UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
FACULTY OF MEDICINE

CONTRIBUTIONS TO THE STUDY OF PATHOGENICAL PATHWAYS OF LIVER FIBROSIS.
A HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

-RESUME-

Scientific leader
Prof. univ. dr. Florin BOGDAN

PhD student
Daniela Elise TACHE

CRAIOVA
2010
Introduction:
Chronic hepatitis represents the substrate for many medical researches, from which emerged various theories about the pathogenesis of liver fibrosis, more or less documented, in our country, as well as abroad.

From these growing body of evidence many pathogenical pathways that are trying to explain liver fibrosis.

Fibrosis is a complex mechanism, initiated to impair lesion and to isolate the impaired tissue, with a systemic response, affecting other tissues and organs [Kisseleva și Brenner, 2008].

Liver fibrosis is the final pathway for chronic liver diseases, regardless their etiology. Chronic viral hepatitis represents an important public health problem worldwide.

The objectives of the research are to stop progression of chronic liver disease to advanced stages of fibrosis, at that point the therapeutical meanings being very limited, conservatory or highly invasive (liver transplant) [Strong, 2001].

Aim: In this thesis we tried to correlate some immunohistochemical markers expression, which underline some of the pathogenical pathways of liver fibrosis, with the stage of stromal changes.

Material and methods:
Liver biopsies from patients diagnosed with chronic viral hepatitis B and C and necroptic fragments from patients with
chronic viral hepatitis B and C and chronic alcoholic liver disease were embedded in paraffin and further used for histological staining and immunohistochemical reactions to detect markers for some of the pathways of liver fibrosis: matrix remodeling, growth factors or oxidative and nitrosative stress.

Liver sections stained with hematoxilin-eosine, trichromic Masson and van Gieson for collagen staining and Gömöri’s silver impregnation for specific liver stroma revealed various degrees of liver fibrosis, noted in the METAVIR scale from 1 to 4, as follows: F0-no fibrosis, F1-portal fibrosis without fibrous septa, F2-portal fibrosis and rare fibrous septa, F3-septa without cirrhosis, F4-cirrhosis.

Immunohistochemical reactions were performed on sections of liver specimens prepared as mentioned before using the following primary antibodies:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal mouse anti-human actin (smooth muscle), 1A4</td>
<td>1:100</td>
<td>Dako</td>
</tr>
<tr>
<td>Monoclonal mouse anti-human TGFβ1, (1A4)</td>
<td>1:300</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Monoclonal mouse anti-human CTGF (6B13), sc -101586</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Monoclonal mouse anti-human MMP1 (3B6), sc-21731</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Monoclonal mouse anti-human MMP2 (8B4), sc-13595</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Monoclonal mouse anti-TIMP1 (2A5), sc-21734</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Monoclonal mouse anti-TIMP2 (3A4), sc-21735</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-human NOS2 (N-20), sc-651</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology</td>
</tr>
</tbody>
</table>

After inhibition of the endogenous peroxidase with hydrogen peroxide in methanol and blockage of nonspecific binding, an overnight incubation with the primary antibodies at 4°C, in a humid chamber, was performed.

The next day reactions were amplified with EnVision-Dual Link System-HRP (Dako) or Avidin-Biotin complex (ABC) and
developed with 3, 3’-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Sigma-Aldrich Co.). Nuclear counterstaining was done with Mayer’s hematoxylin. Slides were observed and registered with a Nikon Eclipse microscope coupled to a digital camera. Images were finally processed using the Microsoft Office Picture Manager. For the negative controls, the primary antibodies were omitted.

Additionally I statistically analyzed the AST/ALT ratio and APRI (aspartate aminotransferase to platelet index ratio) as predictive markers for fibrosis.

**Results:**

*Localization of TGF-β1 positive reaction*

The intensity of the immunohistochemical staining was not equal for the specimens with fibrosis F1 and F2, but had the same localization, in activated cells lining sinusoids, mainly in the portal area. In the group with moderate hepatic fibrosis we noted intense positive reaction for TGF-β1 in perivenular areas, portal spaces and fibrous septa. For the cirrhosis specimens we observed a very intense reaction in the hepatocytes, also in few pro-inflammatory cells, but diminished in the ductal epithelium.

