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Research Article

Analysis of Serum Indoleamine 2, 3-Dioxygenase Activity and Expression in Leukocytes of Chronic Hepatitis C Virus-Infected Patients

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Abstract

More than 170 million people are infected with Hepatitis C Virus (HCV) globally. Indoleamine 2, 3-Dioxygenase (IDO) is an immunosuppressive enzyme associated with chronic HCV infection. The underlying immunosuppressive mechanisms of this disease after acquiring viral infection are still unclear. The current study aims to evaluate the IDO expression in leukocytes and IDO activity in serum of treatment naïve patients with chronic HCV infection. The messenger RNA level of IDO in leukocytes was quantified using Real-Time Polymerase Chain Reaction (RT-PCR) in treatment-naïve chronic HCV infected patients (n=25) and healthy controls (n=25). The IDO enzymatic activity was measured by colorimetric assay in the serum of another group of treatment naïve chronic hepatitis C infected patients (n=45) and healthy controls (n=45). No difference in IDO expression was found among leukocytes of chronic HCV infected patients and healthy controls (P=0.07). However, the IDO enzymatic activity was significantly higher among infected patients than healthy controls (P<0.0001). Our results indicate that there is no significant difference of IDO expression in leukocytes of chronic HCV infected patients and healthy control. IDO enzymatic activity increased in the infected patients and has the potential to be used as a prognostic indicator in chronic HCV infection.

Keywords: Indoleamine 2; 3-Dioxygenase; HCV; Pakistan

Introduction

The Hepatitis C Virus (HCV) is the primary cause of chronic liver disease and associated morbidity worldwide. Over 170 million people are infected by HCV globally [1]. Chronically infected HCV patients develop cirrhosis within 20 years [2]. Liver transplantation is the only choice for patients with decompensated liver cirrhosis or liver carcinoma [3]. Identifying novel therapeutic targets is urgent. HCV-specific CD8+ cells induce clearance of the virus in its acute phase; however, underlying immunosuppressive mechanisms involved in the progression to chronic infection remain elusive [4].

Indoleamine 2, 3-Dioxygenase (IDO) is an intracellular enzyme induced by proinflammatory cytokines such as interferon-gamma and tumor necrosis factor-alpha [5-7]. It converts tryptophan to kynurenine catabolites [8]. Increased IDO activity is reported to provoke tolerogenicity of antigen-presenting cells and tryptophan deprived T-cells, leading to proliferation arrest and T-cell apoptosis [9]. Tryptophan metabolites are reported to have immunomodulatory activity, alone or in combination with other signaling pathways ultimately leading towards immunosuppressive effects at the tissue level [3-10]. However, the exact molecular mechanisms and role of different immune components modulating this suppression remain elusive. Few studies have evaluated the differential expression of IDO in the liver [11] as well as in dendritic cells [12] and further correlated it with the degree of liver inflammation and fibrosis [13].

Leukocytes are the central part of the immune system and actively involved in fighting against infections. Leukocytes are primarily regulated by Dendritic Cells (DCs) that either help to promote or suppress T-cell responses in infection [14-16]. We hypothesized that IDO might have a potential role in the pathogenesis of HCV infection. Therefore, in the present study, we aimed to investigate (i) IDO expression in leukocytes of chronic HCV infected patients and (ii) IDO functional enzymatic activity in serum of chronic HCV infected patients.

Patients and Methods

Subjects

A total of 140 individuals were included in this study from 2013 to 2014. Patients included were referred by the outpatient department of the Center for Liver and Digestive Diseases, Holy Family Hospital, Rawalpindi and Khan Research Laboratories Hospital, Islamabad. Patients recruited in the current study fulfilled the following criteria: (a) HCV enzyme-linked immunosorbent assay positive and active HCV-RNA; (b) negative for hepatitis B virus and/or HIV infection; (c) receiving no immune suppression treatment; and (d) treatmentnaïve. All patients included in the study provided written informed consent. The study was approved by the institutional review board of the Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan. The study was conducted from Jun, 20, 2013 to Sep, 16, 2016.

IDO expression in leukocytes was analyzed in a total of 50 individuals, in treatment-naïve chronic HCV infected patient group

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(n=25) and in the subsequent healthy control group (n=25). Serum samples were available from 90 individuals from another group of treatment-naïve patients with chronic HCV infection (n=45) and healthy controls (n=45). Serum IDO enzymatic activity was measured by colorimetric assay.

