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In the peripheral blood mononuclear cells (PBMCs) of HCV infected patients the expression of STAT1 and IRF-1 is downregulated while that of caspase-3 upregulated

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Summary. – Several peripheral blood mononuclear cell (PBMC) defects have been linked with hepatitis C virus (HCV) infection, including alterations in cytokine secretion and increased cell death. This study was performed to investigate the expression levels of signal transducer and activator of transcription 1 (STAT1), interferon regulatory factor 1 (IRF-1), and caspase 3 in PBMCs of patients infected with HCV. STAT1, IRF-1, and caspase 3 expression levels were compared in PBMCs from 19 untreated (naïve) HCV+ patients, 8 treated (sustained responder [SR]) HCV patients, and 20 HCV– healthy controls. Moreover, PBMCs from naïve HCV+ patients and SR-HCV patients were also evaluated for HCV RNA expression. The expression levels of STAT-1 and IRF-1 were significantly downregulated in PBMCs from naïve HCV+ patients ($P < 0.04$) and SR-HCV patients ($P < 0.05$) compared to HCV-controls. In comparison with HCV– controls, naïve HCV+ and SR-HCV patients showed a significant upregulation in the expression of caspase-3 in their PBMCs ($P < 0.0005$ and $P < 0.03$, respectively). No significant differences were observed in the expression of STAT-1, IRF-1, and caspase-3 between PBMCs from naïve HCV+ and SR-HCV patients. HCV RNA was detected in PBMCs from 18 (94.7%) naïve HCV+ patients as well as 6 (75%) SR-HCV patients. Downregulation of STAT1 and IRF-1 expression levels and upregulation of caspase-3 expression level in PBMCs from HCV-infected patients may contribute to alterations in cytokine secretion and enhanced PBMCs cell death reported in previous studies.

Keywords: PBMC; hepatitis C virus; STAT1; IRF-1; caspase-3

Introduction

Hepatitis C infection is among most common viral infections worldwide (Modi and Liang, 2008). To date, more than 170 million individuals have been infected with hepatitis C virus (HCV) and over 3 million new cases are reported every year. HCV infection causes acute and chronic liver inflammation, which in the majority of cases progresses to cirrhosis and hepatocellular carcinoma (Modi and Liang, 2008; Ashfaq *et al*., 2011). The virus infects and replicates in hepatocytes and other cell types, including peripheral blood mononuclear cells (PBMCs) and bone marrow cells (Darnell *et al*., 1994; Manzin *et al*., 1994; Bare, 2009; Revie and Salahuddin., 2011), resulting in persistence of infection and induction of chronic immune-activation. It has been recently shown that PBMCs from HCV+ patients are sensitized to spontaneous programmed cell death and this phenomenon is associ-

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Abbreviations: HCV = hepatitis C virus; IFN = interferon; IL = interleukin; IRF = interferon regulatory factor; PBMC = peripheral blood mononuclear cell; STAT = signal transducer and activator of transcription; SR-HCV = sustained responder-HCV

ated with reduced levels of interleukin (IL)-8, IL-6, and IL-10 production (Alhetheel *et al*., 2016). The molecular mechanism involved in these defects is still unclear.

Several studies have shown that PBMCs play a critical role in the elimination of pathogens via phagocytic and cytolytic activities. Many of such PBMC functions are mediated through the regulation of different signaling pathways, including the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway (Darnell *et al*., 1994; Imada and Leonard, 2000; Levy and Darnell, 2002). HCV proteins such as the core and E2 polypeptides are capable to affect several signaling pathways including JAK-STAT, mitogen-associated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI3K) pathways in hepatocytes and macrophages. For instance, HCV core proteins have been shown to induce STAT3 and PI3K activation in monocytes and macrophages through the release of IL-6 (Tacke *et al*., 2011). Furthermore, several *in vitro and ex vivo* experiments have demonstrated that HCV and its proteins are capable of inducing apoptosis in hepatocytes and T cells (Bantel and Schulze-Osthoff, 2003). Thus, it is possible that HCV predisposes PBMCs to cell death and impairs their functions directly (by the action of its proteins) or indirectly (by induction of alterations in cytokine secretion via the STAT1 signaling pathway). Moreover, interferon gamma (IFN-γ) plays a critical role in HCV infection because of its anti-viral properties. A strong Th1 response, which is mediated by IFN-γ, plays a critical role in clearance of HCV infection (Saito and Gale, 2008). In addition, hyper-activation of interferon regulatory factor 1 (IRF-1) and elevated STAT1 have been implicated in enhancement of antiviral activity against HCV (Zhang XN *et al*., 2006). Considering the fact that a vast majority of studies have demonstrated the effect of HCV and its proteins on expression levels of STAT1 and IRF-1 using cell lines (Heim *et al*., 1999; Kanazawa *et al*., 2004), in this paper for the first time we describe the expression of STAT1, IRF-1 and caspase-3 in PBMCs from chronically infected HCV patients.

