

## Evidence for the involvement of TGF- $\beta$ 1–CTGF axis in liver fibrogenesis secondary to hepatic viral infection

DANIELA TACHE<sup>1)</sup>, FL. BOGDAN<sup>2)</sup>, CĂTĂLINA PISOSCHI<sup>1)</sup>, MONICA BANIȚĂ<sup>2)</sup>,  
CAMELIA STĂNCIULESCU<sup>1)</sup>, ANA MARIA FUSARU<sup>2)</sup>, VIOLETA COMĂNESCU<sup>3)</sup>

<sup>1)</sup>Department of Biochemistry

<sup>2)</sup>Department of Histology

University of Medicine and Pharmacy of Craiova

<sup>3)</sup>Department of Pathology,

Emergency County Hospital, Craiova

### Abstract

Liver fibrosis is a nonspecific response to injuries, which implies the synthesis of an abnormal extracellular matrix (ECM). TGF- $\beta$  (transforming growth factor  $\beta$ ) is a cytokine involved in regulation of several important processes: cell development and differentiation, apoptosis, synthesis and degradation of ECM. CTGF (connective tissue growth factor) is a cysteine rich peptide that belongs to the CCN family of proteins and plays an essential role in the formation of blood vessels, bone and connective tissue. The purpose of this study was to assess TGF- $\beta$ 1 and CTGF immunohistochemical expression in different stages of liver fibrosis secondary to chronic viral hepatitis. Liver biopsies from patients diagnosed with chronic viral hepatitis B and C were embedded in paraffin and further used for histological staining and immunohistochemical reactions to detect TGF- $\beta$ 1 and CTGF. Liver sections stained with trichromic Masson for collagen staining and Gömöri's silver impregnation revealed various degrees of liver fibrosis, noted in the METAVIR scale from 1 to 4. Sections with discrete degrees of fibrosis revealed the positivity only in the endothelial cells of liver sinusoids and occasionally in proinflammatory cells from the portal tracts, the number of TGF- $\beta$ 1-positive cells being directly proportional to the incidence of liver injury. Positive reaction for TGF- $\beta$ 1 expanded to the cytoplasm of hepatocytes located nearby fibrosis bundles while increasing the parenchymal damage. The expression of CTGF was observed in the classical areas of the hepatic lobule, such as the perisinusoidal spaces around the portal tracts or central veins, but also in the hepatocytes surrounding the fibrotic areas. Regardless of the etiological factor of liver damage, activation of liver cells causes an increased synthesis of TGF- $\beta$ 1 followed by a CTGF overproduction in various polymorphic hepatic structures, in accordance with the degree of fibrosis.

**Keywords:** chronic hepatitis, liver fibrosis, immunohistochemistry, TGF- $\beta$ 1, CTGF.

### Introduction

Fibrosis represents the main complication for a broad spectrum of chronic liver diseases, whatever their etiology. Nowadays, fibrosis is considered a dynamic process of continuous extracellular matrix (ECM) remodeling, even if initially it was thought to be a passive process consisting in the collapse of the liver parenchyma and condensation of the hepatic stroma [1].

Hepatic fibrogenesis is a complex process involving different cell types, a result of an important accumulation of ECM components [2].

The molecular mechanism of fibrosis is not fully understood. However, fibrogenesis requires numerous mediators, among which growth factors play an important role. The hierarchy of pro-fibrogenic growth factors includes platelet derived growth factor (PDGF) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), the latter being considered the “fibrogenic master cytokine” with multiple effects on inflammation, proliferation, apoptosis, carcinogenesis and ECM turnover [3–5].

The balance of TGF- $\beta$ 1 actions is required for maintaining tissue homeostasis.

Aside from this key mediator, other molecules, such

as connective tissue growth factor (CTGF), may play a role in the fibrogenesis process for which the major inducer is TGF- $\beta$ 1.

Substantial evidence exists that hepatic stellate cells (HSC) play a pivotal role, acting as the main ECM-producing cells, so their activation by some molecules such TGF- $\beta$ 1 is a central event of hepatic fibrogenesis [6–8].

In this work, we performed a study to assess the TGF- $\beta$ 1 and CTGF expression and their involvement in liver disease depending on fibrosis stage.

### Materials and Methods

This study included liver biopsy samples from patients infected with hepatic virus C (HVC) (n=15), and with hepatic virus B (HVB) (n=6) collected from subjects which have never received specific antiviral and immunomodulatory therapy; the tissues were obtained from the Pathology Department, Emergency County Hospital, Craiova.

Liver specimens were formalin-fixed, processed for paraffin embedding, cut at 4–5  $\mu$ m, and routinely stained for trichromic Masson and Gömöri's silver impregnation.

