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CXCL10 antagonism and plasma sDPPIV correlate with increasing liver disease in chronic HCV genotype 4 infected patients ☆

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ABSTRACT

Egypt has the highest prevalence of hepatitis C virus infection worldwide. CXCL10 is a potent chemoattractant that directs effector lymphocytes to sites of inflammation. It has been reported that plasma CXCL10 is processed by dipeptidylpeptidase IV (DPPIV) thus leading to the generation of an antagonist form. Using Luminex-based immunoassays we determined the concentration of different forms of CXCL10 (total, agonist, and antagonist). We also evaluated plasma soluble DPPIV (sDPPIV) concentration and plasma dipeptidylpeptidase (DPP) activity. Using flow cytometry and immunohistochemistry, we analyzed the distribution of lymphocyte subsets. Plasma CXCL10 was elevated in chronic HCV patients, however the agonist form was undetectable. Increased sDPPIV concentration and DPP activity supported the NH_2-truncation of CXCL10. Finally, we demonstrated an increased frequency of $CXCR3^+$ cells in the peripheral blood, and low numbers of $CXCR3^+$ cells within the lobular regions of the liver. These findings generalize the observation of chemokine antagonism as a mechanism of immune modulation in chronic HCV patients and may help guide the use of new therapeutic immune modulators.

1. Introduction

Hepatitis C virus (HCV) infection poses a major public health problem with an estimated 130–170 million infected individuals worldwide [1]. Chronic infection can progress to fibrosis, cirrhosis and/or hepatocellular carcinoma. The high incidence of HCV genotype 4 in Egypt (14.7% of the adult population is HCV seropositive) provides the unique opportunity to evaluate HCV disease pathogenesis [2]. The origin of the HCV epidemic in Egypt has been attributed to schistosomiasis treatment campaigns in the 1960s and 1970s [3]. As part of a clinical investigation that evaluated the use of non-invasive methods for evaluating liver fibrosis (ANRS 12184), we conducted a sub-study to define the role of CXCL10 in HCVg4 disease pathogenesis.

CXCL10 (also known as interferon-gamma induced protein 10 or IP-10) has been investigated in the context of HCV disease pathogenesis and noted to be a negative predictor of response to pegylated interferon/ribavirin (peg-IFN/RBV) therapy. Hepatocytes and immune cells present in the inflamed liver have been identified as the dominant source of CXCL10 in patients with chronic HCV infection [4]. Based on the expression pattern of its receptor CXCR3, CXCL10 is presumed to play a major role in the recruitment of activated $CD4^+$ T cells, $CD8^+$ T cells and NK cells to the liver parenchyma [5]. Given the antiviral effector function of such

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[1] These authors are patent holders for the CXCL10 assay used in this study.

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lymphocytes, it was paradoxical to find higher plasma levels of CXCL10 in patients that would ultimately fail peg-IFN/RBV therapy [6–10]. We have previously reported that circulating CXCL10 in HCVg1 patients is catabolized by the enzyme dipeptidylpeptidase IV (DPPIV; also known as CD26), generating an antagonist form of CXCL10 [11]. These results clarified the correlation with treatment failure, and introduced the possibility of inhibiting DPPIV as a mechanism of enhancing treatment response [11].

Herein, we investigated the role of CXCL10-mediated chemokine antagonism in a population of chronic HCVg4 Egyptian patients. Our aim was to extend our prior data and validate DPPIV as a potential therapeutic target in Egyptian patients. Using unique ELISA assays [12], we examined agonist and antagonist forms of CXCL10, as well as soluble DPPIV (sDPPIV) plasma concentration and dipeptidylpeptidase (DPP) activity. These data were correlated with measures of liver fibrosis. Based on our findings, we conclude that CXCL10 chemokine antagonism is a general feature of HCV disease pathogenesis and is associated with liver fibrosis.

2. Materials and methods

2.1. Subjects and sample collections

Seventy patients (Table 1) with chronic HCV were recruited at a single study site, The National Hepatology and Tropical Medicine Research Institute (NHTMRI) in Cairo. Inclusion criteria included age 18–59, positive HCV RNA as confirmed by a qualitative PCR, and being naïve to anti-HCV therapy. Twenty uninfected age-matched individuals were confirmed to be HCV negative and were studied as a control population. Data from one chronic HCV patient was removed as values for all biochemical studies were above the limit of detection. All donors provided informed consent and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. This pathogenesis sub-study was part of the ANRS 12184 study entitled: "Liver fibrosis evaluation among HCV genotype 4 infected patients in Egypt" (approved by local IRB, October 2009). Heparinized blood samples (5 ml) were collected from each donor, and plasma was separated by high-speed centrifugation and stored at −80 °C. Whole blood (3 ml) was also collected in BD P700 tubes (pre-loaded with a DPPIV inhibitor) and plasma was separated by high-speed centrifugation and stored at −80 °C until use. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by density gradient centrifugation over Ficoll–Hypaque (Lonza, BioWhittaker), and used for cytometric studies.