The normal liver architecture in cirrhosis is damaged and for some samples we noted an interesting aspect: positive hepatocytes alternating with negative ones (patchy or “chess table” aspect) (Fig.4).

*Localization of CTGF positive reaction*

For the cases with fibrosis F1 the intensity of the immunohistochemical reaction for CTGF was discrete, in few hepatocytes lining the periportal area. For F2 cases - portal fibrosis and rare fibrous septa - we noted an increase of positive structures for CTGF: beside hepatocytes, positive reaction was present in inflammatory cells and ductal epithelium.

As the stage of fibrosis advances (F3 and F4) the positivity increases in the parenchymal cells and in some cells from the inflammatory infiltrate, such as Kupffer cells or hepatic stellate cells.
Evaluation of $\alpha$-SMA localization

In the patients with minimal portal fibrosis (F1), $\alpha$-SMA localization was faint in perivenular and portal areas. Periportal area showed negative reaction for $\alpha$-SMA in some specimens and moderate positive reaction in two of them. In these cases we noted many hepatic stellate cells lining hepatic sinusoids and no $\alpha$-SMA immunostaining in the lobular parenchyma.

In the group with moderate hepatic fibrosis (F2) we noted intense positive reaction for $\alpha$-SMA in perivenular areas, portal spaces and fibrous septa. The number of $\alpha$-SMA positive cells lining sinusoids from the periportal area was increased.

In cases with severe fibrosis and cirrhosis (F4), $\alpha$-SMA positivity was strong in the portal tracts (in blood vessels, bile ducts and many myofibroblasts) and fibrous septa and also increased in activated hepatic stellate cells in the periportal space and perivenular area in our specimens. In the nodular parenchyma, all hepatocytes showed no $\alpha$-SMA positivity.

Matrix remodeling implication in liver fibrosis progression

We noted positive reaction for MMP-1 only for the specimens characterized by F1 and F2 fibrosis in portal spaces, macrophages and endothelial cells, with no positive reaction whatsoever for the specimens with an advanced degree of fibrosis. For MMP-2 the immunostaining was positive for F1 and F2 specimens, but also for F3 fibrosis, diminished and with perivascular localization. TIMP-1 immunostaining proved to be positive in the inflammatory cells from the sinusoids in F1 fibrosis, for F2 in the extracellular matrix, and for F3 in the inflammatory cells and endothelial cells, with a very discrete positivity in the hepatocytes surrounding the fibrotic area. The positive reaction for TIMP-2 was in the extracellular matrix from portal spaces for F1 and F2 pieces, also in the ductal epithelium for the F3 cases.

Nitrosative stress implication in liver fibrosis progression

For minimal fibrosis (F1 and F2) the positivity for i-NOS was discrete and limited inside the lobule, while for the advanced
fibrosis (F3 and F4) the reaction was intensified in hepatocytes comparing to macrophages.

**Medium values of AST/ALT and APRI**

<table>
<thead>
<tr>
<th></th>
<th>Hepatită cronică virală C</th>
<th>Hepatită cronică virală B</th>
<th>Hepatită cronică alcoolică</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AST/ALT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+F2</td>
<td>1.72±1.03</td>
<td>0.89±0.39</td>
<td>1.27±0.01</td>
<td>1.29±0.47</td>
</tr>
<tr>
<td>F3</td>
<td>1.03±0.54</td>
<td>1.02±0.60</td>
<td>1.11±0.28</td>
<td>1.05±0.47</td>
</tr>
<tr>
<td>F4</td>
<td>1.12±0.52</td>
<td>1.13±0.49</td>
<td>1.42±0.87</td>
<td>1.22±0.62</td>
</tr>
<tr>
<td><strong>APRI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+F2</td>
<td>0.58±0.41</td>
<td>0.51±0.26</td>
<td>0.35±0.11</td>
<td>0.48±0.26</td>
</tr>
<tr>
<td>F3</td>
<td>1.07±0.58</td>
<td>0.68±0.41</td>
<td>0.92±0.92</td>
<td>0.89±0.63</td>
</tr>
<tr>
<td>F4</td>
<td>1.98±1.40</td>
<td>2.12±2.16</td>
<td>2.56±3.02</td>
<td>2.22±2.19</td>
</tr>
</tbody>
</table>

* t Student test for APRI: F1+F2 vs F3, p=0.02; F3 vs F4, p=0.005.

