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Circulating fibrocytes as a marker of liver fibrosis in chronic hepatitis C

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1. ABSTRACT

Surrogate markers of liver fibrosis are needed as an alternative to liver biopsy, which is invasive and life-threatening. Peripheral blood fibrocytes (PBF) are considered to be involved in systemic fibrogenic processes. We measured the level of PBF in patients with chronic hepatitis C by enrolling 70 patients affected with chronic hepatitis C, 20 patients with HCV-positive decompensated cirrhosis and 30 healthy volunteers. All patients underwent liver biopsy and Fibroscan for fibrosis assessment. Patients with chronic hepatitis C had significantly higher levels of PBF in comparison with healthy individuals and decompensated cirrhotics. Patients in the F0-F1 stage had a percentage of PBF of 23.3±4 %, significantly lower ($p<0.001$) than in F2 and F3 stages. Patients in the F4 stage had a PBF rate of 50.6±2% ($p<0.001$ versus the F0, F1 and F2 stages). The percentage of PBF correlated positively with the Metavir score and the liver stiffness as measured by Fibroscan. PBF are increased in patients with HCV infection and correlate with the histological stage of liver fibrosis.

2. INTRODUCTION

Hepatitis C virus (HCV) infection often leads to chronic viral hepatitis, frequently progressing to cirrhosis and hepatocellular carcinoma (1-3). Liver fibrosis is now thought to be a potentially reversible phenomenon that needs to be monitored and quantitated. Liver biopsy followed by histology is the gold standard technique to determine the stage of liver fibrosis. Of importance, liver biopsy not only is subject to sampling error and inter-observer variability but, since it is an invasive technique in its nature, it may lead to complications such as pain and bleeding requiring hospital admission in 1% to 5% of patients with a mortality rate of 1 to 1000 to 1 to 10,000 (1-3).

Several alternative indirect methods have been investigated to predict the stage of liver fibrosis. Fibrotest and Fibrosure, the Forns' index, the Apri test, the Hepascore and the Fib-4 are all indirect markers of liver fibrosis used in clinical practice. Transient elastography (Fibroscan), a technique based on a low frequency vibration transmitted to the liver, is able to measure liver stiffness in kilopascal units. It was found particularly

Table 1. Baseline patients' parameters (± SD)

Total number of patients (n=)	70
Males (n=)	40
Females (n=)	30
Age (years)	52.5±7
BMI	26.0±6
Duration of HCV infection (years)	12.4±6
HCV genotype 1b (n=)	50
HCV genotype 2 (n=)	10
HCV genotype 3 (n=)	10
HCV RNA (x 10^6 UI/ml)	2.8±1.1
ALT (IU/ml)	97.4±30
Bilirubine (mg/dl)	1.8±1
F0-1 by Metavir (n=)	20
F2 by Metavir (n=)	20
F3 by Metavir (n=)	20
F4 by Metavir (n=)	10
Liver stiffness by Fibroscan (kilopascal units)	7,12±6

accurate to detect either early or advanced stages of fibrosis in chronic hepatitis C (1).

Liver fibrosis is mainly characterized by the excess deposition of extracellular matrix (ECM) which is also altered in structure and profile. Extracellular matrix is composed of different types of collagens, glycoproteins, proteoglycans and hyaluronic acid (HA) (a glycosaminoglycan synthesized by the hepatic stellate cells), procollagen type III aminoterminal peptide (PIIINP), type I and type IV collagens, laminin, matrix metalloproteinases (MMPs) and tissue inhibitors matrix metalloproteinases (TIMPs), YKL-40 or chondrex. These are all molecules that participate to the development of liver fibrosis thus their role as potential markers of fibrosis has been studied alone or in combination with other indirect markers (1-4).

Different cells seem to be involved in ECM deposition. In particular, the matrix production seems to be mostly related to fibroblasts and contractile myofibroblasts. Hepatic stellate cells are also thought to play a key role in the pathogenesis of liver fibrosis since they differentiate into myofibroblasts upon several stimuli (such as virus-induced liver necrosis), leading to the production of collagen, glycosaminoglycans, hyaluronan and fibronectin (3).

