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Restoration of albumin production by nucleoside analogue therapy in patients with chronic hepatitis B

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Abstract The clinical course of patients with chronic hepatitis B (CH-B) was greatly changed by the introduction of nucleoside analogues. We often encounter patients where the serum level of albumin recovers quickly following the treatment. In this study, we focused carefully on the changes in serum albumin level noted during nucleoside analogue therapy, in an effort to clarify the mechanism behind the restoration of albumin production. We observed changes in serum albumin levels during nucleoside analogue therapy in 12 patients with CH-B and studied the mechanism behind the restoration of albumin production following the therapy. The serum level of albumin was significantly increased very soon after the treatment was started. Prior to treatment with nucleoside analogues, the albumin signal for mRNA was only slightly seen in the peri-portal area, whereas 12 months after the treatment, the liver tissue presented an obvious signal of albumin mRNA. Serum levels of hepatocyte growth factor (HGF) were significantly decreased 12 months after the treatment. In this study, we demonstrated that nucleoside analogues decrease HGF through the suppression of hepatocyte damage, leading to the restoration of albumin production in patients with CH-B.

Keywords Chronic hepatitis B · Nucleotide analogues · Albumin · Hepatocyte growth factor · In situ hybridization

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Introduction

Hepatitis B virus (HBV) infection has been a major public health problem, with 350–400 million patients worldwide. Chronic hepatitis B (CH-B) can lead to progression of liver diseases with increased risk of cirrhosis and hepatocellular carcinoma (HCC) [1].

The clinical course of CH-B was greatly changed by the introduction of nucleoside analogues. Long-term treatment can reverse, and thereby lead to the recovery of, fibrosis of the liver, even if the liver disease was progressing toward cirrhosis [2, 3].

Since albumin is a ubiquitous protein that is synthesized only by hepatocytes, the serum albumin level is an important factor in the evaluation of liver function. We often encounter patients where the serum level of albumin recovers quickly following treatment with nucleoside analogues.

In this study, we focused carefully on the changes in serum albumin level noted during nucleoside analogue therapy, in an effort to clarify the mechanism behind the restoration of albumin production. Moreover, we attempted to demonstrate the restoration of albumin expression, morphologically.

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Patients and methods

Patients

We investigated the changes in serum albumin level noted in patients with CH-B who were started treatment with lamivudine or entecavir in Fukuoka University Hospital. Twelve of the patients who met the following 5 requirements comprised the object of this study: (1) did not have HCC; (2) did not have esophago-gastric varices requiring treatment; (3) were followed up for more than 12 months; (4) suffered no recurrence of hepatitis within the 12 months period; (5) serum samples are saved. The characteristics of the patients are shown in Table 1.

Quantification of serum levels of cytokines

We investigated the changes in serum levels of human hepatocyte growth factor (HGF) and transforming growth factor beta 1 (TGF-β1). Serum samples before and 1 year after beginning the treatment with nucleoside analogues were obtained from the patients. Quantification of HGF and TGF-β1 in serum was performed using the Quantikine ELISA kit (R&D Systems Co. Ltd., USA) and the procedures were carried out according to the manufacturer's protocol.

Liver biopsy specimens

Liver specimens were obtained from four patients who received needle biopsy both before and 1 year after beginning the treatment with nucleoside analogues. The liver specimens were immersion fixed in 10 % neutral buffered formalin at room temperature for 24 h, and then samples were processed for paraffin embedding. Five-micrometre thick sections were cut and mounted on glass slides that had been previously covered with 3-aminopropyltriethoxysilane (Matsunami Glass Ind., Ltd., Japan). The samples were then used for in situ hybridization.

Table 1 Characteristics of the patients

	Median (min–max)
Age (years)	42.0 (21–68)
Gender (M/F)	10/2
Platelet ($\times 10^4/\mu L$)	14.8 (3.6–27.5)
Albumin (g/dL)	3.70 (2.9–4.3)
AST (IU/L)	50.0 (25–256)
ALT (IU/L)	71.5 (38–380)
GGT (IU/L)	71.0 (22–180)
HBeAg (±)	11/1
HBV DNA (log copy/mL)	7.3 (5.9–8.7)

Albumin probe

Five oligonucleotides labelled with FITC were prepared based on the published sequence of albumin complementary DNA (cDNA) [4]. These included the regions of the cDNA coding for amino acids −17 to −8, −2 to 8, 111 to 121, 291 to 300, and 561 to 570 [5].

