stem/progenitor cells will be useful for future therapies of severe liver diseases.[38]

Expansion of progenitor cells such as oval cells in injured livers is regulated by several soluble factors and cell–cell interactions. Recent studies report that fibroblast growth factor 7 (FGF7) derived from periportal mesenchymal cells is essential for the expansion of progenitor cells and survival in patients with severe liver injuries.[39] Severe and chronic liver damage induces Thy1^{+} mesenchymal cell expansion and the expression of FGF7. Overexpression of FGF7 in livers *in vivo* induces expansion of hepatic progenitor-like cells and suppresses liver damage. In contrast, *Fgf7*-knockout mice exhibited marked suppression of progenitor cell expansion and higher mortality in the severe liver injury model.

DIFFERENTIATION OF HEPATIC STEM/ PROGENITOR CELLS DERIVED FROM PLURIPOTENT STEM CELLS

MECHANISMS UNDERLYING PROLIFERATION and differentiation of human hepatic stem/ progenitor cells remain largely unknown, because of the difficulty associated with analyzing cellular and molecular events *in vivo*. As mentioned previously, ES and iPS cells are multipotent and differentiate into specialized cell types of several organs. Methods to induce differentiation of mouse and human ES and iPS cells into hepatic progenitor cells and mature hepatocytes *in vitro* have been established.[40] Hepatic cells generated from patient-derived iPS cells are considered to be beneficial for the treatment of severe liver diseases, screening of drug toxicities and basic research of several hepatocytic disorders. However, generating iPS cell clones derived from many patients is problematic. These issues include the recruitment of patients, definition of guidelines to validate new clones and the diversity of iPS cell characteristics arising from different genetic backgrounds. For example, donor differences of human iPS cells affect their propensity for hepatic differentiation.[41] As recently described, novel methods to generate human iPS cells carrying genomic mutations have been established using genome editing enzymes such as zinc-finger nucleases, transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9).[42] Human cell line- or pluripotent stem cell-derived disease models carrying site-directed mutations reportedly have been established using TALEN.[43] These models are highly attractive because disease-related iPS cells carrying specific mutations can be easily and rapidly derived from control iPS cells.

Differentiation of pluripotent stem cells into hepatic lineage cells mimics the *in vivo* stepwise developmental processes, including sequential stimulation with several differentiation factors such as activin A, basic fibroblast growth factor, bone morphogenetic protein and HGF.[40] Thus, it is speculated that HPC derived from human iPS cells develop *in vitro* following the same differentiation steps and time-course as normal cells *in vivo*. Our group and others analyzed differentiation of human ES and iPS cells into fetal HPC in these culture systems.[44–46] For example, we found that highly proliferative cells exist in the CD13^{+}CD133^{+} fraction of human iPS cells stimulated by hepatocytic differentiation factors (Fig. 4). As mentioned previously, CD13 and CD133 are cell surface markers of stem/progenitor cells in mouse fetal and adult livers. Individual CD13^{+}CD133^{+} cells formed large colonies containing more than 100 cells and expressed both hepatocytic (α-fetoprotein and hepatocyte nuclear factor 4α) and cholangiocytic marker genes (*Ck7*), suggesting that CD13^{+}CD133^{+} cells derived from human iPS cells in culture exhibit characteristics of HPC. Next, we assessed whether human iPS cell-derived HPC had the potential to differentiate into mature hepatocytic and cholangiocytic cells. 3-D biological structures are important for the induction of mature hepatocytic functions and the hanging drop method is often used to self-assemble hepatic stem/progenitor cells into aggregates called spheroids. We found that several hepatic functional genes, such as cytochrome P450, were induced in human iPS cell-derived HPC spheroids. In addition, we tested whether human iPS cell-derived HPC formed cholangiocytic structures during gel culture. Cholangiocytic cells form cysts with epithelial polarity, demonstrating that *in vitro* tubulogenesis occurs in extracellular matrix gel supplemented with cytokines.[47] Many epithelial cysts were formed in gel cultures of human iPS cell-derived HPC. Several of these epithelial cysts expressed cholangiocytic marker CK7, but did not express hepatocytic marker α-fetoprotein. These results indicate that human iPS cell-derived HPC exhibit a bipotent differentiation ability, forming hepatocytes and cholangiocytes.[45]

Efficient methods to establish functional hepatocytes derived from human ES and iPS cells have yet to be identified. One avenue to establish functional livers derived from human iPS cells is to organize hepatic cells *in vitro* in 3-D. Co-culture of human iPS cell-derived hepatocytic cells with human endothelial and mesenchymal cells reportedly forms specific 3-D organoids *in vitro*.[48] These organoids expressed several functional enzymes and proliferated *in vivo* after transplantation. In

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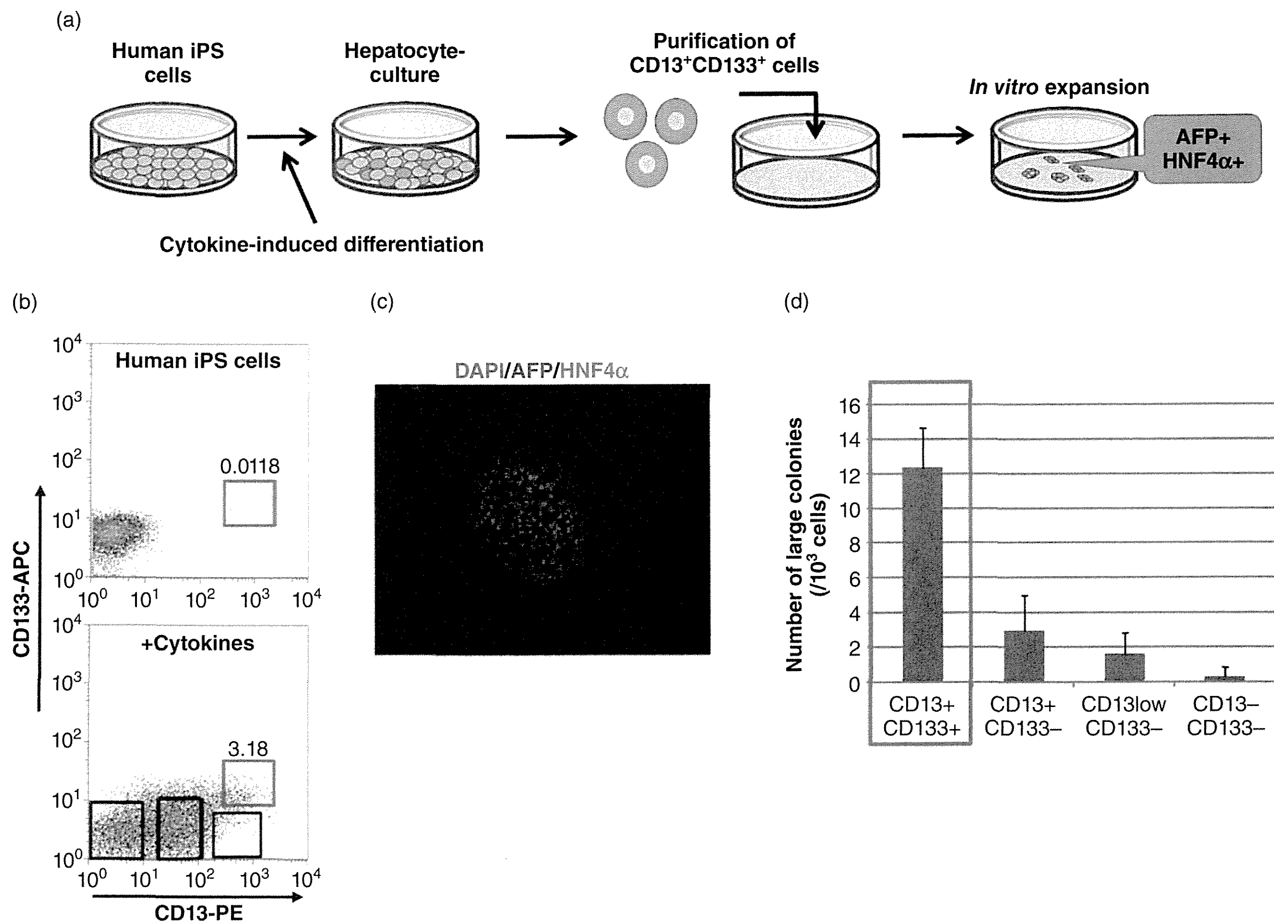

