Histopathological and immunohistochemical study of hepatic stellate cells in patients with viral C chronic liver disease

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Abstract
Objective: Our aim was to establish a correlation between hepatic stellate cells (HSCs) activity within different areas of hepatic tissue and the degree of liver fibrosis, necroinflammation, and steatosis in patients with viral C chronic liver disease. Patients and Methods: We prospectively included 41 liver biopsies from patients with chronic hepatitis C or liver cirrhosis, prior to antiviral treatment. Our control group consisted of seven tissue samples, obtained from 10 to 38 weeks old embryos. We assessed the alpha-smooth muscle actin (α-SMA) expression using immunohistochemistry and a semi-quantitative scoring method. Results: We found a strong correlation (p<0.0001) between the number of activated HSCs and the stage of fibrosis in all examined areas. Necroinflammatory activity was significantly correlated (p<0.05) with the number of activated HSCs in perivenular area (p=0.014) and intermediate area (p=0.018), and strongly correlated (p<0.0001) in periportal and portal tracts and fibrous septa areas. We found no correlation between the degree of steatosis and the number of activated HSCs in the perivenular area (p=0.25), intermediate area (p=0.166) and in the perportal area (p=0.154); however, in the portal tracts and fibrous septa area we observed a significant correlation (p=0.022). Conclusions: The analysis of HSCs activity within specified areas of liver tissue may lead to new perspectives in early diagnosis of liver fibrosis and in the development of future antifibrinogenic therapies.

Keywords: liver fibrosis, hepatic stellate cells, alpha smooth muscle actin, immunohistochemical assessment.

Introduction

Worldwide, chronic viral hepatitis C (VHC) prevalence is around 3% of the population [1], while in Romania it is estimated around 3.5% of total population, genotype I being the most frequent [2, 3]. The prognosis of hepatitis C virus (HCV) infected patients is correlated with liver fibrosis progression towards cirrhosis and the development of hepatocellular carcinoma (HCC).

In early stage of HCV infection, the immune system generates antibodies to eradicate the virus and, once the infection becomes chronic, it inflicts hepatocyte damage through direct cellular toxicity and local stimulation of inflammatory cytokine expression, which triggers liver fibrosis by activating hepatic stellate cells (HSCs) [4, 5].

Normally, HSCs are quiescent, store vitamin A and synthesize collagen types III, IV and, in small quantities, type I [6]. HSCs activation consists of two major stages: initiation triggered by chronic hepatic injury and inflammation mediated through a series of signaling molecules released by inflammatory cells, damaged hepatocytes, as well as other non-parenchymatous cells, mainly Kupffer cells and sinusoidal endothelial cells. The second stage – perpetuation – is defined by HSCs proliferation, followed by an increase in type I collagen synthesis and extracellular matrix (ECM) accumulation, with a reduction in its degradation.

Activated HSCs lose their cytoplasmic lipid droplets, forming multiple microfilaments that consist mainly of alpha-smooth muscle actin (α-SMA). Although activated HSCs may be immunohistochemically stained with a series of antibodies, α-SMA represents a trustworthy marker in emphasizing their filaments [7–9].

HCV replication generates a series of factors that intervene in modulating HSCs synthesis, by increasing type I and III procollagen expression, as well as by inhibiting the fibrinolytic activity of matrix metalloproteinases (MMPs). MMPs represent a large family of...
calcium-dependent enzymes involved in a specific way of degrading of all collagen types and non-collagen sublayers [10].

Although there is an important anatomical correlation between the initial injury location in the liver and the following ECM accumulation, hepatic fibrosis progression is linked to bridging fibrosis development, which leads to fibrous septae formation and regeneration nodules that characterize cirrhosis.

The aim of this study is to establish a correlation between HSCs activity within perivenular, intermediate, periporal and portal tract area of hepatic tissue, and histological parameters in VHC infected patients with minimum, moderate, and severe activity and C hepatic viral cirrhosis.

Patients and Methods

Patients

We have performed a two-year prospective study between 2010–2012 on samples obtained through liver biopsy from a group of 41 VHC infected patients, all under investigation within the Research Centre of Gastroenterology and Hepatology from the University of Medicine and Pharmacy Craiova, Romania, before the antiviral therapy initiation with pegylated interferon and ribavirin.

