HuS-E/2 細胞を HBV 陽性患者血清で処理することで HBV 感染を検討したが、HBV コアタンパク質に対する抗体を用いた間接蛍光抗体法では HBV 感染細胞を検出することができなかった。

2. HuS-E/2 細胞では HBV 受容体分子として報告された NTCP の mRNA 発現量は低レベルであり、2%DMSO を含む培養液を用いて前培養した場合でもその発現量は低かった。

3. HMY1 細胞における AFP 発現は極低レベルであるが、FGF4 および BMP2 を添加して培養することにより 5 日間培養することにより AFP の発現が誘導されることがわかった。

4. HMY1 細胞を無血清肝細胞用培地で培養するとそれまで全く検出できなかったアルブミン mRNA の発現が誘導されるが、その誘導は通常の平面培養に比較して温度感受性ゲル化ポリマーを用いた立体培養では著しく早期に誘導を観察することが可能であった。

5. HYM1 と一ヶ月間無血清肝細胞用培地で培養した HYM1 細胞から総 RNA を抽出し NTCP mRNA の発現量を比較したが、HYM1 においても低いレベルで発現が認められたが、アルブミン mRNA 発現が誘導されている後者では NTCP mRNA 量が高く上昇していることがわかった。

D. 考察

1. HuS-E/2 細胞は HBV 感染増殖能が報告されているが、今回我々の実験条件ではその感染は認められなかった。原因の一つは感染源である HBV が報告では組換え体を用いているが、今回我々は患者血清を用いていることが考えられる。今後はさらに培養条件を検討し、また組換え体 HBV を用いて感染実験をおこなう必要があると考えられた。

2. HYM1 細胞は培養条件次第で肝細胞へと効率良く分化させることが可能であると考えられた。また分化誘導した HYM1 細胞では高い NTCP mRNA の発現が検出されたことから、この細胞は HBV に対して高い感受性を示す可能性が考えられた。

E. 結論

今後、HuS-E/2 細胞に関しては NTCP の発現量を指標として至適な培養条件を検討する必要があると考えられた。また HYM1 細胞に関しては培養条件を検討することで、HBV 感染増殖に至適な培養条件を見出す事が可能になることが考えられた。

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2. 学会発表

なし

G. 知的所得権の所得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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分担研究報告書（平成24年度）

Ｂ型肝炎ウイルスの持続感染を再現する効率的な培養細胞評価系の開発に関する研究

分担研究者：渡士　幸一　国立感染症研究所　ウイルス第二部　主任研究官

分担研究課題：生理活性物質とHBV複製増殖との関連解析

研究要旨：抗HBV作用をもつ低分子化合物あるいは生理活性物質のスクリーニングに適した細胞株を得るために、本研究では既存のHBV複製細胞株から単一細胞をクローニングした。HBVが恒常的に複製しているHepG2.2.15細胞からHepG2.2.15.7細胞を、またテトラサイクリン除去によりHBV複製を誘導できるHepAD38細胞からHep38.7-Tet細胞を得た。親株に比較してHepG2.2.15.7細胞はHBs放出が高く、Hep38.7-Tet細胞はHBV DNAおよびcccDNA発現が上昇していた。また両者は核酸アナログに対する感受性が親株よりも高かった。このようにこれらの細胞株は抗HBV剤探索に有用なツールになりうると考えられた。実際に小規模な化合物スクリーニングをおこなったところ、抗HBV作用を有すると考えられる低分子化合物が同定できた。

A.　研究目的

これまで HBV 複製を再現する実験系として、HBV が恒常的に複製している HepG2.2.15 細胞、あるいはテトラサイクリン除去により HBV 複製を誘導できる HepAD38 細胞などが知られている。しかしながら、抗 HBV 剤の大規模スクリーニングを簡便におこなうには、さらに HBV DNA 産生が高く、また薬剤感受性が高い細胞株を得ることが望ましい。本研究においては、これらの細胞から単一細胞クローニングにより、より抗 HBV 剤スクリーニングに適した細胞株を選別することを目的とした。

B.　研究方法

HepG2.2.15 細胞、HepAD38 細胞を 96 ウェルプレートに 1 ウェルあたり 1/10 細胞になるように播種し、これを長期培養する。3 日に 1 回培地交換し、単一コロニーを形成したウェルから細胞をスケールアップし、各細胞クローンを得た。得られたそれぞれの細胞株から HBV DNA を抽出し、シークエンスした。また各細胞株の培地中 HBs タンパク質および HBV DNA 量、細胞内 HBV DNA および cccDNA を 2, 4, 6, 9 日後と経時的に ELISA およびリアルタイム PCR 法により定量した。