Figure 4 Purification and expansion of hepatic progenitor-like cells derived from human induced pluripotent stem (iPS) cells. (a) Expansion method for hepatic progenitor-like cells. Human iPS cells were differentiated into hepatocytic cells by using cytokines (activin A, fibroblast growth factor, bone morphogenetic protein and hepatocyte growth factor). $CD13^+CD133^+$ cells in the differentiated cell culture were purified and plated onto feeder cells at a low density. (b) Flow cytometry data from human iPS cells and differentiated cells (+Cytokines). (c) Representative colony-derived $CD13^+CD133^+$ hepatic progenitor-like cells. α-Fetoprotein (AFP) and hepatocyte nuclear factor 4α (HNF4α) were detected by immunocytochemistry. (d) The number of large colonies derived from human iPS cells differentiated by cytokines. The $CD13^+CD133^+$ fraction contained many progenitor-like cells. (Reproduced from Yanagida *et al.* with permission.)[45]

addition, organoids were highly vascularized. These results suggested that a suitable environment, such as the presence with non-parenchymal cells, is important for cultured human iPS cells to acquire normal liver functions.

SUMMARY AND FUTURE PERSPECTIVE

IN THIS REVIEW, we present data showing that mesenchymal cells can perform a niche-like function, supporting hepatic stem/progenitor cells in the co-culture system. Recently, we found that small numbers of HPC generated by *in vitro* expansion using the co-culture system could be transplanted into the injured-liver mouse model (unpubl. data). Therefore, stem/progenitor cells in fetal and adult livers are good candidates to be used in regenerative medicine for several liver diseases. Pluripotent stem cells are also potential tools for cell transplantation therapies and drug discovery research. Recently, human ES and iPS cell-derived hepatocytic cells have been established using protocols that mimic the events that occur during normal development. Therefore, culture systems that produce iPS cell-derived hepatic progenitor cells

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may also be useful for the study of human hepatic cell development.

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HEPATOLOGY

Efficacy of continuous plasma diafiltration therapy in critical patients with acute liver failure

Takuya Komura,*,† Takumi Taniguchi,* Yoshio Sakai,† Tatsuya Yamashita,† Eishiro Mizukoshi,† Toru Noda,* Masaki Okajima*† and Shuichi Kaneko†

*Intensive Care Unit, Kanazawa University Hospital, and †Disease Control and Homeostasis, Kanazawa University, Kanazawa, Japan

Key words
acute kidney disease, acute liver failure, blood purification therapy, plasma exchange.

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Correspondence
Dr Shuichi Kaneko, Disease Control and Homeostasis, Graduate School of Medical Science, Kanazawa University, Kanazawa 920-8641, Japan. Email: skaneko@m-kanazawa.jp

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Abstract

Background and Aims: Acute liver failure (ALF) is a critical illness with high mortality. Plasma diafiltration (PDF) is a blood purification therapy that is useful for ALF patients, but it is difficult to use when those patients have multiple organ failure or unstable hemodynamics. In these patients, symptoms are also likely to exacerbate immediately after PDF therapy. We developed continuous PDF (CPDF) as a new concept in PDF therapy, and assessed its efficacy and safety in ALF patients.
Methods: Ten ALF patients (gender: M/F 6/4, Age: 47 ± 14) were employed CPDF therapy. The primary outcomes were altered liver function, measured by the model for end-stage liver disease (MELD) score, and total bilirubin and prothrombin time international normalized ratios (PT-INR), 5 days after CPDF therapy. Secondary outcomes included sequential organ failure assessment (SOFA) scores, 5 days after CPDF therapy, and the survival rate 14 days after this therapy.
Results: The MELD score (34.5–28.0; $P = 0.005$), total bilirubin (10.9–7.25 mg/dL; $P = 0.048$), PT-INR (1.89–1.31; $P = 0.084$), and SOFA score (10.0–7.5; $P < 0.039$) were improved 5 days after CPDF therapy. Nine patients were alive, and one patient died because of acute pancreatitis, complicated by ALF. There were no major adverse events related to this therapy under hemodynamic stability.
Conclusion: In the present study, CPDF therapy safely supported liver function and generally improved the condition of critically ill patients with ALF.

Introduction

Acute liver failure (ALF) is a rapidly progressing critical illness associated with a high mortality rate, and characterized by jaundice, ascites, hepatic encephalopathy, and bleeding due to severe impairment of liver function caused by massive liver necrosis.[1,2] Acute, severe hepatic necrosis releases toxic metabolites such as ammonia from the splanchnic circulation.[3]

The therapeutic strategy for ALF differs between Western countries and Japan. In Japan, an artificial liver support system (ALSS) is commonly the first-line therapy because liver transplantation is usually accomplished with living donors.[4–7] In Western countries, deceased donor liver transplantation is the first-line therapy for ALF.[8]

Plasma exchange (PE) is a fundamental and simple ALSS. However, the procedure is accompanied by adverse effects such as hypernatremia, metabolic alkalosis, citrate poisoning, and abrupt changes in colloid osmotic pressure.[9,10] In Japan, PE is often combined with continuous hemodiafiltration (CHDF), a process in which electrolyte imbalance is corrected and fluids are controlled, simultaneously. PE/CHDF therapy, however, can be expensive because it requires 3.2–4.8 L of fresh frozen plasma (FFP), along with the necessary equipment; a risk of infection is also associated with this therapy.

Plasma diafiltration (PDF or selective plasma filtration with dialysis) has been developed as an alternative to PE/CHDF therapy. PDF is a blood purification therapy in which simple PE is performed with a membrane plasma separator, while dialysate flows outside the hollow fibers.[11] In ALF patients, PDF is a useful bridge therapy for liver regeneration or transplantation.[12,13] However, PDF therapy is difficult to use in critically ill ALF patients with complicated multiple organ failure, especially in those with unstable hemodynamics. Symptoms can also be exacerbated immediately after PDF therapy because this conventional PDF therapy is used intermittently throughout an 8-h day.

In this study, we designed a new PDF therapy concept, termed continuous PDF (CPDF), and conducted an observational study to assess the efficacy and safety of this therapy for patients with ALF.

Materials and methods

The study protocols conformed to the ethical guidelines of the 2008 Declaration of Helsinki. This study was approved by the

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Table 1 Demographics of ten ALF patients

No.	Age (years)	Gender (M/F)	Etiology	MELD score	PT-INR	Total bilirubin (mg/dL)	Encephalopathy grade	M.V.	SOFA score	Total duration (days)	Outcome
1	33	F	HELLP	29	1.81	5.4	—	+	13	9	Alive
2	37	M	Alcohol	21	1.57	11.3	—	–	5	14	Alive
3	60	F	AIH	40	1.90	34	III	–	11	5	Death
4	38	M	Drug	43	4.83	5.1	III	–	8	8	Alive
5	54	M	HBV	34	1.88	28	II	+	14	12	Alive
6	63	F	AIH	26	2.30	15.2	II	+	8	9	Alive
7	26	M	Unknown	36	1.96	10.5	IV	–	11	9	Alive
8	47	F	HBV	51	9.57	4.5	III	+	9	7	Alive
9	34	M	Drug	35	1.76	10.4	II	+	14	14	Alive
10	38	M	Unknown	27	1.57	65.3	—	–	12	11	Alive

—, not detected; AIH, autoimmune hepatitis; ALF, acute liver failure; HBV, hepatitis B virus; HELLP, hemolysis elevated liver enzymes; MELD, model for end-stage liver disease; M.V., mechanical ventilation; PT-INR, prothrombin time international normalized ratios; SOFA, sequential organ failure assessment.

institutional review board of the Kanazawa University Graduate School of Medicine.

Patients. Ten patients in the intensive care unit (ICU) at Kanazawa University Hospital from January 2011 to April 2013 received CPDF therapy. These patients fulfilled the Japanese diagnostic criteria for ALF,[14] which consists of prothrombin time international normalized ratios (PT-INR) of > 1.5 caused by severe liver damage within 8 weeks of onset of the symptoms when prior liver function was estimated as normal. Informed written consent of the patients or responsible family members was obtained prior to enrollment.

