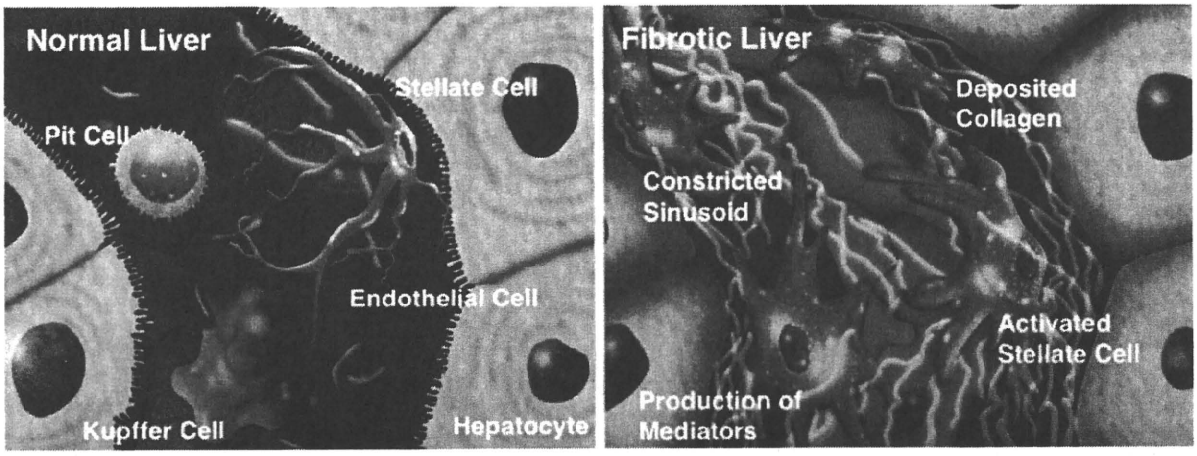

Figure 1 Illustrative demonstration of normal and fibrotic liver. (Left) Hepatic sinusoid of intact liver is composed of endothelial cells with sieve plate, to which Kupffer cells (liver-specific macrophages) and pit cells (natural killer cells) adhere. Stellate cells with vitamin A-droplets in the space of Disse attach to both endothelial cells and hepatocytes with their cytoplasmic processes. (Right) In fibrotic liver, stellate cells become activated and secrete type I collagen that is deposited in the space of Disse and profibrogenic and inflammatory mediators. Activated stellate cells constrict sinusoids, leading to microcirculatory disturbance in advanced fibrosis and ultimately to portal hypertension.

Hepatic stellate cells, which are also called Ito cells, fat-storing cells, lipocytes or perisinusoidal cells, are located in the Disse space in the hepatic sinusoid, encapsulate sinusoidal endothelial cells with their well-developed dendritic processes on one side, and face hepatocytes on the other side.[6] The principal function of HSC is storing vitamin A in their cytoplasm; 50–80% of vitamin A in the body is accumulated in the liver and 90% is stored in HSC, which secrete retinol to portal blood flow when required. Because HSC function also as liver-specific pericytes, their contraction and relaxation in response to vasoactive substances, such as endothein-1, nitric oxide and angiotensin-II, control the diameter of the sinusoidal lumen, resulting in regulation of the local microcirculation.[7] When liver injury takes place, HSC undergo activation and change their function and morphology to myofibroblast-like cells (Fig. 1).[8] Activated Kupffer cells, infiltrating circulating monocytes, activated and aggregated platelets, and damaged hepatocytes are sources of platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) that trigger the initiation of intracellular signaling cascades after their binding to cell surface receptors. Activated HSC lose vitamin A droplets and in contrast increase the expression of cytoskeletous proteins, such as desmin and α-smooth muscle actin, which are associated with their augmented contractile activity, and generate ECM, including types I and III collagens. Activation of HSC is controlled by transcription factors, such as activated protein-1 (AP-1), Jun D, Sp1, Krueppel-like factor 6 (KLF6) and nuclear factor (NF)-κB, leading to transcriptional upregulation of latent TGF-β. In the process, intracellular signaling molecules, such as Smad, Ras, Raf-1 and mitogen-activated protein (MAP) kinase, play important roles.[9] In addition, augmented production of the tissue inhibitor of matrix metalloproteinases (TIMP) hampers the degradation of ECM and conversely stimulates their accumulation in the inflamed liver. Involvement of leptin and other adipocytokines in the HSC activation process is also notable.[10] Activated HSC are characterized by an increased expression of receptors for PDGF, TGF-β, vascular endothelial growth factor (VEGF), angiotensin-II, endothelin-1, and so on (Fig. 2).[8,9]

Although the involvement of HSC in the hepatic fibrotic process has been reported in a large number of publications since the 1980s, recent investigations have revealed the participation of mesenchymal cells originating from bone marrow using rodent models and human damaged livers.[11] Similarly, fibrocytes in the circulation and portal fibroblasts are acknowledged as fibrotic players.[12,13] Furthermore, epithelial–mesenchymal transition (EMT) may be involved in the fibrotic process in the liver as well as in the kidney and lung,[14] although there have been controversial discussions on this issue.[15,16]

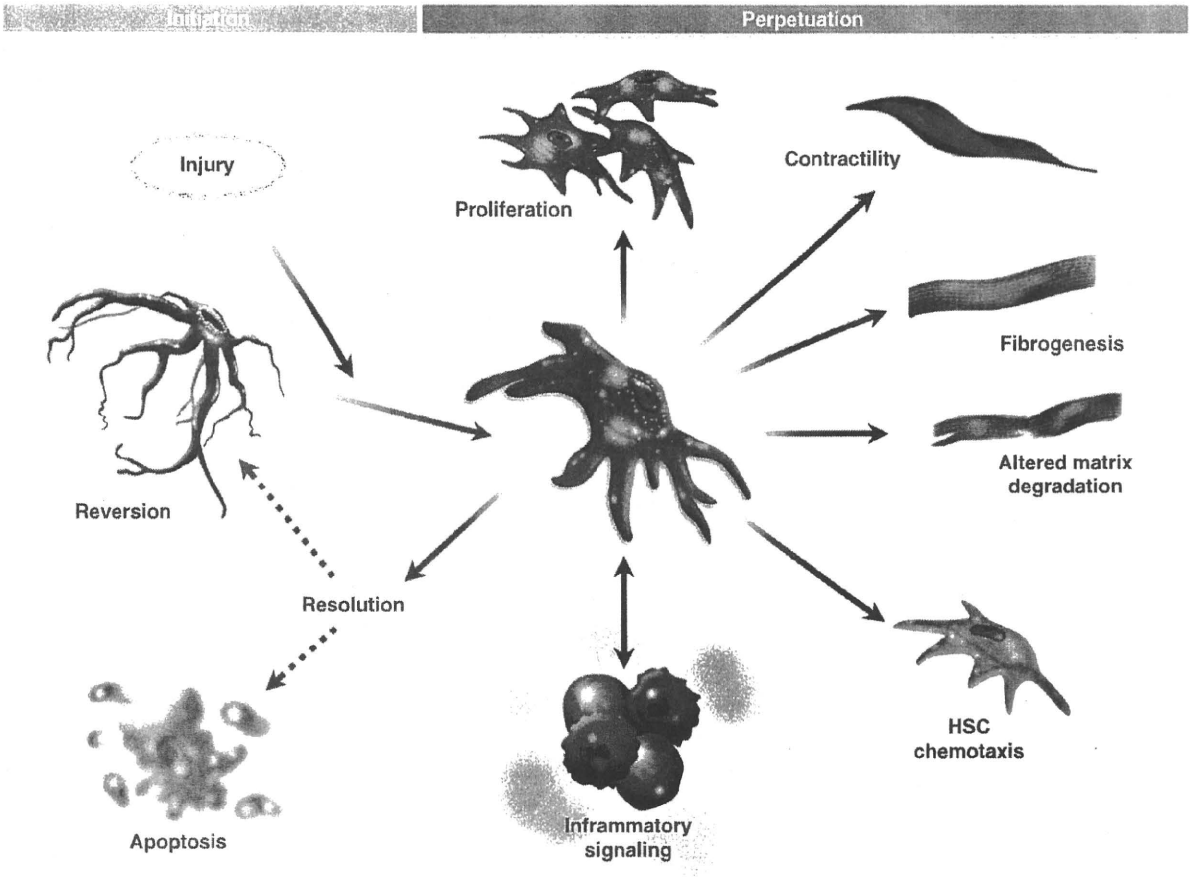

Figure 2 Illustrative demonstration of activation and deactivation of stellate cell and the related cellular function. When liver injury takes place, stellate cells undergo activation and switch their phenotype from a vitamin A-storing one to myofibroblast-like one that expresses α-smooth muscle actin. The latter phenotype of stellate cells proliferate, have contractile activity and produce extracellular matrices including type I collagen, inhibitors of matrix metalloproteinases and pro-inflammatory mediators. In addition, they acquire chemotactic activity. In the resolution stage of liver fibrosis, activated stellate cells undergo either apoptosis or reversion to the original vitamin A-storing phenotype.

