DOCTORAL THESIS

ASSESSMENT OF HEPATIC STELLATE CELLS
ACTIVITY IN THE PROGRESSION OF VIRAL C
CHRONIC LIVER DISEASE

~ ABSTRACT ~

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CRAIOVA
2013
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**Keywords:** chronic hepatitis C, C viral cirrhosis, hepatocellular carcinoma, hepatic stellate cells
STATE OF THE ART

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

Liver fibrosis and cirrhosis are generated after liver aggression induced by HCV. Chronic liver disease is characterized by the excessive accumulation of extracellular matrix (ECM). In injured liver, activated hepatic stellate cells (HSCs) represent the main collagen producing cells. Following liver injury, inactivated HSCs become activated and transdifferentiate in proliferative, fibrogenic and contractile myofibroblast-like cells [3,4].

Liver steatosis may indirectly generate liver fibrosis by amplifying hepatocytes susceptibility both to oxidative stress and viral aggression, being responsible for ECM accumulation through the initiation of HSCs activation [5].

According to World Health Organization, HCC prevalence is more than 600000 now diagnosed cases every year, thus having an ascending trend, both in Europe and United States of America. Worldwide, relative to tumour location, HCC represents the third leading cause of death from cancer and the second cause of death in gastrointestinal cancers [6,7]. Viral liver cirrhosis represents a major risk factor in HCC development. Between 20% and 30% of all patients with VHC will develop liver cirrhosis and, each year, between 3-5% are diagnosed with HCC. Thus, we may estimate that, in a 10 year period, around a third of patients with viral C liver cirrhosis will develop HCC.

Liver carcinogenesis is initiated by the combined effect of several growth factors synthesized by activated HSCs. Also, by amplifying the activity of signalling pathways mediated by NF-kB and ERK, activated HSCs intervene in HCC progression, on one hand by stimulating the proliferation of tumoral cells and, on the other hand, by inhibiting their apoptosis [8]. Previous studies performed in vitro and in vivo have demonstrated that tumoral HCC cells can activate HSCs, which contribute, through growth factors, in the progression and in the aggressiveness of HCC [9], by stimulating the development of intratumoral and peritumoral stroma of HCC [10].
ASSESSMENT OF ACTIVATED HEPATIC STELLATE CELLS ACTIVITY IN PATIENTS WITH VIRAL C CHRONIC LIVER DISEASE

MATERIAL AND METHOD

I have performed a two years prospective study (1st of January 2010 – 31st of December 2011), on samples prelevated through liver biopsy from a group of 41 VHC infected patients, with ages comprised between 27 and 64 years (mean age 53.61 +/- 9.86) and sex ratio F/M = 2.15/1. All patients were under investigation within the 1st Medical Clinic of Emergency Clinical County Hospital from Craiova, before the initiation of antiviral therapy with pegylated interferon and ribavirin. The study focused on the assessment of activated HSCs in four distinct areas of the liver (perivenular area, intermediate area, periportal area and fibrous septa area), in patients with viral C chronic liver disease genotype I.

Chronic liver disease diagnosis was established based both on histopathological assessment performed in the Laboratory of Histological, Histopathological and Immunohistochemical Techniques within the Research Centre in Microscopic Morphology and Immunology of University of Medicine and Pharmacy Craiova, and also on biochemical and virologic tests, according to the same inclusion and exclusion criteria as those for antiviral therapy.

The control group consisted in 7 tissue samples, each from 10 to 38 weeks old embryos. We chose embryos because, in normal fetal liver, α-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.

Samples obtained after liver biopsy were histopathological processed according to the protocol used in the Laboratory of Histological, Histopathological and Immunohistochemical Techniques within the Research Centre in Microscopic Morphology and Immunology of University of Medicine and Pharmacy Craiova [11]. Slices cut from paraffin blocks used initially for histological assessment of necroinflammatory activity and liver fibrosis were subsequently displayed on glass slides which have been previously treated with polylysine, for immunohistochemical staining. In order to determine the number of activated HSCs within the biopsy tissue, this was stained with an anti-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 evaluation of immunostained regions was performed using a semiquantitative method that determined the percentage of immunostained cells from specific areas of biopsy
fragment: perivenular area, intermediate area, periportal area, portal tracts and fibrous septa area. The percentage of immunostained cells was assessed as following: absent = up to 3%; slight = 3-33%; moderate = 34-66%; severe = more than 66%. Steatosis was graded in three stages: slight (0-30% of hepatocytes), moderate (30-60% of hepatocytes) and severe (more than 60% of hepatocytes) [5].