Cellular RNA Extraction and cDNA Synthesis

Leukocytes were separated from the whole blood using red blood cell lysis buffer (NH_4Cl, $KHCO_3$, 5% EDTA, pH 7.4). Total RNA was extracted from these cells using the TRIzol™ LS reagent (Thermo Fisher Scientific) using the manufacturer's protocol. RNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). M-MLV Reverse Transcriptase (Invitrogen) with Oligo(dT)18 Primers (Thermo Fisher Scientific) were used to reverse transcribe equal amounts of RNA (2µg RNA per reaction) for complementary DNA (cDNA) synthesis using the default instructions. A No-Reverse Transcriptase reaction (NRT) was included to rule out any genomic DNA contamination. Subsequently, the cDNA was diluted 1:10 for further downstream reactions [17].

Real-Time Polymerase Chain Reaction for Expression Analysis

Expression of IDO mRNA was quantified viareal-time polymerase chain reaction RT-PCR using an ABI PRISM 7000 Sequence Detection System. Each 20-µl reaction mixture contained 4 µl HOT FIREPolEva Green qPCR Mix with ROX (SolisBioDyne), 12 µl RNase free water, 2 µM of each forward and reverse primer, and 2µl of 1:10 cDNA dilution. The quantitative (qPCR) reaction mix was initially incubated for 5 minutes at 50°C and 10 minutes at 95°C then followed by 45 PCR cycles of 95°C for 15seconds and 60°C for 1 minute. An additional melting/dissociation step was included to monitor amplification specificity. An NRT control (NRTC) and no-template control were run simultaneously. Expression of the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a reference to calculate the relative expression level of the target gene. Primers for IDO-1(Hs00984148_m1) and GAPDH (Hs02758991_g1) were purchased from Applied Biosystems. The results were analyzed via the ABI Sequence Detection Software, and the relative expression levels of IDO-1 were determined by using the formula rE=100 x 2-ΔC_T, where ΔC_T= mean C_T (target gene) - mean C_T (GAPDH) [18].

Colorimetric Assay

Serum kynurenine was measured as described previously [19,20]. In brief, 50µl of 30% trichloroacetic acid was added to 100µl serum, vortexed, and spun in a centrifuge at 10,000 x g for 5 minutes. Seventy-five microliters of the supernatant was mixed with an equal volume of Ehrlich's reagent (100 mg p-dimethylaminobenzaldehyde and 5ml glacial acetic acid) and transferred to a microtiter 96-well plate. The optical density was measured at 492 nm by ELx800 Absorbance Microplate reader (Dynex Technologies, Chantilly, VA, USA). Unknown concentrations were measured using a standard curve of defined kynurenine concentrations (0–100 µM).

Statistical Analysis

GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. Graphical data represent the mean value of an experiment, performed in triplicate, using the Mann-Whitney U test, unless stated otherwise. *P* values < .05 were considered significant.

Results

IDO Expression in Leukocytes

The mRNA levels of IDO relative to GAPDH were quantified in the leukocytes of treatment naïve patients with chronic HCV infection (n=25) and controls. No significant difference was observed in the IDO expression between leukocytes of patients and the healthy controls *P* =0.07; (Figure 1).

IDO Enzymatic Activity in Serum

The IDO enzymatic activity was determined by a standard colorimetric assay in the serum of patients infected with HCV and controls. Significantly higher levels of kynurenine were observed in treatment-naïve patients with chronic HCV infection as compared to the control group *P*<.0001; (Figure 2).

Discussion

In the current study, we investigated IDO expression in leukocytes of treatment naïve patients with chronic HCV infection and healthy controls. There was no significant difference in IDO expression in leukocytes of treatment-naïve patients with chronic HCV infection as compared to controls. However, high IDO enzymatic activity was observed in the serum of patients with chronic HCV as compared to controls. This agrees with previous results [11, 21].

