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Circulating soluble Fas levels in patients with hepatitis C virus infection and interferon therapy

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Background. The clinical relevance of the circulating soluble form of the Fas-Receptor (sFas) was investigated in patients with hepatitis C receiving type 1 interferon (IFN) therapy. ***Methods.*** sFas was quantified by enzyme-linked immunosorbent assay in 66 hepatitis C virus (HCV) carriers and 30 HCV-naive or previously infected controls. The levels were then monitored during enhanced treatment with type 1 IFNs in 15 chronic hepatitis C patients. ***Results.*** The HCV carriers had high levels of sFas compared with controls (3.8 ± 1.3 vs 2.7 ± 0.8 ng/ml; $P < 0.001$). sFas levels in patients with chronic HCV infection were directly related to serum alanine aminotransferase levels ($r = 0.440$; $P < 0.001$) and the histological grade ($r = 0.403$; $P = 0.019$). Among necroinflammatory reactions, only piecemeal necrosis showed a correlation with sFas levels ($r = 0.556$; $P = 0.001$). Pretreatment sFas levels, however, were not predictive of therapeutic outcomes. A sustained virological response to enhanced IFN therapy showed a relation to only the pretreatment HCV load. Interestingly, circulating sFas was upregulated when IFN-β was administered at short intervals (3 MU/every 12 h). This upregulation was accompanied by parallel aminotransferase elevation, which was observed regardless of a virological response. ***Conclusions.*** sFas elevation, in parallel with the severity of liver injury, suggests the possible upregulation of hepatic Fas expression and the Fas-mediated pathway in both HCV- and type 1 IFN-induced liver injury. The essential function of sFas to protect hepatocytes against Fas-mediated liver injury was not evident in these clinical settings.

Key words: hepatitis C, type 1 interferon, apoptosis, soluble Fas

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Introduction

Fas-Receptor/Fas ligand-mediated apoptosis of liver cells plays a significant role in the pathogenesis of hepatitis C. Hepatic upregulation of Fas was found to be correlated with more severe inflammation caused by ongoing hepatitis C virus (HCV) infection.[1,2] Parallel activation of T lymphocytes expressing Fas ligand was detected in liver-infiltrating mononuclear cells, allowing transduction of the apoptotic death signal to Fas-bearing hepatocytes.[3] Interferon (IFN)-α and IFN-β, members of the type 1 IFN family, are currently used for the treatment of chronic hepatitis C. Hepatic injury in patients treated with systemic administration of type 1 IFNs remains an unresolved problem. A recent study showed that apoptosis may also be involved in type 1 IFN-induced hepatotoxicity.[4]

Fas-Receptor (APO-1/CD95) has a single membrane-spanning domain and is expressed in hepatocytes. An alternatively spliced soluble form of the Fas-Receptor (sFas) without the transmembrane-spanning domain is produced by Fas-expressing hepatocytes,[5,6] and upregulation of Fas expression in hepatocytes contributes to the increase in serum sFas levels. It is of interest that sFas may serve to bind Fas ligand on cytotoxic T lymphocytes, thereby minimizing Fas-mediated liver injury.[7–9] However, the clinical relevance of circulating sFas is not yet fully understood in relation to chronic hepatitis C and type 1 IFN therapy. sFas elevation has been reported in chronic HCV infection,[10] but controversy remains. Possible upregulation of sFas during IFN therapy has also been shown, based on sFas levels within 24 h after IFN administration,[11] but the data are limited. To further our understanding of sFas, we studied serum sFas levels in patients with HCV infection as compared with HCV-naive subjects or previously infected controls. sFas levels were then monitored in the course of treatment with type 1 IFNs, and the results were examined in relation to liver injury.

Patients and methods

Patients

The subjects were 66 patients with chronic HCV infection (39 men and 27 women; age, 27–80 years [median, 59 years]) and 30 controls without ongoing HCV infection (21 men and 9 women, age 26–73 years [median, 56 years]). No significant difference was seen in sex (P = 0.426) or age (P = 0.761) distribution between the two groups. The 66 HCV carriers had been persistently positive for serum HCV RNA for more than 6 months. During the entire follow-up, none of the patients displayed any confounding etiology of liver disease, such as hepatitis B virus, autoimmune markers, or alcohol abuse (>25 g/day). Sera were obtained from all patients, and liver biopsy specimens could be obtained from 35 patients at the time of serum collection. After enrollment, 15 of the chronic hepatitis C patients (10 men and 5 women, age 33–63 years [median, 45 years]) were assigned to two different regimens of 4-week enhanced IFN induction therapy. Eight patients (group A) were treated with a high dose of IFN-α2a (Roferon-A; Nippon Roche, Tokyo, Japan; 9 MU/daily for 4 weeks, followed by 9 MU/thrice weekly; total dose, 720–1206 MU [median, 941 MU]). The remaining 7 patients (group B) received a high dose of IFN-β (Feron; Toray Industries, Tokyo, Japan; 3 MU/twice a day for 4 weeks, followed by 6 MU/thrice weekly; total dose, 276–636 MU [median, 384 MU]). Using stored serum samples obtained at 0, 1, 2, 4, and 8 weeks after initiation of the induction therapy, we investigated changes in serum alanine aminotransferase (ALT) activity, HCV RNA, and sFas levels.

