluronic acid (HA) and platelet-derived growth factor (PDGF). Immunohistochemical expression of connective tissue growth factor (CTGF), PDGF and transforming growth factor-β1 (TGF-β1) was also carried out. A significant increase ($p < 0.01$) in serum HA was noticed in CHC, LC and HCC compared to controls. Although, a significant decrease in serum PDGF was detected in CHC and LC compared to controls, HCC values were comparable. A significant up-regulation of CTGF was detected in CHC, LC and HCC ($p < 0.01$) in contrast to its limited mild expression in normal livers. Intense PDGF positive staining was noticed in CHC, LC and HCC compared to scattered faint expression in controls. The significant expression and marked intensity of PDGF staining matched the progress to tumorigenesis. A positive TGF-β1 immunostaining was also noticed in CHC, LC and HCC. An intense and extensive cytoplasmic expression of TGF-β1 was encountered in patients with LC revealing that CTGF, PDGF and TGF-β1 act synergistically in LC. Data revealed that HA and CTGF may be implicated as important diagnostic parameters for assessment of hepatic fibrosis and PDGF for monitoring malignant transformation in CHC.

Keywords Hepatitis C virus (HCV) · Hepatocellular carcinoma (HCC) · Hyaluronic acid (HA) · Platelet-derived growth factor (PDGF) · Connective tissue growth factor (CTGF) · Transforming growth factor-β1 (TGF-β1)

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Introduction

Hepatic fibrosis is a characteristic sequel of chronic liver diseases (CLD) [1]. In hepatitis C virus (HCV) infection, liver injury results in excessive and altered deposition of extracellular matrix (ECM) components into the liver through transition of hepatic stellate cells (HSCs) to myofibroblasts (MFs) [2]. 2–5% of patients with HCV-induced liver cirrhosis develop hepatocellular carcinoma (HCC) [3].

Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan, synthesized by HSCs and degraded by the liver sinusoidal cells in patients with liver disease, particularly cirrhosis [4]. It can specifically facilitate tumor

progression by enhancing invasion, growth and angiogenesis. HA hydrogel is utilized to allow anchorage-independent growth of clusters and colonies of cells or as a milieu to which adhesive motifs are incorporated and enable cell attachment and growth [5]. It can also be used as drug carrier or optical imaging agent for cancer therapy [6]. Connective tissue growth factor (CTGF), a 38 kDa protein, is strongly expressed by HSC during hepatic fibrosis [2]. It is synthesized in hepatocytes where its expression and secretion is strongly dependent on TGF-β1 [7]. It regulates cell adhesion, migration, proliferation, survival and differentiation via integrin and heparin sulfate proteoglycan-mediated adhesive signaling [8]. CTGF was found to be associated with, and released by platelets during the coagulation process. Moreover, thrombocytopenia was also found to be associated with advanced fibrosis and cirrhosis, Therefore, we studied its relationship with platelet derived growth factor (PDGF) which is up-regulated following liver injury [9]. PDGF and transforming growth factor-beta1 (TGF-β1) are the most potent growth factors that greatly contribute to the profibrogenic role of HSCs [10, 11]. PDGF is one of the most reliable markers for assessment of hepatic fibrosis [12]. TGF-β1 favors the transition of HSCs to myofibroblast-like cells [11, 13]. It has been implicated both in the development of liver inflammation and fibrosis and appears to be the major hepatic stellate cell-transformation promoting cytokine of kupffer cells and infiltrating mononuclear cells [14].

The aim of the current study is to assess the possible relationship between potential biomarkers for matrix deposition and viral-mediated hepatic fibrosis caused by HCV genotype 4. Their impact on fibrogenic process and progression to tumorigenesis were also studied.

Subjects and methods

135 subjects, of whom 120 patients with HCV-induced chronic liver disease admitted to Hepato-Gastroenterology Department of Theodor Bilharz Research Institute (TBRI) and 15 age- and sex-matched healthy adults were enrolled in this study. Patients have M/F ratio of 2/1 and mean age of 48.7 ± 7.5 (range 22–60 years). Moreover, the 15 control wedge liver biopsies of individuals underwent laparoscopic cholecystectomy were 10 males and 5 females having a mean age of 45.0 ± 7.5. The study was approved by the Institution's Human Research Ethics Committee. Full medical history, thorough clinical examination, ultrasonography and liver biopsies were conducted using ultrasound-guided percutaneous Menghini-needle.

