Decreased expression of cytochrome P450s 1A2, 2E1, and 3A4; and drug transporters 
\( \text{Na}^+\)-taurocholate cotransporting polypeptide, organic cation transporter 1, and organic 
anion-transporting peptide-C correlates with the progression of liver fibrosis in chronic 
hepatitis C patients

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Abbreviations: LPS, lipopolysaccharide; IL-1β and IL-6, interleukins 1β and 6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; HCV, Hepatitis C virus; CYP, cytochrome P450; PPARα, peroxisome proliferator-activated receptor-α; ROS, reactive oxygen species; PCR, polymerase chain reaction; NTCP, Na+-taurocholate cotransporting peptide, OATP-C, organic anion-transporting peptide-C; OCT1, organic cation transporter 1; MRPs, multidrug resistance-associated proteins; MDRs, multidrug resistance proteins; BSEP, bile salt export pump; BCRP, breast cancer resistance protein; OAT2, organic anion transporter 2; CAR, constitutive androstane receptor; PXR, pregnane X receptor; VDR, vitamin D receptor; UGT1A1, UDP glucuronosyltransferase 1A1; SULTs, sulfotransferases; HNF, hepatocyte nuclear factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; international normalized ratio, INR; GRPs, glucose-regulated proteins; ER, endoplasmic reticulum
Abstract

Patients with chronic hepatitis C viral infection underwent liver biopsies and laboratory studies for evaluation and to determine subsequent treatment. Changes in status of drug metabolism and disposition may vary with chronic hepatitis C stage and should be assessed. Total RNA was extracted from liver biopsy specimens (n=63) and reverse transcribed to yield cDNA. Relative mRNA levels of drug metabolizing enzymes, transporters, nuclear receptors, and pro-inflammatory cytokines were analyzed with normalization to glyceraldehyde 3-phosphate dehydrogenase expression. mRNAs encoding cytochrome P450s (CYPs) 1A2, 2E1, and 3A4, and drug transporters, Na+-taurocholate cotransporting polypeptide, organic anion transporting peptide-C, and organic cation transporter 1 showed remarkable decreases, and tumor necrosis factor-α showed an increase according to fibrosis stage progression. HepG2 cells and primary hepatocytes of two human individuals were treated either with interleukin 1β, interleukin 6, or tumor necrosis factor-α. CYP1A2 and Na+-taurocholate cotransporting polypeptide mRNA levels significantly decreased in HepG2 cells with interleukin 1β and interleukin 6 treatments. CYP2E1 and organic cation transporter 1 mRNA levels significantly decreased with tumor necrosis factor-α treatment only in HepG2. These results suggested that downregulation of CYP1A2, 2E1, and 3A4, and drug transporters, Na+-taurocholate cotransporting polypeptide, organic anion transporting peptide-C, and organic cation transporter 1 manifested in livers of patients with chronic hepatitis C viral infection, was associated, at least in part, with the elevated production of pro-inflammatory cytokines including tumor necrosis factor-α.
Infection and inflammation generally cause a decrease in hepatic capacity for drug metabolism and disposition. Using lipopolysaccharide (LPS) models of hepatic inflammation, a number of investigators have demonstrated decreases in the expression of various drug metabolizing enzymes (Iber et al., 1999; Renton et al., 2004). Bacterial and viral infections are associated with the induction of various cytokines, including interleukins 1β and 6 (IL-1β and IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), which decrease the level of hepatic drug metabolizing enzymes (Iber et al., 1999; Renton et al., 2004). Hepatitis C virus (HCV) infection was reported to cause changes in levels of drug metabolizing enzymes cytochrome P450 (CYP) 2E1 (Gochee et al., 2003), an oxidative stress-related enzyme, and glutathione peroxidase (Levent et al., 2006); and nuclear receptors, including peroxisome proliferator-activated receptor-α (PPARα) (Dharancy et al., 2005). Immune mediated liver damage, viral product mediated cytotoxicity, and oxidative stress have also been documented to play a role in the pathogenicity of HCV infection. Production of cytokines such as TNF-α, which is associated with the generation of reactive oxygen species (ROS), is increased in patients with chronic hepatitis C, causing oxidative stress in these patients (Choi et al., 2004). Despite much accumulated knowledge on the pathophysiology of various infectious diseases, including HCV, there is little information on liver-specific markers that indicate individual drug elimination capacities during HCV infection. In this report, we describe the results of a comprehensive study of variations of mRNA levels of drug metabolizing enzymes and drug transporters in chronic hepatitis C patients. Using quantitative real time polymerase chain reaction (PCR) analysis, liver biopsy samples from 63 Japanese patients were examined. We observed clear correlations between fibrosis stage and mRNA levels of hepatic CYP1A2, CYP2E1, and CYP3A4; and the
drug transporters Na\(^+\)-taurocholate cotransporting peptide (NTCP), organic anion-transporting peptide-C (OATP-C), and organic cation transporter 1 (OCT1). Fibrosis stage-dependent increase in TNF-\(\alpha\) mRNA level was observed in our study population. mRNAs involved in drug metabolism and disposition were also examined in relationship to clinical laboratory data on hepatic and renal function. In addition, exposure of a human hepatoblastoma cell line, HepG2, and human primary haptocytes of two human individuals to TNF-\(\alpha\) partly resulted in a decrease in CYP2E1 mRNA/activity and OCT1 mRNA levels. Exposure of HepG2 cells to IL-1 and IL-6 resulted in a decrease in CYP1A2 and NTCP mRNA levels. The data provide information on fibrosis stage-associated changes in gene expression related to hepatic drug metabolism and disposition, which is useful for drug therapy for patients with chronic hepatitis C infection.
Methods

