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Research Article

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Evolution of hepatitis B genotype C viral quasi-species during hepatitis B e antigen seroconversion

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Background & Aims: Although the evolution of viral quasi-species may be related to the pathological status of disease, little is known about this phenomenon in hepatitis B, particularly with respect to hepatitis B e antigen (HBeAg) seroconversion.

Methods: Nucleotide sequences of the hepatitis B virus (HBV) *X/precore/core* region was analyzed at five time-points in four groups of chronic hepatitis B patients, interferon-induced seroconverters (IS, $N = 9$), interferon non-responders (IN, $N = 9$), spontaneous seroconverters (SS, $N = 9$), and non-seroconverters (SN, $N = 9$) followed during 60 months on an average. Only patients with genotype C were studied.

Results: Analysis of 1800 nucleotide sequences showed that there was no statistical difference between the nucleotide genetic distances of seroconverters (IS and SS; 6.9×10^{-3} substitutions (st)/site and 6.7×10^{-3} st/site, respectively) and those of non-seroconverters (IN and SN; 5.3×10^{-3} st/site and 3.8×10^{-3} st/site, respectively) before seroconversion. Compared to non-seroconverters (IN and SN; 5.1×10^{-3} st/site and 5.9×10^{-3} st/site, respectively), the sequence diversity of seroconverters (IS and SS; 10.9×10^{-3} st/site and 9.9×10^{-3} st/site, respectively) was significantly higher after seroconversion ($p < 0.05$), and was higher in seroconverters after seroconversion than before seroconversion ($p < 0.05$), while this changed very little in non-seroconverters during the observation period. Phylogenetic trees showed greater complexity in secoconverters than non-seroconverters. Parsimony-based estimation of the direction of sequence change between descendants and ancestors before HBeAg seroconversion, revealed higher frequencies of transversional A to T substitution in seroconverters (0.06 *vs.* 0.02, $p = 0.0036$) that coincided with the dynamics of quasi-species possessing A1762T mutation.

Conclusions: The distinctly greater viral diversity in HBeAg seroconverters after seroconversion could be related to escape mutants resulting from stronger selection pressure.

Keywords: Chronic hepatitis B; Quasi-species; Hepatitis B e antigen seroconversion.

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Abbreviations: SC, seroconversion; ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IS, interferon-induced HBeAg seroconverters; IN, IFN non-responders; SS, spontaneous seroconverters; SN, non-seroconverters.

Introduction

Hepatitis B virus (HBV) is a major human pathogen which can cause severe hepatic disease, including chronic hepatitis, cirrhosis (LC), and hepatocellular carcinoma (HCC). Quasi-species comprises a complex and dynamic distribution of non-identical but related genomes [1]. The evolution of viral quasi-species has been reported as important in the pathogenesis of RNA viruses such as hepatitis C virus [2–6] and human immunodeficiency virus [7–10], but little is known about HBV. HBV is a hepatotropic, non-cytopathic DNA virus replicated by an error-prone polymerase through an RNA intermediate. Because of this feature, the replication of HBV lacks fidelity. This results in a complex distributions of genomes with naturally-acquired mutations or mutations selected by either antiviral therapy or the immune response of the host. HBV quasi-species have not been subjected to detailed investigation, especially in the context of hepatitis B e antigen (HBeAg) seroconversion (SC), an immunologically mediated event. Whether there is a causal relationship between HBV seroconversion and HBV quasi-species remains unclear. HBV-related disease is known to be mediated both virologically and immunologically. Several studies have depicted the dynamic evolution of HBV quasi-species during lamivudine resistance or multiple drug resistance. This highlights the importance of HBV molecular evolution in revealing the mechanism of drug resistance [11,12]. HBV-specific cytotoxic T-cells play a significant role in the control of replication of HBV, which has been well documented in the literature [13–16].

Precore/core protein is the target of immunologically mediated HBeAg seroconversion. When the *precore/core* gene in HBV DNA is transcribed and translated, HBeAg is produced and secreted into the circulation [17,18]. But the synthesis and secretion of HBeAg are aborted by the emergence of a point mutation from G to A at nucleotide (nt)1896 (G1896A). Convincing lines of evidence have indicated a close association between HBeAg/anti-HBe seroconversion and the emergence of *precore* and *core* promoter mutations [19,20].

Research Article

The purpose of this study was to elucidate the evolution of HBV quasi-species during HBeAg seroconversion. The results might help us to better understand the pathogenic mechanisms of HBV. We selected patients with well-characterized clinical phenotypes and compared their viral diversity based on the nucleotide sequences of the *X*/precore/core region. *Precore* and *core* promoter mutations were also investigated in detail before and after HBeAg seroconversion.

Materials and methods

Patients

Sera from 36 chronic hepatitis B patients with well-characterized clinical follow-up for >5 years were selected from a chronic hepatitis B database (77 seroconverters and 67 non-seroconverters) at Chiba University Hospital. Only patients with genotype C (subtype C2) were studied to ensure that differences found in viral evolution were not due to genotypic variation. Nine patients in each group were selected randomly if they fulfilled the following criteria and had sufficiently long follow-up. The index group comprised patients with documented HBeAg seroconversion (spontaneous seroconverters, SS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−25.2 ± 6.2/months), time-point II (−11.6 ± 2.7/months), time-point III (1 ± 2.3/months), time-point IV (12.5 ± 3.3/months), and time-point V (25 ± 3.6 months). Untreated control patients included those who were followed for a similar period of time and were persistently HBeAg positive (non-seroconverters, SN), and they were matched for average age of seroconversion and time-point intervals of the SS group. A second index group of patients with interferon (IFN)-induced HBeAg seroconversion (IFN seroconverters, IS), with serum at the following time-points relative to HBeAg seroconversion: time-point I (−24.3 ± 3.1/months), time-point II (−11.2 ± 1.9/months), time-point III (1 ± 1.2/months), time-point IV (12.7 ± 1.7/months), and time-point V (25.4 ± 2.2/months). Control patients were persistently HBeAg-positive despite IFN therapy (IFN non-responders, IN). Controls were matched for the average age of seroconversion, sex and time-point intervals of the IS group.

HBeAg seroconversion was defined as the loss of HBeAg and the development of anti-HBe. The serial serum samples in this study were taken at five time-points for each patient, as described above. This study was approved by the Ethics Committee of Chiba University Hospital.

Serological examination

HBsAg, HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, Chicago, IL). HBV genotype was determined from the patients' sera by ELISA (HBV genotype EIA; Tokushu-Meneki Laboratory, Tokyo, Japan), based on the method described by Usuda et al. [21]. Serum HBV DNA levels were monitored using the Roche Amplicor Monitor test (Roche Diagnostics, Tokyo, Japan), which has a lower detection limit of 2.6 $\log_{10}$ copies/ml, at each time-point.

Cloning and sequencing

Total DNA was extracted from 200 μl of each serum sample using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 200 μl distilled water. Because HBeAg seroconversion is associated with a decrease in HBV DNA levels, nested PCR was performed for all the samples. The primers for the first round of PCR were 5′-TCG CAT GGA GAC CAC CGT GA-3′ (sense, nt1604–1623) and 5′-ATA GCT TGC CTG AGT GC-3′ (antisense, nt 2076–2060). The primers for the second round of PCR were 5′-CAT AAG AGG ACT CTT GGA CT-3′ (sense, nt 1653–1672) and 5′-GGA AAG AAG TCA GAA GGC-3′ (antisense, nt 1974–1957).

