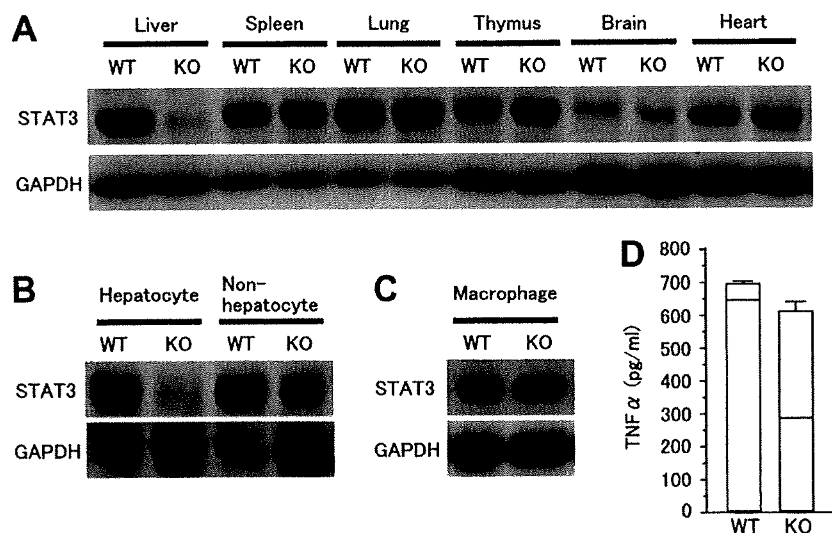

Fig. 1. Hepatocyte-specific STAT3 deficiency in mice. Floxed STAT3 mice were crossed with Alb-Cre transgenic mice. Floxed STAT3 mice having the Alb-Cre transgene were regarded as L-STAT3 KO mice (KO); those not having the Alb-Cre gene were used as a wild-type control (WT). (A) STAT3 expression in a variety of organs from L-STAT3 KO mice and wild-type mice via western blot analysis. Expression of GAPDH was served as a loading control. Representative blots are shown. (B) Expression of STAT3 of isolated hepatocytes and nonhepatocytes. Liver of L-STAT3 KO mice or wild-type mice was collagenase-perfused and separated into hepatocyte and nonhepatocyte fractions. STAT3 expression was determined via western blot analysis. Expression of GAPDH was served as a loading control. Representative blots are shown. (C) Expression of STAT3 in isolated macrophage. Peritoneal macrophage was isolated from L-STAT3 KO mice or wild-type mice and subjected to western blot analysis of STAT3 expression. Representative blots are shown. (D) LPS-stimulated TNF-α production of peritoneal macrophages. Peritoneal macrophages were isolated from L-STAT3 KO mice or wild-type mice (n = 6 for each group) and stimulated with LPS (100 ng/mL) for 24 hours. TNF-α production was determined via ELISA in culture supernatants.

binding to CCAAT enhancer-binding protein (C/EBP) and type 2 IL-6 responsive elements.[21]

To address the issue of whether hepatic STAT3 is involved in the outcome of CLP-induced lethality, we performed CLP blinded to the genetic background and checked the survival of the mice every 6 hours. L-STAT3 KO mice were significantly more vulnerable to CLP-induced lethality than wild-type littermates (Fig. 2C). To examine the possible difference in bacterial infection after CLP, we measured colony forming unit of blood bacteria 24 hours after CLP. Because there was no significant difference in bacterial amount between L-STAT3 KO mice and wild-type mice (Fig. 2D), we considered hepatic STAT3 to have had a beneficial effect on the outcome of septic shock without affecting bacterial infection.

Hepatic STAT3-Deficient Mice Show Exacerbated Liver Injury. To examine liver injury and renal dysfunction in CLP-induced sepsis, we measured ALT and creatinine levels. L-STAT3 KO mice showed increased levels of serum ALT and creatinine compared with wild-type littermates, although the difference in creatinine did not reach a significant level (Fig. 3A). TUNEL of the liver revealed that the number of apoptotic hepatocytes was significantly higher in L-STAT3 KO mice than in wild-type littermates (Fig. 3B,C). However, the liver injury itself presumably is not a direct cause of animal death, because histologic abnormality was modest. Furthermore, LPS injection, which is another model of septic shock, induced more hepatocyte apoptosis than CLP but did not kill any mice tested (Fig. 3A-C and data not shown), supporting the idea that increased liver injury could not explain the increased lethality in L-STAT3 KO mice.

Exacerbated Systemic Inflammatory Response in L-STAT3 KO Mice. Hypercytokinemia underlying systemic inflammatory response syndrome may play an important role in the development of multiple organ dysfunction syndrome and lethality.[9] We measured several circulating cytokines and chemokines in septic mice and found that TNF-α, IFN-γ, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β) had clearly increased 24 hours after CLP in L-STAT3 KO mice. Of importance is the finding that the plasma levels of these cytokines and chemokines were significantly higher in L-STAT3 KO mice than in wild-type mice, although they did not differ before CLP. This result indicates that the increased lethality found in L-STAT3 KO mice is associated with hypercytokinemia (Fig. 4A). Although plasma insulin levels significantly increased 24 hours after CLP, there was no significant difference between L-STAT3 KO mice and wild-type mice, suggesting that insulin levels do not affect the difference in animal lethality (Supplementary Fig. 1).

Given that bacterial infection did not differ between L-STAT3 KO mice and wild-type mice, we examined the

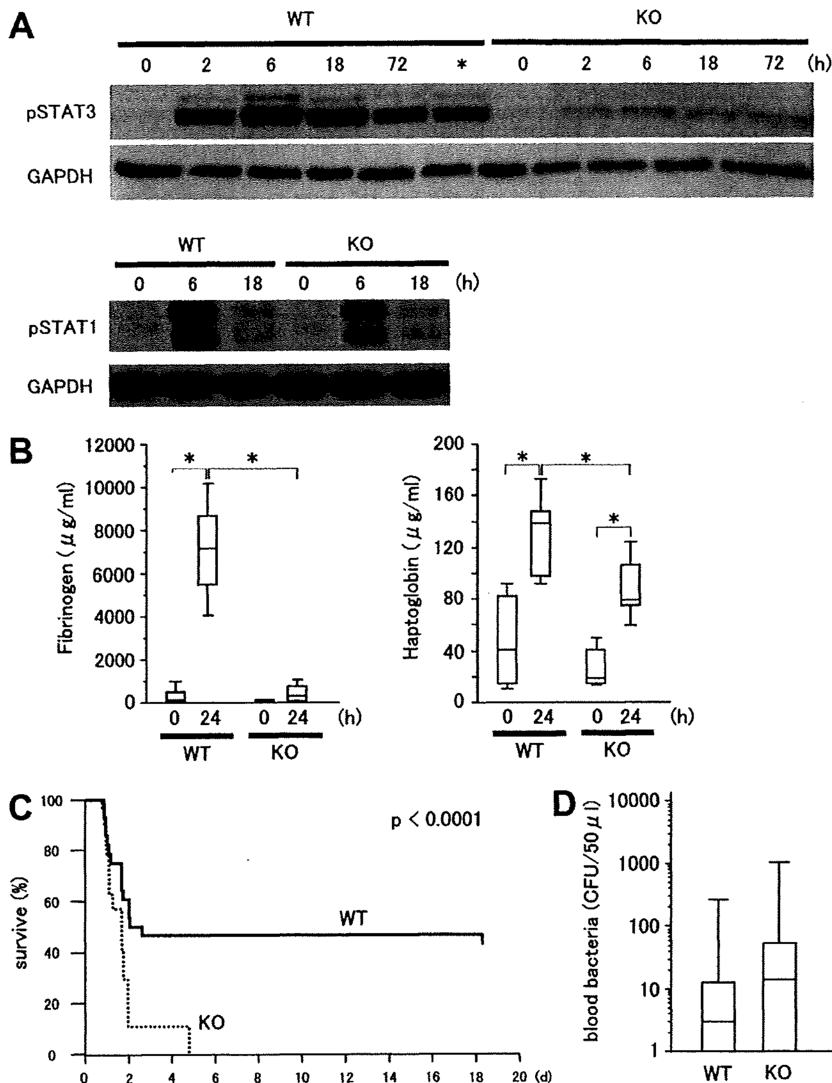

Fig. 2. STAT3 activation, APP production, and survival in CLP mice. (A) Western blot analysis of phosphorylated STAT3 and STAT1 in CLP mice. L-STAT3 KO mice (KO) and wild-type mice (WT) were treated with CLP and sacrificed at the indicated time points. Their liver tissues were subjected to analysis of Tyrosine 705 phosphorylation of STAT3 or Tyrosine 701 phosphorylation of STAT1 via western blot analysis. GAPDH expression served as a control. Representative blots are shown. *7 days. (B) Levels of circulating haptoglobin and fibrinogen before and 24 hours after CLP (n = 8 for each group). *$P < 0.05$. (C) Comparison of survival after CLP between L-STAT3 KO (n = 27) mice and wild-type littermates (n =28). (D) Colony-forming units of blood bacteria after CLP. L-STAT3 KO or wild-type mice were sacrificed 24 hours after CLP Blood samples were subjected to analysis of bacterial growth (n = 10 for knockout mice and n = 9 for wild-type mice).

response of cytokine production upon endotoxin stimulation. To this end, we injected the same amount of LPS to L-STAT3 KO mice and control mice and measured circulating cytokines. LPS injection into L-STAT3 KO mice upregulated those cytokines to a lesser extent than CLP. In agreement with the finding on the CLP model, the levels of TNF-α, IL-10, MCP-1, and MIP-1β were significantly higher in L-STAT3 KO mice than in wild-type mice after LPS injection (Fig. 4B), indicating that L-STAT3 KO mice were highly sensitive to endotoxin and prone to show hypercytokinemia.