また HepG2.2.15, HepG2.2.15.7, HepAD38, Hep38.7-Tet 細胞にラミブジン、エンテカビル、17AAG、カフェイン酸をさまざまな濃度で 4 日および 6 日間処理し、培地中 HBV DNA 量を定量した。また細胞毒性は MTT アッセイで調べた。

さらに約 20 種類の低分子化合物を Hep38.7-Tet および HepG2.2.15.7 細胞に 6 日間処理した後の培地中 HBV DNA を定量することにより、各化合物の HBV 複製に対する効果を検討した。

C. 研究結果

1）単一細胞クローニング

HepG2.2.15 細胞および HepAD38 細胞から単一コロニーを形成した複数の細胞クローンが得られた。これらのうち長期的に増殖し、また細胞凍結緩衝液中で凍結後生存したものとして、HepG2.2.15.7 および Hep38.2-Tet, Hep38.3-Tet, Hep38.7-Tet 細胞が得られた。

2）各細胞クローンの性状解析

各細胞クローン内 HBV DNA をシークエンスしたところ、すべてのクローン株は親株と同じ DNA 配列を有していた。

各細胞クローンの培地中 HBs タンパク質および HBV DNA 量、細胞内 HBV DNA および cccDNA を経時的に測定したところ（図１）、HepG2.2.15.7 細胞から放出された HBs は親株である HepG2.2.15 細胞の約 10 倍であった。また HepAD38 クローン細胞の中では Hep38.7-Tet 細胞における HBV DNA および cccDNA が親株に比較して約 4-10 倍程度高値であった。

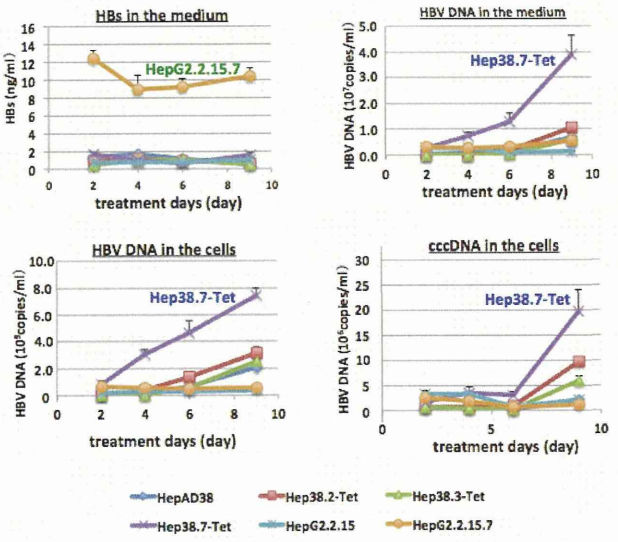

図１　各細胞株における、培地に放出された HBs タンパク質、HBV DNA および細胞内 HBV DNA、cccDNA 量

HBs タンパク質放出は HepG2.2.15.7 細胞で高く、HBV DNA あるいは cccDNA 発現は Hep38.7-Tet 細胞で高かった。

3）既知抗 HBV 剤に対する感受性

HepG2.2.15.7 および Hep38.7-Tet 細胞とそれぞれの親株での既知抗 HBV 剤に対する感受性を比較した。核酸アナログであるラミブジン、エンテカビルに対しては、いずれも HepG2.2.15.7 および Hep38.7-Tet 細胞の方が親株よりも高い感受性を示した。すでにその抗 HBV 効果が報告されている非核酸アナログであるカフェイン酸に対する感受性も同様であった。17AAG 感受性は、HepG2.2.15.7 では HepG2.2.15 細胞よりやはり高かった一方で、Hep38.7-Tet および HepAD38 細胞では細胞毒性を発揮したため、比較が難しかった。

4）低分子化合物のスクリーニング

Hep38.7-Tet 細胞は HBV DNA 産生が高く、また既知抗 HBV 剤に対する感受性が高い傾向にあったため、これを小規模の低分子化合物のスクリーニングに用いた。化合物処理後の培地中 HBV DNA をリアルタイム PCR 法で定量した。約 20 種類の化合物の中で、臨床市販薬として用いられているものとして lovastatin、MPA が HBV DNA 産生を低下させた。このうち少なくとも MPA は southern blot 法においても細胞内不完全二重鎖 DNA 産生量を低下させていた。

D. 考察

本研究で得られた細胞クローンの中には親株と比較して HBs 産生あるいは HBV DNA 発現等で異なる性状を有しているものが見られた。HBV DNA 配列に変異が見られなかったことから、これらの株間での性状の違いは、細胞内環境の違いによるものである可能性が考えられた。また HepG2.2.15.7 および Hep38.7-Tet 細胞では親株とは異なる核酸アナログ感受性が観察されたが、これは薬剤感受性に重要なポリメラーゼの発現量や翻訳後修飾、あるいはその機能を制御する

