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Comparison of the Mechanisms of HBeAg Seroconversion Among Patients with HBV and HCV, and with HBV Alone

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Hepatitis B virus (HBV) occasionally co-infects with hepatitis C virus (HCV). Because replication of HBV is suppressed by HCV core protein in double infection, HBV is usually inactive in such case. In light of these facts, we examined HBV from patients who were negative for hepatitis B virus e antigen (HBe Ag) and positive for anti-HBe for differences in sequence of the core promoter and precore region between patients with and without HCV co-infection. The subjects were 17 HBeAg-negative/anti-HBe-positive chronic hepatitis B patients divided into 2 groups: 10 without HCV, and 7 with HCV. DNA from the core promoter to the core gene was directly sequenced and compared between these 2 groups. There was a significant difference in the frequency of a change from guanine to adenine at nucleotide 1896 between patients with double infection (3 out of 7) and patients infected with HBV alone (9 out of 10) ($p<0.05$). Seven of the patients with HBV alone had genotype C, and 2 had genotype B. Four of the patients with double infection had genoype C, and 2 had genoype B. In the core region, mutations at amino acids 13 and 35 were significantly more frequent in patients with HBV alone. There was no difference in the sequence of the core promoter between the 2 groups. There were significant differences in the sequence of the precore region between patients infected with HBV alone and patients with a double infection of HBV and HCV. This suggests that the mechanisms by which HBV persists after seroconversion of HbeAg differs between patients with double infection and patients with HBV alone.

Introduction

Hepatitis B e antigen (HBeAg) was previously thought to be a marker of active replication. Accordingly, seroconversion from HBeAg to hepatitis B e antibody (anti-HBe) was thought to indicate reduced viral replication and infectivity as a result of the host's immune response. However, HBeAg seroconversion is often associated with a G-to-A mutation at nucleotide (nt) 1896 that prevents production of HBeAg by creating a stop codon in the precore region [1]. Seroconversion due to this mutation in the precore region does not reflect diminishing viral replication, and it has even been detected in cases of fulminant hepatitis [2]. Occurrence of this precore mutation depends on the base at nt 1858, which is the counterpart of nt 1896 in the stem-loop structure [3]. The base at nt 1858 is cytosine (C) in genotypes A and F, and is thymidine (T) in genotypes B, C, D and E [4]. Mutation from guanine (G) to adenine (A) at nt 1896

occurs readily in genotypes B, C, D and E, possibly due to stabilization of the stem-loop structure by the matching of adenine to thymidine [5]. In contrast, G-to-A mutation at nt 1896 is rare in genotypes A and F, and HBeAg seroconversion in these 2 genotypes occurs by the mechanisms described below.

Reduced production of HBeAg is caused not only by precore mutation, but also by mutation at nt 1762 and/or nt 1764 in the precore/core promoter region. Transcription of the precore and core genes is directed from the so-called core promoter region. Okamoto et al were the first to report that mutation from AGG to TGA at nt 1762 to 1764 in the core promoter is associated with HbeAg(−)/anti-Hbe(+) phenotype [6]. However, this core promoter mutation has been detected in both fulminant hepatitis patients [7] and asymptomatic carriers [8], and its function is still uncertain, despite several in vitro transfection experiments [9]~[11]. One possible clue to the role of this mutation in the clinical course of hepatitis B is the fact that it has been detected at a high rate in HBeAg-negative patients with cytosine at nt 1858 [5].

In previous studies of pediatric chronic hepatitis B patients, neither mutation of the core promoter region [12] nor mutation of the precore region [13] was detected at the time of seroconversion from HBeAg to anti-HBe. In a study of adult patients treated with interferon, precore mutation was not detected after HBeAg seroconversion [14]. Thus, in some cases, seroconversion is not associated with core promoter or precore sequences.

Studies have found that double infection with HCV and HBV suppresses replication of HBV by viral interference [15]~[17]. In most such cases, HBeAg is negative and anti-HBe is positive. However, there have been no previous studies of core promoter, precore and core sequences in cases of double infection. In the present study, we sequenced HBV DNA from the core promoter to the core gene, and compared sequences of patients infected with HBV alone to those of patients infected with both HCV and HBV.