Perpetuation of fibrotic response in the liver

Perpetuation of the hepatic fibrotic process is supported by the activation of HSC and the presence of MFB, which are continuously stimulated by growth factors, cytokines and oxidative stress, which are derived from neighboring cells. Damaged hepatocytes are a source of lipid peroxides and reactive oxygen species (ROS) generated from hepatocytic mitochondria, and breakdown products of the hepatocyte membrane activate HSC.[17] Nicotinamide adenine dinucleotide phosphate oxidase is an important component of the ROS-producing system, which could be a target for antifibrotic therapy. This enzyme is supposed to be induced in HSC to dispose of cells undergoing apoptosis, and lowering its expression in HSC by rosaltan, an angiotensin receptor antagonist, results in the decreased production of type I collagen and matrix metalloproteinase (MMP)-2.[18] Alternatively, excess accumulation of Fe^{2+} triggers the Fenton reaction to generate a radical·OH and leads to the apoptosis of hepatocytes, resulting in the activation of HSC and promotion of the fibrotic process.

Transforming growth factor-α, TGF-β, insulin-like growth factor and its binding protein, hepatocyte growth factor (HGF), VEGF and interleukin (IL)-6 are supposed to be candidates released from hepatocytes to

trigger HSC activation. On the other hand, when liver injury takes place, Kupffer cells become activated by oxidative stress and endotoxin lipopolysaccharide (LPS) derived from intestinal flora through Toll-like receptor (TLR)4 and CD14.[19] Macrophage chemotactic protein-1 (MCP-1) and osteopontin derived from activated Kupffer cells are involved in the infiltration of inflammatory cells into the liver.[20] In addition, activated Kupffer cells generate PDGF and TGF-β1, which in turn induce HSC activation. In an animal model, depletion of Kuppfer cells using the administration of gadolinium chloride ameliorates hepatic fibrosis, suggesting the active participation of Kupffer cells in hepatic fibrogenesis. Functional roles of chemokines in liver injury and fibrotic process are also discussed.[21]

Recent investigations revealed that, in addition to macrophages, Kupffer cells and classical lymphocytes (T and B cells), natural killer (NK) cells, NKT cells, dendritic cells and mast cells are important players in liver fibrosis, whose cellular functions are regulated by the TLR system and NF-κB signaling pathways. Namely, interaction between HSC/MFB and these immune-regulatory cells take part in the local infiltration of inflammatory cells in hepatic sinusoids.[22]

It was noted that cannabinoid receptors and their endogenous ligands are new players in liver fibrosis and in the activation process of HSC.[23] Three major components of cannnabinoids are tetrahydrocannabinol, cannabinol and cannabidiol whose receptors are composed of CB1 and CB2. Hemp and marijuana accelerate fibrosis progression in chronic hepatitis C and cannabinoids are also assumed to be associated with non-alcoholic steatohepatitis because CB1 receptor antagonists suppress appetite. HSC express both CB1 and CB2. The CB2-dependent signal initiates the apoptosis of HSC, CB1 level increases depending on HSC activation and regulation of its expression restricts TGF-β production. HSC isolated from CB1 receptor knockout mice exhibit decreased phosphorylation of extracellular signal-regulated kinase (ERK) and Akt.[24]

Analyses using molecular and biological techniques have revealed that the phosphorylation status of several proteins, methylation of target genes, stability of mRNA and microRNA levels are involved in the activation of HSC and MFB. For instance, activation of HSC triggers the phosphorylation of Ser^{536} of the RelA subunit of NF-κB, which prevents the death of HSC. On the other hand, microRNA (miR) 132 hampers the transcription of CpG methylation protein, leading to the suppression of peroxisome proliferator-activated receptor (PPAR)-γ and MFB activation.[25] Mir-29b is reported to attenuate collagen 1A1 transcription in LX-2 cells, a human immortalized stellate cell line, independently of the Smad pathway, by binding to the 3′-untranslated region of its gene and also by downregulating a transcription factor, Sp-1.[26] Elevation of miR-29 was found to be increased in advanced fibrosis in human liver disease.[27]

Furthermore, genetic approaches have uncovered that the mutation of human genes, most frequently SNP, also relates to the progression of liver fibrosis; for instance, among SNP, Leu10Pro of TGF-β, Arg25Pro and G-6A of angiotensin, G-308A of tumor necrosis factor (TNF)-α, and C-592A/-819/G-1082A of IL-10 have been identified.[28] The 7-gene cirrhosis risk score (CRS), composed of seven different SNP, was reported to associate strongly with fibrosis progression.[29] Among the SNP in CRS, DDX5 S480A regulates the transcription of several genes in fibrosis in HSC.[30]

Regression of hepatic fibrosis

It is generally accepted that regression of liver fibrosis happens clinically in patients who achieve a sustained viral response (SVR) after the eradication of HCV by (pegylated) interferon (IFN) (+ ribavirin) therapy or whose HBV viral level is well controlled by using nucleot(s)ide analogs such as lamivudine, adefovir and entecavir. In addition, many clinical data indicate that not only cirrhosis can regress but also that this recovers the function of the liver and improves the prognosis of patients. Thus, the development of antifibrotic therapy is anticipated regardless of the etiology of liver disease.

Regression of liver fibrosis is mechanistically explained by the following four aspects: (i) regeneration of hepatocytes; (ii) retracing of activated and myofibroblast-like HSC to vitamin A-storing quiescent HSC; (iii) removal of MFB by apoptosis; and (iv) lysis of ECM. When hepatocyte necrosis takes place, the remaining hepatocytes undergo proliferation, leading to repair of the local environment. These processes are stimulated by growth factors derived from HSC, such as HGF, epidermal growth factor (EGF), epimorphin and pleiotrophin.[31] It is also indicated that neurotrophin signals are important as a paracrine loop between HSC and hepatocytes, and that Foxf1 forkhead transcription factor is involved in the process. On the other hand, retracing activated HSC to vitamin A-storing quiescent HSC is achieved at least in culture by forced overexpression of PPAR-γ or sterol regulatory element binding protein 1c (SREBP-1c) in activated HSC.[32] Regarding the removal of activated HSC or MFB, cul-

tured cells are sensitive to apoptosis induced by CD95 ligand and NK cell-derived TNF related apostosis including ligand (TRAIL). Apoptosis of activated HSC by hepatocyte-derived nerve growth factor (NGF) is regulated by a signal dependent on serotonin receptor. There is an interesting report describing the participation of NK cells in hepatic fibrogenesis; in a mouse model, depletion of NK cells using anti-asialo-GM1 antibody accelerates liver fibrosis and conversely, NK cell activation using poly I:C, a TLR3 ligand, attenuates the pathological process. Killing by NK cells is restricted to activated HSC which express NK cell-activating receptor NKG2D. Such involvement of NK cells in liver fibrosis is clinically observed in patients receiving liver transplantation; in patients taking immune-suppressing agents, such as cyclosporine and glucocorticoid, liver fibrosis progresses at an increasing rate after liver transplantation.[33]