Materials and Methods

Patient characteristics. This is a cross-sectional study conducted at King Khalid University Hospital in Riyadh, Saudi Arabia, between November 2012 and December 2013. A total of 27 patients infected with HCV were enrolled. These included 19 (13 females and 6 males; mean age, 49 ± 12.8 years) untreated patients (naïve group) and 8 treated patients (three females and five males; mean age, 47 ± 8 years) (sustained responder [SR] group). The average HCV load in the naïve group was 1.35 × 10^6 ± 1.72 × 10^6 IU/ml, while the SR-HCV group had undetectable levels of HCV for at least 6 months prior to the study. In addition, 20 healthy control individuals (all males; mean age, 32 ± 10.6 years) were included. They were negative by serology testing for infection with human immunodeficiency virus, human T-lymphotropic virus, and hepatitis B and C viruses. The therapy of the SR-HCV group comprised the combination of PEGylated-IFN and ribavirin for 1 year. The study protocol was approved by the Institutional Review Board of the College of Medicine, King Saud University (Project# E-11-461), and all patients provided written informed consent.

Isolation of PBMCs, RNA extraction, and real-time polymerase chain reaction (RT-PCR). PBMCs were isolated from whole blood by density gradient centrifugation, as previously described (Alhetheel *et al*., 2017). PBMCs were counted and 4 × 10^5 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum for 24 h. The cells were washed and collected for RNA extraction. Total RNA was extracted from PBMCs using the RNeasy Mini kit (Qiagen, Hilden, Germany). Briefly, PBMCs (5 × 10^5 cells) were homogenized in a denaturation buffer containing guanidine-isothiocyanate, which prevents RNA degradation. Cellular lysate was passed through genomic DNA eliminator spin column to purify RNA from genomic DNA. Equal volume of 70% ethanol was added to the total RNA to enhance its binding to the membrane of the RNeasy spin column during sample application, followed by two washes with a buffer containing ethanol. Total RNA was eluted with 50 µl of RNase-free water. For the detection of HCV RNA from PBMCs, total RNA was

Table 1. Specific primers used in the study

Primers	Sequence	References
HCV	Forward 5'-CGGGAGAGCCATAGTGGT-3' Reverse 5'-CGCGACCCAACACTACTC-3'	Hideshima *et al*., 2007
STAT-1	Forward 5'-GGAACTTGATGGCCCTAAAGGA-3' Reverse 5'-ACAGAGCCCACTATCCGAGACA-3'	Kaiser *et al*., 2006
IRF-1	Forward 5'-GCATGAGACCCTGGCTAGAGAT-3' Reverse 5'-CGGAACAAACAGGCATCCTT-3'	Kaiser *et al*., 2006
Caspase-3	Forward 5'-GCAGCAAACCTCAGGGAAAC-3' Reverse 5'-TGTCGGCATACTGTTTCAGCA-3'	Hideshima *et al*., 2007
GAPDH	Forward 5'-AATCCCATCACCATCTTCCA-3' Reverse 5'-TGGACTCCACGACGTACTCA-3'	Imada and Leonard, 2000; Kanazawa *et al*., 2004

