



Role of Circulating Hematopoietic Fibrocytes in Chronic Hepatitis C Patients Induced Liver Fibrosis

Nora El-Bassiouni¹, Noha Amin^{1*}, S. H. Rizk², M. K. Abo El Azayem², Mona Madkour¹, Hasan Gareem³, Raafat Ibrahim⁴, Ola Abo El Nil¹

¹Department of Haematology, Theodor Bilharz Research Institute, Imbaba, Giza, Egypt; ²Department of Clinical and Chemical Pathology, Cairo University, Cairo, Egypt; ³Department of Hepatology and Gastroenterology, Cairo University, Cairo, Egypt; ⁴Department of Hepatology and Gastroenterology, Theodor Bilharz Research Institute, Imbaba, Giza, Egypt

Abstract

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*Correspondence: Noha Amin, Department of Haematology, Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. E-mail: nesmaea@gmail.com

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BACKGROUND: Bone marrow-derived fibrocytes may play an important role in pathogenesis and resolution of liver fibrosis.

AIM: The aim is to define the circulating fibrocytes proportion with hematopoietic progenitor origin and to assess their possible contributing role in the progression of liver fibrosis.

SUBJECTS AND METHODS: Sixty chronic hepatitis C (CHC) patients were classified according to METAVIR score into 4 stages of liver fibrosis, 15 age- and sex-matched controls were included. Patients with clinical and laboratory evidence of CHC were included in this study. Patients with parasitological, serological, or ultrasonographic findings other than chronic liver disease were excluded from this study. Flowcytometric analysis for circulating levels of fibrocytes was carried out using anti-CD34, CD45, collagen Type I, and CXCR4. Granulocyte macrophage colony-stimulating factor (GM-CSF), transforming growth factor- β (TGF- β), and alpha-smooth muscle actin (α -SMA) were assessed using enzyme-linked immunosorbent assay.

RESULTS: A significant increase in the circulating levels of GM-CSF, TGF- β , and α -SMA, with a significant increase in the percentage of cells express CXCR4 and in cells that coexpress CD34, CD45, and collagen Type I in different groups compared to control, denoting an increased proportion of circulating fibrocytes in peripheral blood of these patients in conjunction with worsening severity of liver disease.

CONCLUSIONS: Liver fibrosis is associated with increased levels of circulating TGF- β 1 and lipopolysaccharide lead to activation of myofibroblasts, and extensive deposition of extracellular matrix, mostly collagen Type I. Increased expression of CXCR4 trigger fibrocyte recruitment to the injured liver, promoting their differentiation into collagen Type I and producing myofibroblast, support that fibrocytes may become a novel target for anti-fibrotic therapy.

Introduction

Hepatitis C is considered the most common cause of chronic hepatitis in Egypt [1]. Inflammation is a crucial physiological event that occurs during chronic hepatitis C virus (HCV) infection. Chronic inflammation is defined by the persistence of inflammatory cells and destruction of liver cells. The liver cells have a unique regenerative capacity and can replace a significant loss of liver cells by compensatory proliferation. However, the chronic liver damage and regeneration results in scarring of liver called liver fibrosis [2]. Hepatic fibrosis is characterized by abnormal excessive accumulation of extracellular matrix (ECM) accompanied by exaggerated cytokine release. Activated myofibroblasts are the most common cell type that produces the excessive ECM. Newly recognized pathogenic mechanisms point to influx of the liver with bone marrow (BM)-derived cells, fibrocytes, circulating peripheral monocytes and myofibroblasts derived from epithelial mesenchymal

transition of hepatocytes and bile duct epithelial cells, all three complementary mechanisms enlarge pool of matrix synthesizing myofibroblasts [3], [4], [5]. BM cells may play an important role in pathogenesis and resolution of liver fibrosis. BM cells contribute to the inflammatory response by activation of liver resident myofibroblasts. Moreover, BM itself can serve as a source of collagen expressing cells, for example, BM-derived fibrocytes and mesenchymal progenitors, which in turn have a potential to in situ differentiation into fibrogenic myofibroblasts and facilitate fibrosis [6]. Fibrocytes are circulating progenitor cells (CD34 positive) of hematopoietic origin (CD45 positive) capable of differentiating into diverse mesenchymal cell types. In addition to CD34 and CD45 fibrocytes can be positively expressed Type I and IV collagen and the CXCR4 chemokine. The determination of the colony stimulating factors (CSF), macrophage CSF (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and transforming growth factor- β (TGF- β) which are increasingly expressed in fibrotic liver tissue and elevated in serum, are possibly involved

