Differential Expression of Toll-like Receptors 7 & 8 mRNA in Monocytes of Patients with Chronic Hepatitis C Infection: Correlation with Interferon and Ribavirin Treatment

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Toll-like receptors (TLRs) have been identified as key regulators of innate and adaptive immune responses in viral infection. The contribution of the host immune response to sustained virologic response is not clear in patients with chronic hepatitis C virus (HCV) infection. This study aimed to investigate the expression of TLRs 7 & 8 mRNA in monocytes of patients with chronic (HCV) infection showing different responses to interferon and ribavirin treatment. The study group was comprised of 21 chronic HCV infected patients. mRNA levels of TLRs 7 & 8 in monocytes were evaluated using real-time PCR before a 48-week treatment with pegylated interferon (PEG-IFN) α-2b and ribavirin. In addition, we studied TNF production in monocytes using three-color immunofluorescence and flow cytometry after TLRs 7 & 8 ligand (R848) stimulation. According to the virological outcome of the treatment, the infected patients were classified into non-responders (NR) and sustained virological responders (SVR). The study determined that mRNA levels of TLRs 7 & 8 were significantly high in SVR compared to NR. Furthermore, after stimulation with R848 the median fluorescence intensity for TNF protein was significantly high in SVR compared to NR. Our data show that a differential mRNA expression of TLRs 7 & 8 is associated with different responses to IFN-based antiviral therapy in patients with chronic HCV. These findings suggest that the TLRs-expression profiles of monocytes from patients with chronic HCV may be useful biomarkers for IFN therapy.
(TLRs) that are expressed on monocytes can signal cytokine release, cellular activation, and upregulation of the MHC Class I or Class II (Takeda et al., 2003), and thus help link the innate and the adaptive immune responses. TLRs 7 & 8 are members of the toll like family of receptors (Medzhitov & Janeway 2000), recognize single-stranded viral RNA (ssRNA) (Diebold et al., 2004), lead to NF-kB activation, and promote the production of cytokines such as IL-12 and TNF-α (Bekeredjian-Ding et al., 2006). TLR7 and TLR9 agonists have been shown to have clinical efficacy against HCV (Horsmans et al., 2005). It is still unknown whether TLRs 7 & 8 expression in monocytes is related to disease progression in HCV infected patients or whether it represents a protective factor in those patients who are slow disease-progressors.

In this study, we investigated relationship of TLRs 7 & 8 expression with the outcome of antiviral therapy in patients with chronic HCV. We measured mRNA expression of TLRs 7 & 8 in monocytes derived from patients with chronic HCV at baseline, and correlated these with the sustained virologic response rates.

**Patients and Methods**

**Patients**

This study was carried out at the Immunology Research Lab, Department of Microbiology and Immunology, Faculty of Medicine, Zagazig, Egypt. Twenty one patients with virologically and biochemically diagnosed chronic hepatitis C (ages 23-48 years, 5 females and 16 males), attending the outpatient clinic of the Internal Medicine, Faculty of Medicine, Zagazig, Egypt. The study was approved by the ethical committee of the hospital and written informed consent was obtained from each patient. A detailed clinical history and a complete physical examination were carried out for each patient. None of patients had received antiviral treatment before entry into the study, in order to avoid the possible effect of therapy on the expression of TLRs (such effect has been described by Vollmer et al., 2004). Routine laboratory methods were used to determine serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and viral load in HCV patients. None of the patients had serological evidence of co-infection with hepatitis B, HIV, or clinical signs of an autoimmune disease prior to treatment with IFN. The characteristics of the patients at baseline are shown in (Table 1). Patients were administered PEG-IFN-α-2b (PegIntron) at a dosage of 1.5 µg/kg per week subcutaneously and ribavirin 15 mg/kg per day orally for 48 weeks. Serum HCV-RNA was measured at the baseline, at the 12th, 24th and 48th weeks during treatment, and after 24 weeks of untreated follow-up (study week 72) using the LightCycler system (Roche Diagnostics, Germany). Sustained virologic responders (SVR) were defined as subjects with undetectable HCV RNA in the serum at 24 weeks post treatment, while subjects with positive HCV RNA at the end of the treatment were defined as non-responders (NR).