Under experimental conditions, bone marrow-derived cells may contribute to the formation of hepatic stellate cells and myofibroblasts in the liver (5,6,7).

Bone marrow-derived myofibroblasts have been found in human liver fibrosis, although, the nature of these cells remains to be clarified (2-6). Fibrocytes are bone marrow-derived mesenchimal progenitors cells representing 0.1-0.5 % of circulating non erythropoietic cells (8). They are characterized by the expression of CD34 and collagen I. These cells also express several other surface molecules such as CD 11b, CD13, the leukocyte common antigen CD45, class II major histocompatibility complex molecules (HLA-DP, -DQ, -DR), the costimulatory molecules CD80 and CD86, the adhesion molecules CD11a, CD54, CD58, and the chemokine receptors CCR3, CCR5, CCR7 and CXCR-4 (7-16). A preliminary study showed that myelogenic fibrocytes are present in the liver and can differentiate into myofibroblasts (7). In detail, fibrocytes acquire the expression of alpha-smooth muscle actin (alpha-SMA) and decrease the expression of CD34 under the influence of Transforming Growth Factor-beta (TGF-beta), thus becoming contractile myofibroblasts and contributing to the development of fibrosis (8-10)

These cells have been associated with hypertrophic scars, keloids, pulmonary fibrosis, nephrogenic systemic fibrosis, human atherosclerotic lesions and scleroderma (6).. The presence of collagen positive, CD34+ fibrocytes was associated with airway fibrosis in endobronchial biopsies of patients with allergen-induced fibrosis (11-13). Moreover, during systemic inflammatory processes, fibrocytes are augmented, reaching the inflammatory site from the bone marrow through the CCR2 pathway (9).

We investigated whether the concentration of PBF is elevated in patients with chronic hepatitis C at different stages of liver fibrosis and whether PBF may act as potential non-invasive markers of liver fibrosis.

3. PATIENTS AND METHODS

Between January 2006 and January 2007 we enrolled 70 patients affected with chronic hepatitis C at the Unit of Infectious Diseases of the ARNAS Garibaldi Hospital, University of Catania. Informed consent was offered to all the enrolled individuals. Patients' characteristics, as collected at enrolment, are reported in Table 1.

When recruited, all patients had normal values of serum alpha-phetoprotein, none had advanced liver disease as assessed by clinical and biochemical parameters, liver ultrasound scan and oesophagogastroscopy. None of them was under interferon and ribavirin treatment. In detail, exclusion criteria were jaundice, ascites, hepatic encephalopathy, variceal bleeding, co-infection with human immunodeficiency virus type I (HIV) and/or hepatitis virus B (HBV), autoimmune active liver diseases or asymptomatic positivity for autoantibodies such as anti nuclear, anti mitochondrial, anti smooth muscle antibody or cryoglobulinemia.

All 70 patients were HCV-RNA positive according to polymerase-chain reaction assay; 50 were infected with genotype 1b, 10 were infected with genotype 2 and 10 with genotype 3; all had persistent elevation of serum alanine aminotrasferase (ALT) above the normal limits for at least 6 months previous to enrolment; all 70 patients had undergone to liver biopsy and Fibroscan exam within 6 months before the onset of the study. Finally, two different control groups were examined: a) 30 healthy adult volunteers (18 males and 12 females) all with normal ALT levels, not affected by HCV, HBV or HIV infection, matched in sex and age distribution with the patients' group. This group served as a healthy control group; b) 20 patients affected with HCV-related decompensated (Child C) liver cirrhosis (11 males and 9 females) were enrolled as

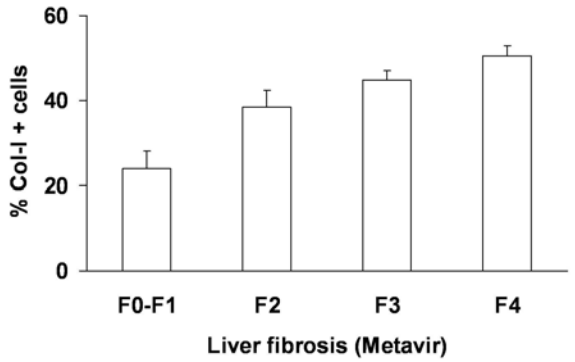

Figure 1. Level of circulating fibrocytes (expressed as percentage of Col-I positive cells) according to the different stages of Metavir score in chronic hepatitis C . F2<0.001 vs F0-1; F3<0.001 vs F0-F1; F4<0.001 vs F0-F1, F2