Matrix-metalloproteinases are calcium-dependent enzymes that digest ECM. MMP are classified into: (i) interstitial collagenases (MMP-1, -8, -13); (ii) gelatinase (MMP-2 and -9); (iii) stromelysin (MMP-3, -7, -10, -11); (iv) membrane type (MMP-14, -15, -16, -17, -24, -25); and (v) metalloelastase (MMP-12). HSC secrete MMP-2, -9 and -13 (in humans, MMP-1), and stromelysin. Because MMP-1 is a key collagenase that metabolizes type I collagen, it is the principal element to the improved fibrotic status of the liver; however, MMP activity also strictly regulates its binding partner, TIMP. Because HSC are able to generate both TIMP-1 and -2, a local balance between MMP and TIMP offer a key to solve the fibrotic process.[34] It is interesting to note that human T-cell-derived microparticles in the blood of hepatitis patients induce fibrolytic activity of HSC.[35]

Therapy for liver fibrosis

Information obtained by molecular analyses of liver fibrogenesis, in particular the activation process of HSC and MFB, facilitates the establishment of regulatory methods of human liver fibrosis and, currently, a clinical trial using a new compound, GI262570, is underway (see http://clinicaltrials.gov/). In chronic hepatitis C, IFN-based therapy has progressed markedly and the SVR rate after therapy has approached 50–60% even in difficult-to-treat patients with genotype 1, high viral load. SVR is expected to reach 70–90% in the near future by using additional protease or polymerase inhibitors. Regarding chronic hepatitis B, liver fibrosis progression is ameliorated in patients treated by nucleot(s)ide analogs, while mutation of HBV by elongation of the therapy period is far from reassuring. However, antifibrotic therapies that are safe, have minimal side-effects and can be given for a long time are definitely required in patients who have failed to achieve SVR with previous IFN therapy for chronic hepatitis C and who have an advanced fibrotic stage, such as cirrhosis, in any kind of liver disease, including NASH, a risk factor for non-B/non-C cirrhosis. These patients should be treated for a better prognosis of cirrhosis and to prevent the occurrence of HCC.

Strategies for antifibrotic therapy include: (i) regulation of the activation of HSC and MFB; (ii) suppression of collagen production by them; (iii) control of their proliferation; and (iv) regulation of stroma and neovascularization. IFN-α and -β, which are clinically used to eradicate HCV and HBV, are known to regulate promoters of collagen gene expression by way of intracellular signaling molecules, such as signal transducers and activators of transcription-1 (Stat-1) and p300.[36] As stated above, IFN is able to regulate collagen gene expression in HSC through inducing microRNA-29b.[26] Pegylated-IFN-α is retained in the circulation for a longer period than the conventional IFN and may activate the type I IFN receptor (IFNAR)-dependent Janus kinase/Stat-1 pathway, resulting in the augmented suppression of HSC.

Newly developed anticancer agents target tumor stroma that support tumor growth and survival through neovascularization.[37] Sorafenib and other multi-kinase inhibitors in clinical trials inhibit receptor-tyrosine kinase and Raf–MEK (MAPK Extracellular Signal-Related Kinase)–ERK signaling pathways that are activated by the binding of VEGF, fibroblast growth factor, PDGF and other growth factors to their individual receptors.[38] The septum of fibrotic liver is composed of capillaries with vascular endothelial cells and "pericytic" activated HSC and MFB. Thus, molecular-targeted anticancer agents themselves are anticipated to have antifibrotic potential.[39]

Evaluation of liver fibrosis

While liver fibrosis research has raised enthusiasm in the area of mechanistic analyses and therapeutic approaches, clinical diagnosis has also made marked progression. Because liver tissues obtained at liver biopsy are equivalent to 1/50 000, this small part of the liver does not always reflect the condition of the whole liver. Thus, it is necessary to solve several problems related to liver biopsy and to develop a method of evaluating the stage of liver fibrosis non-invasively.[40]

NEW TOOLS TO EVALUATE LIVER FIBROSIS

Serum tests

IN CLINICAL PRACTICE, type IV collagen 7 S, type III procollagen N-terminal peptide (P-III-P) and hyarulonic acid are commonly utilized as serum markers for human liver fibrosis, although it is difficult to distinguish each pathological fibrosis stage using one of these ECM products. On the other hand, the European Liver Fibrosis (ELF) study reported a marker composed of the combination of P-III-P, hyarulonic acid, and TIMP-1, which achieved the diagnostic power of area under the curve (AUC) 0.80 for Scheuer 3–4.[41] Independently of these "direct" serum markers originating from ECM degradation, "indirect" serum estimation markers of liver fibrosis have been reported including AAR (aspartate aminotransferase [AST]/alanine aminotransferase [ALT] ratio), APRI (AST-to-platelet ratio index), CDS (cirrhosis discriminant score), fibrotest and the HALT-C model, which are composed of plural parameters commonly measured in clinical practice. Fibrotest is an algorithm composed of six parameters – haptoglobin, α2-macroglobulin, apolipoprotein-A1, γ-glutamyltransferase, bilirubin, and γ-globulin – and its diagnostic power for more than F2-4 was reported to be AUC 0.87. Fibrometer is a numerical formula composed of the platelet count, prothrombin time, AST, hyarulonic acid, α2-macroglobulin, sex and age, and its diagnostic power for more than F2-4 was reported to be AUC 0.89.[42] The advantage of these serum tests is to determine mild fibrosis or cirrhosis at a rate of more than 50% without liver biopsy, while they have less accuracy in the stepwise discrimination of each stage of moderate fibrosis (F1–F2, F2–F3, and F3–F4) (Table 1).[43]

Evaluation of liver fibrosis using ultrasound-based technology

Transient elastography

Transient elastography, FibroScan502, was developed by ECOSENS (Paris, France) to evaluate liver fibrosis non-invasively in a short examination, measuring using ultrasonography, the propagation of low energy 3.5-MHz signals of a mechanical shear wave through liver tissue. This machine evaluates the stiffness of 3 cm^3 liver, implying that it covers at least a 100-times larger volume of liver tissue than liver biopsy. In particular, this device has been used to evaluate liver stiffness in chronic hepatitis C with good correlation to the F stage of the Metavir score and Inuyama classification. A report from Castera *et al.* in 2005 was pioneering;[44] the diagnostic accuracy of liver stiffness measurement as evaluated by Area Under Receiver Operating Characteristic Curve (AUROC) ranges was 0.84 for the diagnosis of significant fibrosis (≥F2), 0.90 for the diagnosis of advanced fibrosis (≥F3) and 0.94 for the diagnosis of cirrhosis. Recent publications have indicated that liver stiffness measurement is also applied for the evaluation of liver fibrosis in hepatitis B and NASH, while careful attention is required in cases of acute inflammation (acute hepatitis) or flares because the value of liver stiffness increases independently of the stage of liver fibrosis in these cases.[45,46]

Real-time tissue elastography

Real-time tissue elastography is a new diagnostic tool for the evaluation of tissue elasticity based on ultrasound technology, and was developed by Hitachi Medical (Tokyo, Japan). Real-time tissue elastography can be performed when patients undergo common B-mode screening of the liver by ultrasonography. It is already utilized to detect space-occupying lesions in mammary glands, the thyroid and prostate because tumors have abnormal elasticity compared to surrounding intact tissue. The computer-assisted apparatus calculates the relative hardness of tissue from the degree of tissue distortion and displays this information as a color image. Although ultrasound elastography does not reflect physical elasticity directly, it indicates the relative degree of tissue strain under subtle compression (Fig. 3). Frriedrich-Rust *et al.* applied this technique to measure liver stiffness and reported its usefulness for the diagnosis of significant fibrosis (for >F2, AUC 0.93) in combination with APRI.[47] The AUROC power of real-time tissue elastography was reported to be similar to that of transient elastography in patients with chronic hepatitis C.[48] Because real-time tissue elastography can be performed simultaneously with B-mode ultrasonography and applied for patients with obesity and ascites, it could be used widely in clinical practice more than transient elastography.