in the mobilization of fibrocytes from the BM and their homing in the liver during fibrogenesis [4]. Furthermore, mechanical force generation by myofibroblasts, which in turn depends on the neo expression of alpha-smooth muscle actin (α -SMA) in stress fibers of these cells, regulates essential phenomena for tissue remodeling, such as cytokine synthesis and ECM component production [7], oxidative stress, resulting from hepatic and mitochondrial injury with increased lipid metabolism, contributes to the initiation of fibrosis with hepatic stellate cells (HSCs) activation and expression of α -SMA in chronic hepatitis C (CHC). That is why the presence of α -SMA positive HSCs in stage 0 fibrosis suggests that stellate cells are activated early in HCV mediated injury, possibly in response to oxidative stress resulting from inflammation and lipid metabolism [8].

We aimed to define the possible contributing role of the circulating fibrocytes with hematopoietic progenitor origin as defined by CD34⁺ CD45⁺ in patients with HCV induced chronic liver disease (CLD).

Patients and Methods

This study was conducted on patients admitted to the Gastroenterology and Hepatology Department and its outpatient's clinic Theodor Bilharz Research Institute (TBRI), Imbaba, Giza. Seventy-five individuals were the subjects of this study. Sixty patients suffering from CHC-related liver fibrosis were selected and then classified into 4 groups according to the stage of liver fibrosis after METAVIR score. Fifteen healthy age- and sex-matched individuals were also selected from medical and paramedical healthy personals and served as a control group. Patients with clinical and laboratory evidences of chronic HCV was included in this study.

Patients were excluded from this study if they had parasitological, serological, or ultrasonographic findings indicative of other etiologies of CLD.

The medical procedures of this study were approved by the Local Ethics Committee of TBRI, Imbaba, Giza. Participation in the study was fully voluntary and anonymous, and written informed consent was obtained from each person prior to the medical examination. Patients were subjected to thorough clinical examination, laboratory investigations including urine and stool analysis, liver function tests, complete blood count, and serologic diagnosis of schistosomiasis and hepatitis markers and abdominal ultrasonography.

Liver function tests were carried out using commercially available kits (ELITechGroup, USA). Hepatitis B markers, including Hepatitis B surface antigen and anti-HBs antibodies, total and IgM class antibodies against Hepatitis B core antigen, hepatitis B e antigen, and anti-HBe antibodies, were tested

using commercially available enzyme immunoassay kits (Abbott Laboratories; North Chicago, Illinois). Circulating anti-HCV antibodies were detected using the Murex enzyme immunoassay kit (Murex anti-HCV, Version V; Murex Diagnostics; Dartford, England). The presence of HCV-RNA in the patient's sera was detected by real-time polymerase chain reaction using the Amplicor test (Roche Diagnostic Systems; Meylan, France). Liver function tests of the normal controls were within the normal range and had no serologic evidence of Hepatitis B and/or C viruses. Patients with clinical and laboratory evidences of chronic HCV was included in this study. Patients were excluded from the study if they had parasitological, serological, histopathological, or ultrasonographic findings indicative of other etiologies of CLD, such as: chronic viral diseases other than HCV, nonalcoholic steatohepatitis (NASH), autoimmune hepatitis, biliary disorders, malignancies, bacterial infection, *Schistosoma* infection, or dual chronic hepatitis Band C infection.