Amplification was performed with 2 μl of DNA template (extracted DNA from serum samples for the first round PCR and the first round PCR products for the second round PCR) in 50 μl reaction under the following conditions: an initial 2 min of denaturation at 94 °C and 36 cycles of 94 °C denaturation for 1 min, annealing at either 54 °C or 52 °C for 1 min, in the first and second round respectively, and 72 °C extension for 1 min. The last cycle was followed by a final extension at 72 °C for 10 min. A 473-base pairs fragment (nt 1604–2076) containing the *X/precore/core* region was amplified.

PCR reactions were followed by cloning using TOPO® TA cloning kits (Invitrogen, Carlsbad, CA). All PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), then cloned into the TOPO vector, and transformed into *Escherichia coli*. At least 15 clones per one cloning for samples from PCR reactions proceeded subsequent to the electrophoretic size separation on 1.2% agarose gel. Ten positive clones per cloning for samples from each PCR reaction were sequenced using BigDye® Terminator and a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The cloning PCR and sequencing primers were M13-forward, 5′-GTA AAA CGA CGG CCA GT-3′, and M13-reverse, 5′-GGA AAC AGC TAT GAC CAT G-3′.

Sequence analysis

The DNAPARS program from PHYLIP v3.65 package, implemented in Simmonic Sequence Editor version 1.5 [22], was used for sequence analysis. To evaluate quasi-species-based evolution of HBV strains in chronic patients, sequences of clones ($N = 10$) isolated at each time-point ($N = 5$) from individual patients ($N = 36$) were subjected to alignment and used to generate one parsimonious ancestral sequence. Maximum nucleotide composition distances were evaluated pair-wise between the ancestral sequence and the sequences of each of the 10 clones with a mean value estimated for each patient at a given time-point (*MEGA* version 4 [23]). All patients were categorized into four groups with respect to seroconversion status and the mean distance value for each group was calculated for each time-point.

The differences in genetic distance among clinical groups and time-points, and diversity at each time-point, were analyzed using ANOVA (analysis of variance). Student's *t*-test was also performed to determine the average of genetic diversities in non-seroconverters. All graphical data are presented as means ± standard deviation (SD). Results were considered statistically significant at $p < 0.05$. The statistical analysis was performed with SPSS (2004; SPSS Inc., Tokyo, Japan).

Construction of phylogenetic trees

To examine the evolution of the viral sequence and whether this evolution was elicited by quasi-species or mutagenesis, phylogenetic trees were constructed using the Neighbor-Joining (NJ) model with the Simmonic Sequence Editor version 1.5, based on the genomic sequences of HBV. Moreover, to investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 were further analyzed phylogenetically. Neighbor-Joining trees were constructed at time-points 1 and 2 (Fig. S1 and S2, respectively) using all groups of sequences.

Results

Baseline clinical characteristics of the patients and sequential levels of serum ALT and HBV DNA

The clinical and laboratory characteristics of all patients are listed in Table 1. The levels of alanine aminotransferase (ALT) and HBV DNA over time are illustrated in Fig. 1A and B, respectively. Serum ALT levels, a marker of hepatocyte damage, normalized after seroconversion and, for all groups except the interferon non-responders, were <40 IU/L at the end-point of observation. HBV DNA loads decreased markedly in seroconverters (<3 $\log_{10}$ copies/ml, $p < 0.0001$) but changed very little in non-seroconverters. It is noteworthy that, at the second year after seroconversion, serum HBV DNA loads increased in interferon-induced seroconverters compared to spontaneous seroconverters, without statistical significance ($p^H = 0.1087$) (Fig. 1B).

Viral nucleotide sequence diversity

Viral sequence diversity, phylogenetic trees, and mutation pattern based on 1800 HBV nucleotide sequences from clones of the *X/precore/core* region, were analyzed among selected patients.

Table 1. Baseline clinical features of patients.

	IFN Seroconverters (IS)	IFN Non-seroconverters (IN)	Spontaneous Seroconverters (SS)	Spontaneous Non-seroconverters (SN)
Age (y)	40 ± 8	40 ± 11	29 ± 10	34 ± 6
Male : Female	6:3	8:1	5:4	7:2
HBV DNA (log_{10} copies/ml)	6.8 ± 0.9	6.8 ± 1.0	6.8 ± 1.2	7.1 ± 0.8
ALT (IU/L)	88.3 ± 48.6	94.3 ± 144.4	89.8 ± 71.4	67.6 ± 48.7

Note: The IFN-induced group (seroconverters and non-responders) was older than the spontaneous group (seroconverters and non-responders). Males were the majority in all groups. Baseline serum HBV DNA and ALT levels are similar among the four groups. Data are shown as mean ± SD.

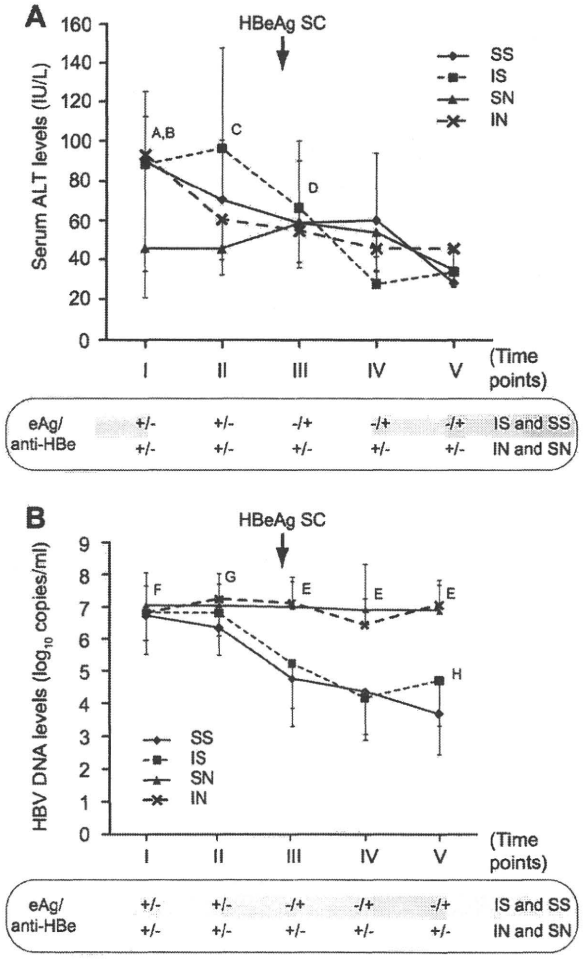

Fig. 1. Serum ALT and DNA levels in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk, and non-seroconverters controls (SN) is a solid line triangle. (A) $p^A = 0.0234$ comparing time-point I with time-point IV, $p^B = 0.0028$ comparing time-point I with time-point V, $p^C = 0.007$ comparing time-point II with time-point V, $p^D = 0.0068$ comparing time-point III with time-point V. (B) $p^E < 0.0001$ comparing seroconverters with non-seroconverters, $p^F < 0.0001$ comparing time-point I with III, IV, V, $p^G < 0.0001$ comparing time-point II with the other time-points, $p^H = 0.1087$ at time-point V in seroconverters.