HSP90 など宿主因子の発現や活性が異なるためである可能性が考えられた。

E. 結論

以上のように本研究では、恒常的に HBV 複製がおこなわれている、あるいはテトラサイクリン除去により HBV 複製を誘導できる新たな細胞クローンを樹立した。このうち HepG2.2.15.7 細胞は高い HBs タンパク質放出を示したため、HBs 動態、代謝研究に有用であると考えられる。一方で Hep38.7-Tet 細胞は親株よりも HBV DNA および cccDNA 発現が高く、また核酸アナログに対して高感受性であったため、抗 HBV 剤スクリーニングに有用であると期待された。Hep38.7-Tet 細胞を用いた小規模の低分子化合物スクリーニングではいくつかの化合物が HBV DNA 量を低下させるものとして得られた。この細胞は今後より大規模な抗 HBV 剤の探索に有用であると期待される。

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G. 知的所得権の所得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

III. 研究成果の刊行一覧

研究成果の刊行に関する一覧表

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＊ Ⅳ. 研究成果の別冊あり

IV. 研究成果の刊行物・別冊

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Sequential immunological analysis of HBV/HCV co-infected patients during Peg-IFN/RBV therapy

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Abstract
Background The immunopathogenesis of dual chronic infection with hepatitis B virus and hepatitis C virus (HBV/HCV) remains unclear. The in vivo suppressive effects of each virus on the other have been reported. In this study we aimed to analyze the virological and immunological parameters of HBV/HCV coinfected patients during pegylated interferon/ribavirin (Peg-IFN/RBV) therapy.
Methods One patient with high HBV-DNA and high HCV-RNA titers (HBV-high/HCV-high) and 5 patients with low HBV-DNA and high HCV-RNA titers (HBV-low/HCV-high) were enrolled. Twenty patients monoinfected with HBV and 10 patients monoinfected with HCV were enrolled as control subjects.. In vitro cultures of Huh 7 cells with HBV/HCV dual infection were used to analyze the direct interaction of HBV/HCV.
Results Direct interaction of HBV clones and HCV could not be detected in the Huh-7 cells. In the HBV-high/HCV-high-patient, the HCV-RNA level gradually declined and HBV-DNA gradually increased during Peg-IFN/RBV therapy. Activated CD4- and CD8-positive T cells were increased at 1 month of Peg-IFN/RBV-therapy, but HBV-specific IFN-γ-secreting cells were not increased and HBV-specific interleukin (IL)-10 secreting cells were increased. The level of HBV- and HCV-specific IFN-γ-secreting cells in the HBV-high/HCV-high-patient was low in comparison to that in the HBV- or HCV-monoinfected patients. In the HBV-low/HCV-high-patient, HCV-RNA and HBV-DNA rapidly declined during Peg-IFN/RBV therapy. Activated CD4- and CD8-positive T cells were increased, and HBV- and HCV-specific IFN-γ-secreting cells were also increased during Peg-IFN/RBV-therapy.
Conclusion The immunological responses of the HBV-high/HCV-high patient were low in comparison to the responses in HBV and HCV monoinfected patients. Moreover, the response of immune cells in the HBV-high/HCV-high patient during Peg-IFN/RBV therapy was insufficient to suppress HBV and HCV.

Keywords Dual infection · HBV · HCV · Immunopathogenesis

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Introduction

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are noncytopathic viruses that cause chronic hepatitis and hepatocellular carcinoma (HCC) [1, 2]. HBV now affects more than 400 million people worldwide, and persistent infection develops in ~5 % of adults and 95 % of neonates who become infected with HBV [3]. HCV infects about 170 million people worldwide and is a major cause of chronic hepatitis, cirrhosis, and HCC [4]. Some groups have mentioned that dual infection with HBV/HCV is not uncommon in Asian patients [5, 6]. The prevalence of patients with dual HBV/HCV infection is approximately 10–15 %, although it likely differs among countries [7–9]. Co-infection with HBV/HCV has been associated with severe liver disease and frequent progression to cirrhosis [10]. Moreover, a significantly higher incidence of HCC and liver-related mortality was noted in patients with HBV/HCV co-infection [11, 12]. However, some groups reported, based on a meta-analysis, that dual infection with HBV/HCV did not increase the risk of HCC [13, 14]. These contradictory reports could be explained by the rarity of dual infection with HBV/HCV in patients without clinically evident liver disease. It might be difficult to estimate the hepatocarcinogenic risk of dual infection compared with that of either infection alone in such clinical settings [15].