Flow cytometric analysis

The assessment of percentage of circulating BM-derived fibrocytes of hematopoietic origin in different groups studied was carried out by immunophenotype characterization by flow cytometric (EPICS[®] XL-MCL, Brea, CA, USA) analysis using anti- D45/PE, anti-CD34/FITC (Beckman Coulter, Marseille, France) and purified Type I collagen (1ry antibody) and goat anti-mouse/PerCP (2ry antibody) (Bio-Rad, Germany) as follows: 10 μ l of the appropriate mAb (anti-CD45/PE, - anti-CD34/FITC) was added to 100 μ l of fresh blood with ethylenediaminetetraacetic acid (EDTA) and mixed gently then incubated for 20 min, in the dark, at room temperature. 25 μ l of PerFix-nc Buffer 1 (Fixative Reagent) (Beckman Coulter, Marseille, France) was added and immediately mixed gently and similarly incubated for 30 min. 300 μ l of PerFix-nc Buffer 2 (Permeabilization Reagent) (Beckman Coulter, Marseille, France) was added. 10 μ l of primary mAb for collagen Type I was added and incubated for 30 min. After incubation, 10 μ l of goat anti-mouse MAb (2ry antibody)/PerCP was added for 30 min. Finally, 3 ml of pre-diluted PerFix-nc Buffer 3 (Final 10x Reagent) (Beckman Coulter, Marseille, France) was added and mixed gently. The surface expression of CD34, CD45, and intracellular protein collagen Type I of peripheral blood fibrocytes was detected on gated CD45⁺ lymphocytes cells by flow cytometric analysis. Prism is used to analyze multicolor immunofluorescence samples. Using a multicolor immunofluorescence analysis, a cell is either positive or negative for each of 2 or 3 cell surface markers. A particular combination is called a phenotype. The Prism parameter allows displaying the percentages of all the phenotypic populations in a single histogram. The identification of the percentage of fibrocyte that positively express chemokine receptor (CXCR4) was performed using

rabbit anti-human polyclonal antibodies CXCR4/PE (Beckman Coulter, France) as follows: 50 μ l of fresh blood with EDTA were dispensed, 10 μ l of the appropriate polyclonal antibody (anti-CXCR4/PE) were added and incubated for 20 min, in the dark. 500 μ l of red blood cells (RBCs) lysis buffer (eBioscience, San Diego, CA, USA) were added to eliminate RBCs and were mixed gently then incubated for 10 min. The sample tubes were then introduced and processed, the surface expression of CXCR4⁺ cells was detected on gated lymphocyte.

Enzyme-linked immunosorbent assay (ELISA)

Quantitative determination of serum levels of TGF- β (DRG Diagnostics, Marburg, Germany), Granulocyte M-CSF (GM-CSF) (eBioscience, San Diego, CA, USA) and α -SMA (CUSABIO, BIOTECH Co., Ltd., P.R.C) was conducted using ELISA to identify myofibroblasts activation. The procedures were performed according to manufacturer's instructions.

Statistical analysis

The data were analyzed using statistical package SPSS version 18.0 for windows (SPSS Inc., Chicago, IL, USA). Diagnostic parameters of subjects were compared using the independent sample (*t*) test. A *p* < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curve analysis was performed on the ELISA results to determine the optimal cutoff point (at which the sum of the sensitivity and specificity values is highest) for distinguishing between positive and negative results. The area under the ROC curve (AUC), which can be used as a measure of the accuracy of the test, was also calculated.

Results

The surface expression of CD34, CD45, and detection of intracellular protein collagen Type I of circulating blood fibrocyte of hematopoietic origin were detected on gated CD45⁺ cells. Different combinations of percentage of circulating BM-derived fibrocytes of hematopoietic origin surface markers and intracellular

protein were studied in healthy controls and patients with CHC with different stage of fibrosis.

The proportion CD34⁺ fibrocytes demonstrated a statistically significant increase in different groups studied. Patients with stage F1 (*p* > 0.01), F2 (*p* < 0.05), F3, F4 (*p* < 0.01) fibrosis showed a marked increase in the percentage of CD34⁺ fibrocytes compared to healthy subjects. A marked progressive increase in the percentage of CD34⁺ fibrocytes was also detected in patients with stage F3 (*p* < 0.01) fibrosis compared to those with stage F1 fibrosis moreover, the marked increase in their percentage was mostly noticed among patients with stage F4 fibrosis (*p* > 0.01) (Table 1).