STAT3-Regulated Soluble Factors Produced by Hepatocytes Suppress Cytokine Production From Immune Cells. To examine the underlying mechanisms of the hyperimmune response in L-STAT3 KO mice, we hypothesized that STAT3-mediated soluble factors from hepatocytes repress cytokine production from immune cells. We isolated hepatocytes from L-STAT3 KO mice and control mice and stimulated them with or without IL-6, collecting the conditional medium of hepatocytes. Wild-type hepatocytes displayed STAT3 activation in primary culture without stimulation, but the levels increased upon IL-6 exposure, whereas KO hepatocytes did not show any STAT3 activation (Fig. 5A). Consistent with this was the finding that the wild-type hepatocytes produced more haptoglobin than KO hepatocytes, even in the absence of IL-6 (Fig. 5B).

Next, we cultured RAW cells, a murine macrophage cell line, in the presence or absence of culture supernatant of hepatocytes. RAW cells produced TNF-α, IL-6, and IL-10 but not IFN-γ upon stimulation of LPS, and hepatocyte culture supernatant suppressed the production of these cytokines (Fig. 5C). Importantly, the suppression was significantly weaker in the presence of conditional medium of KO hepatocytes than in the presence of conditional medium of wild-type hepato-

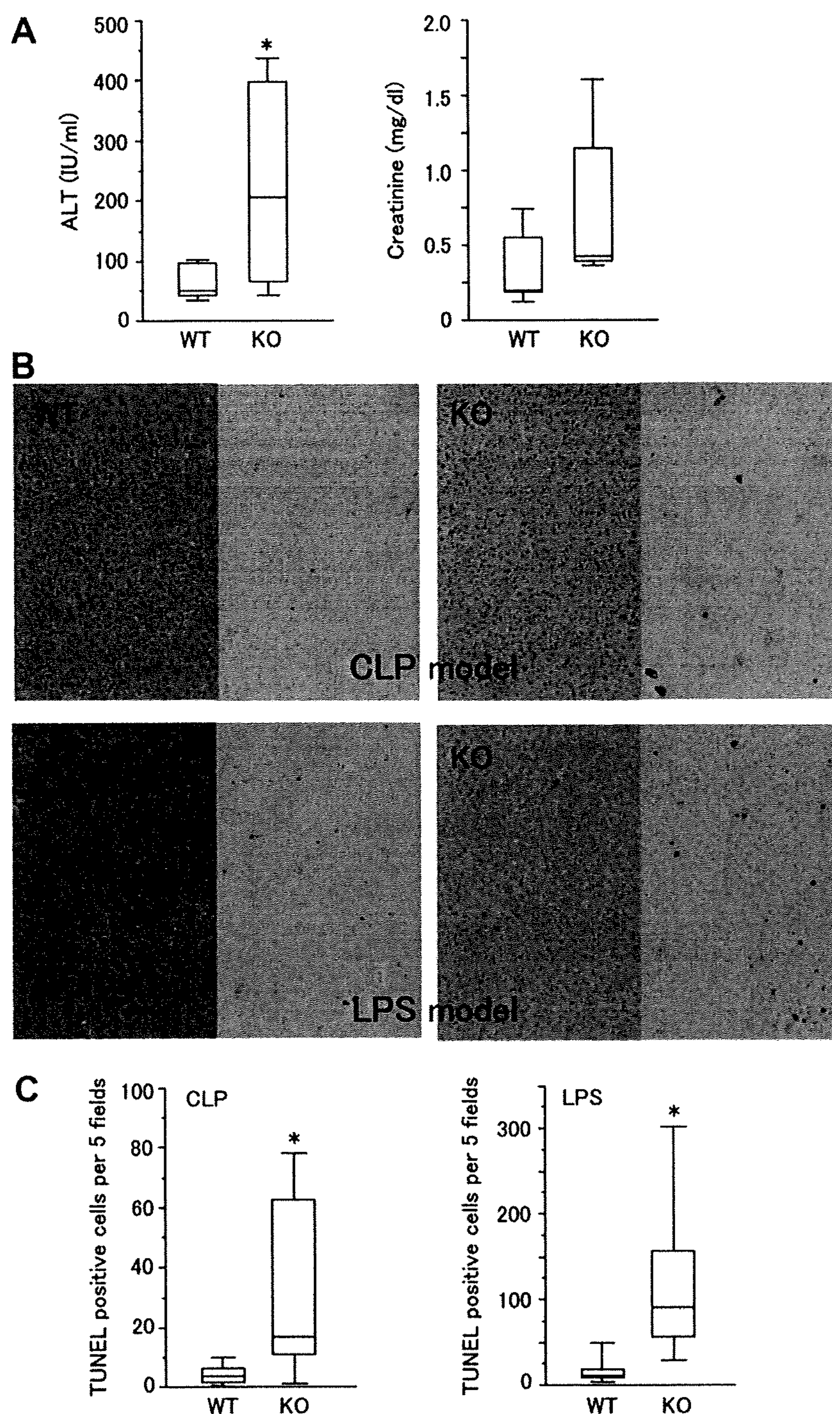

Fig. 3. Organ injury in septic mice. (A) Serum ALT and creatinine levels in L-STAT3 KO mice (KO) and control mice (WT) 24 hours after CLP. *$P < 0.05$. (B) Representative histology (left part of each panel) and TUNEL (right part of each panel) of liver sections 24 hours after CLP or LPS injection. (C) Comparison of TUNEL-positive hepatocytes for at least 9 mice in each group. *$P < 0.05$.

cytes. Furthermore, murine primary splenocytes produced IFN-γ upon LPS stimulation, and the production was also suppressed in the presence of conditional medium of hepatocytes. Again, IFN-γ production was significantly higher in splenocytes cultured with KO hepatocyte supernatant than in those with wild-type hepatocyte supernatant (Fig. 5D). These data indicate that soluble substances from hepatocytes suppressed activation of immune cells, which was critically dependent on STAT3.

Discussion

The present study clearly demonstrated that the absence of STAT3 in hepatocytes leads to high levels of circulating cytokines and increased mortality of CLP-in-