We established the diagnosis of chronic liver disease on both histopathological assessments performed in the Laboratory of Histological, Histopathological and Immunohistochemical Techniques within the Research Centre in Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova, as well as biochemical and virological tests. Our study used the same inclusion criteria as those for antiviral therapy: virological criteria – detectable RNA HCV, anti-HCV antibodies; biochemical criteria – increased ALT levels for more than six months; morphological criteria – chronic hepatitis – Metavir score $A \geq 1$ and $F \geq 1$. We excluded patients with neurological diseases, mental disorders, diabetes mellitus, autoimmune diseases, ischemic coronary disease or uncontrolled severe heart failure, uncontrolled severe respiratory diseases, hemoglobin level below 11 g/dL, leukocytes below 5000/mm$^3$, polymorphonuclears below 1500/mm$^3$.

Our control group consisted in seven tissue samples, each from 10 to 38 weeks old embryos. We chose embryos because, in normal fetal liver, a-SMA immunostains only tunica media of arteries, fusiform cells around bile ducts and centrilobular vein. Since for embryos most HSCs are in quiescent state, only few of them are immunostained.

All 41 patients gave their consent regarding their participation in this study and we obtained all necessary approvals from the hospital Ethical Commission for the study protocol.

Histopathological study

We obtained hepatic tissue fragments through transcutaneous liver biopsy with an automatic Autovac gun with Tru-Cut$^\text{®}$ needle with a diameter of 1.4 mm. In our study, we included only samples longer than 20 mm and with more than eight portal spaces. Coagulation tests and platelets count performed prior to liver biopsy were all inside normal range for all biopsied patients.

Liver biopsy fragments were formalin fixed and paraffin embedded. Slices of 4–5 μm were subsequently stained using Hematoxylin–Eosin (HE) technique, Van Gieson technique, Masson’s trichrome. We evaluated biopsy samples according to Metavir scores for necro-inflammatory activity and fibrosis [11].

According to Metavir score, we graded the stage of fibrosis on a scale from 0 to 4: stage 0 – without fibrosis; stage 1 – slight/moderate fibrosis at portal space level; stage 2 – marked portal or periporal fibrosis, with few septa, but without structural alterations; stage 3 – numerous septa with portal-portal or portal-central bridging fibrosis, structural alterations, but without cirrhosis; stage 4 – cirrhosis. The activity was also graded on a scale from 0 to 3: A0 – no activity, A1 – mild activity, A2 – moderate activity, A3 – severe activity [11].

Hepatic inflammation was also quantified with the modified HAI Knodell score that takes into account the following markers: periportal and/or bridging necrosis (graded from 0 to 10), intralobular degeneration and focal necrosis, portal inflammation and fibrosis (all three graded from 0 to 4) [12].

Steatosis was graded in three stages: slight (1–30% of hepatocytes), moderate (30–60% of hepatocytes) and severe (more than 60% of hepatocytes) [13, 14].

An experienced pathologist (MCV) performed the histopathological assessment.

Immunohistochemical study

Immunohistochemical evaluation of paraffin blocks was performed in the Laboratory of Histological, Histopathological and Immunohistochemical Techniques within the Research Centre in Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova.

Slices cut from paraffin blocks used initially for histological assessment of necroinflammatory activity and liver fibrosis were subsequently displayed on glass slides that have been previously treated with poly-L-lysine, for immunohistochemical staining. In order to determine the number of activated HSCs within the biopsy tissue, this was stained with an anti-a-SMA antibody (DAKO, Carpinteria, CA). The HHF35 clone, anti-human mouse, diluted 1:200, was used as a primary antibody, and the IgG horse anti-mouse, diluted 1:500, represented the second antibody. The immunostaining obtained with the help of DAB chromogen was counterstained with Hematoxylin.