An inverse relationship in the replicative levels of the two viruses has been noted, suggesting direct or indirect effects in vivo [16]. More recently, some groups have reported, using an in vitro infection system, that there is little direct interaction of HBV/HCV in coinfected hepatocytes [17, 18]. Therefore, the viral interference observed in coinfected patients is probably due to indirect mechanisms mediated by the innate and/or adaptive host immune responses.

The cellular immune response to HBV and HCV plays an important role in the pathogenesis of chronic hepatitis, cirrhosis, and HCC [19–21]. Hyporesponsiveness of HBV- or HCV-specific T-helper 1 cells and excessive regulatory function of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) in peripheral blood have been shown in patients with chronic hepatitis B and C [22–34]. Recently, we reported that HBV replication stress could enhance the suppressive activity of Tregs via TLR2 [35]. However, little is known about the immunopathogenesis of HBV/HCV dual infection.

Dual infection can be classified into several groups (e.g., group A: HBV active and HCV active; group B: HBV inactive and HCV active; and group C: HBV active and HCV inactive) [36]. HCV is reported to be the dominant virus in HBV/HCV dual infection, but the dominance of either virus might be due to the genotypes of each virus and/or ethnic differences that could affect the proliferative activity of the viruses [36]. In this study, we investigated immunopathogenesis in a group A patient and in group B patients during therapy with pegylated interferon-α2b (Peg-IFN-α2b) plus ribavirin.

Patients, materials, and methods

Patients

One patient with high HBV-DNA and high HCV-RNA titers (HBV-high/HCV-high; patient A) and 5 patients with low HBV-DNA and high HCV-RNA titers (HBV-low/HCV-high) were enrolled (one of these patients, whose results were analyzed in detail, was termed patient B; see findings below in the "Results"). Twenty patients monoinfected with HBV and 10 patients monoinfected with HCV were enrolled as control subjects. None of the patients had liver disease due to other causes, such as alcohol, drugs, congestive heart failure, or autoimmune diseases. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006-194). Written informed consent was obtained from all the participants enrolled in this study. Participants were monitored for two years. At each assessment, patients were evaluated by biochemical laboratory tests, immunological analysis, and virological tests. Liver histology was analyzed at the start of Peg-IFN/RBV therapy by using laparoscopic liver biopsy samples and by employment of the METAVIR score.

Detection of interleukin (IL)-28B polymorphism

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using an automated DNA isolation kit. Then polymorphism of IL-28B (rs8099917) was analyzed using real-time polymerase chain reaction (PCR) (TaqMan SNP Genotyping Assay, Applied Biosystems, CA, USA). Detection of the IL-28B polymorphism was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-323).

Isolation of peripheral blood mononuclear cells (PBMCs) and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation (Amersham Bioscience, Uppsala, Sweden). PBMCs were stained with CD3, CD4, CD8, CD19, CD25, CD40, CD56, CD86, HLA-DR, NKG2D, and isotype control antibodies (Becton Dickinson, NJ, USA) for 15 min on ice to analyze the frequency

of $CD3^+CD4^+HLA\text{-}DR^+$ cells, $CD3^+CD8^+HLA\text{-}DR^+$cells, $CD4^+CD25^+$ Tregs, $CD3^-CD16^-CD56^{high}$natural killer (NK) cells, and $CD3^-CD16^+CD56^{dim}$ NK cells. The frequencies of the immune subsets were analyzed by flow cytometry using FACS Canto-II (Becton Dickinson, NJ, USA).

ELISPOT assay

The detection of IFN-γ and IL-10 was performed using an ELISPOT Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cultures of PBMCs were established in triplicate on round-bottomed 96-well plates for all time points investigated, at a concentration of 3×10^5 cells per well in 100 µl RPMI 1640 containing 10 % fetal bovine serum (FBS). Positive spots were detected using an automated counting machine.

Detection of HBV-DNA and determination of HBV genotype

DNA was extracted from 100 µl of serum using SMITEST EX-R&D (Medical & Biological Laboratories, Nagoya, Japan) and dissolved in 20 µl of nuclease-free distilled water. The DNA preparation thus obtained (10 µl) was subjected to nested PCR with primers targeting the S gene of the HBV-DNA, as described previously [37]. Briefly, first-round PCR was carried out for 35 cycles (98 °C for 10 s, 55 °C for 15 s, and 72 °C for 1 min, with an additional 7 min in the last cycle) in the presence of PrimeSTAR HS DNA Polymerase (TaKaRa Bio, Shiga, Japan) and primers HB095 (sense, 5′-GAG TCT AGA CTC GTG GTG GAC-3′) and HB184 (antisense, 5′-CGA ACC ACT GAA CAA ATG GCA CCG-3′), for 25 cycles. This was followed by a second-round PCR consisting of 25 cycles using the same conditions as in the first round, with primers HB097 (sense, 5′-GAC TCG TGG TGG ACT TCT CTC-3′) and S2-2 (antisense, 5′-GGC ACT AGT AAA CTG AGC CA-3′). The HBV genotype was determined by phylogenetic analysis of the S gene sequence (437 nt) of the HBV isolates.