None of the subjects had joint injuries, based on clinical examination and inflammatory markers; or renal impairment based on normal creatinine clearance.

Laboratory investigations

Liver function tests and serologic diagnosis of viral hepatitis were carried out indicating presence of chronic HCV infection. The cases had reactive anti-HCV antibodies for more than 6 months (using Murex enzyme immunoassay kit, Dartford, England); a positive HCV-RNA by PCR according to Hodinka [15]; absence of other CLD as hepatitis B (defined as negative reaction to HBV surface antigen and HBV core antibody), autoimmune hepatitis (negative reaction to anti-nuclear, anti-smooth muscle, anti-mitochondrial and anti-liver-kidney microsomal antibodies), schistosomiasis *mansoni* (no previous history and negative stool examination); no previous history of regular use of hepato-toxic drugs or alcohol abuse. Patients previously treated for hepatitis C were also excluded.

Serum levels of HA were measured (Corgenix HA Test Kit, Corgenix Inc. Westminster, Colorado 80234 USA) following the manufacturer's instructions with the patients in the fasting conditions and no physical efforts. Each HA level was measured in duplicate (range 1–871 μg/l) and a pool control set was used. All serum samples were obtained on the day of liver biopsy. HA normal values was <50 ng/ml.

PDGF-AB levels were determined by quantitative sandwich enzyme immunoassay technique (Quantikine, R&D System Inc. Mckinley Pleco NE, Minneapolis, MN 55413 USA).

Histopathologic study

The 15 control cases were found to be histopathologically free from any hepatic lesion. Liver biopsies from the studied patients (120) were assessed histopathologically for the grade of inflammation, stage of fibrosis and grade of tumor differentiation. Serial sections (5 μm thick) from formalin-fixed, paraffin-embedded blocks were stained with hematoxylin/eosin and Masson trichrome stains. The stage of hepatic fibrosis in each HCV-infected patient was determined using Ishak scoring scale [16]. Accordingly, cases were categorized apart from healthy controls into: mild fibrosis (Stage 1–2, 30 cases), moderate fibrosis (Stage 3–4, 30 cases), and liver cirrhosis (Stage 5–6, 30 cases). According to Colecchia et al. [17], cases with HCC (30 specimens) are classified into well- and poorly-differentiated tumors.

Immunohistochemistry

The 5-μm thick sections from formalin-fixed, paraffin-embedded blocks were collected on 3-amino propyl triethoxysilane- coated glass slides (Sigma Chemicals; St. Louis, USA) to properly fix tissue sections on the slides

and to minimize staining artifacts. Following deparaffinization, rehydration and endogenous peroxidase inactivation, antigen retrieval was performed by microwaving in 10 mM citrate buffer, pH 6.0 (Dako, Denmark). Non-specific antibody binding was hindered by pre-incubation with 100 µl blocking serum for 30 min at room temperature. Liver sections were incubated overnight, at 4°C, with the primary mouse anti-human monoclonal antibodies for CTGF, PDGF, TGF-β1 (Dako, Santa Cruz Biotechnology, Inc, USA) at 1:100, 1:40, 1:100 dilutions, respectively. After thorough rinsing in PBS, the biotinylated secondary antibody was applied followed by streptavidin peroxidase conjugation. Peroxidase activity was developed using diamino-benzidine as chromogen, and Mayer's hematoxylin as counter stain. Negative controls were stained appropriately and run with each setting. The intensity of expression of CTGF [18] and PDGF [11] in the immunohistochemical assay was graded as (0) negative, (+) mild, (++) moderate or (+++) severe. If the stain was weaker than mild, it was considered (0/+) faint. However, biopsies were classified as altered if more than 50% of the cells showed immunoreactivity to TGF-β1, the cut-off point between the diseased and normal tissue [19].

Statistical analysis

The statistical package for social sciences (SPSS) for windows (version 11) computer program was used for statistical analysis. Means of different groups were compared using one-way ANOVA. Comparison between percent positive cases was calculated by χ^2 test. A p value < 0.05 was considered statistically significant. Pearson correlation coefficient r was used to measure the relationship between two variables.

