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Connective tissue growth factor (CTGF/CCN2) in serum is an indicator of fibrogenic progression and malignant transformation in patients with chronic hepatitis B infection

Olav A. Gressner [a,*,1], Meng Fang [b,1], Hui Li [b], Lun Gen Lu [c], Axel M. Gressner [a], Chun Fang Gao [b,**]

[a] Wisplinghoff Medical Laboratories, Cologne, Germany
[b] Department of Laboratory Medicine, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China
[c] Department of Gastroenterology, Shanghai First People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

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ABSTRACT

Still a challenging medical problem is the non-invasive monitoring of patients with a variety of chronic liver diseases being on risk to develop fibrosis, cirrhosis, and, finally, primary liver cell carcinoma. Previously, we have shown that CTGF/CCN2, a down-stream mediator of TGF-β, in serum might be a promising non-invasive biomarker of fibrosis, which is extended in the following study to cirrhosis and liver cell carcinoma.

Healthy individuals (n = 56), as well as fibrotic (n = 77), cirrhotic (n = 17), and HCC-patients (n = 72) with chronic hepatitis B (HBV) infection, clinically, biochemically and histopathologically well characterized and classified, were included for the measurements of CTGF-concentrations in serum using a newly developed CTGF-enzyme immunoassay.

A statistical significant increase of the mean serum CTGF-concentrations was associated with different stages of fibrosis, ranging from 15.9 μg/L (S0), 20.3 μg/L (S1/2) to 36.9 μg/L (S3/4). The highest CTGF-concentrations were measured in cirrhotic patients (43.6 μg/L), compared to healthy subjects (17.7 μg/L), followed by a decrease in cirrhotic HCC-patients (38.5 μg/L; $p = 0.001$). Of note, HCC patients without underlying cirrhosis (n = 8) had CTGF levels (13.5 ± 13.2 μg/L) comparable to those in healthy controls. No statistical relation between CTGF levels and parameters of liver injury (e.g. AST, ALT) was noticed, but CTGF levels are correlated negatively with serum albumin levels ($p = 0.007$) and platelet counts ($p = 0.0032$), respectively. The latter was negatively correlated with the stage of fibrosis ($p = 0.025$). In HCC patients, CTGF concentrations decreased with tumor progression and size, with lower levels in TNM stage II (30.5 μg/L) and stage III (33.6 μg/L) compared to TNM stage I (41.6 μg/L).

Our data suggest a valuable diagnostic impact of CTGF in serum for the follow-up of patients suffering from chronic liver diseases developing fibrosis, cirrhosis and finally HCC. CTGF serum levels in HCC are most likely due to underlying fibrosis/cirrhosis but not due to malignancy per se.

1. Introduction

Chronic liver diseases are the fifth most frequent cause of death in the European Union and the United States, as they entail multiple risks, such as portal hypertension, ascites, spontaneous bacterial peritonitis, hepatorenal and hepatopulmonary syndromes, hepatic encephalopathy and, of course, hepatocellular carcinoma (HCC) [1].

Liver fibrosis, and ultimately liver cirrhosis, are the common end-stage of all chronic liver diseases. At the beginning of fibrogenesis stands a chronic inflammatory condition. But it is not the virus- or toxin-induced hepatocellular damage that primarily causes tissue-destruction and the formation of granulation tissue, but the activation of immunocompetent cells (e.g. Kupffer-cells) and the release of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6 and IL-12. These mediators and the accumulation of potentially toxic free fatty acids generate highly reactive oxygen species (ROS), which

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; APRI, ALT-platelet-ratio index; AST, aspartate aminotransferase; BMP, bone morphogenetic proteins; CLD, chronic liver diseases; CTGF, connective tissue growth factor; ECM, extracellular matrix; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcAb, hepatitis B core antibody; anti-HCV, anti-hepatitis C virus antibody; PLT, platelets, thrombocytes; TGF-β, transforming growth factor type β; TNM, tumor, nodes (lymph nodes), metastasis.