Subjects. Ultrasound-guided or laparoscopic liver biopsy was performed on 63 patients with chronic hepatitis C at Toranomon Hospital, Tokyo, Japan. Fibrosis staging was divided into 4 classes: F0, no fibrosis; F1, periportal expansion; F2, portoportal septa; and F3, portocentral linkage or bridging fibrosis (Desmet et al., 1994). No patients with liver cirrhosis (F4) were included in this study. Inflammatory activity was divided into 4 classes: A0, no necroinflammatory reaction; A1, mild necroinflammatory reaction; and A2, moderate necroinflammatory reaction (Desmet et al., 1994). No A3 patients with severe necroinflammatory reaction were included in this study (Table 1). Eight liver biopsy samples were excluded because of degraded RNA. RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Hachioji, Japan) (Fig.1).

Clinical laboratory data were collected (Table 2) from patients who were evaluated on the same or previous day that the liver biopsy was performed. Clinical data and history were obtained from each patient’s chart, which was stored at Toranomon Hospital.

Non-cancerous liver tissues, which were resected at Dokkyo Medical University School of Medicine from 21 patients (13 males and 8 females, aged 39-82) with metastatic liver cancer originated from colon cancer, were also used. We obtained 3 non-cancerous liver samples taken from patients (2 males and 1 female, aged 61-71) with metastatic liver cancer originated from colon through Health Science Research Resources Bank (Osaka, Japan). These donor patients were free from both chronic hepatitis B and C viruses. RNA quality was also evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Hachioji, Japan). The study protocol was approved by an independent ethics committee from Toranomon Hospital, Dokkyo Medical University School of Medicine, the National Institute of Health Sciences, and Meiji Pharmaceutical.
University, and conformed to the 1975 Declaration of Helsinki ethical guidelines. Written informed consent was obtained from all patients participating in the study.

**RNA extraction, reverse transcription, and real-time polymerase chain reaction (PCR).** The analyzed liver tissues of chronic hepatitis C patients weighed 1-25 mg (mean±SD = 5.9±5.4 mg), and weight of those of non-hepatitis C patients ranged 50-750 mg, and samples were stored at -70ºC until used. The liver specimens were homogenized three times for 15 sec in Lysis/Binding Solution from an RNA isolation kit (RNAqueous, Ambion Inc., Austin, TX), using a KINEMATICA POLYTRON homogenizer (PT10-35, Kinematika, Lucerne, Switzerland). Total RNA (200 ng) was reverse transcribed to yield cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Real time PCR was performed with probe and primer sets (TaqMan® Gene Expression Assays) available from Applied Biosystems (Applied Biosystems, Foster City, CA) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) for the quantitative analysis of mRNA encoding the following drug metabolism enzymes, drug transporters, nuclear receptors and pro-inflammatory cytokines: CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5; multidrug resistance-associated proteins (MRPs) MRP1, MRP2, MRP3; multidrug resistance proteins (MDRs) MDR1, MDR3; NTCP; OATP-A, OATP-B, OATP-C; bile salt export pump, (BSEP); breast cancer resistance protein (BCRP); OCT1; organic anion transporter 2 (OAT2); constitutive androstane receptor (CAR); pregnane X receptor (PXR); vitamin D receptor (VDR); UDP glucuronosyltransferase 1A1 (UGT1A1); PPARα; sulfotransferases (SULTs) SULT2A1 and SULT2B1; and hepatocyte nuclear factor (HNF) HNF4α and HNF1α dimerization cofactor; IL-1β, IL-6,
mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ABI PRISM 7700 Sequence Detection System automatically created a standard curve by plotting the Ct values against each standard dilution of a known standard human liver poly (A)+ RNA concentration (Beckton Dickinson, Japan). Amounts of poly (A)+ RNA used as a calibration standard that contained the same amounts of transcripts of interest (drug metabolizing enzyme and so on) in the certain amount (200 ng) of patients’ total RNAs were calculated, and were given as A. The same procedure was performed for amounts of poly (A)+ RNA that contained the same amounts of GAPDH transcripts in 200 ng of patients’ total RNAs, and were given as B. Relative mRNA levels were calculated as A/B, which were shown throughout in the present study, and were compared among various liver samples. In principle, A is dependent on the mRNA copy numbers contained in the poly (A)+ RNA used as a calibration standard. Therefore, comparison of A/B cannot be made between different genes. In our preliminary experiments, GAPDH and β-actin mRNA levels well correlated (r=0.7975, p<0.001), indicating that using GAPDH and β-actin gave quite similar results. We decided to use GAPDH for normalization, although justification of GAPDH usage has not fully been verified. All amplification reactions were carried out in duplicate. All mRNA level measurements were performed in at least two separate experiments, which had deviations within 7% of the mean values.