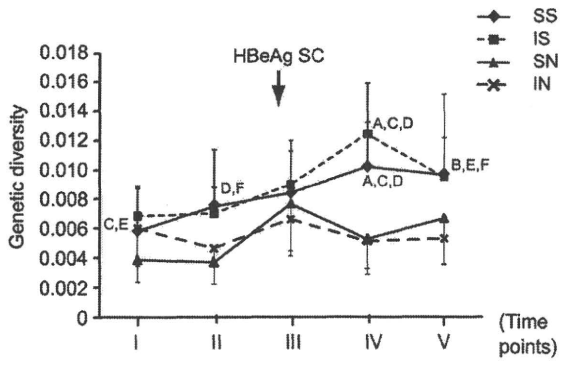

Fig. 2. Viral genetic diversity in the four groups. The group of spontaneous seroconverters (SS) is a solid line diamond, IFN-induced seroconverters (IS) is a broken line square, IFN non-responders (IN) is a broken line asterisk and non-seroconverters controls (SN) is a solid line triangle. $p^A < 0.0001$ comparing seroconverters with non-seroconverters at time-point IV, $p^B = 0.0301$ comparing seroconverters with non-seroconverters at time-point V, $p^C = 0.0013$ and $p^D = 0.0025$ comparing I and II with time-point IV in seroconverters. $p^E = 0.0121$ and $p^F = 0.021$ comparing time-points I and II with V in seroconverters.

Striking differences in nucleotide sequence diversity were revealed between seroconverters and non-seroconverters before and after seroconversion (Fig. 2). The nucleotide sequence diversity of seroconverters was similar to that of non-seroconverters before seroconversion. Analysis of genetic distance showed that the viral sequence diversity of seroconverters was significantly greater than that of non-seroconverters after seroconversion (Fig. 2, $p^A < 0.0001$ at time-point IV, $p^B = 0.0301$ at time-point V) and was greater in seroconverters after seroconversion than before (Fig. 2, $p^C = 0.0013$ and $p^D = 0.0025$), while almost no changes were observed in non-seroconverters during the observation period.

It is noteworthy that, in interferon-induced seroconverters at the last time-point of observation, the nucleotide sequence diversity was less, although this increased clearly at the first year after seroconversion. This tendency of reversed change at the last two time-points was also seen in HBV DNA loads (Fig. 1B), namely, increase or decrease of the genetic diversity accompanied by decrease or increase of the viral load in interferon-induced seroconverters. On the other hand, the nucleotide sequence diversity increased continuously in spontaneous seroconverters, accompanied by a concurrent decrease of viral loads (Fig. 1B) during the follow-up period. Amino acid sequence diversity had an almost

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Table 3. *Core* promoter and *precore* mutations in seroconverters (IS and SS).

Patients	CP (ntA1762T/G1764A) (percent)			PC (ntG1896A) (percent)			DNA Loads (log_{10} copies/ml)			Histological diagnosis
	I	III	V	I	III	V	I	III	V	
IS1	100	100	100	0	0	70	5.7	3.8	4.8	CHB
IS2	100	100	100	90	100	90	7.6	7.2	7.6	HCC
IS3	70	100	100	10	0	10	6.5	5.2	5.5	CHB
IS4	90	100	10	0	10	0	7.6	6.2	3.3	CHB
IS5	100	40	20	0	0	0	7.6	3.6	4.1	CHB
IS6	70	90	90	20	10	90	5.7	4.1	4.5	HCC
IS7	100	100	90	0	10	10	7.2	3.1	3.4	LC
IS8	80	100	60	0	0	60	7.6	4.0	4.5	CHB
IS9	100	100	10	0	0	0	6.0	4.5	4.8	HCC
SS1	0	60	0	80	0	80	7.6	4.2	5.4	HCC
SS2	80	100	90	10	90	10	6.6	7.6	5.9	ASC
SS3	100	90	60	10	0	0	6.5	4.3	2.8	ASC
SS6	30	100	10	0	0	10	3.9	4.4	4.1	CHB
SS7	80	100	100	0	0	0	7.6	2.8	2.6	ASC
SS8	0	100	90	0	20	0	7.6	5.4	3.6	CHB
SS9	0	80	20	0	10	0	7.6	4.0	2.6	CHB
SS10	50	20	40	0	0	40	7.3	3.9	2.6	CHB
SS11	100	100	100	0	0	0	6.1	6.3	3.8	CHB

IS: interferon induced seroconverter; SS: spontaneous seroconverter; ASC: asymptomatic carriers; CHB: chronic hepatitis B; LC: cirrhosis; HCC: hepatocellular carcinoma.

ratio of T1762A quasi-species between seroconverters and non-seroconverters, indicating that it might be a marker preceding seroconversion in HBV/genotype C-infected patients as reported previously [35–37].

HBeAg seroconversion is an incomplete marker of immune control, although most patients experience some clinical benefit from it [38,39]. Previous studies have shown that the average rate of spontaneous HBeAg seroconversion in patients with chronic hepatitis B is about 10% per year [40,41]. HBeAg seroconversion associated with incomplete viral suppression may result in the emergence of the *precore* mutant and attendant chronic sequelae. Mutations in the *precore* and *core* promoter regions of the HBV genome have been reported in many HBeAg-negative CHB patients. Longitudinal studies found that the A1896 mutation emerges or is selected around the time of HBeAg seroconversion, and high *precore* mutant ratios have been associated with persistent hepatitis after anti-HBe seroconversion [42]. Patients who continued to have high HBV DNA titres after HBe seroconversion had a lower genetic heterogeneity but more often had the *precore* mutant.

The limitations of this study were, the small size of study group, only 10 clones per sample, and a small region for analysis of genetic distance. In addition, the *X/precore/core* region is a highly conserved region, investigation of another region of the HBV genome, such as the polymerase, might help us to better understand the evolution of quasi-species of HBV.

In conclusion, the distinctly greater viral diversity after seroconversion in HBeAg seroconverters could be related to increased HBV-specific T-cell responses and escape mutants which arise from selective pressure caused by host immune activity. Long-term follow-up is required to determine whether hepatitis B viral diversity decreases or remains at a high level. Further study will be needed to elucidate the relationship between seroconversion and viral quasi-species in relation to antiviral therapy.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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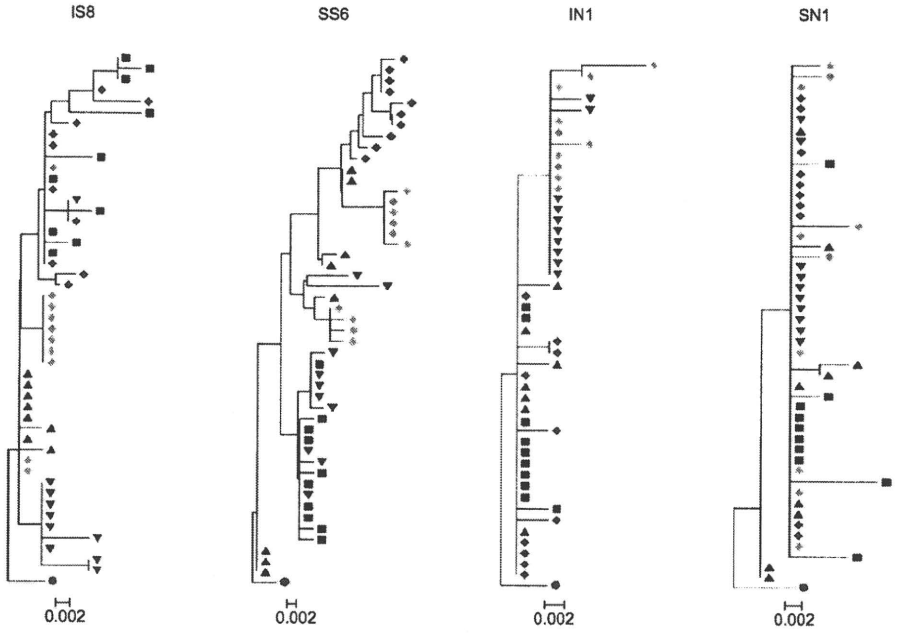