CPDF decision process and implementation. The decision to employ CPDF for each patient included fulfilling the diagnostic criteria of ALF; complicated renal dysfunction that compromised fluid management; and ongoing fluid management concerns including significant ascites, edema, and/or fluid overload. The patient characteristics are listed in Table 1. CPDF therapy was performed using an Evacure EC-2A plasma separator (Kuraray, Tokyo, Japan) at a blood flow rate of 80 mL/min. Filtered replacement fluid for artificial kidneys (Sublood-BS; Fuso Pharmaceutical, Osaka, Japan) was infused at a dialysate flow rate of 400 mL/h and a replacement flow rate of 280 mL/h. FFP was infused intravenously at 120 mL/h, and nafamostat mesilate (Futhan; Torii Pharmaceutical, Tokyo, Japan) was used as an anticoagulant.

The CPDF column was replaced every 24–48 h unless disabled. Patients were monitored closely for signs and symptoms of adverse effects or complications during this therapy. We decided that the criteria for discontinuation of CPDF was a total bilirubin of < 5.0 mg/dL and PT-INR < 1.2, and the point in time at which the survival rate improved.

The primary outcomes were altered liver function measured by the model for end-stage liver disease (MELD) score, total bilirubin, and PT-INR 5 days after CPDF therapy. This time point was chosen because patients with ALF are generally re-assessed for liver transplantation every 5 days. Secondary outcomes included sequential organ failure assessment (SOFA) scores 5 days after CPDF therapy and survival rate 14 days after CPDF therapy. SOFA is a scoring system to determine the extent of a person's multiple organ function or rate of failure based on six different scores, such as respiratory, cardiovascular, hepatic, coagulation, renal, and neurological systems.[15]

Statistical analysis. Data are expressed as medians and interquartile ranges. Differences in variables before and after CPDF therapy were examined by paired Student's *t*-test after a symmetrical distribution was confirmed. $P < 0.05$ indicated statistical significance. We also considered clinical efficacy analyzed by effect size (ES) using Cohen's *d*, which measures the strength of the relationship before and after CPDF therapy due to the low number of patients in this study. We determined that ES > 0.2 = small, ES > 0.5 = moderate, and ES > 0.8 = large efficacy of this therapy based on Cohen's criteria.

Results

Demographics. We assessed 10 patients with ALF. All patients were diagnosed with ALF and received CPDF therapy. The characteristics of the patients are shown in Table 1. The etiology for ALF was variable, and the average age of patients was 47 ± 14 years (range, 26–64). Seven ALF patients had overt hepatic encephalopathy. Five ALF patients received mechanical ventilation therapy, while there was no patient with inotropic and vasopressor support.

Primary outcomes. The MELD score improved significantly from 34.5 to 28.0 ($P = 0.005$; Fig. 1a), resulting in high clinical effectiveness (ES = 0.78; Table 2) after CPDF therapy. Total bilirubin also significantly improved from 10.9 to 7.25 mg/dL ($P = 0.048$; Fig. 1b), resulting in moderate clinical effectiveness (ES = 0.65; Table 2) after CPDF therapy. PT-INR had a tendency to improve from 1.89 to 1.31 ($P = 0.084$; Fig. 1c), resulting in high clinical effectiveness (ES = 0.92; Table 2) after CPDF therapy.

Secondary outcomes. The SOFA score decreased from 10.0 to 7.5 ($P < 0.039$; Fig. 1d), resulting in moderate clinical

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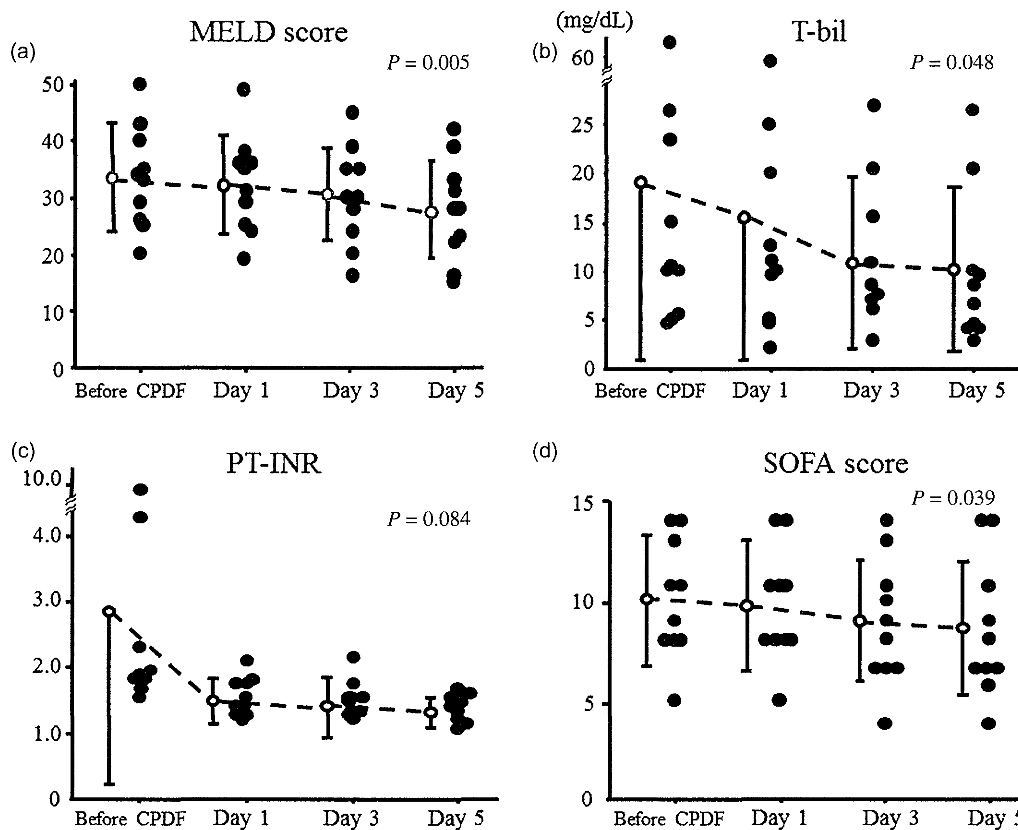

Figure 1 ALF patient parameters. (a–c) Primary study outcomes of this study. MELD score (a), total bilirubin value (b), and PT-INR (c) were improved 5 days after CPDF therapy. (d) Secondary study outcomes of this study. SOFA score (d) were improved after 5 days CPDF therapy. Each value are plotted, and also expressed as means ± SD.

Table 2 Parameter alteration 5 days after CPDF Treatment

Variables	Before CPDF	After CPDF	Effect size	95% CI	*P* value
SOFA score	10 (5–14)	7.5 (4–14)	0.47	0.085–2.715	0.039
MELD score	34.5 (21–51)	28 (14–42)	0.78	2.611–10.789	0.005
Total bilirubin (mg/dL)	10.9 (4.5–65.3)	7.25 (3.4–27.7)	0.65	0.076–17.82	0.048
Ammonia (mg/dL)	119 (60–316)	143 (58–407)	0.46	−115–24.2	0.174
Creatinine (mg/dL)	1.44 (0.47–4.79)	1.36 (0.27–3.12)	0.37	−0.176–1.198	0.127
PT-INR	1.89 (1.57–9.57)	1.31 (1.17)	0.92	−0.258–3.384	0.084
ALT (IU/L)	123 (21–10 892)	39 (17–482)	0.77	−743–495	0.129
AST (IU/L)	109 (60–15 183)	52.5 (28–436)	0.82	−759–6440	0.108
Albumin (g/dL)	3.1 (2.6–3.7)	2.7 (2.3–3.2)	1.08	−0.017–0.777	0.059
MAP (mm Hg)	82 (62–112)	89.5 (72–115)	0.23	−22.4–3.42	0.131
Heart rate (beat/min)	89.5 (76–122)	92 (57–135)	0.34	−5.49–17.49	0.268
PaO_2/FiO_2 ratio	330 (188–583)	383 (240–567)	0.38	−139–59.4	0.195

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; CPDF, continuous plasma diafiltration; MAP, mean arterial pressure; MELD, model for end-stage liver disease; PT-INR, prothrombin time international normalized ratios; SOFA, sequential organ failure assessment.

effectiveness (ES = 0.47; Table 2) after CPDF therapy. Nine patients were alive and discharged from the ICU, and one patient died due to acute pancreatitis complicated by ALF. CPDF therapy had no major adverse effect, including bleeding, and maintained hemodynamic stability.