In situ hybridization

Liver sections were dewaxed and incubated in 0.2 N HCl for 10 min at room temperature, and washed with PBS 3 times, each time for 5 min. The sections were digested with proteinase K (final concentration, 2.5 μg/ml, SIGMA, St. Louis, MO, USA) for 30 min at 37 °C. Sections were fixed with 4 % paraformaldehyde (Wako, Japan) for 5 min, and then washed 3 times with PBS for 5 min. Sections were dehydrated and dried with cool air. Nonspecific probe binding was blocked using a prehybridization buffer [20 × SSC, dextran sulphate sodium, formamide (deionized), mix well all components, vortex and then add poly A (10 mg/ml), ssDNA (10 mg/ml), tRNA (5 mg/ml), 1 M DTT and 50 × Denhardt's solution were added], and this was incubated for 30 min at 37 °C. A labelled probe was added to the hybridization buffer with a final concentration of 2.5 ng/μl, and the sections were hybridized overnight at 37 °C in a humid chamber.

Visualization of albumin mRNA

After hybridization, slides were incubated twice for 15 min at 37 °C in a solution of formamide 50 % in buffer 2 × SSC, then washed twice with 2 × SSC in 2-mercaptoethanol (Wako, Japan) for 5 min. Sections were washed and blocked with 5 % skim milk for 30 min. Alkaline phosphatase-conjugated polyclonal rabbit anti-FITC (Dako Cytomation, Japan) antibody diluted 1:50 was applied for 1 h at room temperature. To enable the sections to develop, they were incubated overnight at 4 °C with BCIP/NBT substrate system (Dako Cytomation). Sections were washed with distilled water and counterstained with methyl green solution (Wako, Japan) for 30 min at room temperature, washed with distilled water and covered with Aquatex mounting media (Merck, Germany). For a negative control, we used one slide cover only with hybridization buffer omitting the probe. For a positive control, we used normal liver tissue.

Statistical analyses

Statistical analyses were performed with the statistical software package JMP version 5.1.2. Values are expressed as mean ± S.D. The changes in biochemical parameters

and cytokines were evaluated using a paired t test. A p value of <0.05 was considered to be statistically significant.

Results

Biochemical parameters and platelet count

The serum level of albumin before treatment was 3.70 ± 0.44 g/dl and was significantly increased very soon after the treatment was started. The levels after treatment for 3, 6, 9, and 12 months were 3.88 ± 0.29, 4.07 ± 0.27, 4.08 ± 0.31 and 4.18 ± 0.28 g/dl, respectively. In contrast, the serum level of ALT was significantly decreased very soon after the treatment was started.

As for, platelet count, this increased only gradually. The level of platelet count before treatment was $15.5 \pm 7.1 \times 10^4/\mu l$ and the levels after treatment for 3, 6, 9, and 12 months were 15.2 ± 6.7, 15.5 ± 7.8, 15.7 ± 7.3 and $17.0 \pm 8.4 \times 10^4/\mu l$, respectively (Fig. 1).

In situ hybridization

Staining for albumin mRNA was considered to be positive when a distinct fine granular cytoplasmic purple staining was observed. In the positive control liver sections, we observed abundant cords of hepatocytes stained for albumin mRNA. These were close to the portal areas. However, in the negative controls where the albumin probe was omitted, the signal for albumin mRNA was absent in the hepatocytes (Fig. 2).

In patients prior to treatment with nucleoside analogues, important areas of fibrosis and hepatic necrosis with inflammatory infiltrate were seen surrounding the portal areas and the albumin signal for mRNA was only slightly seen. Twelve months after the treatment, the liver tissue presented an obvious signal of albumin mRNA extensively in the hepatic lobes (Fig. 3).