Results

The HA levels were significantly elevated ($p < 0.01$) in CHC and HCC patients compared to controls. The highest values were mostly encountered among cases with liver cirrhosis (Table 1). HA levels were found to be significantly correlated with the grade of inflammation and stage of fibrosis ($p < 0.01$) in CHC patients. Moreover, it was positively correlated with the grade of tumor differentiation in HCC cases ($r = 0.56$; $p < 0.05$) (Table 4).

Decreased levels of circulating PDGF were detected in CHC and cirrhotic patients compared to controls ($p < 0.01$) (Table 1). A significant inverse correlation was detected between serum PDGF and grade of inflammation ($r = -0.43$; $p < 0.01$) or stage of fibrosis ($r = -0.74$; $p < 0.01$) (Table 4).

CTGF revealed granular and diffuse cytoplasmic immunostaining with perinuclear reinforcement. Mild staining was detected in portal tracts of 33.3% of normal livers with no staining in the hepatic lobules or central veins. CTGF immunostaining was observed in patients with CHC in portal tracts at areas of ductal proliferation, fibrous septa, in sinusoidal cells close to the fibrous septa and areas of interface hepatitis (Fig. 1). No or mild staining was observed in the lobular necro-inflammatory foci. Furthermore, moderate or strong CTGF immunostaining was observed in 40% of cases with no or mild activity and 60% with moderate or severe activity. Although a significant correlation was found between CTGF expression and the stage of fibrosis ($r = 0.88$; $p < 0.01$), no significant correlation was detected between its expression and CHC grade of inflammatory activity or grade of HCC tumor differentiation (Table 4). CTGF expression in CHC was absent in 18 cases, mild in 5 cases, moderate in 17 cases and strong in 20 cases. The intensity of expression was significantly increased with the development of cirrhosis ($p < 0.01$) (Table 2).

Three control cases (20%) showed faint PDGF cytoplasmic immunostaining in few scattered cells. In contrast,

Table 1 Serum level of HA and PDGF in all studied cases

Diagnosis (n)	Serum HA (µg/l) Mean ± SE	Serum PDGF (µg/l) Mean ± SE
Controls (15)	20.66 ± 0.92	263.2 ± 55.09
CHC (60)	216.0 ± 60.77[a]	146.0 ± 48.80[d]
LC/HCV (30)	435.47 ± 44.9[a,b]	104.9 ± 21.07[d]
HCC/HCV (30)	113.81 ± 41.24[a,c]	305.0 ± 65.08

n number of cases, *SE* standard error

[a] Significant increase compared to control cases ($p < 0.01$)

[b] Significant increase compared to CHC ($p < 0.01$)

[c] Significant decrease compared to LC ($p < 0.01$)

[d] Significant decrease compared to control cases ($p < 0.01$)

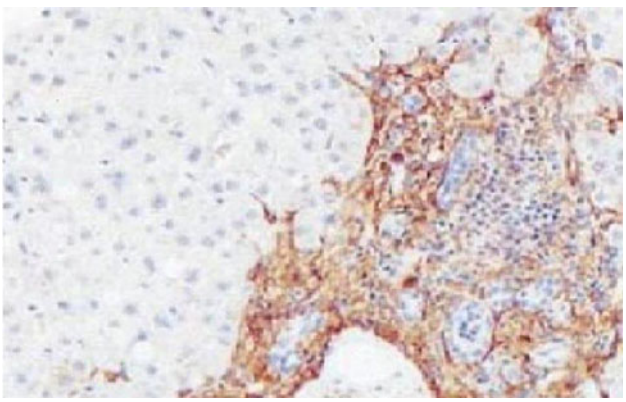

Fig. 1 Strong CTGF expression is detected in the ECM of the portal tract in chronic hepatitis C. Immunostaining for CTGF is observed in the fibrous septa and in the sinusoidal lining at the interface between fibrous tissue and liver parenchyma (Immunostaining ×20)

Table 2 Percent immunohistochemical expression of CTGF in liver tissue of all studied cases