Other outcomes. Parameters of hepatocyte injury such as aspartate aminotransferase/alanine aminotransferase also decreased (ES = 0.82 and 0.77, respectively) by CPDF therapy. The creatinine value (ES = 0.37) had a small clinical effectiveness (Table 2).

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Five ALF patients with overt encephalopathy were controlled by CPDF therapy, while two patients with uncontrolled encephalopathy were treated with CPDF therapy combined with hemodialysis to control overt encephalopathy. Circulation parameters such as mean arterial pressure and heart rate were maintained (Table 2) without inotropic and vasopressor support during CPDF treatment period. Oxygenation index (PaO_2/FiO_2) as a measure of pulmonary function increased after this treatment (Table 2). In this study, mechanical ventilation was indicated not only by hepatic encephalopathy but also by impaired respiratory function because of massive ascites or pleural effusion, or an unstable hemodynamic state. Two patients were withdrawn from mechanical ventilation after pulmonary function improved.

Moreover, we could employ this treatment without any adverse events, such as infections and unstable hemodynamics.

Discussion

In the present study, we observed that CPDF therapy improved liver function in critical ALF patients with beneficial effects on renal, pulmonary, and hemodynamic function that led to an improved SOFA score which reflected the severity of critical illness without any adverse effects.

ALSS is the first-line therapy for ALF patients until liver regeneration or transplantation because only a small proportion of ALF patients can receive deceased donor liver transplants, in Japan.[4,6] Recently, several types of ALSS methodologies such as Prometheus or the Molecular Adsorbent Recirculating System have been developed, primarily to eliminate toxic substances. However, these therapies require complex equipment and are expensive.[16,17]

Conventional PDF therapy is simple, less expensive, and results in fewer adverse events than other therapies. Consequently, this therapy has been demonstrated to be one of the most useful blood purification therapies for ALF patients.[11-13] However, conventional PDF therapy, which is used intermittently throughout an 8-h day, does not usually maintain hemodynamic stability. Because we dealt with critical patients in ALF, a group in whom hemodynamic instability and a high SOFA score implying high mortality in this observational study, we employed CPDF therapy, which can maintain stable hemodynamics in most cases.

In this study, we showed that the efficacy of CPDF liver support. Moreover, CPDF therapy provides some of the characteristics of renal replacement treatment for patients with ALF; these patients frequently have renal functional impairment. CPDF therapy, as well as CHDF, avoid abrupt changes and successively remove toxic substances while managing fluid balance.[18] This reduces pulmonary edema and the exacerbation of impaired respiratory function and satisfactorily supports liver function.[19,20] In fact, CPDF therapy improved a pulmonary function in several patients of this study. Thus, CPDF therapy can improve the function of multiple organs, possibly making this therapy superior to conventional PDF therapy. Moreover, these benefits suggest that CPDF therapy is cost-effective and helps avoid the possibility of infection.

Whether CPDF therapy can effectively remove toxic substances (e.g. ammonia) during rapid disease progression, caused by hepatic encephalopathy, remains to be determined. Similarly, an assessment of how CPDF therapy maintains decreased plasma albumin value also remains to be determined, as we did not observe improvements in ammonia values. This study included two patients with high ammonia values and uncontrolled encephalopathy; these patients employed CPDF, combined with hemodialysis, to control the encephalopathy. The plasma albumin value was decreased during the CPDF therapy because the sieving coefficient of 0.3 for albumin selectively removed low- and intermediate-molecular weight, albumin-bound substances in the plasma separator (Evacure EC-2A). Albumin loss was managed by intravenously administering 12.5–25 g of albumin to maintain the plasma albumin level.

Some of the limitations of this study should also be considered. The first is that the therapeutic strategy for ALF is different, in Japan, from that in Western countries because few deceased donor liver transplants are performed. The second is that although our study was prospective, it was not a randomized controlled study (RCT), and the sample size was small. An RCT should be explored to determine the effects of CPDF in patients with ALF.

In summary, CPDF therapy, a new concept in ALSS, improved liver function in critically ill ALF patients and had beneficial effects on multiple organ functions, suggesting that it may be an alternative or at least one of the useful and desirable forms of ALSS for ALF patients.

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Original Article

Characteristics and prediction of hepatitis B e-antigen negative hepatitis following seroconversion in patients with chronic hepatitis B

Susumu Morita,[1]* Akihiro Matsumoto,[1]* Takeji Umemura,[1] Soichiro Shibata,[1] Nozomi Kamijo,[1] Yuki Ichikawa,[1] Takefumi Kimura,[1] Satoru Joshita,[1] Michiharu Komatsu,[1] Kaname Yoshizawa[1,2] and Eiji Tanaka[1]

[1]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, and* [2]*Department of Gastroenterology, National Hospital Organization Shinshu Ueda Medical Center, Ueda, Japan*

Aim: We analyzed the characteristics of alanine aminotransferase (ALT) abnormality after achieving hepatitis B e-antigen (HBeAg) seroconversion (SC) and other factors associated with the occurrence of HBeAg negative hepatitis.

Methods: We followed 36 patients with chronic hepatitis B from 3 years prior to at least 3 years after SC (mean, 11.6 years) and examined ALT, hepatitis B virus (HBV) DNA, HB surface antigen, HB core-related antigen (HBcrAg) levels and mutations related to HBeAg SC.

Results: ALT normalization (<31 IU/L for at least 1 year) was primarily observed until 2 years following SC, after which it became more infrequent. We next divided patients into abnormal (≥31 IU/L, $n = 20$) and normal (<31 IU/L, $n = 16$) groups based on integrated ALT level after the time point of 2 years from SC, and considered the former group as having HBeAg negative hepatitis in the present study. Although changes in median levels of ALT and HBcrAg differed significantly between the groups, multivariate analysis showed ALT normalization within 2 years after SC to be the only significant determining factor for this disease ($P = 0.001$). We then assessed the 19 patients whose ALT was normal at 2 years following SC, four of whom developed HBeAg negative hepatitis. Increased levels of HBV DNA ($P = 0.037$) and HBcrAg ($P = 0.033$) were significant factors of potential relevance.

Conclusion: ALT abnormality after 2 years of SC may be evaluated as HBeAg-negative hepatitis. ALT, HBV DNA and HBcrAg levels may be useful in predicting the outcome of patients who achieve HBeAg SC.

Key words: hepatitis B core-related antigen, hepatitis B virus, reactivation, seroconversion

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern with an estimated 350–400 million carriers worldwide. Whereas acute infection in adults is generally self-limiting, that during early childhood develops into persistent infection in most individuals, which can lead to chronic hepatitis and eventually liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] The natural history of chronic HBV infection can be classified into several phases based on levels of alanine aminotransferase (ALT) and HBV DNA, hepatitis B e-antigen (HBeAg) status and estimated immunological status.[4] In the immune tolerance phase, HBeAg is positive, ALT level is normal, histological evidence of hepatitis is absent or minimal, and HBV DNA level is elevated. The chronic hepatitis B phase is characterized by raised ALT and HBV DNA levels. In this phase, the host's immune system initiates a response that results in active hepatitis. In patients who are HBeAg positive, active hepatitis can be prolonged and may result in cirrhosis. However, chronic hepatitis B eventually transitions into an inactive phase with a loss of HBeAg positivity in the majority of patients. Seroconversion (SC) of HBeAg to HBe antibodies and the fall of HBV DNA level result in the disappearance of disease activity despite persisting hepatitis B surface antigen (HBsAg) and low HBV DNA level. The SC of

Correspondence: Dr Takeji Umemura, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Email: tumemura@shinshu-u.ac.jp
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HBeAg marks the transition from the hepatitis phase to the inactive carrier phase, which is generally thought to be a benign course for the HBV carrier, although hepatitis can sometimes reactivate spontaneously.[5]

Patients experiencing HBV reactivation undergo another transition characterized by increases in HBV DNA and ALT levels and disease activity without the reappearance of HBeAg. This phase is referred to as HBeAg negative chronic hepatitis B. Occasional severe hepatitis B flare-ups with moderate HBV DNA level occur in this phase.[6,7] It is thought that HBeAg negative chronic hepatitis B is caused by mutant strains of HBV that are unable to produce HBeAg[6,8] and tends to develop into cirrhosis and HCC more frequently than does HBeAg positive chronic hepatitis B.[9–13] Therefore, it is important to identify patients who are likely to develop HBeAg negative hepatitis after HBeAg SC from those who can maintain an inactive carrier phase. In the present study, we evaluated 36 patients with HBeAg SC to examine the effects of host factors and viral factors, including serum quantitative HBsAg, hepatitis B core-related antigen (HBcrAg), HBV DNA, PC (A1896) mutation and BCP mutations (T1762 and A1764) before, during and after SC.