Detection of HCV RNA

RNAs were extracted from 250 µl of serum using TRIzol LS (Invitrogen, Tokyo, Japan). They were divided into two aliquots and each was assayed by reverse transcription (RT)-PCR with nested primers derived from the core region and NS5A interferon sensitivity determining region (ISDR) of the HCV genome. Nested PCR of the core region of the HCV genome was carried out with primers C008 (sense, 5′-AAC CTC AAA GAA AAA CCA AAC G-3′) and C011 (antisense, 5′-CAT GGG GTA CAT YCC GCT YG-3′) in the first round and C009 (sense, 5′-CCA CAG GAC GTY AAG TTC CC-3′) and C010 (antisense, 5′-AGG GTA TCG ATG ACC TTA CC-3′) in the second round. Nested primers that were derived from NS5A-ISDR of the HCV genomes were designed to amplify a 188-bp product with C004 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and C005 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) in the first round, and C006 (sense, 5′-ACC GG A TGT GGC AGT GCT CA-3′) and C007 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) in the second round.

Analysis of nucleotide and amino acid sequences

The PCR products were sequenced directly on both strands using a BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Genetyx-Mac ver. 12.2.6 (Genetyx, Tokyo, Japan) and ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) [38]. Sequence alignments were generated using CLUSTAL W (Version 1.8) [39]. The phylogenetic tree was constructed by the neighbor-joining method [40]. The reliability of the phylogenetic results was assessed using 1000 bootstrap replicants [41]. The final tree was obtained with the Njplot program (version 2.2) [42].

Plasmid construction

HBV expression plasmids were constructed by previously published methods. Serum samples were obtained from two patients infected with HBV genotype Bj and two patients infected with HBV genotype C. HBV-DNA was extracted from 100 µl serum using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into a pGEM-T Easy Vector (Promega, Madison, WI, USA) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). Briefly, at least 5 clones of each fragment were sequenced and the consensus sequence was identified and used as a template for 1.24-fold the HBV genome of different genotypes (B1 indicates the genotype Bj35 clone; B2 indicates the genotype Bj56 clone; C1 indicates the genotype C-AT clone; and C2 indicates the genotype C-22 clone). The HCV-JFH-1 strain was provided by Dr. T. Wakita (National Institute of Infectious Diseases, Japan).

HCV and HBV expression in Huh 7 cells

Cell-culture-derived infectious HCV was generated as described previously [43]. The HCV was quantified as

follows: RNA was extracted from the Huh-7 culture supernatant using a QIAamp Viral RNA Kit (Qiagen, Valencia, CA, USA). The HCV RNA was quantified by real-time RT-PCR, using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems) according to the manufacturer's protocol, using the published primers and probe [44]. The filtered (0.45 μm) culture supernatant of HCV-infected Huh-7 cells containing 2×10^8 HCV RNA copies/ml [equivalent to 9.7×10^4 focus-forming units (ffu)/ml] was used for the experiments. To analyze HCV-RNA in the supernatant, Huh-7 cells (2×10^5 cells in a 6-well plate) were infected with JFH-1 (multiplicity of infection [MOI] = 0.01) and after 4 h the cells were washed twice with phosphate-buffered saline (PBS). The supernatants were then collected and the cells were reseeded at 2×10^5 cells per 6-well plate. Then the HBV expression and mock plasmid were transfected by FuGENE6 (Roche Applied Science, IN, USA). The supernatant of the culture medium was collected 72 h after transfection. Quantification of HBV-DNA and HCV-RNA was carried out using real-time PCR.

IFN-α was added 24 h after the transfection of the HBV plasmids, and the supernatant of the culture medium was then collected 48 h after the addition of the IFN-α.

Results

Clinical characteristics of patients A and B

Patient A (high HBV-DNA titer and high HCV-RNA titer)

Patient A was a 44 year-old man with a high aspartate aminotransferase/alanine aminotransferase (AST/ALT) level. The prothrombin time-international normalized ratio (PT-INR) was in the normal range. Patient A had high HBV-DNA titers and high HCV-RNA titers (Table 1). His liver histology was classified as A2/F3 (Fig. 1). The laparoscopic analysis indicated moderate inflammation and intermediate fibrosis. The liver surfaces of the right lobe and left lobe were almost the same phenotype. Polymorphism of IL-28B (rs8099917) was T/G (hetero allele).