Acoustic radiation force impulse (ARFI)

Most recent technology is ARFI. This examination is performed at the time of B-mode observation of ultrasonography, similarly to real-time tissue elastography. This method is based on the generation and spreading of the shear wave within tissues after giving a "pushing pulse" of focused, impulsive and acoustic radiation force impulse. The harder the tissue is, the faster the shear wave spreads. Previous reports have indicated that

Table 1 Serum fibrosis markers

Index	Parameters	CLD and number of patients	Calculation	Interpretation	PPV/NPV (%)	AUC
Foms	Age, plt, γGT, cholesterol	HCV: t = 351 v = 125	7.811 – 3.131 × ln (plt) + 0.781 × ln (γGT) + 3.467 × ln (age) – 0.014 (cholesterol)			
APRI	AST, plt	HCV: t = 192 v = 78	([AST / ULN] / plt [×10⁹/L]) × 100	>1.5 = Ishak 3–6 ≤0.5 = Ishak 0–2	PPV = 91 NPV = 90	t = 0.80 v = 0.88
FT, FS	Haptoglobin, α2-MC, apo-A1	HCV, HBV: t = 205 v = 134	Logistic regression index (proprietary)	0.75–1.00 = F4 0.73–0.74 = F3–F4 0.59–0.72 = F3 0.49–0.58 = F2 0.32–0.48 = F1–F2 0.28–0.31 = F1 0.22–0.27 = F0–F1 0.00–0.21 = F0	PPV = 78 PPV = 76 PPV = 76 PPV = 67 PPV/NPV = 61/85 NPV = 91 NPV = 92 NPV = 94	≥F2–F4 t = 0.83 v = 0.87
Fibroindex	Plt, AST, γGT	HCV: t = 240 v = 120	1.738 – 0.064 (plt [×10⁴/mm³]) + 0.005 (AST [IU/L]) + 0.463 × (γGT [g/dL])	≤1.25 = F0–F1 ≥2.25 = F2–F3	NPV = 61.7 PPV = 90	t = 0.83 v = 0.82
FIB-4	Plt, AST, ALT, age	HCV or HIV: t = 555 v = 277	(Age × AST) / (plt count × ALT$^{1/2}$)	<1.45 = Ishak <4–6 >3.25 = Ishak ≥4–6	NPV = 90 PPV = 65	0.76
Virahep-C model	AST, plt, ALP, age	HCV: 399	1 / (exp [–y] + 1) with y = –5.17 + 0.20 × race + 0.07 × age (years) + 1.19 × ln (AST – 1.76) × ln (plt [×10³/mL]) + 1.38 × ln (ALP)	>0.55 = Ishak 3–6 ≤0.22 = Ishak 0–2	NPV = 85 PPV = 81	0.83
GUCI	AST, INR, plt	HCV: 179	AST × prothrombin-INR × 100 / plt (×10³/L)	>1.0 = Ishak ≥4–6	NPV = 31 PPV = 97	0.85
Hui	BMI, bilirubin, plt, albumin	HBV: t = 150 v = 85	exp (3.148 + 0.167 × BMI + 0.088 × bilirubin [μM] – 0.151 × albumin [g/L] – 0.019 × plt [×10⁹/L]) / (1 + exp (3.148 + 0.167 × BMI + 0.088 × bilirubin [μM] – 0.151 × albumin [g/L] – 0.019 × plt [×10⁹/L])	>0.5 = Ishak 3–6 ≤0.15 = Ishak 0–2	NPV = 81 PPV = 53 NPV = 92 PPV = 38	t = 0.80 v = 0.77
Mohamadenjad	Albumin, plt, ALP, HBV DNA	HBV: t = 130 v = 99	10 + (0.771 × $\log_{10}$ HBV DNA copies/mL) + (3.828 × $\log_{10}$ (ALP / ULN) (1.066 albumin g/dL) – (0.011 [plt/1000 μL])	<4.72 = Ishak <4–6 >7.75 = Ishak ≥4–6	NPV = 99 PPV = 26 NPV = 93 PPV = 75	t = 0.91 v = 0.85

α2-MC, α2-macroglobulin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; CLD, chronic liver disease; γGT, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus; INR, international normalized ratio; NPV, negative predictive value; plt, platelets; PPV, positive predictive value; ULN, upper limit of normal.

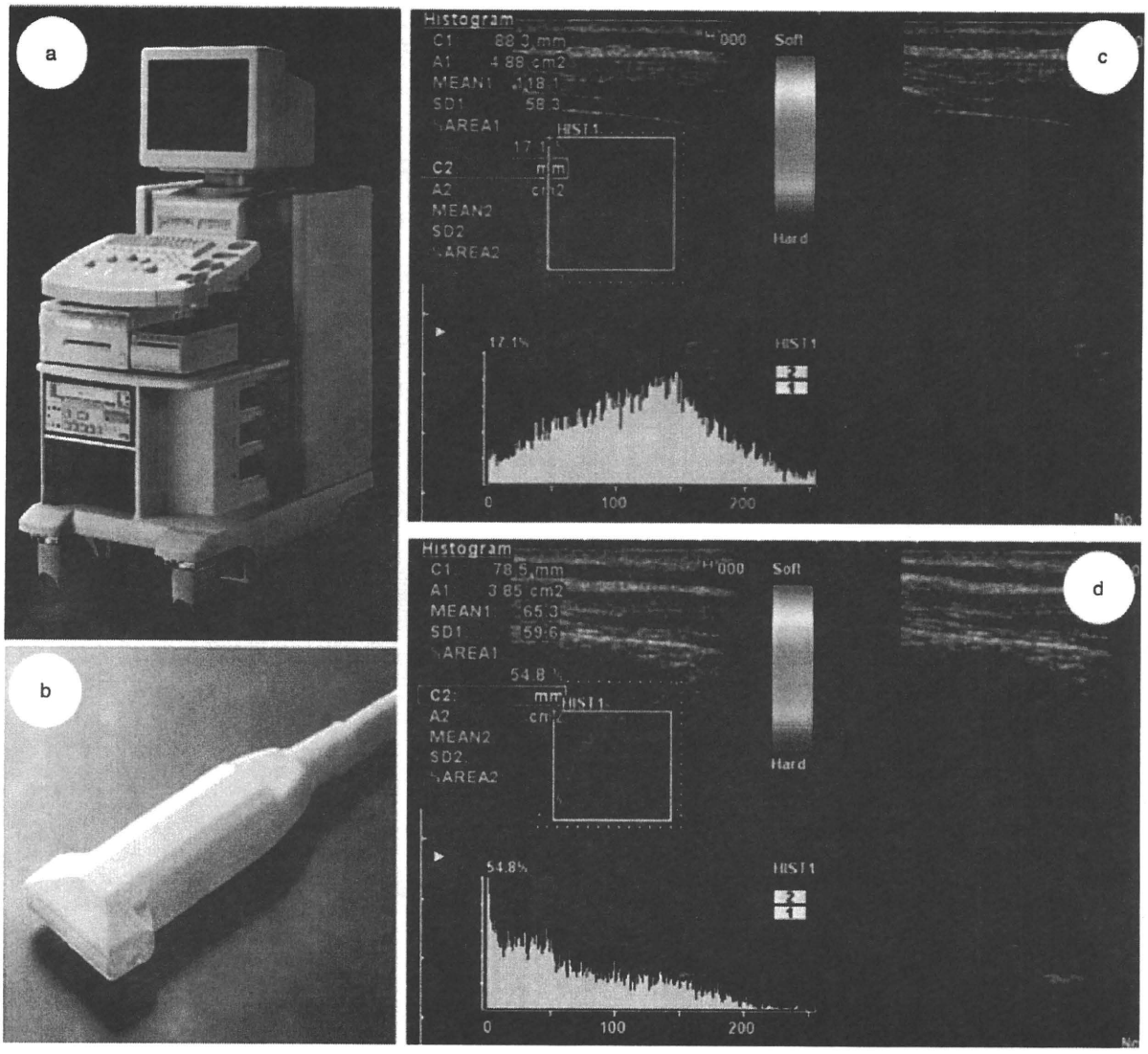

Figure 3 Real-time tissue elastography. (a) Ultrasonography machine. (b) Probe for real-time tissue elastography. (c) RTE color image of F1 patient with liver stiffness of 6.9 kPa obtained by transient elastography (FibroScan). (d) RTE color image of F4 patient with liver stiffness of 33.3 kPa obtained by transient elastography (FibroScan). RTE, real-time tissue elastography.

the diagnostic power of ARFI for the staging of liver fibrosis is the same as that of transient elastography.[49]