For both studies, I used a Nikon Eclipse E200 microscope 10x, 20x and 40x with magnification. Images were captured using a Nikon DS-Fi1 digital camera and the LUCIA NET software application version 1.16.5.

I’ve 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 semiquantitative analysis of α-SMA positive cells, were evaluated using Kendal correlation test. A $p$ value $< 0.05$ was considered statistically significant.

**RESULTS**

Within the study group, I have noticed a predominance of female patients, both in urban area (ratio F/M = 1.33/1) and rural area (ratio F/M = 12/1). I have also observed that most patients had ages comprised within the interval 51 – 65 years old. Most of the female patients, both in urban area (ratio F/M = 1.57/1) as well as in rural area (ratio F/M = 7/1), had increased BMI values.

Biopsy samples were evaluated according to Metavir scores for necroinflammatory activity and fibrosis. Thus, I divided the study group into sub-groups, based on the activity degree, thus: 9 patients had chronic hepatitis with minimum activity, 17 patients had chronic hepatitis with moderate activity, 11 patients had chronic hepatitis with severe activity and 4 patients had cirrhosis.

In the group of patients with minimum active hepatitis, the semiquantitative 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. 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. For patients with severe active hepatitis, the semiquantitative analysis of α-SMA positive cells was predominantly moderate in perivenular, intermediate and
periportal areas, and severe in portal tracts and fibrous septa area. For the group of patients with cirrhosis, α-SMA immunostaining was mostly moderate and severe for all areas.

Following statistical analysis, I 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. I 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. I 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, I found a significant correlation between steatosis and the number of α-SMA positive cells (p = 0.022).

For the same study group, I have also performed a statistical analysis in order to assess potential correlations between the number of activated HSCs immunostained with α-SMA in the four studied areas and liver function, expressed by specific parameters for the following syndromes: hepatic cytolysis, liver failure, mesenchymal activation and bile-secretion syndrome. I have found a statistic correlation between the number of activated HSCs in perivenular and intermediate areas with several parameters quantified within the liver failure (albumin, prothrombin time), mesenchimal activation (γ-globulins, IgG) and bile-secretion syndromes (total bilirubin).

In periportal area, I have noticed a strong correlation between the number of activated HSCs and a series of parameters quantified within hepatic cytolysis (ALT), mesenchymal activation (γ-globulins, IgG) and bile-secretion syndromes (total bilirubin, alkaline phosphatase).

In portal tracts and fibrous septa area, the number of activated HSCs was correlated with almost all studied parameters within hepatic cytolysis (ALT, AST), liver failure (total proteins, prothrombin time), mesenchymal activation (γ-globulins, IgG) and bile-secretion syndromes (total bilirubin, alkaline phosphatase), which pleads to an interdependence between activated HSCs and the progression of chronic liver disease.

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.

ASSESSMENT OF ACTIVATED HEPATIC STELLATE CELLS ACTIVITY IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

MATERIAL AND METHOD
The second prospective study (1\textsuperscript{st} of January 2010 – 31\textsuperscript{st} of December 2011), was performed on samples obtained at necropsy from a group of 20 patients which had HCC of C viral etiology, with ages comprised between 39 and 78 years (mean age 64.95 +/- 9.89) and sex ratio M/F=2.33/1. All patients were initially under observation within the 1\textsuperscript{st} Medical Clinic of Emergency Clinical County Hospital from Craiova.

For the group of patients with HCC, the semiquantitative assessment aimed to identify the percentage of immunostained cells with $\alpha$-SMA (HSCs), CD20 (B lymphocytes), CD45RO (T lymphocytes), and NK1 (natural killer cells) in 3 specific areas, in the samples obtained at necropsy: tumour, thickness of tumoral capsule, for patients with encapsulated tumours, or the transition area in patients with not encapsulated tumours, as well as in the proximity tissue (2-5 mm). The percentage of immunostained cells was assessed as following: absent = up to 3%; slight = 3-33%; moderate = 34-66%; severe = more than 66%.

RESULTS
Within the study group of patients with HCC, I have noticed a predominance of male patients, both in urban area (ratio M/F = 1.33/1) and rural area (ratio M/F = 3.33/1). I have also observed that most patients had ages comprised within the interval 61 – 80 years old. The number of males from the same age interval was greater than the number of female patients (ratio M/F = 1.8/1).

