るとの報告がある[20].

われわれは*in vitro*において単球由来樹状細胞(MoDC)の成熟化が，細胞外のBCAA特に，バリンにより影響をうけること(図4)，肝硬変患者では健常人と比較しMoDC機能が抑制されており，細胞外のバリン濃度を高めることにより一部改善できること(図5)を報告した[10]．さらに，*ex vivo*の検討として，早朝空腹時に非代償性肝硬変患者にBCAA顆粒を経口内服させ，内服前の血漿下で末梢血単核球を刺激した場合と内服後の血漿下で刺激した場合で産生するサイトカインを比較したところ，内服後では有意にIFN-γの産生が増加した(図6)．

おわりに

近年，前向き研究によりBCAA製剤は肝硬変患者に対して肝不全イベントの低下，QOLの改善，さらに発癌リスクを低下させる効果を持つことが報告され[21)-25)]，*in vivo*でのBCAA製剤の有効性が証明されている．今後，これらのエビデンスを示す基礎的研究のよりいっそうの充実が必要と考える．

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肝硬変でのアミノ酸代謝異常が樹状細胞に与える影響

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索引用語：肝硬変，樹状細胞，分岐鎖アミノ酸，栄養，免疫

はじめに

厚生労働省の報告によると平成18年の死亡原因において肝疾患は16,248人，肝臓癌は男22,754人，女11,086人と肝臓関連死の割合は極めて高く，慢性C型肝炎患者の高齢化によりこの状況は今後もしばらく継続すると考えられる．慢性肝炎が持続すると，肝硬変・非代償性肝硬変・肝不全へと進行する．臨床的には，肝硬変に対するインターフェロン治療は奏効率・副作用の面で困難であり[1]，肝庇護療法や栄養療法が治療の主体となる．肝硬変では，糖・蛋白・アミノ酸・脂質・ビタミン・ミネラルなど極めて多岐に渡る栄養代謝障害が出現する．一方で，進行した肝硬変では発癌・肝不全だけでなく肺炎・特発性細菌性腹膜炎などの感染症が予後に深く関わることが知られている．これは，肝硬変では免疫機能低下から易感染性宿主となることに由来する

自然免疫・獲得免疫の最も重要な細胞の一つである樹状細胞(DC)は主要な抗原提示細胞であり，全身の臓器・組織・器官に存在し病原体や腫瘍に対して免疫反応を誘導・仲介する．典型的には，未熟DCは抗原を捕獲しペプチドに分解し，主要組織適合性複合体(MHC)クラスⅡもしくはⅠ分子により提示する．通常，末梢組織に存在するDCは侵入抗原から刺激を受けた後，所属リンパ節に移動して抗原をリンパ球に提示する[2]．最近の報告では，末梢組織から再度血中に戻り他の遠隔組織に移行するcirculating示唆されている[3,4]．

現在，基礎的研究では分子生物学的手法を用いた樹状細胞の細胞内シグナル解析や共刺激分子を介した他細胞との詳細な抗原提示メカニズムまで解析が進められている．

当章では肝硬変(肝臓癌)患者では健常人と

Eiji KAKAZU et al : An imbalance of plasma amino acids influences the function of dendritic cells in cirrhotic patients

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表1　慢性C型肝炎・肝硬変・肝臓癌患者の樹状細胞機能

慢性C型肝炎患者の樹状細胞機能低下	機能障害	使用培地	掲載
Kanto T	リンパ球刺激能・IL-12，IFN-γ産生低下（刺激後のCD86の低下）	血清含	J Immunol 162 : 5584–5591, 1999
Bain C	リンパ球刺激低下，表現系は差	血清含	Gastroenterology 120 : 512–524, 2001
Auffermann-Gretzinger S	リンパ球刺激能・成熟化抑制（刺激後のCD1a CD54 CD83 HLA-DR低下）　＊患者背景に肝硬変が多い	無血清	Blood 97 : 3171–3176, 2001
Longman RS	機能低下なし	血清含	Blood 103 : 1026–1029, 2004

肝硬変・肝癌患者の樹状細胞機能低下	機能障害		掲載
Ninomiya T	リンパ球刺激能・IL-12産生低下・未熟DCのHLA-DR発現低下	血清含	J Hepatology 31 : 323–331, 1999
Kakumu S	リンパ球刺激能・IL-12産生低下	血清含	J Gastroenterology and Hepatology 15 : 431–436, 2000
Beckebaum S	IL-10の産生増加	無血清	Clin Cancer Res 10 : 7260–7269, 2004
Li L	成熟化抑制	血清含	Immunol Lett 114 : 38–45, 2007
Ohno T	IFN-γの産生低下	血清含	Hepatol Res 37 : 276–285, 2007
Kakazu E	リンパ球刺激能・成熟化抑制（刺激後のCD40 CD83 HLA-DR低下）	無血清	J Immunol 179 : 7137–7146, 2007

比較して樹状細胞にどのような変化が生じているのかを過去の文献から考察するとともに，肝硬変で出現する栄養代謝異常が樹状細胞にいかなる影響を与えるのかをわれわれの施設で中心的に研究を進めているアミノ酸インバランスを中心に解説する．

2 肝硬変（肝臓癌）ではどのような樹状細胞の機能抑制が生じるのか

1999年に考藤らが，慢性C型肝炎患者の樹状細胞機能低下を報告[5]して以来，各施設から同様な報告が追従した．健常人とC型慢性肝炎患者の樹状細胞機能を比較したものを表にした（表1）．

臨床的に慢性肝炎の進行により汎血球減少が出現することはよく経験するが，その中でも樹状細胞の割合が慢性C型肝炎では低下しているとの報告が多い[6～8]．

機能に関してして樹状細胞のallostimulative capacity，IL-12産生低下，共刺激分子の発現低下などが報告されている．*in vitro*においてはHCVのコア蛋白，NS3蛋白が樹状細胞の分化を抑制する報告が存在し[9]，さらに最近ではJFH-1株を用いた樹状細胞機能解析も行われている[10]．しかし慢性C型肝炎での樹状細胞機能抑制はサブセットにより若干結果が異なり，一部には健常人と機能は変わらないとの報告も存在する[7,11]．一方肝硬変