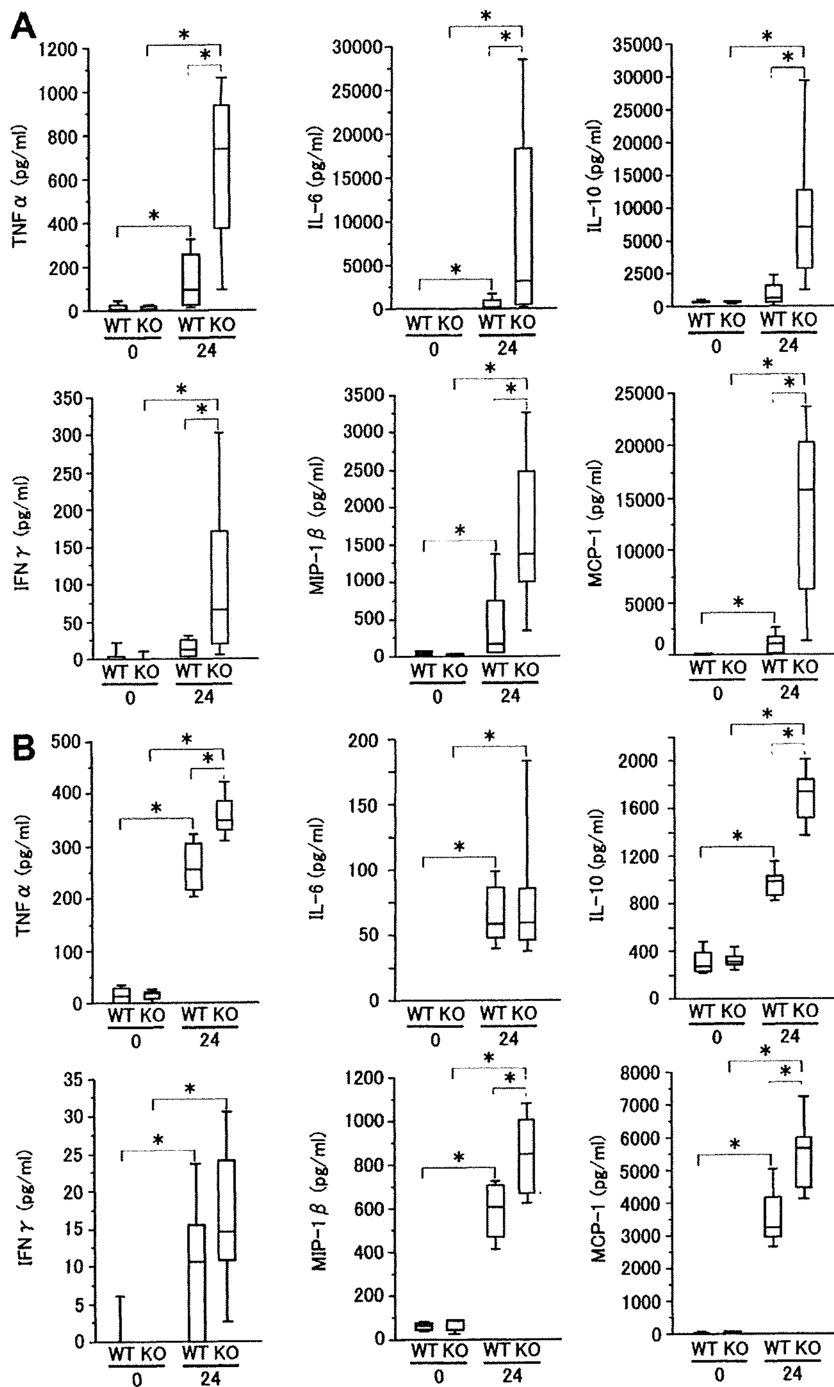

Fig. 4. Circulating cytokines before and after CLP or LPS injection. (A) L-STAT3 KO mice (KO) or wild-type littermates (WT) were treated with CLP ($n = 8$ in each group). Before and 24 hours after CLP, blood samples were obtained from mice and subjected to analysis of each cytokine indicated. $*P < 0.05$. (B) Mice were injected with 4 mg/kg of LPS ($n = 8$ in each group). Before and 24 hours after LPS injection, blood samples were obtained and subjected to the analysis of each cytokine indicated. $*P < 0.05$.

duced septic mice without affecting bacterial infection. L-STAT3 KO mice produced high levels of cytokines when injected with LPS, confirming that the absence of STAT3 signaling within hepatocytes induces a hyperinflammatory response even if the extent of the input stimuli remains constant. This phenomenon is similar to a previous report of macrophage-specific disruption of STAT3 in which serum cytokines such as TNF-α, IL-6, and IL-10 increased upon LPS stimulation.[22] In those mice, immune cells could not respond to IL-10, which potentially inhibit TNF-α production via STAT3 signaling, and thus produced high levels of TNF-α. Further study revealed those mice to be vulnerable to CLP-induced sepsis.[23,24] However, in our L-STAT3 KO mice, the levels of STAT3 in macrophage did not differ from control mice and produced the same amount of TNF-α in response to LPS (Fig. 1C-D). Thus, suppression of the inflammatory response in wild-type mice was critically

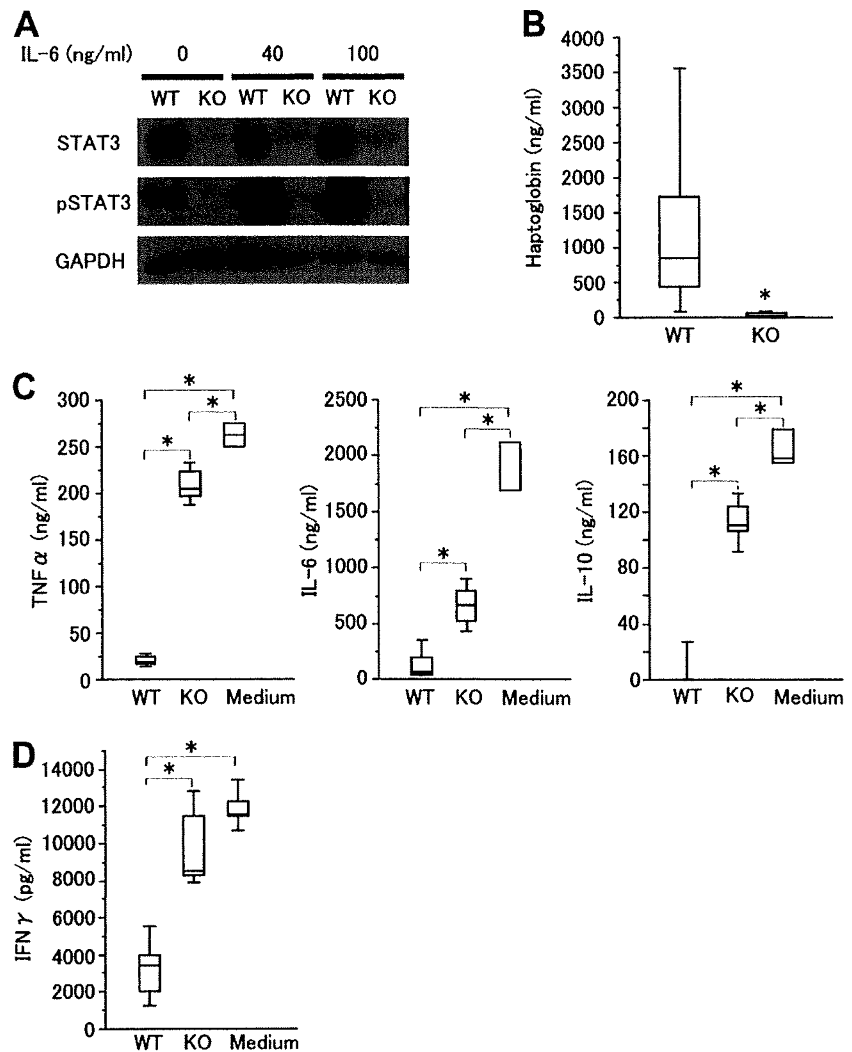

Fig. 5. Suppression of cytokine production from immune cells by hepatocyte culture supernatant. Hepatocytes were isolated from L-STAT3 KO (KO) or wild-type (WT) mice and cultured in the presence or absence of IL-6 for 2 hours (for western blot analysis) or 48 hours (for collection of culture supernatants). (A) STAT3 phosphorylation (pSTAT3) and STAT3 expression of hepatocytes via western blot analysis. GAPDH expression served as a control. Representative blots are shown. (B) Haptoglobin production from primary hepatocytes. Haptoglobin concentration was determined in the hepatocyte supernatants via ELISA. Comparison of haptoglobin production between knockout hepatocytes and wild-type hepatocytes (n = 5 mice/group) cultured in the absence of IL-6. $*P < 0.05$. (C,D) Suppression of cytokine production in RAW cells or splenocytes by the hepatocyte supernatants. Hepatocytes were cultured for 48 hours. RAW cells (C) or splenocytes freshly isolated from wild-type mice (D) were cultured in the presence (KO or WT) or absence (Medium) of hepatocyte supernatants for 24 hours and then stimulated with LPS for another 24 hours. TNF-α, IL-6, IL-10, and IFN-γ production was determined via ELISA. $*P < 0.05$.

dependent on hepatic STAT3 signaling. Indeed, in vitro analysis revealed that soluble factors from hepatocytes repress cytokine production from activated macrophage and splenocytes in a hepatic STAT3-dependent manner. Whereas research has established that STAT3 mediates a variety of effects on hepatocytes, including proliferation,[5] apoptosis protection,[6] and glucose metabolism,[7] the present study reveals that hepatic STAT3 has an important extrahepatic effect. This effect is activated by a variety of cytokines produced from immune cells such as IL-6 but, in turn, suppresses immune cell activation via production of soluble factors, providing a negative feedback loop. Thus, the present study describes a role of hepatic STAT3 in maintaining host homeostasis by negatively regulating the immune system.

APPs are liver plasma proteins whose levels of expression are either positively or negatively regulated by cytokines during inflammation. It has been established that STAT3 regulates the expression of most, if not all, APPs in the liver.[19] Consistent with this, L-STAT3 KO mice displayed impaired production of APPs in response to CLP (Fig. 2B). Some APPs such as C-reactive protein,[25] serum amyloid P,[26] and α2-macroglobulin[27] have been shown to bind bacteria and to positively or negatively affect their eradication. Several reports also suggest that APPs exert proinflammatory as well as anti-inflammatory effects.[25,28] C-reactive protein binds to the phosphocholine of some foreign pathogens as well as phospholipid constituents of damaged cells and can activate the complement system, whereas the antioxidants haptoglobin and hemopexin protect against reactive oxygen species. Thus, each APP has a unique role in the complex mechanism controlling infection-induced inflammation. The L-STAT3 KO mice used in the present study offer a unique model for identifying the net effect of STAT3-regulated APPs during the septic condition. Our work has revealed that the most prominent effect of STAT3-regulated APPs is suppression of the hyperinflammatory re-

sponse and lethality without an effect on bacterial infection. The soluble factors from hepatocytes that suppress cytokine production from immune cells are still unknown. Although there may be several substances involved in this phenomenon, one candidate might be haptoglobin, which was recently demonstrated to suppress TNF-α, IL-12, and IL-10 from human peripheral blood mononuclear cells in vitro.[29] We also obtained a similar finding that RAW cells produced a lesser amount of TNF-α upon LPS stimulation in the presence of haptoglobin (Supplementary Fig. 2). Identification of these substances may have important therapeutic implications for controlling the hyperinflammatory condition. Further study is needed to clarify this point.