The fibrosis stage was considered according to the METAVIR scoring system, using histological staining 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 above using the following primary antibodies: monoclonal mouse anti-human TGF- $\beta$ 1 (Santa Cruz Biotechnology Inc.), 1:200 dilution, and monoclonal mouse anti-human CTGF (Santa Cruz Biotechnology Inc.), 1:200 dilution. After inhibition of the endogenous peroxidase with hydrogen peroxide in methanol and blocking 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 performed 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.

## Results

The histological analysis allowed us to establish a fibrosis stage for each liver specimen, according to the METAVIR scoring system, as follows: F1 (n=4), F2 (n=6), F3 (n=8), F4 (n=3).

### Localization of TGF- $\beta$ 1 positive reaction

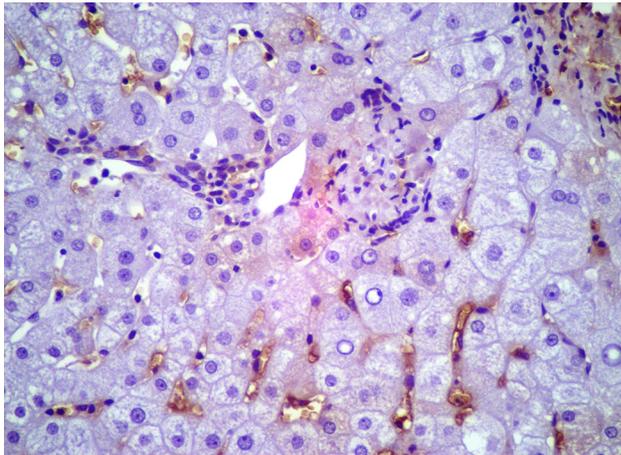
The histological aspect and the immunohistochemical staining was the same, regardless the infection with HVB or HVC.

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 (Figure 1).

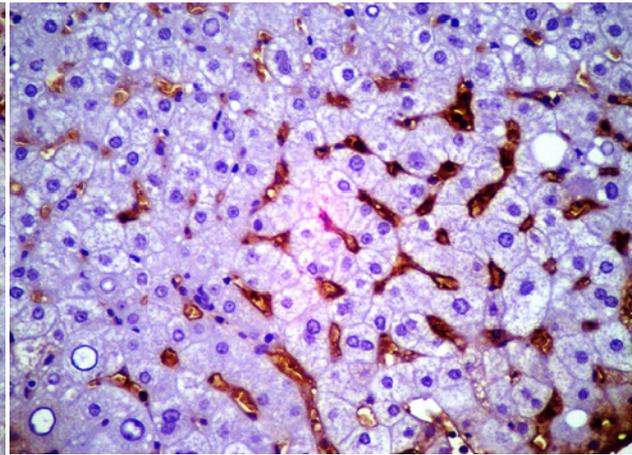
In the group with moderate hepatic fibrosis, we noted intense positive reaction for TGF- $\beta$ 1 in perivenular areas, portal spaces and fibrous septa (Figure 2).

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 (Figure 3).

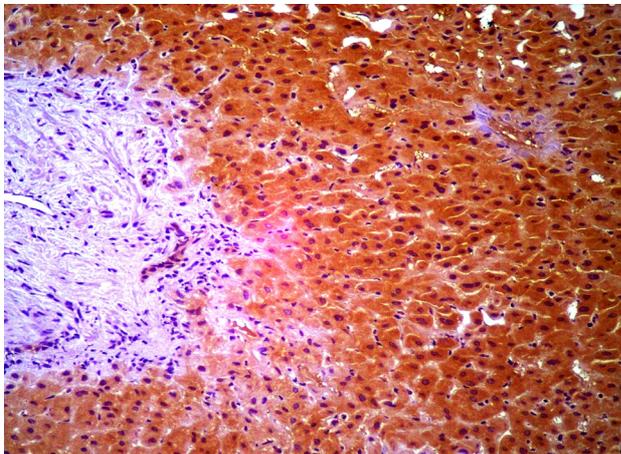
The normal liver architecture in cirrhosis is damaged and for some samples, we noted an interesting aspect: positive hepatocytes alternating with negative ones (patchy or "chess table" aspect) (Figure 4).