* Correspondence to: O.A. Gressner, Wisplinghoff Medical Laboratories, Classen-Kappelmann-Str. 24, 50931 Cologne, Germany. Fax: +49 221 940 505 100.
** Correspondence to: C.F. Gao, Department of Laboratory Medicine, Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, China. Fax: +86 21 65562400.
E-mail addresses: o.gressner@wisplinghoff.de (O.A. Gressner), gaocf1115@163.com (C.F. Gao).

[1] OAG and MF contributed equally.

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expose the hepatocyte to an oxidative stress, which, primarily via peroxidation of membrane lipids and DNA damage, leads to hepatocellular injury. In the meantime, it comes to an activation of mesenchymal cells, resulting in an increased synthesis and interstitial deposition of extracellular matrix components [2]. These mesenchymal cells, hepatic stellate cells (HSC), also known as Ito cells, are pericytes found in the perisinusoidal space of the liver also known as the space of Disse. Following liver injury, HSC undergo "activation" which connotes a transition from quiescent vitamin A-rich cells into proliferative, fibrogenic, and contractile myofibroblasts (MFB). This pathway has long been, and probably still is, considered as the "canonical" pathway in the pathogenic understanding of liver fibrogenesis. The major phenotypic changes after activation include proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss, and white blood cell chemoattraction [2].

In the western countries, most frequent causes of chronic liver failure are of nutritive-toxic origin: chronic alcohol abuse, followed by virus hepatitides. Hereditary causes (e.g. hemochromatosis or Morbus Wilson), autoimmune processes (e.g. primary biliary cirrhosis, primary sclerosing cholangitis or autoimmune hepatitis), metabolic disorders, venous obstruction/liver congestion are in the minority.

More than 50% of all patients with complicated liver cirrhosis die within the first 17 years following diagnosis, mostly from HCC. In more than 90% of all cases, the HCC develops within a cirrhotic liver. Therefore, attenuation of the fibrogenic process can significantly lower morbidity [1].

This urgently requires reliable tools for early diagnosis and continuous monitoring of patients at risk [3]. Due to its highly invasive nature and serious analytical limitations the histological evaluation of liver biopsy specimens is no longer recommended for this purpose [4]. As an alternative, non-invasive procedures like liver elastography to measure the increasing stiffness of the tissue due to accumulating extracellular matrix (ECM) [5], various imaging methods [6], and multi-parametric biochemical scores [7–9] have been developed. About 20 of these algorithms, mostly based on routine biochemical and hematological parameters, are presently recommended for the follow-up of patients at risk to develop fibrosis, cirrhosis and, finally, primary hepatocellular carcinoma (HCC). Previously, we have shown that the diagnostic value of multi-parametric panels is limited due to analytical imprecision and globally unstandardized methods for the measurement of biochemical routine parameters [10]. Thus, both the comparability and reproducibility of grading the activity and staging the extent of fibrotic tissue transition might scatter considerably between the various investigators, which hamper their large-scale application and comparison. We therefore have focused our efforts on finding a single biochemical parameter, which per se is directly involved in the pathogenesis of liver inflammation and fibrogenesis.

CYR61-CTGF-NOV (CCN) 2/connective tissue growth factor (CTGF), a member of the CCN superfamily of secreted, cysteine-rich glycoproteins, has been implicated in the pathogenesis of hepatic fibrosis and is currently suggested to be an important downstream amplifier of the effects of the profibrogenic master cytokine transforming growth factor (TGF)-β [11–13]. Its molecular mechanism of action is still not known in detail, but it very likely strengthens the binding of TGFβ1 to its cognate receptors. Its crucial role in fibrogenesis is documented by strong upregulation in fibrotic liver tissue, and even more importantly by recent studies, in which knock-down of CCN2/CTGF by siRNA leads to substantial attenuation of experimental liver fibrosis (summarized in [14]). We were among the first to identify that hepatocytes (PC) substantially synthesize CCN2/CTGF in cell culture and in injured liver, and that CCN2/CTGF is sensitively up-regulated by TGFβ1 [15].

Significant increases of CTGF in serum/plasma of patients with fibrogenic CLD were shown by us previously using an in-house immunoassay for CTGF [16,17]. Thus, there is good evidence for CTGF as a diagnostic relevant fibrogenic master switch in fibrotic CLD [18].