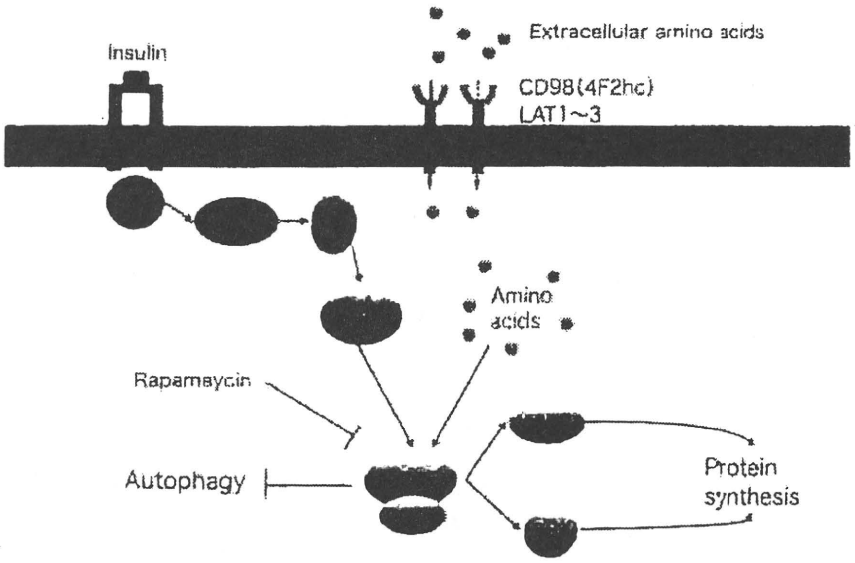

図1　mTORを中心とした栄養感受性シグナル

または肝臓癌では樹状細胞の細胞数低下，分化・成熟抑制による共刺激分子・MHCクラスⅡ発現低下，リンパ球刺激能の低下が生じることは各施設の報告で比較的一致しており，肝炎の進行・発癌により樹状細胞の数・機能低下が明瞭となる可能性が示唆される．もちろん，肝硬変とはさまざまな慢性肝疾患の最終的な病態のことであり，その病因(HCV，HBV，アルコール，NASH，肝臓癌の有無など)が樹状細胞に与える影響を解析することが優先される．しかし，肝硬変という病態が樹状細胞に与える影響を解析する際には細胞自体だけでなく細胞外環境による影響も十分考慮しなければならない．なぜなら，樹状細胞の分化・成熟は周囲環境にも大きく左右されるからである．例えば樹状細胞の培養にはヒト・ウシ血清を10％程度添加した血清培地がよく用いられるが，当然のごとく血清にはさまざまなサイトカインや未知の活性化物質が含まれそのロットにより大きく機能が左右される．健常人と患者の樹状細胞機能を比較する際に血清を用いた場合本来の樹状細胞の機能が血清によりマスクされる可能性もある．機能解析に無血清培地を用いたAuffermann-Gretzinger, S.の結果とわれわれの施設の結果が非常に類似していることからもこのことが示唆される．つまり培地に添加する糖・アミノ酸・アルブミン・脂質・PH・電解質などさまざまな細胞外の要素により樹状細胞の機能は修飾を受けるため，この点を考慮しなければ肝硬変の病態が樹状細胞機能に与える影響を説明するのは不可能である．さらに，これまでの多くの報告は末梢血単球から人工的に誘導した単球由来樹状細胞もしくは末梢血中を循環している樹状細胞を用いた解析であり，各種肝炎・肝硬変の病態を研究するには実際の肝臓内・所属リンパ節の樹状細胞機能を解析する必要がある．

3　肝硬変で出現するアミノ酸代謝異常が樹状細胞にいかなる影響を与えるのか

肝臓は栄養・代謝を司る生体内の最大の臓器であり肝硬変では糖質，蛋白・アミノ酸，

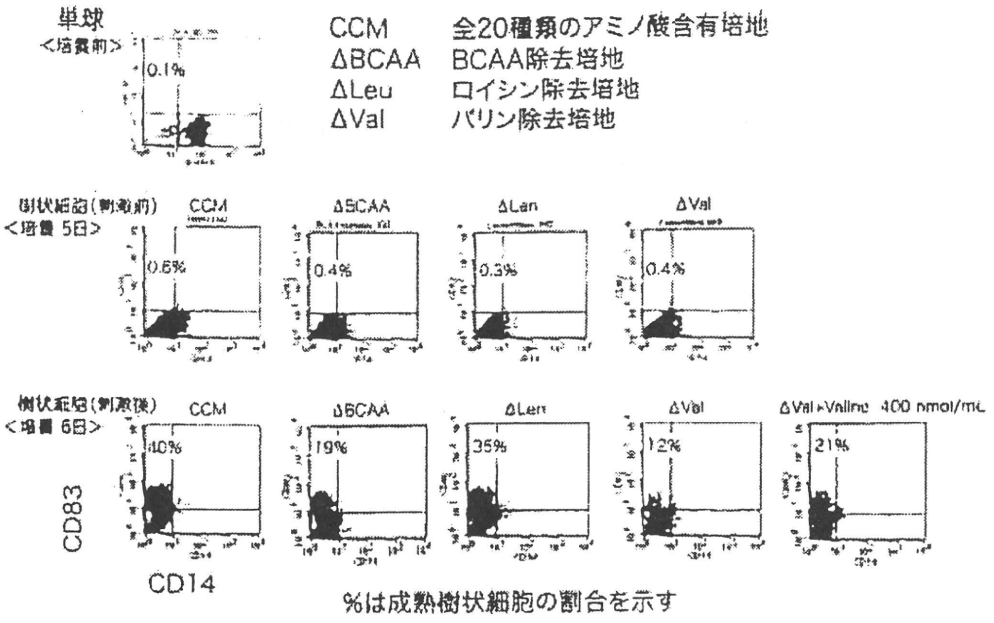

図2　分岐鎖アミノ酸の単球由来樹状細胞の分化・成熟に与える影響　(文献22より引用)

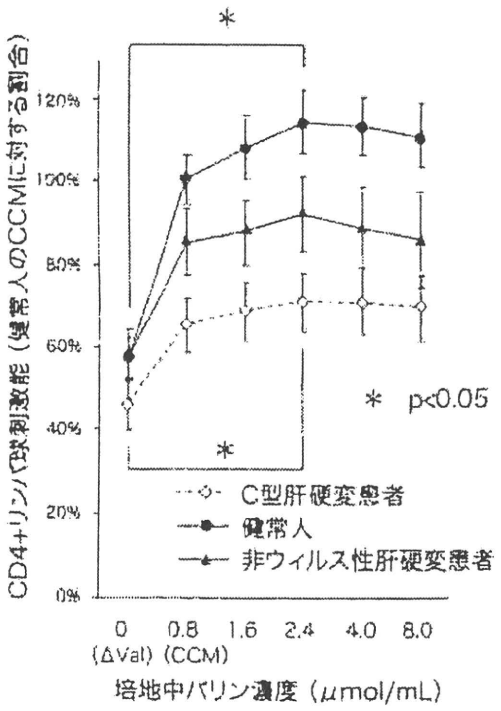

図3　培地中のバリン濃度に対する健常人と肝硬変患者の樹状細胞リンパ球刺激能
(文献22より引用)

脂質，ビタミン，微量元素など極めて多岐にわたる栄養代謝異常が認められる．アミノ酸代謝異常に関しては肝硬変では血漿アミノ酸濃度の不均衡が出現することは当時から報告[12,13]されており，芳香族アミノ酸(AAA：aromatic amino acid)が増加し分岐鎖アミノ酸(BCAA：branched chail amino acid)が低下する．最近では，肝臓の線維化に伴いこれらの異常が出現するため，肝硬変の線維化の指標として有用であるとの報告もある[14]．このような肝硬変患者のアミノ酸代謝異常を補正するBCAA製剤は最も中心的な栄養療法のひとつである．必須アミノ酸のLロイシン，Lイソロイシン，Lバリンから構成され，肝性脳症・低アルブミン血症・腹水出現予防などの効果が得られる．近年，前向き研究によりBCAA製剤は肝硬変患者に対して肝不全イベントの低下，QOLの改善，さらに発癌リスクを低下させる効果を持つことが報告され[15～17]，*in vivo*でのBCAA製剤の有効性が証明されている．しかし，BCAA製剤がなぜこのような効果をもたらすのかの実験レベルでのエビデンスは少ない．近年*in vitro*では

表2　当研究で使用した無血清培地のアミノ酸組成(単位nmol/mL)