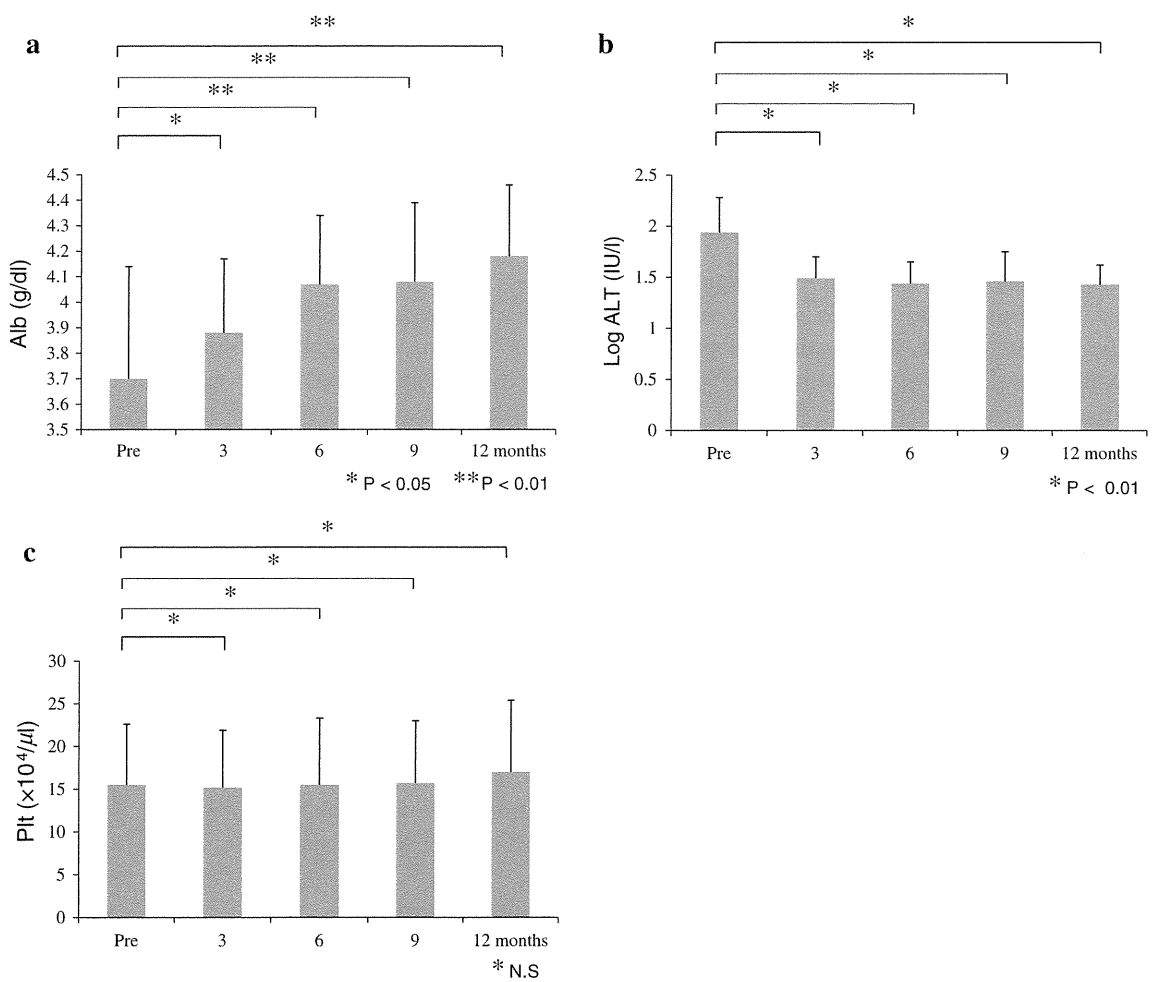

Fig. 1 **a** The serum level of albumin was significantly increased very soon after the treatment was started. **b** In contrast, the serum level of ALT was significantly decreased very soon after the treatment was started. **c** Platelet count increased only gradually

