Mutations of Glucocorticoid Responsive Element of HBV DNA

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Summary: The mutation of glucocorticoid responsive element (GRE) of HBV DNA obtained from a patient with chronic hepatitis B was evaluated. This patient showed fatal course by glucocorticoid administration. The HBV DNA from this patient (GRE-M) and two patients with HBeAg positive chronic hepatitis B (GRE-W1,2), whose HBV DNA have few mutations, were examined. The 212 bp region from nt.274 to nt.485 (GRE region) was amplified by PCR and the nucleotide sequence was determined. A base mismatched sequence of the latter half of the GRE consensus sequence was confirmed at nt.296-301 (G1), nt.347-352 (G2), nt.359-364 (G3), and nt.473-478 (G4). Also one base mismatched sequence of the AP-1 response element was detected at nt.331-337 (A1). The nucleotide substitutions in GRE-M generate three putative loop formation sites, four bases in length, from nt.22 to nt.31 (L1), nt.35 to nt.42 (L2), and nt.74 to nt.83 (L3). The L1 was located just upstream of the G1. The L2 was located between the A1 and the G2. These mutations followed by three dimensional form change may affect the responses to glucocorticoid.

Key words glucocorticoid responsive element (GRE), hepatitis B virus (HBV), mutation, AP-1

INTRODUCTION

There are several reports indicating that the mutations in the HBc gene region and the PreC region play roles in pathological conditions in patients with HBe antibody positive chronic hepatitis B [1-3]. However, recently these mutations were found to be common phenomena in HBV DNA from patients aged over 30 or more. Consequently, these mutations have lost their importance when considering the cause of the clinical aspects of anti-HBe positive chronic hepatitis B [4-5]. At present, mutations in the regulatory elements such as enhancer I, enhancer II, promoters, and GRE are considered to play an important role in influencing viral replication or clinical conditions. Tur-Kaspa et al. found that HBV DNA contained GRE, which up-regulated its own enhancer I [6,7]. Chou et al. reported that glucocorticoid stimulated HBV gene expression in cultured human hepatoma cells [8]. Furthermore, there are reports of severe hepatitis or fatal chronic hepatitis B induced by the administration of glucocorticoid agents [9,10]. In these cases, decreased hepatic reserve or underlying conditions may have contributed to the poor clinical course. In addition to those conditions, a mutation in the GRE, which could raise the sensitivity to glucocorticoid, might play a role in the high replication of HBV leading to poor clinical courses. In the present study, the mutations of the GRE region of HBV DNA isolated from a patient who died while receiving predonisolone were evaluated.

MATERIALS AND METHODS

The patient died (age 47, male) while receiving predonisolone as an immunosuppresant after renal transplantation. Two patients aged 22-24 years old with HBeAg positive chronic hepatitis B who showed no elevation of serum transaminase were
examined as controls. Sera (100 μl) were treated with 400 μl of protein digestion buffer (100 μg/ml proteinase K, 0.5% SDS, 0.5 mM EDTA, 10 mM Tris-HCl PH 7.8) and DNA was isolated by the phenol chloroform method. DNA was precipitated by ethanol with 0.2 M sodium chloride, rinsed with 70% ethanol twice and dried.

**PCR procedure and cloning**

The primers used for PCR were as follows; forward primer (5'-CGTGGTGACTCCTCTC-3': nt.255-nt.272) and reverse primer (5'-TGCTGGTGATGTGC-3': nt.489-nt.472) (nt.1 was the EcoRI site of the HBV ADR). The reaction mixture contained 10 pM each of primer, 0.2 mM dNTPs, 10 mM Tris-HCl (PH8.3), 50 mM KCl, 1.5 mM MgCl2, and 2.5 U Taq DNA polymerase (Takara Shuzo Co., LTD, Shiga, Japan). Thirty-five cycles of the reaction were performed and each cycle included denaturation at 95 °C for 60 sec, annealing at 55 °C for 60 sec, and extension at 72 °C for 60 sec. PCR products were electrophoresed in 1.2% agarose gel and the amount of the products was estimated by the intensity of the ethidium bromide staining. The cloning of the PCR products was performed with One Shot T-A Cloning Kit (Invitrogen, Inc., San Diego, USA). The molecular ratio of the vector and the PCR product was 1:1-4. Ligation was performed at 14 °C overnight.

