regeneration or repair of various impaired organs. Mesenchymal tissue from bone marrow, umbilical cord, and adipose tissue are relatively enriched with pluripotent stem cells.[12] Since the pathophysiological features of liver cirrhosis are a consequence of chronic hepatic inflammation, MSCs are especially suited to enhance regeneration and/or repair of damaged cirrhotic liver.

We have established a clinically relevant NASH cirrhotic murine model by feeding animals an atherogenic high-fat (Ath+HF) diet.[13] In this study we examined whether adipose-tissue-derived stem cells (ADSCs) can regenerate and/or repair the cirrhotic liver. We observed that injected ADSCs resided in the liver and expressed albumin, leading to restored albumin expression in hepatic parenchymal cells. ADSCs also ameliorated advanced fibrosis. Moreover, ADSCs suppressed the underlying persistent inflammation contributed by granulocytes, phagocytic cells, and T cells. These results suggest that treatment of patients with cirrhosis with ADSCs is a potentially novel approach for regenerating and/or repairing damaged cirrhotic liver tissue to restore hepatic function.

Materials and Methods

Culture of ADSCs. ADSCs were prepared as described.[14] Briefly, adipose tissue was obtained from the inguinal subcutaneous region of 10-week-old GFP-Tg male mice (a gift from Professor Okabe, Osaka University, Japan). The stem cell fraction was isolated from adipose tissue using type-I collagenase (Wako Pure Chemical Industries, Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium: nutrient mixture F-12 supplemented with 10% heat-inactivated bovine serum albumin and 1% antibiotic-antimycotic solution. Cell culture reagents were purchased from Life Technologies (Carlsbad, CA).

NASH Murine Model. Female 8-week-old C57Bl/6J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were fed an Ath+HF diet composed of cocoa butter, cholesterol, cholate, and corticotropin-releasing factor-1 (Oriental Yeast Co., Tokyo, Japan) to induce steatohepatitis as reported previously.[13] Our Institutional Review Board approved the care and use of laboratory animals in all experiments.

ADSC Treatment of NASH Mice. ADSCs were harvested after six to eight passages in culture by treatment with trypsin/EDTA (Life Technologies) and passed through a 100-μm Cell Strainer mesh (BD Biosciences, San Jose, CA). Laparotomy was performed to inject 1×10^5 ADSCs or phosphate-buffered saline (PBS) into the splenic subcapsule. After ADSC treatment, the mice were anesthetized with pentobarbital (40 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan), after which the liver was perfused with PBS and dissected. A portion of liver tissue was homogenized and incubated with type I collagenase (Wako Pure Chemical Industries), and hepatic parenchymal cells and inflammatory cells were separated with Percoll (GE Healthcare UK, Buckinghamshire, UK). $CD4^+$ T cells were isolated from hepatic inflammatory cells using a magnetic sorting system, the $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec, Gladbach, Germany).

Histology and Immunohistochemical Staining. Liver tissue was preserved with formalin for paraffin embedding or embedded in OCT compound and frozen for sectioning (Sakura Finetek Japan, Tokyo, Japan). The frozen liver sections were fixed in acetone and endogenous peroxidase activity blocked with 3% hydrogen peroxide solution. After washing in PBS, the sections were incubated with a rabbit anti-CD11b antibody (BD Pharmingen, San Diego, CA) and a rabbit anti-Gr-1 antibody (eBioscience, San Diego, CA) overnight at 4°C. The slides were then washed and incubated with Histofine mouse MAXPO (Nichirei Bioscience, Tokyo, Japan) for 1 hour at room temperature. The immune complex was visualized by incubating with diaminobenzidine for 5 minutes. The paraffin-embedded sections were stained with a rabbit-anti-GFP antibody (Millipore, Billerica, MA), a rabbit anti-α-smooth muscle actin (α-SMA) antibody (Abcam, Cambridge, UK), and a rabbit anticollagen IV antibody (Abcam). Secondary antibody development was performed with diaminobenzidine as described above. In some experiments, the sliced

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Address reprint requests to: Shuichi Kaneko, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan. E-mail: skaneko@m-kanazawa.jp; fax: +81-76-234-4250.

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sections were double-stained with a combination of a goat antimouse serum albumin antibody (Abcam) and a rabbit anti-GFP antibody followed by the secondary antibody and development as described above. To quantify fibrosis, paraffin-embedded sections were stained with Azan and viewed microscopically, after which the stained area was calculated using an image-analysis system (BIOREVO BZ-9000 and BZ-H1C, Keyence Japan, Osaka, Japan).

Flow Cytometry. Isolated hepatic inflammatory cells were incubated in PBS supplemented with 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) for 10 minutes at 4°C. The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (eBioscience) and phycoerythrin (PE)-conjugated anti-CD8 antibodies (eBioscience) for 30 minutes at 4°C before examination using a FACSCalibur cytometer (BD Biosciences). Similarly, ADSCs were incubated with PE-conjugated CD90 (Beckman Coulter, Fullerton, CA), or PE-conjugated CD105 (Miltenyi Biotec). The data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

DNA Microarray Analysis. Isolated RNAs were amplified and labeled with Cy3 using a QuickAmp Labeling Kit (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer's protocol. cRNA (825 ng) was hybridized onto a Whole Mouse Genome 4 × 44K Array (Agilent Technologies). The hybridized microarray slide was scanned using a DNA microarray scanner (model G2505B; Agilent Technologies).

Gene expression analysis was carried out using GeneSpring analysis software (Agilent Technologies). Each measurement was divided by the 75th percentile of all measurements in that sample to normalize per chip. Hierarchical clustering and principal component analysis of gene expression was performed. Welch's t test with Benjamini and Hochberg's false-discovery rate were used to identify differentially expressed genes in the groups of interest. Analysis of biological processes was performed using the MetaCore software suite (GeneGo, San Diego, CA). BRB array tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) were also used for unsupervised clustering or one-way clustering analysis. Microarray data were deposited in the NBCI Gene Expression Omnibus (GSE ID: GSE40395).

Statistical Analysis. GraphPad Prism (v. 5.0; GraphPad Software, La Jolla, CA) was used to perform a Mann-Whitney U test to compare data between two groups, and differences were considered statistically significant at $P < 0.05$.

All other materials and methods are described in the Supporting Information.