Histopathologic diagnosis (*n*)	Positive expression (%)	Mild (+) *n* (%)	Moderate (++) *n* (%)	Marked (+++) *n* (%)
Control (15)	5 (33.3)	5 (33.3)	0 (0)	0 (0)
CHC (60)	42 (70)[a]	5 (8.3)	17 (28.3)	20 (33.4)
LC/HCV (30)	30 (100)[ab]	0 (0)	5 (16.7)	25 (83.3)[c]
HCC/HCV (30)	25 (83.3)[a]	5 (16.7)	10 (33.3)	10 (33.3)

All positive control cases revealed mild (+) cytoplasmic expression of CTGF. 83% of cirrhotic cases revealed marked staining intensity of CTGF

[a] Significant increase compared to control cases ($p < 0.01$)

[b] Significant increase compared to CHC ($p < 0.05$)

[c] Significant increase compared to CHC & HCC ($p < 0.01$)

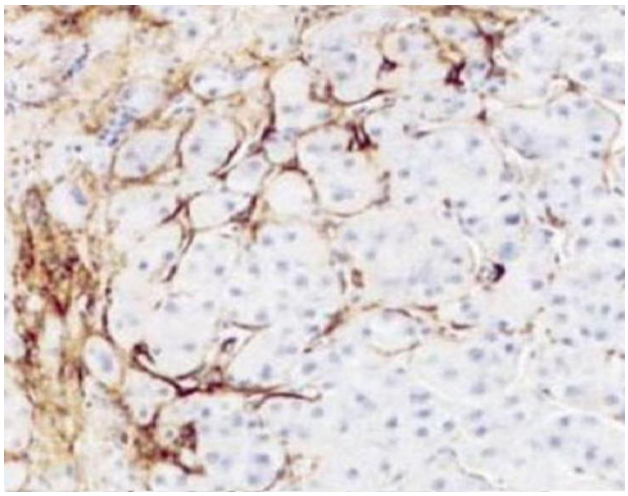

Fig. 2 PDGF expression is detected in the periportal and perisinusoidal cells in chronic hepatitis C (Immunostaining ×20)

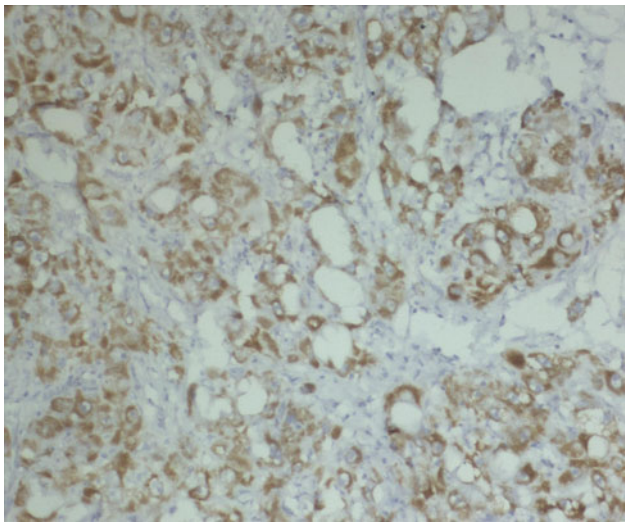

Fig. 3 Strong PDGF expression with malignant transformation in a case of HCC-acinar pattern (Immunostaining ×10)

patients' with CHC revealed positive PDGF cytoplasmic immunostained cells in portal areas. Inside the lobules, the stained cells were seen in regions of focal spotty necrosis. The increased expression paralleled the increase in liver damage. Myofibroblast-like cells which are abundant in the periportal and perisinusoidal areas showed strong positive PDGF immunostaining (Fig. 2). PDGF expression and intensity of immuno-staining matched the advancement of CHC–LC–HCC (Fig. 3; Table 3). Moreover, PDGF expression was found to be strongly correlated ($r = 0.66$; $p < 0.01$) with the grade of tumor differentiation in HCC cases (Table 4).

Transforming growth factor-β1 revealed diffuse granular cytoplasmic staining. The faint expression in less than 50% of cells was considered negative in normal liver sections. Data demonstrated that TGF-β1 was expressed in hepatocytes of CHC patients. Marked staining was detected in 50% of non-cirrhotic compared to 93.3% of cirrhotic cases (Fig. 4). Moreover, TGF-β1 was positively expressed in 66.6% of patients of HCC/HCV that ranked between marked (46.6%) (Fig. 5) and moderate (20%) staining (Table 5).