Patient B (low HBV-DNA titer and high HCV-RNA titer)

Patient B was a 63 year-old man with a low AST/ALT level. PT-INR was in the normal range. Patient B had low HBV-DNA titers and high HCV-RNA titers. The liver histology was classified as A2/F1 (Fig. 1). The liver surface showed moderate inflammation and was smooth. The polymorphism of IL-28B (rs8099917) was T/T (major homo allele).

Biopsy samples from patients with dual HBV and HCV infection were collected at the main liver centers in Miyagi prefecture. Fifteen HBV/HCV dual-infected patients were found in this study (Supplementary Table 1). Many of these patients had HCV-dominant infection and undetectable levels of HBV replication (10/15 patients). Most of the patients were HB envelope antigen (eAg)-negative and HBe antibody (Ab)-positive (14/14 patients). All HBV/HCV dual-infected patients who had received Peg-IFN-based

Table 1 Background of HBV/HCV dual-infected patients

	Patient A HCV high titer/ HBV high titer	Patient B HCV high titer/ HBV low titer	Normal range
Gender	Male	Male	
Age (years)	44	63	
HCV-RNA	6.5	5.5	log copies/ml
HCV genotype	1b	1b	
HBV-DNA	5.5	3.5	log copies/ml
HBV genotype	C	Bj	
HBe-Ag	129.5	0.5	0–0.9 index
HBe-Ab	0.1	99.3	0–49 %
Total bilirubin	0.7	1.2	0.2–1.2 mg/dl
Direct bilirubin	0.1	0.1	0–0.3 mg/dl
γ-GTP	208	31	8–57 IU/l
AST	138	33	12–30 IU/l
ALT	256	38	8–35 IU/l
Hb-A1c	5.3	5.4	4.3–5.8 %
Glu	103	83	68–106 mg/dl
BMI	25.34	18.75	
T-cho	160	195	128–220 mg/dl
LDL-cho	69	93	70–139 mg/dl
HDL-cho	37	67	36–89 md/dl
WBC	7800	5100	3200–9600/μl
RBC	491	446	428–566 × 10^4/μl
Hb	17.1	14.1	13.6–17.4 g/dl
PLT	169000	176000	155000–347000/μl
PT-INR	0.87	0.96	0–1.15 INR
Liver histology	A2/F3	A2/F1	METAVIR score
IL-28B SNP (rs8099917)	T/G	T/T	

HCV hepatitis C virus, *HBV* hepatitis B virus, e-*Ag* envelope antigen, *e-Ab* envelope antibody, *γ-GTP* γ-guanosine triphosphate, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Hb* hemoglobin, *Glu* glucose, *BMI* body mass index, *T-cho* total cholesterol, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *PLT* platelets, *PT-INR* prothromin time-international normalized ratio, *IL* interleukin, *SNP* single-nucleotide polymorphism