Fig. 3. Representative Neighbor-Joining phylogenetic trees of HBV sequences for each clinical group showing complex trees in seroconverters. HBV *X/precore/core* sequences from time-points I (purple filled triangle), II (blue filled inverted triangle), III (green filled square), IV (red filled diamond) and V (sky blue filled diamond) serum samples are analyzed phylogenetically and their positions are displayed on the trees. A sequence retrieved from the time-point I (red dot) of each group as outgroup in the trees, respectively. Scale bar represents 0.002% genetic variation. Seroconversion patients (IS, SS) show relatively complex branching patterns, forming clusters over time. With the pressure of seroconversion, the genetic diversity increased. In contrast, patients without seroconversion (IN, SN) were simply branching patterns and the genetic diversity in these patients changed very little over time.

identical pattern to that of DNA nucleotide sequence diversity (data not shown).

Construction of phylogenetic trees

Phylogenetic trees were complex for secoconverters and comparatively simple for non-seroconverters. In seroconverters (IS and SS), the arrangement and branch lengths of the trees were consistently more complex and longer than those for non-seroconverters. The genetic diversity was great after seroconversion in seroconverters (IS and SS) and less in non-seroconverters (IN and SN) (Fig. 3).

To investigate viral genetic features possibly associated with seroconversion, sequences isolated at time-points 1 and 2 (before seroconversion) were further analyzed phylogenetically. Trees were reconstructed using Neighbor-Joining, ML (data not shown), and PAML methods (data not shown). In general, no clusters were seen to be supported by robust bootstrap values for any group or particular patient quasi-species. This indicates that the region of the HBV genome studied does not contain patterns of variability sufficient for robust phylogenetic relation reconstruction. However, variability of branch lengths in the tree indicated that seroconversion patient groups exhibit greater diversity of the quasi-species compared to patients without seroconversion. This is in agreement with the genetic distance plot (Fig. 2), showing greater deviation from the mean values observed in patients with seroconversion. The IN group exhibited least deviation on the distance plot (Fig. 2) and shortest branch lengths on the trees (Fig. 3).

Interclonal differences of the quasi-species

To investigate whether a particular mutation pattern of evolution of the quasi-species is associated with seroconversion, we further analyzed the sequence changes in all patients at time-points 1 and 2, corresponding to the time before seroconversion. Parsimony-based ancestral sequences were generated using the Simmonic Sequence Editor. Aligned sequences of time-points 1 and 2 from a single patient were used as the input. Frequencies of changes in 12 types of mutations, including 4 transitions (CT, TC, AG, and GA) and 8 transversions (AT, TA, AC, CA, CG, GC, GT, and TG) were evaluated between generated descendants and ancestral sequences for each clone of the patient. Statistical *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups is summarized in Table 2 and Supplementary Table 1.

Analysis of sequence changes indicated a higher frequency of transversional A to T in spontaneous seroconverters (SS *vs.* SN = 0.06 *vs.* 0.02, $p = 0.04$) and IFN-induced seroconverters (IS *vs.* IN = 0.05 *vs.* 0.01, $p = 0.05$) and A to C changes in IFN-induced seroconverters (IS *vs.* IN = 0.025 *vs.* 0.006, $p = 0.04$) before seroconversion. Comparison of seroconversion groups (SS and IS) indicated a higher frequency of transversional A to T mutation pattern ($p = 0.003$, Table 2) and the trend of G to A mutation is higher in seroconversion groups (SS and IS) (Table 2). Subsequently, alignments of the clones were generated. Visual inspection of the alignments indicated variation in the ratio of A1762T mutation in clones isolated from each patient at time-points 1 and 2 (Fig. 4). In contrast to non-seroconverters, seroconverters

Table 2. *t*-test comparison of mean values of nucleotide changes between seroconversion and non-seroconversion groups.

	Seroconversion (n = 18)	Non-seroconversion (n = 18)	*p*
CT	0.117033	0.103750	0.637023
TC	0.156706	0.201328	0.155252
AG	0.125483	0.148372	0.498916
GA	0.196722	0.124511	0.073433
AT	0.061194	0.022128	0.003665
TA	0.049372	0.045417	0.778612
AC	0.027944	0.012550	0.145158
CA	0.017128	0.011094	0.523868
CG	0.009439	0.007744	0.835337
GC	0.018167	0.014894	0.748267
GT	0.009839	0.019217	0.272185
TG	0.041783	0.035528	0.731324

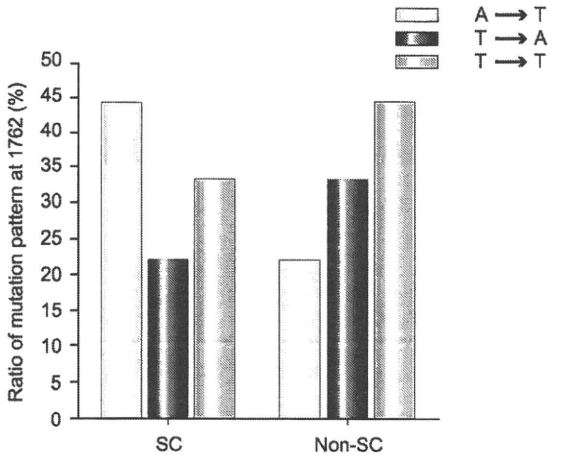

Fig. 4. The evolution of the *core* promoter mutation (A1762T) between seroconversion and control groups from time-point I to II. SC indicates seroconversion and non-SC, non-seroconversion. Alignment of the clones was carried out and the frequency of A1762T mutation in clones isolated from each patient at time-points 1 and 2 was determined. Subsequently, the evolutionary ratio of mutation from time-point I to II was calculated.

showed a higher frequency of A to T mutation pattern in the core promoter region from time-point I to II.