**Isolation, culture, and stimulation of monocytes**

At baseline, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of heparinized blood by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation; monocytes were further isolated using CD14+ conjugated magnetic beads (Miltenyi Biotech, Germany) for the positive selection according to the manufacturer’s instructions. The purity of the monocytes population was > 97% as determined by flow cytometry with Fluorescein isothiocyanate (FITC) -conjugated anti-CD14 antibody (Beckman Coulter, Germany) and phycoerythrin (PE) -conjugated anti-CD16 antibody (BD Biosciences). For direct determination of absolute numbers of cells, we used CountBright1 absolute counting beads (Invitrogen, Karlsruhe, Germany) (Heimbeck et al., 2010). Monocytes were cultured in RPMI 1640 (Sigma Diagnostic Louis, Mo.) supplemented with L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen cooperation, Grand Island, NY.) and 10% FCS. Monocytes were stimulated with TLRs 7 & 8 ligand R848 (made up at a concentration of 1 mg/ml in sterile endotoxin-free water; InvivoGen, San Diego, California) at 1 µg/ml (Bekeredjian-Ding et al., 2006) or medium alone (controls) in cultures at 37°C, 5% CO2 for 6 h in the presence of 10 µg/ml Brefeldin A (Sigma, Germany). The stimulated cells were permeabilized with Perm/Wash solution (BD Biosciences, PharMingen) for 5 min at room temperature. For detection of intracellular TNF, we added PE-conjugated anti-TNF Ab (Caltag, Germany) or as isotype control PE-conjugated rat IgG1 (Caltag, Germany) both at 10 µg /ml for 20 min (Fig. 1). The
cells were resuspended in 400 µl of PBS for acquisition and analysis by flow cytometry.

Measurement of TLRs 7 & 8 mRNA expression

Total RNA was extracted from monocytes using the RNeasy Protect Mini Kit (QIAGEN) according to the manufacturer’s protocol and reverse-transcribed as for conventional RT-PCR (Roche Diagnostics, Germany). cDNA were used for amplification in the SYBR green format using the LightCycler FastStart DNA Master SYBR Green (Roche Diagnostics, Germany). The following primer pairs were used: TLR7 5’ primer, 5'-TTA ACC TGG ATG GAA ACC AGC TAI 3' and 3’ primer, 5'-TCA AGG CTG AGA AGC TGT AAG CTA-3' and TLR8 5’ primer, 5'-TGT TAT GAC AGC AGA GGG TGA TG-3' and 3’ primer, 5'-GAG TTG AAA AAG GAG GTT ATA GGA TAA ATCI3'. As an internal control the housekeeping gene αIenolase was amplified (Belge et al., 2002).

Statistical Analysis

The computer program SPSS was used to analyze the data statistically. Student’s t test was used to determine the significance of differences between patients in this study. The results were considered statistically significant if the p-value was <0.05.

Results

Differential expression of TLRs 7 & 8 mRNA between treatment non-responders and SVR at baseline

Chronic HCV infection is associated with increased serum levels of inflammatory cytokines (Neuman et al., 2002). Blood monocytes and tissue macrophages are major sources of these cytokines. It has been reported that increased monocyte production of TNF-α in patients with chronic HCV infection (Dolganiuc et al., 2004). In this study, we used real-time PCR to assess whether TLRs 7 & 8 mRNA expression in monocytes of HCV-infected patients before treatment was associated with sustained virologic response; patients were divided into two groups according to their outcome of therapy as described in patients and methods. The baseline characteristics of patients were comparable between NR (n=8) and SVR (n=13) (Table 1). CD14++ monocytes were purified from PBMC by positive selection using MACS (Fig.1). To detect TLRs 7 & 8 mRNA in the monocytes, we used mRNA analysis by RT-PCR using the LightCycler technology (Fig. 2). The average mRNA level was calculated to be 3.5 fold higher for TLR7 and 2 fold higher for TLR8 in SVR as compared with NR at baseline. TLRs 7 & 8 mRNA levels in SVR were significantly higher at baseline as compared with NR (p < 0.05).