The results of this study showed a significant increase in the proportion of collagen Type I⁺ fibrocytes in different groups studied, it was found in the groups of patients with stage F1 (*p* < 0.05), F2, F3, and F4 (*p* < 0.01) fibrosis compared to healthy subjects. Data also revealed a marked progressive increase in the proportion of collagen Type I⁺ fibrocytes in the diseased groups which parallel the severity of the disease. This up-regulation (*p* < 0.01) was noticed in patients with stage F2 and F3 fibrosis compared to patients with stage F1 fibrosis and the highest percentage (*p* < 0.01) was mostly encountered among patients with stage 4 fibrosis (Table 1).

The percentage of fibrocytes that co-express CD34 and collagen Type I showed a marked increase in patients with stage F1 (*p* < 0.01), F2, F3 (*p* < 0.05) and F4 (*p* < 0.01) fibrosis compared to healthy controls. Moreover, a marked progressive increase in the percentage of CD34⁺ and collagen Type I⁺ fibrocytes was also detected in patients with stage F2 (*p* < 0.01) and F3 (*p* < 0.05) fibrosis compared to those with stage F1 fibrosis. The marked increase (*p* < 0.01) was mostly found among patients with stage F4 fibrosis (Table 1).

CXCR4⁺ peripheral blood fibrocytes were markedly increase (*p* < 0.01) in patients with stage F1, F2, F3, and F4 compared to healthy controls. Data also revealed a marked progressive increase in the percentage of CXCR4⁺ lymphocytes in patients with stage F2 and F3 (*p* < 0.01) fibrosis compared to those with stage F1 fibrosis and in patients with stage F3 (*p* < 0.05) fibrosis compared to patients with stage F2 fibrosis. The increase in their percentage was mostly encountered among patients with stage F4 fibrosis compared to those with stage F1, F2 (*p* < 0.01), and F3 (*p* < 0.05) fibrosis (Table 1).

In this study, the serum levels of α -SMA, GM-CSF, and TGF- β revealed marked progressive

Table 1: Flow cytometric analysis of surface expression of CXCR4⁺, CD34⁺Cells and CD34⁺COLI⁺Cells on peripheral blood lymphocytes of patients with HCV induced chronic liver disease and healthy subjects

Groups	Controls (n = 15)	F1 fibrosis (n = 15)	F2 fibrosis (n = 15)	F3 fibrosis (n = 15)	F4 fibrosis (n = 15)
CXCR4	39.02 \pm 14.25	49.50 \pm 6.95 ^b	57.60 \pm 7.02 ^{bd}	63.10 \pm 9.32 ^{bde}	71.70 \pm 11.36 ^{bdfg}
CD34 ⁺ Cells	2.69 \pm 1.70	5.00 \pm 1.80 ^b	10.40 \pm 3.40 ^{bd}	12.21 \pm 2.60 ^{bc}	21.90 \pm 3.90 ^{bdfh}
COL I ⁺ Cells	2.86 \pm 2.04	5.40 \pm 1.99 ^a	11.30 \pm 3.50 ^{bd}	13.40 \pm 2.50 ^{bc}	22.40 \pm 3.9 ^{bdfh}
CD43 ⁺ COLI ⁺ Cells	4.80 \pm 2.70	10.60 \pm 3.90 ^b	12.90 \pm 3.30 ^a	14.80 \pm 4.70 ^{bd}	24.90 \pm 3.90 ^{bdfh}

Data are represented as mean \pm SD. ^a*p* < 0.05, ^b*p* < 0.01: Controls versus other groups, ^c*p* < 0.05, ^d*p* < 0.01: Stage F1 versus other groups, ^e*p* < 0.05, ^f*p* < 0.01: Stage F2 versus other groups, ^g*p* < 0.05, ^h*p* < 0.01: Stage F3 versus other groups. SD: Standard deviation.

increases, which parallel the severity of the disease (Table 2). A statistically significant increase in the circulating levels of α -SMA, GM-CSF, and TGF- β was detected in patients with stage F2, F3 ($p < 0.05$) fibrosis, and F4 ($p < 0.01$) fibrosis compared to healthy controls. Although an increase in α -SMA level was also noticed in patients with stage F1 fibrosis compared to controls, the results were comparable ($p > 0.05$). A statistically significant increase ($p < 0.05$) was noticed in stage F2 and F3 fibrosis compared to a patient with stage F1 fibrosis and in patients with stage F3 fibrosis ($p < 0.01$) compared to those with stage F2 fibrosis. The marked progressive increase ($p < 0.01$) in these markers levels were mostly encountered among patients with stage F4 fibrosis. Moreover, these marked progressions were found to match the progression of the disease (Table 2).