**Discussions:**

In chronic liver viral diseases, hepatic alteration is not specific reported to HVB or HVC presence and it is due to the necroinflammatory activity and fibrosis.

Liver fibrogenesis is a complex process implying a large accumulation of pathological ECM. HSC are the major source of ECM and their activation is an important step in liver fibrogenesis [Safadi and Friedman, 2002, Battaler and Brenner, 2005, Kisseleva and Brenner, 2006].

In chronic viral injury, Kupffer cells, hepatocytes, endothelial cells, monocytes, release a lot of proinflammatory cytokines such as platelet derived growth factor, PDGF, the main mediator of proliferation, and TGF-β1, the most important profibrogenic cytokine [Yoshui et al., 2006].

In our study, we observed TGF-β1 positive cells in the periportal and perisinusoidal areas, precisely in the same zones were fibrosis started, as well as in some portal spaces for the F2, F3 and cirrhosis specimens. In cirrhosis, positive and negative cells were alternated in the parenchyma, creating a patchy aspect. Most of the hepatocytes from these specimens were damaged, possibly apoptotic cells.

HSCs activation consecutively to TGF action consists of their transformation into myofibroblasts, able to express desmin,
vimentin, smooth muscle actin (α-SMA), but also neuroendocrine markers [Moreira, 2007]. In a vicious circle, activated HSCs are able to express TGF-β1. Another biologic effect of TGF-β1 is to inhibit the activity of matrix metalloproteinases (MMP) and to increase the activity of their tissue inhibitors (TIMP), and by that the extracellular matrix homeostasis is imbalanced and the degree of fibrosis increases.

Another way to influence the collagen type I and matrix proteins synthesis is through CTGF activation.

CTGF has been recently described as a novel profibrotic factor that mediates some TGF-β1 responses, including apoptosis and fibrosis [Perbal, 2004].

Body of evidence suggests that TGF-β1 and CTGF synergize to promote chronic fibrosis [Leask and Abraham, 2003, 2006]. CTGF has been described to bind directly to TGF-β1, leading to an enhancement of TGF-β1 activity, by increasing the affinity of TGF-β1 to its receptors [Abreu et al., 2002], by blocking the negative feed-backs for TGF-β1, perpetuating its signaling activation [Wahab et al., 2005] or by stimulating fibroblasts mitosis [Gressner et al., 2002, Abou-Shady et al., 2000].

Our study allowed us to detect the presence of CTGF protein along the fibrosis areas and portal spaces, with intensity depending on the degree of fibrosis (more intense for the specimens with extended fibrous septa and cirrhosis).

Unlike previous studies that referred the presence of CTGF only perisinusoidal and in myofibroblasts from the portal spaces [Paradis et al., 1999] or in vascular elements or ductal epithelium [Abou-Shady et al., 2000], our data showed a restricted positivity in the hepatocytes lining the fibrosis septa, correlating with the expression of TGF-β1, in accordance with more recent studies, which clearly demonstrate CTGF expression in parenchymal cells, sensitively upregulated by exogenous TGF-β1 [Gressner et al., 2007, Weng et al., 2007].

The presence of CTGF in the same regions a TGF-β1 for specimens with various chronic disease suggests that CTGF, as
well as TGF-β1, is a key mediator of fibrosis regardless its etiology.

If further research will confirm the fact that CTGF plays exclusively a profibrotic role in the liver, blocking the pathogenic pathway of TGF-β1-CTGF might represent a more efficient therapeutic strategy in the treatment of liver fibrosis, excluding the adverse effects of medicines targeting directly TGF-β1.

Recently, myofibroblasts have been recognized as antigen presenting cells [Inanue, 2007] transdifferentiation of HSCs in myofibroblast-like cells being an important step in this cascade.

According to reported data, in normal liver specimens, activated HSCs, can be found in portal and perivenular areas [Carpino et al., 2005] because action of some molecules that not leads to necrosis could be related to their activation.

In our study, the number of α-SMA positive cells was directly correlated with the stage of fibrosis, being localized mainly in the portal spaces, periportal and perisinusoidal areas and fibrous septa. Our observations are in accordance with recent reported findings, even at the past wasn’t a scientific agreement on the fact that exists a direct correlation between the number of activated HSCs and the stage of fibrosis [Kisseleva and Brenner, 2007, Chu et al., 2008].

