Increased Spontaneous Programmed Cell Death Is Associated with Impaired Cytokine Secretion in Peripheral Blood Mononuclear Cells from Hepatitis C Virus-Positive Patients

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Abstract

Peripheral blood mononuclear cells (PBMCs) play a critical role in clearing hepatitis C virus (HCV). PBMC defects have been linked with HCV infection; however, the underlying mechanisms remain obscure. We hypothesized that PBMCs of HCV-infected patients are more susceptible to programmed cell death (PCD), and are therefore unable to clear HCV. We compared spontaneous PBMC PCD and cytokine [interleukin (IL)-1, -6, -8, -10, and -12] secretion between untreated (naïve) HCV⁺ and treated [sustained responder (SR)] patients with HCV, and HCV⁻ healthy controls. Spontaneous PBMC PCD was assessed by annexin-V fluorescein isothiocyanate/propidium iodide staining, and cytokine levels were measured by cytometric bead array. Differences between groups were analyzed through paired and nonpaired *t* tests and Mann–Whitney *U* test. The rate of spontaneous PCD was higher in PBMCs of naïve HCV⁺ patients ($p < 0.0001$) and SR-HCV patients ($p < 0.002$) than in HCV⁻ controls. Significantly low levels of IL-8, -6, and -10 were detected in the supernatant of cell cultures of PBMCs from naïve HCV⁺ ($p < 0.05$) and SR-HCV ($p < 0.05$) patients relative to HCV⁻ controls. There was no difference between the naïve HCV⁺ and SR-HCV groups in terms of PBMC PCD rate or cytokine levels. The present findings indicate that HCV infection is associated with increased PBMC susceptibility to PCD and decreased production of IL-8, -6, and -10.

Keywords: programmed cell death, PBMCs, HCV, cytokines

Introduction

INFECTION WITH HEPATITIS C virus (HCV) is a major public health problem worldwide (15). More than 170 million people are believed to be currently infected with HCV, with more than 3 million new cases reported annually. HCV infection usually manifests as acute or chronic liver inflammation, with 85% of cases progressing to cirrhosis and hepatocellular carcinoma (4,15). HCV replicates not only in hepatocytes but also in peripheral blood mononuclear cells (PBMCs) and bone marrow cells (7,9,13,21,28), resulting in viral persistence and chronic immune activation. The cellular functions of immune cells are consequently impaired because of alterations in cytokine networks and signaling pathways. HCV infection is known to be associated with elevated levels of interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), TNF-related apoptosis-inducing ligand, and Fas (22,26,27); however, the impact of these HCV-related alterations on PBMCs remains elusive.

Programmed cell death (PCD) through apoptosis plays a key role in the maintenance of cellular homeostasis; dysregulation of apoptotic processes may lead to malignancies and/or immune dysfunction (1). Apoptosis is triggered either by cell surface death receptors (extrinsic pathway) or by disruption of the mitochondrial membrane potential (intrinsic pathway). During apoptosis, downstream effector caspases 3, 6, and 7 are activated, resulting in nuclear poly (ADP-ribose) polymerase cleavage, DNA fragmentation, and cell death (1). Several studies have demonstrated a role for PCD in HCV pathogenesis during liver inflammation and fibrosis, or its progression to hepatocellular carcinoma (6,8,10,18).

The phagocytic and cytolytic activities of PBMCs are critical for pathogen clearance. After activation, increased production of cytokines such as interleukin (IL)-1 α and β , -2, -6, -10, -12, and -18 by PBMCs is crucial for induction of the adaptive immune response. We hypothesized that PBMCs of HCV-infected patients exhibit elevated susceptibility to PCD, and are therefore unable to clear HCV. In this work, we

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investigated whether PBMCs of chronic HCV patients are more susceptible to PCD than those of healthy controls by comparing spontaneous PCD and cytokine secretion between PBMCs from HCV⁺ patients and those from HCV⁻ healthy controls.