The liver is one of the target organs of sepsis-induced multiple organ dysfunction syndrome. Evidence for this comes from the fact that CLP mice or LPS mice showed liver injury as evidenced by increases in serum ALT and TUNEL-positive hepatocytes scattered in the liver lobule. Furthermore, L-STAT3 KO mice displayed more hepatocyte apoptosis in mice subjected to CLP or LPS injection. Previous research has indicated that the absence of hepatic STAT3 renders hepatocytes more vulnerable to Fas-mediated apoptosis.[6] It is possible that STAT3-null hepatocytes are more vulnerable to apoptosis in the septic model. However, at the same time, L-STAT3 KO mice showed higher levels of proinflammatory cytokines such as TNF-α, which is a direct inducer of hepatocyte apoptosis. In our model, it is difficult to differentiate which contributed more to increased liver injury: the decrease in apoptosis resistance or the increase in proinflammatory cytokine. It can be said that the increase of proinflammatory cytokines is presumably one of the causes, but not a result, of liver injury. In addition, as discussed in the Results section, liver injury was relatively modest and probably not a direct cause of animal death.

In the present study, the lack of hepatic STAT3 caused increased mortality in CLP mice. Although we did not address the direct link between hypercytokinemia and animal death, accumulating evidence suggests that an increase in a variety of cytokines is involved in lethality in CLP mice. For example, it was shown that IL-6 plays an important role in the increased expression of the C5a receptor in the lung, liver, kidney, and heart during the development of sepsis in CLP mice and that interception of IL-6 leads to reduced expression of the C5a receptor and improved survival.[30] In addition, enforced expression of the IL-6 gene in wild-type mice led to high mortality (unpublished data). TNF-α and other cytokines increase expression of inducible nitric oxide synthase, and increased production of nitric oxide causes further vascular instability and may also contribute to the direct myocardial depression that occurs in sepsis.[31] Thus, dysregulation of cytokines may be harmful for host organs and is probably linked to animal death.

The present study revealed an important role of hepatocytes in repressing the hyperinflammatory response in pathologic conditions. This raises the possibility that hyperinflammation may be ill-controlled when liver function is severely impaired. Although sepsis itself is not a frequent cause of liver failure, it is a serious complication of acute or chronic liver failure. Systemic inflammatory response syndrome is an important determinant of prognosis in fulminant hepatitis.[12] Sepsis originating from spontaneous bacterial peritonitis or renal infection is one of the causes of patient death with decompensated cirrhosis.[13] In patients with limited function of the liver, possible impairment of STAT3-regulated hepatocyte function may be involved in their poor prognosis when complicated with severe inflammation. Careful liver-supporting therapy or early liver transplantation should be considered not only for maintaining liver function but also from the aspect of controlling dysregulated hyperinflammatory responses.

In conclusion, hepatic STAT3 represses systemic hyperinflammatory response by stimulating hepatic production of soluble substances that can attenuate immune cell overactivation and also improves host survival during septic condition. This sheds light on hepatocytic STAT3 as a negative regulator for immune cell overactivation and its role in host defense during systemic severe inflammation.

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Type B Fulminant Hepatitis Is Closely Associated with a Highly Mutated Hepatitis B Virus Strain

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Key Words

Hepatitis B virus · Type B acute self-limited hepatitis · Type B fulminant hepatitis · Hepatitis B virus mutation · Full-length hepatitis B virus nucleotide sequencing

Abstract

Objective: Genome-wide sequences of hepatitis B virus strain associated with type B fulminant hepatitis have not been compared with those of acute self-limited hepatitis. We carried out full-length sequencing analysis of viral strains derived from patients with type B acute liver injury. ***Methods:*** Nine acute self-limited hepatitis and 6 fulminant hepatitis patients were the subjects of this study. Full-length sequencing analysis of viral DNA was done by PCR-direct sequencing. ***Results:*** Higher frequencies in fulminant hepatitis strains compared with acute hepatitis ones were observed in the T1762/A1764 ($p < 0.05$), A1896 ($p = 0.09$) and M1753 (M = C or A) ($p = 0.09$) mutations. Viruses related to fulminant hepatitis possessed the higher number of nucleotide substitutions than those related to acute hepatitis in the whole virus genome ($p < 0.01$) and various regions including preS/S gene ($p < 0.05$), precore/core gene ($p < 0.01$), polymerase gene ($p < 0.05$) and basic core promoter/core upstream regulatory sequence ($p < 0.01$). The high number of nucleotide substitutions in viruses related to fulminant hepatitis was predominantly non-synonymous in the preS/S and precore/core genes. ***Conclusion:*** Development of type B fulminant hepatitis may be associated with a highly mutated hepatitis B virus strain.

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Introduction

Hepatitis B virus (HBV) is a double-stranded, circular, hepatotropic DNA virus of approximately 3.2 kb in length and causes both acute and chronic liver diseases. Acute HBV infection results in various liver diseases ranging from acute self-limited hepatitis (AH) to fulminant hepatitis (FH). FH develops in approximately 1% of patients with acute type B liver disease [1]. It is believed that a hyperimmune response to viral antigens and/or enhanced viral replicative activity may be relevant to the development of type B FH.

Processes in HBV replication include a reverse transcription step, and hence, HBV is more susceptible to mutations than other DNA viruses because the viral reverse

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transcriptase lacks proofreading activity. During the past 15 years, a great number of reports have revealed a relationship between the particular mutations within the HBV genome and various clinical manifestations during the course of HBV infection. Mutations within the precore and basic core promoter (BCP) regions have been documented with regard to comparison between AH and FH. The A1896 mutation, which produces the in-frame stop codon in the distal precore gene and results in the incapability to synthesize hepatitis B e antigen (HBeAg) [2], has been shown to be more frequently found in viruses from FH patients than in those from AH ones [3, 4]. Patients with FH have also been reported to possess the virus with T1762/A1764 double mutation in the BCP, which is another type of mutation associated with the HBeAg-negative phenotype through reduced synthesis of precore mRNA [5, 6], more frequently than those with AH [7]. In addition, more frequent detection of the V1753 or V1754 (V = C, G or A) mutation has been reported in the BCP in the FH-related virus than in the AH-related one [8]. The presence of these mutation hotspots associated with FH in the precore and BCP regions of HBV has recently been verified in a large-scale study including approximately 300 patients with acute HBV infection [9]. However, genomic changes occurring in other regions of HBV associated with the development of FH have not been extensively studied.

There have been several reports that have determined the full-length nucleotide sequences of the HBV DNA strains in a single patient or a limited number of patients with FH [10–15]. It has been shown that most of these HBV strains revealed considerable differences in nucleotide and amino acid sequences, compared with the previously reported consensus viral strain [10–15]. However, these studies have analyzed the full-length viral sequences only for the FH-related strain and did not directly compare the viral sequences derived from AH and FH patients. Thus, peculiarities over the entire HBV genome have not been clarified in strains derived from FH patients in comparison to those derived from AH patients.

To better understand the differences between AH and FH strains, we conducted sequencing analysis of full-length HBV DNA derived from patients with HBV-related acute liver injury and compared the nucleotide sequences between viral strains from AH and FH patients.

Patients and Methods

Patients

The subjects in this study were 15 consecutive Japanese patients with HBV-related acute liver injury, who had been admitted to Osaka University Hospital, Osaka Koseinenkin Hospital and National Hospital Organization Osaka Minami National Hospital. They were 12 males and 3 females, ranging in age from 17 to 72 (median 49) years. None of them had been previously found to have abnormal liver function test results. All of them displayed a sudden onset of the clinical signs of acute liver injury. Of the 15 patients, 6 were diagnosed as FH, because they fulfilled the criteria of fulminant hepatic failure as proposed by Trey and Davidson [16] with the minor modifications of the Inuyama Symposium 1981 (Aichi, Japan); i.e., a prothrombin time ≤40% of control and coma ≥ grade II having developed within 8 weeks after the onset of illness. The remaining 9 patients showed neither encephalopathy nor coagulopathy and were diagnosed as AH. 13 patients tested positive for the immunoglobulin M antibody to hepatitis B core antigen (IgM anti-HBc) and were considered to be infected with HBV for the first time. The possibility of acute exacerbation from a chronic carrier state of HBV could not be excluded in the remaining 2 patients belonging to the FH group, who were hepatitis B surface antigen (HBsAg)-positive but IgM anti-HBc-negative. All patients tested negative for IgM antibody to hepatitis A virus, and antibodies to hepatitis C and human immunodeficiency viruses. None of them showed evidence of other kinds of liver diseases, such as alcoholic liver disorder, autoimmune hepatitis and drug-induced liver injury. As for the prognosis, 4 FH patients, 1 of whom received living donor transplantation, died of hepatic failure, whereas the remaining 11 patients recovered without transition into chronicity. The patient clinical characteristics are summarized in table 1. There was no significant difference in age, gender ratio, AST, ALT and HBV DNA between the AH and FH patients. Those with FH did have a higher total bilirubin than those with AH (median 9.1 [range 4.9–17.3] mg/dl vs. median 3.0 [range 0.6–6.8] mg/dl, $p < 0.01$). The prothrombin time was lower in patients with FH (median 22 [range 15–31] % of the control) than in those with AH (median 79 [range 45–111]% of the control) ($p < 0.002$). Serum samples from the patients on admission were collected and stored at –80°C until use. Informed consent was obtained from the patient or patient's family member.

HBV Markers

HBsAg, antibody to HBsAg (anti-HBs), HBeAg, and antibody to HBeAg (anti-HBe) were examined by chemiluminescent immunoassay or enzyme immunoassay. IgM anti-HBc was measured by radioimmunoassay. Serum HBV DNA was detected quantitatively by the PCR-based assay (Amplicor HB Monitor, Roche) [17].

