BRIEF REPORT

Altered Expression of SHIP, a Toll-like Receptor Pathway Inhibitor, Is Associated With the Severity of Liver Fibrosis in Chronic Hepatitis C Virus Infection

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Hepatitis C-related fibrogenesis has been shown to involve complex interactions between peripheral and hepatic immune responses. Peripheral whole blood (PB) samples from patients with chronic hepatitis C (n = 36) were subjected to microarray analysis in order to identify gene expression patterns associated with immune pathways in PB and hepatic fibrosis. Distinct regulation of gene expression of inositol polyphosphate-5-phosphatase/145kDa (INPP5D or SHIP), a TLR2/TLR4-inhibitor, and heat shock protein 8/22kDa (HSPB8), an endogenous TLR4-ligand, during fibrogenesis was identified and could be confirmed by quantitative reverse-transcription polymerase chain reaction. These results suggest a potential link between peripheral activity of the TLR4-pathway, peripheral SHIP-dependent immune regulation, and liver fibrosis.

Hepatitis C virus (HCV) is estimated to chronically infect ≥170 million people worldwide [1]. In chronic HCV infection (cHCV), persistent inflammation is one major determinant of liver fibrosis progression toward cirrhosis and reportedly regulated by the innate immune system through toll-like receptor (TLR)-dependent signaling [2]. Upon challenge with specific ligands, for example, HCV core protein (TLR2), dsRNA (TLR3), lipopolysaccharide (LPS, TLR4), or ssRNA (TLR7/8), activation of most TLR pathways results in production of proinflammatory cytokines [2]. To date, a key role of TLR2/TLR4 in human liver fibrosis has been suggested by recent studies [2]. However, fibrogenic mechanisms modulated by the TLR system are still not fully understood, in particular, how peripheral TLR-mediated inflammatory responses are related or contribute to hepatic fibrogenesis during cHCV.

We previously reported in peripheral whole blood (PB) of therapy-naive patients with CHC-related early fibrosis (EF) the existence of a gene expression signature involved in regulation of (1) T-helper 1 (Th1), (2) interferon-α (IFN-α), and (3) TLR-dependent responses [3]. Here we identified gene expression patterns significantly associated with these pathways (items 1–3) and the histological stages of liver fibrosis in order to better characterize molecular events reflective of altered pathway activity in PB potentially linked to hepatic fibrogenesis. Our results based on microarray analysis and follow-up quantitative reverse-transcription polymerase chain reaction (qRT-PCR) demonstrate significant inverse correlation between gene expression of SHIP, a negative regulator of TLR2- and TLR4-induced functions [4], and HSPB8, an endogenous TLR4-ligand [5], and the severity of fibrosis. These findings suggest induction of TLR4-pathway activity in PB in association with more advanced HCV-related liver fibrosis.

METHODS

Study Subjects. Therapy-naive Caucasian patients with cHCV (n = 36) received Peg-IFN-α2a (Cohort-1: Pegasys/Roche; 180 μg once weekly, n = 21) or Peg-IFN-α2b (Cohort-2: PEG-Interon/Schering-Plough; 1.5 mcg/kg/week, n = 15) and daily Ribavirin (RBV) (Rebetol/Schering-Plough, 1000–1200 mg/day) for 48 weeks (HCV-genotype 1, n = 30) or 24 weeks (HCV-genotype 3, n = 6) and were followed for 24 weeks post-treatment at the University Hospital in Essen, Germany. All donors signed informed consents approved by the local ethical committee.

Liver Biopsy. Liver biopsies were obtained from all 36 patients prior to enrollment as part of their standard clinical assessments. Specimens were evaluated using the Batts-Ludwig Grading (BL-G) and Staging (BL-S) system for viral hepatitis [6]. Based on BL-S for fibrosis, patients were assigned into
Laboratory Studies. Quantitative HCV RNA was performed 12 hours before and 36 hours after administration of the first dose of Peg-IFN-α and later during each study visit. Liver chemistry and safety laboratory tests were performed 12 hours prior to treatment initiation and during each study visit.

Microarray Analysis. RNA (PAXgene-Blood-RNA-Kit, Qiagen) isolated from PB collected 12 hours before the first injection of Peg-IFN-α directly into PAXgene-Blood-RNA-Tubes (Qiagen). Total RNA, cRNA synthesis and labeling, and hybridization to the U133A_2 (n = 21, Cohort-1) or U133A (n = 15, Cohort-2) arrays (Affymetrix/St.Clara were performed according to the manufacturer’s protocol. Gene expression values (log2) were determined using a correction for probe GC content, RMA background subtraction, and quantile normalization.