By means of the ROC curves, the accuracy of GM-CSF and TGF- β ELISA and α -SMA tests were evaluated, and we found that the sensitivity, specificity, and AUC of the GM-CSF ELISA test were (60%, 66.7%, and 0.57) for Stage 1, (53%, 100% and 0.64) for Stage 2, (100%, 93% and 0.97) for Stage 3 and (66.7%, 100% and 0.87) for Stage 4 of the liver fibrosis, as well as the specificity, sensitivity, and AUC of the TGF- β ELISA test was (100%, 100% and 1) for Stage1, (80%, 100% and 0.95) for Stage 2, (66.7%, 93.3%, and 0.9) for Stage 3 and (100%, 93%, and 1%) in the liver fibrosis Stages 4 (Table 3 and Figure 1).

Moreover, the specificity, sensitivity, and AUC of the α -SMA ELISA test were 80%, 60%, and 0.75% for Stage 1; 100%, 73.3%, and 0.87% for Stage 2; 80%, 66.7%, and 0.85% for Stage 3; and 80%, 60%, and 0.91% in the liver fibrosis Stage 4 (Table 3 and Figure 1).

Discussion

Fibrocytes are primarily located in the BM, where they comprise a small subset (0.1%) of mononuclear cells which proliferate and transmigrate within the bloodstream in response to injury [9]. Under physiological conditions simply a proliferative stimulus can regulate the functional activity of these cells.

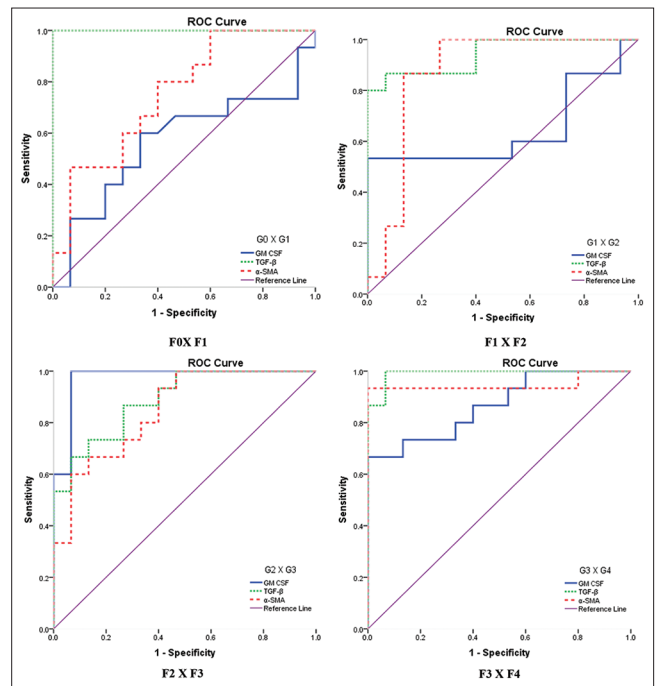


Figure 1: ROC Curves of the studied biomarkers in all studied groups.