	CCM	Δ BCAA	Δ Val	Δ Leu	Δ Ile	HCM	ACM
Glycine	400	400	400	400	400	251	280
L-Alanine	400	400	400	400	400	366	307
L-Serine	400	400	400	400	400	118	151
L-Threonine	800	800	800	800	800	128	138
L-Cystine 2HCl	200	200	200	200	200	39	67
L-Methionine	200	200	200	200	200	30	75
L-Glutamine	4000	4000	4000	4000	4000	563	689
L-Asparagine	400	400	400	400	400	71	64
L-Glutamic Acid	400	400	400	400	400	38	53
L-Aspartic Acid	400	400	400	400	400	2	4
L-Valine	800	0	0	800	800	227	175
L-Leucine	800	0	800	0	800	124	100
L-Isoleucine	800	0	800	800	0	78	53
L-Phenylalanine	400	400	400	400	400	59	99
L-Tyrosine	400	400	400	400	400	65	133
L-Tryptophan	80	80	80	80	80	56	45
L-Lysine-HCl	800	800	800	800	800	175	184
L-Arginine-HCl	400	400	400	400	400	94	92
L-Histidine HCl-H2O	200	200	200	200	200	76	85
L-Proline	400	400	400	400	400	175	176
Fisher's rate	3.00	0.00	2.00	2.00	2.00	3.45	1.42

アミノ酸はただ単に細胞骨格やさなざなな代謝経路の基質となるだけでなく，インスリンと共にmTORを介した栄養感受性シグナル経路(図1)に作用し，細胞機能を調節することが解明されてきている[18~20]．しかし，そのメカニズムに関しては依然として不明な点が多く特に分岐鎖アミノ酸が免疫機構に与える影響については現在までほとんど知られていない[21]．

最近，われわれは単球由来樹状細胞(MoDC)の成熟化が，細胞外のBCAA特に，バリンにより影響をうけること(図2)，肝硬変患者では健常人と比較しMoDC機能が抑制されており，細胞外のバリン濃度を高めることにより一部改善できること(図3)を報告した[22]．

*in vivo*の実験では，BCAAの制限食を与えたマウスと制限しないマウスにSalmonella合に前者で死亡率が有意に上昇するとの報告がある[23]．

現在，われわれの施設では健常人と非代償性肝硬変の血漿中アミノ酸濃度に一致させた2種類の無血清培地(HCM : Healthy control medium, ACM : Advanced cirrhotic mediumu)を新たに作製し(表2)，より生体内に近い環境で樹状細胞を中心とした免疫細胞機能の解析を進めている(図4)．非代償性肝硬変の血漿中のアミノ酸濃度と一致した培地において樹状細胞の成熟化・IL-12産生が低下すること，さらにこのことにより末梢血単核球(PBMC)からのIFN-γの産生が低下することが明らかとなった．これらの結果は肝細胞

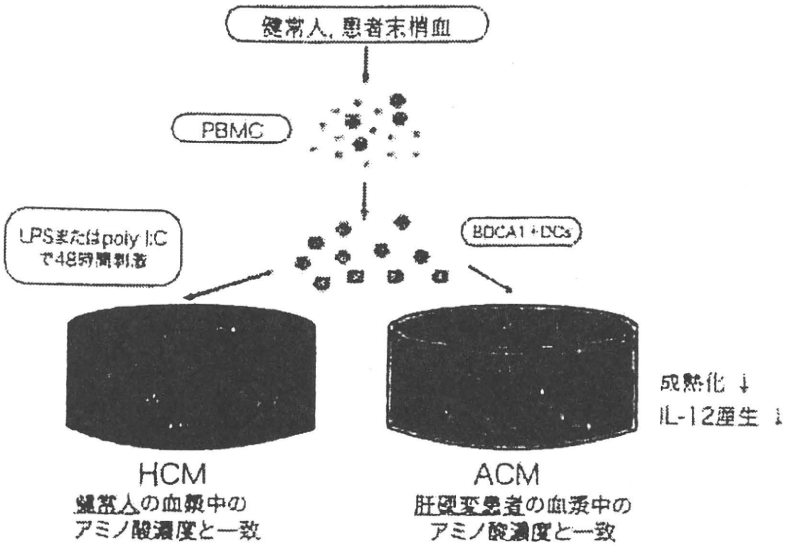

図4　健常人と肝硬変患者の血漿アミノ酸濃度と一致した培地下での樹状細胞機能

癌の有無にかかわらず，非代償性肝硬変で出現する血漿アミノ酸インバランスが樹状細胞をはじめとした免疫細胞全体の反応を抑制している可能性が示している．

細胞外アミノ酸濃度が免疫細胞機能に大きな影響を与えていることは確実であるが，それを解析する際にいくつかの問題が存在する．

①生体内の蛋白質を構成するアミノ酸は20種類存在し，それらのどのアミノ酸がどのような割合で存在するときに樹状細胞機能はじめ免疫反応全体に最も影響を与えるのか依然として不明である．また，機能解析の際に一般的に用いられるRPMI 1640，IMDM，D-MEMの組成の培地は各種アミノ酸濃度が生体内の濃度よりもはるかに高濃度であるためこの中で機能解析はできない．

②生体内においては，食事，体内水分量，さらにその局在(末梢血，門脈血，各種臓器・リンパ節など)により細胞外のアミノ酸濃度は変化し，それらが免疫細胞にいかなる影響を与えているのかを解析するのは困難な課題である．

③BCAAをはじめとした各種アミノ酸の生体に対する効果は，短期的な効果と長期的な効果(アルブミンの増加など付随的な効果)をそれぞれ分けて解析する必要がある．

以上これらの課題を解決していくことが重要である．

4 おわりに

2004年10月にペグインターフェロン＋リバビリン併用療法が保険適応になって以降多くの慢性C型肝炎患者の根治が可能となった．しかし今後は患者の高齢化により，肝硬変・非代償性肝硬変に対する加療(栄養療法)の重要性が増していくことは明白である．BCAAをはじめとした遊離アミノ酸の免疫細胞に与える影響がより詳細に解析されれば，先に述べた肝硬変患者の①感染症予防②発癌抑制③インターフェロン療法の奏功率増加などにつながる可能性がある．さらに，アミノ酸と免疫の研究は肝臓分野にとどまらず，

①免疫細胞療法において，各種癌種に対するより有効な細胞誘導法の樹立

②炎症性腸疾患に対するより有効な経腸栄

製剤の開発
に発展する可能性もある.

Lシステイン, Lトリプトファンなどの一部の単独アミノ酸の過剰投与において毒性を有するものも存在するが, アミノ酸は基本的に少量であれば副作用はほとんどないことも長所である.

近年, 樹状細胞機能とmTORシグナルの解析がトップジャーナルに掲載されている[24~26]. ようやく, 遊離アミノ酸⇒mTOR⇒樹状細胞機能解析の流れができはじめ, 今後は細胞内外の遊離アミノ酸が樹状細胞をはじめとした免疫細胞に与える影響を解析することに重要性が高まると考える.

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Add-on combination therapy with adefovir dipivoxil induces renal impairment in patients with lamivudine-refractory hepatitis B virus

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SUMMARY. Combination therapy with adefovir dipivoxil (ADV) and lamivudine (LAM) is recommended for patients infected with LAM-refractory hepatitis B virus (HBV). However, the effects of such therapy on renal function and serum phosphorus levels have not been fully evaluated. Combination therapy with ADV and LAM was given to 37 patients infected with LAM-refractory HBV, including 17 with hepatic cirrhosis. Serum HBV DNA levels decreased to below 2.6 $\log_{10}$ copies/mL in 23 (62%) of 37 patients at 12 months, 25 (78%) of 32 patients at 24 months, and 16 (84%) of 19 patients at 36 months. Except for one cirrhotic patient, serum alanine aminotransferase levels were below 50 IU/L in all patients during combination therapy. Serum creatinine levels increased in 14 (38%) of 37 patients, and serum phosphate levels decreased to below 2.5 mg/mL in 6 (16%) of 37 patients during combination therapy. Patients who received combination therapy for 36 months or longer had a significantly incidence of elevated serum creatinine levels. Fanconi syndrome occurred in a 57-year-old woman with cirrhosis after ADV was added to LAM. Combination therapy with ADV and LAM can maintain biochemical remission in patients with LAM-refractory HBV. However, the dosing interval of ADV should be adjusted according to renal function and serum phosphate levels in patients receiving long-term treatment.