PCR Amplification and Direct Sequencing of Full-Length HBV DNA

The predominant nucleotide sequences of full-length HBV DNA in each of the patients were determined by the PCR-direct sequencing method. Briefly, DNA was extracted from serum samples by the standard procedure with Tris-Cl/EDTA/SDS/proteinase K solution. The first round PCR was undertaken using the primers BF1 (5'-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3', nt 1821–1841) and BR1 (5'-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3', nt 1825–1806), which were previously designed by Günther et al. [18]. This PCR reaction yielded the amplification of full-length HBV DNA. The second round PCR reactions were done using three sets of primers to obtain mutually overlap-

Table 1. Clinical and virological characteristics of patients with AH and FH

	Age/gender	AST IU/l	ALT IU/l	Total bilirubin mg/dl	Pro-thrombin time, %	HBsAg/anti-HBs	HBeAg/anti-HBe	IgM anti-HBc	Geno-type	T1762/A1764	A1896	M1753	HBV DNA log_{10} copies/ml	Liver trans-plantation	Pro-gnosis
AH patients (n = 9)															
1	47/M	602	1,519	6.4	89	+/–	–/+	+	C2/Ce	–	–	–	3.6	no	surviving
2	37/M	810	4,132	6.8	45	–/+	–/+	+	C2/Ce	+	+	+ (C1753)	3.4	no	surviving
3	43/M	164	587	2.0	61	+/–	–/+	+	C2/Ce	+	–	–	5.8	no	surviving
4	17/F	120	439	0.6	109	+/–	+/+	+	C2/Ce	–	–	–	3.1	no	surviving
5	57/M	902	2,170	1.4	67	+/–	+/–	+	C1/Cs	–	–	–	7.5	no	surviving
6	62/M	140	236	0.9	68	+/–	+/–	+	B2/Ba	–	–	–	>7.6	no	surviving
7	69/M	505	1,905	1.8	111	+/–	–/+	+	C2/Ce	–	–	–	5.8	no	surviving
8	72/M	387	818	0.6	76	+/+	+/–	+	A2/Ae	–	–	–	7.6	no	surviving
9	53/M	806	1,782	6.1	89	+/–	+/–	+	A2/Ae	–	–	–	6.6	no	surviving
FH patients (n = 6)															
10	56/F	1,751	3,561	6.6	15	–/+	+/+	+	C2/Ce	+	–	+ (C1753)	5.3	no	died
11	48/M	9,657	4,825	4.9	31	+/–	+/–	+	C2/Ce	+	+	+ (C1753)	7.0	no	surviving
12	52/M	313	497	11.2	29	+/–	–/+	+	B1/Bj	–	+	–	5.5	no	surviving
13	48/F	1,361	2,872	7.2	22	–/+	–/+	+	B1/Bj	+	+	+ (A1753)	4.6	yes	died
14	49/M	595	1,025	17.3	18	+/–	+/–	–	C2/Ce	+	–	–	5.6	no	died
15	38/M	329	1,127	7.6	15	+/–	–/+	–	C2/Ce	+	+	+ (C1753)	5.9	no	died

ping HBV DNA fragments. The primers were BF1s (5′-TTT TTC ACC TCT GCC TAA TCA-3′, nt 1821–1841), BR5 (5′-AAC TGG AGC CAC CAG CAG GA-3′, nt 74–55), BF4 (5′-GTC ACC ATA TTC TTG GGA AC-3′, nt 2816–2835), BR7 (5′-GGG TTC AAA TGT ATA CCC AA-3′, nt 839–820), BF7 (5′-TAT TGG GGG CCA AGT CTG TA-3′, nt 752–771) and BR1s (5′-AAA AAG TTG CAT GGT GCT GG-3′, nt 1825–1806). All PCR reactions were performed using the Expand High Fidelity PLUS PCR system (Roche). Under this PCR condition, the detection limit of the full-length HBV DNA was approximately 3.0–3.5 log_{10} copies/ml. After the purification, the BF1s/BR5, BF4/BR7 and BF7/BR1s DNA fragments were subjected to direct sequencing reaction using the BigDye Terminator Version 3.1 Cycle Sequencing Kit and 3100 or 3730 Genetic Analyzer (Applied Biosystems). The cycle sequencing reaction was conducted using primers, BF2 (5′-CAG ACA ACT ATT GTG GTT TC-3′, nt 2191–2210), BF3 (5′-TCT TTA ATC CTG AGT GGC AA-3′, nt 2512–2531), BF5 (5′-AAG AGA CAG TCA TCC TCA GG-3′ nt 3183–3202), BF6 (5′-CCT CCA ATT TGT CCT GGC TA-3′, nt 350–369), BF8 (5′-TTT ACC CCG TTG CCC GGC A-3′, nt 1142–1160), BR2 (5′-CAG AAT AGC TTG CCT GAG TG-3′, nt 2080–2061), BR3 (5′-TTC CCG AGA TTG AGA TCT TC-3′, nt 2440–2421), BR4 (5′-GAC CAA ATG CTC CCG CTC CT-3′, nt 3040–3021) and BR6 (5′-GAG CAG GGG TCC TAG GAA TC-3′ nt 193–174), as well as the above-mentioned primers, BF1s, BF4, BF7, BR5, BR7 and BR1s. The nucleotide sequences of HBV strains determined in this study are shown in the international DNA database with accession numbers of AB300359 to AB300373.

Molecular Genetic Analysis

DNA sequences of the 15 HBV strains derived from the FH and AH patients in this study and 5 representative HBV strains of subgenotypes A2/Ae, B1/Bj, B2/Ba, C1/Cs and C2/Ce (GenBank Accession No. X02763, D00330, D00329, AF223954 and X01587) [19–22] were aligned using the CLUSTALW software. A phylogenetic tree analysis was conducted using Kimura's two-parameter method and the neighbor-joining method [23, 24], and illustrated as a rectangular cladogram using the TreeView software. To quantitatively evaluate the degree of mutation in each of the HBV strains obtained in this study, the number of nucleotide substitutions per site (evolutional distance) was calculated in comparison with the reference HBV strain using Kimura's two-parameter method [23]. The Nei-Gojobori's method [25] with Jukes-Cantor's correction was also used to calculate the numbers of synonymous (silent) substitutions per synonymous site (d_S) and non-synonymous (amino acid-substituting) substitutions per non-synonymous site (d_N) between each HBV strain and the reference strain. In the present study, the above-mentioned HBV strains, X02763, D00330, D00329, AF223954 and X01587 [19–22], of subgenotypes A2/Ae, B1/Bj, B2/Ba, C1/Cs and C2/Ce were used as the references to standardize the value of the number of nucleotide substitutions in different subgenotypes of HBV strains. These analyses were conducted at the homepage of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) or using MEGA version 3.1 software [26].

Statistical Analysis

Mann-Whitney's non-parametric U test and Fisher's exact probability test were used for the group comparison. A p value < 0.05 was considered to be statistically significant.

Results

The nucleotide sequences of full-length HBV DNA derived from the 15 patients with AH and FH were 3158–3227 bp in length. Figure 1 represents the result of the phylogenetic tree analysis including the 15 HBV strains

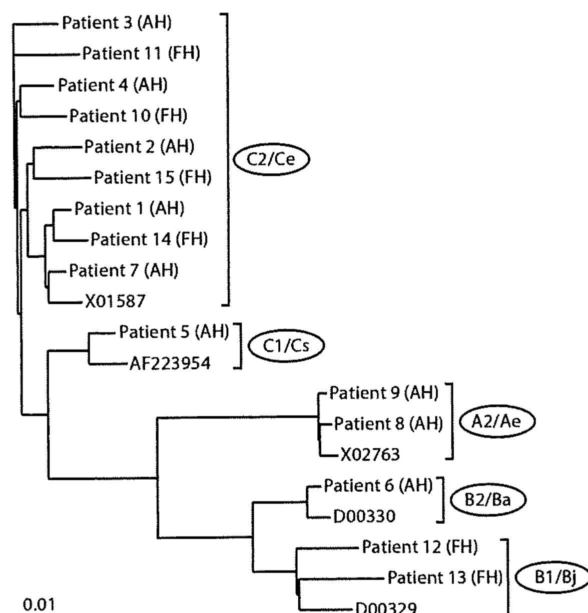

Fig. 1. Phylogenetic tree analysis of 15 HBV strains derived from the AH and FH patients and 5 representative HBV strains of subgenotypes A2/Ae, B1/Bj, B2/Ba, C1/Cs and C2/Ce.

obtained in this study and five representative (reference) HBV strains of subgenotypes A2/Ae, B1/Bj, B2/Ba, C1/Cs and C2/Ce. Among the 15 strains determined in this study, nine from 5 AH and 4 FH patients belonged to subgenotype C2/Ce, the most prevalent type in Japan. There were two subgenotype A2/Ae strains from AH patients, two subgenotype B1/Bj strains from FH patients, one subgenotype B2/Ba strain from an AH patient and one subgenotype C1/Cs strain from an AH patient.