In the current work, immunophenotypic characterization of CD34 on CD45 positive peripheral blood gated lymphocytes revealed an increase in its expression in different groups of patients with HCV-induced CLD compared to normal subjects. These results are in accordance with those of Di Carlo *et al.* [10] who found that liver sinusoids affected by different degrees of chronic active hepatitis showed absence or focal immunostaining for CD34. The authors noticed that the immunoreactivity, however, increase in the periportal sinusoids of the cirrhotic nodules, and, therefore concluded that the positive immunoreactivity for CD34 represents an effective method to evaluate angiogenesis. The increased expression of CD34⁺ on CD45⁺ fibrocytes match the severity of the disease, and the stage of liver fibrosis, the highest levels of CD34⁺ on CD45⁺ fibrocytes were mostly detected among patients with stages F3 and F4 liver fibrosis. A similar increase in the expression of CD34 positive sinusoidal endothelial cells was reported in patients with HCC-, HBV-, and HCV-associated CLD [11]. The authors suggested that the increase in the expression of CD34 may be a risk factor for HCC in patients with HCV-associated CLD. In the present study, we found a marked increase in the percentage of fibrocytes that co-expression of CD34, CD45, and collagen Type I in different groups of patients with HCV-induced CLD compared to controls with marked progressive increase in their percentage found to be parallel the advancement of the disease and

Table 2: Circulating levels of granulocyte macrophage colony stimulating factor (pg/ml), transforming growth factor- β (pg/ml) and of alpha-smooth muscle actin (ng/ml) in patients with hepatitis C virus induced chronic liver disease and healthy subjects

Groups	F4 fibrosis (n = 15)	F3 fibrosis (n = 15)	F2 fibrosis (n = 15)	F1 fibrosis (n = 15)	Controls (n = 15)
GM-CSF (pg/ml)	3.48 ± 0.41	3.58 ± 0.49	8.07 ± 9.8 ^{bd}	33.34 ± 13.00 ^{bd}	91.79 ± 28.90 ^{bdg}
TGF- β (pg/ml)	2.59 ± 1.10	10.63 ± 3.96 ^{bd}	22.68 ± 6.66 ^{bc}	32.80 ± 5.34 ^{bd}	61.86 ± 12.37 ^{bdgh}
α -SMA (ng/ml)	2.11 ± 1.10	3.00 ± 1.70	5.00 ± 0.40 ^{bc}	7.70 ± 1.60 ^{bd}	17.00 ± 5.00 ^{bdh}

Data are represented as mean ± SD. ^a $p < 0.05$, ^b $p < 0.01$: Controls versus other groups. ^c $p < 0.05$, ^d $p < 0.01$: Stage F1 versus other groups. ^e $p < 0.05$, ^f $p < 0.01$: Stage F2 versus other groups. ^g $p < 0.05$, ^h $p < 0.01$: Stage F3 versus other groups. GM-CSF: Granulocyte macrophage colony stimulating factor, TGF- β : Transforming growth factor- β , α -SMA: Alpha-smooth muscle actin, SD: Standard deviation.

Table 3: Diagnostic performance of the studied markers

Parameters	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	95% CI		x
								Lower	Upper	
F0×F1										
GM CSF	3.5	60.0	66.7	64.3	62.5	63.3	56.7	35.0	78.3	0.5
TGF β	4.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	< 0.001
α-SMA	0.1896	80.0	60.0	83.0	57.0	71.0	75.1	57.6	92.6	0.02*
F1×F2										
GM CSF	4.2	53.3	100.0	100.0	68.2	76.7	64.4	42.6	86.3	0.2
TGF β	16.1	80.0	100.0	100.0	83.3	90.0	94.2	86.6	100.0	< 0.001**
α-SMA	0.2963	100.0	73.3	100.0	71.5	82.0	87.1	72.3	100.0	0.001**
F2×F3										
GM CSF	11.6	100.0	93.3	93.8	100.0	96.7	97.3	92.1	100.0	< 0.001**
TGF β	29.7	66.7	93.3	90.9	73.7	80.0	88.9	78.1	99.6	< 0.001**
α-SMA	0.6067	80.0	66.7	77.5	64.5	82.0	84.9	71.4	98.4	0.001**
F3×F4										
GM CSF	83.0	66.7	100.0	100.0	75.0	83.3	86.7	74.1	99.2	< 0.001**
TGF β	38.0	100.0	93.3	93.8	100.0	96.7	99.1	97.4	100.0	< 0.001**
α-SMA	0.1896	80.0	60.0	78.0	57.0	86.5	91.0	87.5	100.0	0.001**