Results

Characteristics of the NASH Mouse Model. The pathological and clinical features of cirrhosis in patients are not well replicated by the majority of chemically induced murine cirrhotic liver models. We have established steatohepatitis as a cirrhotic liver mouse model by feeding mice an Ath+HF diet.[13] When mice were fed this diet for 34 weeks, hepatocytes developed steatosis, Mallory-Denk bodies, and ballooning (Fig. 1A,B), which are identical to typical pathological features of clinical NASH.[15] Albumin expression in parenchymal cells of the cirrhotic liver significantly decreased in mice fed the Ath+HF diet for 24 weeks (Fig. 1C), while alpha-fetoprotein (AFP) expression was not affected (Fig. 1D). Fibrosis developed and reached maximal levels after 34 weeks of feeding the Ath+HF diet (Fig. 1E,F). Immunohistochemical staining for immunomodulatory cells showed an increased number of Gr-1^+ cells in the liver of the steatohepatitis mice fed the Ath+HF diet for 12, 34, and 70 weeks (Fig. 2A,B). The number of CD11b^+ cells in the liver also increased and reached maximal levels after 34 weeks of feeding the Ath+HF diet (Fig. 2C,D). Thus, the murine cirrhosis model established by an Ath+HF diet mimics the features of clinical NASH.

Effect of ADSCs Treatment on Liver Albumin Expression and Fibrosis. Adipose tissue contains MSCs, which have the potential to differentiate into several types of cell lineages[10,14] and to act as immunomodulators.[11] In this study, we isolated stromal cells from inguinal adipose tissue of GFP-expressing transgenic (GFP-Tg) mice as ADSCs and expanded them in culture. The majority of these cells expressed CD90 and CD44, known surface markers of mesenchymal cells (Supporting Fig. 1A). A proportion of the expanded ADSCs also expressed CD105 (Supporting Fig. 1B), which has been recognized as a representative surface marker of MSCs.[11]

We evaluated whether ADSCs could provide a therapeutically beneficial treatment for liver cirrhosis in steatohepatitis mice. We injected 1×10^5 GFP-ADSCs by way of the spleen/portal vein in mice fed the Ath+HF diet for 32 weeks. We observed that the GFP-ADSCs resided in all lobes of the liver at 3, 7, and 14 days after injection (Fig. 3A,B). Importantly, immunohistochemical staining showed that GFP$^+$ cells in the cirrhotic liver expressed higher levels of albumin than did the surrounding parenchymal cells (Fig. 3C).

We also injected 1×10^5 or 2×10^4 GFP-ADSCs twice every 2 weeks by way of the splenic/portal vein

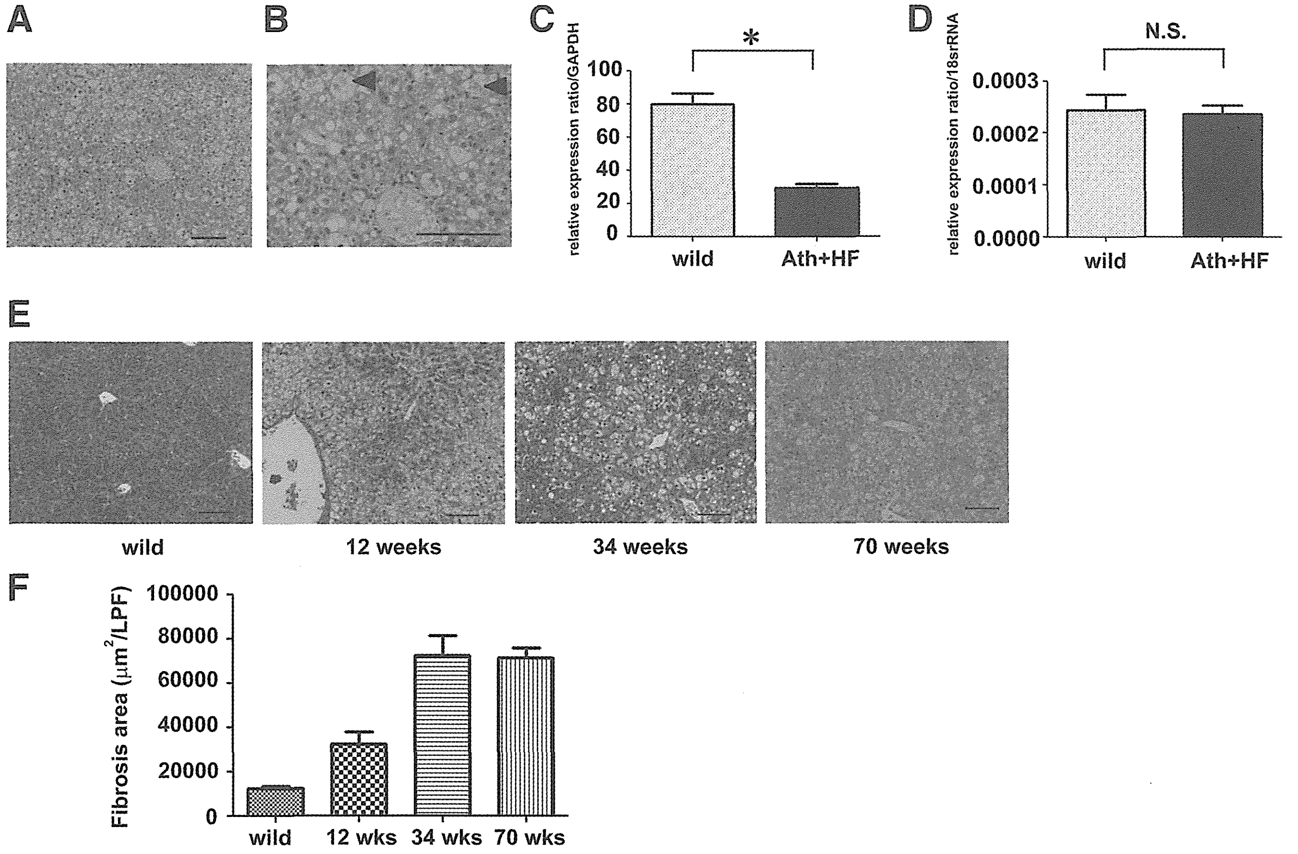

Fig. 1. Characteristics of the steatohepatitis murine model. Eight-week-old C57Bl/6 female mice were fed an Ath+HF diet. Liver tissue was obtained after 34 weeks, sectioned, and histologically examined with hematoxylin and eosin staining in (A,B) mice fed an Ath+HF diet for 34 weeks. Arrowheads indicate a Mallory-Denk body in a hepatocyte with ballooning. Parenchymal cells were isolated from 32-week-old C57Bl/6 wild-type female mice or Ath+HF mice that started the diet at 8 weeks old and continued for 24 weeks. Expression of (C) albumin and (D) AFP was assessed by reverse-transcription polymerase chain reaction (RT-PCR), n = 4, $*P < 0.05$. (E) Fibrosis was histologically examined with Azan staining in liver tissue of mice fed the Ath+HF diet for 12, 34, and 70 weeks. (F) Fibrosis areas of mice at 12, 34, and 70 weeks per ×100 low-power field were calculated for five visual fields. Bars: standard error. Scale bars = 100 μm.

in mice fed an Ath+HF diet for 32 or 36 weeks, respectively. Two weeks after the last injection the mice were euthanized and the therapeutic effects were assessed. The expression of albumin (Fig. 4A) was restored in hepatic parenchymal cells of cirrhotic mice at 2 weeks after the last injection, suggesting that ADSC treatment restored parenchymal cell function. The expression of AFP was also increased by ADSC treatment (Fig. 4B), implying enhanced regeneration of hepatic parenchymal cells. Similar effects were observed with a reduced number of (2×10^4) GFP-ADSCs (Supporting Fig. 2A,B).