Keywords: adefovir dipivoxil, Fanconi syndrome, hepatitis B virus, lamivudine.

INTRODUCTION

Hepatitis B virus (HBV) is a widely prevalent pathogen that causes chronic hepatitis, hepatic cirrhosis, and hepatocellular carcinoma (HCC) [1]. Interferon and nucleos(t)ide analogues are used for antiviral therapy in patients infected with chronic hepatitis B infection [2,3]. Lamivudine (LAM) is the first nucleoside analogue approved for the treatment of HBV-infected liver disease. LAM suppresses HBV replication in most patients and improves hepatic inflammation [4]. However, more than 60% of patients with HBV who receive long-term LAM therapy become resistant to the drug within 4 years after starting treatment [5]. For LAM-resistant HBV, a switching-to adefovir dipivoxil (ADV) or entecavir (ETV) treatment is a choice of treatment [6,7]. However, in ADV monotherapy for LAM-refractory chronic hepatitis B, virological breakthrough due to development of ADV resistant mutations occurred in three (21%) of 14 patients within 18 months [8]. In another study, ETV-resistant mutations developed in 12 (8%) of 151 patients with LAM-refractory chronic hepatitis B who received 1 mg ETV once daily for 2 years [7]. In contrast, combination therapy with ADV and LAM decreased HBV DNA levels in patients with LAM-resistant HBV and maintained the effect without virological and biochemical breakthroughs for 3 years [8,9]. In one study performed in Japan, ADV-resistant mutations occurred in only 2 of 129 patients with LAM-refractory chronic hepatitis B who received ADV plus LAM for 2 years [10].

Abbreviations: AD, adefovir dipivoxil; ALT, alanine aminotransferase; anti-HBc, antibodies to HBcAg; ETV, entecavir; HBcAg, hepatitis B c antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; LAM, lamivudine; TDF, tenofovir disoproxil fumarate.

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These studies concluded that combination therapy with ADV and LAM was the treatment of choice for patients with LAM-resistant HBV.

An important limitation of previous studies of combination therapy with ADV and LAM is the lack of adequate safety data. Monotherapy with LAM is given to more than 30 000 patients with HBV-related chronic liver disease in Japan. LAM has not been reported to induce serious adverse effects, except for the emergence of LAM-resistant HBV. On the other hand, nephrotoxicity is the dose-limiting adverse effect in the use of ADV. In phase III, randomized, controlled studies, there were no increases from baseline of 0.5 mg/dL or greater in the serum creatinine level and no confirmed instances of serum phosphate levels below 2.0 mg/dL during 48 weeks of monotherapy with 10 mg ADV [11]. However, the renal safeness of combination therapy with ADV and LAM in long-term use is not enough to be evaluated. In particular, there are few reports about decrease of serum phosphate during the combination therapy. In our hospital, an open-label study of long-term add-on treatment with ADV in patients with LAM-refractory HBV has been in progress since 2003.

In the present study, we investigated the incidence of serum creatinine increase and hypophosphorus in patients with HBV-related chronic liver diseases during long-term ADV and LAM combination therapy. In addition, clinical characteristics of patients in whom mild renal impairment was observed were evaluated, since early detection of adverse event is important.

METHODS

Patients

The study group comprised 37 consecutive Japanese patients with LAM-refractory HBV who received a combination of 100 mg of LAM plus 10 mg of ADV daily for more than 1 year in Osaka City University Hospital between September 2002 and November 2008 (Table 1). All patients had a 1.5-$\log_{10}$ copies/mL or greater increase in the serum HBV DNA level during LAM treatment. No patient had a history of treatment with other nucleoside analogues, such as ETV and famciclovir. Patients were excluded if they had antibodies to hepatitis C virus or human immunodeficiency virus (HIV). Serum creatinine levels were under 1.2 mg/dL in all patients, and creatinine clearance was over 50 mL/min in all patients except one, who had a value of 45.5 mL/min. Before adding ADV to LAM, 10 patients received curative treatment for HCC. Liver biopsy was performed in 25 patients. Hepatic cirrhosis was histologically diagnosed in 13 patients and clinically diagnosed in 4 patients with oesophageal varices. All patients gave written informed consent to undergo viral sequencing and to participate in this study.

Analysis of serological markers for HBV

Hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and antibodies to HBeAg (anti-HBe) in patient sera were tested by enzyme immunoassay, radioimmunoassay, or both, using commercially available kits (Dainabott, Tokyo, Japan).

Analysis of DNA markers for HBV

Genotypes of HBV were identified by enzyme-linked immunosorbent assay with monoclonal antibodies to type-specific epitopes in the preS2-region (Institute of Immunology, Tokyo, Japan), as described elsewhere [12]. HBV DNA was measured by transcription-mediated amplification (TMA) with a hybridization protection assay (Chugai Diagnostics, Tokyo, Japan) [13]. The detection range of the TMA assay was between 3.7 and 8.7 $\log_{10}$ copies/mL of HBV DNA. If HBV DNA was not detected by this method, we tried again, using the polymerase chain reaction (PCR)-based Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan) [14]. The detection range of the PCR assay was between 2.6 and 7.6

Table 1 Patients' characteristics at the start of adding adefovir to lamivudine

Average age years (min–max)	55 (33–69)
Gender (male/female)	25/12
Prior LAM therapy duration (min–max)	30 months (8–64)
ADV treatment duration (min–max)	38 months (15–68)
Presence of cirrhosis	17
Past history of HCC treatment	10
HBV genotype: A, B, C	2, 1, 34
HBeAg positive/negative	25/12
HBV DNA Log/mL (min–max)	6.6 (4.2–8.6)
ALT IU/L (min–max)	149 (30–397)
Histological examination (F1, F2, F3, F4, not done)	(6, 6, 0, 13, 12)
Phosphate mg/dL (min–max)	3.4 (2.6–4.6)
Creatinine mg/dL (min–max)	0.79 (0.52–1.1)
Creatinine clearance in mL/min (min–max)	87.4 (45.5–136.2)

$\log_{10}$ copies/mL. From 1 March 2008, HBV DNA was measured by the Taqman HBV test (Roche Diagnostics, Tokyo, Japan). The detection range of the Taqman HBV test is between 1.8 and 8.8 $\log_{10}$ copies/mL of HBV DNA [15].

LAM-resistant mutations in the tyrosine–methionine–aspartate–aspartate motif of the HBV polymerase gene, L80I, and ADV-resistant mutations were examined by a line-probe assay (INNO-LiPA HBV DR, Innogenetics NV, Belgium) [16].

Chemical markers in serum

Levels of alanine aminotransferase (ALT), creatinine, and phosphate were examined before and after combination therapy with ADV and LAM. Creatinine clearance was calculated with Cockcroft's formula before add-on treatment with ADV [17]. An increase in the serum creatinine level was defined as an increase equivalent to more than 130% of the creatinine level at the start of ADV add-on therapy, with no decrease in the absence of additional treatment.

Statistical analysis

Statistical analysis was performed with the Statview SE+Graphics program, version 5.0 (SAS Institute, Cary, NC, USA). The Mann–Whitney *U*-test was used to compare two continuous variables, and the chi-square test was used to compare two categorical variables. All tests were two-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 (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine.