mRNA levels for genes involved in drug metabolism and disposition in HepG2 cells and primary human hepatocytes treated with either IL-1β, IL-6 or TNF-α. HepG2 cells, a human hepatoblastoma cell line, which were routinely cultured in Dulbecco’s Minimum Essential Medium supplemented with 10% fetal bovine serum, were treated
with either recombinant IL-1β (4 ng/mL, PeproTech EC, London, UK), recombinant IL-6 (50 ng/mL, PeproTech EC, London, UK), or recombinant TNF-α (0.25 ng/mL, Upstate, Lake Placid, NY, USA) at 37°C for 24 h. These cytokine concentrations did not show any cytotoxicity for up to 72 h. The cells were washed twice with 5 ml of phosphate buffered saline (pH 7.4) after cytokine exposure, and total RNA was purified using the RNeasy Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer’s instructions. Two different human primary hepatocyte monolayers (Human long-term hepatocytes in monolayer derived from a 73 years old female (hepatocytes #1) and a 72 years old male (hepatocytes #2)) were purchased through KAC Co. (Kyoto, Japan) from Biopredic (Renne, France). They were provided in a 24-well plate. The medium was replaced with long term culture medium as indicated by the manufacturer and cultured for 2 days at 37°C. Then, the cells were treated with either recombinant IL-1β (4ng/mL, PeproTech EC, London, UK), recombinant IL-6 (50 ng/mL, PeproTech EC, London, UK), or recombinant TNF-α (0.25 ng/mL, Upstate, Lake Placid, NY, USA) at 37°C for 24 h. Total RNAs were purified using RNeasy Mini Kit (Qiagen, GmbH, Germany) as in case of HepG2 cells. Total RNA was then reverse-transcribed by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and subjected to real-time PCR analyses for levels of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 mRNA by TaqMan Gene Expression Assay using an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). The RNA levels were normalized to GAPDH. The relative mRNA levels were determined in triplicate for HepG2 experiments. The results were expressed as mean±SD. Data are expressed as mean±SD in four separate human hepatocyte experiments.
Measurement of CYP2E1 activity. CYP2E1 activity in human primary hepatocytes was determined according to the method by Ito et al. (Ito et al., 2007). In brief, cultured primary hepatocytes were incubated in a fresh media with 0.5 mM p-nitrophenol at 37°C for 60 min. The supernatant was assayed for 4-nitrocatechol by the addition of 10M NaOH (1:10) and immediate determination of the absorbance at 546 nm, Activity after cytokine exposure was compared with that of control.

Determination of Viral Load. HCV RNA viral load in serum samples was calculated by the Amplicor quantification method (Amplicor HCV Monitor assay version 2.0, Roche Diagnostics, Tokyo).

Statistical analysis. Statistical analysis was performed using the Kruskal-Wallis test, one-way analysis of variance (ANOVA) with a post hoc test (Tukey’s multiple comparison test), the Mann-Whitney test, Chi-square test, and the Spearman rank correlation using GraphPad PRISM version 4.02 (GraphPad Software Inc., San Diego, CA). In each test, $p<0.05$ was considered statistically significant.
Results

Patient characteristics. Table 1 describes the histopathological features of the liver samples, together with the age, sex, and smoking, drinking habits and major medications of the chronic hepatitis C patients recruited in the present study. Liver biopsy was performed to decide subsequent therapy. Therefore, no patient received interferon before liver biopsy. There were 34 males and 29 females; and 20 smokers. There were no heavy drinkers, although 21 reported alcohol consumption. The patient distribution by fibrosis stages was: F₁ = 35, F₂ = 11 and F₃ = 17. The distribution of inflammatory activity was: A₁ = 38 and A₂ = 25. The laboratory data from chronic hepatitis C patients are shown in Table 2. The mean HCV RNA viral load was 777±596 (KIU/mL), and HCV genotypes were divided into 4 classes (n):1a (1), 1b (42), 2a (13), and 2b (7)). Most of the Japanese chronic hepatitis C patients were infected with genotype 1b viruses, and the genotype 1b patients had high HCV RNA viral loads. The prothrombin times (international normalized ratio, INR) and hyaluronic acid levels were significantly higher in F₃ patients than in F₂ patients (Fig. 2). Most of the F₃ patients had hyaluronic acid levels that were greater than the highest normal values (Fig. 2B). No correlation was observed between viral load and fibrosis stage, which was consistent with a previous report (Gronbaek et al., 2005).