The assessment of immunostained regions was evaluated using a semi-quantitative method that determined the percentage of immunostained cells from specific areas of biopsy fragment: perivenular area, intermediate area, periporal area, portal tracts and fibrous septa area. The percentage of immunostained cells was categorized as following: absent = up to 3% (marked as 0); slight = 3–33% (marked as 1); moderate = 34–66% (marked as 2); severe = more than 66% (marked as 3).

We used a Nikon Eclipse E200 microscope with 10×, 20×, and 40× magnification objectives. Images were...
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Statistical analysis

We employed the XLSTAT suite for Microsoft Excel for statistical analysis. The correlations between the necroinflammatory activity, stage of fibrosis, steatosis, architectural distortions in fibrous arrangement and cirrhotic nodules, as well as semi-quantitative analysis of α-SMA positive cells, were evaluated using Kendal correlation test. A p-value <0.05 was considered statistically significant.

Table 1 – Individual characteristics in the study group

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Histopathological aspects

We used Metavir scores to evaluate the fragments obtained after liver biopsies. Although the distribution of inflammatory cells varied in each case, it was characterized by the presence of a dense monocytary infiltrate, located mainly in interlobular spaces. In patients with minimal or moderate hepatitis, the interlobular spaces were not affected at all (Figure 1, a and b). During the examination, we noticed some interlobular spaces of normal size, while others were enlarged, due to the presence of an increased number of inflammatory cells. We also noted an expansion of portal fibrous stroma that pushed neighboring structures without affecting them.

The inflammatory infiltrate consisted of lymphocytes and plasmocytes. In interlobular spaces, we identified macrophages containing intracellular necrotic detritus.

A hallmark for fibrosis was the presence of expansions from the portal spaces, with or without septa formation. Fibrous septa formed bridges between the central veins and adjacent portal spaces or portal veins (Figure 1c).

In cirrhosis, liver architecture is altered and is characterized by the replacement of normal liver tissue with fibrosis, and by the presence of regenerating nodules (Figure 1d). Fibrosis was characterized by the presence of septa disposed as thick collagen cords and fibroblasts that surround groups of hepatocytes, vessels, biliary ducts and, according to the activity degree of cirrhosis, with or without the presence of a lymphoplasmacytic infiltrate. Regenerating nodules appeared in the form of islands, beaches, or cordons, surrounded by fibrosis, and present dilated sinusoids, mainly in periphery. Liver hepatic alteration due to the compression exerted by nodules and fibrosis, leads to vascular alterations and was accompanied by biliary ducts proliferation and cholestasis.

Macrovesicular steatosis was indicated by the presence in hepatocytes cytoplasm of well-delimited optic hollow vesicles. Following the progressive accumulation of lipids, the nucleus of these hepatocytes was pushed towards the periphery.

Fetal liver contained small hepatocytes with smooth membrane, prominent nucleus and well-delimited nuclear membrane. Portal spaces were small, delimited by two branches of hepatocytes; the sinusoids were covered by elongated endothelial cells with small nucleus without nucleoli. Basal membrane was absent.

Immunohistochemical aspects

For the control group, α-SMA immunostains only tunica media of arteries, fusiform cells around bile ducts and centrlobular vein, with few HSCs, located along the sinusoids, mainly in the peripheral areas of the hepatic lobule, in the proximity of portal spaces, in terminal venules and in perivascular areas (Figure 2a).

In each sub-group, after the semi-quantitative analysis of α-SMA positive cells, we found different results in each examined area, according to the degree of hepatitis. For patients with cirrhosis, we observed in all areas the most significant alterations.
In the group of patients with minimum active hepatitis, the semi-quantitative analysis of α-SMA positive cells was predominantly slight in perivenular and intermediate areas, absent in periportal area, and slight in portal tracts and fibrous septa area (Figure 2b). Patients with moderate active hepatitis presented predominantly a slight α-SMA immunostaining in perivenular, intermediate and periportal areas, while in portal tracts and fibrous septa area it was mostly moderate (Figure 2c).

For patients with severe active hepatitis, the semi-quantitative analysis of α-SMA positive cells was predominantly moderate in perivenular, intermediate and periportal areas and severe in portal tracts and fibrous septa area (Figure 2d). For the group of patients with cirrhosis, α-SMA immunostaining was mostly moderate and severe for all areas (Figure 2e).