Patients and Methods

Patients

The subjects were 17 chronic hepatitis patients (Table). Group A consisted of 10 chronic hepatitis B patients (8 male and 2 female) infected with HBV only, with the HBeAg(−)/anti-HBe(+) phenotype. Group B consisted of 7 chronic hepatitis patients (5 male and 2 female) co-infected with HBV and HCV, with the HBeAg(−)/anti-HBe(+) phenotype. Mean age for group A and group B was 62.1 and 56.1, respectively. Serum alanine aminotransferase (ALT) concentration was higher in group B (64.4 ± 28.7) than in group A (45.1 ± 23.4), but the difference was not significant ($p = 0.147$). HBV DNA was not detected by transcription-mediated amplification (TMA) in 3 group A patients and 4 group B patients. HCV RNA was detected by Amplicor PCR in 4 group B patients: 1b genotype in 3 patients, and 2b genotype in 1 patient.

Amplification of HBV DNA by PCR and sequencing of PCR products

Serum (50 μL) was diluted with 50 μL of sterile distilled water and mixed with 100 μL of a mixture containing 25 mM sodium acetate, 2.5 mM EDTA (pH 8.0), 1% SDS, 2 μg/mL of proteinase K, and 10 μg/mL of yeast t-RNA as a carrier. Following digestion at 65 ℃ for 2 hr, viral nucleic acids were extracted twice with phenol-chloroform and once with chloroform, ethanol-precipitated, and suspended in water. The core promoter and precore regions, consisting of a 1359-bp sequence extending from nt 1435 to nt 2793, were amplified by polymerase chain reaction (PCR) using the extracted HBV DNA. The primers were 5′-CGTCGGCGCTGAATCC-3′ (sense; nt 1435 to 1450) and 5′-CGAGGCGAGGGAGTTCTTCTTC-3′ (antisense; nt 2793 to 2771). Five microliters of the ex-

Table Profile of viral markers of 17 patients in the present study

Case no.	Age	Sex	ALT (IU)	HbsAg/ HbsAb	HbeAg/ HbeAb	HBV DNA (LGE/mL)	HBV genotype	anti-HCV	HCV RNA (KIU/mL)	HCV genotype
1	69	F	20	+ / −	− / +	< 3.7	C	−	n.d.	n.d.
2	71	M	49	+ / +	− / +	4.8	C	−	n.d.	n.d.
3	67	M	36	+ / −	− / +	< 3.7	C	−	n.d.	n.d.
4	70	M	80	+ / −	− / +	6.7	B	−	n.d.	n.d.
5	61	M	20	+ / −	− / +	< 3.7	C	−	n.d.	n.d.
6	77	M	60	+ / −	− / +	5.8	C	−	n.d.	n.d.
7	58	M	63	+ / −	− / +	6.7	B	−	n.d.	n.d.
8	47	M	72	+ / −	− / +	5.7	C	−	n.d.	n.d.
9	44	F	13	+ / −	− / +	7.0	n.d.	−	n.d.	n.d.
10	59	M	38	+ / −	− / +	6.7	C	−	n.d.	n.d.
11	61	M	38	+ / −	− / +	< 3.7	C	+	81	1b
12	48	M	55	+ / −	− / +	5.7	B	+	< 0.5	n.d.
13	59	F	89	+ / +	− / +	< 3.7	n.d.	+	10	1b
14	69	M	103	+ / −	− / +	< 3.7	C	+	< 0.5	n.d.
15	49	F	22	+ / +	− / +	< 3.7	C	+	470	2b
16	52	M	63	+ / −	− / +	4.6	C	+	< 0.5	n.d.
17	58	M	81	+ / −	− / +	5.9	B	+	690	1b

ALT: alanine aminotransferase, n.d.: not detected.

tracted DNA was mixed with 45 μL of a PCR reaction mixture containing 400 nM of the primers, and amplified by 35 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 3 min in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR products were directly sequenced by dideoxy-chain termination using an AutoRead DNA Sequencing Kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) and one of the primer used in the PCR reaction.

Statistical analysis

All results are expressed as mean ± SD, unless otherwise indicated. The data of the patients were compared using unpaired 2-tailed Student's t test or the χ^2 test. A p value of <0.05 was considered to indicate statistical significance.

Results

Comparison of clinical data between patients infected with HBV alone and double-infected patients

There was no difference in distribution of age or sex between the 2 groups. Serum ALT was higher in group B, but not significantly. Both HBV and HCV were detectable in 1 group B patient (case no. 17), and neither HBV nor HCV was detectable in another group B patient (case no. 14). In the remaining group B patients, only HBV (2 patients) or only HCV (3 patients) was detectable. Using the TMA method, HBV DNA was detected in 7 group A patients and 3 group B patients. Genotype B was detected in 2 group A patients and 2 group B patients. Genotype C was detected in 7 group A patients and 4 group B patients. Genotype A was not detected in any patients.