Materials and Methods

Patient characteristics

This cross-sectional study was performed at King Khalid University Hospital (KKUH), Riyadh. A total of 27 HCV patients were included in the study (Table 1); of these, 19 patients were recruited before initiation of antiviral therapy (naive HCV⁺ group) and had a relatively high viral load (mean = $1.35 \times 10^6 \pm 1.72 \times 10^6$ IU/mL), whereas 8 patients had undergone successful antiviral treatment [sustained responder (SR)-HCV group] and had not displayed detectable HCV RNA for at least 6 months before enrollment in the study. Patients who were coinfecting with hepatitis B virus (HBV) or the human immunodeficiency virus (HIV), or had detectable HCV viral loads after 6 months, were excluded from the study. In addition, 20 healthy volunteers with no evidence of current or past infection with HIV, human T-lymphotropic virus, or HBV and HCV were included as a control group. The antiviral treatment regimen was a combination of pegylated IFN and ribavirin for 12 months. All experiments were performed in compliance with relevant regulations and institutional guidelines, and in accordance with the ethical standards set by the Declaration of Helsinki. The protocol of the study was approved by the Institutional Review Board of the College of Medicine, King Saud University. All patients were enrolled in the study after providing written informed consent.

Quantification of viral load

HCV load in serum samples was measured using the COBAS TaqMan analyzer and the AmpliPrep/COBAS TaqMan HCV Quantities test v 2.0 (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol.

PBMC isolation and cell viability assay

Whole blood was collected in 10-mL vacutainer tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ). PBMCs were isolated from whole blood by density gradient centrifugation. The blood was layered on top of 4 mL of Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) in 15-mL tubes and centrifuged for 30 min at $400 \times g$ with the brake set to off. PBMCs in the interphase layer were col-

lected and washed twice with sterile phosphate-buffered saline (GIBCO Invitrogen), followed by complete culture medium [RPMI 1640 medium (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (GIBCO Invitrogen)]. Equal number (4×10^5 cells) of PBMCs were left uncultured (designated as "0h" sample) or cultured in complete medium for 24 h and counted after the culturing with no significant increase in the number of cells. Cells were then washed with annexin-binding buffer (ABB): 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4, and then resuspended in 100 μ L of the same buffer. Annexin V-fluorescein isothiocyanate (FITC), 5/100 μ L of cell suspension, and propidium iodide (PI), 0.1 μ g/100 μ L of cell suspension (Molecular Probes, Eugene, OR) were added to the cells, followed by incubation in the dark at 25°C for 15 min. After addition of ABB (300 μ L/sample), samples were analyzed by flow cytometry on an FACSCalibur instrument (BD Biosciences, Great Lakes, NJ). Data were analyzed using Cell-Quest software (BD Biosciences). The percentage of cell death was calculated by adding the percentages of annexin V-FITC single-positive and annexin V-FITC/PI double-positive cells.

Cytometric bead array for human inflammatory cytokine detection

Supernatants from purified PBMCs (4×10^5 cells) cultured in complete medium for 24 h were collected and assayed with the cytometric bead array (CBA) human inflammatory cytokine kit (BD Biosciences) according to the manufacturer's instructions as previously described (2). Cytokine levels (pg/mL) were determined using the FCAP Array software (BD Biosciences).

Statistical analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad, Inc., San Diego, CA). Paired *t* tests were used to assess the statistical significance between two different time points of the same patient, whereas nonpaired *t* test and Mann-Whitney *U* test were employed to evaluate the significance of differences between different groups; $p \leq 0.05$ was considered statistically significant.

Results

Increased spontaneous PCD in PBMCs from HCV-infected patients

We hypothesized that PBMCs from HCV patients become sensitized by endogenous cytokines and are, therefore,

TABLE 1. CHARACTERISTICS OF PATIENT STUDY GROUPS

Study groups	Controls	Naive-HCV ⁺ patients	SR-HCV
Gender (M/F)	20/0	6/13	5/3
Age (mean $\pm$ SD) [range], years	31.6 $\pm$ 10.56 [19–51]	49.75 $\pm$ 12.76 [23–71]	47.25 $\pm$ 8.05 [32–58]
Mean viral load $\pm$ SD [range], IU/mL	ND	$1.3 \times 10^6 \pm 1.7 \times 10^6$ [200–6,819,886]	HCV not detected
HCV genotype (no. of cases)	ND	Type 1 (4), type 1a (1), type 2 (1), type 4 (12), and types 1 and 4 (1)	ND
Mean ALT $\pm$ SD, U/L	ND	84.10 $\pm$ 75.17	ND
Mean AST $\pm$ SD, U/L	ND	54.42 $\pm$ 39.87	ND
Mean ALP $\pm$ SD, U/L	ND	146.26 $\pm$ 44.10	ND
Treatment	No	Pegylated IFN and ribavirin/12 months	No

