Non-invasive method for the evaluation of IL-6 and IL-10 levels in patients with chronic hepatitis C

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Abstract
With over 180 million people affected worldwide, hepatitis C is considered a global problem. In order to establish chronic infections, the virus interferes with cellular mechanisms coordinated by cytokines’ network. Chronic HCV infection is associated with oral manifestations including periodontal disease. The aim of this study was to evaluate the levels of IL-6 and IL-10 in gingival crevicular fluid (GCF) versus plasma, in patients with chronic hepatitis C, in order to estimate the potential of oral fluids as substitute of blood for the evaluation of immune responses in this disease.

Material and methods. Sample-pairs consisted of blood and GCF harvested from 32 anti-HCV positive patients (age range 35-74 y.o) and from control group (10 subjects without HCV infection), which were tested for anti-HCV antibodies, HCV viral loads and IL-6 and IL-10 levels. The dental evaluation was based on CPITN criteria.

Results. All investigated patients were anti-HCV positive in plasma and GCF and HCV RNA was detected in 20/32 sample pairs. Significant differences were observed for IL-10 in plasma versus GCF (p=0.0025), but not for IL-6 (p= 0.44). The expression of IL-10 in GCF displays a significant correlation with the CPITN index.

Conclusions. Elevated GCF levels of IL10 correlated with higher CPITN index and viremia, accounting for usefulness of GCF as an alternative non-invasive source for IL-10 detection in HCV positive cases.

Introduction
Hepatitis C virus (HCV) infection is a global health problem that affects 180 million people worldwide, near four million people being infected yearly (M. BERENGUER & al. [1.]). HCV causes acute liver disease of which near 80% leads to chronic infection with risk of subsequently development of cirrhosis and hepatocellular carcinoma (U.A. ASHFAQ & al. [2]). HCV infection induces both humoral and cellular immune response whose amplitude might lead to viral clearance or persistence. On the other hand, to circumvent the immune response and establish chronic infections, HCV acts either through high mutational rate in its genome (due to low fidelity of RNA polymerase) or through interferences with cellular mechanisms coordinated by cytokines’ network (P. FALLAH & al. [3]). The large family of cytokines is divided according to the role they perform in the immune system regulation as pro-inflammatory (IL-1, IL-6, tumor necrosis factor (TNF)-alpha); T-helper 1 cytokines, (interferon-gamma, IL-12, IL-18) and T-helper 2 cytokine (IL-4, IL-5, IL-10). In liver tissue, cytokines have a dual role, coordinating both physiological (growth and regeneration) and pathological (fibrosis, cirrhosis) processes (A.R. ZEKRI & al. [4]). In HCV infection, pro-inflammatory cytokines induced by viral proteins (like NS5B) and viral genome (dsRNA) (G.Y. YU & al.[5]) create an inflammatory environment in hepatic parenchyma, leading to a
chronic inflammation. In turn, chronic inflammation induced by this type of cytokines (like IL-6) promotes the progression of liver disease and increase the risk for hepatocarcinoma development (V.W. WONG & al. [6]). On the other hand, T-helper 2 (Th 2) cytokines can augment the humoral response. Elevate levels of Th 2 cytokines (like IL-10) are associate with persistent HCV infection and chronic disease (X.G. FAN & al. [7]). Therefore the pattern of cytokines secreted in HCV infection might have a great influence on final outcome (M.S. EBEID & al.[8], (P. FALLAHI & al. [3]). On the other hand, chronic HCV infection is associated with several oral manifestations like mucosal membrane jaundice, bleeding disorders, gingivitis, gingival bleeding (V.E. PANOV [9]). Among these manifestations, periodontal disease might be linked to viral chronic hepatitis. The pattern of cytokines from oral fluids might be influence by both HCV infection and oral diseases. Moreover, oral fluids like saliva and gingival crevicular fluid (GCF) proved to be alternate biological samples for detection of anti-HCV antibodies (V. GONZALEZ & al. [10]) and HCV RNA (T. SUZUKI & al. [11]). GCF is an ultra-filtrate of plasma that contains low concentration of IgG and IgM playing a major role in antimicrobial defence of the periodontium. The aim of this study was to evaluate the levels of IL-6 and IL-10 in GCF versus plasma, in patients with chronic hepatitis C, in order to estimate the potential of oral fluids as substitute of blood for investigations related to immune responses in HCV infection.

Material and Methods

**Subjects.** 32 patients, age range 35-74 years old, anti-HCV positive were recruited from Bals Infectious Disease Clinic, Colentina Hospital and a private dental office. The subjects were clinically and para-clinically evaluated, including liver function tests. Also, a control group consisting of 10 subjects without HCV infection (negative for HCV by both anti-HCV-Ab and HCV RT-PCR) was selected. All the included subjects underwent an oral cavity examination. The dental evaluation was based on CPITN criteria (a screening procedure for clinical assessment of periodontal pockets, calculus and gingival bleeding (J. AINAMO & al.[12]). CPITN index is used to quantify the degree of periodontal damage, giving information on diagnosis and treatment. All the teeth were examined and the samples were harvested from the sextant with the highest score. From each subject a sample-pair consisted of blood and GCF was collected after written informed consent was obtained.