APPLICATION OF LIVER STIFFNESS MEASUREMENT FOR THE SCREENING OF HCC AND CIRRHOSIS COMPLICATIONS

EVALUATION OF LIVER fibrosis in chronic liver disorders is useful in determining disease progression and assessing complications, such as HCC and esophageal varices. Foucher *et al.* reported that, in 144 chronic hepatitis C patients with fibrosis stages F3 or F4, the cut-off values of liver stiffness (kPa) measured by transient elastography were 27.5, 49.1, 53.7 and 62.7 for the appearance of esophageal varices (stage 2/3), ascites, HCC and rupture of esophageal varices.[50]

Masuzaki *et al.* prospectively observed that, in 866 patients with chronic hepatitis C, HCC developed within 3 years of observation in 77 cases, and liver stiffness at entry was less than 10 kPa in 0.4% and more

than 25 kPa in 38.5%, indicating the usefulness of liver stiffness measurement as a tool for forecasting the development of HCC.[51]

CONCLUSION

LIVER FIBROSIS RESEARCH was initiated to explore the unknown functions of HSC and MFB, has advanced simultaneously in both basic and clinical aspects, and has achieved the non-invasive assessment of the diagnosis of liver fibrosis, identifying a new compound that is therapeutically appropriate. Future progression and expansion of this research field is eagerly anticipated.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Prospective study of reactivation of hepatitis B virus in patients with rheumatoid arthritis who received immunosuppressive therapy: evaluation of both HBsAg-positive and HBsAg-negative cohorts

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Abstract
Background Screening and prophylactic treatment for hepatitis B virus (HBV) reactivation is recommended for patients who receive immunosuppressive or cytotoxic therapy. The aim of this study was to clarify the prevalence of HBV reactivation in rheumatoid arthritis (RA) patients who had received more than 1 year of immunosuppressive therapy. This study also evaluated guidelines for determining HBV reactivation in patients with RA.
Methods This was a prospective non-randomized, non-controlled study. We enrolled 50 patients with RA who had antibodies against hepatitis B core antigen (anti-HBc) and who had started treatment with disease-modifying anti-rheumatic drugs, including those who had additionally received anti-tumor necrosis factor-α (anti-TNF-α). HBV DNA levels were measured every 2–3 months by a real-time, polymerase chain reaction-based method. Entecavir was administered to patients with HBV DNA levels >2.1 log/ml.
Results The mean observation period was 23 months (range 12–32 months). HBV reactivation occurred in 2 of 5 patients with HBV surface antigen (HBsAg) and in 1 of 45 patients without HBsAg. In patients who received anti-TNF-α therapy, antibodies against HBsAg decreased significantly. Entecavir therapy inhibited HBV amplification and prevented HBV-associated flares of hepatitis.
Conclusions The incidence of HBV reactivation was low in RA patients in whom HBV infection had been resolved. Screening for HBV reactivation and prophylactic therapy with entecavir were effective means of preventing HBV-associated hepatic failure in patients with HBsAg, as well as in those with only anti-HBc who received immunosuppressive therapy for RA.

Keywords Anti-tumor necrosis factor-α · Anti-HBs · Entecavir · HBV reactivation · Rheumatoid arthritis

Abbreviations

ALT	Alanine aminotransferase
Anti-HBc	Antibodies against hepatitis B core antigen
Anti-HBs	Antibodies against HBsAg
Anti-TNF-α	Anti-tumor necrosis factor-α
DMARDs	Disease-modifying anti-rheumatic drugs
HBV	Hepatitis B virus
HBsAg	HBV surface antigen
MTX	Methotrexate
RA	Rheumatoid arthritis

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Introduction

Hepatitis B virus (HBV) is a circular DNA virus with approximately 3000 bases that causes liver disease in humans, including acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1, 2]. Reactivation is one of the unique pathogenetic mechanisms of HBV [3]. HBV reactivation means a consecutive increase in the serum HBV DNA level by more than 1 log in patients with previously inactive or resolved HBV infection [4]. In patients with HBV reactivation, hepatitis flare can occur, potentially leading to hepatic failure. De-novo hepatitis is defined as a hepatitis flare caused by HBV reactivation in patients who are negative for hepatitis B virus surface antigen (HBsAg). HBV reactivation is known to occur occasionally in patients who receive immunosuppressive or cytotoxic therapy [5]. In particular, rituximab, an anti-CD20 monoclonal antibody used for combined chemotherapy of malignant lymphoma, frequently induces HBV reactivation not only in HBsAg-positive patients, but also in HBsAg-negative patients [6–8]. Several studies have reported that infliximab, a monoclonal antibody against tumor necrosis factor-α (anti-TNF-α), induced HBV reactivation in HBsAg-positive patients with Crohn's disease or rheumatoid arthritis (RA) [9–11]. Recent case reports showed that HBV reactivation occurred in HBV carriers with RA who received etanercept, adalimumab, or rituximab [12, 13]. HBV reactivation has also occurred in HBsAg-positive patients with RA who received low doses of methotrexate (MTX) [14, 15]. Two case reports have documented HBV reactivation in HBsAg-negative RA patients who received MTX or anti-TNF-α [16, 17]. Disease-modifying anti-rheumatic drugs (DMARDs), such as MTX, are standard initial therapy for RA [18, 19]. The availability of anti-TNF-α has increased the number of patients with RA that is resistant to conventional DMARDs whose disease can be satisfactorily controlled, making the remission of RA an increasingly realistic aim [20]. Recently, there has been a shift towards early aggressive treatment with anti-TNF-α for RA patients with milder disease activity [21]. More RA patients are thus expected to receive anti-TNF-α, necessitating the establishment of treatment protocols to ensure its safe use.

Several groups of hepatologists have recommended that patients who receive immunosuppressive therapy undergo screening and prophylaxis for HBV reactivation [22–25]. One meta-analysis suggested that prophylactic treatment with lamivudine, a nucleotide analogue for anti-HBV replication, might reduce the risk of HBV reactivation and decrease HBV-associated morbidity and mortality in patients with neoplasia [26]. In general, immunosuppressive therapy for RA should be continued not only in patients with active disease, but also for those in remission. Guidelines of the American Association for the Study of Liver Diseases recommend that adefovir or entecavir should be given to patients who are likely to require long-term immunosuppressive therapy [24].

To our knowledge, two prospective studies of HBV replication in RA and other rheumatoid diseases have been reported. Vassilopoulos et al. [27] reported that HBV reactivation did not occur in 19 patients with resolved HBV infection who received anti-TNF-α therapy. However, Urata et al. [28] detected HBV DNA amplification in 7 of 135 patients with resolved HBV infection. The results of these two studies are thus inconsistent.

The present study was designed to clarify the prevalence of HBV reactivation in RA patients receiving long-term immunosuppressive therapy. The usefulness of screening and a prophylactic protocol for HBV reactivation with entecavir were also assessed.

Methods

Study design and patient population

We organized a project team to prospectively study HBV reactivation in patients with RA at Osaka City University Hospital in 2007. Before starting treatment with DMARDs or additionally receiving anti-TNF-α, patients were tested for the presence of HBsAg and antibodies against hepatitis B virus core antigen (anti-HBc) in serum by chemiluminescent enzyme immunoassay (CLEIA; Fujirebio, Tokyo Japan). Patients who tested positive for HBsAg or anti-HBc were enrolled in this study. Patients with hepatitis C virus infection, alcoholic liver disease, primary biliary cirrhosis, or autoimmune liver disease were excluded. All enrolled patients were additionally tested for antibodies against HBsAg (anti-HBs) and HBV DNA in serum. Anti-HBs was tested by CLEIA, and the results were expressed in units of mIU/ml. HBV DNA levels were tested every 2–3 months by a real-time polymerase chain reaction (real-time PCR)-based method (COBAS TaqMan PCR version 2, Roche Diagnostics, Tokyo, Japan) [29]. The quantifiable range of the real-time PCR assay was between 2.1 and 9 log copies/ml. In patients with HBV DNA, HBV genotypes were determined by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against type-specific epitopes in the preS2-region (Institute of Immunology, Tokyo, Japan), as described elsewhere [30]. Patients with HBV DNA levels >2.1 log copies/ml received 0.5 mg of entecavir per day to prevent HBV-reactivation-associated hepatitis. In the present study, HBV reactivation was defined as an increase in the HBV DNA level by more than

1.0 log copy/ml as compared with the level at enrollment, or as an HBV DNA level >2.1 copies/ml. Hepatitis flares were defined as an elevation of the alanine aminotransferase (ALT) level to 400 IU/L, equivalent to tenfold the upper limit of normal, or higher. In accordance with the guidelines of the Italian Association for the Study of the Liver [22], HBV-DNA levels were monitored at intervals of 2–3 months in the present study. Our protocol differed from the guidelines recommended by the Intractable Liver Diseases Study Group of Japan and the Japanese Study Group of the Standard Antiviral Therapy for Viral Hepatitis because our prospective study was started before the guidelines were published in 2009 [25].