The specific mutations that significantly differed between viral strains derived from the AH and FH patients were first examined. Throughout the HBV genome, there were only three mutations correlated with the severity of the acute type B liver disease. The occurrence of the T1762/A1764 mutation in the BCP was significantly higher in the FH-related strains (5 of 6, 83%) than in the AH-related ones (2 of 9, 22%) ($p < 0.05$). Patients with FH tended to be more frequently infected with the virus possessing the precore-defective A1896 mutation and the M1753 (M = C or A) mutation in the BCP than those with AH (67 vs. 11%, $p = 0.09$ for the A1896 and 67 vs. 11%, $p = 0.09$ for the M1753), though the difference did not reach a statistically significant level.

Next, we tried to evaluate the degree of mutation in HBV strains obtained from the patients with AH and FH. For this purpose, the number of nucleotide substitutions per site (evolutional distance) of each HBV strain was calculated in comparison with the reference HBV strain. To standardize the values of the number of nucleotide substitutions in different subgenotypes of HBV strains, the respective reference HBV strains of subgenotypes A2/Ae, B1/Bj, B2/Ba, C1/Cs and C2/Ce were employed. These reference HBV strains, except for the subgenotype C1/Cs AF223954 strain, had been cloned in the 1980s by means of a non-PCR method and originated from the plasma/serum sample of a single or a plural number of highly viremic chronic HBV carrier(s) [19, 20, 22]. The remaining subgenotype C1/Cs reference strain has recently been identified by PCR from an HBeAg-positive HBV carrier [21]. Thus, all reference HBV strains used for this study had been derived from patients at an early phase of chronic HBV infection, and did not appear to have mutations accompanied by the disease progression. Then, the number of nucleotide substitutions of each HBV strain was calculated for comparison with the corresponding reference HBV strain of the same subgenotype.

The results of the comparison in the number of nucleotide substitutions of the whole or portions of the HBV genome between AH- and FH-related HBV strains are shown in figure 2. The number of nucleotide substitutions in the whole HBV genome was significantly higher in strains from FH patients (median 0.0295 [range 0.0186–0.0347]/site) than in those from AH ones (median 0.0157 [range 0.0072–0.0292]/site) ($p < 0.01$). When the number of nucleotide substitutions in various regions of the HBV genome was studied, the higher number of nucleotide substitutions in the FH-related strains than in the AH-related ones was also observed for the preS/S gene (median 0.0185 [range 0.0150–0.0211]/site vs. median 0.0101 [range 0.0075–0.0203]/site, $p < 0.05$), precore/core gene (median 0.0354 [range 0.0174–0.0742]/site vs. median 0.0110 [range 0.0047–0.0271]/site, $p < 0.01$) polymerase gene (median 0.0266 [range 0.0172–0.0315]/site vs. median 0.0164 [range 0.0067–0.0294]/site, $p < 0.05$) and BCP/core upstream regulatory sequence (CURS) [27] (median 0.0333 [range 0.0188–0.0429]/site vs. median 0.0093 [range 0.0046–0.0285]/site, $p < 0.01$), but not in the X gene.

The codon-by-codon numbers of nucleotide synonymous and non-synonymous substitutions (d_S and d_N) between each of the HBV strains obtained in this study and the reference strain were studied further (table 2). In the preS/S gene, the d_N value but not the d_S value was significantly higher in strains from the FH patients than in

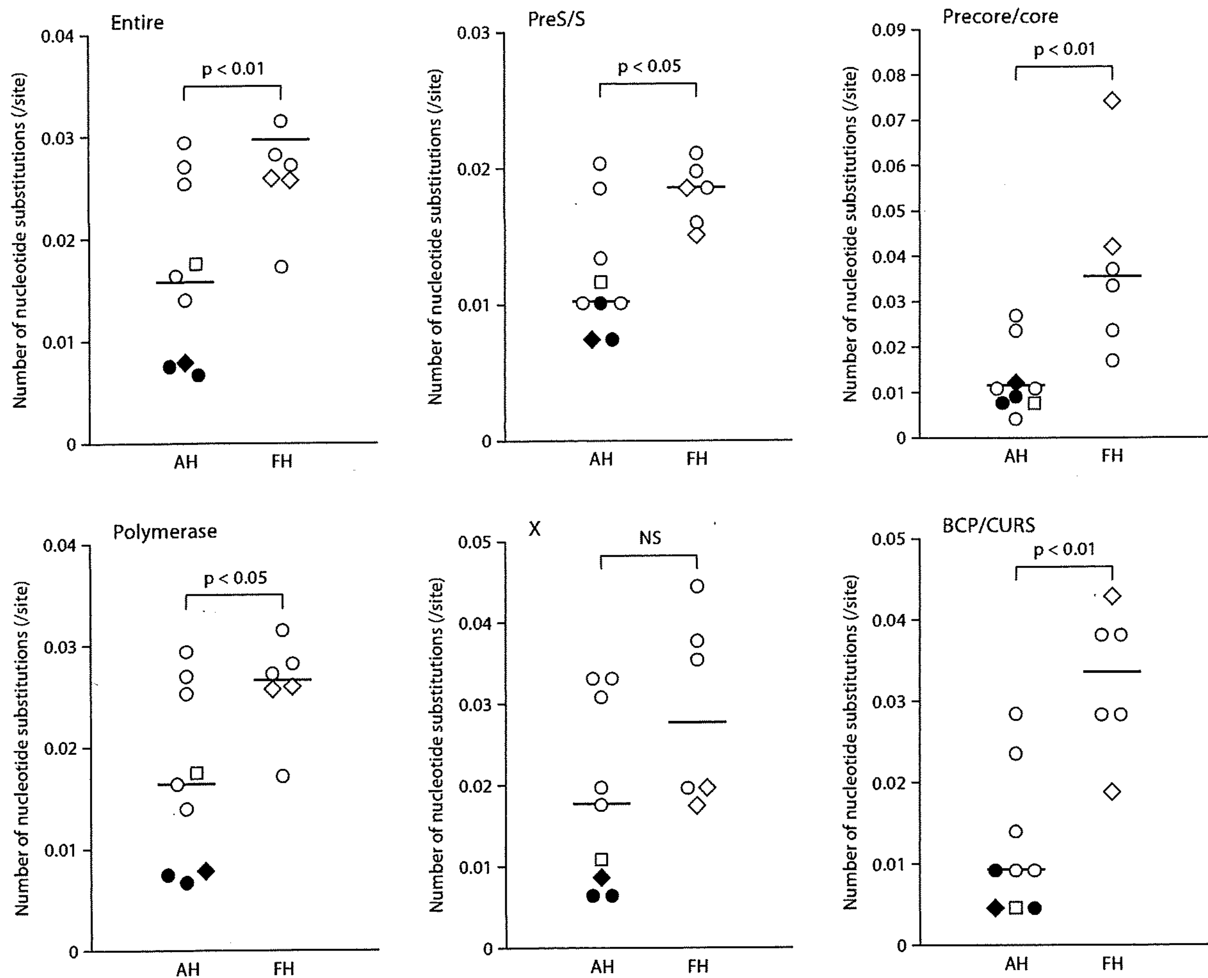

Fig. 2. Number of nucleotide substitutions per site in the entire and various regions of HBV genome in strains from patients with AH and FH. ○ = Subgenotype C2/Ce HBV strains; ●, ◇, ◆, □ = HBV strains of subgenotypes A2/Ae, B1/Bj, B2/Ba and C1/Cs, respectively.

Table 2. Numbers of nucleotide synonymous and non-synonymous substitutions in various genes of AH- and FH-related HBV strains

Genomic regions of HBV	Number of synonymous substitutions (d_S) (/synonymous site)			Number of non-synonymous substitutions (d_N) (/non-synonymous site)		
	AH	FH	p value	AH	FH	p value
PreS/S	0.0271 (0.0101–0.0516)[1]	0.0351 (0.0270–0.0410)	NS[2]	0.0067 (0.0033–0.0146)	0.0121 (0.0082–0.0150)	<0.05
Precore/core	0.0418 (0.0185–0.0683)	0.0771 (0.0515–0.1311)	<0.05	0 (0–0.0032)	0.0184 (0.0064–0.0546)	<0.005
Polymerase	0.0361 (0.0096–0.0646)	0.0611 (0.0353–0.0681)	<0.05	0.0099 (0.0047–0.018)	0.0150 (0.0112–0.0216)	NS
X	0.0344 (0–0.0704)	0.0503 (0.0252–0.0796)	NS	0.0148 (0–0.0209)	0.0187 (0.0089–0.0331)	NS

[1] Values are expressed as median (range). [2] NS = not significant.

those from the AH ones. The FH-related strains showed both higher d_S and d_N values than the AH-related ones in the precore/core gene ($p < 0.05$ for the d_S value and $p < 0.005$ for the d_N value), and the difference of the d_N value was more prominent than that of the d_S value between strains from AH and FH patients. As for the polymerase gene, only the d_S value was significantly higher in viruses infecting FH patients than in those infecting AH ones ($p < 0.05$). Neither the d_S value nor the d_N value in the X gene differed between the strains from the AH and FH patients.

Discussion

In the present study, we carried out sequencing analysis of full-length HBV DNA derived from a considerable number of patients with type B AH and FH. We attempted to validate genome-wide features in the FH-related HBV strains. The mutation hotspots associated with FH were first investigated through screening of the entire HBV genome. It was found that FH-related viral strains tended to have the T1762/A1764 mutation in the BCP, the A1896 mutation in the precore gene and the M1753 mutation in the BCP more frequently than the AH-related ones. This is consistent with previous reports [3, 4, 7–9], though it has not been fully understood why the viruses with these mutations link to the serious disease course in the acute HBV infection. It is also of note that no other particular mutations related to development of FH were observed throughout the HBV genome.