Relationships between relative mRNA expression for CYPs, drug transporters, and nuclear receptors, and fibrosis staging and inflammation grading in chronic hepatitis C patients. We analyzed trends in mRNA expression of drug metabolizing enzymes, drug transporters, nuclear receptors, and pro-inflammatory cytokines according to the progression of chronic hepatitis C measured by histological staging and
grading. Samples of fifty-five of 63 chronic hepatitis C patients (8 samples were excluded because of RNA degradation, Fig. 1) were evaluated by a pathologist from F1 to F3: F1=31, F2=10, and F3=14; and from A1 to A2: A1=34 and A2=21. mRNA levels of drug metabolizing enzymes (CYP1A2 (Fig. 3A), CYP2E1 (Fig. 3B), and CYP3A4 (Fig. 3C)), and drug transporters, NTCP (Fig. 3D), OATP-C (Fig. 3E), and OCT1 (Fig. 3F) showed remarkable decreases as the fibrosis stage progressed. These parameters failed to correlate with increased inflammation, except for CYP1A2. The relative CYP1A2 mRNA levels showed marginal statistical significance with increased inflammation (plot not shown, \( p =0.0369 \) by non-parametric Mann-Whitney test). No fibrosis stage-dependent differences were observed for all other genes studied (see Materials and Methods). For example, mRNA levels for CYP2D6 and MDR1 did not correlate (plots not shown, \( p =0.7059 \) and \( p =0.9932 \), respectively). For reference, mRNA levels for CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 in the non-cancerous and non-hepatits C liver tissues from 24 human individuals, who suffered from colon cancer and underwent resection of liver metastasis. In case of CYP3A4 and OATP-C, mean mRNA levels of the non-hepatits C livers were higher that those of F1 patients, but not in regard to other four mRNAs. Although determinations of mRNA levels of interest were performed in the same way after checking the quality of RNAs as in hepatitis C cases, the non-hepatits C livers were derived from tissues surrounding tumors, not from healthy subjects. Therefore, some mRNAs may be downregulated in the liver tissues surrounding cancerous tissues as in case of hepatits C patients. Alternatively, CYP1A2, CYP2E1, NTCP, and OCT1 might be once upregulated in the initial F1 stage after hepatitis C viral infection, and thereafter, their expression levels gradually decrease to those in the non-HCV patients. One-way ANOVA analyses of these gene expressions
indeed revealed statistically significant elevation of CYP1A2 (p<0.01), CYP2E1 (p<0.01), NTCP (p<0.01), and OCT1 (p<0.05) mRNA levels in the patients of the F1 stage as compared with those in the non-HCV patients. Those in the F3 patients were not statistically different from those in the non-HCV patients. This hypothesis should be clarified by further studies performed using liver samples obtained in one hospital.

mRNA levels of TNF-α, but not those of IL-1β, increased with statistical significance as the fibrosis stage progressed. As illustrated in Fig. 4, TNF-α mRNA levels in F3 patients were significantly higher that those of F1 patients (one-way ANOVA, p<0.05). IL-6 mRNA levels were too low to evaluate in relation to fibrosis stage: IL-6 was detected in 2 of 31 subjects with F1 stage, 1 in 10 (F2), and 4 in 14 (F3) (chi-square test, p>0.05).