RESULTS

Baseline characteristics of patients with LAM-refractory HBV

The HBV genotype was A in 2 patients, B in 1, and C in 34 (Table 1). At the start of add-on treatment with ADV, HBeAg was positive in 25 of the 37 patients. On analysis of the LAM resistant motif, M204I mutations were detected in 12 patients, and M204V mutations were detected in 12. In 11 patients, both mutated motifs of HBV were detected. In one of the patients with both mutations, an additional mutation (L80I) was found. LAM-resistant motifs were not examined in the other two patients. ADV-resistant mutations (A181V/T or N236T) were not detected before the start of ADV add-on treatment in any patient with LAM-resistant HBV.

Virological response to combination therapy

HBV DNA decreased to below 2.6 $\log_{10}$ copies/mL in 23 (62%) of 37 patients with LAM-refractory HBV at 12 months, 25 (78%) of 32 patients at 24 months, 16 (84%) of 19 patients at 36 months, and 8 (80%) of 10 patients at 48 months (Fig. 1). In three patients with HBeAg, the HBV DNA level did not decrease to below 4 $\log_{10}$ copies/mL during more than 30 months of combination therapy. In two of these patients, who did not have cirrhosis, A181T mutations were detected 18 months after the start of ADV add-on therapy. A181V/T or N236T mutation was not detected in the other patient, who had cirrhosis and genotype C (Table 2). Combination therapy reduced the HBV DNA level to below 2.6 $\log_{10}$ copies/mL in 10 (59%) of 17 patients with hepatic cirrhosis at 12 months, and in 12 (80%) of 15 patients with hepatic cirrhosis at 24 months. Among the 10 patients who received curative treatment for HCC before add-on treatment with ADV, combination therapy reduced the HBV DNA level to below 2.6 $\log_{10}$ copies/mL in 6 (60%) of 10 patients at 12 months and 5 (83%) of 6 patients at 24 months.

Biochemical and serological responses to combination therapy

Serum ALT levels decreased to below 50 IU/L in 26 (70%) of 37 patients with LAM-resistant HBV at 6 months, 27 (73%) of 37 patients at 12 months, 26 (81.2%) of 32 patients at 24 months, 17 (89.4%) of 19 patients at 36 months, and 9 (90%) of 10 patients at 48 months. Except for one patient with hepatic cirrhosis, serum ALT levels fell to below 50 IU/L in all patients who received ADV and LAM combination therapy (Fig. 1). HBeAg became undetectable in 6 (24%) of 25 patients at 12 months, 10 (48%) of 21 patients at 24 months, and 5 (38%) of 13 patients at 36 months.

Incidence of HCC

In 2 of 10 cirrhotic patients who received curative treatment for HCC, secondary HCC appeared during combination therapy with ADV and LAM (Table 2). One patient with HCC recurrence continuously had a serum HBV DNA level of more than 4 $\log_{10}$ copies/mL. In 4 (14.8%) of 27 patients with LAM-refractory HBV, primary HCC appeared after adding ADV to LAM treatment. Two of the four patients in whom primary HCC developed had hepatic cirrhosis at the start of add-on treatment with ADV. In one patient with cirrhosis, the serum HBV DNA level exceeded 4 $\log_{10}$ copies/mL at the time of diagnosis of HCC. In the other three patients, serum HBV DNA levels remained below 2.6 $\log_{10}$ copies/mL on the occurrence of HCC. One patient died of advanced HCC 33 months after the start of combination therapy with ADV and LAM.

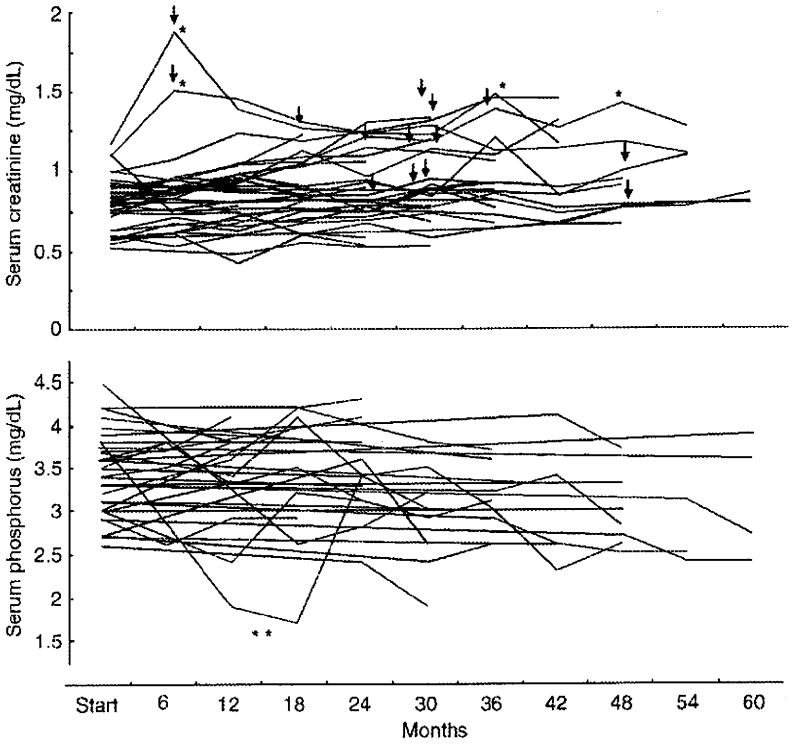

Fig. 1 Clinical courses after adding ADV to LAM in 37 patients with LAM-refractory HBV. In two patients with cirrhosis and two without cirrhosis whose serum creatinine levels rose to higher than 1.4 mg/dL, treatment with ADV was reduced from daily to every 2 days. Subsequently, the serum creatinine level decreased without reactivation of HBV replication. *Arrow* shows the time of an increase equivalent to more than 130% of the creatinine level at the start of ADV add-on therapy. *The times of adjusting the dose of ADV in the four patients with increased serum creatinine levels. **The time of adjusting the dose of ADV in a patient with Fanconi syndrome.

Table 2 Comparison of clinical characteristics and events between the cirrhotic and non-cirrhotic group during combination therapy

	Cirrhotic group	Non-cirrhotic group	*P*-value
n	17	20	
Average age years (min–max)	57 (47–69)	53 (33–68)	0.16
Gender (male/female)	13/4	10/7	0.48
Prior LAM therapy duration (min–max)	25 months (8–48)	34 months (11–64)	0.09
ADV treatment duration (min–max)	41 months (19–68)	36 months (15–65)	0.25
Past history of HCC treatment	8	2	0.03
HBeAg positive/negative	11/6	14/6	0.99
HBV DNA Log/mL (min–max)	6.4 (4.2–8.3)	6.9 (4.2–8.6)	0.28
Phosphate mg/dL (min–max)	3.3 (2.7–4.6)	3.4 (2.7–4.2)	0.61
Creatinine mg/dL (min–max)	0.78 (0.56–1.1)	0.82 (0.52–1.1)	0.42
Creatinine clearance mL/min (min–max)	85.9 (45.5–130.1)	86.4 (53.6–136.2)	0.95
Emergence of ADV-resistant HBV after adding ADV	1	2	0.88
Incidence of HCC (primary/secondary) after adding ADV	5 (2/3)	2 (2/0)	0.28

Renal impairment and hypophosphataemia during combination therapy

Serum creatinine levels gradually increased after the start of add-on treatment with 10 mg of ADV in 14 (38%) of 37 patients. In patients who received combination therapy for longer than 36 months or longer, the incidence of elevated serum creatinine levels increased significantly (Table 3). Serum creatinine did not increase in three patients whose HBV DNA level remained above 4 $\log_{10}$ copies/mL during more than 30 months of combination therapy. Except for these patients, there were no differences in clinical course between patients with creatinine increase and patients without it. In four patients (11%) whose serum creatinine levels increased to above 1.4 mg/dL, the dosing interval of ADV was adjusted to every 2 days. After this adjustment, serum creatinine levels decreased without reactivation of HBV replication. Two of these patients had progression to