Core promoter (A1762T/G1764A) and precore (G1896A) mutations

Given that the *core* promoter/*precore* mutations influenced virus replication and HBeAg seroconversion, we analyzed the sequential change of *core* promoter (A1762T/G1764A)/*precore* (G1896A) mutations over time (Table 3). After seroconversion, patients with more than 50% *precore* mutant clone had higher HBV DNA loads than those with less than 50% of *precore* mutant clone (*precore* wild type) virus at time-point V [5.4 ± 1.3 (n = 5) *vs.* 3.8 ± 1.1 (n = 13), p = 0.0185] and 8 patients with a HBV DNA load less than 4.0 $\log_{10}$ copies/ml had all *precore* wild-type virus at time-point V (Table 3). Clinical progress of these patients was investigated over 10 years as median (range 1–20 years) after HBeAg seroconversion. HCC developed in 3 of 5 patients with *precore* mutant virus, compared to 1 of 13 patients with *precore* wild-type virus at time-point V (p = 0.017). On the other hand, 3 patients with ASC had all *precore* wild-type virus at time-point V (Table 3).

Discussion

In this study, analysis of 1800 nucleotide sequences from 36 HBV carriers showed that the viral diversity of seroconverters (IS and SS) after seroconversion was significantly greater than that of non-seroconverters (IN and SN) (Fig. 2, p <0.05) and was higher after seroconversion than before, in the seroconverters (Fig. 2, p <0.05). Phylogenetic analysis also generated complex trees for seroconverters and relatively simple trees for non-seroconverters. Analysis on interclonal differences in the quasi-specie showed a higher frequency of transversional A to T mutation pattern in seroconverters that coincided with the A1762T core promoter mutation. These findings suggested that HBeAg seroconversion involves dynamic shifts of the serum HBV quasi-species.

Osiowy et al. [24] examined viral quasi-species in eight HBeAg-negative patients at two time-points 25 years apart and obtained the evolutionary rate. Their results suggested that HBV diversity may be generated more rapidly than those estimated previously [25–29]. The higher evolutionary rate may be related to the seroconversion event driving quasi-specie complexity and diversification [24]. Our phylogenetic study showed that viral quasi-species populations appear to be replaced by new populations arising from a different clade after seroconversion.

Increased immune responses are accompanied by the reduction of viral loads and stronger immune pressure induces the selection of escape mutations, which leads to greater viral diversity [30]. According to this scenario, in our study, non-seroconverters have a high viral load and low quasi-species diversity and they obviously have a weak immune response.

Lim et al. [31] reported that viral genetic diversity in genotype B CHB patients was 2.4-fold greater in HBeAg seroconverters (spontaneous or IFN-induced) than in non-seroconverters before seroconversion. In this study of genotype C CHB patients, the nucleotide genetic distance was 1.49-fold greater in seroconverters (IS and SS) than in non-seroconverters before seroconversion but there was no statistical difference. This discrepancy might be due to the smaller region for analysis of genetic distance in our study than that of Lim et al. Another interpretation is that the host's immune response to the selection of mutant virus might differ between genotype B and genotype C. The natural course of CHB and the response to treatment could be affected by HBV genotype and there are some lines of evidence that indicate that the prevalence rates of *precore* and *core* promoter mutations vary among patients infected with HBV strains of different genotypes [32–34].

T-test comparison of mean values of nucleotide changes (Table 2) and linear logistic regression univariate analysis of mutations associated with seroconversion between seroconverters and non-seroconverters (data not shown) indicated a variation in the AT mutation pattern in the former (p = 0.003 and p = 0.006, respectively). This coincided with differences in the

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Patients	CP (ntA1762T/G1764A) (percent)			PC (ntG1896A) (percent)			DNA Loads (log_{10} copies/ml)			Histological diagnosis
	I	III	V	I	III	V	I	III	V	
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IS7	100	100	90	0	10	10	7.2	3.1	3.4	LC
IS8	80	100	60	0	0	60	7.6	4.0	4.5	CHB
IS9	100	100	10	0	0	0	6.0	4.5	4.8	HCC
SS1	0	60	0	80	0	80	7.6	4.2	5.4	HCC
SS2	80	100	90	10	90	10	6.6	7.6	5.9	ASC
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SS9	0	80	20	0	10	0	7.6	4.0	2.6	CHB
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IS: interferon induced seroconverter; SS: spontaneous seroconverter; ASC: asymptomatic carriers; CHB: chronic hepatitis B; LC: cirrhosis; HCC: hepatocellular carcinoma.

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今月のテーマ●B型慢性肝炎に対する最新の治療

ラミブジンとアデホビル併用不応例に対するアデホビルとエンテカビル併用療法

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要旨：ラミブジン（LAM）とアデホビル（ADV）併用療法を12カ月以上行い，HBV DNAが3log copies/ml以上を示したB型慢性肝疾患18例を対象とし，48週以上ADVとエンテカビル（ETV）の併用療法を行いウイルス動態についての検討を行った．LAM耐性例，ADV耐性例，ETV耐性例，多剤耐性例はそれぞれ100%，27.8%，33.3%，55.6%であった．平均HBV DNAはベースラインで4.1log copies/mlより48週の時点で2.9log copies/mlと低下した．ETV耐性を有する症例でHBV DNA減衰量は低下した．本併用療法による副作用は出現せず，48週の経過で新たに獲得したアナログ耐性は認めなかった．テノホビル（TDF）が使用できない本邦の現状ではLAMとADV併用不応例に対して，ADVとETV併用療法は試みるべき治療と思われた．

索引用語：ラミブジン，アデホビル，エンテカビル，B型肝炎ウイルス，耐性変異

はじめに

B型肝炎ウイルスによる持続感染の患者は世界で約3億5千万人いるといわれており[1)]，このウイルスによる持続感染はしばしば肝硬変，肝不全を惹起し，肝細胞癌の発生の原因となる[2)]．インターフェロン（interferon；IFN）製剤はB型肝炎ウイルスの増殖を抑制し，肝炎の鎮静に有効であるが，その効果は限定的であり，ペグインターフェロン（pegylated IFN；PEG-IFN）は30～40%の患者でsustained responseを達成するとされている[3)4)]が，本邦では現在治験中である．核酸アナログ製剤はB型肝炎ウイルスのDNAポリメラーゼを抑制してDNA合成を阻害し，ウイルス増殖を抑える薬剤であり，血液生化学検査値，肝組織所見の改善を促す[5)6)]．長期の核酸アナログ投与は肝硬変の進展や肝細胞癌の発生を抑制し，長期予後を改善する可能性が指摘されている[7)]．一方で長期に及ぶ核酸アナログ投与は薬剤に対する変異株の発生を促し，しばしば，ウイルス学的ブレイクスルーを引きおこす[8)]．実際に長期のラミブジン（lamivudine；LAM）投与は高率にLAM耐性ウイルスの出現を促した[8)9)]．近年登場した新規の核酸アナログ製剤であるエンテカビル（entecavir；ETV）はLAMと比較して耐性ウイルス

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Efficacy of entecavir and adefovir combination therapy in patients with chronic hepatitis B refractory to lamivudine and adefovir combination therapy

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Table 1. 背景因子

性別		男性：13　女性：5
年齢（歳）	Mean＋/－SD	59.6＋/－9.0
肝硬変（あり）	(No. [%])	10 (55.6)
肝癌既往（あり）	(No. [%])	5 (27.8)
LAM＋ADV 治療期間（月）	Mean＋/－SD	29.1＋/－13.1
HBV genotype		Bj：1　C：17
HBV DNA (log_{10} copies/m*l*)	Mean＋/－SD	4.10＋/－1.18
HBeAg（＋）	(No. [%])	13 (72.2)
ALT (IU/L)	Mean＋/－SD	35.9＋/－17.7
LAM 耐性	(No. [%])	18 (100)
ADV 耐性	(No. [%])	5 (27.8)
ETV 耐性	(No. [%])	6 (33.3)
多剤耐性	(No. [%])	10 (55.6)