**Blood** samples harvested on EDTA-treated tubes were depleted through centrifugation for 15 minutes at 2000 x g and resulted plasma were transferred into Eppendorf tubes.

**GCF** samples were collected with filter cones, carefully to avoid gingival bleeding and saliva contamination. Blood contaminated cones were removed. All samples were collected in the same day and stored at – 80 C before use.

**Anti-HCV antibodies detection** in plasma and GCF was performed with DIA.PRO (Diagnostic Bioprobes SRL, Milano, Italia), according to the manufacturer’s instructions.

**HCV RNA load** was determined by Geno-Sen’s HCV real-time PCR Kit (Professional Biotech Pvt. Ltd, India) according to the manufacturer’s instructions. The PCR was performed using Rotor Gene (Corbett Research, Australia). The RT-PCR program consisted of 15 minutes at 50°C (for cDNA synthesis), 10 minutes at 95°C (for initial denaturation) and 45 cycles, including denaturation of 95°C for 15 seconds, 20 seconds annealing at 55°C and an extension for 15 seconds at 72°C. The kit uses a set of standards for the delivery of the calibration curve in order to determine the viral load of the analyzed samples. The detection limit of the kit is 30 IU/ml HCV RNA.

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**IL-6 and IL-10 assays** were performed by enzyme immunoassay (Mabtech, Sweden) according to manufacturer's instructions. The results were calculated based on comparisons with standard curves.

**Statistical methods:** Results were analyzed using GraphPad Prism 5.0 software. Column statistics was performed to generate mean value (min÷max), and Mann-Whitney test (t-test for column analyses) with a confidence interval of 95% was used to compare each set of cases with the controls. According to P value (statistic significance), the difference between cases and controls was considered to be non significant (NS) when >0.05, significant (S) when < 0.05 and highly significant (HS) p< 0.01. Linear regression was performed for correlations between the cytokines levels in GCF and CPITN index.

**Results and discussions**

The investigated group consisted of 32 subjects (mean age of 55.4 years), 18 woman (mean age 58.16±10.43) and 14 men (mean age 51.25±14.95). All the plasma and GCF samples from infected patients were positive in ELISA, with an immunoreactivity of 4.28 ± 3.24 and 2.94 ±2.32 respectively. HCV RNA levels were detected in 20/32 sample pairs of plasma and GCF (with a detection limit of 30IU/ml). The mean values for viral load in plasma and GCF are presented in Table 1.

<table>
<thead>
<tr>
<th>Table 1. The viral load levels of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral load</strong></td>
</tr>
<tr>
<td>Male (n=14)</td>
</tr>
<tr>
<td>GCF (mean)</td>
</tr>
<tr>
<td>Plasma (mean)</td>
</tr>
<tr>
<td>p values (plasma vs GCF)</td>
</tr>
</tbody>
</table>

The results regarding the investigated ILs showed that the mean values in GCF were 195.37 pg/ml for IL-6 and 44.65 pg/ml for IL-10. By comparison, the mean values of plasmatic levels were 319.33 pg/ml for IL6 and 94.05 pg/ml for IL10. Significant differences were observed for IL-10 in plasma versus GCF (p=0.0025), but not for IL-6 in plasma versus GCF (p= 0.44). On the other hand, in control group the mean values for IL-6 were 25.2 pg/ml in GCF and 35.3 pg/ml in plasma. Mean values of IL-10 were 23.41 pg/ml in GCF and 53.8 pg/ml in plasma. Overall, IL-6 and IL-10 levels in plasma versus GCF from patients with chronic hepatitis C are presented in figure 1.

**Figure 1.** IL 6 (a) and IL 10 (b) levels in GCF versus plasma harvested from subjects with chronic hepatitis C. IL-10 levels in plasma and GCF of patients and controls are shown in figure 2.
Taking into account the role of dental status on ILs profile, further in this study the patients were divided in four groups, according to CPITN index: group I- CPITN 1 (no bags, tartar, mild bleeding on palpation; incipient gingivitis), group II- CPITN 2 (cases with pockets less than 3 mm, with calculus scaling; gingivitis installed), group III- CPITN 3 (cases with 4-5 mm pockets and a mild form of periodontal disease) and group IV- CPITN 4 (cases with pockets greater than 6mm and installed periodontal disease). Moreover, as not all the subjects from groups I and II presented detectable levels of HCV RNA, they were subdivided according to this parameter (table 2).