Table 3 Comparison of clinical characteristics between patients with and those without an increase in serum creatinine levels

	Presence of creatinine increase	Absence of creatinine increase	*P*-value
N	14	23	
Average age years	59	53	0.07
Gender (male/female)	10/4	15/8	0.98
ADV treatment duration	45 months	34 months	0.02
Presence of cirrhosis (+/−)	9/5	8/15	0.16
HBV genotype: A, B, C	1, 0, 13	1, 1, 21	0.65
HBeAg positive/negative	11/3	14/9	0.45
HBV DNA Log/mL (min–max)	7	6.4	0.22
ALT (IU/L)	173	129	0.26
Creatinine (mg/dL)	0.82	0.77	0.35
Creatinine clearance (mL/min)	80.6	91.6	0.19

hepatic cirrhosis at the beginning of add-on treatment with ADV. The other patient without cirrhosis had hypertension as a complication. Creatinine clearance in this patient had decreased to 53.6 min/mL at the beginning of add-on treatment with ADV. The left patient did not have other complications.

Serum phosphate levels decreased to below 2.5 mg/mL in 6 (16%) of 37 patients. Serum creatinine levels increased in all six of these patients. No other variables correlated with decreased serum phosphate levels.

Case presentation

In December 2005, ADV was added to LAM therapy in a 57-year-old woman infected with YIDD-mutated HBV. She was given a clinical diagnosis of hepatic cirrhosis with no other complications, including HCC. Before combination therapy, HBeAg was negative, and the HBV DNA level was 6.5 $\log_{10}$ copies/mL. Creatinine and phosphate levels in serum were 0.56 and 3.8 mg/dL, respectively. The creatinine clearance was 56.7 mL/min. Nine months after starting combination therapy, severe lumbago developed. At 14 months, oedema of the legs and joint pain of the feet occurred. The serum alkaline phosphatase level increased to 800 IU/L, and she was admitted to our hospital. The serum HBV DNA level had decreased to less than 2.6 $\log_{10}$ copies/mL (Fig. 2). The serum phosphate level had decreased to 1.9 mg/dL, and the serum creatinine level was 0.88 mg/dL. Bone scintigraphy showed multiple-hot spots (Fig. 3). Urinalysis revealed glucosuria, proteinuria and generalized aminoaciduria. In

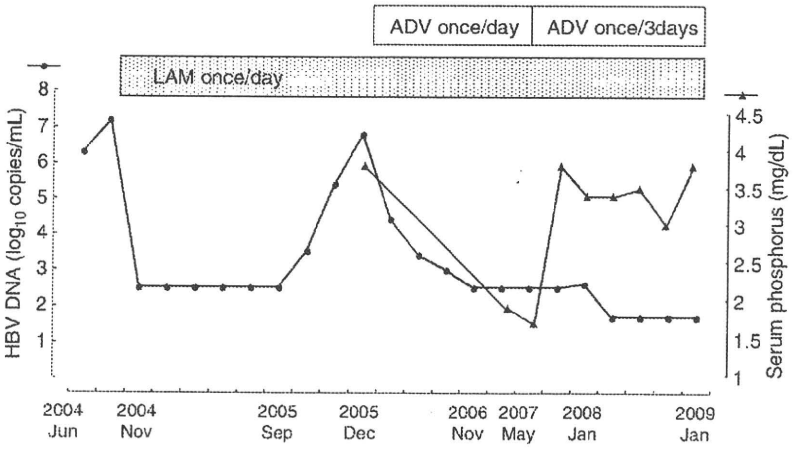

Fig. 2 Clinical course of a cirrhotic patient in whom Fanconi syndrome was induced by combination therapy. LAM treatment for HBV was started in November 2004. After 11 months, HBV DNA gradually increased, and LAM resistant YIDD-mutation was detected. In December 2005, 100 mg of ADV per day was added to LAM therapy. In May 2007, the HBV DNA level had decreased to less than 2.6 $\log_{10}$ copies/mL. However, the serum phosphate level fell to 1.9 mg/dL. After the dosing interval of ADV was adjusted to once every 3 days, the serum phosphate level increased to the normal range. The serum HBV DNA level has remained below 1.7 $\log_{10}$ copies/mL.

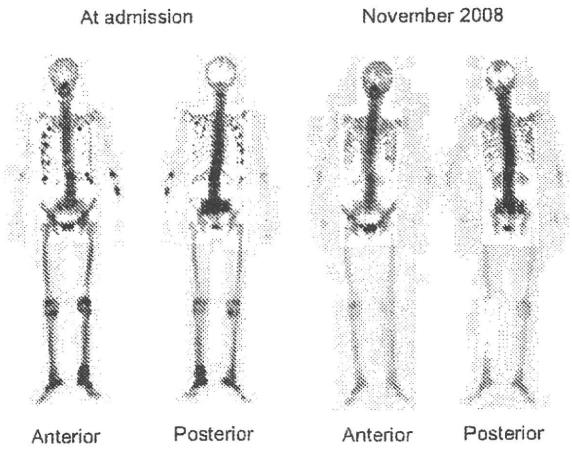

Fig. 3 Bone scintigrams of the patient in whom Fanconi syndrome was induced by combination therapy. At admission, there were many abnormal hot spots in the ribs, spine, sacrum, left humerus, and both legs. After reducing the dose of ADV and replenishment of phosphate, the abnormal spots disappeared except for the spine, which showed a compression fracture.

addition, the serum fibroblast growth factor 23 level had decreased to 3 pg/mL (normal range, 10–50 pg/mL). On the basis of these results, the patient was given a clinical diagnosis of osteomalacia due to secondary (drug-induced) Fanconi syndrome. The dosing interval of ADV was adjusted to once every 3 days, with replenishment of phosphate. After 1 month, the serum phosphate level increased dramatically to the normal range. The serum HBV DNA level has been maintained below 1.7 $\log_{10}$ copies/mL for 12 months with a combination of LAM daily and ADV every 3 days. In November 2008, the abnormal spots in bone improved without the continuous replenishment of phosphate.

DISCUSSION

Our study showed that the addition of ADV to LAM therapy decreased serum HBV DNA levels and improved elevated ALT levels in patients with LAM-refractory HBV. Combination therapy with ADV and LAM continued to be effective for more than 3 years in 19 patients. In 2 (5%) of 37 patients with LAM-resistant HBV, ADV-resistant mutants (A181T) were detected 18 months after the start of combination therapy. The effects of ADV add-on therapy in the present study were consistent with the results of previous studies [9,10]. In particular, our data showed that the antiviral effects of combination therapy in patients with hepatic cirrhosis and those who received treatment for HCC were not inferior to the effect in patients with chronic hepatitis. Three patients have received ADV add-on combination therapy for more than 5 years without elevation of HBV DNA levels. The latest examinations showed that ALT has remained below 40 IU/mL in 36 patients, including three infected with AVD-resistant HBV, during combination therapy. Although the sample size was small, our results suggest that combination therapy suppressed LAM-refractory HBV DNA levels in patients with cirrhosis or HCC and consistently improved elevated ALT levels, even after the emergence of A181T mutants.