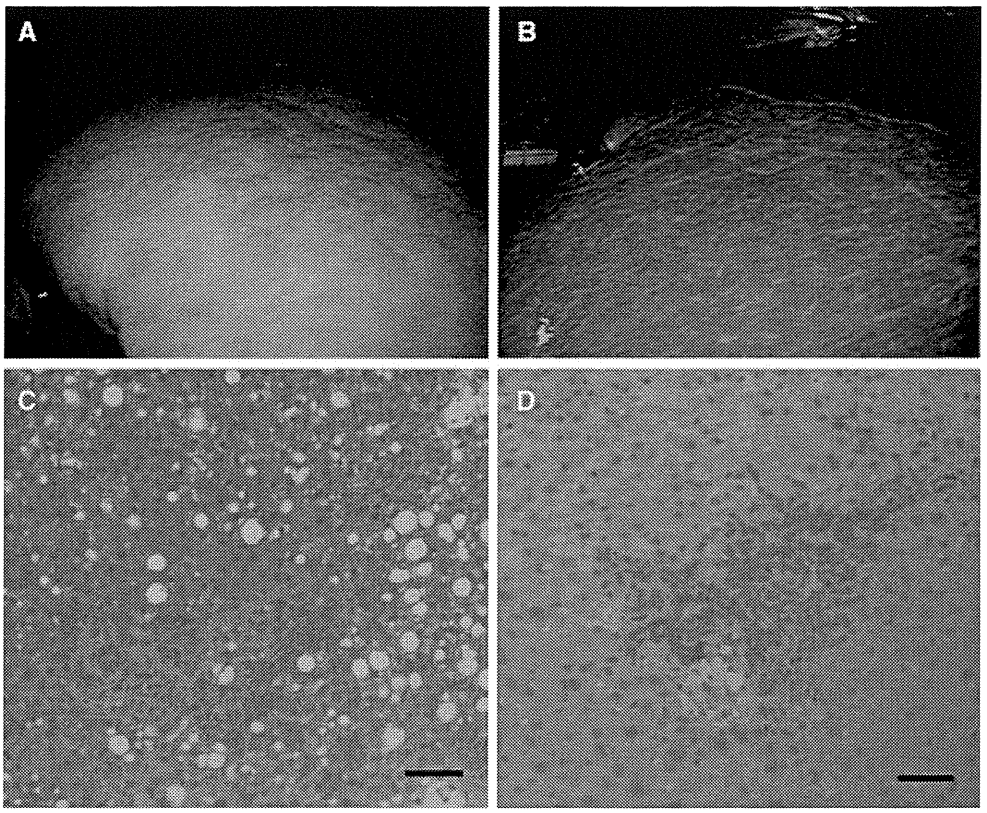

Fig. 1 Laparoscopic liver biopsy. The laparoscopic images of the liver surfaces of patient A (**a**) and patient B (**b**) are shown. Histopathology of patient A (**c**) and patient B (**d**) is also shown. *Bars* = 50 μm

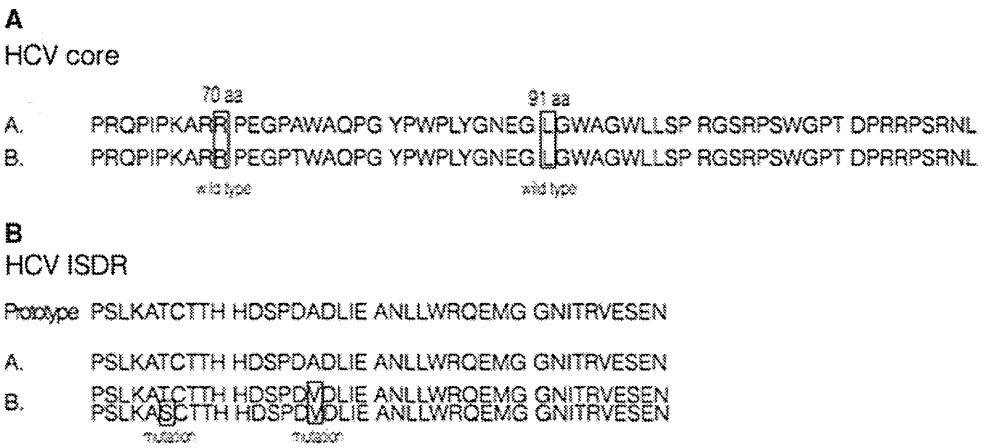

Fig. 2 Virological analysis of hepatitis B virus (HBV) and hepatitis C virus (HCV) in HBV/HCV dual infection. The amino acid sequences of the HCV-core region including core-70 and core-91, which were previously reported as determinants of the sensitivity to pegylated interferon/ribavirin (Peg-IFN/RBV) therapy, in patient A and patient B are shown (**a**). The amino acid sequences of the interferon sensitivity determining region (*ISDR*), which were previously reported as determinants of the sensitivity to IFN, in patients A and B are shown (**b**)

treatment achieved a sustained viral response (SVR) (5/5 patients). These data indicated that HCV-dominant dual-infected patients had good responses to treatment for HCV infection.

Virological analysis of HBV/HCV in patients A and B

The HCV genotype in both patient A and patient B was 1b. The sequences of amino acids in the ISDR region and HCV

core-70 and core-91 amino acids were analyzed by direct sequencing. Both patients had wild-type core-70 and core-91 amino acids (Fig. 2a). None of the mutations of the ISDR region was detected in patient A, but two of the mutations of the ISDR region were detected in patient B (Fig. 2b). The genotypes of HBV in patients A and B were analyzed by direct sequencing and phylogenetic tree analysis. The genotype of HBV in patient A was genotype C, which has been reported as difficult-to-treat HBV. The genotype of HBV in patient B was genotype Bj, which has been reported as easy-to-treat HBV in comparison to genotype C [45–47].

Sequential analysis of biochemical and virological data during Peg-IFN/RBV therapy

Patient A

In patient A, HCV-RNA gradually declined during Peg-IFN/RBV therapy. On the other hand, the HBV-DNA gradually increased during Peg-IFN/RBV therapy (Fig. 3a). The amount of HBeAg started to increase 9 months after the start of Peg-IFN/RBV therapy. HCV-RNA started to increase 12 months after the start of Peg-IFN/RBV therapy, although Peg-IFN/RBV was still being administered up to 18 months after the start of Peg-IFN/RBV therapy (Fig. 3a).