METHODS

Patients

A TOTAL OF 36 patients with sustained HBeAg SC (24 men and 12 women; median age, 38 years [range, 23–65]) were enrolled in this study after meeting the following criteria: (i) follow ups for at least 3 years before and after HBeAg SC; and (ii) serum samples at several time points before, during and after SC available for testing. HBeAg SC was defined as seroclearance of HBeAg with the appearance of anti-HBe that was not followed by HBeAg reversion or loss of anti-HBe. All patients were seen at Shinshu University Hospital from 1985 to 2009. The median follow-up period after SC was 11.6 years (range, 3.2–26.0). HBsAg was confirmed to be positive on two or more occasions at least 6 months apart in all patients. No patients had other liver diseases, such as alcoholic or non-alcoholic fatty liver disease, autoimmune liver disease or drug-induced liver injury. Patients who were complicated with HCC or who showed signs of hepatic failure were excluded from the study. HBV genotype was C in all patients, who were also negative for antibodies to hepatitis C virus and HIV. Nucleoside/nucleotide analog (NUC) therapy was introduced in 14 patients after HBeAg SC on physicians' decision, and then follow up was stopped. No patient was treated with interferon during the study period. ALT, albumin, bilirubin, platelet and other relevant biochemical tests were performed using standard methods.[14] The integration value of ALT after SC was calculated using the method described by Kumada *et al.*[15] (median determination frequency, 4.7/year per person [range, 1.6–13.9]) because a previous study showed integration values to be more meaningful than arithmetic mean values in long-term follow-up cohorts.[16] As guidelines released by the Ministry of Health, Labor and Welfare of Japan advise consideration of antiviral therapy for patients with ALT levels of 31 IU/L or more,[17] an ALT integration value of less than 31 IU/L was defined as normal in this report. Serum samples were stored at −20°C until tested. Liver biopsies were performed by percutaneous sampling of the right lobe with a 14-G needle in eight patients with HBeAg negative hepatitis, as reported previously.[14] All biopsies were 1.5 cm or more in length. Liver histological findings were scored by the histology activity index of Knodell *et al.*[18] The protocol of this study was approved by the ethics committee of our university and was in accordance with the Declaration of Helsinki of 1975. Informed consent was obtained from each patient.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and anti-HBe, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan).[19] Quantitative measurement of HBsAg was done using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan).[20] The assay had a quantitative range of −1.5 to 3.3 log IU/mL. Serum HBcrAg level was measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio, Tokyo, Japan) as described previously.[21] We expressed HBcrAg level in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL. End titers of HBsAg and HBcrAg were determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. HBV DNA level was measured using an Amplicor monitor assay with a dynamic range of 2.6–7.6 log copies/mL.[22] Six major genotypes (A–F) of HBV were determined using the method reported by Mizokami *et al.*,[23] in which the surface gene sequence amplified by polymerase chain reaction was analyzed by restriction fragment length polymorphism.

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The PC and BCP mutations of HBV were assessed as previously described. Briefly, the stop codon mutation in the PC region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Roche Diagnostics, Tokyo, Japan) with a sensitivity of 1000 copies/mL. The results were expressed as the percent mutation rate as defined by Aritomi *et al.*[24] The PC mutation was judged to exist when the mutation rate exceeded 50% in the present study because the mutation rate would increase to 100% once surpassing this value.[25] The BCP double mutation was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories) with a detection limit of 1000 copies/mL.[24] The BCP mutation was judged to exist for all classifications of mutant in the present study.

Statistical analysis

Clinical factors were compared between patients with and without HBeAg negative hepatitis after SC using the χ^2-test and Fisher's exact test, and group medians were compared using the Mann–Whitney *U*-test. Receiver–operator curves (ROC) with Youden's index were used to decide each cut-off point for predicting HBeAg negative hepatitis after SC. Differences between the analyzed groups were assessed using Kaplan–Meier analysis and the log–rank test. Sex, age at SC, HBcrAg level, ALT level, HBV DNA level, HBsAg level, PC mutation and BCP mutation were all suspected to be associated with ALT elevation after SC. Factors attaining a *P*-value of less than 20% in univariate analysis were used in multivariate analysis that employed a stepwise Cox proportional hazard model. These included level of serum albumin and platelet count at SC, levels of ALT at 0, 1, 2 and 3 years after SC, and levels of HBcrAg at 1, 2 and 3 years after SC. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan, Tokyo, Japan). *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Baseline characteristics of patients

ALL 36 PATIENTS enrolled showed abnormal levels of ALT before SC, with the majority showing normalization around the time of SC. We defined ALT normalization as a decrease in ALT level to less than 31 IU/L for at least 1 year. The change in ratio of patients not achieving normalization over time revealed two distinct phases (Fig. 1): the first was a fast decline phase from 2 years before SC to 2 years afterwards, and the second

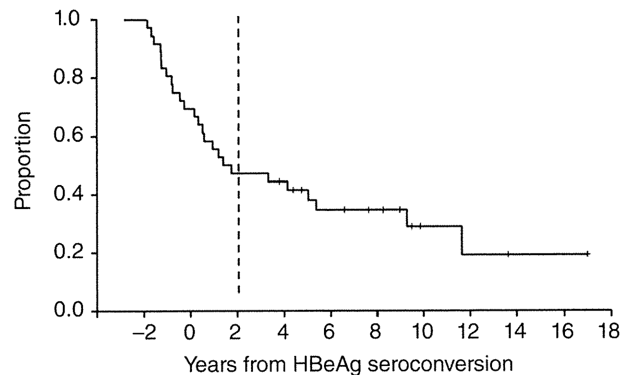

Figure 1 Changes in the proportion of patients with alanine aminotransferase (ALT) abnormality. ALT normalization was defined as ALT level decreasing to lower than 31 IU/L and maintained for at least 1 year. These data reveal two distinct time frames: a fast decline phase around the seroconversion (SC) period until 2 years afterwards, and a slow decline phase from 2 years after SC to the end of follow up. The vertical broken line at 2 years after SC indicates the borderline between the two phases. HBeAg, hepatitis B e-antigen.

was a slow decline phase from 2 years after SC to the end of follow up. Normalization of ALT during the fast phase was presumed to be associated with HBeAg SC, which was seen in 53% (19/36) of total patients. Based on this, we analyzed the risk factors associated with ALT abnormality after the time point of 2 years from SC by calculating integrated ALT levels (Fig. 2). We defined patients whose integrated ALT level exceeded 30 IU/L as having HBeAg negative hepatitis in the present study. Serum HBV DNA of over 4.0 log copies/mL was observed in all patients with HBeAg negative hepatitis.

Of the 36 patients enrolled, 20 (56%) developed HBeAg negative hepatitis and 16 (44%) did not. ALT normalization within 2 years after SC was significantly less frequent in patients with HBeAg negative hepatitis (Table 1). Median age, sex distribution and follow-up period did not differ between the two groups. Median albumin level tended to be lower in patients with HBeAg negative hepatitis, but only modestly. Eight of 20 HBeAg negative hepatitis patients underwent liver biopsy after SC. All had necroinflammatory activity. Initiation of NUC therapy was more common in the HBeAg negative hepatitis group.