The number of α-SMA positive HSCs cells increased as a response to different stimuli. TGF-β, the most potent fibrogenic cytokine, is one of them, TGF-β being able to induce expression of ECM proteins and to stimulate production of inhibitors of ECM degradation.

In our study, in four specimens with moderate fibrosis, TGF-β1 positive cells were localized mainly in the periportal and perisinusoidal areas and few in the portal spaces. These were the cases showing a more active disease. A strong reaction for this protein was observed in specimens with severe fibrosis and cirrhosis, in this stage even a great number of hepatocytes from the liver parenchyma revealing intense positivity.
Important research data have demonstrated the crosstalk among the signaling pathways able to enhance or inhibit TGF-β responses in damaged liver [Gressner et al., 2007].

Our observation that in the majority of the specimens with an active disease we noted a correlation between the areas of detection of TGF-β1 and α-SMA and because both showed intense positive reaction at sites of fibrosis confirm that TGF-β1 is produced in HSCs and have an autocrine control in HSCs activation, migration and their involvement in progression of fibrosis during the development of the disease. Besides this action, TGF-β is the main mediator of constant liver mass supporting the idea that parenchymal TGF-β acts in an autocrine regulation of hepatocyte proliferation and apoptosis.

**General conclusions**

- Liver fibrosis is a dynamic process of continuous remodeling of extracellular matrix (EMC), mediated by various pathways.
  - For evaluation of these pathways, the initial step was to establish the degree of fibrosis and its echo on the parenchyma.
  - Aspartate aminotransferase to platelet ratio index (APRI) is a marker for predicting advanced liver fibrosis or cirrhosis in chronic liver disease.
  - Fibrogenesis dynamics is realized by all liver cellular elements, parenchymal and stromal.
  - Hepatic stellate cells have a major role in fibrogenesis, as the main ECM producing cell, their activation beeing a key step of this process.
  - Myofibroblasts have an active role in ECM remodeling, by synthesis of various collagen types and by matrix metalloproteinases (MMP) and their tissue inhibitors, TIMP.
  - The unbalance between MMP and TIMP activity is an important pathway for EMC accumulation in liver fibrosis.
  - TGF-β has a pivotal role in liver fibrogenesis by controlling myofibroblasts activation and by augmenting CTGF action.
• In chronic viral hepatitis, as well in alcoholic liver disease, the expression of nitric oxide is intensified by increasing the activity of nitric oxide synthase in hepatocytes, suggesting that its overexpression has an important role in evolution of chronic hepatitis to cirrhosis.

• Nitrosative stress mediators may activate contradictional signaling pathways, liver final answer depending on the balance between them; they can determine either hepatic cells’ proliferation, either their fibrogenic response

Selective bibliography
10. KISSELEVA T, BRENNER DA, Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. J. Gastroenterol. Hepatol., 2007, 22, Suppl.1, S73-78

CURRICULUM VITAE

Surname: TACHE
First name: Daniela Elise
Birth date and place: 25.06.1977, Craiova, Dolj, România
Citizenship: Romanian
Mail: danatache@rdslink.ro

STUDIES
- 1991-1995 National College „Carol I”, mathematics-physics specialization
- June 1995 – Baccalaureate - National College „Carol I”, Craiova – grade 9,32

ACADEMIC STUDIES
- 1995-2001 - The Faculty of Medicine of Craiova, University of Craiova, license grade 9,92

PROFESSIONAL EXPERIENCE
• 26.02.2006- present – Assistant Professor at The Faculty of Medicine, University of Medicine and Pharmacy of Craiova– Biochemistry Department
• 01.01.2003 – 31.04.2008 – Resident Physician in Pediatrics – Emergency County Hospital, Craiova
• 01.05.2008 – present – Specialist Physician in Pediatrics - Emergency County Hospital, Craiova

MEMBER OF SCIENTIFIC SOCIETIES
• Romanian Society of Cellular Biology
• Romanian Society of Biochemistry and Molecular Biology

SCIENTIFIC ACTIVITY:
• Articles published in ISI indexed journals: 1
• Articles published in CNCSIS indexed journals: 4
• National and international scientific presentations: 27
• Co-author: 1 book