の出現が少なく，抗ウイルス作用が強いことがいくつかの臨床試験で明らかとされた[10]~[12]．本邦においてもETVは2007年の承認後LAMに代わり第一選択の核酸アナログ製剤となった．しかし，既に世界中で多くのLAM耐性患者を認めており，これらの症例に対して2009年の米国肝臓病学会（American Association for the Study of Liver Disease；AASLD）はアデホビル（adefovir dipivoxil；ADV），あるいはテノホビル(tenofovir disoproxil fumarate；TDF）のLAMとの併用投与，あるいはemtricitabine（FTC）とTDFの併用投与への切り替えを推奨した[13]．同様にヨーロッパ肝臓病学会（European Association for the Study of the Liver；EASL）からはTDFの併用[14]が，本邦からはADVの併用が推奨された[15]．LAM耐性例に対するLAMとADV併用療法（以下LAM/ADV療法）による抗ウイルス効果の発現は緩徐であり，大多数のLAM耐性患者に有効であるが，少数例でHBV DNAの低下量が不十分であることが報告されている[16][17]．今回われわれはLAM/ADV療法不応例に対するADVとETV療法（ADV/ETV療法）48週の成績を検討したので報告する．

I 対象と方法

LAM/ADV療法を少なくとも1年以上行い，HBV DNAが3log copies/m*l*（以下log）以上を示した18例を対象とした．自己免疫性肝炎，アルコール性肝障害，うっ血性肝障害の合併例，C型肝炎ウイルスあるいはヒト免疫不全ウイルスの併発例，黄疸・腹水・脳症・消化管出血をともなう患者は除外した．18例中6例はLAM耐性に対するETV投与の既往を有した．2名の患者がADV投与中に血清クレアチニン上昇をきたしたため，ADVは隔日投与が行われていた．

HBV DNAはTaqMan PCR法(Roche Diagnostics，Tokyo，Japan)，耐性ウイルスの検討はINNO-LiPA HBV DR version 2, version 3(Innogenetics Gent，Belgium）を用いた[18]．

2群の検定にはStudent's t test，Mann-Whitney U test，chi-squared test，Fisher's exact testを用い，$p<0.05$を有意とした．

II 結　果

18例の背景因子をTable 1に示す．5例で肝癌の既往を認め，1例はADV/ETV療法中に肝癌を発症したが，肝部分切除あるいは経皮的ラジオ波焼灼療法で根治的な治療を受けた．10例は代償性肝硬変の状態で，遺伝子型では1例がBj型，17例がC型を示し，HBe抗原陽性は13例(72.2%）であった．LAM耐性は18例全例(100%)，ADV耐性は5例（27.8%），ETV耐性

Table 2. ベースライン，48週の時点におけるHBV DNA，HBe抗原，ALT値の推移とINNO-LiPA法によるベースラインのアナログ耐性

Case	HBV DNA (log copies/ml)			HBeAg (S/CO)		ALT (IU/L)		Resistance Mutation		
	0W	48W	0W-48W	0W	48W	0W	48W	LAM	ADV	ETV
1	7.6	3.1	4.5	1.7	3.4	74	39	+	+	
2	5.03	3.69	1.34	44	18	32	28	+		
3	3.09	1.8	1.29	—	—	31	16	+		+
4	4.12	2.51	1.61	245	106	27	20	+		+
5	4.9	4.6	0.3	528	359	49	36	+		+
6	3	1.8	1.2	3.7	—	27	28	+	+	
7	5.2	3.53	1.67	—	—	39	47	+		
8	3.87	2.93	0.94	1043	927	15	15	+		
9	4.93	3.91	1.02	87	39	28	25	+		+
10	5.24	4.17	1.07	161	121	48	43	+		
11	4.76	2.64	2.12	1.9	1.3	40	36	+		
12	3.46	3.36	0.1	—	—	37	30	+		
13	3	1.8	1.2	7.5	4.8	10	11	+	+	
14	3.61	2.51	1.1	3.9	3	28	25	+		
15	3.07	1.8	1.27	5.7	5.1	21	22	+		
16	3.96	3.11	0.85	164	94	73	138	+		+
17	3.17	1.8	1.37	—	—	28	42	+	+	
18	3.59	2.89	0.7	—	—	14	16	+	+	+

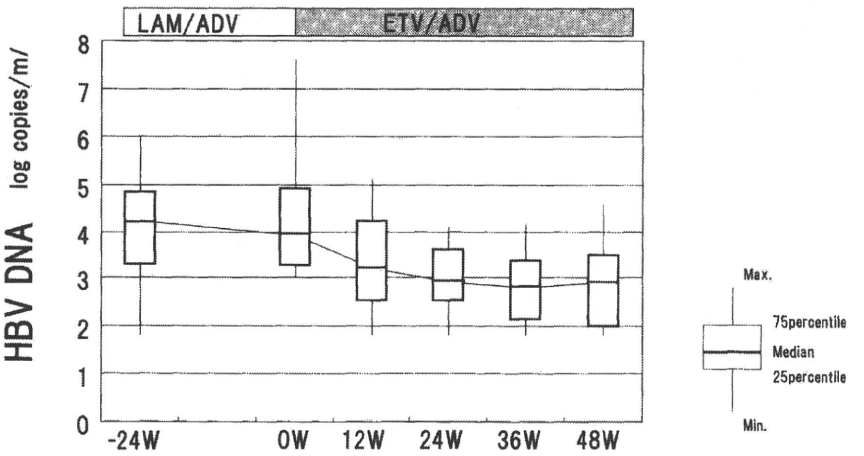

Figure 1. LAM/ADV療法・ADV/ETV療法によるHBV DNAの推移：HBV DNAはベースライン4.1log copies/mlから48週2.9log copies/mlと，48週で1.2log copies/ml低下した．

は6例（33.3%），10例（55.6%）は3剤耐性を認めた（Table 2）．

平均HBV DNAはADV/ETV療法にてベースライン4.1log，12週3.3log，24週3.0log，36週2.8log，48週2.9logと緩徐に低下した（Figure 1）．18例中5例が48週の治療中に2.1log未満を呈した．18例中13例は48週で1log以上のHBV DNA量の低下を示したが，残る5例の低下量は1log未満であった．HBe抗原陽性例・陰性例で治療48週のHBV DNA低下量に差を認めなかった．アナログ耐性別の治療48週でのHBV DNAの減衰量はLAM耐性で1.2log，LAM耐性＋