Clinical and virological profiles

Changes in median levels of ALT, HBV DNA, HBsAg and HBcrAg during the course of SC have been compared between patients with and without HBeAg negative

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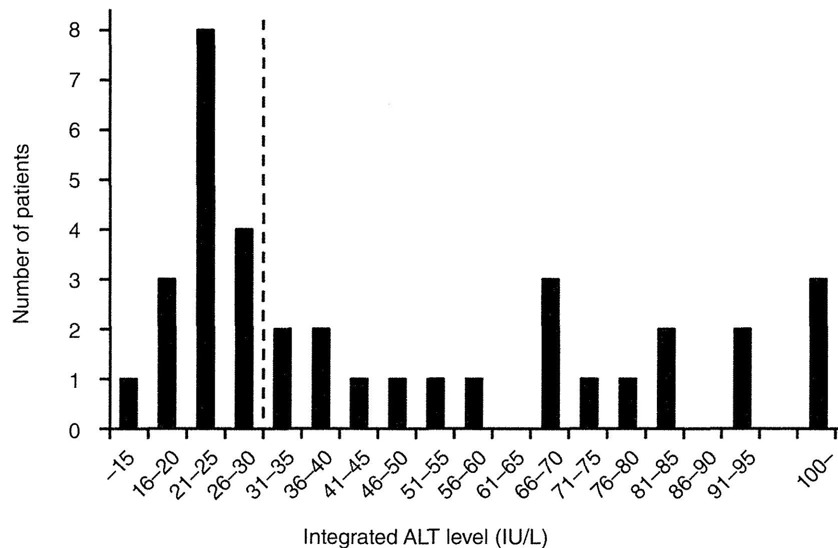

Figure 2 Distribution of integrated alanine aminotransferase (ALT) level from the time point of 2 years after seroconversion (SC) to the end of follow up.

hepatitis in Figure 3. We observed that median ALT level decreased around the time of SC in patients without HBeAg negative hepatitis, but did not in the other group. Overall, median ALT differed significantly between the two groups at the time of SC (43.0 vs 21.5 IU/L; $P = 0.009$) and at 1 (67.0 vs 15.0 IU/L; $P = 0.001$), 2 (52.0 vs 14.5 IU/L; $P < 0.001$) and 3 years (41.5 vs 15.0 IU/L; $P < 0.001$) afterwards (Fig. 3a). Median HBV DNA level decreased similarly in both groups around the time of SC (Fig. 3b). Median HBsAg level was unchanged or minimally decreased in both groups around the time of SC, but was significantly lower in patients with HBeAg negative hepatitis at 1 (3.9 vs 3.2 log IU/mL; $P = 0.025$) and 2 years (3.9 vs 3.2 log IU/mL; $P = 0.045$) before SC and at 2 years (3.7 vs 3.0 log IU/mL; $P = 0.023$) after SC (Fig. 3c). Median HBcrAg level decreased in both groups around the time of SC, but this decline was more gradual in patients with HBeAg negative hepatitis, becoming significantly higher at 1 (5.2 vs 3.9 log U/mL; $P = 0.011$), 2 (4.6 vs 3.5 log

Table 1 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis among total patients

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present (*n* = 20)	Absent (*n* = 16)	
Age at SC (years)†	40 (23–64)	38 (24–65)	0.504
Sex (male : female)	15:5	9:7	0.298
Follow-up period (years)†	10.6 (3.8–26.0)	12.4 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.1 (3.6–4.6)	4.3 (3.7–4.8)	0.030
Bilirubin (mg/dL)†	1.0 (0.4–2.6)	0.8 (0.5–1.3)	0.319
Platelets (/μL)†	13.9 (8.5–24.3)	18.1 (9.6–22.9)	0.187
ALT normalization within 2 years after SC‡	4 (20)	15 (94)	<0.001
Events during follow-up period			
Initiation of NUC therapy‡	12 (60)	2 (13)	0.006
Development of HCC‡	2 (10)	1 (6)	1.000

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

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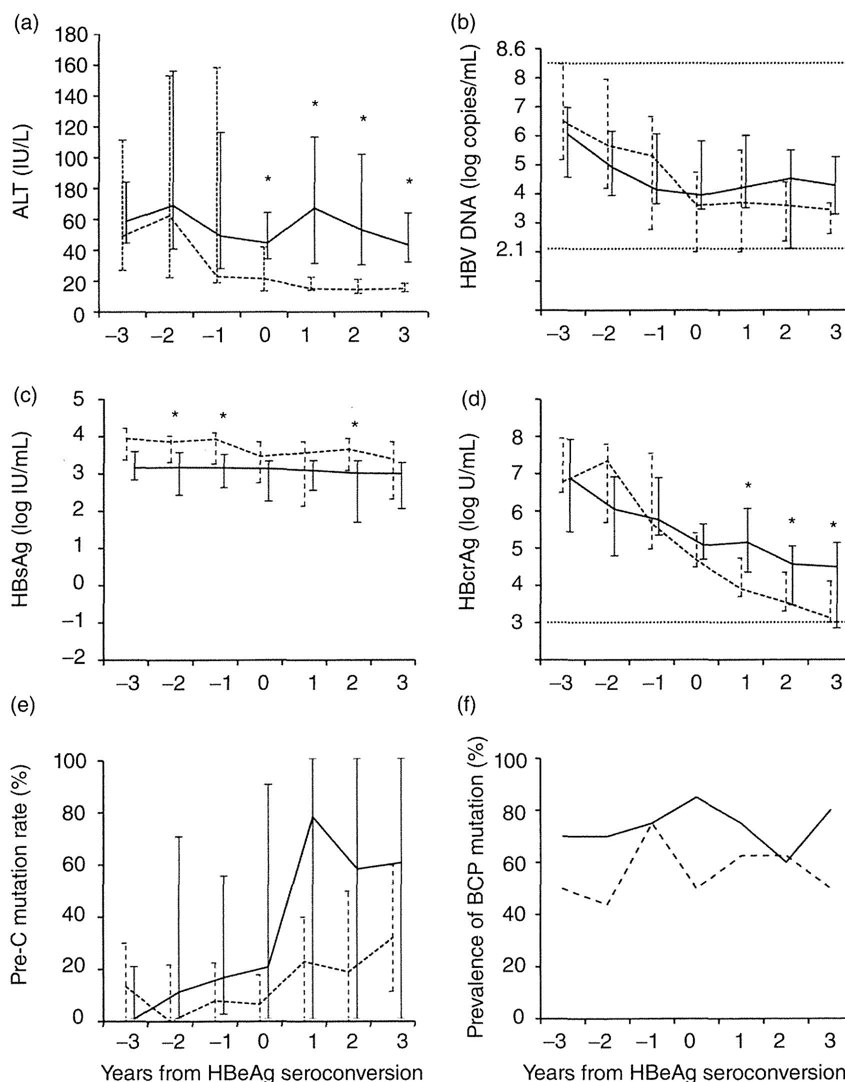

Figure 3 Changes in median levels of serum alanine aminotransferase (ALT) (a), hepatitis B virus (HBV) DNA (b), hepatitis B surface antigen (HBsAg) (c), hepatitis B core-related antigen (HBcrAg) (d) and PC mutation rate (e) are compared between patients with and without the occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis. A similar comparison is made for prevalence of patients with BCP mutations (f). Solid lines indicate patients with HBeAg negative hepatitis ($n = 20$) and broken lines indicate those without ($n = 16$). Data are shown as median values with 25% and 75% ranges at each point for (a–e). Horizontal broken lines in (b) and (d) indicate the upper and lower detection limits of the corresponding markers. $^*P < 0.05$.

U/mL; $P = 0.041$) and 3 years (4.6 vs 3.1 log U/mL; $P = 0.016$) after SC (Fig. 3d). PC mutation rate increased similarly in both groups during the course of SC (Fig. 3e), and the prevalence of BCP mutation positive patients remained comparatively high in both groups throughout the study period (Fig. 3f).

All factors that were associated with the occurrence of HBeAg negative hepatitis were evaluated for independence by multivariate analysis. We found that only abnormal level of ALT (≥31 IU/L) at 2 years after SC (odds ratio, 42.0; 95% confidence interval, 4.3–405.4; $P = 0.001$) was an independent predictive factor. Therefore, we examined for factors associated with the occurrence of HBeAg negative hepatitis in the 19 patients whose ALT level had normalized by 2 years after SC. Four (21%) of these patients developed HBeAg negative hepatitis and the remaining 15 (79%) did not. We found no significant differences between the two groups with regard to age at SC, sex or laboratory data (Table 2). We next analyzed HBV DNA, HBsAg and HBcrAg levels at 2 years after SC to see if these factors could discriminate between patients with and without the development of HBeAg negative hepatitis. Cut-off values for each factor were determined by ROC analysis. As shown in Figure 4, serum levels of HBV DNA (7% vs 60%; $P = 0.037$) and HBcrAg (0% vs 44%; $P = 0.033$) were significant factors indicating susceptibility, but HBsAg was not.