**Expression of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 mRNAs in HepG2 cells and primary human hepatocytes treated with pro-inflammatory cytokines.** HepG2 cells were exposed to either 4 ng/mL recombinant IL-1β, 50 ng/mL recombinant IL-6, or 0.25 ng/mL recombinant TNF-α at 37°C for 24 h, all of which did not show any appreciable cytotoxicity. mRNA levels of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C and OCT1 decreased as the fibrosis-stage progressed and were measured after 24 h cytokine exposure using real-time PCR as described in Methods. As shown in Fig. 5, CYP1A2 and NTCP mRNA levels were significantly decreased by IL-1β and IL-6 (by 26.1% and 37.1% for CYP1A2; and 89.6% and 96.4% for NTCP, respectively). CYP2E1 and OCT1 mRNA levels were significantly decreased by TNF-α treatment by 50.4% and 35.5%, respectively. IL-6 showed a tendency to decrease CYP2E1, CYP3A4 and OCT1 mRNA levels, which did not, however, reach statistical
significance. No remarkable alteration of OATP-C mRNA was observed by exposure to IL-1β, IL-6, or TNF-α. Human primary hepatocytes of two different individuals were treated with either 4 ng/mL recombinant IL-1β, 50 ng/mL recombinant IL-6, or 0.25 ng/mL recombinant TNF-α at 37°C for 24 h exactly in the same way as HepG2 cells. Our preliminary experiments on p-nitrophenol oxidation using human hepatocyte #2 showed that IL-1β and TNF-α almost completely suppressed the activity, whereas IL-6 showed only 44% inhibition in spite of no appreciable change in mRNA expression. Apparently, regulatory mechanisms of CYP2E1 seemed quite different between HepG2 and human primary hepatocytes. Expressed levels of drug transporters, NTCP, OATP-C and OCT-1, after normalization by GAPDH were roughly 100-, 1000- and 10-fold higher in primary hepatocytes than HepG2, respectively. Our preliminary data suggested IL-6-downregulation by larger than 45% of CYP1A2, CYP3A4, NTCP, OATP-C, and OCT1 in the hepatocytes of the two human individuals. Our present in vitro study using HepG2 and primary hepatocytes may indicate both systems should be used complementarily to clarify mechanisms for cytokine-mediated downregulation of drug metabolism and disposition genes.
Discussion

There has been little information published on which genes involved in drug metabolism and disposition undergo alteration of their expression during chronic hepatitis C viral infection. Since data have been accumulating on substrate specificities and function for a number of cytochrome P450s and drug transporters, it is valuable to elucidate the changes in gene expression for those CYPs and transporters for which information is available. We have examined chronic hepatitis C-related changes in the expression of drug metabolism and disposition genes using total RNA extracted from liver biopsy samples from 63 Japanese patients who were diagnosed with chronic hepatitis C at Toranomon Hospital, Tokyo, Japan. The patients recruited in this study exhibited a tendency toward higher values of prothrombin times (INR) and hyaluronic acid levels with HCV-related liver fibrosis progression (Fig. 2).

In agreement with these clinical laboratory observations, a remarkable decrease in CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 mRNA levels was observed in relationship to liver fibrosis progression (Fig. 3). Hepatic expressions of CYP1A2, CYP2E1, NTCP and OCT1 in initial fibrosis stage (F1) of hepatitis C infection seemed to be once upregulated as compared with non-HCV patients according to the statistical analyses throughout non-HCV and F1-F3 HCV patients (Fig. 3). This hypothesis should be evaluated by further studies using liver specimens obtained in one hospital. These mRNA levels overall showed negative correlations with clinical laboratory results of aspartate amino transferase, prothrombin time (INR), and hyaluronic acid (Nakai, unpublished). However, no fibrosis stage-dependent decrease was observed for any of the other genes studied, including CYP2D6 and MDR1 (plots not shown). Only some of drug metabolism and disposition gene expressions showed
fibrosis-stage dependent decrease. During fibrosis progression, fibrotic hepatocytes would be replaced by fibroblasts. This might lead an assumption that not a few hepatic genes would be simultaneously downregulated. The present study excludes this most trivial explanation on mechanisms of downregulation of hepatic genes. In conjunction with inflammatory state of hepatitis C infection, an increase in TNF-α mRNA level was manifested with the progress of liver fibrosis staging (Fig. 4).

We found a clear relationship between the decrease in hepatic CYP1A2 expression and fibrosis stage progression (Fig. 3A) and inflammation grading (plot not shown) in patients with chronic HCV infection, which is generally consistent with observations by Congiu et al. (Congiu et al., 2002). CYP1A2, but not CYP4A, decreased at both the mRNA and protein levels during sepsis progression in the rat. Cytochrome P450 blockade by pretreatment with 1-aminobenzotriazole exacerbated the inflammatory response in sepsis (Crawford et al., 2004). These results, together with the finding that CYP1A2 has a protective role against ROS production (Shertzer et al., 2004), indicate that reduction in CYP1A2 expression during HCV infection state might be deleterious. A number of reports in the literature observed that there is a reduction of CYP2E1 expression in chronic HCV infection (Gochee et al., 2003; Asselah et al., 2005; Bieche et al., 2005). The results of this study are consistent with those reports.