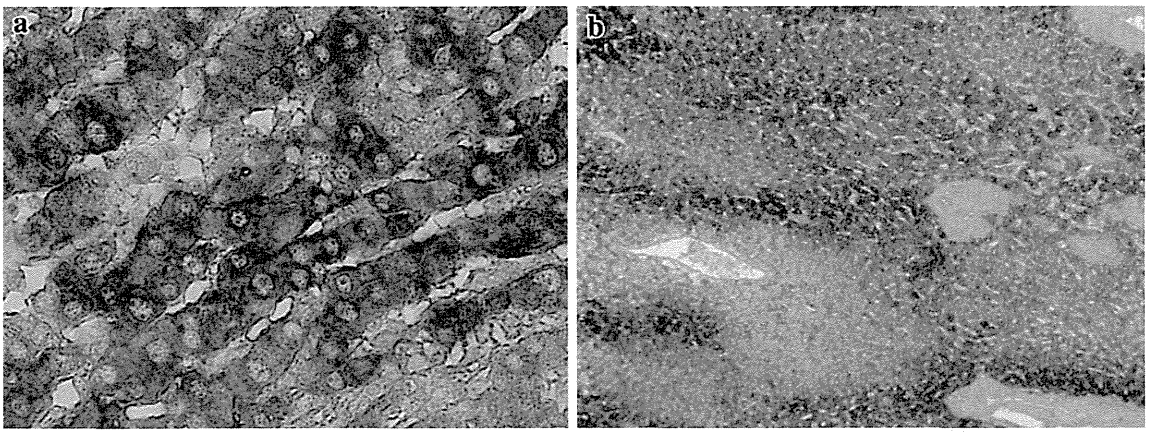

Fig. 2 In the positive control liver sections, we observed abundant cords of hepatocytes stained for albumin mRNA (**a**) (×400). These were close to the portal areas (**b**) (×100)

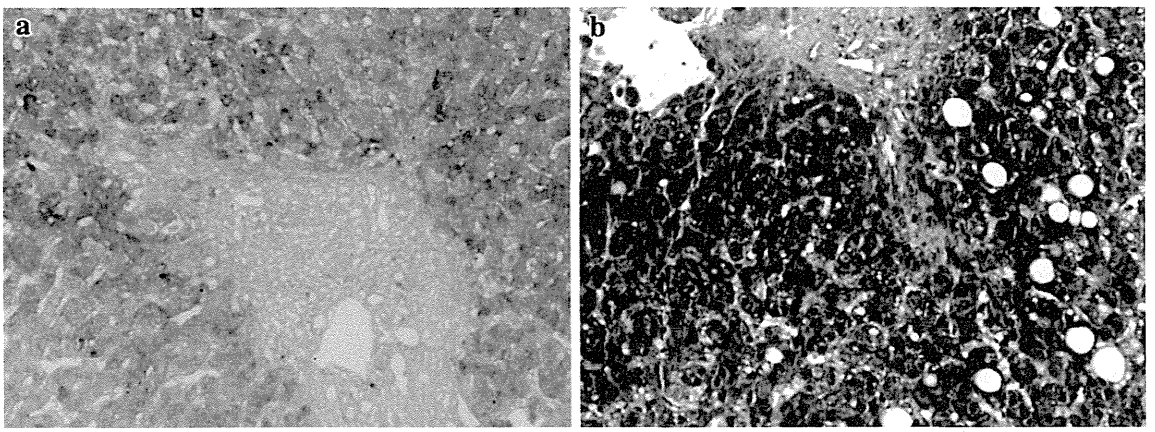

Fig. 3 In a patient (moderate fibrosis and moderate activity) prior to treatment with nucleoside analogue, fibrosis and hepatic necrosis with inflammatory infiltrate were seen surrounding the portal area and the albumin signal for mRNA was only slightly seen (**a**). After the treatment, the liver tissue presented an obvious signal of albumin mRNA extensively in the hepatic lobes (**b**) (×200)

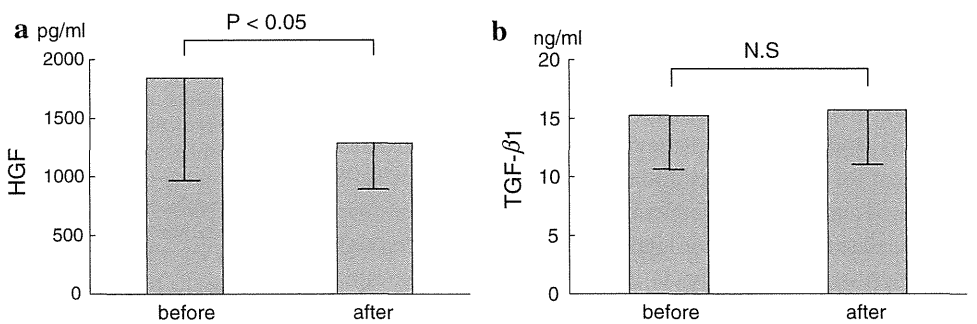

Fig. 4 **a** The serum level of HGF was significantly decreased 12 months after the treatment, **b** whereas the TGF-β1 level did not show any obvious change

Changes in serum levels of HGF and TGF-β1

The serum level of HGF before treatment was 1,845.3 ± 930.6 pg/ml. This was significantly decreased to 1,287.3 ± 443.7 pg/ml 12 months after the treatment, whereas the TGF-β1 level did not show any obvious change (Fig. 4).