belonging to a disease control group. Peripheral blood fibrocytes were identified by flow cytometry. Venous peripheral blood samples were drawn into sterile tubes containing an EDTA and 50 µl were stained with anti-CD34-PE(Beckman Coulter Immunotech, Marseille France) and anti-CD45-PC5 (Beckman CoulterImmunotech, Marseille France) for 30 min at room temperature in the dark. Next, the samples were fixed and permeabilized using IntraPrep Permeabilization Reagent (Beckman Coulter, Immunotech,Marseille France) prior to intracellular staining of collagen-I (Col-I, Chemicon International, Inc.). Briefly, 100 µl of reagent 1 (Fixation Solution) were added to the tubes and incubated for 15 min at room temperature in the dark; then, 1 ml of PBS was added to each tube and centrifugated for 10 min at 300 x g. The supernatant was removed by aspiration and the cell pellet was resuspended in 200 µl of the reagent 2 (Permeabilization Solution) and incubated for 5 min at room temperature in the dark. The cells were incubated with primary antibody, Col-I (final concentration 0,25:100) for 15 minutes at room temperature in the dark. After washing, the secondary antibody, fluorescein (FITC)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was added for 30 min at room temperature. Thereafter, the cells were washed with PBS and the pellet was resuspended in PBS and 0.5-0.8 % paraphormaldeide (PFA). Samples were analyzed using a Coulter Epics Elite ESP flow cytometer (Coulter Corporation, Miami, FL, US) according to the ISHAGE protocol for staminal cells (CD45+/CD34+). The rate of peripheral circulating fibrocytes was defined as the percentage of Col I positive cells over the total number of CD34 positive/CD45 positive cells

HCV-RNA was tested by a polymerase-chain reaction assay (Cobas amplicor HCV Monitor Test, version 2,0 Roche Diagnostics). The HCV genotypes were evaluated with a line-probe assay (Inno LiPA HCV II, Innogenetics).

Fibroscan (Echosens, Paris, France) evaluation was performed by a single expert physician. Up to 10 consecutive successful measurements were performed on each patient. The results were expressed in kilopascal units (KPa). Arbitrarily, three groups were identified on the basis of liver stiffness value: one group with a stiffness value lower than 7 kilopascal units, one group with a stiffness value ranging from 7 to 12 kilopascal units and a latter group with a stiffness value higher than 12 kilopascal units.

Liver biopsy specimens were obtained using Menghini 16G disposable needles, then fixed in 4% buffered formalin and embedded in paraffin. Liver fibrosis stages were evaluated semiquantitatively according to Metavir scoring system, by using a 5-point (F0-F4) scale: stage F0 indicated absence of fibrosis; F1 expressed portal fibrosis without septa; F2 was equal to portal fibrosis with few septa; F3 indicated numerous septa without cirrhosis and F4 was equivalent to liver cirrhosis. All biopsies were analyzed by a single experienced pathologist who was blinded to patient's data. All biopsy specimens contained at least 10 portal tracts and were at least 1.5 cm in length.

By means of Student's *t* test we compared the concentration of peripheral blood fibrocytes between anti-HCV positive patients and either healthy controls or cirrhotics. Within the group of patients affected with chronic hepatitis C, we compared the rate of circulating fibrocytes to the different stages of liver fibrosis and to the different values of liver stiffness by using the one-way analysis of variance (ANOVA) followed by Newman Keuls'multiple comparison test. Finally, *r* Spearman correlation analysis was used to correlate values of liver stiffness, (as measured by Fibroscan), Metavir score and fibrocytes' rate. The study was approved by the local ethics committee and all patients gave their informed consent before undergoing any invasive procedure.

4. RESULTS

Patients affected with HCV-positive biopsy-proven chronic hepatitis showed a significantly higher rate of circulating fibrocytes in comparison with both the healthy control group (31,3% versus 17.59%, respectively; p=0.040) and the disease control group formed by decompensated cirrhotics (31.3% versus 18.21%, p=0.041).