Discussion

Our findings demonstrate increased levels of HA concentrations in patients with CHC and HCC compared to controls that agree with other series [20–22]. The increase in HA levels parallel the advancement of liver fibrosis suggesting that defective hepatic clearance may play a role in this respect. HA levels were found to be correlated with clinical severity [23]. Accordingly, progressive liver damage can be identified and monitored by serum HA evaluation in patients with CLD [24]. Diagnostic accuracy of serum HA levels increases gradually with the hepatic fibrosis stage and it was found to be better than other simple non-invasive indexes using parameters easily available in routine clinical practice for the diagnosing of cirrhosis [25]. We agree with other study [26] suggesting

Table 3 Percent of immunohistochemical expression of PDGF in liver tissue of all studied cases

Histopathologic diagnosis	Number (%) of positive cases	Intensity of expression		
		Mild (+) *n* (%)	Moderate (++) *n* (%)	Marked (+++) *n* (%)
Control (15)	3 (20)	0	0	0
CHC (60)	40 (66.7)[a]	22 (36.7)	9 (15)	9 (15)
LC/HCV (30)	25 (83.3)[a,b]	4 (13.3)	6 (20)	15 (50)[c]
HCC/HCV (30)	29 (96.7)[ab]	0 (0)	8 (26.7)	21 (70)[cd]

Three control cases revealed faint cytoplasmic expression in few scattered cells

[a] Significant increase compared to control cases $p < 0.01$

[b] Significant increase compared to CHC $p < 0.05$

[c] Significant increase compared to controls ($p < 0.01$)

[d] Significant increased compared to LC ($p < 0.05$)

Table 4 Correlation analysis of all cases studied

Parameters	*r*	*p*
sHA # Stage of fibrosis	0.682	0.01
sHA # Grade of inflammation	0.662	0.01
sHA # Grade of HCC differentiation	0.562	0.05
sPDGF # Stage of fibrosis	−0.735	0.01
sPDGF # Grade of inflammation	−0.434	0.01
CTGF # Stage of fibrosis	0.881	0.01
CTGF # Grade of inflammation	0.232	NS
CTGF # Grade of HCC differentiation	0.234	NS
PDGF # Grade of HCC differentiation	0.661	0.01
TGF-β1 # Stage of fibrosis	0.888	0.01
TGF-β1 # Grade of inflammation	0.332	NS
TGF-β1# Grade of HCC differentiation	0.334	NS
CTGF # TGF-β1	0.735	0.01
CTGF # PDGF	0.678	0.01
PDGF # TGF-β1	0.430	0.05

NS non-significant

means versus

that HA levels can be utilized as a marker for distinguishing hepatic fibrosis.

Increased matrix production is the most direct way by which hepatic fibrosis generates [27]. Clinical studies have demonstrated that CTGF expression is increased in fibrotic human liver and has been linked to TGF-β1 pathways in fibro-proliferative diseases [28]. CTGF; one of 6 members of cysteine-rich secreted, heparin-binding proteins with a modular structure; is recognized as an important player in fibrogenic pathways. It acts as a mediator of fibre–fibre, fibre–matrix and matrix–matrix interactions, and as an enhancer of profibrogenic TGF-β1 with its several secondary effects [29]. Moreover, CTGF is proposed as a fibrogenic master switch for epithelial-mesenchymal transition (EMT) since newly recognized pathogenic mechanisms point to influx of the liver with bone marrow derived

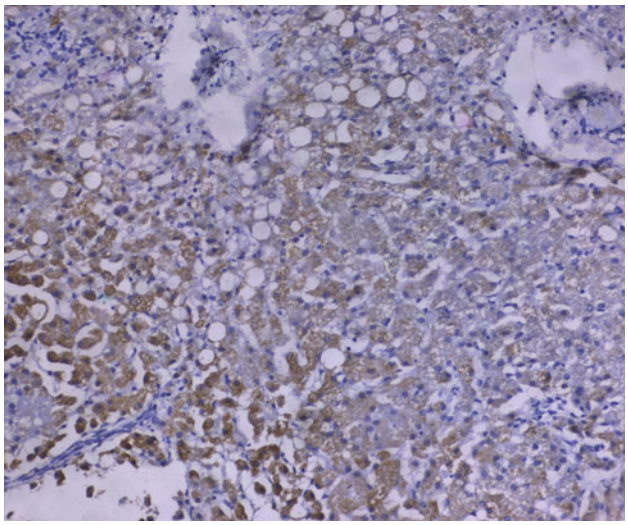

Fig. 4 Strong TGF-β1 cytoplasmic expression in the hepatocytes of CHC (Immunostaining ×10)