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; HCV, hepatitis C virus; IFN, interferon; ND, not done; SD, standard deviation; SR, sustained responder.

more susceptible to cell death. We evaluated PCD in PBMCs (4×10^5 cells) that were freshly isolated (0h) or cultured for 24h by annexin V-FITC/PI staining and flow cytometry. The percentage of PBMCs undergoing spontaneous PCD was significantly higher in naive HCV⁺ patients (mean = $41.9\% \pm 13.1\%$) than in HCV⁻ donors (mean = $20.6\% \pm 5.4\%$, $p < 0.002$) (Fig. 1A). Spontaneous PCD rate was also higher in PBMCs of the SR-HCV group (mean = $35.7\% \pm 19.3\%$) than those from HCV⁻ donors (mean = $20.6\% \pm 5.4\%$), but the difference was not statistically significant. After 24h of culture, the percentage of PBMC death further increased in the naive HCV⁺ group (mean = $54.5\% \pm 13.9\%$, $p < 0.0001$) and the SR-HCV group (mean = $45.7\% \pm 17.9\%$, $p < 0.001$), but not in the control group (mean = $22.5\% \pm 6.4\%$) (Fig. 1A). The rate of spontaneous PBMC death was the highest in naive HCV⁺ patients (mean = $53.6\% \pm 14.6\%$, $p < 0.0001$), followed by SR-HCV patients (mean = $41.2\% \pm 17.4\%$, $p < 0.002$) and HCV⁻ controls (mean = $23.9\% \pm 5.1\%$) (Fig. 1B). The difference between PBMC spontaneous PCD rates in naive HCV⁺ patients and in SR-HCV patients was not statistically significant (Fig. 1B).

FIG. 1. Spontaneous PCD in PBMCs from the patient study groups. Purified PBMCs (4×10^5 cells) were left uncultured or cultured in complete medium for 24h. Subsequently, cells were stained with annexin-V-FITC and PI and analyzed by flow cytometry. The percentage cell death in PBMCs of each group was graphed. (A) Each pair of columns represents data for a given group at 0 and 24h of culture. (B) The percentage cell death for each group at 24h: control group ($n=20$), naive-HCV⁺ group ($n=19$), SR-HCV group ($n=8$). The p values are indicated where significant. FITC, fluorescein isothiocyanate; HCV, hepatitis C virus; PBMCs, peripheral blood mononuclear cells; PCD, programmed cell death; PI, propidium iodide; SR, sustained responder.

Reduced levels of IL-8, -6, and -10 secretion in PBMCs of HCV-infected patients

Given the observed increase in spontaneous PBMC death rate in HCV⁺ patients, we investigated the effect of HCV on cytokine production by PBMCs. Purified PBMCs (4×10^5 cells) were cultured for 24h, and the supernatant was collected for assessment of cytokines by CBA and flow cytometry. All proinflammatory cytokines, including IL-8, -6, -10, -1 β , and -12p70, were detected in the supernatant of PBMC cultures from all groups. The levels of IL-8, -6, and -10 were significantly lower in the PBMC culture supernatants from naive HCV⁺ (mean values: 821.4 ± 629.9 , 15.9 ± 13.8 , and 4.1 ± 0.5 , respectively) and SR-HCV patients (mean values: 1287.8 ± 1034.1 , 14.5 ± 8.2 , and 4.1 ± 0.7 , respectively) than those from HCV⁻ controls (mean values: 2581.5 ± 1670.1 , 30.2 ± 16.9 , and 4.8 ± 0.7 , respectively) ($p < 0.05$; Fig. 2). Cytokine levels of HCV⁺ and SR-HCV patients were similar. No statistically significant difference was observed in the concentrations of IL-1 β and IL-12p70 among the three study groups.

Discussion

PBMCs from naive HCV⁺ and SR-HCV patients exhibited significantly higher rates of spontaneous PCD than those from HCV⁻ controls. These observations were consistent with other studies (1,16,24). Upon starvation, monocytes and T lymphocytes from chronic HCV⁺ patients and HCV⁺ patients with liver cirrhosis or hepatocellular carcinoma have been reported to display higher death rates than controls (16). This increase in spontaneous PCD rates may be attributed to the upregulation of apoptotic genes in the PBMCs of HCV⁺ patients, such as *Fas*, *CASP3*, *CASP8*, and *CASP9* (1).