A total of 50 patients (41 females and 9 males) with anti-HBc were enrolled from November 2007 through October 2009. Their mean age was 59 years (range 15–73 years). Five patients were positive for both HBsAg and anti-HBc. Among the 45 patients who were negative for HBsAg, 36 were positive for anti-HBs, and 9 were positive for anti-HBc only (Table 1). Anti-HBs was examined every 2–3 months in the 36 patients who were positive for anti-HBs. Among the 5 patients with HBsAg, 3 received DMARDs, and 2 additionally received anti-TNF-α therapy. Of the 45 patients without HBsAg, 3 received DMARDs, and 42 additionally received anti-TNF-α therapy. In the present study, 4–10 mg/m^2 of MTX per week or 1000 mg per day of sulfasalazine, or both, were used as DMARD therapy. Anti-TNF-α therapy comprised 3 mg/kg of infliximab every 8 weeks, 25 mg of etanercept twice every week, or 40 mg of adalimumab every 2 weeks. The drug used for anti-TNF-α therapy was not randomly assigned. Before treatment with entecavir, the absence of human immunodeficiency virus co-infection was confirmed. The mean observation period was 23 months (range 12–32 months).

Statistical analysis

Statistical analysis was performed with the Statview SE + Graphics program, version 5.0 (SAS Institute, Cary, NC, USA). The paired t test was used to compare 2 continuous variables. All tests were 2-sided, and p values of <0.05 were considered to indicate statistical significance.

Ethical considerations

The study protocol complied with the ethical guidelines of the Declaration of Helsinki (2000) and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine. Written informed consent was obtained from all enrolled patients.

Results

HBsAg-positive patients

In all 5 HBsAg-positive patients, hepatitis B e antigen (HBeAg) was negative and anti-HBe was positive at enrollment (Table 2). Prophylactic treatment with entecavir was started in 3 of these HBsAg-positive patients at enrollment (Fig. 1). Two of these patients had an HBV DNA level higher than 2.1 log copies/ml before additionally receiving anti-TNF-α therapy, and the other patient had an HBV DNA level of 4.2 log copies/ml before receiving MTX. The HBV genotypes in these 3 patients were type A, B, and C, respectively. The HBV DNA level decreased to below 2.1 log copies/ml 3 months after starting entecavir. During treatment with entecavir, the HBV DNA level remained below 2.1 log copies/ml, and immunosuppressive therapy for RA could be continued without hepatitis flares. In the 2 HBsAg-positive patients who had not received prophylactic treatment with entecavir, the HBV DNA level was below 2.1 log copies/ml before MTX was begun. In 1 patient, the HBV DNA level increased to 2.5 log copies/ml at 19 months after enrollment, and in the other the HBV DNA level increased to 3 log copies/ml at 14 months. Prophylactic treatment with entecavir was therefore started. After 2 months of treatment, the HBV DNA level decreased to below 2.1 log copies/ml without hepatitis flares. Anti-TNF-α and/or DMARD treatment could be continued in addition to entecavir in patients with HBsAg.

HBsAg-negative patients

In 45 patients without HBsAg, HBV DNA levels were below 2.1 log copies/ml at enrollment (Fig. 1). There was no significant difference in age, gender, therapy for RA, or observation period between the 36 anti-HBs-positive patients and the 9 patients in whom only anti-HBc was positive. In 1 of the 3 patients who received DMARD therapy including MTX, HBV DNA reactivation occurred. These 3 patients did not receive anti-TNF-α therapy. The patient in whom HBV DNA reactivation occurred was a 73-year-old woman with an anti-HBs level of 9.9 mIU/ml. Her medical records in our hospital showed that HBsAg had been positive in 1986. Since 1993, the results of serum tests for HBsAg had been negative, without treatment. At enrollment in the present study, the HBV DNA level was below 2.1 log copies/ml, but HBV DNA tested positive on real-time PCR. After treatment with a low dose of MTX, anti-HBs disappeared. After 10 months of MTX treatment, the HBV DNA level increased to 4.7 log copies/ml (10000 IU/ml), and the results of serum HBsAg testing became positive. Entecavir was immediately administered.

Table 1 Characteristics of the 50 enrolled patients

	HBsAg-positive patients	HBsAg-negative patients
n	5	45
Age, years (mean)	58	59
Gender (M/F)	3/2	6/39
HbeAg-positive (*n*)	0	0
Anti-HBc-positive (*n*)	5	45
Anti-HBs-positive (*n*)	0	36
HBV DNA level	3 patients: >2.1 log/ml, 2 patients: not detected	45 patients: <2.1 log/ml 44 patients: not detected
ALT (IU/l), mean (range)	22 (10–31)	27 (9–85)
Treatment for RA (*n*)		
Prednisolone	3	34
DMARDs	4	42
Anti-TNF-α		
Infliximab	1	21
Etanercept	1	19
Adalimumab	0	2

HBV hepatitis B virus, *HBsAg* HBV surface antigen, *HBeAg* HBV e antigen, *anti-HBc* antibodies against hepatitis B core antigen, *anti-HBs* antibodies against HBsAg, *ALT* alanine aminotransferase, *RA* rheumatoid arthritis, *DMARDs* disease-modifying anti-rheumatic drugs, *anti-TNF-α* anti-tumor necrosis factor-α

Table 2 Clinical outcomes in patients treated with entecavir

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6[a]
Age (years)	56	63	67	33	69	73
Gender	Male	Male	Male	Female	Female	Female
HBsAg	Positive	Positive	Positive	Positive	Positive	Negative
HBeAg	Negative	Negative	Negative	Negative	Negative	Negative
Anti-HBe (% inhibition)	100	99	97	100	100	77
HBV DNA level at enrollment	4.8 log/ml	Undetectable	Undetectable	3.6 log/ml	4.2 log/ml	<2.1 log/ml
HBV DNA level at reactivation (log/ml)	–	2.5	3	–	–	4.7
HBV DNA level after ETV therapy	Undetectable	Undetectable	<2.1 log/ml	<2.1 log/ml	Undetectable	Undetectable
HBV genotype	B	C	C	A	C	C
ALT (IU/ml) at enrolment	10	35	31	12	21	21
ALT (IU/ml) after ETV therapy	6	33	29	27	19	19
Treatment for RA	PSL, etanercept	PSL, MTX	PSL, MTX	MTX, infliximab	MTX	MTX
Observation period (months)	31	29	20	18	14	26
Period of entecavir treatment (months)	31	9	9	18	14	16

ETV entecavir, *PSL* prednisolone, *MTX* methotrexate

[a] Patient 6: HBsAg changed to positive at HBV reactivation

The HBV DNA level and HBsAg titer decreased to undetectable levels after 4 months of entecavir treatment. Hepatitis flares occurred after the HBV DNA level decreased in response to the entecavir treatment (Fig. 2). Finally, the ALT level had improved to the normal range and serological testing showed that anti-HBs had reappeared. The genotype of the reactivated HBV was C.