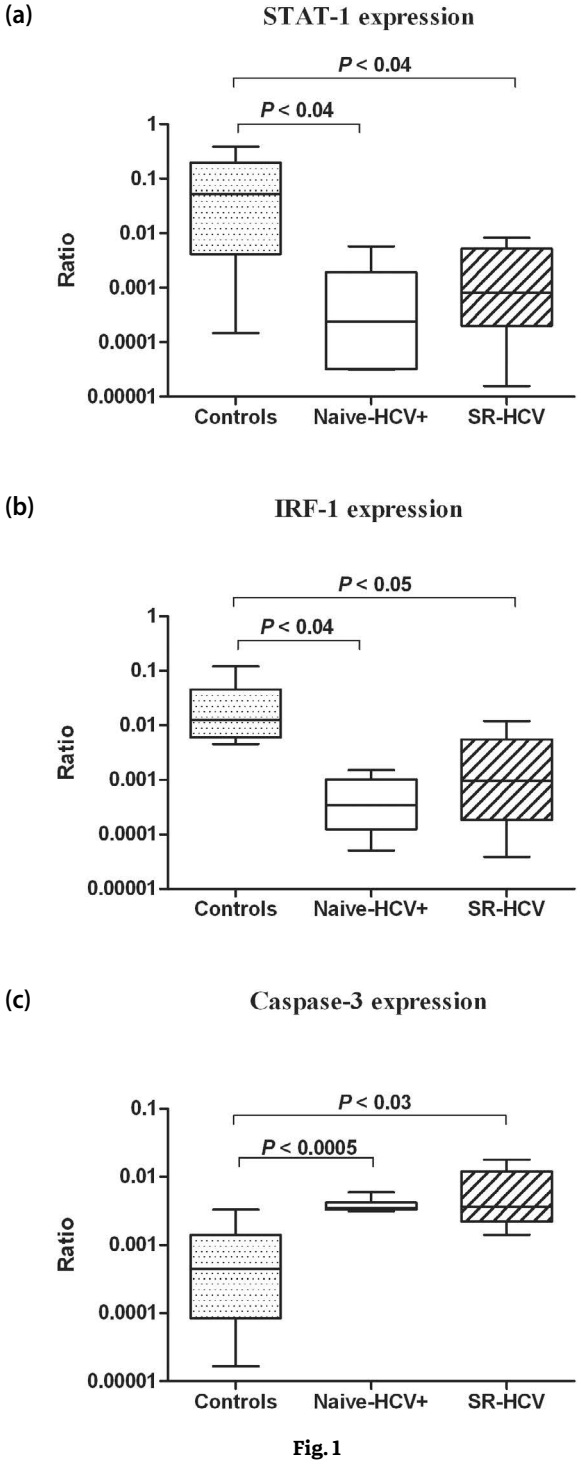

Fig. 1

Expression levels of STAT1, IRF-1, and caspase-3 in PBMCs from study groups

Purified PBMCs (4×10^5 cells) were cultured for 24 h and total RNA was extracted from the cells. The expression levels of STAT1, IRF-1, caspase-3, and GAPDH were measured using RT-PCR. The ratio of STAT1 **(a)**, IRF-1 **(b)**, and caspase-3 **(c)** expression levels for each patient group was plotted after normalization with GAPDH expression. All data were analyzed by Student's *t*-test. Significant differences are indicated with *P* values.

reverse-transcribed using the QuantiTect Reverse Transcriptase kit (Qiagen) according to the manufacturer's protocol. cDNA was amplified in the thermocycler T100 (Bio-Rad, Hercules, CA, USA) using HCV-specific primers indicated in Table 1, as per the previously described protocol (Hideshima *et al.*, 2007). PCR amplicons were visualized on a 1.5% agarose gel. The positive amplicons were sequenced using ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For positive results, nucleotide sequences were blasted against NCBI database.

To evaluate the expression of STAT1, IRF-1, and caspase-3 genes, the cDNA was amplified using primers specific for STAT1, IRF-1, and caspase-3 along with the housekeeping gene encoding glyceraldehyde-3-phosphate (GAPDH) (Table 1) with Rotor Gene Q real-time PCR System (Qiagen), as previously described (Hideshima *et al.*, 2007; Albertoni *et al.*, 2012).