We also assessed the effect of ADSC injection on fibrosis in cirrhotic mice. Liver tissue stained with Azan and anticollagen type IV antibody showed that ADSC administration reduced fibrosis compared to control animals (Fig. 5A,B; Supporting Fig. S3A,B). We also evaluated immunohistochemical staining of α-SMA, a marker of stellate cells, which are largely responsible for developing fibrosis. These results demonstrated that the number of α-SMA$^+$ cells was reduced by ADSC treatment (Fig. 5C-E), suggesting that ADSCs suppress the activity of stellate cells and ameliorate liver fibrosis.

Gene Expression Profiling of Cirrhotic Livers Following ADSC Treatment. We examined the gene expression profile of the livers in the NASH mouse model of cirrhosis by DNA microarray to determine whether administration of ADSCs was therapeutically beneficial. We identified expression of 1,249 gene probes that were significantly affected by ADSC injection. Clustering analysis of gene expression using these gene probes distinguished between ADSCs-treated mice and PBS-treated mice (Fig. 6A). Among 1,249 genes, 797 were up-regulated and 452 were down-regulated by ADSC treatment. Regarding matrix metalloproteinase (MMP), expressions of MMP-8 and MMP-9 were enhanced in the liver of NASH mice treated with PBS compared to the wild type; this enhancement was removed by ADSC treatment

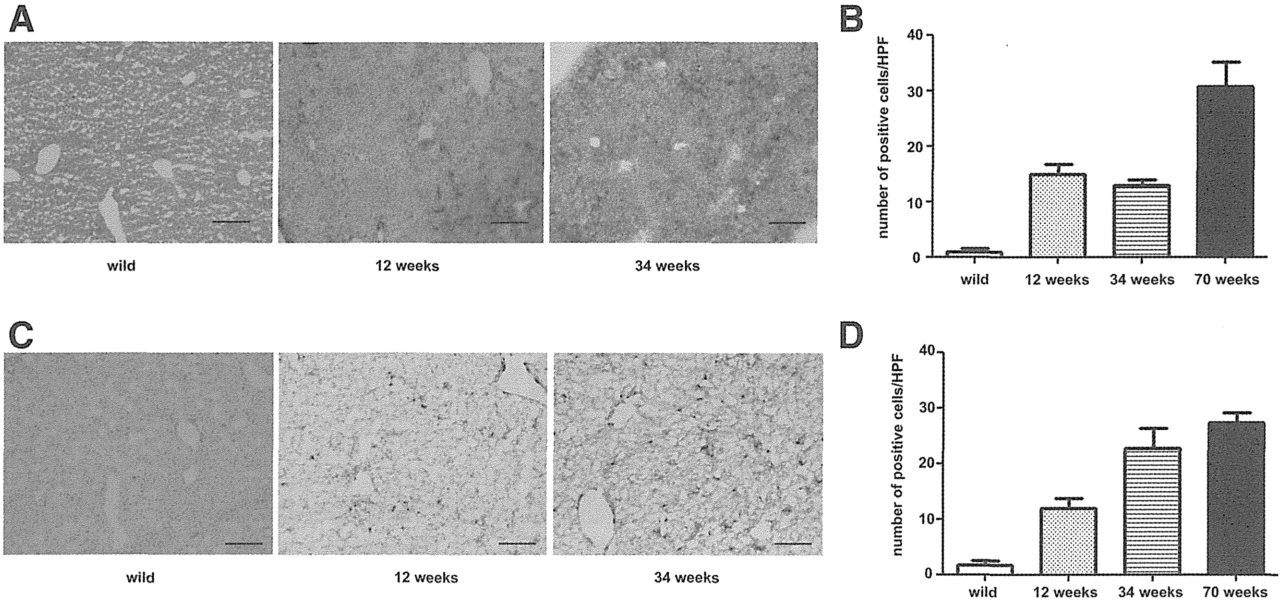

Fig. 2. Immunohistochemical staining of a steatohepatitis liver. Eight-week-old female C57Bl/6 female mice were fed an Ath+HF diet. Liver tissue was obtained from these mice or from wild-type animals after 12, 34, and 70 weeks. Immunohistochemical staining was performed for (A) Gr-1$^+$ or (C) CD11b$^+$ cells and the number of positive cells in a high-power field was counted for five visual fields for (B) Gr-1 or (D) CD11b at each timepoint.