In 42 patients, anti-TNF-α therapy was added to DMARD therapy, including MTX. The mean observation period in these patients was 24 months (range 14–31 months). At the time of this writing, HBV reactivation has not occurred. Eight patients did not have anti-HBs, 9 had anti-HBs levels lower than 100 mIU/ml (low-titer group), 9 had anti-HBs levels between 100 and 800 mIU/ml (middle-titer group), and 16 had anti-HBs levels higher than 800 mIU/ml (high-titer group). We compared anti-HBs titers at enrollment with those at the latest examined point. In the high-titer group, anti-HBs levels remained high, as compared with the titers at enrollment. In contrast, in the middle-titer and low-titer groups, anti-HBs levels decreased significantly (Fig. 3), but did not become negative in any patient. In a 73-year-old woman treated with etanercept, anti-TNF-α therapy was discontinued for 4 months because of cholecystitis. Her anti-HBs titer was

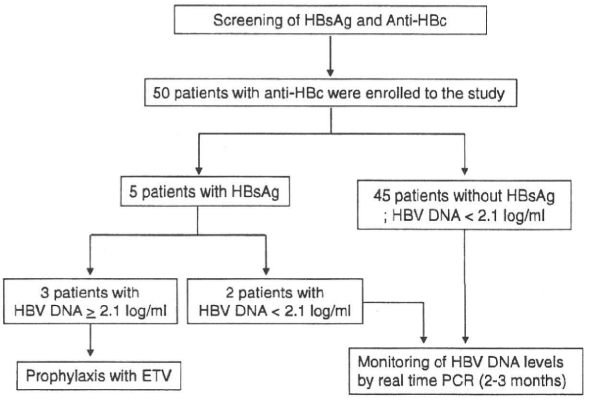


Fig. 1 Screening of hepatitis B virus (*HBV*) surface antigen (*HBsAg*) and antibodies against hepatitis B core antigen (*anti-HBc*) in patients who received immunosuppressive therapy. Prophylactic treatment with entecavir (*ETV*) was given to patients with HBV DNA levels >2.1 log/ml. *PCR* Polymerase chain reaction

224 mIU/ml at enrollment, 116 mIU/ml before stopping anti-TNF-α therapy, 144 mIU/ml during discontinuation of treatment, and 100 mIU/ml after restarting anti-TNF-α therapy. In the low- and middle-titer groups (Fig. 4), the mean anti-HBs level decreased from 219 to 145 mIU/ml in 9 patients treated with etanercept ($p = 0.025$) and decreased from 134 to 128 mIU/ml in 8 patients treated with infliximab ($p = 0.095$). The mean duration of infliximab therapy was 25.6 months (range 17–31 months). The mean duration of etanercept therapy was 25.4 months (range 14–30 months).

Discussion

Several case reports have documented HBV-associated hepatic failure in inactive HBV carriers who received DMARDs or infliximab [31]. Retrospective studies have also suggested that prophylactic treatment with lamivudine is needed to prevent HBV reactivation in RA patients who receive immunosuppressive therapy [22, 23]. The present prospective study showed that HBV reactivation occurred in HBsAg-positive RA patients who received immunosuppressive therapy, including MTX and prednisolone. In patients with HBV DNA levels >2.1 log copies/ml at enrollment, prophylactic therapy with entecavir reduced such levels. Consequently, immunosuppressive therapy for RA could be continued without HBV-associated hepatitis flare. In patients with HBV DNA levels <2.1 log copies/ml at enrollment, monitoring HBV DNA levels at 2 month intervals facilitated the detection of HBV amplification before hepatitis flare. However, our result in which there were only two patients in whom hepatitis was successfully prevented cannot guarantee the safety of our protocol. The Japanese guidelines recommend that prophylactic treatment with entecavir should be started before immunosuppressive therapy for all patients with HBsAg [25].

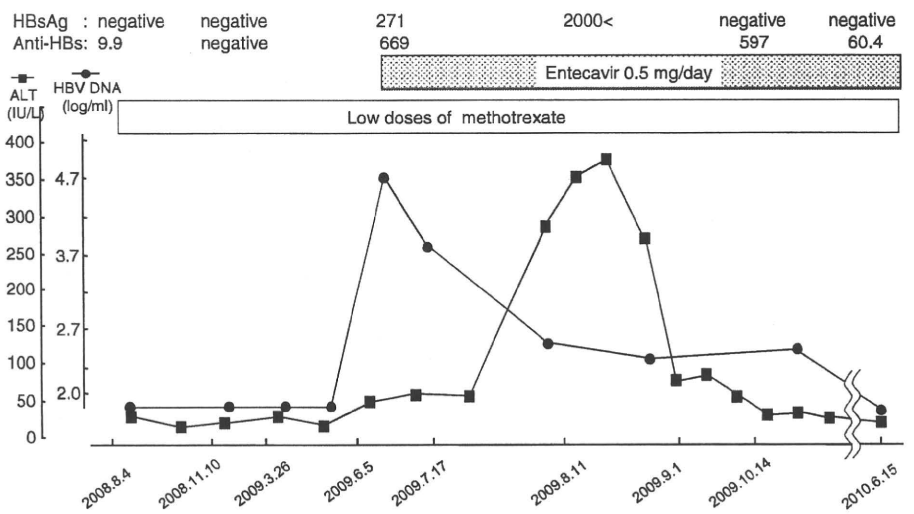


Fig. 2 Clinical course of an HBsAg-negative patient with HBV reactivation. In a 73-year-old woman with a level of antibodies against HBsAg (*anti-HBs*) of 9.9 mIU/ml, HBV DNA increased after methotrexate (MTX) treatment. At enrollment in the present study, the HBV DNA level was below 2.1 log copies/ml, but HBV DNA tested positive on real-time PCR. After treatment with a low dose of MTX, anti-HBs disappeared. After 10 months of MTX treatment, the HBV DNA level increased to 4.7 log copies/ml, and the results of serum HBsAg testing became positive. Treatment with entecavir decreased the HBV DNA level and HBsAg titer to below the detection limits. Alanine aminotransferase (*ALT*) increased transiently after the HBV DNA level decreased in response to the entecavir treatment. MTX therapy has been continued

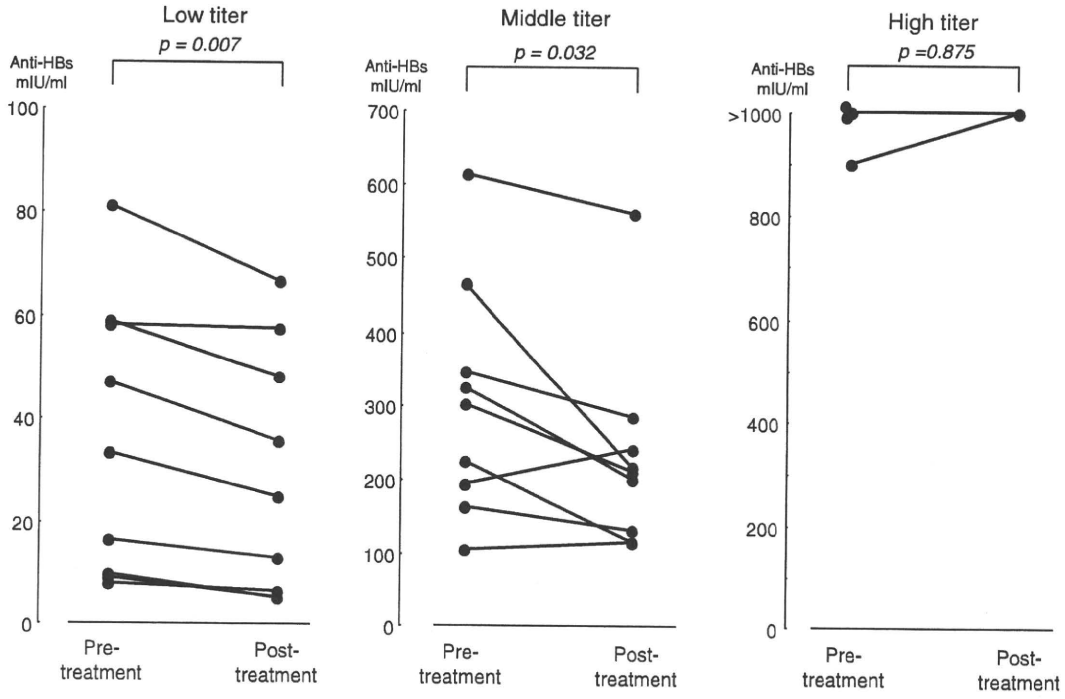
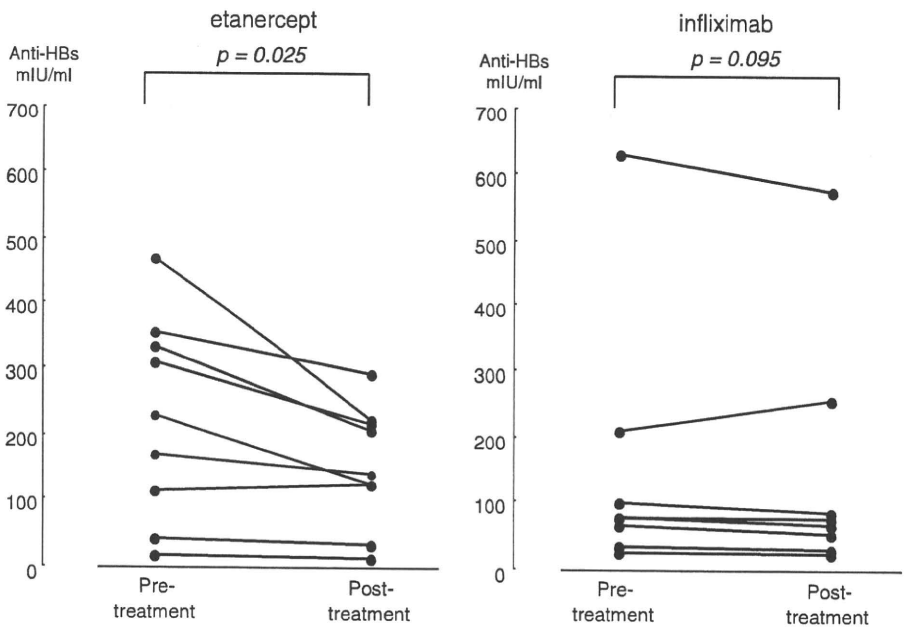