CYP2E1 has been documented to have a role in ROS generation during exposure of mammalian cells to ethanol, a CYP2E1 substrate/inducer (Navasumrit et al., 2000). Singlet oxygen (\(^1\)O\(_2\)) has been suggested to be involved in CYP2E1 function (Hayashi et al, 2005). Oxidative stress by ethanol has been associated with ethanol-induced and ethanol-metabolizing CYP2E1, converting it to more reactive intermediates (Cederbaum and Kessova, 2003). The HCV core protein, in coordination with ethanol,
was reported to increase oxidative stress (Wen et al., 2004). The findings using CYP2E1-overexpress cells have been reviewed regarding the toxicological properties of CYP2E1 in conjunction with alcohol (Caro and Cederbaum, 2004). Mitochondrial ROS species production is synergistically induced by HCV core protein and CYP2E1, resulting in a reduction of mitochondrial antioxidant capacity, and sensitivity to oxidants and TNF-α (Otani et al., 2005). CYP2E1-mediated oxidative stress reportedly downregulates glucose-regulated proteins (GRPs) 78 and 94 that reside in endoplasmic reticulum (ER) and protect ER from CYP2E1-dependent oxidative stress (Dey et al., 2006). Taken together, CYP2E1 downregulation in the chronic hepatitis C-infected liver may be a protective response of liver cells against oxidative stress. The CYP3A4 mRNA titer in blood showed a tendency to decrease with the progression of viral liver disease, which is consistent with our present study (Horiike et al., 2005). PXR, controlling CYP3A4 expression, failed to change in accordance with fibrosis stage progression (Nakai, unpublished). The biological significance of decreases in liver CYP3A4 levels and drug transporters is, at present, difficult to interpret, due to little knowledge on biological function of CYP3A4 and drug transporters, and the mechanisms and pathophysiology of chronic hepatitis C.

Genome-wide analyses of gene expression in liver biopsy specimens from patients with mild or early stage fibrosis caused by chronic hepatitis C have been reported. Upregulation in comparison with normal liver patients of IL-6 and TNF and down-regulation of CYP2E1 was observed as compared with normal liver patients (Bieche et al., 2005; Asselah et al., 2005). It was also shown that IL-6 and TNF levels in patients with liver fibrosis stage 2-4 were significantly higher than those in patients with stage 1 fibrosis (Asselah et al., 2005). In our study population, upregulation of
TNF-α was consistent overall with the studies by Asselah et al. (Asselah et al., 2005), but IL-6 mRNAs were below the limit of detection in many subjects. IL-6 has been shown to down-regulate CYP1A1, CYP1A2, and CYP3A4 in human hepatoma cells (Fukuda et al., 1992); and to down-regulate CYP2E1 (Hakkola et al., 2003) and CYP3A4 (Jover et al., 2002). Abdel-Razzal et al. showed that IL-6, IL-1β, and TNF-α downregulated expression of human CYP1A1, 1A2, and 3A in adult human hepatocytes in primary culture (Abdel-Razzak, 1993). These results were consistent overall with our experiments using HepG2 cells and human primary hepatocytes (Fig. 5). IFN-γ also suppressed CYP1A2 and CYP2E1 in the human primary hepatocyte system (Abdel-Razzak et al., 1993). Similarly, in human primary hepatocyte cultures, cytokines negatively affected inducible expression of CYP1A1, CYP1A2, and CYP3A4 (Muntane-Relat, 1995). With respect to drug transporters, murine Ntcp was shown to be downregulated by IL-1β in vivo (Geier et al, 2005). Very recently, Le Vee et al. reported repression of NTCP mRNA and protein expression by IL-1β in human primary hepatocytes (Le Vee et al., 2008). IL-1β was reported to inhibit CAR-induced expression of hepatic CYP3A4 (Assenat et al., 2005). A decrease in CYP3A4 is consistent with the present results, but a decrease in CAR mRNA levels has not been related to fibrosis stage progression (Nakai, unpublished). Gene promoter analysis of human OATP-C expression revealed that HNF1α stimulated OATP-C expression (Jung et al., 2001). HNF1α transcriptional activator did not strikingly correlate with fibrosis staging (Nakai, unpublished). Expression of human OCT1 was activated by HNF4α, according to a study that used a luciferase reporter assay (Saborowski et al., 2006). Expression of HNF4α was also not correlated with fibrosis staging, (Nakai, unpublished), which suggests that HNF4α by itself does not fully explain the
mechanisms involved in the decrease in OCT1 expression in chronic hepatitis C patients. TNF-α repressed CYP2E1, CYP3A4, and OCT1 expression in HepG2 cells. Our preliminary results showed IL-6-downregulation of CYP1A2, CYP3A4, NTCP, and OCT1 expression in the human hepatocytes of both two individuals. Our present results on fibrosis stage-dependent decreases in the expression of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 suggest that these decreases were likely controlled, at least in part, by mechanisms associated with the elevated cytokine production of TNF-α.