Quantitative Reverse-Transcription Polymerase Chain Reaction. Real-time detection of target gene messenger RNAs (mRNAs) with one-step quantitative PCR was performed on the Rotor-Gene 2000 real-time amplification system (Corbett Research). One-step qRT-PCR was carried out with the QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer’s instructions. For the mRNA population per 1 gene, copy numbers were normalized to the number of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and/or tyrosine 3-monooxygenase/tryptophan-5-monooxygenase activation protein (YWHAZ) transcripts. Furthermore, the HSPB8, SIGIRR, and SHIP gene transcripts were quantified by qRT-PCR using specific QuantiTect Primer Assays (Qiagen). One sample showed overall atypical melt-curves and had to be excluded from the analysis.

Gene Expression Analysis and Statistics. One-way analysis of variance (ANOVA) was performed for pair-wise group comparison between EF and LF and/or for linear regression analysis; for comparisons including both microarray types (n = 36), chip-type related batch effect was removed by regression analysis (PARTEK Genomics Suite). All statistical comparisons and graphics were performed using PARTEK Genomics Suite. The significance cutoff was chosen not to exceed 5% (P < .05).

RESULTS

We previously discovered by microarray analysis and confirmed by qRT-PCR that CCL5 gene expression is significantly associated with detectable Th1 response and up-regulated in PB from therapy-naïve patients with EF versus those with LF during cHCV (P < .005, cohorts 1 and 2 [3]). In this study, we analyzed microarray data to identify genes that significantly correlated (P < .05) with (1) CCL5 mRNA levels (log2; qRT-PCR), (2) histological stages of liver fibrosis (BL-S: 0–4), and (3) were significantly deregulated (P < .05) between EF and LF. Selection of genes that passed all criteria above (1–3) and were associated with key terms Th1, IFN-α, or TLR in the EntrezGene database (Supplementary List 1) resulted in identification of a 4-gene signature including HSPB8, CCL5, TYK2, and SIGIRR (5 probe sets in total; Supplementary Figure 1A). Among these, gene expression of HSPB8, a known endogenous TLR4-ligand [5], showed a significant positive correlation with liver fibrosis (BL-S: 0–4, P < .025; Supplementary Figure 1B). A similar correlation trend was observed for HSPB8 and SIGIRR, a known negative regulator of the TLR4-mediated signalling [7], revealed an inverse correlation slightly above the significance cutoff (P = .057). We detected an inverse correlation between liver fibrosis (BL-S: 0–4) and SIGIRR gene expression using microarray results (P < .01; Supplementary Figure 1B), however, we did not by qRT-PCR at baseline (P = .12).

We further extended our analysis on gene expression profiles in PB of negative regulators of TLR4-mediated functions: INPP5D (SHIP), TWIST, PI3K, and TTP [2]. Of those, SHIP gene expression showed a significant inverse correlation with liver fibrosis (BL-S: 0–4) at baseline (P < .015; Figure 1A). These results were confirmed by qRT-PCR (P < .01; Figure 1B). Interestingly, we found a significant inverse correlation between HSPB8 and SHIP gene expression by both microarray (P < .02) and qRT-PCR (P < .05; Figure 2A and 2B). However, we observed no correlation between gene expression levels (microarray) of TLR4 and TLR2, signaling of which is affected by SHIP [4, 8], with liver fibrosis (BL-S: 0–4) in PB at baseline.

Taken together, gene expression data (qRT-PCR) of inhibitory (SHIP) and stimulatory molecules (HSPB8) of the TLR4-pathway showing biologically consistent patterns in correlation with fibrosis suggest that progression of fibrogenesis during cHCV correlates with enhanced TLR4-dependent proinflammatory signaling in PB in vivo.

It is noteworthy that baseline gene expression of HSPB8 (qRT-PCR) was inversely correlated (P < .03) with absolute viral load decline (HCV-RNA_log10: 36 hours—baseline; Supplementary Figure 2).

DISCUSSION

This study demonstrates that peripheral gene expression profiles consistent with an induction of the TLR4 pathway correlate with advanced stages of liver fibrosis in patients with cHCV. Notably, SHIP, a potent negative regulator of the PI3K pathway that restrains LPS (TLR4-ligand)-induced proinflammatory cytokine production [9], correlates inversely with histological stages of liver fibrosis in vivo. Thus, increased gene expression of SHIP in PB appears as a marker of a clinical state characterized by
favorable liver outcomes during cHCV. Interestingly, previous studies showed that SHIP-deficient (SHIP−/−) mice were more sensitive to LPS-induced mortality [8], while others discovered that SHIP negatively regulated TLR2-dependent induction of proinflammatory cytokines and chemokines in neutrophils, resulting in SHIP−/− animals developing more severe lung injury [4]. Advanced lung pathology was also reported in other studies with SHIP−/− mice [9]. Taken together, these data not only solidly support the herein suggested role of SHIP as a biomarker associated with less severe clinical disease but also imply that SHIP can be casually involved in regulation of immune-related liver pathogenesis due to its anti-inflammatory attributes. In addition, a significant correlation found in vivo between elevated SHIP protein levels and the duration of endotoxin (TLR4-ligand) tolerance [8, 9], thereby blunting the proinflammatory response [10], along with studies stating that persisting inflammation causes progression of liver fibrosis in cHCV [2], add more support to the proposed causal relation between SHIP and hepatic outcome. Moreover, a recent work studying single-nucleotide polymorphisms (SNPs) with respect to spontaneous clearance of HCV identified the SHIP gene region as likely involved in HCV clearance or persistence in European Americans [11], suggesting that SHIP can functionally alter the immune response to HCV.