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Table 2 Comparison of host and viral factors between patients with and without HBeAg negative hepatitis in 19 patients whose ALT levels were normal at 2 years after SC

Clinical characteristics	HBeAg negative hepatitis		*P*
	Present (*n* = 4)	Absent (*n* = 15)	
Age at SC (years)†	41 (30–43)	37 (23–65)	0.549
Sex (male : female)	2:2	8:7	1.000
Follow-up period (years)†	9.1 (8.3–14.1)	12.2 (3.2–23.1)	0.610
Laboratory data at SC			
Albumin (g/dL)†	4.3 (3.8–4.3)	4.3 (3.7–4.7)	0.364
Bilirubin (mg/dL)†	1.0 (1.0–1.3)	0.8 (0.5–1.3)	0.083
Platelets (/μL)†	14.9 (13.3–16.4)	16.9 (9.6–22.5)	0.667
Events during follow-up period			
Initiation of NUC therapy‡	3 (75)	2 (13)	0.037
Development of HCC‡	1 (25)	1 (7)	0.386

†Data are expressed as median (range).
‡Data are expressed as number of patients (%).
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; NUC, nucleoside/nucleotide analog; SC, seroconversion.

DISCUSSION

ALTHOUGH ACTIVE HEPATITIS usually subsides following HBeAg SC, it recurs in a considerable proportion of patients several years afterwards. Hsu *et al.*[5] followed 283 patients with HBeAg SC for a median follow-up period of 8.6 years and observed that ALT elevation of over twice the upper limit of normal occurred in 94 patients (33%). Of these, 68 (72%) were considered to have HBeAg negative hepatitis B because HBV DNA was detectable without the reappearance of HBeAg at the time of ALT elevation. HBeAg negative hepatitis is a major health concern because its occurrence is closely associated with progression to cirrhosis and development of HCC,[9–12] and thus prediction of its onset is important. Hsu *et al.*[5] found that patients with more frequent acute exacerbations of hepatitis before HBeAg SC and those with cirrhosis at the time of HBeAg SC had a higher risk of developing HBeAg negative hepatitis. Although significant, these factors were insufficient to accurately predict the occurrence of the disease.[26–30] Therefore, we analyzed several additional factors, including HBV DNA, HBsAg and HBcrAg levels, as well as viral mutations that halt HBeAg production.

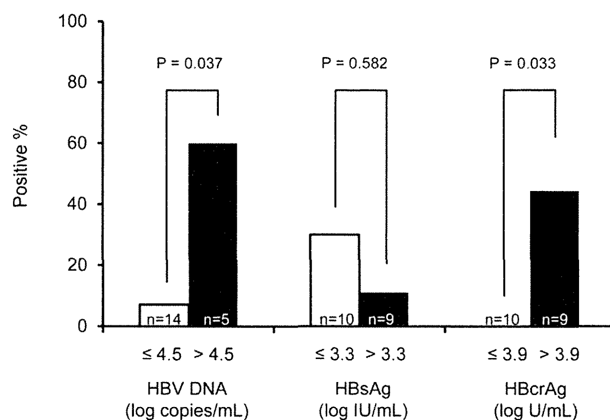

Figure 4 Occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis is compared among patients using higher and lower levels of corresponding markers at 2 years after seroconversion (SC). The cut-off value for each marker was determined by receiver–operator curve analysis. HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

In the present study, we found that the majority of patients with HBeAg SC achieved normalization of ALT within 2 years following SC, after which such normalization became relatively rare. Abnormal ALT was determined using the distribution of integrated ALT level from 2 years after SC to the end of follow up, which clearly showed the existence of two groups. We defined patients with an abnormal integrated level of ALT as having HBeAg negative hepatitis because this abnormality tended to persist and was preceded by HBV DNA elevation. Our result also conferred the important realization that ALT abnormality within 2 years after SC may not necessarily indicate the occurrence of HBeAg negative hepatitis, which has a poor prognosis. NUC

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therapy was not available for patients with chronic hepatitis B in Japan when our subjects began follow up. Hence, the natural history of SC has been evaluated in this cohort. Follow up stopped in this study when NUC therapy was commenced. Currently, we perform NUC therapy on patients with HBe negative hepatitis based on age and ALT activity, as advised by the Ministry of Health, Labor and Welfare.[17]

Many host and viral factors were also analyzed to predict the occurrence of HBeAg negative hepatitis in the current study. Host factors, including age and sex, did not differ between the groups with and without HBeAg negative hepatitis, but changes in median ALT level around SC clearly differed between the two groups. Specifically, ALT level did not decrease even after SC in patients with HBeAg negative hepatitis, while it normalized during the SC period in those without. Viral factors were analyzed at several time points around SC. Among them, median HBcrAg level clearly differed between the groups; HBcrAg showed a steep decrease around the SC period in patients without HBeAg negative hepatitis, while it exhibited a significantly slower decline in those with. Similarly to earlier reports, median levels of HBV DNA and HBsAg showed some differences between the two groups, but these were not remarkable when analyzed chronologically. Negative results were also seen in the analyses of PC and BCP mutations. Multivariate analysis showed that abnormal ALT level at 2 years after SC was the only significant factor to predict the occurrence of HBeAg negative hepatitis among the factors analyzed. Because patients with normal ALT had maintained that level for at least 1 year, this result may indicate that continuous normalization of ALT is rare in patients with HBeAg negative hepatitis after SC and that ALT abnormality is associated with higher levels of HBcrAg and HBV DNA.

Because ALT level was closely related to the occurrence of HBeAg negative hepatitis, we next analyzed for predictive factors in patients whose ALT level was normal (<31 IU/L) at 2 years after SC. We observed that increased HBV DNA and HBcrAg levels at 2 years after SC were significant factors for predicting the occurrence of HBeAg negative hepatitis, but that HBsAg level was not. Single or combined monitoring use of HBV DNA and HBcrAg levels may therefore be useful to predict the recurrence of hepatitis in patients whose ALT level normalizes following HBeAg SC. However, further studies are required to verify this in the clinical setting.

Whereas HBsAg is a serum marker commonly used for the diagnosis of HBV infection, HBcrAg assays measure serum levels of HBc, HBe and the 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[31] Because the latter assay measures all antigens transcribed from the precore/core gene, it is regarded as core-related.[21] It has been suggested that viral antigen levels, including those of HBsAg and HBcrAg, are differently associated with HBV activity from HBV DNA and ALT levels, and thus are useful for predicting the future activity of hepatitis B. For example, HBcrAg level was seen to predict hepatitis relapse after discontinuation of NUC therapy,[32,33] and HBsAg level has been reportedly associated with the response to pegylated interferon therapy differently from HBV DNA.[34,35] Both antigen levels are believed to be related to intracellular levels of HBV cccDNA. However, it is possible that levels of HBsAg and HBcrAg have different roles in monitoring viral activity because the transcription of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.[1] The serum level of HBcrAg was more useful than that of HBsAg to predict the occurrence of HBeAg negative hepatitis in the present study. This difference may be attributed to the fact that the production of all antigens that constitute HBcrAg is regulated by the same system as that of HBeAg, while the production of HBsAg is not.

Lastly, it is reasonable to presume that the PC and BCP mutations which halt HBeAg production are associated with integrated values of ALT elevation because the disease is essentially caused by HBV containing these mutations.[8,10] However, the prevalence of either mutation did not differ between the groups at any time point during the study. Our results showed that almost all patients had PC and/or BCP mutations, especially after SC, and implied that the existence of these mutations alone was not sufficient for developing ALT elevation. HBV genotype is also closely associated with HBeAg SC,[36] but we could not include genotype as a factor because our entire cohort was genotype C.