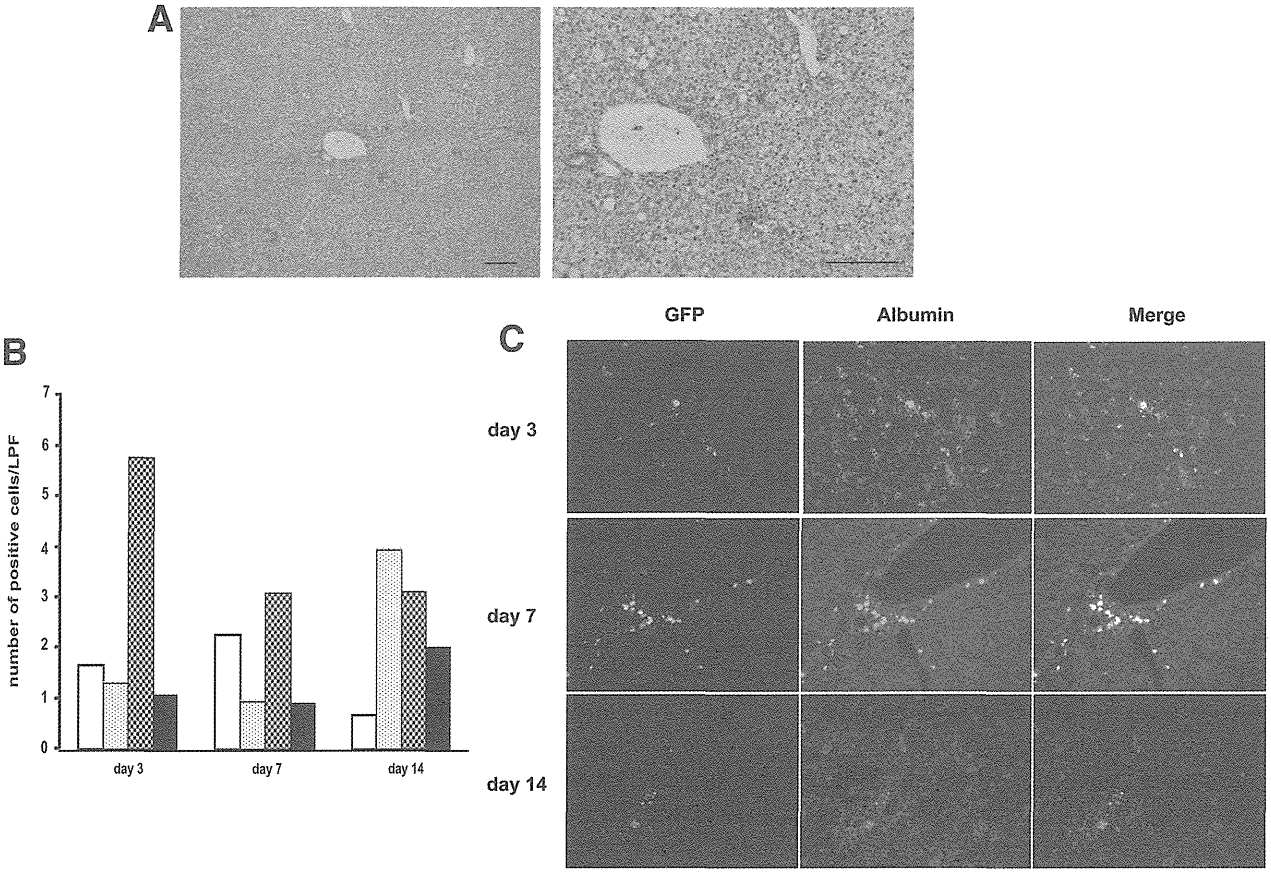

Fig. 3. Distribution of ADSCs and albumin expression in the livers of steatohepatitis mice. ADSCs from GFP-Tg mice (1×10^5) were injected into the splenic subcapsule of cirrhotic C57Bl/6 mice fed the Ath+HF diet for 32 weeks. After 3, 7, and 14 days, liver tissue was obtained and examined by immunohistochemical staining for (A) GFP; Scale bars = 100 μm. (B) GFP$^+$ cells in the liver were counted per ×100 low-power field and five visual fields were calculated. White bar, caudate lobe; dotted bar, left lobe; hatched bar, middle lobe; black bar, right lobe. (C) Immunohistochemical staining for GFP or albumin antibody. Magnification, ×100.

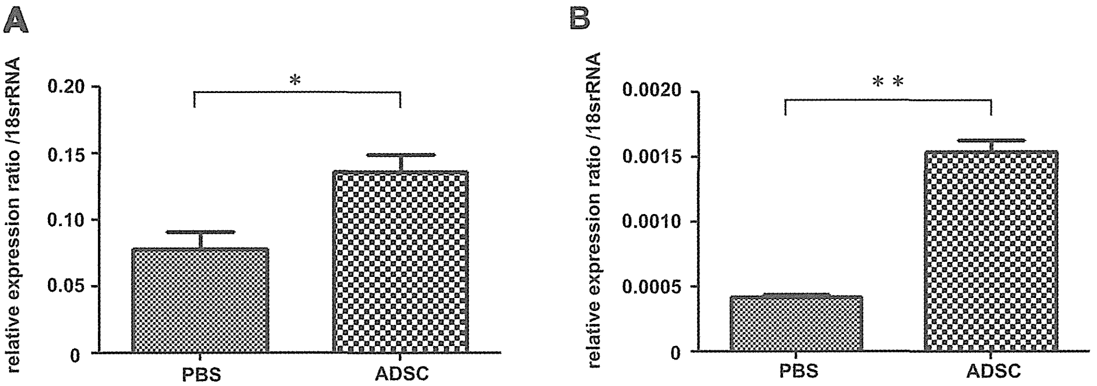

Fig. 4. Albumin and AFP expression in hepatic parenchymal cells. ADSCs from GFP-Tg mice (1 × 10^5) were injected twice every 2 weeks into the splenic subcapsule of cirrhotic C57Bl/6 mice fed an Ath+HF diet for 32 weeks. Control mice received PBS injections. Two weeks after the last injection, liver tissue was obtained and parenchymal cells were isolated for real-time PCR. Expressions of (A) albumin and (B) AFP were normalized relative to that of 18S ribosomal RNA (rRNA); *$P < 0.05$, **$P < 0.01$.