Our 15 HBV strains encompassed five different subgenotypes, A2/Ae, B1/Bj, B2/Ba, C1/Cs and C2/Ce. It has recently been shown in a large-scale study from Japan that patients with FH were infected less frequently with the subgenotype A2/Ae virus and more frequently with the subgenotype B1/Bj virus than those with AH [9]. Indeed, the subgenotype A2/Ae was detected in only two AH-related strains, whereas the subgenotype B1/Bj was seen in only two FH-related strains in this study. Thus, the HBV subgenotype may be one of the determining factors for prognosis of the acute HBV infection.

Next, we evaluated the number of nucleotide substitutions as an index of the degree of the mutation in each of the viral strains obtained in this study in comparison with the reference HBV strain. The values of the number of nucleotide substitutions were standardized by setting up the respective reference HBV strain of each subgenotype to overcome sequence variability among subgenotypes. The number of nucleotide substitutions was shown to be significantly higher in the FH-related strain than in the AH-related ones for the whole genome and various regions (preS/S, precore/core, polymerase and BCP/CURS) of HBV. Our finding indicates that development of type B FH may be closely associated with a highly mutated HBV strain, whose mutations occur in a wide range of the viral genome. Several investigators have examined the nucleotide sequences of full-length HBV strains derived from a limited number of FH patients, and most of these FH-related HBV strains differed considerably in nucleotide and amino acid sequences when they were compared with the previously reported consensus HBV strain [10–15]. However, these previous studies were not comparative ones and did not examine sequence information of the viral genome derived from AH patients. As the present study analyzed the HBV sequences derived from a considerable number of patients of both AH and FH, our findings should offer more reliable information regarding the difference between AH and FH strains. Our observations indicate that a highly mutated HBV strain may be one of the important etiologic causes of type B FH.

The BCP/CURS region [27], which overlaps the enhancer II region [28, 29], is known to regulate the viral transcription via interaction of cellular transcription factors with their specific DNA elements. In this study, the number of nucleotide substitutions in the BCP/CURS region was found to be higher in viruses from the FH than AH patients. This region includes three mutation hotspots (nt positions 1753, 1762 and 1764) associated with FH as described above. In addition, a few investigators have identified the strain-specific mutation/insertion occurring in the BCP other than the hotspot mutations as the accountable genomic change for development of FH [11, 15, 30]. Baumert et al. [30] have demonstrated that the HBV strain derived from an FH patient [11] showed more than tenfold higher viral replication, and that the enhanced viral encapsidation activity of the strain was caused by the T1766/A1768 mutation. More recently, transfection with the HBV strain having the T1766/A1768 mutation has also been reported to induce more apoptosis in primary *Tupaia* hepatocytes than that with the wild-type HBV strain [31]. It has also been shown by Pult et al. [15] that the HBV strain isolated from a heart transplant recipient who developed the transplant-transmitted FH had the 11-bp insertion in the BCP, leading to enhanced viral transcription and replication via generation of the novel hepatocyte nuclear factor 1 binding site. Taken together, the hypermutation in the BCP/CURS region including the hotspot mutations and other types of genomic changes may be involved in the pathogenesis of type B FH.

In the present study, we further studied the numbers of nucleotide synonymous and non-synonymous substitutions (d_S and d_N) in each viral strain in the four open reading frames of HBV. The higher number of nucleotide substitutions in the FH-related strains compared with the AH-related ones was found to be predominantly non-synonymous in the precore/core and preS/S genes. By contrast, in the polymerase and X genes, no significant differences in the number of non-synonymous substitutions were observed between strains from AH and FH patients. The precore/core and preS/S proteins are known to include epitopes against the humoral and cellular immune responses of the host. The high incidence of the amino acid substitutions in these viral structural proteins may cause a hyperimmune response, resulting in enhanced hepatocyte injury and development of FH.

It is debatable whether many viral mutations occurring in the FH-related strains would appear before infection or during development of FH after infection. It is currently conceivable that the high incidence of non-synonymous mutations compared to synonymous ones reflects the positive selection. In the precore/core and preS/S genes, the difference in the d_N value the between AH- and FH-related strains was more prominent than that in the d_S value, suggesting that the strong positive selection in these genes may occur in the FH patients than in the AH ones. With respect to this, the possibility cannot be excluded that mutations occurred in the precore/core and preS/S genes may be rapidly emerged in the acutely infected patients as a consequence of the strong intrahost selection. On the other hand, the d_S value also tended to be higher in the viruses derived from FH patients than in those from AH ones, especially in the polymerase gene. This indicates that at least a portion of the mutations in the FH-related viral strain may occur presumably in the HBV carrier of an infectious source before transmission of HBV, because synonymous mutations generally occur in time irrespective of the selective pressure within the host.

We also showed that a substantial number of mutations can occur even in the viral strains derived from AH patients, compared with the reference strain. Some of the AH-related strains possessed a considerably high rate of mutations in the HBV genome. According to this, a highly mutated HBV strain may be a necessary, but not a sufficient, factor for development of FH. Accumulation of various viral and host factors may be involved in the pathogenesis of type B FH. Further studies are required to clarify this.

In summary, our full-length sequencing study of HBV strains derived from a considerable number of patients with AH and FH revealed a close relationship of the highly mutated HBV strain with the development of FH. In particular, our findings suggest that the hypermutation in the BCP/CURS region and the high incidence of the amino acid-substituting mutation in the precore/core and preS/S genes may be relevant to the pathogenesis of type B FH.

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Original Article

Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C

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Aim: Ribavirin, used to treat chronic hepatitis C, can induce hemolytic anemia, forcing the discontinuance of treatment. To establish a predictive measure to help circumvent this, we evaluated the relationship of hemoglobin (Hb) decline with the discontinuance of treatment during the progression of ribavirin-induced anemia.

Methods: One hundred and sixteen patients (71% male) with genotype 1 chronic hepatitis C were treated with pegylated interferon (PegIFN) α-2b and ribavirin. The mean age was 50.6 years and 55% were IFN naïve. A decline of Hb concentration by 2 g/dL at two weeks from the start of the treatment ("2 by 2" standard) was adopted as the predictive factor for the progression of anemia.

Results: By applying the "2 by 2" standard, with $\Delta Hb \geq 2$ g/dL (34%, $n = 39$), treatment was discontinued in 12 cases (31%), three of which (8%) because of severe anemia. For $\Delta Hb < 2$ g/dL (64%, $n = 76$), treatment was discontinued in 11 (14%) cases; none due to severe anemia. Ten percent (4/39) of patients showed the minimum $Hb \leq 8.5$ g/dL in the $\Delta Hb \geq 2$ g/dL group, with none in the $\Delta Hb < 2$ g/dL group ($P = 0.001$). Furthermore, the patients with minimum $Hb \leq 8.5$ g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Conclusion: Monitoring the Hb decline using the "2 by 2" standard can identify patients who are prone to developing severe anemia. Further prospective studies are needed using ribavirin reduction based on the "2 by 2" standard.

Key words: "2 by 2" standard, chronic hepatitis C, pegylated interferon and ribavirin combination therapy, progression of anemia

INTRODUCTION

THE AIM OF antiviral therapy for hepatitis C virus (HCV) is to obtain a sustained viral response (SVR) and to reduce the occurrence rate of hepatocellular carcinoma or hepatic disease-related mortality.[1,2] The current optimal therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PegIFN) and ribavirin. This combination can significantly improve the SVR rate and is recommended as a standard regimen worldwide.[3–8] However, the SVR rates for the combination therapy of ribavirin with PegIFN for naïve patients with HCV genotype 1 has been reported to be 42–52%,[6,9,10] which means that eradication of HCV is not complete in approximately half of these patients. Recently, long-term treatment[11] and a higher dosage

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of drugs[12,13] have been used to try to raise the SVR rate for patients with HCV genotype 1. However, it remains to be established what constitutes satisfactory efficacy. In this study we focused on a treatment strategy to enable the prediction of severe side-effects in order to avoid the need to discontinue treatment and raise the SVR rate by PegIFN and ribavirin combination therapy. It is important that ribavirin, the key drug for eradicating HCV, is continued until the end of treatment in order to attain the maximum SVR rate. Hemolytic anemia induced by ribavirin is known as one of the most important adverse effects in the combination therapy of PegIFN and ribavirin.[14–17] To decrease the discontinuance rate of ribavirin due to severe anemia, epoetin alfa has been used for patients with progressing anemia, which can maintain the dose level of ribavirin as well as the quality of life of the patients.[18–20] However, from a cost-effectiveness standpoint, it would be difficult for this treatment strategy to become standard. Also, side-effects other than anemia arising from an overload of ribavirin mainly due to renal dysfunction cannot be avoided by the additional administration of epoetin alfa.