Renal impairment is one of the most important adverse effects of ADV. The dosing interval of ADV should therefore be adjusted according to the creatinine clearance of patients. However, guidelines for dosage adjustment in patients given ADV plus LAM are lacking. In the present study, we evaluated the safety of treatment with ADV 10 mg daily added to LAM. Creatinine clearance was above 50 mL/min in all except one patient. In four (11%) patients, including one with a low creatinine clearance, the serum creatinine level increased to more than 1.4 mg/dL. After the interval between doses of ADV was adjusted to every 2 days, serum creatinine levels improved, with no increase in HBV DNA levels. A long-term study safety and efficacy study of ADV monotherapy showed that the serum creatinine level increased by at least 0.5 mg/dL as compared with the baseline value in 5 (8%) of 65 patients at 240 months [18]. In previous studies of ADV plus LAM combination therapy, daily treatment with ADV was shifted to every 2 days in 4 (3%) of 132 patients or 10 (7%) of 145 patients because the serum creatinine level rose by more than 0.5 mg/dL [9,10]. To evaluate slight alterations in renal function, we defined elevation of the serum creatinine level as a 30% increase from the baseline value. Elevations of serum creatinine were detected in 14 (38%) patients. The incidence of elevated serum creatinine levels was significantly higher in patients who received ADV plus LAM combination therapy for 36 months or longer. These results suggested that patients who receive long-term combination therapy are at risk for renal impairment. In the present study, 19 of 37 patients, including 17 with cirrhosis and 2 without cirrhosis who had received treatment for HCC, had a high risk of HCC. Computed tomography with contrast medium was repeatedly performed to detect the onset or recurrence of HCC. In addition to ADV, contrast medium might have contributed to renal impairment.

Some drugs have been reported to induce renal proximal tubulopathy in association with decreased reabsorption of phosphate. Serum phosphate concentrations were not enough to be evaluated in patients given ADV and LAM combination therapy. In our study, the serum phosphate level decreased to below 2.5 mg/mL in 6 (16.2%) of 37 patients during combination therapy. Serum creatinine levels increased in all six of these patients. It was suggested that decreased phosphate levels were accompanied by increased creatinine levels. In particular, Fanconi syndrome developed in one patient in whom the serum phosphate level decreased to 1.9 mg/dL. To our knowledge, this is the first case of combination therapy-related Fanconi syndrome to be reported. Tenofovir disoproxil fumarate (TDF), an anti- HIV drug, was approved for the treatment of patients with HBV

in the United States [19,20]. This is an acyclic nucleotide analogue with a molecular structure related to that of ADV. Recent study showed that 300 mg of TDF treatment had superior antiviral effect to patients with chronic hepatitis B compared to 10 mg of ADV treatment. The serious clinical adverse event related to TDF did not occurred during 48 weeks of the administration [21].

However, Fanconi syndrome was reported to have developed in a 45-year-old cirrhotic woman coinfected with HIV and HCV during treatment with TDF [22]. To quantify the risk of Fanconi syndrome, renal proximal tubulopathy should be assessed in large numbers of patients with HBV during nucleotide therapy, including a combination of ADV and LAM.

In conclusion, our study showed that combination therapy with ADV and LAM effectively suppressed HBV replication and maintained biochemical remission in patients who have chronic liver disease associated with LAM-refractory HBV. However, it is important to closely monitor renal function and serum phosphate levels in patients with cirrhosis, as well as those who receive long-term antiviral therapy. Renal impairment improved without increased HBV replication after adjusting the dosing interval of ADV.

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CONFLICT OF INTEREST

None.

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Original Article

Usefulness of transient elastography for assessment of liver fibrosis in chronic hepatitis B: Regression of liver stiffness during entecavir therapy

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Aim: The usefulness of transient elastography remains to be validated in chronic hepatitis B, particularly as a tool for monitoring the degree of liver fibrosis during treatment.

Methods: The subjects were 50 patients with chronic hepatitis B virus infection. Liver biopsy was performed in 38 patients, and in 12 patients with platelet counts of 50×10^9/L or less, cirrhosis was clinically diagnosed on the basis of specific signs of portal hypertension. Liver stiffness was measured by transient elastography at baseline and after 12 months of treatment in 20 nucleos(t)ide-naïve patients who started entecavir within 3 months after study entry.

Results: Twenty (40%) patients were classified as F1, 10 (20%) as F2, 5 (10%) as F3, and 15 (30%) as F4 (cirrhosis). Median liver stiffness (interquartile range) was 7.0 kPa (5.6–9.4), 9.8 kPa (5.6–14.7), 9.8 kPa (7.6–12.9), and 17.3 kPa (8.2–27.6) in fibrosis stages F1 to F4, respectively. Liver stiffness significantly correlated with fibrosis stage ($r = 0.46$; $P = 0.0014$). Of the patients who started entecavir, median liver stiffness significantly decreased from 11.2 kPa (7.0–15.2) to 7.8 kPa (5.1–11.9; $P = 0.0090$) during 12 months of treatment. Median levels of amino-terminal peptide of type III procollagen and type IV collagen 7S domain in serum significantly decreased from 0.9 (0.6–1.3) to 0.6 (0.5–0.7) U/mL ($P = 0.0010$) and from 5.0 (4.4–6.7) to 3.9 (3.2–4.4) ng/mL ($P = 0.015$), respectively.

Conclusion: Liver stiffness measurement can be useful for monitoring regression of liver fibrosis during entecavir treatment in patients with chronic hepatitis B virus infection.

Key words: chronic hepatitis B virus, entecavir, FibroScan, liver stiffness, transient elastography.

INTRODUCTION

INFECTION WITH HEPATITIS B virus (HBV) remains an important public health problem and a leading cause of liver-related morbidity worldwide.[1] Currently available antiviral therapy for chronic HBV includes the immunomodulator interferon and oral nucleos(t)ide analogues.[2,3] Entecavir, a cyclopentyl guanosine analogue, is the most potent agent against HBV among licensed nucleos(t)ide analogues and is used as the first-line treatment of choice for chronic HBV infection. Randomized controlled trials have demonstrated not only virological and biochemical responses, but also a histological response (defined as 2-point reduction in the Knodell necroinflammatory score without progression to fibrosis) in patients treated with entecavir.[4,5] A *post hoc* descriptive analysis of the trials showed that the stage of fibrosis was improved in 58% of nucleos(t)ide-naïve patients with advanced liver fibrosis by 52 weeks of entecavir treatment.[6]

Liver biopsy is considered the gold standard for diagnosing chronic liver disease, grading necroinflammatory activity, and staging liver fibrosis. However, sampling error can lead to underestimation of the degree of liver fibrosis, especially when biopsy specimens are small or fragmented. In addition, interpretation of the results is subject to significant intra- and inter-observer

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variability. Moreover, liver biopsy is not suitable for repeated evaluations, because it is invasive and can cause major complications (0.3–0.5%), including death (0.03–0.1%).[7,8] Several surrogate serum markers and laboratory indices/scores thus have been proposed as alternative techniques for the non-invasive assessment of liver fibrosis.[9,10] More recently, transient elastography (FibroScan; Echosens, Paris) has been introduced and found to be a rapid, objective, and promising technique for staging liver fibrosis by measuring liver stiffness.[11,12] In a meta-analysis of 50 studies done mainly in chronic hepatitis C, the mean areas under the receiver operating characteristic curve for the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis were 0.84, 0.89, and 0.94, respectively.[13]

Compared to hepatitis C virus, the usefulness of transient elastography has been less extensively studied and validated in chronic HBV. In particular, transient elastography has not been evaluated as a tool for monitoring regression of liver fibrosis during antiviral treatment for chronic HBV, although patients' acceptance of repeated evaluations is excellent.