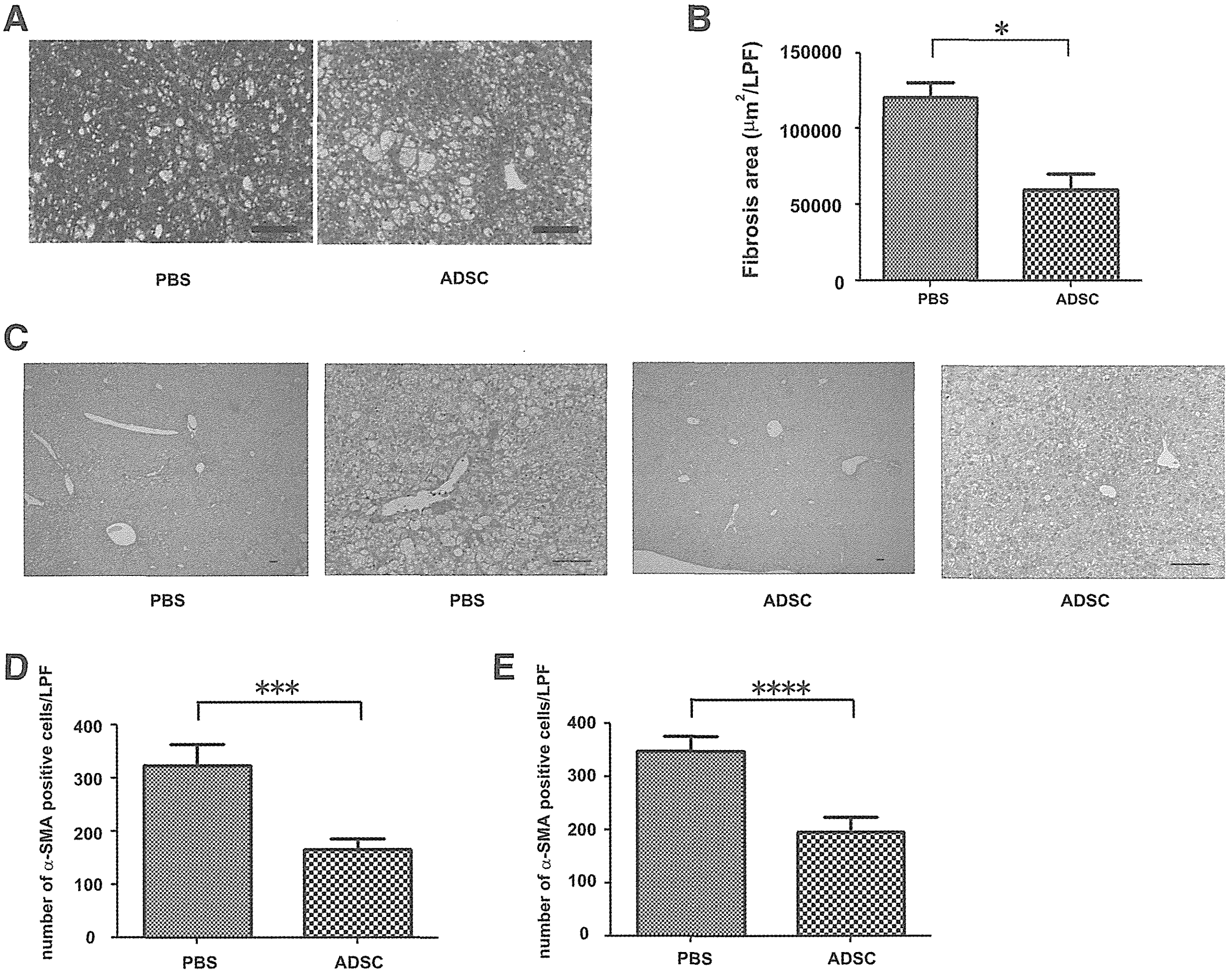

Fig. 5. Effect of ADSCs on liver fibrosis. ADSCs from GFP-Tg mice (1 × 10^5) were injected twice every 2 weeks into the splenic subcapsule of cirrhotic C57Bl/6 mice fed the Ath+HF diet for 32 weeks. Control mice received PBS injections. (A) Two weeks after the last injection, liver tissue was obtained, sectioned, and histologically examined with hematoxylin and eosin staining. (B) Fibrosis was examined by Azan staining and fibrotic area was quantified by image-analysis. (C) Immunohistochemical staining of liver sections for α-SMA. Scale bars = 100 µm. (D,E) The number of α-SMA+ cells in liver tissues obtained 1 (D) or 2 weeks (E) after the last ADSC injection determined by microscopy of five low-power fields (×100); ***$P < 0.005$, ****$P = 0.0001$.

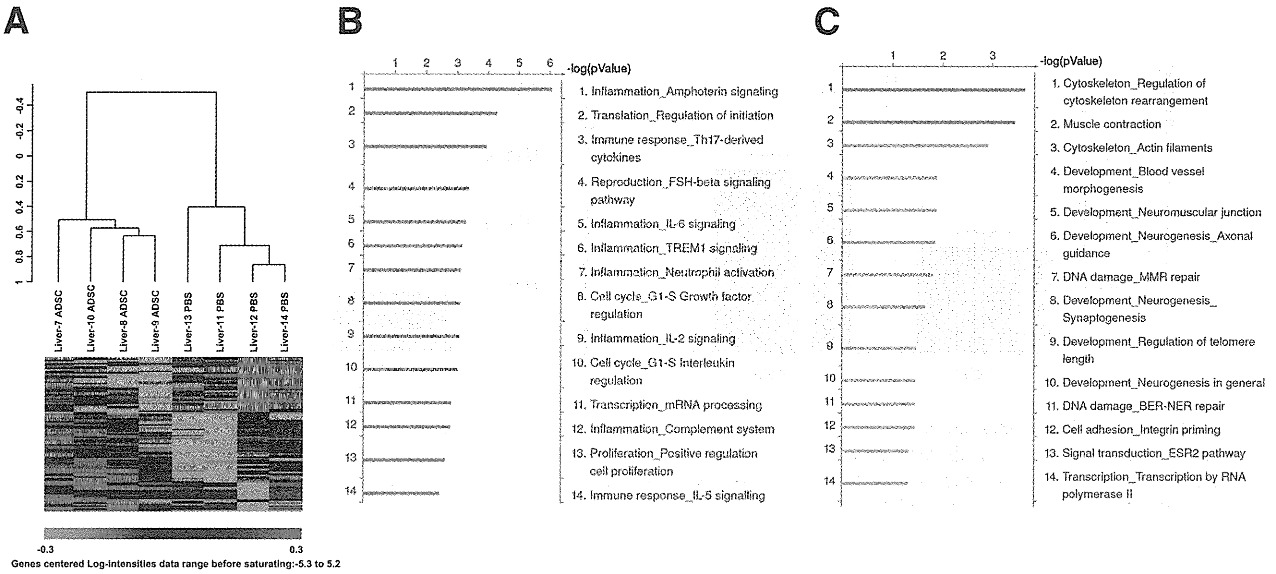

Fig. 6. Hepatic gene expression analysis. ADSCs from GFP-Tg mice (1×10^5) were injected twice every 2 weeks into the splenic subcapsule of cirrhotic C57Bl/6 mice fed an Ath+HF diet for 40 weeks. Control mice received PBS injections. Two weeks later, liver tissue was subjected to RNA isolation and gene expression using DNA microarrays. (A) Unsupervised clustering analysis was performed using probes for 1,249 genes whose expression differed significantly between the PBS and ADSC groups. (B) The biological processes of 452 genes whose expression was down-regulated in the ADSCs group compared to the PBS group were analyzed. (C) The biological processes of 797 genes whose expression was up-regulated in the ADSCs group compared to the PBS group were analyzed.

(Supporting Fig. S4). Biological process analysis indicated that the down-regulated genes were primarily related to inflammation and the immune response (Fig. 6B), and the up-regulated genes were related to tissue construction and development (Fig. 6C). Thus, gene expression analysis of liver tissue demonstrated that ADSCs treatment caused anti-inflammatory effects, as well as regeneration/repair effects, in the livers of a NASH mouse model of cirrhosis.

Anti-inflammatory Effects of ADSC Treatment. The fundamental underlying pathophysiology of steatohepatitis-induced cirrhosis is persistent hepatic inflammation caused by steatosis in hepatocytes.[16] We examined how ADSCs affected persistent inflammation of the liver in NASH mice at 2 weeks after the last injection of ADSCs. Immunohistochemical staining showed that the number of CD11b$^+$ cells accumulating in the livers of cirrhotic mice decreased with ADSC treatment compared to those of PBS-treated mice (Fig. 7A). The number of Gr-1$^+$ cells in cirrhotic liver also decreased with ADSC treatment (Fig. 7A), suggesting that ADSCs affect granulocytes and antigen-presenting cell lineage.