**Sequencing**

Four clones from each subject were sequenced using Cy5 Auto Read Sequencing Kit and ALFred DNA sequencer (Pharmacia LKB Biotech, Tokyo, Japan).

**RESULTS**

**Homology search for GRE consensus and AP-1 responsive element sequence (Fig. 1)**

A base mismatched sequence (TGTCCT) to the latter half of the GRE consensus sequence (AGAACAnnnTGTCCTT) was confirmed at nt.296 (G1 site), nt.347 (G2 site), nt.359 (G3 site), and nt.473 (G4 site). Also one base mismatched sequence (TCACTCA) to the AP-1 responsive element sequence (TGACTCA) was detected at nt.331 (A1 site).

**Nucleotide sequence of GRE region (Fig. 1)**

In GRE-W1 and 2, none to two nucleotide substitutions were observed, and they varied with clones. Nucleotide homology of the GRE region to the prototype HBV subtype ADR was 98.7% in the average. On the other hand in the GRE-M, nucleotide sequence of the GRE region of the four clones was identical. Ten nucleotides were substituted and the homology to prototype HBV subtype ADR was 93.7%.

**Putative loop formation sites (Figs 1, 2)**

A putative loop formation site which met the conditions of minimum stem length: 4 bases, maximum loop length: 3 bases, no mismatch allowed, was found from nt.187 to nt.196 (W-L1 site) in GRE-W. As a result of nucleotide substitutions in GRE-M, this site diminished. In contrast, new putative loop formation sites were generated from nt.22 to nt.31
Fig. 2. Schema of the putative loops in GRE-M. The numbers below indicate the start and end points of each loop. The loops met the conditions of minimum stem length: 4 bases, maximum loop length: 3 bases, no mismatch allowed.

(M-L1 site), nt.35 to nt. 42 (M-L2 site), and nt.74 to nt.83 (M-L3 site). The M-L1 site was located just upstream of the G1 site. The M-L2 site was located between the A1 and the G2 site.

DISCUSSION

Generally, enhancers, promoters, silencers, and GREs were well known cis-regulatory elements. HBV DNA possesses these regulatory elements [11-13]. Furthermore, the HBx gene product is a potent trans-activator [14,15]. However, the functions of these regulatory elements may change according to the nucleotide substitutions in their regions.

In some cases of chronic hepatitis B, the amount of HBV DNA increased after the administration of glucocorticoid, and in another case, HBV showed no replication despite glucocorticoid administration. The mutation in the GRE region which affects the responses to glucocorticoid, may explain this discrepancy. Glucocorticoid agents are frequently administered, and doctors may have the opportunity to treat HBV carriers with these agents. Thus, it is important to examine the relationship between viral replication and mutation in the GRE region. In the present study, GRE-W showed diversity in its DNA sequences. Similar to HCV, HBV exists as a quasispecies to some extent. On the other hand, the four clones isolated from the GRE-M were identical. This suggests that in GRE-M glucocorticoid stimulates viral replication resulting in monoclonal growth. The sequence of TGTCCT, a base mismatched sequence of the latter half of the GRE consensus sequence, was highly conserved in both GRE-W and GRE-M, and a base mismatched sequence of AP-1 responsive element was found with in close proximity to the G2 site. As AP-1 is thought to be a co-operative factor of GRE [16,17], the portion from the A-1 site to the G2 site may be an important region for determining the reaction to glucocorticoid. Some regulatory elements show their action through three dimensional form change. If dimensional form change actually occurs in GRE-M, the G-1, the A-1 and the G-2 sites, which are considered to be important to GRE functions, may be affected.

HBV DNA has several complicated factors. For example the coding regions of HBV DNA overlap their regulatory elements or each other. Thus to examine the relationship between viral replication and mutation in GRE, it is necessary to extend the objective DNA sequence range. To assess whether the mutations found in the present study really showed high sensitivity to glucocorticoid, further functional transient transfection assays are considered to be necessary.

REFERENCES