Comparison of core promoter sequences between patients infected with HBV alone and double-infected patients

Double mutation of A to T at nt 1762 and G to A at nt 1764 was detected in 8 group A patients and 5 group B patients (Fig. 1). There was no other mutation in the core promoter region that showed a significant difference in prevalence between the 2 groups.

Comparison of precore and core sequences between patients infected with HBV alone and

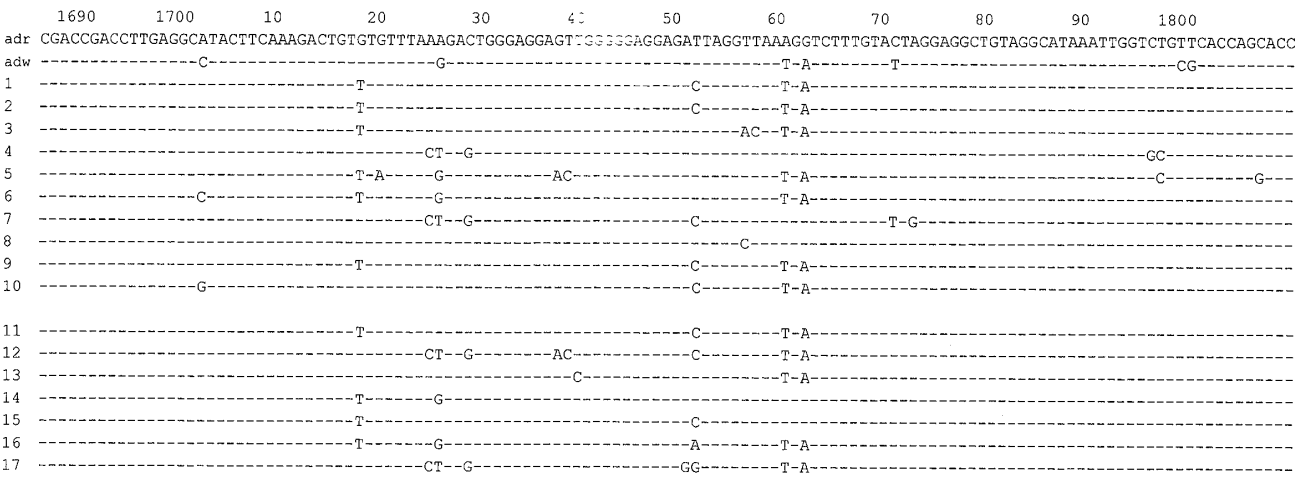

Fig. 1 Nucleotide sequences of the core promoter region from 17 patients, compared with reference sequences[30] indicated at the top.

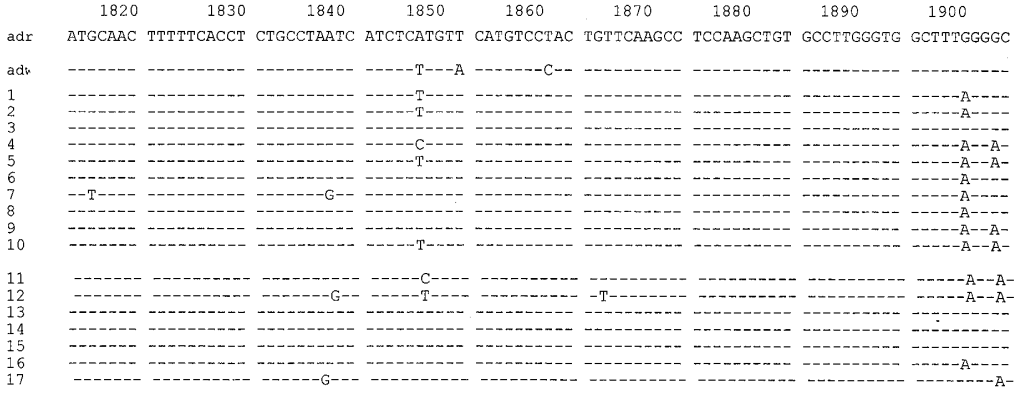

Fig. 2 Nucleotide sequences of the precore region from 17 patients, compared with reference sequences[30] indicated at the top.

double-infected patients

There was significant difference in frequency of mutation of G to A at nt 1896, which was detected in 9 group A patients (90%) and 3 group B patients (43%) ($p = 0.036$, Fig. 2). Additionally, mutation from G to A at nt 1899 was detected in 4 group A patients and 3 group B patients. All patients in both groups had thymidine at nt 1858, which is the counterpart of 1896 in the stem-loop structure. In the core region, mutations at amino acids 13 and 35 were more frequent in patients with HBV alone ($p = 0.024$ and $p = 0.008$, respectively; Fig. 3). The number of mutations in the core region was 9.0 ± 4.2 in group A and 6.9 ± 4.8 in group B. Thus, the number of mutations was higher in group A, but the difference was not significant ($p = 0.28$).