A recent review by Papatheodoridis *et al.*[37] showed that histologically significant liver disease is rare in HBeAg negative patients with persistently normal ALT based on stringent criteria and serum HBV DNA of 20 000 IU/mL or less. They suggest that such individuals can be considered as true inactive HBV carriers, who require continued follow up rather than liver biopsy or immediate therapy. On the contrary, liver biopsy samples obtained from eight of our patients with HBeAg negative hepatitis having elevated ALT levels after SC revealed necroinflammatory activity. Hence, it remains controversial if histological findings are important for diagnosis of HBeAg negative hepatitis.

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This study has the main limitations of a retrospective design and a small cohort size. However, our findings from careful extended follow up indicate that ALT abnormality after 2 years from SC can be considered to be HBeAg negative hepatitis, and that HBcrAg and HBV DNA levels may be useful for predicting the long-term outcome of patients who achieve HBeAg SC and ALT normalization.

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Original Article

Serum levels of interleukin-22 and hepatitis B core-related antigen are associated with treatment response to entecavir therapy in chronic hepatitis B

Sadahisa Okuhara,[1] Takeji Umemura,[1] Satoru Joshita,[1] Soichiro Shibata,[1] Takefumi Kimura,[1] Susumu Morita,[1] Michiharu Komatsu,[1] Akihiro Matsumoto,[1] Kaname Yoshizawa,[1] Yoshihiko Katsuyama,[2] Masao Ota[3] and Eiji Tanaka[1]

[1]*Department of Medicine, Division of Hepatology and Gastroenterology,* [3]*Department of Legal Medicine, Shinshu University School of Medicine, and* [2]*Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan*

Aim: We sought to clarify the associations between serum cytokines and chemokines, hepatitis B surface antigen (HBsAg), hepatitis B core-related antigen (HBcrAg), and hepatitis B virus (HBV) DNA and response to entecavir therapy in chronic hepatitis B.

Methods: We analyzed six cytokines (interleukin [IL]-2, IL-6, IL-10, IL-12p70, IL-21 and IL-22) and five chemokines (CCL2, CCL3, CXCL9, CXCL10 and CXCL11) before and at 6, 12 and 24 months during entecavir therapy in 48 chronic hepatitis B patients. Quantitative measurement of HBsAg, HBcrAg and HBV DNA was performed. A virological response (VR) was defined as serum HBV DNA of less than 2.1 log copies/mL by treatment month 24.

Results: Thirty-nine patients (81%) achieved a VR. Serum IL-6 ($P = 0.031$), CXCL-9 ($P = 0.002$), and CXCL-10 ($P = 0.001$) were high in chronic HBV and correlated positively with transaminases and bilirubin. Before treatment, elevated IL-22 ($P = 0.031$) and lower HBsAg ($P = 0.001$) and HBcrAg ($P < 0.001$), but not HBV DNA, were associated with a favorable treatment outcome. In multivariate analysis, high IL-22 (hazard ratio = 13.67, $P = 0.046$) and low HBcrAg (hazard ratio = 10.88, $P = 0.048$) were independently associated with a VR. The levels of IL-22 ($P < 0.001$), HBsAg ($P < 0.001$), and HBcrAg ($P < 0.001$) all decreased from baseline to 24 months of treatment in virological responders.

Conclusion: Serum IL-22 and HBcrAg are predictive markers of a VR to entecavir therapy in patients with chronic hepatitis B.

Key words: entecavir, hepatitis B core-related antigen, hepatitis B surface antigen, hepatitis B virus, interleukin-22

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is the primary cause of cirrhosis and hepatocellular carcinoma (HCC) and is one of the major causes of death globally.[1,2] Because high plasma HBV DNA concentrations and quantitative hepatitis B surface antigen (HBsAg) levels are associated with progression to cirrhosis and development of HCC,[3,4] viral suppression by means of nucleoside/nucleotide analog therapy has shown clinical benefits via a reduction in hepatic decompensation and lower HCC rates.[5–7]

Cytokines and chemokines are involved in cell-mediated and humoral immune responses as well as in antiviral activity, viral clearance, apoptosis and fibrogenesis. As the control of cytokine production is highly complex and their effects widespread throughout multiple regulatory networks, it would seem that screening for multiple biomarkers may best clarify the immunopathogenesis of this disease and predict responses to antiviral therapy. Our previous studies have shown that several cytokines and chemokines are associated with treatment outcome in patients with chronic hepatitis C using bead-based multiplex immunoassays.[8–10] Although other reports have demonstrated an association between individual cytokines and clinical outcome in subjects with HBV,[11–18] the

Correspondence: *Dr Takeji Umemura, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Email: tumemura@shinshu-u.ac.jp*
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relationship between multiple cytokines and chemokines and response to nucleoside/nucleotide analog therapy in chronic hepatitis B patients has not yet been examined in the Japanese population.

The objective of this study is to determine which cytokines and chemokines in chronic hepatitis B are related to the clinical and virological characteristics of hepatitis and how they affect the HBV response to entecavir (ETV) treatment.

METHODS

Subjects

WE ENROLLED 48 consecutive patients with chronic hepatitis B in this study. All patients were treatment naïve at the time of commencing ETV at a daily dose of 0.5 mg for a duration of at least 24 months. Clinical and laboratory data of the patients were analyzed at baseline and at months 6, 12 and 24 of therapy. Chronic hepatitis B was based on HBsAg positivity for at least 6 months. No patients had a history of organ transplantation, decompensated cirrhosis, HCC or the concurrent use of immunomodulatory drugs or corticosteroids. Patients who were co-infected with the hepatitis C virus (HCV) or who exhibited evidence of other liver diseases, such as primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease and non-alcoholic liver disease, were excluded from this study. A group of 10 healthy individuals negative for HBV and HCV serology and normal transaminase levels was used as the control. All patients and subjects were negative for antibodies to HIV type 1. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine. All patients provided written informed consent.

Laboratory testing

Hepatitis B surface antigen, hepatitis B e-antigen (HBeAg), anti-HBe, anti-HCV and anti-HIV-1 were determined using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan).[19] Serum levels of HBV DNA were quantified using the COBAS TaqMan HBV Test v2.0 (Roche Diagnostics, Tokyo, Japan) that had a dynamic range of 2.1–9.0 log copies/mL. Quantitative measurement of HBsAg was performed using an HISCL HBsAg assay based on the chemiluminescence enzyme immunoassay (CLEIA; Sysmex, Kobe, Japan) which had a quantitative range of –1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range. Serum HB core-related antigen (HBcrAg) levels were measured using a CLEIA-based HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio, Tokyo, Japan). We expressed HBcrAg level in terms of log U/mL with a quantitative range set at 3.0–6.8 log U/mL. HBV genotypes were determined using commercially available ELISA kits (HBV GENOTYPE EIA; Institute of Immunology). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and other relevant biochemical tests were performed using standard methods.[20]

Definitions

A virological response (VR) was defined as a HBV DNA level that was undetectable by real-time polymerase chain reaction (<2.1 copies/mL) at 24 months. A virological breakthrough was defined as an increase in HBV DNA level by 1 log copies/mL or more above nadir while on treatment following an initial decline to 2 log copies/mL or more.

Detection of cytokines and chemokines

Six cytokines (interleukin [IL]-2, IL-6, IL-10, IL-12p70, IL-21 and IL-22) and five chemokines (CCL2/MCP-1, CCL3/MIP-1α, CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC) were quantified using Luminex Multiplex Cytokine Kits (Procarta Cytokine Assay Kit) for serum samples obtained before the start of treatment and at weeks 24, 48 and 96 as reported previously.[8,9] These markers had been implicated in HBV pathogenesis in earlier reports.[11–16,18] All collected samples were immediately stored at –70°C and remained in storage until testing.

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to analyze continuous variables where appropriate. The Friedman test was employed to evaluate changes in serum cytokine levels over time. Spearman's rank correlation coefficients were adopted to evaluate the relationship between pairs of markers. The χ^2-test with Yates's correction was used for the analysis of categorical data. In cases where the number of subjects was less than five, we employed Fisher's exact test. $P < 0.05$ was considered statistically significant. To predict treatment outcome, cut-off points for continuous variables were decided by receiver–operator curve (ROC) analysis with Youden's index. Factors attaining a *P*-value of less than 0.1 in univariate analysis were evaluated by multivariate analysis using a stepwise logistic regression model. These

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