We further examined whether ADSC treatment affected the lymphocyte lineage of T cells, since they also play an important role in immune regulation of steatohepatitis.[17] We isolated lymphocytes from the livers of mice treated with ADSCs and examined the CD4$^+$ and CD8$^+$ T cells using flow cytometry. CD8$^+$ T cells were found predominantly in cirrhotic mice treated with PBS (Fig. 7B,C). However, when the mice were treated with ADSCs the number of CD4$^+$ T cells increased and was comparable to that of CD8$^+$ T cells, indicating that ADSC treatment affected T-cell subpopulations.

Gene Expression Profiling of Hepatic Inflammatory Cells Following ADSC Treatment. We further examined how injected ADSCs affected hepatic inflammatory cell gene expression by using DNA microarrays. By filtering the results from 5,065 gene probes, completely discernible clusters of gene expression were formed between ADSC- and PBS-treated animals (Fig. 8A). We identified the expression of 873 genes that were significantly up-regulated at least 2-fold with ADSC injection and 658 genes that were down-regulated. Most of the chemokines and cytokines whose expression was significantly affected by ADSCs were down-regulated (Supporting Table S1). Using the publicly available gene expression database for hematopoietic cells (GSE27787) and various types of helper T cells (GSE14308), we examined features of these affected genes in the context of immunomodulatory cells. Among the hematopoietic cells, genes with available symbol annotation were predominately Gr-1$^+$ and CD11b$^+$ cells from granulocyte and macrophage lineages (Fig. 8B). Among helper T-cell populations,

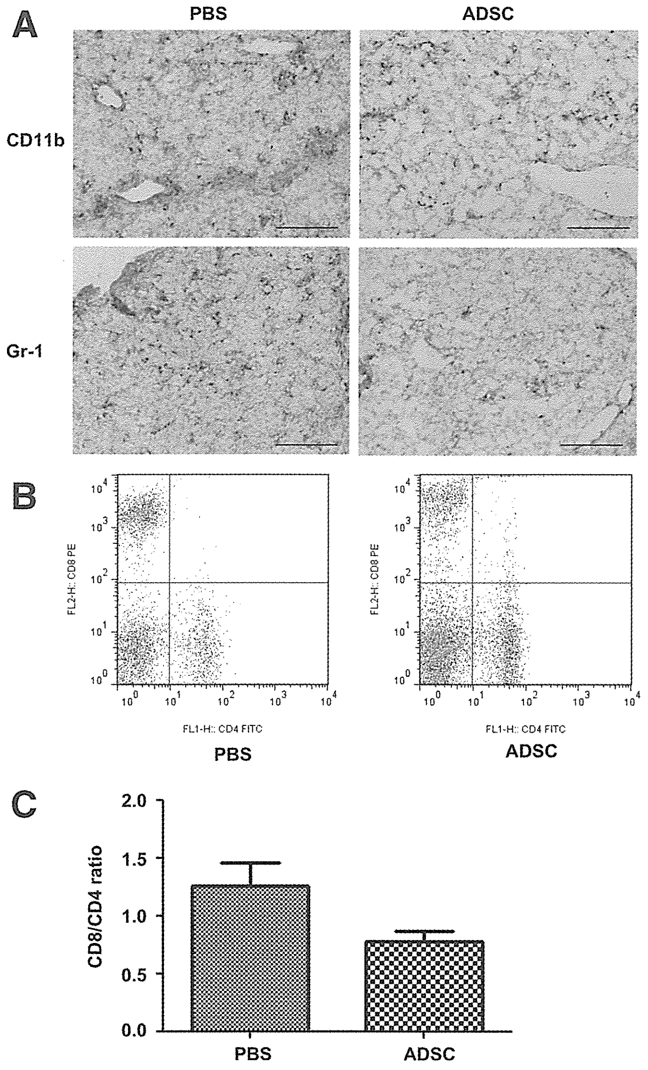

Fig. 7. Effect of ADSCs on inflammatory cells in the cirrhotic liver. ADSCs from GFP-Tg mice (1 × 10^5) were injected twice every 2 weeks into the splenic subcapsule of cirrhotic C57Bl/6 mice fed the Ath+HF diet for 32 weeks. Control mice received PBS injections. Two weeks later, liver tissue was obtained and immunohistochemical staining of (A) CD11b$^+$ and (B) Gr-1$^+$ cells was performed. Inflammatory cells in the liver were also isolated and stained with FITC-labeled anti-CD4 and PE-labeled CD8 antibodies. (C) The ratio of CD8$^+$ cells to CD4$^+$ cells was calculated. N = 4 ± standard error.

annotated genes included activated Th1, Th2, and Th17 cell types (Fig. 8C). We also isolated CD4$^+$T cells from hepatic inflammatory cells obtained from NASH mice fed an Ath+HF diet for 12 weeks, then treated with ADSC. Expressions of the Th1, Th2, and Th17 cytokines, interferon-γ, interleukin (IL)−4, IL-10, and IL-17, the Th17-related cytokine transforming growth factor beta (TGF-β), and Foxp3, a representative transcription factor of regulatory T cells, were down-regulated by ADSC treatment (Supporting Fig. S5).

These results suggest that ADSC treatment suppresses inflammation in the NASH mouse model primarily by down-regulating granulocytes, antigen-presenting cells, and activated helper T cells.

Discussion

This study investigated the therapeutic effect of ADSCs in a NASH murine model of cirrhosis. This model is relevant to clinical NASH, with similar pathological features established by an atherogenic high-fat diet, including the appearance of steatosis, ballooning, and Mallory-Denk bodies in hepatocytes, infiltration of inflammatory cells, and pericellular fibrosis. Our results demonstrate that ADSC injection is therapeutically beneficial for cirrhosis in this murine model through restoration of albumin expression in hepatic parenchymal cells, amelioration of fibrosis, and suppression of persistent hepatic inflammation.

Gene expression analysis of the liver in this cirrhotic mouse model revealed that ADSC injection affects biological processes relating to anti-inflammatory and regeneration/repair pathways. The anti-inflammatory effects are mediated by ADSC targeting of Gr-1$^+$, CD11b$^+$, and helper T-cell lineages. In patients with clinical NASH, the ratio of neutrophils to lymphocytes increases,[18] suggesting that granulocytes are involved in the pathogenesis of NASH. The NASH murine model used in this study produced an increased CD8$^+$/CD4$^+$ T-cell ratio, which is also comparable to clinical NASH patient pathology.[19] Gene expression analysis of liver tissue and hepatic inflammatory cells from NASH mice showed that Th1-, Th2-, and Th17-related genes were down-regulated by ADSC treatment. Helper T-cell activation skewed to produce Th1 cytokines is pathogenic in steatohepatitis.[20,21] In particular, Th17 is emerging as an important source of IL-17 family cytokines[22] and is involved in the hepatic inflammation in NASH.[23] Helper T cells producing Th2 cytokines such as IL-4, 5, and 13 contribute to fibrosis.[24] We conclude that activated T helper cells are responsible for the pathogenesis of steatohepatitis in the NASH murine model used in this study and that ADSCs suppress pathogenic helper T-cell activation. However, the suppression of miscellaneous effector and regulatory helper T cells by ADSCs should be further evaluated with regard to prevention of hepatocellular carcinoma, a frequent sequela to cirrhosis, since Th1 promotes antitumor immunity and Th2 down-regulates antitumor immunity.