CYP1A2 mRNA levels significantly correlated with CYP2E1 (r=0.705, P <0.0001) and CYP3A4 (r=0.535, P <0.0001), but not with CYP2D6 (r=0.231, P >0.05). In addition, OCT1 mRNA levels significantly correlated with NTCP (r=0.634, P <0.0001), but not with MDR1 (r=0.231, P >0.05). These results are consistent with our hypothesis that inflammatory cytokines are involved in the downregulation of the expression of these genes. The precise molecular mechanisms of fibrosis stage-dependent decreases in the expression of the CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 genes governing drug metabolism and transport should be further clarified. The data, providing information on fibrosis stage-associated changes in the gene expression related to hepatic drug metabolism and disposition, is useful for drug therapy for patients with chronic hepatitis C infection. In fact, NTCP reportedly has an important role in hepatic uptake of ursodeoxycholic acid (Mita et al., 2006). Recent report indicated INR of prothrombin time negatively correlated with antipyrine clearance, informative indices for hepatic impairment in hepatitis C patients (Mahmoud et al., 2007). The results were quite consistent with our results, as antipyrine was shown to be a substrate for CYP1A2 and CYP3A4 (Rendic, 2002).

In conclusion, our results indicate that in the relatively early stages of chronic
hepatitis C infection without cirrhosis, factors such as cytokines are likely to affect the expression of drug metabolism enzymes and drug transporters, such as CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C and OCT1.
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Foot notes

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Legends for figures

Figure 1. Analysis for RNA degradation by Agilent 2100 Bioanalyzer.

Total RNA extracted from 63 Japanese patients was subjected to electrophoretic analysis. As shown in sample A, 28S and 18S rRNAs were detected as sharp peaks, while in sample B, both rRNAs sizes were completely degraded. Of the 63 samples, 8 samples showed a high degree of RNA degradation, and therefore were excluded from subsequent analyses.

Figure 2. Relationships between disease progression (fibrosis) and prothrombin time (INR) and hyaluronic acid level.

Clinical laboratory data were plotted in relationship to fibrosis stage. Black lines represent means for each category. Red lines represent the highest limit of normal values. The statistical significance of the differences in the values between fibrosis stages was evaluated by the Kruskal-Wallis test and shown as P values. INR = international normalized ratio.

Figure 3. Reduced expression of CYP1A2 (A), CYP2E1 (B), CYP3A4 (C), NTCP (D), OATP-C (E), and OCT1 (F) in relationship to the progression of liver fibrosis in chronic hepatitis C patients. Relative mRNA levels for drug metabolism and disposition genes in 55 biopsy liver samples are plotted (closed circles) according to the disease stage defined by liver fibrosis. The methods for the determination of the relative mRNA levels are described in the Methods. Degrees of statistical significance were analyzed by one-way ANOVA with a post hoc test (Tukey’s multiple comparison test). NS = not significant (P > 0.05). For reference, relative mRNA levels involved in drug
metabolism and disposition in 24 liver non-cancerous samples derived from human individuals, who underwent resection of metastatic liver cancer (originated from colon cancer), are plotted (open circles). Degrees of statistical significance were analyzed by one-way ANOVA with a post hoc test (Dunnet’s multiple comparison test) throughout non-HCV and F_1-F_3 HCV patients. Means of CYP1A2, CYP2E1, NTCP, and OCT1 were significantly different ($P<0.001$). Statistical significance between values of non-HCV and each of F_1-F_3 patients is shown on the graph in case of significant difference.

Figure 4. Expression of pro-inflammatory cytokines TNF-$\alpha$ (A) and IL-1$\beta$ (B) in relationship to the progression of liver fibrosis in chronic hepatitis C patients. Relative mRNA levels for TNF-$\alpha$ (A) and IL-1$\beta$ (B) genes in 55 biopsy liver samples are plotted according to the disease stage defined by liver fibrosis. Approaches for the determination of the relative mRNA levels are described in the Methods. Degrees of statistical significance were analyzed by one-way ANOVA with a post hoc test (Tukey’s multiple comparison test). NS = not significant ($P>0.05$).

Figure 5. Expression of CYP1A2 (A), CYP2E1 (B), CYP3A4 (C), NTCP (D), OATP-C (E), and OCT1 (F) mRNA levels in HepG2 cells after 24 h-exposure to pro-inflammatory cytokines IL-1$\beta$, IL-6, or TNF-$\alpha$.

HepG2 cells were exposed to either IL-1$\beta$, IL-6 or TNF-$\alpha$ at 37°C for 24 h as described in the Methods. Data are expressed as mean±SD from three separate experiments.