In the present study we evaluated a new commercial ELISA for CTGF which is based on our previous assay to measure CTGF concentrations in the serum of patients with various stages of developing fibrotic liver diseases and, for the first time, HCC. The data suggest CTGF in serum as a promising single-type biochemical parameter for the diagnosis and follow-up of patients with CLD.

2. Materials and methods

2.1. Patients

A total of 222 serum samples, including patients with liver fibrosis (n = 77), liver cirrhosis (n = 17), HCC (n = 72), and healthy control subjects (n = 56), were collected in this study. Patients with HBV-infected liver cirrhosis and HBV-related HCC were recruited from the Eastern Hepatobiliary Hospital, Shanghai, China. All enrolled patients with HCC were diagnosed with histological confirmation, while liver cirrhosis was diagnosed by the physical condition of the patient and by imaging techniques. The HCC stage was classified according to the TNM-criteria [19] and the liver function was scored according to the Child–Turcotte–Pugh classification [20]. Patients with liver fibrosis suffering from chronic hepatitis B virus (HBV) infection were recruited from the first people's hospital, Shanghai Jiaotong University, China. All selected patients received liver biopsy directed by ultrasonography within 1 week after admission, using a needle with an internal diameter of 1.4 mm (G14, Quick-Cut; Hakko. Company, Japan). A minimum length of at least 1.0 cm of the liver biopsy and at least 6 portal tracts were required for diagnosis. Specimens were fixed in 10% formalin, embedded in paraffin, followed by hematoxylin-eosin (HE) staining and Masson's trichrome staining. Histological staging was blindly and independently determined by two pathologists using Scheuer's classification from stage 0 to stage 4 [21]. Moreover, patients with HAV, HCV, HEV, or HIV infection, alcoholic liver disease, autoimmune liver disease, and drug-related liver disease were excluded from the study. 56 cases of sex and age matched healthy subjects were recruited from Eastern Hepatobiliary Hospital, Shanghai, China and served as a control group. The study protocol was approved by the Chinese Ethics Committee of Human Resources, Eastern Hepatobiliary Hospital. Additionally, informed consent was obtained from all participants for the use of their blood in this study.

Table 1
Compilation of personal and laboratory data (mean ± SD) of the patient study and control groups (n = 225).

	n	Male(%)	Age	Bilirubin [μmol/L]	Albumin [g/L]	ALT [U/L]	AFP [μg/L]	PLT [$\times 10^9$/L]
Normal	56	35 (62.5)	50.93 ± 6.2	13.0 ± 4.8	47.6 ± 2.4	20.2 ± 9.8	3.4 ± 2.6	226 ± 43
Fibrosis stage (Scheuer)								
0	9	5 (55.6)	36.44 ± 8.9	17.4 ± 7.7	45.3 ± 5.5	54.1 ± 27.9	2.65 ± 1.4	180 ± 60
1	18	13 (72.2)	34.2 ± 5.9	21.6 ± 25.2	42.5 ± 2.9	174.3 ± 86.8	3.4 ± 0.1	186 ± 59
2	19	15 (78.9)	31.7 ± 11.1	32.4 ± 37.7	41.2 ± 4.1	348.3 ± 428.8	11.8 ± 10.1	198 ± 51
3	15	14 (93.3)	32.7 ± 6.6	49.3 ± 52.5	39.1 ± 4.3	328.8 ± 252.3	61.8 ± 78.6	184 ± 59
4	16	16 (100)	39.4 ± 9.6	40.0 ± 56.2	38.7 ± 7.0	253.6 ± 612.8	55.4 ± 83.4	118 ± 53
Cirrhosis	17	14 (82.4)	48.8 ± 8.5	35.7 ± 19.6	34.8 ± 7.3	158.3 ± 468.2	25.2 ± 37.9	71 ± 36
HCC	72	61 (84.7)	49.3 ± 10.5	15.4 ± 6.2	41.7 ± 4.1	44.6 ± 26.4	199.8 ± 311.2	157 ± 67