Figure 2 – (a) Fetal liver. Alpha-SMA immunostaining, ×100. (b) Minimal active hepatitis. Alpha-SMA immunostaining, ×200. (c) Moderate active hepatitis. Alpha-SMA immunostaining, ×200. (d) Severe active hepatitis. Alpha-SMA immunostaining, ×200. (e) Liver cirrhosis. Alpha-SMA immunostaining, ×40.

Statistical analysis

We observed a significant correlation ($p<0.05$) between the necroinflammatory activity and the number of α-SMA positive cells, both in perivenular area ($p=0.014$) and intermediate area ($p=0.018$), as well as a strong correlation ($p<0.0001$) in periportal and fibrous septa areas. We also found a strong correlation between the number of α-SMA positive cells both with the stage of fibrosis, and with architectural distortions in fibrous arrangement and cirrhotic nodules, in all four areas. We found no correlation between the degree of steatosis and the number of α-SMA positive cells in the perivenular area ($p=0.25$), intermediate area ($p=0.166$)
and in the periportal area ($p=0.154$). Only in the fibrous septa area we found a significant correlation between steatosis and the number of $\alpha$-SMA positive cells ($p=0.022$) (Table 2, Figure 3).

**Table 2 – Statistical results**

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![Image of Table 2]

**Figure 3 – Statistical results.**

**Discussion**

The activation of innate immune system inflammatory cells at hepatic level represents one of the first alterations induced by the hepatic injury, which triggers liver fibrosis, followed by subsequent activation of other inflammatory cells. Kupffer cells are activated following the chronicisation of liver inflammation, and release locally proinflammatory cytokines like tumoral necrosis factor (TNF)-$\alpha$ and interleukins-1$\beta$ and -6 [15].

HSCs activation is initiated on one hand by Kupffer cells that amplify the activity of nuclear factor kappa-B and, on the other hand, by the increased release of proinflammatory cytokines, including the monocyte chemoattractant protein and TNF-$\alpha$ [16].

In our study, we followed the presence of activated HSCs in perivenular, intermediate, periportal, and fibrous septa areas, knowing that, in healthy liver, HSCs are in quiescent state and are located in the perisinusoidal space of Disse, where their main role is storing retinoids [17].

The activation of hepatic myofibroblasts, including HSCs, plays a major role in the feedback mechanism that regulates injury and tissular regeneration.
HSCs activation takes place in two major phases: initiation and perpetuation, involving a series of modifications in cellular behavior, like proliferation, contractility, fibrogenesis, impairment of ECM degradation, chemotaxis, and loss of retinoid [17].

In the activation of HSCs, transforming growth factor (TGF)-β is considered an important mediator of liver fibrogenesis, while platelet-derived growth factor plays a major role in HSCs proliferation.

Previous studies have reported that patients with chronic hepatitis C presented an increased expression of TGF-β, associated with increased levels of mRNA for this cytokine, all this being correlated with the number of activated HSCs and the degree of liver fibrosis [18, 19].

Using α-SMA immunostaining techniques, several studies have emphasized an increase in the number of activated HSCs according to the severity of chronic liver injury, in patients infected with HCV, emphasizing a correlation between HSCs activity and the degree of liver fibrosis. In our study, we found a strong correlation between the number of activated HSCs and liver fibrosis in all four studied areas (perivenular, intermediate, periportal and fibrous septa areas), which is in accordance with most studies in literature which demonstrate the role of HSCs in the development of liver fibrosis [20–26].

Following hepatic injury, HSCs are activated and transdifferentiate into contractile and proliferative myofibroblasts. They play a role in the increase of ECM synthesis, which is a characteristic process of liver fibrosis. Early stages of fibrosis are defined by an accumulation of fibronectin and types III and IV collagen [27]. The ECM accumulation is concomitant with the progression of liver fibrosis, due to an increased synthesis of types I and IV fibrillar collagen, as well as elastin. These increase the deposition of ECM and reduce its degradation, contributing to the formation and accumulation of scar tissue [28].