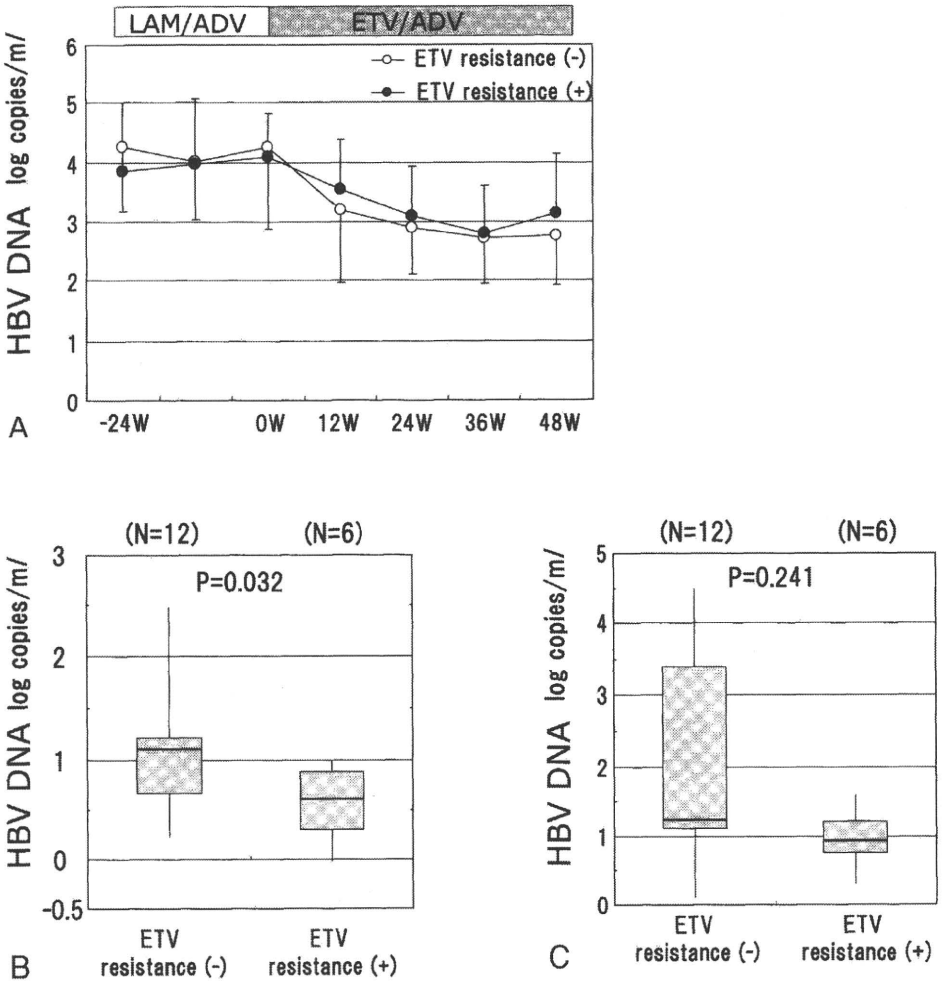

Figure 2. ETV耐性の有無によるLAM/ADV療法・ADV/ETV療法によるHBV DNAの推移 **A**）HBV DNAの推移．**B**）ADV/ETV療法ベースライン・12週後のHBV DNAの減衰量の比較．**C**）ADV/ETV療法ベースライン・48週後のHBV DNAの減衰量の比較．ETV耐性を有する症例でHBV DNA低下量が乏しい傾向（12週 $p=0.032$, 48週 $p=0.241$）を示した．

ADV耐性で2.1log，LAM耐性＋ETV耐性で1.0log，3剤耐性で0.7logであった．ETV耐性を有する6例と有さない12例の投与12週，48週のHBV DNA減衰量を比較すると，ETV耐性を有する例で減衰量が低下した（ETV耐性なし vs ETV耐性あり 12週 1.1log vs 0.6log，$p=0.032$，48週 1.5log vs 1.0log，$p=0.241$）（Figure 2）．

ベースラインでHBe抗原陽性を示した13例中1例が治療8週の時点で陰性となり，1例を除くとHBe抗原量が低下した．ALTについてはベースライン，治療後で有意な変化を認めなかった（Table 2）．

INNO-LiPA法による耐性部位の検出では，治療48週において，新たな耐性の出現を認めなかった．一部の症例でコドン181のA/VがA，コドン236のTがNに変化するなどの耐性クローンの消失が認められた．ウイルス量の低下にともない1例でINNO-LiPA法による検出が不能となった（Table 3）．

本研究中に有害事象の出現による中止例は認めなかった．2例がLAM/ADV療法の時点で腎障害のため既にADVが隔日投与となっていたが，この2例を含めてADV/ETV療法に移行後の腎障害の増悪例は認めなかった．

Table 3. ETV/ADV 療法ベースライン，48 週における耐性部位の検出

A) Baseline											
Case	Codon	80	173	180	204	181	233	236	184	202	250	194
	1	L	V	L	I	A	I	T	T	S	M	A
	2	L	V	L/M	I	A	I	N	T	S	M	A
	3	L	V	L/M	V	A	I	N	T/SCGA	S	M/V	A
	4	L/I	V	L/M	V/I	A	I	N	T	S	M/L	A
	5	L	V	L/M	V	A	I	N	T/ILFM	S/G	M	A
	6	L	V	L	M/I	A/T	I	N	T	S	M	A
	7	I	V	L/M	V/I	A	I	N	T	S	M	A
	8	L/I	V	L/M	M/I	A	I	N	T	S	M	A
	9	L	V	L/M	V	A	I	N	T	S/G	M	A
	10	L	V	L/M	M/V/I	A	I	N	T	S	M	A
	11	L	V	M	V	A	I	N	T	S	M	A
	12	L	V	L/M	M/V/I	A	I	N	T	S	M	A
	13	L	V/L	L/M	M/V/I	A/T	I	N	T	S	M	A
	14	L	V/L	L/M	V/I	A	I	N	T	S	M	A
	15	L/I	V	L/M	M/V/I	A	I	N	T	S	M	A
	16	L	V	L/M	M/V/I	A	I	N	T/ILFM	S/G	M	A
	17	L/I	V	L/M	M/I	A/V	I	N	T	S	M	A
	18	L	V	L/M	V	A	V	N	T/SCGA	S	M	A
B) Week 48												
Case	Codon	80	173	180	204	181	233	236	184	202	250	194
	1	L	V	L	*	A	I	N	T	S	M	A
	2	L	V	L/M	*	A	I	N	T	S	M	A
	3	L	V	M	V	A	I	N	GA/IL	S	M/I	A
	4	L/I	V	L/M	I	A	I	N	T	S	M/L	A
	5	L	V	M	V	A	I	N	T/ILFM	S/G	M	A
	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	7	I	V	L/M	I	A	I	N	T	S	M	A
	8	L/I	V	L/M	I	A	I	N	T	S	M	A
	9	L	V	M	V	A	I	N	T	G	M	A
	10	L	V	L/M	V	A	I	N	T	S	M	A
	11	L	V	M	V	A	*	N	T	S	M	A
	12	L	V	M	V	A	I	N	T	S	M	A
	13	L	L	M	V	A/T	I	N	T	S	M	A
	14	L	L	L/M	V	A	I	N	*	S	M	A
	15	L/I	V	L/M	V/I	A	I	N	T	S	M	A
	16	L	V	M	V	A	I	N	T/ILFM	S/G	M	A
	17	I	V	L	I	A	I	N	T	S	M	A
	18	L	V	M	V	A	V	N	SCGA	S	M	A

A：alanine，C：cysteine，G：glysine，F：phenylalanine，I：isoleucine，L：leucine，M：methionine，N：asparagine，S：serine，T：threonine，V：valine.
ND：not detected，*：impossible to judge.