Among HCV-positive subjects with an histological Metavir F0-1 stage, the percentage of circulating fibrocytes was 23.3 ± 4% whereas patients in the Metavir F2 and F3 stage had a mean rate of circulating fibrocytes of 38.44 ± 4.01% and 44.88 ± 2.25%, respectively (p<0.001 versus F0-1). Patients with a severe histological fibrosis (F4 Metavir stage) had a percentage of circulating fibrocytes equal to 50.62 ± 2.26%, which resulted significantly higher (p<0.001) than that found in groups F0/1 and F2, respectively (Figure 1).

As refers to Fibroscan results: 38 patients with a stiffness value lower than 7 kilopascal had a mean rate of circulating fibrocytes of 25.96±5.86%; 21 patients with a stiffness ranging from 7 to 12 kilopascal units had a percentage of 38.66±9.50%; finally, the level of circulating fibrocytes among 11 patients with a liver stiffness higher than 12 kilopascal, was 50,40± 2.30% (p<0.001 versus the other two groups) (Figure 2)

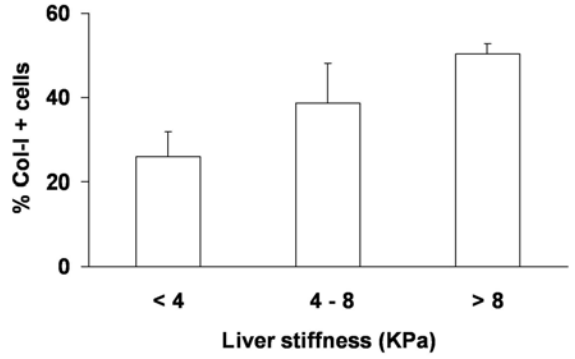

Figure 2. Level of circulating fibrocytes (expressed as percentage of Col-I positive cells) in chronic hepatitis C, according to the value of liver stiffness (in kilopascal units) as measured by Fibroscan. . >12 KPa: p< 0.001 vs <7 KPa; >12 KPa: p< 0.001 vs 7- 12 Kpa

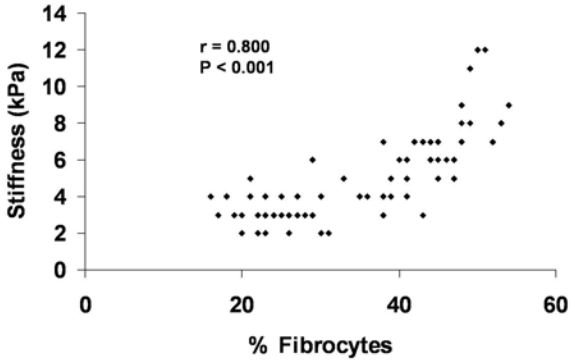

Figure 3. Correlation between circulating fibrocytes' level (expressed as the percentage of Col I positive cells over the total number of CD34 positive/CD45 positive cells) and liver stiffness values as measured (in kilopascal units) by the Fibroscan test *(r* Spearman correlation analysis).

When circulating fibrocytes values were analyzed by *r* Spearman correlation analysis, we found a statistically significant correlation either with the stiffness as measured by Fibroscan (r:0.724) or with the Metavir fibrosis score (r: 0.711) (Figure 3). On the contrary, no correlation was found between fibrocytes' mean rate and HCV RNA and ALT levels (r: 0.188 and r: 0.213, respectively).

5. DISCUSSION

In the present study, we investigated the role of PBF as a potential and easy-to-perform marker of liver fibrosis in patients with chronic hepatitis C. We found that the level of PBF in patients with HCV-related chronic liver disease was higher than that observed in healthy individuals and in patients affected with advanced (Child C) liver cirrhosis. These data suggest that these cells, thought to play a role in ECM remodelling, could really be involved in the fibrogenic process. In fact, in those cases where fibrogenesis is absent (healthy individuals) or exhausted (advanced cirrhotics), the level of circulating fibrocytes results remarkably lower.