The aim of this study was to determine whether liver stiffness as measured by transient elastography can be used for on-treatment monitoring of the effects of entecavir on liver fibrosis in patients with chronic HBV. First, we evaluated the correlation between liver stiffness and the stage of fibrosis in 50 patients with chronic HBV. Second, in 20 patients who started entecavir within 3 months after study entry, liver stiffness, as well as serum levels of liver fibrosis markers, was measured at baseline and after 12 months of treatment.

METHODS

Patients

THE FLOW OF the participants through the trial is shown in Figure 1. Between April 2005 and June 2008, 38 patients with chronic HBV in whom liver biopsy was clinically indicated were admitted to our hospital. During the same period, 12 other patients with chronic HBV in whom percutaneous liver biopsy was contraindicated by a low platelet count ($\leq 50 \times 10^9$/L) received a clinical diagnosis of cirrhosis on the basis of specific signs of portal hypertension, such as esophageal varices.

A total of 50 consecutive patients with chronic HBV were included in this study. We excluded patients who had antibodies to hepatitis C virus and other likely causes of chronic liver diseases; ascites and other clinical signs of decompensated liver disease; acute exacerbations of liver disease, defined as a rise in alanine aminotransferase (ALT) to 10-times higher than the upper limit of normal; or hepatocellular carcinoma detectable by ultrasonography in the target area for liver stiffness measurement. Informed consent was obtained from each patient. The procedures of the study were in accordance with the Helsinki Declaration of 1975 (2000 revision).

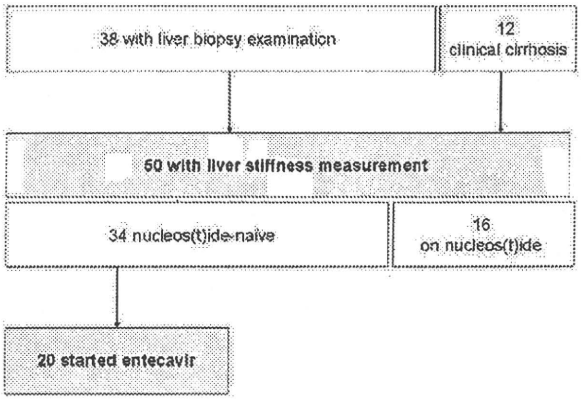

Figure 1 Flow of participants through the trial. Of the 50 patients studied, 16 were receiving nucleos(t)ide analogue treatment at entry. Of the remaining 34 nucleos(t)ide-naïve patients, 20 patients started entecavir within 3 months of entry.

Liver histology and quantification of liver fibrosis

Liver biopsies were performed with a 15-gauge Tru-Cut needle (Hakko, Tokyo) under ultrasound guidance. Liver tissues were fixed in formalin immediately after biopsy and embedded in paraffin. Four-micrometre-thick sections were stained with hematoxylin-eosin and Sirius red and immunostained with α-smooth muscle actin (Dako, Glostrup). The stage of liver fibrosis and grade of necroinflammatory activity were evaluated semiquantitatively according to the METAVIR scoring system as follows: F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[14] METAVIR activity scores were defined as follows: A1, mild; A2, moderate; and A3, severe. A liver sample was considered adequate if it was longer than 15 mm and contained six or more portal tracts.

In some biopsy specimens, morphometric image analysis was performed with a computerized system, consisting of a photomicroscope, digital camera, and

LuminaVision 2.4 bio-imaging software (Mitani, Tokyo), to quantitatively assess fibrosis. The proportion of area stained with Sirius red or α-smooth muscle actin in liver-biopsy sections was calculated as the sum of the pixel-wise bound stain measurements divided by the number of summed pixels.

Liver stiffness measurement

Liver stiffness was measured by transient elastography (FibroScan; EchoSens)[11,12] within 3 months of study entry. Briefly, this system is equipped with a probe including an ultrasonic transducer mounted on the axis of a vibrator. The subject laid down on a bed in the horizontal supine position with the right arm in maximal abduction, and a probe was placed on the skin above the right intercostal space. A vibration transmitted from the vibrator toward the tissue induces an elastic shear wave that propagates through the right lobe of the liver. These propagations are followed by pulse-echo ultrasound acquisitions, and their velocity, which is directly related to tissue stiffness, is measured. The median of ten successful measurements was expressed in units of kilopascals (kPa) and used as the liver stiffness for a given subject. Performance was considered optimal when the rate of successful measurements to the total number of acquisitions was at least 60% and the ratio of the interquartile range to the median value did not exceed 30%.

Biochemical, hematological, and virological examinations

The following variables were determined at baseline: serum ALT activity, platelet count, HBV surface antigen, HBV e antigen (HBeAg), anti-HBe, HBV genotypes, and HBV DNA levels. HBV surface antigen, HBeAg, and anti-HBe were detected by chemiluminescence enzyme immunoassay. Genotypes of HBV were identified by enzyme-linked immunosorbent assay (Institute of Immunology, Tokyo).[15] HBV DNA was measured by transcription-mediated amplification with a hybridization protection assay (Chugai Diagnostics, Tokyo); the detection range was 3.7–8.7 $\log_{10}$ copies/mL.[16] If HBV DNA was not detected by this method, a PCR-based Amplicor Monitor test (Roche Molecular Systems, Pleasanton, CA) was utilized; the detection range was 2.6–7.6 $\log_{10}$ copies/mL.[17]

Surrogate serum markers of liver fibrosis

In patients who started entecavir within 3 months of entry, two serum markers of liver fibrosis were measured at baseline and after 12 months of treatment. Serum levels of amino-terminal peptide of type III procollagen (PIIINP) were measured by radioimmunoassay (Nihon Schering K.K., Osaka), with a normal range of 0.3–0.8 U/mL. Serum levels of type IV collagen 7S were measured by radioimmunoassay (Mitsubishi Kagaku Iatron, Tokyo), with a normal range of not more than 6 ng/mL.

Statistical analysis

Statistical analysis was performed with the Statview SE + Graphics program, version 5.0 (SAS Institute, Cary, NC). Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Differences in proportions were evaluated by Fisher's exact test. The significance of correlation was tested by Spearman's rank analysis. The significance of changes in values between two time points was evaluated by the Wilcoxon signed-rank test. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of patients

TABLE 1 SHOWS the baseline characteristics of the enrolled patients with chronic HBV. Mean body mass index was 22.4 kg/m^2; six patients were overweight (25–30 kg/m^2), and no patient was obese (>30 kg/m^2). Among all 50 subjects, 16 were receiving nucleos(t)ide analogue treatment at entry: 5 were receiving lamivudine, 2 lamivudine plus adefovir dipivoxil, and 9 entecavir (Fig. 1). Among the remaining 34 nucleos(t)ide-naïve patients, 20 started entecavir at an oral dose of 0.5 mg once daily within 3 months after entry. The 20 patients who started entecavir had significantly higher ALT ($P = 0.018$) and HBV DNA levels ($P = 0.011$) and a lower stage of fibrosis ($P = 0.0016$) than the other 30 patients.

Liver stiffness and fibrosis stage

The liver stiffness measurements are shown according to the stage of fibrosis in Figure 2. Of the 50 enrolled patients with chronic HBV, 20 (40%) were classified as F1, 10 (20%) as F2, 5 (10%) as F3, and 3 (6%) as F4 on liver biopsies; cirrhosis was clinically diagnosed in 12 (24%) patients. When clinical cirrhosis was combined with histological F4, median liver stiffness (interquartile range) was 7.0 kPa (5.6–9.4), 9.8 kPa (5.6–14.7), 9.8 kPa (7.6–12.9), and 17.3 kPa (8.2–27.6) in fibrosis stages F1 to F4, respectively. Liver stiffness significantly correlated with the stage of fibrosis ($r = 0.46$;