We also observed that ADSC treatment ameliorated fibrosis and decreased the number of α-SMA$^+$ stellate

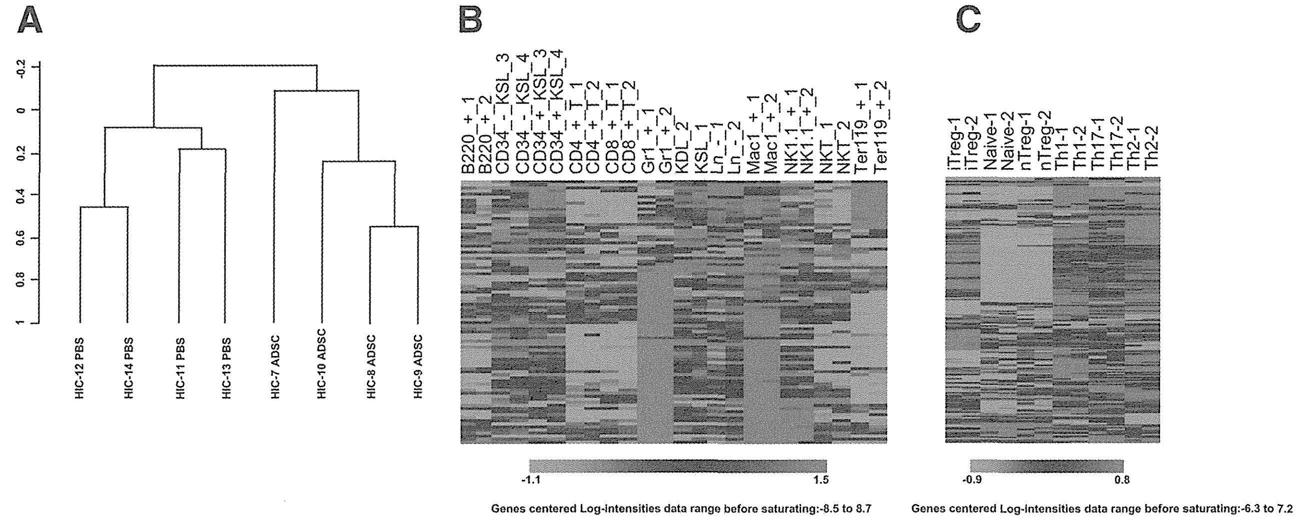

Fig. 8. Gene-expression analysis of intrahepatic inflammatory cells. ADSCs from GFP-Tg mice (1 × 10^5) were injected twice every 2 weeks into the splenic subcapsule of cirrhotic C57Bl/6 mice fed an Ath+HF diet for 40 weeks. Control mice received PBS injections. Inflammatory cells were isolated from the liver and gene expression examination was performed using DNA microarrays. (A) Unsupervised clustering analysis using the filtered 5,065 gene probes. HIC; hepatic inflammatory cells. (B) One-way clustering analysis using a publicly available database of hematopoietic cells (GSE27787) of 658 genes whose expression was down-regulated by ADSC treatment with available gene symbol annotations. (C) One-way clustering analysis using publicly available database of different helper T subsets (GSE14308) of 658 genes whose expression was down-regulated by ADSCs treatment with available gene symbol annotations.

cells in cirrhotic liver. When inflammation persists in the liver, fibrosis progresses due to these activated stellate cells, which are almost identical to myofibroblasts and produce extracellular matrix. Stellate cells are activated by miscellaneous factors including TGF-β and platelet-derived growth factor,[25] produced mostly from Kupffer cells. Helper T cells expressing Th2 cytokines are also involved in the development of fibrosis. Gene expression analysis of the cirrhotic livers indicated that ADSC treatment suppressed Th2-type helper T cells. Although details of how these molecules mediate fibrosis development have yet to be examined in the current NASH murine model, the antifibrotic effect of ADSCs is achieved in part by suppressing Th2-type helper T cells. We found that MMP-8 and MMP-9 enhancement in the NASH-cirrhotic liver was ameliorated by ADSC treatment. MMP-9 expression is related to the inflammation typical of steatohepatitis[26] and can ameliorate the hepatic fibrosis induced by carbon tetrachloride.[27] Further studies are needed to clarify the role of MMPs in the pathogenesis of cirrhosis as well as to explore novel therapies for this condition.

Pluripotent MSCs differentiate into several cell lineages and are a promising avenue for regenerative therapy of various impaired organs, including the liver. Although ADSCs were observed in cirrhotic livers at up to 2 weeks after injection and expressed albumin, the numbers of resident cells were not sufficient to supplement hepatic function. Therefore, pluripotency, as well as the anti-inflammatory and antifibrotic effects of ADSCs, are important for their regenerative/repair effects in liver cirrhosis. Rather than studying the effects of ADSCs on early-stage steatohepatitis, we treated mice with endstage cirrhosis with ADSCs to observe their therapeutic effects. Our results demonstrated that ADSCs can effectively resolve chronic fibrosis and decrease inflammation, thereby restoring hepatic function in endstage cirrhotic mice, implying the usefulness of this therapy as an alternative to liver transplantation.

In conclusion, ADSCs proved therapeutically beneficial and clinically relevant in regenerative therapy of a murine steatohepatitis-cirrhosis model. Clinical application of ADSCs in the treatment of cirrhosis is expected to provide a novel alternative regenerative/repair therapy for patients with cirrhosis.

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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

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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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*These authors contributed equally to this study.

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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.

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 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.