We further analyzed the percentage of PBF in patients with biopsy-proven chronic hepatitis C stratified according to the Metavir stage of liver fibrosis. Higher values of PBF were detected in patients belonging to the F2 and F3 groups compared with those in the F0-F1 group. Patients belonging to the F4 Metavir group showed, on their turn, a higher level of PBF as compared to patients in lower Metavir stages.

Similarly, a higher rate of circulating fibrocytes was found among those patients with a liver stiffness superior to 12 kilopascal (as measured by Fibroscan). We have demonstrated, for the first time, not only that PBF levels are increased in chronic hepatitis C, but also that these levels directly correlate with the stage of liver disease.

There is an increasing body of literature reporting the role of fibrocytes in fibrotic disorders, both *ex vivo* and *in vitro* (8-13). They have been identified in human atherosclerotic lesions, nephrogenic systemic fibrosis, hypertrophic scars and keloids. Of interest, the appearance of fibrocytes was demonstrated in the airways of patients with allergic asthma after allergen inhalation (14). Moreover, in patients with interstitial pulmonary fibrosis, the concentration of peripheral blood fibrocytes was more elevated than in healthy donors (15).

A recent experimental study showed that fibrocytes not only are present in the liver, but can further transform into myofibroblasts under the influence of TGF beta, as such potentially being able to contribute to ECM production leading to liver fibrosis (7). Of importance, plasma and liver TGF beta concentrations are elevated in patients with chronic hepatitis C (3). Other factors known to be over-expressed in fibrotic livers, such as M-CSF, G-CSF, and GM-CSF, could be involved either in the mobilization of fibrocytes from bone marrow or in their homing to the liver (3).

To our knowledge, this is the first study to demonstrate that peripheral blood fibrocytes are increased in chronic hepatitis C with progressively higher levels according to fibrosis progression.This preliminary study suggests that PBF not only play a role in the pathogenesis of fibrogenic processes but also that they could be used as potential, easy-to-perform, inexpensive and not invasive biomarkers of liver fibrosis to monitor disease progression, somewhat comparable to the role played by CD4 T-lymphocyte count in HIV-1 infected individuals.

Accurate determination of the presence and degree of liver fibrosis is essential for prognosis and treatment decisions concerning patients with chronic hepatitis C. Liver biopsy remains the gold standard technique to quantify liver fibrosis although invasive, potentially burdened by severe complications and not easily accepted by patients. Inter-observer variability and sampling error are also to be taken into consideration. Furthermore, it should be considered that the dynamic process of liver fibrosis results from an average of progression and regression phenomena which cannot be quantified merely by liver biopsy.

FibroScan is a novel non-invasive method that has been proposed for the assessment of hepatic fibrosis in patients with chronic liver diseases, by measuring liver stiffness. FibroScan is a rapid and user-friendly technique that can be easily performed at the bedside or in the outpatient clinic with immediate results and good reproducibility. Failure occurs in around 5% of cases, mainly in obese patients and in those with acute liver necrosis. Fibroscan results excellent for the diagnosis of severe fibrosis or cirrhosis compared to mild fibrosis though less relevant for the distinction of two adjacent stages of fibrosis. Probably the combination of two non-invasive methods (such as Fibroscan plus one of the known biomarkers) can reliably differentiate between minimal and significant fibrosis, thereby avoiding liver biopsy in a significant number of patients.

Currently, there is an increased need of indirect markers to quantitate and continuously assess liver fibrosis in patients with chronic liver diseases in order to overcome the invasiveness of liver biopsies. Measurement of fibrocytes' blood level seems a promising method.

We believe that further longitudinal studies on a higher number of patients are needed to better characterize the real potential of this test in the management and follow up of chronic hepatitis C.

6. ACKNOWLEDGMENT

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Abbreviations: Peripheral blood fibrocytes:PBF, Hepatitis C virus:HCV, Extracellular matrix:ECM, Transient elastography:Fibroscan, Transforming Growth Factor - beta:TGF-beta, Alanine aminotrasferase:ALT, Human immunodeficiency virus type I:HIV, Hepatitis virus B:HBV

Key Words: Hepatitis C virus, HCV, Fibrosis, Fibroscan, Fibrocytes, Liver biopsy

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