Discussion

We successfully showed that albumin production is quickly restored following treatment with nucleoside analogues in both serum and liver tissue. We speculate that the decrease in HGF level brought about by the treatment directly led to

the restoration of albumin production. Although there were few cases, it has been proved with the significant difference statistically and the result of in situ hybridization was almost same in all the examples.

Nucleoside analogues block the reverse transcription of the HBV gene in hepatocytes, leading to a decrease in HBV replication. In most patients with CH-B who receive treatment with nucleoside analogues, serum levels of transaminase are normalized within a few months. Moreover, long-term treatment has been reported to lead to the recovery of fibrosis in the liver [6, 7]. The fibrosis stage in CH-B was reported to recover after a few years from the commencement of treatment, but it should be noted that the restoration of the serum level of albumin was seen much earlier than that.

Albumin synthesis is reported to be regulated by several cytokines, such as IL-6, HGF and TGFβ [8–13]. IL-6, an inflammatory cytokine which is released from lymphocytes or macrophages, increases serum C-reactive protein especially in case of bacterial infection. Under such conditions, albumin synthesis in the liver is suppressed. HGF and TGFβ are related to hepatocyte regeneration following liver damage and hepatic fibrosis, respectively.

Among the above cytokines, HGF is the most potent in regulating albumin synthesis. In general, HGF stimulates the albumin synthesis of hepatocytes, as well as DNA synthesis. However, HGF stimulates both albumin and DNA syntheses of hepatocytes, in a reciprocal relationship. Namely, when the DNA synthesis of hepatocytes is vigorous, HGF suppresses albumin synthesis [11]. In patients with viral hepatitis, serum level of HGF increases at the stage of exacerbation and reduces with recovering of serum level of ALT. A serum level of alpha-fetoprotein (AFP), one of proliferation markers of hepatocytes, is variable in parallel to serum level of HGF, and serum level of albumin rises contrary to the reduction of the AFP level at the healing stage.

In the present study, serum levels of HGF before treatment were high, suggesting that HGF may act to stimulate the DNA synthesis of hepatocytes for liver regeneration, resulting in the albumin synthesis of hepatocytes being suppressed. After treatment for hepatitis was initiated, the serum level of HGF decreased and albumin production was recovered. Therefore, we speculate that nucleoside analogues decrease HGF through the suppression of hepatocyte damage, leading to the rapid restoration of albumin production.

TGF-β1 is also an important cytokine in the albumin synthesis. Flisiak et al. [14] reported that lamivudine treatment for 6 months decreases plasma level of TGF-β1 in patients with CH-B. However, our results showed that TGF-β1 is not changed during treatment for 6 months. In our study, patient's age and TGF-β1 levels before treatment were different than the previous report. Thus, our cases were older and the liver fibrosis was more severe. We speculate that longer period of treatment with nucleotide analogues is required for reduction of TGF-β1 level in patients with CH-B with severe fibrosis.

There have been several studies using albumin in situ hybridization in HCC and also showing its use as a diagnostic method [15]. However, there have been only a few studies regarding albumin in situ production in hepatitis B and C which have mentioned the correlation between albumin serum level and the prognosis of the disease. However, the precise mechanism behind the production of albumin protein by hepatocytes during viral liver disease and after treatment still needs further clarification.

We showed that the mRNA of albumin was present in hepatocytes close to the portal area. Since inflammation mainly occurs at the portal area, we speculate that the HGF induced by inflammation was able to suppress albumin production in non-treated patients with CH-B.

In conclusion, our results lead us to believe that nucleoside analogues decrease HGF through the suppression of hepatocyte damage, thereby leading to the rapid restoration of albumin production in patients with CH-B. The significance of albumin production in regenerated liver tissue requires further investigation.

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