Degree of statistical significance of the difference of mRNA levels between control (solvent) and each cytokine-treated group was analyzed by one-way ANOVA with Dunnet post hoc test. Statistical significance is indicated by symbols, *, and **, which
represent $p<0.05$ and $p<0.01$, respectively.
Table 1. Histopathological features and demographics in chronic hepatitis C infection

<table>
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<tr>
<th>Patients with chronic hepatitis C (n=63)</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Sex (M/F)</td>
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<tr>
<td>Smoking (n)</td>
<td>20</td>
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<td>Alcohol (n)</td>
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<td>Major medications</td>
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<tr>
<td>Glycyrrhizic acid/Ursodeoxycholic acid</td>
<td>F1 (6), F2 (2), F3 (4)</td>
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<tr>
<td>Amlodipine/Nicardipine/Atenolol</td>
<td>F1 (5), F2 (2), F3 (2)</td>
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<td>Brotizolam/Alprazolam/Triazolam</td>
<td>F1 (4), F2 (1), F3 (0)</td>
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<td>Glimepiride/Nateglinide</td>
<td>F1 (4), F2 (1), F3 (1)</td>
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<td>Pathological class</td>
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<tr>
<td>Fibrosis staging (n)</td>
<td>F0 (0), F1 (35), F2 (11), F3 (17), F4 (0)</td>
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<tr>
<td>Inflammation grading (n)</td>
<td>A0 (0), A1 (38), A2 (25), A3 (0)</td>
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</table>

No subject received interferon therapy before liver biopsy.
Table 2. Chronic hepatitis C patient laboratory data

<table>
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<tr>
<th>Clinical laboratory data</th>
<th>Number or value (mean±SD; Range)</th>
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<td>Viral load (KIU/mL)</td>
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<td>1b</td>
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<td>2a</td>
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<td>2b</td>
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<td>ALT (IU/L)</td>
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<tr>
<td>AST (IU/L)</td>
<td>88±53; 23-276</td>
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<td>γ-GTP (IU/L)</td>
<td>89±73; 17-264</td>
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<td>ALP (IU/L)</td>
<td>218±78; 81-430</td>
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<tr>
<td>LDH (IU/L)</td>
<td>159±27; 104-256</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.9±0.3; 0.4-2.2</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>165±27; 109-222</td>
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<tr>
<td>Serum triglycerides (mg/dL)</td>
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<td>HDL (mg/dL)</td>
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<td>Total protein (g/dL)</td>
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<td>Prothrombin time (INR)</td>
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<td>AFP (ng/mL)</td>
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<td>Fe (µg/dL)</td>
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<td>UIBC (µg/dL)</td>
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<td>Ferritin (ng/mL)</td>
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<td>Hyaluronic acid (µg/L)</td>
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HCV = hepatitis C virus, ALT = alanine aminotransferase; AST = aspartate aminotransferase, γ-GTP = γ-glutamyl transpeptidase, ALP = alkaline phosphatase, LDH = lactatedehydrogenase, HDL = high density lipoprotein, INR = international normalized ratio, AFP = α-fetoprotein; UIBC = unsaturated iron binding capacity.
Table 3. Probe and primer sets (TaqMan® Gene Expression Assays) used in this study

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*a Dimerization cofactor of HNF1α (TCF1) 2
Fig. 2

**A. Prothrombin time (INR)**

**B. Hyaluronic acid**

$p = 0.0112$  

$p = 0.0010$
*Non-HCV livers were derived from non-cancerous liver tissues from individuals, who suffered from metastatic liver cancer originated from colon cancer.
Fig. 4

A

\[ p < 0.05 \]

\[ \text{NS} \]

\[ \text{NS} \]

\[ \text{TNF-\(\alpha\)/GAPDH} \]

\[ \text{F1} \]

\[ \text{F2} \]

\[ \text{F3} \]

\[ p = 0.0352 \]

\[ \text{One-way ANOVA} \]

B

\[ p > 0.05 \]

\[ \text{IL-1\(\beta\)/GAPDH} \]

\[ \text{F1} \]

\[ \text{F2} \]

\[ \text{F3} \]

\[ \text{NS} = p > 0.05 \]
Fig. 5

A

B

C

D

E

F

Control
IL-1β (4 ng/mL)
IL-6 (50 ng/mL)
TNF-α (0.25 ng/mL)

Control
IL-1β (4 ng/mL)
IL-6 (50 ng/mL)
TNF-α (0.25 ng/mL)

Control
IL-1β (4 ng/mL)
IL-6 (50 ng/mL)
TNF-α (0.25 ng/mL)

Control
IL-1β (4 ng/mL)
IL-6 (50 ng/mL)
TNF-α (0.25 ng/mL)

Control
IL-1β (4 ng/mL)
IL-6 (50 ng/mL)
TNF-α (0.25 ng/mL)

Control
IL-1β (4 ng/mL)
IL-6 (50 ng/mL)
TNF-α (0.25 ng/mL)