We report here for the first time that increased susceptibility of PBMCs to spontaneous PCD was associated with decreased levels of IL-6, -8, and -10 in supernatants of PBMC cultures from HCV⁺ patients. These findings substantiate earlier observations suggesting that increased syntheses of IL-10 and IL-8 are predictors of unfavorable prognosis of IFN therapy (14). Moreover, increased production of IL-8 has been linked with histological evidence of progressed inflammation, and persistently elevated levels of IL-8 are believed to be a strong predictor of progression to cirrhosis (17). Even though low levels of supernatant proinflammatory cytokines could indicate a favorable outcome, they may have an adverse effect on the survival of PBMCs.

We have previously shown that plasma levels of IL-1, -6, -8, and -10 are higher in naive HCV⁺ patients than in HCV⁻ donors (2). Several other studies have also reported increased blood levels of IL-6, -8, and -10 among patients infected with HCV (3,19,20,25,29). These cytokines appear to be critical for PBMC survival as recombinant IL-8 inhibits apoptosis of endothelial cells by enhancing the expression of antiapoptotic genes such as B cell lymphoma 2 (*Bcl-2*) and extra large *Bcl-xL* (12). Similarly, IL-10 has been shown to block spontaneous T cell apoptosis in infectious mononucleosis (23). IL-6 prevents cluster of differentiation 3-induced T cell death and hydrogen peroxide-induced apoptosis of alveolar epithelial cells (5,11). These data indicate that despite high blood levels of antiapoptotic cytokines such as IL-8, IL-10, and IL-6, PBMCs from HCV-infected patients are more prone to

FIG. 2. Human inflammatory cytokine levels in supernatant of PBMCs from patient study groups. Patient PBMCs (4×10^5 cells) were cultured in complete medium for 24 h. Supernatants were collected and analyzed using the CBA human inflammatory kit and flow cytometry. (A) IL-8, (B) IL-6, (C) IL-10, (D) IL-1 β , and (E) IL-12p70 levels in PBMC supernatant from patient study groups were graphed. The p values are indicated where significant. CBA, cytometric bead array; IL, interleukin.

cell death. It is possible that factors related to the physical presence of HCV are critical for the enhanced susceptibility to cell death observed in this study. However, this was a cross-sectional study limited by the small number of patients studied. A large-scale longitudinal study is recommended to validate the current findings.

Conclusion

In this study, PBMCs from HCV⁺ patients were compared with those from HCV⁻ healthy controls to determine whether the former exhibited increased susceptibility to PCD. Our findings revealed that PBMCs from HCV⁺ patients were

more susceptible to spontaneous PCD than those from HCV⁻ controls. This difference was associated with impaired IL-6, -8, and -10 secretion in HCV⁺ patients and may thus contribute to failure of HCV clearance.

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Author Disclosure Statement

No competing financial interests exist.