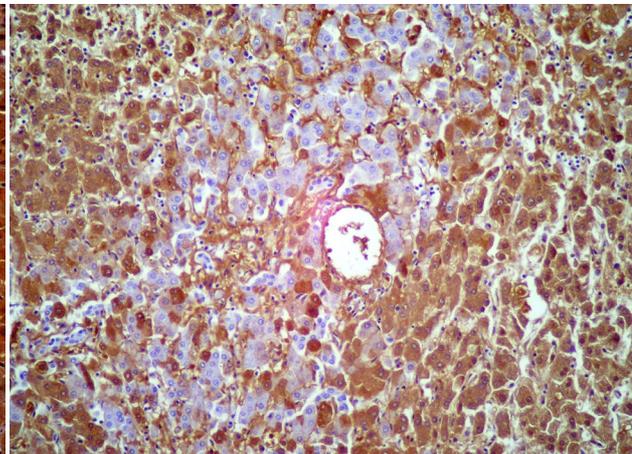
**Figure 1** – Chronic hepatitis with minimal fibrosis. Positivity for TGF- $\beta$ 1 in sinusoidal cells surrounding the portal area and also in some hepatocytes (ob.  $\times 20$ ).



**Figure 2** – Chronic hepatitis with moderate fibrosis. Increased number of TGF- $\beta$ 1-positive activated hepatic stellate cells (ob.  $\times 20$ ).



**Figure 3** – Liver cirrhosis. Intense positive reaction for TGF- $\beta$ 1 in hepatocytes and in ductal epithelium (ob.  $\times 10$ ).

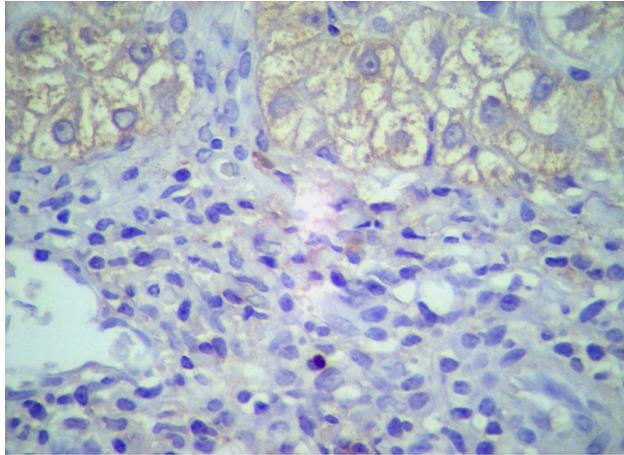


**Figure 4** – Liver cirrhosis. Alternating positive and negative reaction in hepatocytes (patchy aspect) (ob.  $\times 10$ ).

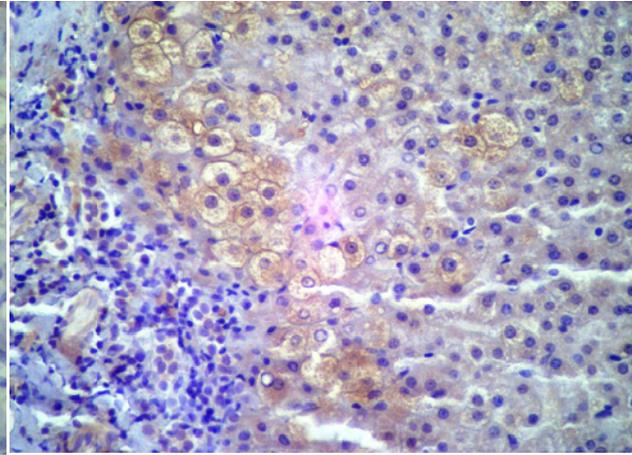
### Localization of CTGF-positive reaction

For the cases with fibrosis F1, the intensity of the immunohistochemical reaction for CTGF was discrete, in few hepatocytes lining the periportal area (Figure 5).

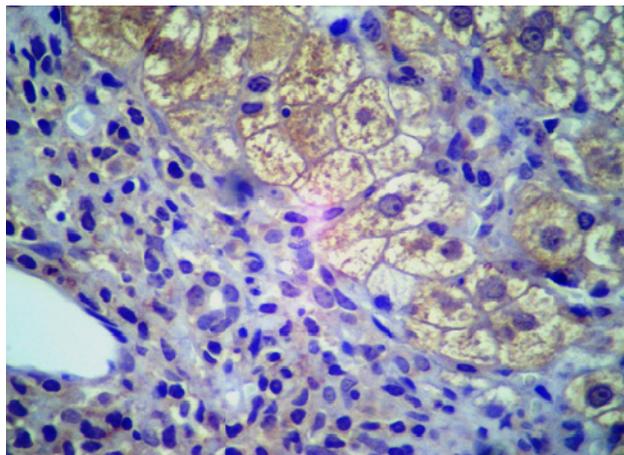
For F2 cases – portal fibrosis and rare fibrous septa – we noted an increase of positive structures for CTGF:



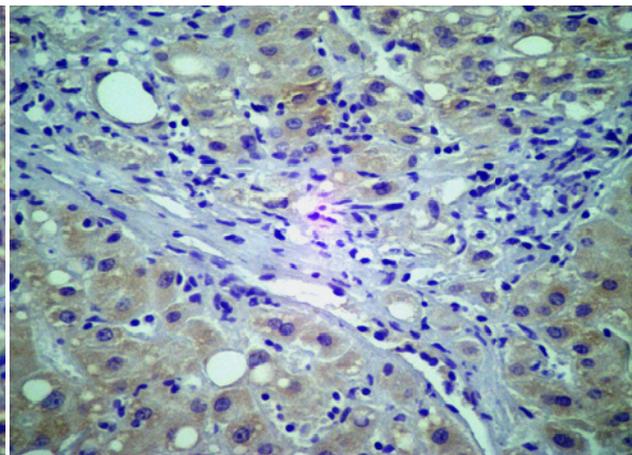
**Figure 5** – Chronic hepatitis with fibrosis F1. CTGF-positive reaction in hepatocytes in the proximity of the portal area (ob.  $\times 20$ ).



**Figure 6** – Chronic hepatitis with fibrosis F2. More intense positivity for CTGF on the periphery of the hepatic lobule (ob.  $\times 20$ ).



**Figure 7** – Chronic hepatitis with fibrosis F3. Parenchymal and inflammatory infiltrate with positivity for CTGF (ob.  $\times 40$ ).



**Figure 8** – Liver cirrhosis. Intense positive reaction for CTGF in hepatocytes (ob.  $\times 20$ ).

### Discussion

In chronic liver viral diseases, hepatic alteration is not specific according to HVB or HVC presence and it is due to the necro-inflammatory 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 [6, 8, 9].

In chronic viral injury, Kupffer cells, hepatocytes, endothelial cells, monocytes, release many proinflammatory cytokines such as platelet-derived growth factor, PDGF, the main mediator of proliferation, and TGF- $\beta$ 1, the most important profibrogenic cytokine [10].

In our study, we observed TGF- $\beta$ 1-positive cells in the periportal and perisinusoidal areas, precisely in the same zones where fibrosis started, as well as in some portal spaces for the F2, F3 and cirrhosis specimens. In cirrhosis, positive and negative cells alternated in the

parenchyma, creating a patchy aspect. Most of the hepatocytes from these specimens were damaged, possibly apoptotic cells.

HSCs activation consecutive to TGF action consists of their transformation into myofibroblasts, able to express desmin, vimentin, smooth muscle actin (alpha-SMA), but also neuroendocrine markers [1]. In a vicious circle, activated HSCs are able to express TGF- $\beta$ 1. Another biologic effect of TGF- $\beta$ 1 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 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- $\beta$ 1 responses, including apoptosis and fibrosis [11].

Body of evidence suggests that TGF- $\beta$ 1 and CTGF synergize to promote chronic fibrosis [12, 13]. CTGF has been described to bind directly to TGF- $\beta$ 1, leading to an enhancement of TGF- $\beta$ 1 activity, by increasing the affinity of TGF- $\beta$ 1 to its receptors [14], by blocking the negative feed-backs for TGF- $\beta$ 1, perpetuating its signaling activation [15] or by stimulating fibroblast mitosis [4, 16].

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

Unlike previous studies that referred to the presence of CTGF only in the perisinusoidal area and in myofibroblasts from the portal spaces [17] or in vascular elements or ductal epithelium [16], our data showed a restricted positivity in the hepatocytes lining the fibrous septa, correlating with the expression of TGF- $\beta$ 1, in accordance with more recent studies, which clearly demonstrate CTGF expression in parenchymal cells, sensitively up-regulated by exogenous TGF- $\beta$ 1 [18, 19].

The presence of CTGF in the same regions a TGF- $\beta$ 1 for specimens with various chronic disease suggests that CTGF, as well as TGF- $\beta$ 1, is a key mediator of fibrosis regardless of its etiology.

Further research will confirm the fact that CTGF plays exclusively a profibrotic role in the liver, blocking the pathogenic pathway of TGF- $\beta$ 1-CTGF might represent a more efficient therapeutic strategy in the treatment of liver fibrosis, excluding the adverse effects of drugs targeting directly TGF- $\beta$ 1.

## ☒ Conclusions

During the progression of liver injury, there is a permanent interrelation between stromal and parenchymal elements, regardless its etiology.

Hepatocytes have a dual role, being the source of many growth factors and their target for autocrine action as well.

TGF- $\beta$  has a pivotal role in liver fibrogenesis by directly controlling EMC synthesis and indirectly by increasing CTGF action.

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## Corresponding author

Daniela Tache, Assistant Professor, MD, Department of Biochemistry, University of Medicine and Pharmacy of Craiova, 2–4 Petru Rareș Street, 200349 Craiova, Romania; e-mail: danatache@rdslink.ro

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