III 考 察

B型慢性肝疾患に対する核酸アナログ療法の最も重要な問題は耐性ウイルスの出現である．ETV やTDFの登場により耐性ウイルスの出現率は低下した[19]が，既にLAM耐性を獲得してしまった多くの患者が全世界中に存在している[9]．日本肝

臓病学会ではLAM耐性例に対してはADVを併用するよう推奨している[15]．このLAM/ADV療法は多くの患者に有効であるが，一部の患者ではHBV DNAの低下量が不十分であり，HBV DNAの陰性化が得られない[16][17]．また，LAM耐性例ではLAM/ADV療法中にADV耐性が出現することが報告[20][21]されており，HBV DNAが陰性化しない，いわゆる不応例においては，新規の治療法が望まれてきた．以前われわれはLAM耐性例に対するLAM/ADV療法中にADV耐性を獲得した1例を経験したが，この症例はウイルス学的ブレイクスルーを発症し，軽度であるが肝炎の増悪をきたした．LAMを中止し，ADVは継続したままでETVを追加したところ，良好な抗ウイルス効果が得られ，ALT値も正常化した（Table 2, case 1）．この症例の経験を踏まえて，LAM/ADV療法不応例に対するADV/ETV療法の臨床研究を行った．ETVはLAM耐性とADV耐性例に[22][23]，ADVはLAM耐性とETV耐性例に対して抗ウイルス効果を発揮する[24]ことが示されており，LAM/ADV療法不応例に対するADV/ETV療法はTDFが承認されていない本邦の現状を考慮すると，また，交叉耐性を考えても理にかなった治療法と考えられる．

ADV不応例に対するETV療法の報告は近年散見されるが，いずれも症例数が少なく，短期間の成績が示されるにすぎない．40例のADV不応例（14例でADV耐性あり）に対してETVを投与した報告では，HBV DNA陰性化率が10%と低率で，6例（15%）にETV耐性の出現を認めた[25]．一方でHBe抗原陽性ADV不応14例（3例でADV耐性あり）に対するETV投与では，HBV DNA陰性化率は低いものの，15カ月の経過でHBV DNAはLAM投与歴なしで3.4log，LAM投与歴のあるもので3.9log低下し，この報告ではETV耐性の出現は認めなかった[26]．LAMとADVの2剤耐性を有する50例に対するETV投与では48週の経過でHBV DNA陰性化率は10%，HBV DNA量はベースライン6.90logより2.96logと低下した．ETV耐性はわずか1例(2%)で出現した[27]．ADV投与歴を有する症例に対するETV投与ではLAM投与歴を有する24例（9例がADV耐性あり）でHBV DNA陰性化率は42%であり，17%の症例でETV耐性が出現した[28]．報告によりウイルス陰性化率やHBV DNA低下量に差があるのは，症例数が少ないことや，人種や遺伝子型などの対象症例が異なること，過去に受けた核酸アナログの治療内容や期間に差があるためと思われる．

今回われわれが行ったLAM/ADV不応例に対するADV/ETV療法48週では平均でHBV DNAは1.2log低下した．低下量は少ないが，18例中5例が48週の治療中に2.1log未満を呈した．先に記したが，ADV不応例に対するETV単独療法によりETV耐性出現が報告されている[25][27][28]が，ADV/ETV療法では，更なる耐性の出現は認めなかった．ADVの併用投与がETV耐性を抑制した可能性が示唆された．

ADV/ETV療法中，脱落例・中止例は認めなかった．ADVによる腎障害の報告が散見される[29][30]が，ETVも腎排泄型のため注意が必要である．血清クレアチニンによる腎機能のモニターを定期的に行い，必要に応じて投与量の調節を行うことが重要である．

おわりに

LAM/ADV療法不応例に対するADV/ETV療法の成績を示した．経過観察期間が短く，少数例の検討ではあるが，HBV DNAは低下し，新たなアナログ耐性の出現は認めなかった．今後，観察期間を延長し，ADV/ETV療法の効果と安全性を検証する必要があると思われた．

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Original Article

Differential evaluation of hepatocyte apoptosis and necrosis in acute liver injury

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Aim: The significance of cytokelatin-18 fragment cleaved by caspase-3 (CK-18-fr) and high mobility group box-1 (HMGB-1) were evaluated experimentally and clinically for the differential evaluation of hepatocyte apoptosis and necrosis in patients with acute hepatic injury (AHI).

Methods: In this study, typical apoptosis and necrosis were induced in HepG2 cells by staurosporin (STS) and hydrogen peroxide, respectively. Intracellular generation of CK-18-fr and extracellular leakage of CK-18-fr and HMGB-1 were determined. In the clinical study, serum CK-18-fr and HMGB-1 levels in 84 patients with AHI of varied severity and etiology were measured and compared with conventional liver tests.

Results: In the experimental study, CK-18-fr was rapidly increased after STS stimulation, and peaked after 6 h inside the cells but increased in the medium 12 h after stimulation, while hydrogen peroxide stimulation caused no increase either in- or outside the cells. Extracellular HMGB-1 levels markedly increased after hydrogen peroxide stimulation, but did not change after STS stimulation. In the clinical study, serum CK-18-fr increased in correlation with serum aminotransferase, but not other liver tests or markers of disease severity of AHI,. Serum HMGB-1 levels mildly increased without any correlation to liver test or disease severity. Serum HMGB-1 levels in patients with circulation disturbance was significantly higher than that in patients with other etiologies.

Conclusion: The simultaneous determination of the serum CK-18-fr and HMGB-1 may be useful in the differential diagnosis of hetocellular death in AHI, which is primarily due to apoptosis except in patients with circulation disturbance.

Key words: apoptosis, cytokelatin -18, HMGB-1, oncotic necrosis

INTRODUCTION

FULMINANT HEPATIC FAILURE (FHF) develops in 1– 2% of acute hepatic injury (AHI) cases[1] and it affects approximately 400 patients in Japan per year.[2] In addition, the proportion of survival in FHF patients remains around 30% without liver transplantation,[3–5] whereas most patients with conventional AHI spontaneously recover without any specific therapy.[6,7] The major pathological difference between the two disease types, AHI and FHF, is the degree of hepatocellular death; focal and extensive (submassive to massive) hepatocullular death in conventional AHI and FHF, respectively.[8] The mechanism by which focal hepatocellular death progresses to massive hepatocellular death is under elucidation but is generally considered as a complex process including excessive inflammation due to excess viral load,[9] an excessive response of proinflammatory cytokines,[10–12] and a microcirculation disturbance resulting from intravascular coagulation in the hepatic sinusoid.[13]

Two distinct mechanisms, namely oncotic necrosis (necrosis) and apoptosis, are involved in the hepatocellular death.[14] Excessive inflammation through proinflammatory cytokines and acquired immune response may cause apoptotic hepatocellular death, while anoxia through microcirculation disturbance may lead to necrosis. However, it is unclear which of them is dominantly responsible for the extensive hepatocyte death in FHF. Because the predominance of the two mechanisms is supposed to depend on many clinical factors including the etiology of the liver injury and the course and severity of the disease, the differential estimation of the implication of those mechanisms in each case may provide valuable information for the treatment of liver

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