Furthermore, we discovered a significant inverse correlation between PB gene expression levels of SHIP and HSPB8, a member of the small heat shock protein (HSP) family [12], along with a positive correlation trend between peripheral

![Figure 1](image1.png)

**Figure 1.** At baseline, SHIP gene expression levels correlated inversely with the histological stages of fibrosis. Results are for n = 36 patients (microarray, A) and n = 35 patients (qRT-PCR, normalized against GAPDH, B).

![Figure 2](image2.png)

**Figure 2.** At baseline, a significant inverse correlation between gene expression of HSPB8 (endogenous TLR4-ligand) and SHIP (TLR2,4-pathway inhibitor) was observed. Results are for n = 36 patients (microarray, A) and n = 35 patients (qRT-PCR, normalized against YWHAZ, B).
HSPB8 gene expression and liver fibrosis. To date, induction of expression levels of numerous HSPs has been described in patients with cHCV and/or HCV-related hepatocellular carcinoma (HCC) [12, 13] (Supplementary Information; online only). Of those, HSP27, another small HSP family member, showed increased expression in peripheral lymphocytes and monocytes from cirrhotic patients in correlation with advanced stages for HCC and/or poor HCC-related survival [12]. Similarly, HSPB8 gene expression in PB appears to correlate with more advanced liver disease in our study. In this light, we speculate that both increased and decreased biosynthesis of HSPB8 and SHIP, respectively, may synergistically render patients more susceptible for emergent severe liver fibrosis in cHCV. Recently, HSPB8 has been identified as an endogenous TLR4-ligand that induces production of proinflammatory cytokines in systemic autoimmune diseases [5], which supports its potential role in hepatic fibrogenesis. Moreover, the decreasing SHIP gene expression toward complete fibrosis/cirrhosis in PB suggests an emergent loss of tolerance to TLR2/TLR4-ligands in cHCV-patients with advanced liver disease. This constellation of molecular events supports aggravation of inflammatory responses in the peripheral as well as the hepatic compartment as (1) endotoxin plasma levels are elevated in cHCV-patients [2], (2) homo-tolerance to TLR4-ligands (eg, endotoxin, LPS) and TLR2-ligands (eg, HCV core protein) or LPS-rendered hetero-tolerance to TLR(1-3)-ligands and TLR(6-8)-ligands have been more rarely found in monocytes from patients with cHCV relative to those from healthy donors [2], and (3) in vivo endotoxemia has been shown to lead to loss of tolerance to subsequent TLR stimuli in Kupffer cells, the resident macrophages of the liver [2]. Hence, increasing biosynthesis of HSPB8 along with decreasing hepatic clearance of enteric bacterial antigens (endotoxin or LPS) may lead to a systemic overaccumulation of TLR4-ligands and (given the TLR-tolerance loss frequently occurring during cHCV [2]) favor profibrogenic immune profile. Finally, the significant inverse correlation found between HSPB8 mRNA levels at baseline and the absolute decline of HCV viral load within 36 hours after initiation of treatment further supports the role of increased HSPB8 mRNA levels in PB as an indicator for the occurrence of negative rather than positive clinical trends/events. However, it remains to be determined if small HSPs (ie, HSP27 or HSPB8) have the potential to affect the life cycle of HCV [14].

In summary, we discovered distinctly regulated gene expression profiles of SHIP (TLR2/TLR4-inhibitor) and HSPB8 (endogenous TLR4-ligand) in PB during HCV-related fibrogenesis and validated these results with qRT-PCR. The observed relation between SHIP or HSPB8 to liver fibrosis and to each other complements the biological context of previous research; however, it needs to be validated in larger cohorts. Furthermore, our study indicates a biological link between the peripheral TLR4-pathway, peripheral SHIP-dependent immune regulation, and liver fibrosis, which implies that mechanisms of systemic inflammation with detectable activity in the peripheral circulation can be linked to liver pathogenesis as it has been described for intestinal injury [15]. These results further provide a rational for developing/optimizing therapies that target the SHIP/P13K axis and/or HSPB8 for the treatment of cHCV-related chronic inflammation and fibrosis.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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