As DCs are the key players in the regulation of leukocytes, it might be possible that, during chronic HCV infection, defective DCs are involved in the impairment of leukocytes. This may explain the insignificant difference between IDO expression in leukocytes of patients with chronic HCV as compared to controls. The DCs of patients with chronic HCV are less efficient in stimulating T-helper-1

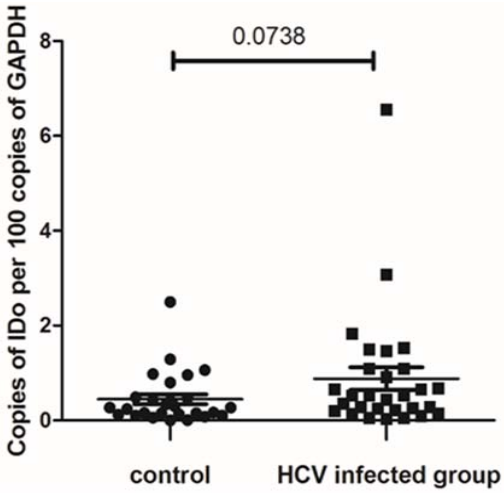

Figure 1: IDO expression in leukocytes of control vs. infected individuals. IDO mRNA in leukocytes of healthy controls (n=25) and treatment-naïve patients with chronic HCV infection (n=25) was quantified by real-time PCR. The difference in the mRNA expression in both groups was insignificant as depicted by P values (Mann-Whitney U test) (P=0.037). Abbreviations: IDO: Indoleamine 2, 3-Dioxygenase; mRNA: Messenger RNA; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HCV: Hepatitis C virus; PCR: Polymerase Chain Reaction.

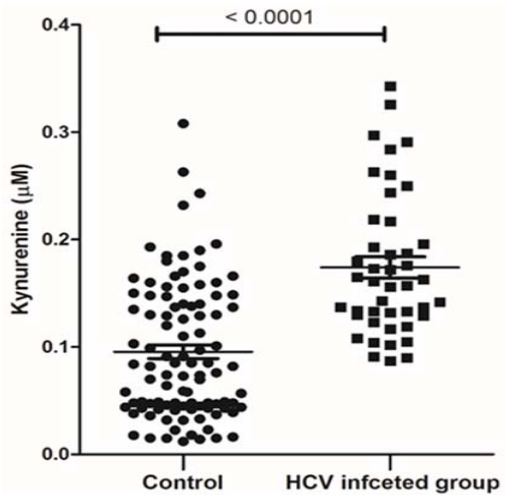

Figure 2: Serum IDO enzymatic activity in terms of kynurenine levels. IDO enzymatic activity in healthy controls (n=45) and treatment naïve patients with chronic HCV (n=45). Kynurenine levels were significantly elevated in HCV infected individuals compared to healthy controls (P<0.0001) as depicted by P values (Mann-Whitney U test). Abbreviations: IDO: Indoleamine 2, 3-Dioxygenase; HCV: Hepatitis C Virus.

polarization than healthy controls [14,15]. DCs have been shown to exert their immune-suppressive effect by the expansion of the T regulatory cells populations [9,16]. Furthermore, both immature and mature DCs constitutively express IDO protein that suppresses T-cell proliferation in vitro and in vivo [22]. Leukocytes are the key immune players regulated by DCs. Thus, it is imperative to study the IDO expression in leukocytesas they constitute an immunologically important cellular compartment. Further studies are required to elucidate the regulation of leukocytes through the maturation of DCs in chronic HCV infection.

HCV-induced immune suppression has a critical role in the establishment of chronic infection. Elucidation of these suppressive mechanisms is an essential step forward for better understanding the pathogenesis of disease. One limitation of this study was that all patients were treatment-naïve and did not belong to the same group. Future studies focusing on the impact of different treatment regimens on IDO expression and achievement of sustained virological response will be of great importance.

The specific role of IDO in HCV infection remains to be elucidated. Our understanding of the immunosuppressive nature of IDO during chronic HCV infection is in its infancy. The data presented in this study will certainly serve as a useful addition to the already available knowledge. Moreover, our results are in favor of the previous speculation that serum IDO activity could be a potential biomarker to differentiate individuals with chronic HCV infection from healthy individuals. These results give a new dimension to the current understanding of in vivo IDO regulatory mechanisms.

Conclusion

Our results indicate that there is no significant difference of IDO expression in leukocytes of chronic HCV infected patients and healthy control. IDO enzymatic activity increased in the infected patients and has the potential to be used as a prognostic indicator in chronic HCV infection.

Conflict of Interest

The authors declare no conflict of interest.

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