Hepatitis C viral load in patients' sera was evaluated using the COBAS TaqMan analyzer and COBAS AmpliPrep/COBAS TaqMan HCV Quantities test v.2.0 (Roche Diagnostics, Manheim, Germany) as previously described (Alhetheel *et al.*, 2016).

Statistical analyses. Data were analyzed using GraphPad Prism 5 software (GraphPad Inc., San Diego, CA). The non-paired *t*-test was used to evaluate the significance between different groups. A value of $P \leq 0.05$ was considered statistically significant.

Results

Expression levels of STAT1, IRF-1, and caspase-3 in PBMCs from study groups

We cultured PBMCs (4×10^5 cells) from naïve HCV+ patients, SR-HCV patients, and healthy controls for 24 h. Cellular RNA was extracted and reverse-transcribed into cDNA. The expression levels of STAT1, IRF-1, and caspase-3 were measured by real-time PCR. The expression level of STAT1 was significantly downregulated in naïve HCV+ patients ($P < 0.04$) and SR-HCV patients ($P < 0.04$) as compared with healthy controls (Fig. 1a). We found a similar significant downregulation in the expression level of IRF-1 in naïve HCV+ patients ($P < 0.04$) and SR-HCV patients ($P < 0.05$) as compared with healthy controls (Fig. 1b). On the other hand, the expression level of caspase-3 was significantly upregulated in naïve HCV+ patients ($P < 0.0005$) and SR-HCV patients ($P < 0.03$) as compared with healthy controls (Fig. 1c). No significant differences were observed in the expression levels of STAT1, IRF-1, and caspase-3 between PBMCs from naïve HCV+ and SR-HCV patients (Fig. 1).

Detection of HCV RNA in PBMCs from naïve and SR-HCV patients

We cultured PBMCs (4×10^5 cells) from naïve HCV+ and SR-HCV patients for 24 h. Cells were subjected to RNA

Table 2. HCV-RNA in naïve and sustained responders HCV patients

Study group	Patient #	HCV in serum (IU/ml)	HCV in PBMCs	Genotype	Duration of HCV negativity of serum
Naïve HCV patients	P1	625762	positive	4	0
	P2	517632	positive	4	0
	P3	1091129	positive	4	0
	P4	1516317	positive	1	0
	P5	251634	positive	4	0
	P6	2100027	positive	1	0
	P7	1511113	positive	4	0
	P8	907450	positive	1b	0
	P9	380036	positive	1	0
	P10	318792	positive	4	0
	P11	200	positive	1 & 4	0
	P12	2052906	negative	2	0
	P13	179514	positive	4	0
	P14	685043	positive	1	0
	P15	4543161	positive	4	0
	P16	1988858	positive	4	0
	P17	191963	positive	4	0
	P18	24351	positive	4	0
	P19	6819886	positive	4	0
Sustained-responder HCV patients	P20	Not detected	negative	NA	7 years
	P21	Not detected	positive	NA	5 years
	P22	Not detected	positive	NA	3 years
	P23	Not detected	positive	NA	6 years
	P24	Not detected	positive	NA	6 years
	P25	Not detected	positive	NA	3 years
	P26	Not detected	positive	NA	3 years
	P27	Not detected	negative	NA	6 years

extraction and HCV RNA detection using PCR analysis. All treatment-naïve HCV+ patients were detected positive for HCV RNA, with the serum viral load ranging from 200 to 6,819,886 IU/ml. On the other hand, 18 treatment-naïve patients (94.7%) were positive and one patient (5.3%) was negative for HCV RNA in PBMCs (Table 2). No detectable HCV RNA expression was observed in serum samples from all SR-HCV patients; however, six SR-HCV patients (75%) were positive and two patients (25%) were negative for HCV RNA in PBMCs (Table 2). The detection of HCV in PBMCs was confirmed from the sequencing of PCR products.