TNF production by monocytes in response to R848 and correlation with treatment in SVR and NR

A key event in TLRs signalling is induction of inflammatory cytokines with activation of innate and adaptive immunity (Takeda et al., 2003). To evaluate the relationship between TNF production by monocytes and TLRs 7 & 8 mRNA, we measured baseline TNF expression of monocytes in HCV-infected patients after R848 stimulation at 1µg/ml for 6 h in the presence of Brefeldin A and stained the cells for CD14 and TNF. As shown in Fig. 3, the average mean fluorescence intensity was 67.9 ± 20 channels in the NR monocytes while the SVR monocytes expressed TNF with a mean fluorescence intensity of 153 ± 58 channels (p < 0.05); i.e., the signal for TNF protein was 2.5-fold higher in SVR monocytes. We did not observe any correlation between TLRs 7 & 8 and ALT, viral load or TNF in the subjects studied.
Table 1. Characteristics of patients at baseline

<table>
<thead>
<tr>
<th></th>
<th>Non responders (n = 8)</th>
<th>Sustained virologic responders (n = 13)</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>34.5 (23-47)</td>
<td>32 (25-48)</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>6/2</td>
<td>10/3</td>
<td></td>
</tr>
<tr>
<td>HCV RNA X10^6 (IU/ml)</td>
<td>0.7 (0.04-5.9)</td>
<td>0.4 (0.03-18.9)</td>
<td>NS</td>
</tr>
<tr>
<td>ALT levels (IU/ml)</td>
<td>47.5 (40-85)</td>
<td>40 (32-86)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data expressed as median and total range
*P >0.05 is not significant (NS).

Figure 1. TNF expression in purified monocytes. (A) R1 was around the purified cells and R2 was around Flow-Count Fluorospheres in the FS/SS dot plot. (B) R3 was around purifiedCD14^{++} monocytes. The purity of CD14^{++} monocytes was > 97% relative to all Mo. (C) Anti-TNF antibody staining (red curve) vs the isotype control (green curve) for R3. Shown are data from one of the experiments.
Figure 2. Expression of TLR7 mRNA in monocytes. mRNA was prepared from SVR and NR monocytes before the treatment with pegylated interferon and ribavirin (purity > 97%) and cDNA was amplified in the presence of SYBR green in the LightCycler. Curves were analyzed by determining the cycle number at which the amplification curves intersect with the horizontal line. In this example, (A) in the case of TLR7: the SVR curve intersected at 23 cycles while the NR curve intersected at 27 cycles. (B) Both cells amplification curves were at 14 cycles for the housekeeping gene. Shown are data from one of the experiments.
Differential Expression of TLRs 7 & 8 mRNA in Monocytes of Patients with Chronic HCV Infection

Figure 3. TNF induction in monocytes of NR and SVR. Monocytes were stimulated with R848 at 1µg/ml for 6 hr in the presence of Brefeldin A. Then the cells were stained for CD14 and TNF. Shown was TNF expression by monocytes in a NR (left panel) and SVR (right panel). Shown are data from one of the experiments.

Discussion

HCV load, genotype, and fibrosis have been listed as factors that influence the effectiveness of IFN therapy (Martinot-Peignoux et al., 1995), but these factors are not sufficient, and other predictive factors are needed. TLRs 7 & 8 are members of TLR family which is directly involved in the recognition of specific viral components, regulation of immune response and clearance of the invading organisms and their activation by microbial components leads to the production of cytokines and interferons. It has been shown previously that the TLRs 7 & 8 and their ligands play important roles in host-pathogen interaction and prediction of HCV outcomes (He et al., 2006; Thomas et al., 2007). TLRs 7 & 8 are stimulated by synthetic compounds imiquimod and resiquimod (R848). Lund et al., 2004 showed that TLR7 recognizes the single-stranded RNA viruses, vesicular stomatitis virus and influenza virus. The recognition of these viruses by plasmacytoid dendritic cells and B cells through TLR7 results in cellular activation and the production of cytokines. HCV is a single stranded RNA virus, thus, it could theoretically act as a ligand for TLR7.