Hemolysis induced by ribavirin has been suggested to be related to a high plasma concentration of ribavirin.[21] The apparent clearance of ribavirin (CL/F), which reflects its plasma concentration at four weeks after the start of combination therapy, has been used as a predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[22–24] However, the progression of hemolytic anemia occurs due not only to hemolysis, but also impaired hematogenous function. On the other hand, hemoglobin (Hb) dynamics directly reflect the degree of progression of anemia. We have reported that the early decline of Hb correlates with the progression of anemia during IFN and ribavirin combination therapy.[25] It is necessary to verify that a similar early predictor for the progression of anemia can be adopted in PegIFN and ribavirin combination therapy, since PegIFN is known to induce less depression of bone marrow function than usual IFN.

In this study, we evaluated the utility of the early decline of Hb in comparison with the CL/F to predict the progression of anemia in the combination therapy of PegIFN and ribavirin.

METHODS

Patients

THIS STUDY WAS conducted at 12 institutions in Japan. A total of 116 patients with chronic hepatitis C were enrolled and treated with a combination of PegIFN and ribavirin. All patients were anti-hepatitis C virus antibody positive, had HCV-RNA detectable in their serum by the polymerase chain reaction (PCR) method, and showed elevated serum alanine transaminase (ALT) (above the upper limit of the normal), serum Hb concentration ≥12 g/dL, neutrocytes ≥1500/mm^3 and platelets ≥10^5/mm^3 within six months before the treatment. Exclusion criteria were the presence of hepatitis B surface antigen, antihuman immunodeficiency virus antibody and other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis).

Table 1 Patient characteristics

Age (years)	50.6 ± 10.1 (24–70)
Gender (male/female)	82/34 (male 70.7%)
Body weight (kg)	64.5 ± 11.1
Previous IFN therapy (naïve/relapser/no responder)	64/38/14
HCV-RNA level (KIU/L) (<500/500–850/850<)	18/27/71
ALT (IU/L)	110 ± 60 (33–76)
Crnn (mg/dL)	0.9 ± 0.2
Liver histology	
Fibrosis (F1/F2/F3/unknown)	35/49/31/1
Activity (A1/A2/A3/A4)	15/33/56/12
WBC (/mm^3)	5317 ± 1207
Neutrocytes (/mm^3)	2778 ± 902
Platelets (×10^4/mm^3)	17 4 ± 4.0
RBC (×10^4/mm^3)	459 ± 41
Hemoglobin (g/dL)	14.5 ± 1.2

Data are given as the mean ± SD.
ALT, alanine transaminase; RBC, red blood cells; WBC, white blood cells.

The baseline characteristics of the patients are shown in Table 1. The mean age was 50.6 ± 10.1 years, and 71% (82 patients) were male. All patients had HCV-RNA with genotype 1 and high viral loads (more than 10^5 copies/mL serum by Amplicor-HCV monitor assay). The mean ALT level was 110 ± 60 IU/L. Sixty-four patients (55%) were IFN naïve and the others were undergoing retreatment.

Treatment schedule

All patients were treated with a combination of PegIFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) for 48 weeks. PegIFN was administered at a mean of 1.5 μg/kg body weight subcutaneously once a week. Ribavirin was given orally twice a day for the total dose. Dosages of both medications were decided based on the

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body weight of the patients: those with a body weight of 40–60 kilograms (kg) were given PegIFN 75 μg/body and ribavirin 600 mg/day, those with a body weight of 60–80 kg were given PegIFN 105 μg/body and ribavirin 800 mg/day, and those with a body weight of 80–100 kg were given PegIFN 135 μg/body and ribavirin 1000 mg/day. The PegIFN dose was reduced by 50% if the neutrocyte count was below 750/mm^3 or the platelet (Plt) count was below 8×10^4/mm^3. The PegIFN was discontinued if the neutrocyte count was below 500/mm^3 or the Plt count was below 5.0×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dL and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dL, in accordance with the drug information for ribavirin. No ferric medicine or erythropoietin to prevent anemia was administered.

Patients with persistently undetectable HCV-RNA six-months after the end of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level, hematological and biochemical tests just before therapy, at the end of week 2 and every four weeks during the treatment. When the treatment was completed, the patients were assessed every four weeks up to 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar *et al.*, CL/F at the start of the treatment was calculated as follows: CL/F (L/h) = $32.3 \times \text{BW} \times (1 - 0.0094 \times \text{age}) \times (1 - 0.42 \times \text{sex})/\text{Scr}$ (BW, body weight; sex = 0 for male and 1 for female; Scr = serum creatinine).[17]

Definition of "severe anemia" leading to the discontinuance of ribavirin

In this study, the "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dL or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dL from the start of the combination therapy.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, white blood cell count, red blood cell count, Hb concentration, Plt, serum ALT levels and serum creatinine are expressed as mean ± SD. The SVR rate was evaluated using the intention-to-treat analysis (ITT analysis). The differences in proportions were tested by the χ^2-test and Mantel–Haenszel χ^2-test. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed by SAS program 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Frequency and reasons for dose reduction or discontinuance of PegIFN and/or ribavirin

OF THE 116 patients, 92 completed 48 weeks of therapy, but 24 patients (21%) had to discontinue both PegIFN and ribavirin. Thirty-nine patients (34%) completed the entire treatment schedule without reduction or discontinuance of either drug. The ribavirin dose was decreased for 39 patients (34%) and the PegIFN dose was decreased for 33 patients (28%), including 19 patients for whom both drugs had to be reduced. The reasons for discontinuance of both drugs included anemia, thyroid dysfunction, skin eruption and neutropenia, with the major reasons being anemia (17%) and thyroid dysfunction (17%).

Efficacy of the combination therapy with dose reduction or discontinuance of PegIFN and/or ribavirin

The SVR rate was 57% (66/116) for all according to ITT analysis. According to the category of response to previous IFN therapy, the SVR rates were 43% (6/14) in

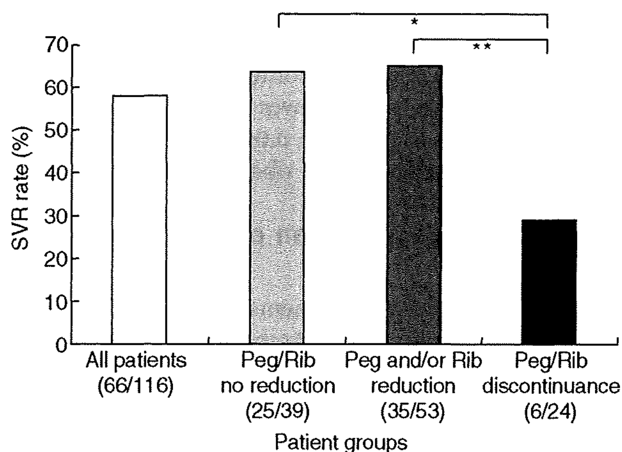

Figure 1 SVR rate due to PegIFN/ribavirin dose reduction or discontinuance. (□), All patients; (▤), patients without dose reduction; (▩), patients with dose reduction; (■), patients with drug discontinuance. Significant levels: $*P = 0.003$; $**P = 0.001$.

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Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
20 ≤ CL/F (n = 12)	67% (8/12)	25% (3/12)	8% (1/12)	0
15 ≤ CL/F < 20 (n = 23)	57% (13/23)	30% (7/23)	13% (3/23)	0
10 ≤ CL/F < 15 (n = 39)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
CL/F < 10 (n = 42)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

P = 0.031 (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
20 ≤ CL/F (n = 12)	92% (11/12)	12% (1/12)	0
15 ≤ CL/F < 20 (n = 23)	83% (19/23)	17% (4/23)	0
10 ≤ CL/F < 15 (n = 39)	72% (28/39)	23% (9/39)	5% (2/39)
CL/F < 10 (n = 42)	50% (21/42)	43% (18/42)	7% (3/42)

P = 0.009 (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs (P = 0.003), and the group with reduction of PegIFN and/or ribavirin (P = 0.001).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the CL/F ≥ 20, 13% (3/23) for the 15 ≤ CL/F < 20, 23% (9/39) for the 10 ≤ CL/F < 15, and 26% (11/42) for the CL/F < 10 group. Ribavirin did not have to be discontinued due to severe anemia among patients with 15 ≤ CL/F, but did for the 18% (2/11) of those with CL/F < 10 and 22% (2/9) of those with 10 ≤ CL/F < 15. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of CL/F < 10, and 5% (2/39) of the group of 10 ≤ CL/F < 15. No patients of the group of CL/F ≥15 showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four-weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin (n = 53), 1.6 ± 1.2 g/dL among those with dose reduction (n = 39), and 1.8 ± 1.0 g/dL among

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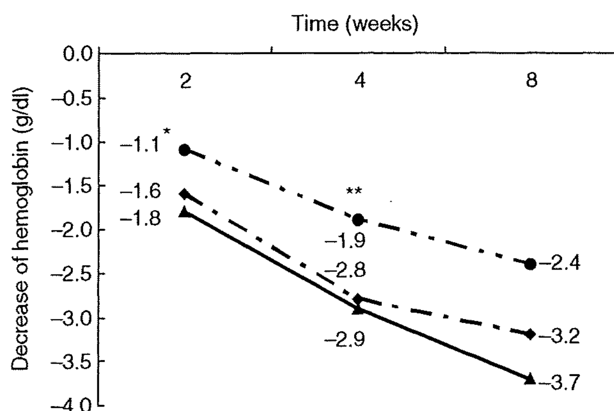

Figure 2 Course of ΔHb in the initial phase. (—··—), No reduction; (—·—·), reduction; (——), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel–Haenszel χ^2-test).

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