Discussion

In this study, we examined differences in mechanisms of HBeAg seroconversion between hepatitis B patients with and without HCV infection. Prevalence of mutation in the core promoter and precore region after seroconversion differed among the genotypes detected. In a previous study, precore stop codons were only detected in HBV-infected patients with thymidine at nt 1858 (87% vs. 0%)[5]. In the present study, all HBV-infected patients had thymidine at nt 1858, and belonged to genotype B or C. Of the patients infected with HBV alone, 9 (90%) had a HBV pre-

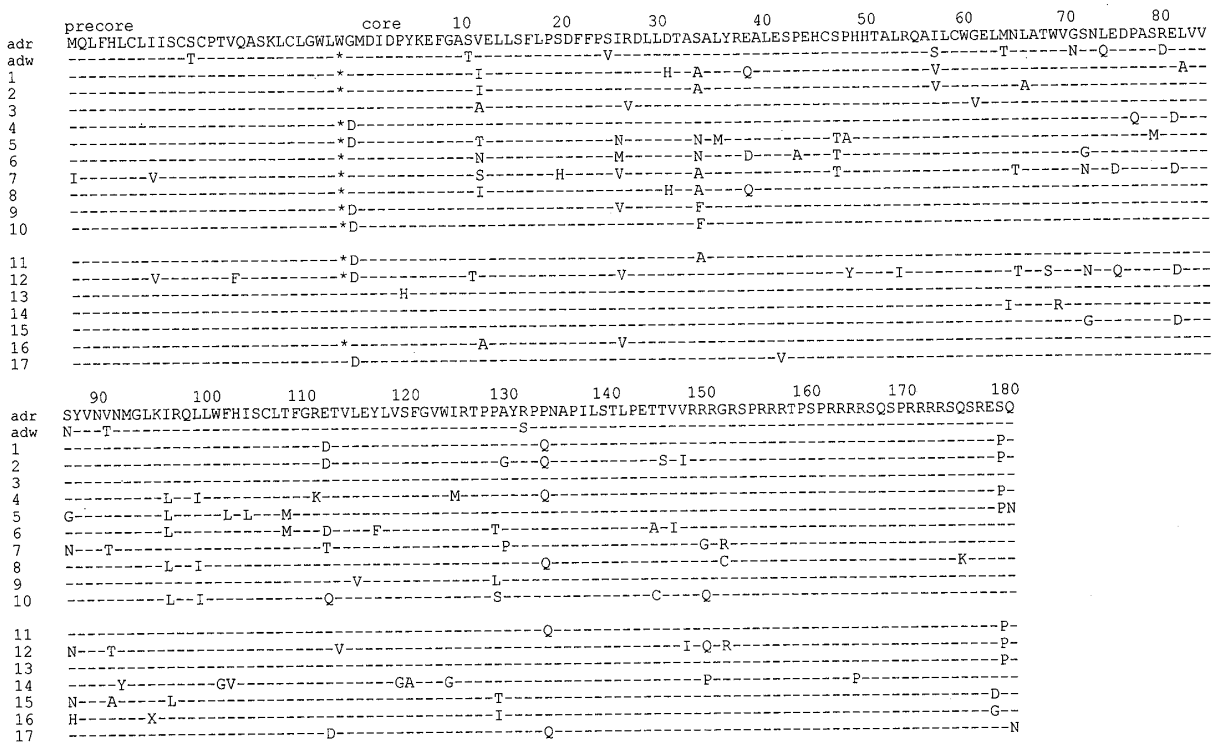

Fig. 3 Amino acid sequences of the precore and core region from 17 patients, compared with refernce sequences[30] indicated at the top.

core stop codon. In contrast, only 3 of the patients with double infection (43%) had a HBV precore stop codon, although all double-infected patients had thymidine at nt 1858 of HBV. The significance of the precore stop codon in HBeAg seroconversion is not fully understood. Maruyama et al reported that seroconversion was not induced by the precore stop codon, because seroconversion from HBeAg to anti-HBe often occurred before genomic conversion[17]. Their conclusion is supported by another study, in which seroconversion was found to precede emergence of the precore stop codon [18]. These findings support the theory that the precore stop codon acts as an escape mechanism after the emergence of anti-Hbe antibody.