**Table 2. IL-6 and IL-10 levels in GCF and plasma, according to CPITN index and viremia**

<table>
<thead>
<tr>
<th>CPITN Index</th>
<th>Patients</th>
<th>Controls</th>
<th>HCV RNA</th>
<th>IL-6 [pg/ml]</th>
<th>IL-10 [pg/ml]</th>
<th>HCV RNA [IU/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cases</td>
<td></td>
<td></td>
<td>GCF</td>
<td>Plasma</td>
<td>GCF</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td></td>
<td>negative</td>
<td>(min-max)</td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td></td>
<td>positive</td>
<td>(min-max)</td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td></td>
<td>Positive</td>
<td>(min-max)</td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td></td>
<td>positive</td>
<td>(min-max)</td>
<td>mean</td>
<td>mean</td>
</tr>
<tr>
<td>0-1</td>
<td>4</td>
<td></td>
<td>positive</td>
<td>(min-max)</td>
<td>mean</td>
<td>mean</td>
</tr>
</tbody>
</table>

**In GCF, elevated levels of IL-6 and IL-10 were found in subjects with higher CPITN index and viremia. Comparing the groups of patients with control, significant differences were found for group IV (p=0.007). Analyzing the plasmatic levels of interleukins, it was**
found that IL-6 displayed higher levels in patients with HCV RNA. As IL-6 levels were higher in GCF comparing with plasma, it is to assume that the amounts of this cytokine reflect both the viral disease and the dental status. Plasmatic IL-10 levels were higher than those from GCF, suggesting that the expression of this cytokine is less related oral health status but to viremia. However, using linear regression it was found that the expression of IL-10 in GCF displays a significant correlation with the CPITN index (fig. 3).

Figure 3. The correlation between GCF levels of IL-6 (a) and IL-10 (b) and CPITN index

The data obtained showed that IL-6 presented elevated levels in both GCF and plasma as compared to control. In both cases its expression seems to correlate with HCV RNA as high levels of serum IL-6 were reported in chronic liver disease of viral ethiology (N.E. SPANAKIS & al. [13], Y. OYANAGI & al14) Moreover, Malaguarnera et al reported that in chronic HCV patients, serum IL-6 levels correlate with viral load and histological index (M. MALAGUARNERA & al. [15]). On the other hand, lower levels of IL-6 strongly associated with sustained virologic response (SVR), mainly in men (M. UEYAMA & al. [16]). In our group of patients, we found no significant differences between plasmatic levels of IL-6 in men versus women (p=0.77). Also for plasmatic IL-10 we failed to find differences between genders (p=0.79). In this study that comprises subjects with chronic HCV infection, IL-6 levels are higher in GCF comparing to plasma, with augmented GCF levels in patients with severe CPITN index. As IL-6 is secreted in response to inflammation and local infection its amounts in GCF seems to reflect inflammation related to local oral status and viral disease. In this context, GCF seems not to be a suitable sample for IL-6 quantification in order to evaluate SVR or the resistance to antiviral therapy (PEG-IFN/RBV).

As mentioned, Th2 immune response has been associated with the persistence of HCV infection, IL-10 (as a Th2 type cytokine) displaying suppressive function against the pro-inflammatory responses (W. OUYANG & al.[17]). In our study, IL-10 presented higher amounts in plasma versus GCF. Plasmatic levels of IL-10 were higher in patients comparing to control group but not as elevated as other studies reported (I.M. EL_KADY & al.[18]). However, comparative studies regarding plasmatic levels of IL-10 in HCV positive cases versus control groups are contradictory. It is suggested that high levels of serum IL-10 correlate with poor responses to antiviral therapy (D.G. BROOKS & al[19]). (K.K. FLYNN & al. [20]). In our study, the plasmatic levels of IL-10 in patients seem to be correlated with
disease progression. IL-10 levels in GCF are significantly higher than in control and correlated with CPITN index. Our data correlate with study of Teppe and colab, who found that high levels of IL-10 in oral fluid and oral tissue may play important role in mechanisms of periodontal diseases (E. TEPPE & al. [21]).

There are conflicting data regarding the magnitude of cytokines’ levels in GCF. A comparative study on GCF and serum harvested from pregnant women with periodontal disease revealed that all investigated cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α), except IL-10, were significantly higher in GCF than serum. On the other hand, periodontal depth and bleeding were found to be significantly associated with GCF IL-6 levels but not to serum levels (T. FIORINI & al. [22]). Another study conducted on IL-6 and IL-8 in GCF of patients with chronic periodontitis, failed to found a strong relationship between cytokines amounts and periodontal destruction and inflammation.

Conclusions

Regarding the ILs, higher levels of IL-6 were found in patients with HCV RNA, without significant differences between plasmatic levels in men versus women. Higher levels of IL-6 in GCF associated with poor oral health. On the other hand, no differences between genders was observed for IL-10. IL-10 elevated levels correlated with higher CPITN index and viremia, accounting for usefulness of IL-10 as marker of disease progression to chronic HCV infection. The obtained data suggest that GCF might be used as an alternative source for IL-10 detection in HCV positive cases.

References

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