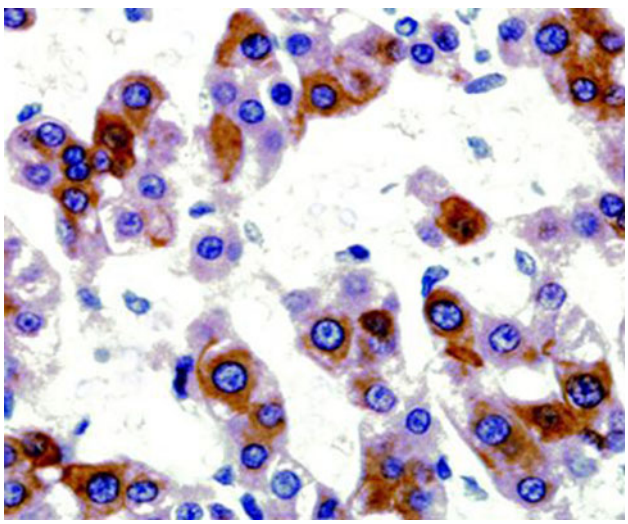

Fig. 5 A case of HCC/HCV showing marked immunoreactivity for TGF-β1 in the hepatocytes with intact intervening stroma (immunoperoxidase staining ×40)

Table 5 Percent immunohistochemical expression of TGF-β1 in liver tissue of all studied cases

Histopathologic diagnosis	Positive expression (%)	Frequency of TGF-β1 expression		
		Mild 50–60%	Moderate 61–80%	Marked >80%
Control (15)*	0 (0)	0 (0)	0 (0)	0 (0)
CHC (60)	60 (100)	3 (5)	27 (45)	30 (50)
LC/HCV (30)	30 (100)	0	2 (6.7)	28 (93.3)[b]
HCC/HCV (30)	20 (66.6)[a]	0	6 (20)	14 (46.6)

All sections from histologically normal livers displayed faint expression of TGF-beta1 in <50% of the cells that considered negative

[a] Significant decrease compared to CHC and LC ($p < 0.05$)

[b] Significant increase compared to CHC and HCC ($p < 0.01$)

cells (fibrocytes), circulating peripheral monocytes and MFs derived from EMT of bile duct epithelial cells [30, 31].

Our data showed a marked simultaneous increase in the expression of both CTGF and TGF-β1 which was strongly correlated with stage of liver fibrosis. This is consistent with the findings of Weng et al. [29]. Immunohistochemical study showed that CTGF was predominantly located in the ECM of portal tracts and fibrous septa. This predominant extracellular localization was expected, since CTGF was initially described as a peptide secreted by vascular endothelial cells in culture [32]. The overall regulation process of fibrogenesis is complex and requires a large number of factors, including cytokines and various growth factors. Although our results provide evidence for the involvement of CTGF in liver fibrogenesis, its role in the multifactorial model of ECM gene regulation remains to be clarified. Since data revealed that patients with chronic HCV showed a strong correlation between TGF-β1 level and severity and progression of fibrosis, we hypothesize that CTGF expression may be up regulated in HSC through endogenous TGF-β1. These cells will then be able to produce and secrete CTGF, which in turn, will accumulate in the surrounding ECM. Via a putative receptor present on the HSC cell membrane, CTGF could act as an autocrine stimulator to increase ECM component expression.