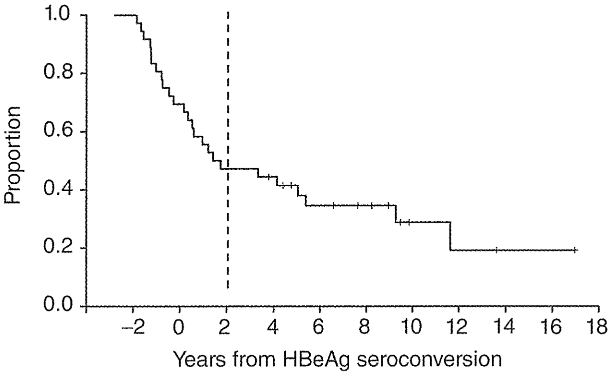

Figure 1 Changes in the proportion of patients with alanine aminotransferase (ALT) abnormality. ALT normalization was defined as ALT level decreasing to lower than 31 IU/L and maintained for at least 1 year. These data reveal two distinct time frames: a fast decline phase around the seroconversion (SC) 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.

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

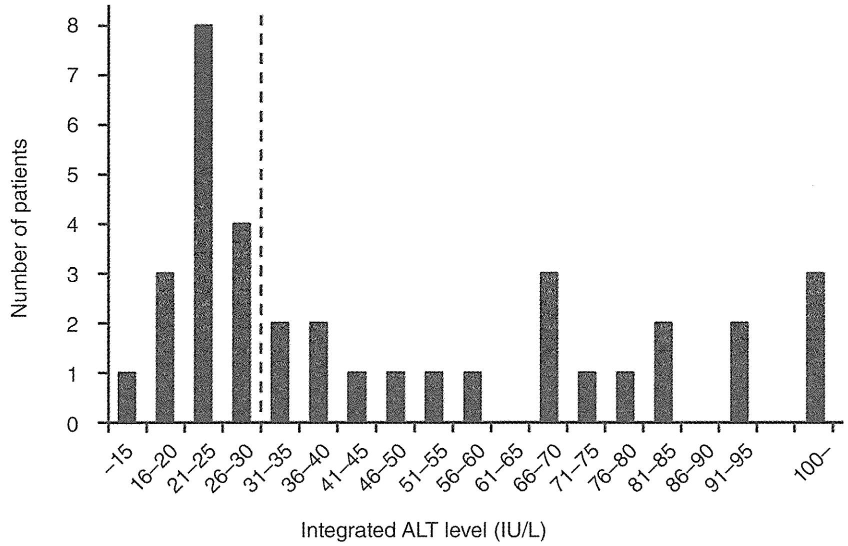

Figure 2 Distribution of integrated alanine aminotransferase (ALT) level from the time point of 2 years after seroconversion (SC) to the end of follow up.

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.

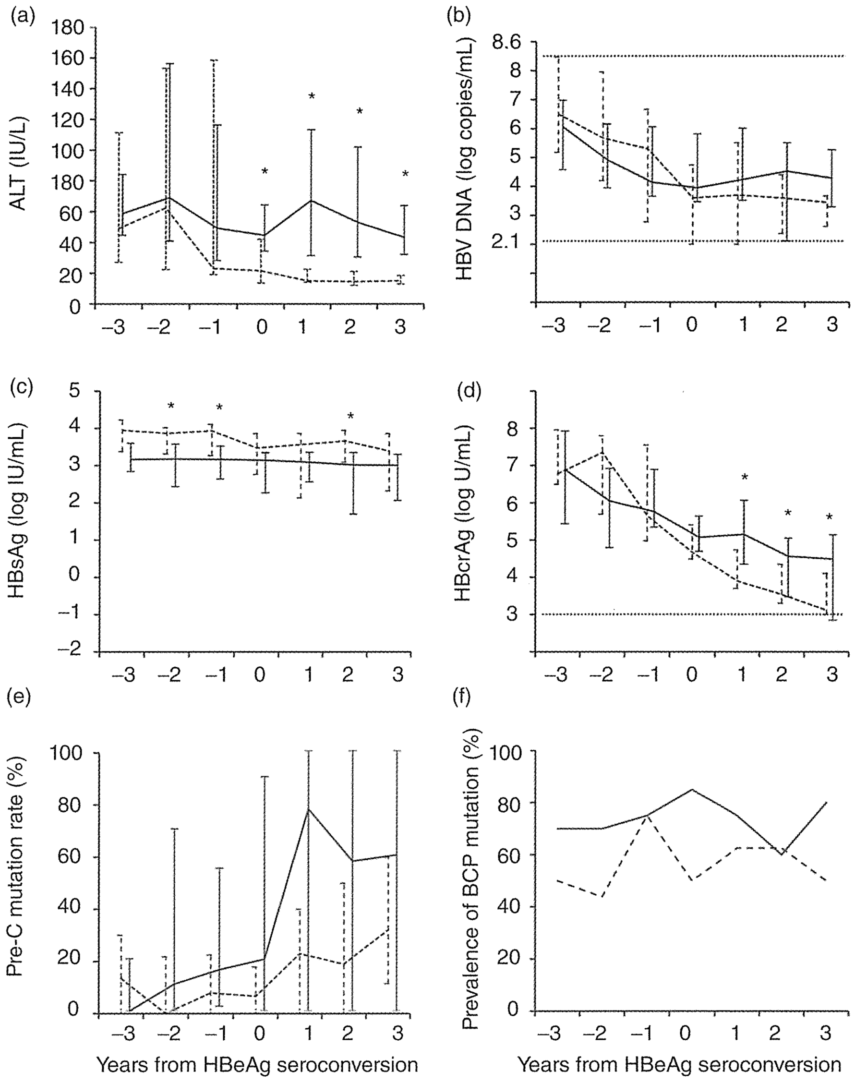

Figure 3 Changes in median levels of serum alanine aminotransferase (ALT) (a), hepatitis B virus (HBV) DNA (b), hepatitis B surface antigen (HBsAg) (c), hepatitis B core-related antigen (HBcrAg) (d) and PC mutation rate (e) are compared between patients with and without the occurrence of hepatitis B 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.

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.

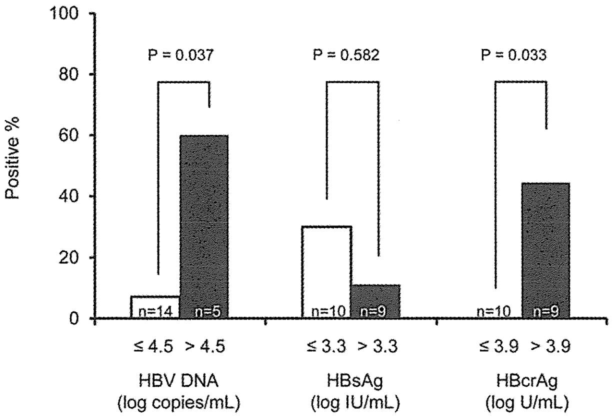

Figure 4 Occurrence of hepatitis B e-antigen (HBeAg) negative hepatitis is compared among patients using higher and lower levels of corresponding markers at 2 years after seroconversion (SC). The cut-off value for each marker was determined by receiver–operator curve analysis. HBcrAg, hepatitis B core-related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

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

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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