Discussion

This study demonstrates that 94% of naïve HCV+ patients and 75% of SR-HCV patients carry HCV RNA in their PBMCs. This observation is consistent with the findings of previously published data supporting the presence of HCV in the PBMCs of viremic and non-viremic individuals (Bronowicki *et al*., 1998; Cavalheiro *et al*., 2007; Mohamad *et al*., 2011). A number of other studies has also reported evidence not only for the presence of HCV in hematopoietic cells but also for the ability of the virus to replicate in these cell types (Lerat *et al*., 1998; Laskus *et al*., 2000; Castillo *et al*., 2005). On the contrary, there are sufficient published data refuting these claims. A study by Manzin *et al*. (1994) reported that PBMCs and bone marrow cells from all five naïve HCV patients tested negative for HCV RNA (Manzin *et al*., 1994). Another study investigating 69 non-viremic and 56 viremic individuals failed to document the presence of HCV RNA in PBMCs in all participants in question. Therefore, they proposed that PBMCs are unlikely reservoir for prolonged persistence of HCV infection (Bernardin *et al*., 2008). Similarly, a follow up study has reported that the PBMC-associated HCV RNA after treatment could possibly represent either viral particles non-specifically attached to blood cells or regarded the low-level replication of HCV in PBMCs as of

minor quantitative importance for systemic replication (Kaiser *et al.*, 2006). Despite the controversies, it has been proposed that the persistence of HCV infection in PBMCs as an extra-hepatic source of HCV infection remains a potent threat for reactivation of the virus after termination of successful anti-viral therapy (Pham *et al.*, 2004; Radkowski *et al.*, 2005).

Here we showed for the first time a significant downregulation in STAT1 and IRF-1 expression levels in PBMCs from treatment-naïve as well as SR-HCV patients. Several studies have demonstrated the effect of HCV and its proteins on expression levels of STAT1 and IRF-1 in human hepatocellular carcinoma (Huh7 cells) and other cells (Heim *et al.*, 1999; Kanazawa *et al.*, 2004; Lin *et al* 2005; Ciccaglione *et al.*, 2007; Kumthip *et al.*, 2012). On the contrary, a previous study demonstrated upregulated STAT1 expression in hepatocytes from chronic HCV patients with viremia (El-Saadany *et al.*, 2013). STAT1 and IRF-1 are transcription factors involved in different immune functions and dysregulation in the expression of these proteins may compromise the immune system (Darnell *et al.*, 1994; Imada and Leonard, 2000; Taniguchi *et al.*, 2001; Levy and Darnell, 2002). IFN-α and IFN-γ co-treatment in human hepatoma cells has been shown to upregulate the expression of STAT1 and IRF-1 that contributes to the enhanced interferon stimulated gene (ISG) expression with robust anti-viral activity (Zhang *et al.*, 2006). Downregulation of STAT1 and IRF-1 expression in PBMCs of HCV patients observed in the present study is consistent with a previous report where HCV-transfected cell lines were shown to promote viral replication (Zhang *et al.*, 2006). It is therefore possible that persistent HCV infection is most likely due to virus-induced suppression of IFN expression. Moreover, since interferon therapy upregulates expression of STAT1 and IRF-1, monitoring the expression of these two genes may serve as useful marker for assessing the therapeutic response.

We observed a significant upregulation in caspase-3 expression in PBMCs from treatment-naïve and SR-HCV patients. This observation is in line with the results of our previous study, wherein an increase in the spontaneous programmed cell death was observed in PBMCs from treatment-naïve and SR-HCV patients as compared to PBMCs from HCV– healthy controls (Alhetheel *et al.*, 2017). Moreover, upregulation of caspase-3 has previously been shown to induce apoptosis in PBMCs among patients with high and low viral loads (Albertoni *et al.*, 2012). In addition, a number of studies have already implicated caspase-3 in PBMCs dysfunction and death (Taniguchi *et al.*, 2000; Nakamoto *et al.*, 2002; Bantel and Schulze-Osthoff, 2003). Our study was limited by low patient numbers. Large scale follow-up studies are recommended to validate the findings of this study and to gain a better insight for understanding of the PBMC dysfunction and increased cell death among patients with HCV infection.

Conclusion

Downregulation of STAT1 and IRF-1 expression levels and upregulation of caspase-3 expression level in PBMCs from HCV-infected patients may contribute to alterations in cytokine secretion and enhanced PBMCs cell death reported in previous studies.

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