The number of HSCs immunostained in the tumour was mainly moderate and severe; the number of immunostained T lymphocytes was mostly slight and moderate; the number of immunostained natural killer cells was preponderant slight and absent; the number of immunostained B lymphocytes was slight for most of the patients.

In the transition area (tumoral capsule), the number of immunostained HSCs was mainly severe and moderate (all 3 patients with encapsulated tumours presented a severe
immunostaining). Most patients presented a slight immunostaining for T lymphocytes, mostly slight and absent for natural killer cells, while it was preponderant slight for B lymphocytes.

For the proximity tissue, the semiquantitative analysis of activated HSCs was slight and moderate; in the case of T lymphocytes – moderate and severe, while for natural killer cells and B lymphocytes – slight and moderate.

The statistical analysis, performed for all 3 studied areas, between the number of activated HSCs and the number of T lymphocytes (in tumour $p = 0.0036$, in the transition area $p = 0.0345$, in the proximity tissue $p = 0.0473$), as well as the number of natural killer cells (in tumour $p = 0.0007$, in the transition area $p = 0.0247$, in the proximity tissue $p = 0.0388$), reported an inverse correlation between these parameters. This demonstrates the inhibitory effect generated by activated HSCs upon T lymphocytes and natural killer cells, thus contributing to the progression and invasion of HCC.

I have not identified any relation between the number of activated HSCs and B lymphocytes, in all 3 studied areas (in tumour $p = 0.1683$, in the transition area $p = 0.2031$, in the proximity tissue $p = 0.8760$).

Although active chronic viral hepatitis is commonly associated to a significant increase of activated T lymphocytes, immunosuppression is still present in the liver. HSCs activation is the main cause of liver immunosuppression, by triggering activated T lymphocytes apoptosis, which favours HCC’s progression and migration. Natural killer cells exert a protective role by inhibiting tumoral cells proliferation and liver fibrosis, by directly inducing activated HSCs apoptosis. Natural killer cells stimulate apoptosis of senescent HSCs or in early stages of activation, but not for quiescent HSCs or fully activated, which are resistant to their action. Intratumoral activated HSCs inhibit T lymphocytes response.

Quantifying peritumoral activated HSCs may have a prognostic role in assessing the evolution of HCC patients, as well as in the development of intrahepatic metastasis, or tumoral recidive after surgical resection.

A better understanding of physiopathological mechanisms through which activated HSCs are involved in liver carcinogenesis, can lead to the development of new therapeutic targets that aim to inhibit HSCs activation.
CONCLUSIONS

- The number of activated HSCs and necroinflammatory activity were significantly correlated in perivenular and intermediate areas, and strongly correlated in periportal and fibrous septa areas.
- The degree of steatosis was not correlated with the number of activated HSCs in perivenular, intermediate and periportal areas, while I’ve found a significant correlation in the fibrous septa area.
- In perivenular and intermediate areas, I’ve found a significant correlation between the number of activated HSCs and the liver failure, mesenchymal activation and bile-secretion syndromes.
- In periportal area, I’ve identified a significant correlation between the number of activated HSCs and hepatic cytolysis, mesenchymal activation and bile-secretion syndromes.
- The number of activated HSCs in portal tracts and fibrous septa area was correlated with hepatic cytolysis, liver failure, mesenchymal activation and bile-secretion syndromes.
- In tumour, the number of immunostained HSCs was mostly moderate and severe; for T lymphocytes was mainly slight and moderate; for natural killer cells was preponderant slight and absent, while for B lymphocytes was slight for most patients.
- In the transition area (tumoral capsule), the number of immunostained HSCs was mostly moderate and severe (all 3 patients with encapsulated tumours presented a severe immunostaining). The number of immunostained T lymphocytes was mainly slight; while the number natural killer cells was preponderant slight and absent, and the number of B lymphocytes was mostly slight.
- In the proximity tissue, the number of immunostained HSCs was mostly slight and moderate. Immunostained T lymphocytes were mainly moderate and severe; while the number of immunostained natural killer cells and B lymphocytes was slight and moderate.
- The statistical analysis performed between the number of activated HSCs and the number of T lymphocytes and natural killer cells, in all 3 examined areas, identified an inverse correlation between them.
- I have not identified a correlation between the number of activated HSCs and B lymphocytes, in any of the examined areas.
REFERENCES