*p < 0.05 is significant, **p < 0.01 is highly significant. AUC: Area under curve, CI: Confidence interval, NPV: Negative predictive value, PPV: Positive predictive value, GM-CSF: Granulocyte macrophage colony stimulating factor, TGF-β: Transforming growth factor-β, α-SMA: Alpha-smooth muscle actin.

the progress of liver fibrosis. The role of fibrocytes in the pathogenesis of fibrosis was recently suggested by a study in which fibrocyte precursors that were identified by co expression of CD34, CXCR4, and collagen I found with increased frequency in the blood and ocular lesions of patients with thyroid associated ophthalmopathy. These cells were similar to orbital fibroblasts in that they expressed the thyroid-stimulating hormone receptor, which is a potential target of autoimmunity in this disease [12]. Thus, the contribution of fibrocytes to Graves' disease seems to be similar to that of both fibroblasts and macrophages, in terms of their ECM-producing and pro-inflammatory properties, respectively. Several studies revealed that high numbers of fibrocytes were detected in the lungs of patients with pulmonary fibrosis, the intensity of which correlate with the severity of the disease [13], [14], [15], [16]. The increased percentage of circulating fibrocytes in peripheral blood of these patients implicate that circulating fibrocytes may serve as a biomarker of pulmonary fibrosis progression. Similar findings were reported in patients with kidney fibrosis [17], bronchial asthma, burns [18], and Crohn's disease [19]. The percentage of peripheral blood fibrocytes was also found to be positively correlated with both the METAVIR score and the liver stiffness as measured by Fibroscan, denoting that the proportion of peripheral blood fibrocytes are increased in patients with HCV infection and correlate with the histological stage of liver fibrosis [20]. It was found that myofibroblasts have become an attractive target for anti-fibrotic therapy aimed to eliminate the source of activated hepatic myofibroblasts [21], [22]. Moreover, fibrocytes was also implicated as a novel target for anti-fibrotic therapy [23].

In the present study, immunophenotypic characterization of CXCR4 on peripheral blood gated lymphocytes revealed an increase in its expression in different groups of patients compared to controls. These results are in agreement with Hong *et al.* [24] who found an increased expression of CXCR4 on peripheral blood in patients with HCV cirrhosis. Data also revealed a marked increase in percentage of CXCR4⁺ cells in peripheral blood, which was mostly noticed in patients with stages F3 and F4 liver fibrosis, indicating that the progressive increase in CXCR4 expression on

peripheral blood lymphocytes parallels the progress of liver disease. These findings confirm those of others [24] who demonstrated that the expression of CXCR4 is increased in patients with HCV cirrhosis denoting that its expression increases with stellate cell activation. In addition, an increase in CXCR4 levels in the lungs and plasma of patients with pulmonary fibrosis was found demonstrating that the levels of CXCR4 correlated with the concentrations of circulating fibrocytes [14], [25]. Moreover, data revealed that the CXCR4⁺ cells were positively correlated ($r = 0.358$, $p < 0.01$) with that of BM-derived CD45⁺ CD34⁺ collagen Type I⁺ fibrocytes. These results are in accordance with those of others [26], [27] who found that hepatic fibrocytes express and further upregulate CXCR4 and other chemokine receptor in response to injury, indicating that these receptors may have an important impact on regulation of fibrocyte expansion in the BM, egress from the BM, and trafficking and extravagation from the blood stream into the fibrotic liver.

In the present work, a marked increase in GM-CSF levels was noticed in different groups of patients with HCV-induced CLD compared to controls. These results agree with those of Tan-Garcia *et al.* [28] who found that, a marked increase in GM-CSF levels was noticed in different groups of patients with HCV-induced CLD compared to controls. Therefore, circulating GM-CSF levels may correlate with the degree of liver fibrosis and systemic inflammation in CLD of different etiologies including HCV infection and NASH. Moreover, our findings are in agreement with Al-Barzinji and Ahmed [29] who demonstrated significant increase in GM-CSF levels in patients in the acute phase of hepatitis compared to those with chronic hepatitis and control subjects. Our findings also revealed a progressive increase in GM-CSF levels which match the severity of the disease and the stage of liver fibrosis. These results coincide with those of Kubota *et al.* [30] and Gressner *et al.* [4] who found that GM-CSF concentrations are increasingly expressed in fibrotic liver tissue and are elevated in serum implicating a role for GM-CSF in the mobilization of fibrocytes from the BM and their homing in the liver during fibrogenesis.