Fig. 3 Changes in anti-HBs titers in patients who received anti-tumor necrosis factor-α (anti-TNF-α) and disease-modifying anti-rheumatic drug (DMARD) therapy. In 9 patients with anti-HBs levels lower than 100 mIU/ml (*low titer group*) and 9 patients with anti-HBs levels between 100 and 800 mIU/ml (*middle titer group*), anti-HBs levels decreased significantly. However, anti-HBs did not become negative in any patient. In contrast, anti-HBs levels remained high in 16 patients with anti-HBs levels higher than 800 mIU/ml (*high titer group*)

Fig. 4 In the present study, treatment with anti-TNF-α was not randomly assigned. In the low and middle titer groups, anti-HBs levels decreased significantly in 9 patients treated with etanercept. In addition, anti-HBs levels changed from 134 to 128 mIU/ml in 8 patients treated with infliximab



Entecavir was given prophylactically in the present study because, as compared with lamivudine, entecavir rapidly reduces HBV DNA levels in patients with or without HBeAg [32, 33]. In addition, the cumulative probability of drug-resistant HBV was reported to be 57% for 3 years of lamivudine therapy, as compared with only 1.2% for 5 years of entecavir therapy in nucleotide-naïve patients with HBeAg [34, 35]. In the present study,

HBsAg-positive patients continued to receive entecavir therapy, without the emergence of drug-resistant mutants. Our protocol did not include criteria for the cessation of entecavir in immunosuppressed patients with HBV. One disadvantage of entecavir, however, is the high cost as compared with lamivudine. To our knowledge, the emergence of drug-resistant HBV after prolonged prophylactic therapy with lamivudine has not been studied previously. It remains to be clarified which nucleos(t)ide analogue is better for preemptive therapy for HBV reactivation in patients with RA.

HBV reactivation occurred in 1 of the 45 HBsAg-negative patients in our study. This patient was previously an HBV carrier and received low doses of MTX and prednisolone. At enrollment, HBV DNA tested positive on real-time PCR. However, treatment with entecavir was not started because HBV DNA levels remained below 2.1 log copies/ml. One case report has documented the occurrence of de-novo hepatitis in a 66-year-old woman with RA who received low doses of MTX and prednisolone [14]. After the ALT level in that patient increased, the initiation of lamivudine therapy could not rescue the patient. In the present study, prophylactic treatment with entecavir reduced the HBV DNA level from 4.7 log copies/ml to under the detection limit and rescued the patient with HBV reactivation. However, entecavir could not prevent the elevation of ALT. There are two possible explanations for the elevation of ALT in the present patient. First, the prevention of ALT elevation by entecavir may have been delayed. The other possibility is "on-treatment ALT flare." Sudden increases in ALT levels during the first 4–6 weeks of nucleotide analogue therapy have been reported, apart from a reduction in HBV DNA levels [36, 37]. Such exacerbations, termed "on-treatment ALT flare," are uncommon, but have occurred in patients given lamivudine, famicovir, or entecavir [36]. In the present case, ALT elevation occurred after the HBV DNA level had decreased to 2.5 log copies/ml. We therefore attributed the ALT elevation to on-treatment ALT flare, not directly related to HBV amplification. Another message came from this case. Tests for HBV markers showed that the patient was an occult HBV carrier. Therefore, she should have been classified as an HBV carrier, not as a case of resolved HBV infection. If she had been classified as an HBV carrier, the patient should have received prophylactic treatment with entecavir in accordance with the 2009 Japanese guidelines before starting immunosuppressive therapy.

In a previous case report, anti-TNF-α therapy induced HBV reactivation followed by hepatic exacerbation in an HBsAg-negative patient [17]. However, our results, based on approximately 2 years of follow-up, suggest that HBV reactivation rarely occurs in HBsAg-negative, anti-HBc-positive patients who receive DMARDs and anti-TNF-α therapy. Another prospective study showed a high incidence of HBV reactivation in patients with resolved HBV infection who were followed up for 1 year. In particular, HBV reactivation was reported in 6 (16%) of 38 patients treated with etanercept [28]. In the present study, HBV DNA was not detected in 19 patients who received etanercept for several years. The prevalence of HBV reactivation in RA patients with resolved HBV infection thus remains controversial. The follow-up period in the present study was limited. It is necessary to monitor HBV DNA in patients with resolved HBV infection for a longer time.

In the present study, after adding anti-TNF-α, serum anti-HBs levels gradually decreased in patients with low or middle titers of anti-HBs. In addition, anti-HBs levels in patients treated with etanercept significantly decreased. A recent study has reported that the anti-HBs titer decreased during anti-TNF-α therapy in 19 HBV-vaccinated patients with RA [27]. In the present study, there were no vaccinated patients because universal vaccination for HBV was not performed in Japan. In patients who recover from acute HBV infection, anti-HBs develops in serum and persists for life at a high titer [38]. However, we did not have useful data on the natural course of the anti-HBs titer that we could compare with the data of the present study. It remains unclear whether the decrease in the anti-HBs titer in the patients with low and medium titers contributed definitively to the response to immunosuppressive therapy for RA. Basic research has suggested that TNF-α is one of the most important molecules participating in host defense mechanisms against external pathogens. TNF-α induces the proliferation of HBV-specific cytotoxic T lymphocytes and inhibits the suppressive effect of regulatory T cells on the HBV-specific immune response [39, 40]. In addition, TNF-α plays an important role in the acquisition of anti-HBs by HB vaccination [41]. It remains to be clarified whether a longer period of anti-TNF-α therapy in RA patients induces the disappearance of anti-HBs followed by HBV reactivation. The answer to this question would require studies of patients who receive aggressive immunosuppressive therapy for prolonged periods.

In conclusion, our prospective study suggested that screening for HBV DNA and prophylactic therapy with entecavir might be effective in patients with HBsAg or those with anti-HBc alone who receive immunosuppressive therapy for RA. The incidence of HBV reactivation was low in RA patients with resolved HBV infection. Future studies should evaluate anti-HBs as a monitoring marker. Our results might be useful for planning studies of HBV reactivation in patients with Crohn's disease who receive immunosuppressive therapy with anti-TNF-α.

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Conflict of interest All authors declare that we have no conflict of interest in relation to the submission of this manuscript.

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Original Article

Down-regulation of cyclin E1 expression by microRNA-195 accounts for interferon-β-induced inhibition of hepatic stellate cell proliferation

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