References

- Albertoni G, Arnoni CP, Latini FR, *et al.* Altered of apoptotic markers of both extrinsic and intrinsic pathways induced by hepatitis C virus infection in peripheral blood mononuclear cells. *Virol J* 2012;9:314.
- Alhethel A, Albarrag A, Shakoor Z, *et al.* Assessment of pro-inflammatory cytokines in sera of patients with hepatitis C virus infection before and after anti-viral therapy. *J Infect Dev Ctries* 2016;10:1093–1098.
- Antonelli A, Ferri C, Ferrari SM, *et al.* Serum levels of proinflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor alpha in mixed cryoglobulinemia. *Arthritis Rheum* 2009;60:3841–3847.
- Ashfaq UA, Javed T, Rehman S, *et al.* An overview of HCV molecular biology, replication and immune responses. *Virol J* 2011;8:161.
- Ayrolidi E, Zollo O, Cannarile L, *et al.* Interleukin-6 (IL-6) prevents activation-induced cell death: IL-2-independent inhibition of Fas/fasL expression and cell death. *Blood* 1998;92:4212–4219.
- Bantel H, and Schulze-Osthoff K. Apoptosis in hepatitis C virus infection. *Cell Death Differ* 2003;10 Suppl. 1:S48–S58.
- Bare P. Hepatitis C virus and peripheral blood mononuclear cell reservoirs Patricia Bare. *World J Hepatol* 2009;1:67–71.
- Calabrese F, Pontisso P, Pettenazzo E, *et al.* Liver cell apoptosis in chronic hepatitis C correlates with histological but not biochemical activity or serum HCV-RNA levels. *Hepatology* 2000;31:1153–1159.
- Castillo I, Rodriguez-Inigo E, Bartolome J, *et al.* Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. *Gut* 2005;54:682–685.
- El-Domyati M, bo-Elenin M, El-Din WH, *et al.* Expression of apoptosis regulatory markers in the skin of advanced hepatitis-C virus liver patients. *Indian J Dermatol* 2012; 57:187–193.
- Kida H, Yoshida M, Hoshino S, *et al.* Protective effect of IL-6 on alveolar epithelial cell death induced by hydrogen peroxide. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L342–L349.
- Li A, Dubey S, Varney ML, *et al.* IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol* 2003;170:3369–3376.
- Manzin A, Candela M, Paolucci S, *et al.* Presence of hepatitis C virus (HCV) genomic RNA and viral replicative intermediates in bone marrow and peripheral blood mononuclear cells from HCV-infected patients. *Clin Diagn Lab Immunol* 1994;1:160–163.
- Mirodzhov GK, Odinaev RI, and Sattarova MI. [Cytokines in chronic hepatitis C]. *Klin Med (Mosk)* 2009;87:13–17.
- Modi AA, and Liang TJ. Hepatitis C: a clinical review. *Oral Dis* 2008;14:10–14.
- Nakamoto Y, Kaneko S, and Kobayashi K. Increased susceptibility to apoptosis and attenuated Bcl-2 expression in T lymphocytes and monocytes from patients with advanced chronic hepatitis C. *J Leukoc Biol* 2002;72:49–55.
- Neuman MG, Benhamou JP, Marcellin P, *et al.* Cytokine—chemokine and apoptotic signatures in patients with hepatitis C. *Transl Res* 2007;149:126–136.
- Otsuka M, Kato N, Taniguchi H, *et al.* Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. *Virology* 2002;296:84–93.
- Par G, Szereday L, Berki T, *et al.* Increased baseline proinflammatory cytokine production in chronic hepatitis C patients with rapid virological response to peginterferon plus ribavirin. *PLoS One* 2013;8:e67770.
- Polyak SJ, Khabar KS, Rezeiq M, and Gretch DR. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* 2001;75:6209–6211.
- Revie D, and Salahuddin SZ. Human cell types important for hepatitis C virus replication in vivo and in vitro: old assertions and current evidence. *Virol J* 2011;8:346.
- Spanakis NE, Garinis GA, Alexopoulos EC, *et al.* Cytokine serum levels in patients with chronic HCV infection. *J Clin Lab Anal* 2002;16:40–46.
- Taga K, Chretien J, Cherney B, *et al.* Interleukin-10 inhibits apoptotic cell death in infectious mononucleosis T cells. *J Clin Invest* 1994;94:251–260.
- Taya N, Torimoto Y, Shindo M, *et al.* Fas-mediated apoptosis of peripheral blood mononuclear cells in patients with hepatitis C. *Br J Haematol* 2000;110:89–97.
- Yoneda S, Umemura T, Katsuyama Y, *et al.* Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. *J Infect Dis* 2011;203:1087–1095.
- Zekri AR, AlamEl-Din HM, Bahnassy AA, *et al.* Serum levels of soluble Fas, soluble tumor necrosis factor-receptor II, interleukin-2 receptor and interleukin-8 as early predictors of hepatocellular carcinoma in Egyptian patients with hepatitis C virus genotype-4. *Comp Hepatol* 2010;9:1.
- Zhang L, Miao L, Han F, and Dou XG. [Cytokine levels in serum of patients with chronic hepatitis C and its significance.]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2011;27:301–303.
- Zignego AL, Macchia D, Monti M, *et al.* Infection of peripheral mononuclear blood cells by hepatitis C virus. *J Hepatol* 1992;15:382–386.
- Zimmermann HW, Seidler S, Gassler N, *et al.* Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis. *PLoS One* 2011;6:e21381.

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