Data also showed a marked elevation in TGF- β levels in different groups of patients with HCV-induced CLD compared to healthy subjects which parallel the advancement of the disease and the stage of liver fibrosis. The increase in TGF- β levels was mostly encountered among patients with stage 4 fibrosis. Similarly, plasma and liver TGF- β were found to be elevated in patients with CHC [2]. The development of liver fibrosis was also found to be strongly associated with elevated levels of TGF- β [31]. Our findings also revealed that the levels of TGF- β are strongly correlated ($r = 0.901$, $p < 0.01$) with the percentage of BM-derived CD45⁺ CD34⁺ and collagen Type I⁺ fibrocytes in patients with different stages of liver fibrosis. Our results are in accordance with those of Scholten *et al.* [32] who proved that TGF- β induces differentiation of BM collagen Type I⁺ and CD45⁺ cells in blood, they also demonstrated that TGF- β and lipopolysaccharide (LPS) induce migration of fibrocytes *in vivo* and in culture, implicated that accelerated fibrosis correlate with increased recruitment of fibrocytes to fibrotic liver. Under physiological conditions TGF- β 1 regulates tissues remodeling and apoptosis to maintain cellular homeostasis [33]. Under pathological conditions, TGF- β 1 is the strongest known inducer of fibrosis [34]. In response to injury, TGF- β 1 orchestrates a cross talk between parenchymal, inflammatory, and myofibroblast cells. Signaling by the cytokine TGF- β 1 plays a pivotal role in growth and differentiation, maintenance of liver homeostasis, terminal differentiation of hepatocytes, other epithelial cells and cytokine-mediated mitogenic signaling [35], [36].

A marked increase in α -SMA levels was noticed in different groups of patients with HCV-induced CLD compared to controls. Our results confirm those of others [37] who found that α -SMA levels increase in relevance to the fibrotic stages of liver disease. Data also demonstrated that the marked increase in α -SMA levels matched the advancement of the disease and the stage of liver fibrosis. The highest values were mostly encountered among patients with F3 and F4 stages of liver fibrosis. Our findings are in accordance with others studies [38], [39] who found that the percentage of α -SMA⁺ HSCs was significantly higher in HBV and HCV cirrhosis groups and demonstrated that HSCs activation to fibrosis was positively correlated to both the percentage of α -SMA⁺ HSCs and the extent of fibrosis. Moreover, a highly significant correlation ($r = 0.790$, $p < 0.01$) was noticed with in the current study between the levels of α -SMA and the percentage of circulating BM-derived fibrocytes of hematopoietic origin. Mechanical force generation by myofibroblasts, which in turn depends on the neo expression of α -SMA in stress fibers of these cells, regulates essential phenomena for tissue remodeling, such as cytokine synthesis and ECM component production [7]. Oxidative stress, resulting from hepatic and mitochondrial injury with increased lipid metabolism, contributes to the

initiation of fibrosis with HSC activation and expression of α -SMA in CHC [8]. That is why the correlation of α -SMA levels with the percentage of circulating BM fibrocyte of hematopoietic origin in different stages of liver fibrosis suggests that the circulating BM fibrocytes are activated early in HCV-mediated injury, possibly in response to oxidative stress resulting from inflammation and lipid metabolism. Moreover, high levels of α -SMA may be used, not only as an early marker of hepatic fibrogenesis necessary to begin a treatment before the process become irreversible, but also as a marker for monitoring the efficacy of therapy.

Conclusion

Liver fibrosis is associated with increased levels of circulating TGF- β 1 and LPS, activation of myofibroblasts, and extensive deposition of ECM, mostly collagen Type I. TGF- β and CXCR4 play a critical role in fibrogenesis and trigger fibrocyte recruitment to the injured liver promoting their differentiation into collagen Type I producing myofibroblast, supporting that fibrocytes may become a novel target for anti-fibrotic therapy.

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