Table 1
Summary of chronic HCV cohort patient data.

	Chronic HCV patients
Age in years: median (range)	38 (19–58)
Gender	
Male	43
Female	27
ALT median IU/ml (range)[a]	44 (19–245)
BMI median kg/ml2 (range)[b]	26.3 (18.1–36.6)
Fibrosis stage	
Metavir F score (0/1/2/3/4)[c]	1/28/19/15/7
Actitest score median (range)[e]	0.33 (0.07–0.8)
Fibrotest score median (range)[d]	0.51 (0.04–0.98)
Liver sections	
Biopsy size median cm (range)[e]	1.5 (0.6–2)
No portal tracts median (range)[e]	12 (5–20)
Viral genotype	
HCV genotype 4	68
Not typed	2

[a] Data available for 62 patients.
[b] Data available for 53 patients.
[c] Data available for 69 patients.
[d] Data available for 51 patients.
[e] Data available for 61 patients.

2.2. Flow cytometry

Freshly isolated PBMCs were resuspended (10^6 cells per ml) in FACS buffer and stained. Antibodies used included CD3-FITC, CD19-PECy5 (eBioscience, San Diego, CA, USA) and CXCR3-PE (BD Biosciences, San Jose, CA, USA). Appropriate isotype-matched monoclonal antibodies were used to establish gating parameters. Data was collected using a four-colour Guava cytometer (Merck Millipore, France) and analysis was performed using FlowJo software (TreeStar, La Jolla, USA).

2.3. Measurement of CXCL10, sDPPIV and DPP activity

Total, agonist (1–77a.a.) and NH_2-truncated (3–77a.a.) forms of CXCL10 were measured in the plasma isolated from the BD P700 tubes [11]. Briefly, plasma samples were clarified by a high-speed centrifugation and analyzed using Luminex xMAP technology. Samples were measured by a CLIA certified diagnostic lab, (validated using guidelines set forth by the Clinical and Laboratory Standards Institute), *Myriad-Rules Based Medicine* (Austin, TX, USA). The least detectable dose (LDD) was determined as the mean + 3 standard deviations of 20 blank readings [12]. sDPPIV levels were measured in heparinized plasma using human DPPIV (CD26) DuoSet ELISA (R&D Systems, UK). DPP activity was assessed using DPP-Glo™ Protease assay (Promega, Madison, WI, USA). For the latter assay, titration studies were performed in order to ensure that samples were assayed in the linear range of the test; a final concentration of 0.5% plasma diluted in 0.01% Prionex/PBS was used.

2.4. Histological analyses

Liver biopsy was performed on all patients and Metavir and Actitest score was determined. METAVIR fibrosis scoring system was applied as previously reported [13]. Briefly fibrosis stage was ranked F0–F4; F0: no fibrosis, F1: portal tract expansion by fibrosis, F2: <50% bridging fibrosis, F3: >50% bridging fibrosis (including incomplete cirrhosis), F4: established cirrhosis. From 61 of the study patients, biopsy material was available for research study. 1.5 cm Length of tissue (or an area encompassing a minimum of 10 portal areas) samples were fixed in 10% formalin from which paraffin blocks were prepared. Immunohistochemical staining was applied to coated slides with antibodies against CD3, CD20, CD4, CD8 (Dako, Glostrup, Denmark), DPPIV (Thermo-Fisher, Waltham, MA, USA), CXCR3 (BD Biosciences, San Jose, CA), and CXCL10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) applied for 30 min at room temperature. The biotin-free EnVision detection kit (Dako), 3,3′-diaminobenzidine (DAB; brown colour) and Chromogen (red colour) were used to identify positive antibody labelling. For enumeration of staining all cells observed in portal areas were counted and the mean was reported, for lobular areas three lobules per section were counted and the mean was reported.

2.5. Statistical analyses

Data was analyzed using Prism 5 software (GraphPad, La Jolla, CA). Patient and control groups were compared using a two-tailed Mann–Whitney test. According to the null hypothesis, p-values less than 0.05 were considered significant. Correlations between parameters were determined using Spearman's correlation