Patient B

In patient B, HCV-RNA and HBV-DNA rapidly declined after the start of Peg-IFN/RBV therapy (Fig. 3b). HCV-RNA could not be detected in peripheral blood 2 months after the start of Peg-IFN/RBV therapy. Peg-IFN/RBV was administered up to 12 months after the start of the Peg-IFN/RBV therapy. The amounts of HBeAb and HBeAg did not change during the Peg-IFN/RBV therapy (Fig. 3b).

Sequential immunological analysis during Peg-IFN/RBV therapy

We analyzed various subsets of immune cells that could affect the immunopathogenesis of HBV/HCV dual infection. NK cells ($CD3^-CD16^-CD56^{high}$ and $CD3^-CD16^+CD56^{dim}$) and NK-T cells ($CD3^+CD56^+CD16^+$, $CD3^+CD56^+CD16^-$ and $CD3^+CD56^-CD16^+$) were analyzed (Supplementary Fig. 1A). The $CD3^-$ gated lymphocytes were separated into 4 groups (a, b, c, and d). For these subsets, (a) indicated the presence of $CD3^-CD16^-CD56^{high}$ NK cells that could produce various cytokines vigorously and had low cytotoxic activity. Subset (b) showed $CD3^-CD16^+CD56^{dim}$ NK cells that had weak cytokine production ability and high cytotoxic activity.

The $CD3^+$ gated lymphocytes were separated into 3 groups (a, b, and c). The activated $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells were analyzed (Supplementary Fig. 1B). HLA-DR^+ activated $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells could be clearly distinguished by FACS analysis. Additionally, representative dot plots of Tregs and B cells were created (shown in Supplementary Fig. 1C). The frequencies of $CD3^-CD16^+CD56^{dim}$ NK cells, $CD3^+CD16^-CD56^+$ NK-T cells, activated $CD3^+CD4^+$ T cells, and activated $CD3^+CD8^+$ T cells fluctuated similarly during Peg-IFN/RBV therapy in patient A (Supplementary Fig. 1D). Activated T cells were increased at one month of Peg-IFN/RBV therapy, and the above subsets of lymphocytes gradually decreased up to 3 months of Peg-IFN/RBV therapy. After that, these cells gradually increased again up to 9 months of Peg-IFN/RBV therapy. In patient A, after 9 months of Peg-IFN/RBV therapy, these cells had decreased again (Supplementary Fig. 1D). The frequency of Tregs and activated B cells (data not shown) did not change during Peg-IFN/RBV therapy in patient A (Supplementary Fig. 1D). On the other hand, in patient B, the frequencies of $CD3^-CD16^+CD56^{dim}$ NK cells, $CD3^+CD16^-CD56^+$ NK-T cells, activated $CD3^+CD4^+$ T cells, and activated $CD3^+CD8^+$ T cells were increased and sustained during Peg-IFN/RBV therapy (Supplementary Fig. 1E). Five HCV monoinfected patients were analyzed by the same protocol (Supplementary Fig 1F). The mean frequency of various kinds of immune subsets was analyzed (Supplementary Fig 1F). The tendency of immunological reactions during Peg-IFN/RBV therapy in these five patients was similar to that in patient B.

Analysis of HBV- and HCV-specific immune responses

The analysis of HBV- and HCV-specific-immune responses was carried out by ELISPOT assay. Representative spots of IFN-γ are shown in Fig. 4a. In patient A, HCV- and HBV-specific IFN-γ secretion activities were remarkably low in comparison to the IL-10 secretion activity. Moreover, in patient A, the induction of IFN-γ-secreting cells could not be detected after Peg-IFN/RBV therapy, especially in regard to HBV-core specific IFN-γ secretion in PBMCs (Fig. 4b). On the other hand, in patient B, the HBV-core specific IFN-γ-secreting cells were high in comparison to those in patient A (Fig. 4c). Moreover, the induction of IFN-γ-secreting cells could be detected during Peg-IFN/RBV therapy in patient B (Fig. 4c). The mean numbers of IFN-γ- and IL-10-secreting spots in HBV-dominant dual-infected patients, patients with monoinfection with HBV genotype Bj ($HBeAb^+$), Bj ($HBeAg^+$), C ($HBeAb^+$), C ($HBeAg^+$), or HCV genotype 1b are shown in Fig. 4d. In patient A, HB core antigen (HBcAg)-specific IFN-γ secretion was weaker than that in