Several studies have examined the prevalence of HCV infection in HBV-infected patients [19)~21)]. Results of these studies indicate that HCV superinfection in patients with chronic HBV infection may exert a viral interference effect that suppresses or terminates the HBV carrier state [22)~24)]. It has been reported that the mechanism of this suppression is binding of HBV RNA by HCV core protein in the nucleus, resulting in suppression of HBV expression and replication [25)]. In the present study, HBV DNA was undetectable in 4 of the 7 patients with dual infection, using the TMA method. In the remaining 3 patients, 2 had the precore stop codon. Three of the 4 patients with undetectable HBV DNA had the wild-type precore sequence. These results indicate that mutation of the precore gene sequence is not necessary for termination of HBeAg production in patients with dual infection, because HBV-related protein expression may be suppressed by HCV core protein in such patients.

In the present study, the only patient infected with HBV alone who did not have the precore stop codon (case no. 3) had the double mutation of A to T at nt 1762 and G to A at nt 1764. This double mutation is reportedly associated with

HBeAg negativity[6)~8)]. However, of the 4 double-infected patients who did not have the precore stop codon, although 2 had the above-mentioned double mutation, the remaining 2 patients (case no. 14 and no. 15) were wild-type at nt 1762, 1764 or 1896. Persistence of HBV without mutations in core promoter or precore sequences (without the precore stop codon) in the anti-HBe-positive phase in case no. 14 and no. 15 may have been made possible by suppression of HBeAg production by HCV core protein.

In the present study, mutations at core amino acids 13 and 35 were more frequently detected in patients with HBV alone than in patients with double infection. Previously, it has been reported that mutations in the core region are associated with HBeAg seroconversion [26)]. However, there have been no previous reports of frequent mutations at core amino acids 13 and 35, which are located outside a previously described CTL epitope. Thus, the clinical significance of these 2 amino acid substitutions in the core region is unclear. We found that patients infected with HBV alone had a greater number of mutations in the core region, but the difference was not significant. This may be due to suppression of HBV replication by HCV core protein in patients with double infection, because the resultant decrease in DNA polymerization would reduce the number of opportunities for misreadings.

It has been reported that the prevalence of hepatocellular carcinoma (HCC) is greater for patients infected with both HBV and HCV than for patients infected with either virus alone[19)27)]. Furthermore, risk of HCC for patients with type C liver cirrhosis is higher if they continue to have detectable levels of anti-HBc antibody and HBV DNA (detected by PCR) after remission of cirrhosis symptoms [28)~29)]. More research is necessary to clarify the influence of HBV co-infection on hepatocarcinogenesis in type C liver disease.

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HBV と HCV の重複感染者と HBV 単独感染者間の HBeAg セロコンバージョンの機構の比較

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HBV と HCV の重感染者におけるセロコンバージョン（SC）の機序を明らかにすることを目的に，HBV 単独感染者と HBV と HCV の重複感染者において，E-II/CP 領域と preC/C 領域の sequence を比較した．対象は HBsAg 陽性，HBeAg 陰性，HBeAb 陽性，HCV-RNA 陽性の慢性肝炎患者 7 例と HBsAg 陽性，HBeAg 陰性，HBeAb 陽性，HCV-RNA 陰性の慢性肝炎患者 10 例である．保存血清より DNA を抽出し direct sequence 法で E-II/CP 領域と preC/C 領域の遺伝子解析をした．単独感染者の 7 例はジェノタイプ C で，2 例はジェノタイプ B であった．重複感染者の 4 例はジェノタイプ C で，2 例はジェノタイプ B であった．1896 番の塩基のグアニンからアデニンへの変異は，重複感染者の 7 例中 3 例，単独感染者の 10 例中 9 例で認め，2 群間で有意差があった．また，コア領域の 13 番と 35 番のアミノ酸変異は単独感染者に有意に認めた．コアプロモーター領域の塩基変異は 2 群間で差はなかった．プレコア領域の塩基変異には有意差を認めた．このことは，2 群間における HBeAg の SC 後の HBV の存在様式が異なることを示唆した．