Imiquimod, a TLRs 7 & 8 ligand, has been approved for the therapy of genital warts (Dockrell & Kinghorn 2001), and there are several trials to be used as adjuvant therapy in HCV patients (Pockros et al., 2007). If TLRs 7 & 8 are associated with the recognition of specific viral components, it is important to characterize their expression in the monocytes of patients with chronic HCV infection to further understand pathogen-host interactions and predicted the outcomes. This study to our knowledge represents the first analysis of the expression of TLRs 7 & 8 in monocytes of the Egyptian patients with chronic HCV. Unlike liver-biopsy specimens, monocytes can be easily collected, collection can be repeated as necessary and they exhibit a broader repertoire and express higher levels of TLRs mRNA compared with other tissues in humans (Zarember & Godowski 2002). Therefore, we analysed mRNA expression levels of TLRs 7& 8 and TNF production in monocytes of HCV infected patients before the initiation of ribaverin and interferon therapy.

Our results demonstrate that TLRs 7 & 8 mRNA levels are significantly high in SVR compared to NR. This result is in agreement
with the result of Taylor et al., 2007 who demonstrated decreased level of TLR 7 in non-responder patients. On the other hand, the result of our study is different from the study of Dolganuic et al., 2006 who showed that TLR 7 mRNA is significantly down regulated in chronic HCV patients. This difference could be explained by the difference in viral genotypes, other associated viral infection, different methodological approaches, and different clinical stages. The expression of gene is a yield of multiple processes which is reflected by post transcriptional process and other regulatory influences (Chen et al., 2002). Consequently, mRNA levels of TLRs do not usually represent the actual expressed protein level (McKimmie et al., 2005), so it recommended to study the TLRs protein expression level as well.

Then, we analyzed whether the variation of TLRs 7 & 8 mRNA is correlated with the levels of serum HCV RNA. Our result shows that patients with SVR have a tendency of lower viral load in comparison to patients with NR but it is not statistically significant. This result is in agreement with the result of He et al., 2006 who found no association between viral load and the expression level of TLRs.

Given that TLRs 7 & 8 receptors on monocytes can recognize viral ssRNA and thus mediate antiviral immune response. We considered whether the stimulation of TLRs 7 & 8 with R848 can initiate differential immune responses between patients with SVR and NR. Several studies reported conflicting results about the pro inflammatory cytokines at the vitro levels or the circulating levels during HCV infection (Xagorari & Chlichlia 2008). Therefore, we studied TNF production in monocytes of SVR and NR before interferone therapy after stimulation with R848. The median fluorescence intensity for TNF protein was significantly high in SVR compared to NR. Our result is different from the previous result by Marei et al., 2011 who found that TNF production in the NR patients was around one and half increase than that shown by SVR. This difference could be explained by the difference of ligand used for stimulation (LPS) of monocytes and studied monocytes subsets.

In this study we have shown that TLRs 7 & 8 have different levels in HCV patients with different outcome of therapy. In these regards, it seems that TLRs 7 & 8 have great importance in controlling HCV infection and the responses to IFN therapy could be predicted on the basis of changes in gene expression by monocytes. However, the data presented here should be considered as a preliminary report from a work in progress that we are currently developing at our laboratory, but they point to the fact that measuring the expression of TLRs could provide a new set of molecular markers for the prognosis of the HCV infection. Nevertheless, further research covering other stages of disease at large number and at different monocytes subsets levels will be needed to confirm their real value.

References


during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy. J Virol, 81, 3391-3401.