Angiogenic factors may contribute to fibrogenesis by acting as hypoxia-inducible, autocrine and paracrine factors able to recruit myofibroblastic-like cells. Moreover, HSC/MFs, in addition to their established pro-fibrogenic role, may also contribute to neo-angiogenesis during chronic hepatic wound healing [33]. PDGF is known to be among the most potent stellate cell mitogens. Stellate cells display increased PDGF production as well as up-regulation of PDGF receptors during liver injury. Our data also revealed a strong correlation between CTGF and PDGF expression in HCV infection and their significant relation with the stage of fibrosis. This cytokine probably acts on myofibroblast-like mesenchymal cells, and accordingly may be implicated in liver fibrosis in chronic liver disease. Up-regulation of PDGF receptors following liver injury enhances responsiveness to autocrine PDGF, whose expression is, also, increased [34]. Furthermore, transgenic over-expression of PDGF promotes fibrogenesis and its neutralization by antisense technologies inhibits liver scarring in vivo and interference with PDGF is likely to modulate the natural history of liver fibrosis in HCV infection [35]. Moreover, TGF-β activate monocytes, stimulate synthesis of a variety of cytokines (such as interleukin-1β and tumor necrosis factor-α), chemokines (such as monocyte chemo attractant protein-1), and growth factors (including basic fibroblast growth factor and platelet-derived growth factor-BB and increasing integrin expression [36]. Because ECM through an interaction with integrins affects cell structure and function, induces gene expression and stimulate cellular proliferation, it is theoretically possible that the reduced fibrosis may have led to an attenuation of the hepatic injury at a later stage in the disease process and thus to lessening of liver dysfunction. This agreed with Dooly et al. [37]. In liver fibrosis, TGF-β1 secreted by HSCs represents the highest impact on collagen over-production and accumulation [38].

An important role in the process of fibrogenesis is played by reactive oxygen species (ROS). ROS-sensitive cytokines contribute to HSC activation during inflammation through paracrine signals released from immune cells. The activated HSCs become responsive to PDGF and TGF-β1 that facilitates the accumulation of hydrogen peroxide in HSCs. Specifically PDGF induced collagen deposition and liver fibrosis is markedly reduced by treatment with antioxidant drugs. Moreover, TGF-β1 increases ROS production and decrease the concentration of glutathione [39]. In CHC and cirrhosis, chronic inflammation with fibrosis continues by infection with the virus. These pathologic changes cause remodeling of blood vessels and capillarization of sinusoids in the liver. Pathologic change in blood vessels in patients with chronic liver disease, and alteration of angiogenic factors is predicted not only in the tissue but also, in circulating blood [40]. Based on this consideration, we continue to determine the plasma level of PDGF in

CHC patients and study their relation with progression of the liver disease and HCC development. Liver sections from patients with HCC showed increased TGF-β1 expression compared with histologically normal liver, suggesting a possible role of this cytokine in carcinogenesis. This may be attributed to either loss of TGF-β1 tumor-suppressor activity or action of this protein as a tumor-promoting oncogene. While fibroblasts can have tumor suppressing activity the phenotype of the fibroblast changes to a tumor promoting state as carcinogenesis progresses [41, 42]. TGF-β1 has the ability to induce malignant behavior of normal fibroblasts and is the key factor in uncoupling cells from normal growth control to become tumorigenic [43]. The tumor-promoting effect of TGF-β1, enhancement of ECM deposition, degradation, angiogenesis, and conversion of transformed epithelial cells to more invasive mesenchymal phenotype are preserved even when growth inhibitory responses are lost, and this, in turn, may enhance tumor development [44].

Conclusion

The notion of routinely measuring a marker that reflects the function of the sinusoidal endothelial cells rather than the hepatocytes themselves is an exciting concept. HA may be an accurate prognostic parameter for progress to liver cirrhosis and may be clinically useful with high diagnostic accuracy in screening the stage of hepatic fibrosis. CTGF may be used for determination of hepatic fibrosis and especially in patients with chronic HCV infection to provide information on fibrogenic activity. The development of selective antagonists of CTGF may specifically affect the fibrogenesis process. The fibrogenic master cytokines, TGF-β1 and PDGF, may also play a role in hepatic cell damage in HCV infection, which emphasize their usage as prognostic markers for liver cell injury. Moreover, they may further play a crucial role in hepatocarcinogenesis. Regulation of TGF-β1 activation and modulation of its signal transduction pathway may prevent the progression of hepatic damage and development of HCC in chronic HCV infection. The combined expression of these factors in HCV-related HCC suggests their synergistic action in the pathophysiology of hepatocarcinogenesis.

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