Activated HSCs stimulate the expression of tissue inhibitors of matrix metalloproteinases (TIMPs) [29]. Consequently, the balance between the degrading action exerted by MMPs and their inhibitors is altered, leading to ECM accumulation. The reversibility of liver fibrosis is accompanied by the initiation of activated HSCs apoptosis, whose main effect is the simultaneous removal of an important ECM and TIMPs producing source [30].

A series of authors have demonstrated that chronic HCV infection leads to the activation of HSCs, through two specific pathways. The main pathway concerns necroinflammation following chronic liver injury, which stimulates the activation of neighboring HSCs; the second pathway involves HSCs direct activation by HCV. Falcón V et al. identified HCV core protein in activated HSCs obtained through liver biopsy from HCV infected patients [31]. An in vitro study reported that HCV core and non-structural proteins induce fibrogenic effects in activated HSCs by regulating specific biologic functions [32].

Considering the necroinflammatory activity and the number of activated HSCs, we identified a significant correlation in perivenular and intermediate areas, and a strong correlation in periportal and fibrous septa areas. Our results are in accordance with other studies, which concluded that in chronic viral hepatitis, HSCs activation is a common event that correlates significantly with necroinflammation and fibrosis [20–22, 26, 33]. On the other hand, there are authors that have not found a correlation between activated HSCs and necroinflammatory activity, probably because most HSCs were directly activated by HCV, while necroinflammatory activity played only a second role [24].

However, we consider that the necroinflammatory process is involved in liver fibrogenesis, as activated HSCs are present around areas with necroinflammatory lesions, where there is an increased expression in proinflammatory cytokines.

Liver fibrosis can be indirectly generated by hepatic steatosis, which increases the susceptibility of hepatocytes to oxidative stress and viral aggression. C virus infection generates oxidative stress that, together with steatosis, increases ECM accumulation subsequent to the activation of HSCs.

A series of studies have found different signaling pathways involved in steatosis-related fibrogenesis, by increasing hepatocytes susceptibility to apoptosis, amplifying the response to oxidative stress, inducing alterations in cellular response to injury, activating and signaling of peroxisome proliferators-activated receptor (PPAR), or by the impairment in leptin synthesis and signaling [34, 35].

A meta-analysis performed on different groups of patients in Europe and United States demonstrated that steatosis is significantly and independently correlated with liver fibrosis in chronic hepatitis C [36].

Despite the numerous studies upon the role of steatosis in the progression of liver fibrosis in patients infected with HCV, results are still inconclusive and no clear correlation can be determined [20, 26, 36, 37]. In our study conducted upon patients with genotype 1 C hepatitis, we found no correlation between steatosis and the number of activated HSCs in perivenular, intermediate and periportal areas; however, a significant correlation was found in the fibrous septa area.

In the control group, α-SMA immunostained few HSCs along the sinusoids, especially in the peripheral areas of the hepatic lobule, in the proximity of portal spaces, in terminal venules and in perivascular areas. HSCs represent the most important cells that play a major role in the development of liver fibrosis. Identifying the main phenomena modulating physiopathological mechanisms involved in early stages of liver injury, which leads to HSC activation, followed by the initiation and development of liver fibrosis, new antifibrotic therapies can be developed. A potential therapeutic strategy may target the inhibition of activated HSCs response to inflammatory cytokine and growth factor stimulation, with a subsequent reduction in ECM production and an improvement in the severity of liver fibrosis and possible implications in the prevention of HCC [38].

Conclusions

Alpha-SMA is still a reliable immunohistochemical marker for identifying activated HSCs. In patients with minimal, moderate, and severe VHC and with liver
This study found that stellate cells activity is correlated with the degree of fibrosis and necroinflammatory activity in all studied areas, while with steatosis a correlation was found only in fibrous septa area. The immunohistochemical analysis of HSCs activity within specified zones of liver tissue may open new perspectives in early diagnosis of liver fibrosis, as well as in the development of future antifibrogenic therapies.

**Contribution Note**
All authors had an equal contribution to this study.

**References**


Histopathological and immunohistochemical study of hepatic stellate cells in patients with viral C chronic liver disease


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