Sera were also obtained from the 30 control subjects. The controls consisted of 8 healthy individuals without serum HCV RNA and HCV antibody, 8 serum HCV RNA-negative but HCV antibody-positive healthy individuals with no history of IFN therapy, and 14 nonviremic HCV antibody-positive patients who had been successfully treated with IFN therapy for chronic hepatitis C. Patients in the last group had remained negative for serum HCV RNA for 0.7–8.0 years (median, 4.0 years) after the end of previous IFN therapy. All serum samples were stored at −80°C without thawing until use. The study was approved by the local Research Ethics Committee, in accordance with the 1975 Declaration of Helsinki, and all the patients provided written informed consent.

Laboratory testing

HCV antibody was tested with a third-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostic Systems, Tokyo, Japan). Serum HCV RNA was detected by a polymerase chain reaction assay (Amplicor HCV Monitor; Roche Diagnostics, Tokyo, Japan) and quantified using a branched DNA (bDNA) assay (Quantiplex HCV-RNA; Chiron, Emeryville, CA, USA). HCV RNA-positive serum samples were subjected to HCV genotyping with the genotype-specific NS4 antibody assay (Immucheck-HCV Gr; International Reagent, Kobe, Japan), which is based on a comparison between antibody responses to the two genotype-specific NS4 antigens (C14-1 and C14-2).

Detection of sFas

Serum sFas levels were measured by ELISA (Medical and Biological Laboratories [MBL], Nagoya, Japan).[12,13] Serum sFas was first captured by polyclonal antibody to an intracellular sequence of human Fas (KDITSDSENSNFRNEIQSLV), and then detected by monoclonal antibody to an extracellular epitope (KCRCKPNFFC). Thus, the assay is theoretically expected to detect a complete sFas molecule composed of the extracellular and intracellular domains of Fas-Receptor, but is unlikely to detect incomplete cleaved forms of Fas-Receptor, which can be released following the destruction of Fas-expressing cells. In order to quantify the sFas levels, human Fas (GenBank accession no. CAA45250) was expressed in WR19L-12a cells, and standard Fas protein was purified from the membrane fraction. The assay was performed according to the manufacturer's instructions. In brief, a 96-well plate coated with the polyclonal antibody was incubated with 100 μl of fivefold diluted serum samples and standard Fas protein (0–2 ng/ml) at room temperature for 1 h. After thorough washing, the plate was further incubated with the peroxidase-conjugated monoclonal antibody at room temperature for 1 h. The plate was then washed, and a solution containing tetramethyl benzidine was added. After 30-min incubation, the reaction was stopped, and the optical density at 450 nm was measured. All assays for sFas were done in duplicate, and the concentration of sFas was determined from the standard curve. High levels of sFas were quantified using diluted serum samples.

Histological evaluation

Liver biopsy specimens were fixed in formalin and embedded in paraffin for routine staining with hematoxylin-eosin. All specimens were examined by the same experienced pathologist, who was unaware of the biochemical, serological, and virological data. Biopsy specimens were semiquantitatively evaluated by the histological activity index described by Knodell et al.[14]

Statistical analysis

All data values are expressed as means ± SD. For numeric variables, group comparisons were performed by the Wilcoxon nonparametric test. The χ^2 test or Fisher's exact test was used for categorical variables. Correlations between the variables were calculated using Spearman rank order correlations. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. For multivariate analysis, multiple logistic regression analysis, with a stepwise procedure, was performed with the computer program, SAS 8.02 (SAS Institute, Cary, NC, USA).

Results

Figure 1 shows serum sFas levels in relation to the presence of ongoing HCV infection. The 66 chronic HCV carriers had significantly higher levels of sFas (3.8 ± 1.3 ng/ml) than the 30 controls without ongoing HCV infection (2.7 ± 0.8 ng/ml; $P < 0.001$). In the control group, remote history of HCV infection exerted no impact on sFas levels. Serum sFas levels were 2.5 ± 0.3 ng/ml for the serum HCV RNA-negative but HCV antibody-positive healthy individuals who were spontaneously cleared of HCV. The levels were compatible with those for healthy individuals with no HCV markers (3.1 ± 0.9 ng/ml). sFas levels were equally low in chronic hepatitis C patients who had been clear of serum HCV RNA for 0.7–8.0 years (median, 4.0) after the end of IFN therapy (2.5 ± 0.9 ng/ml). No relation of sFas levels with time after IFN therapy was evident in these patients. No correlation was seen between sFas levels and age in the chronic HCV carriers ($r = 0.007$; $P = 0.954$) or the control subjects ($r = 0.103$; $P = 0.579$).

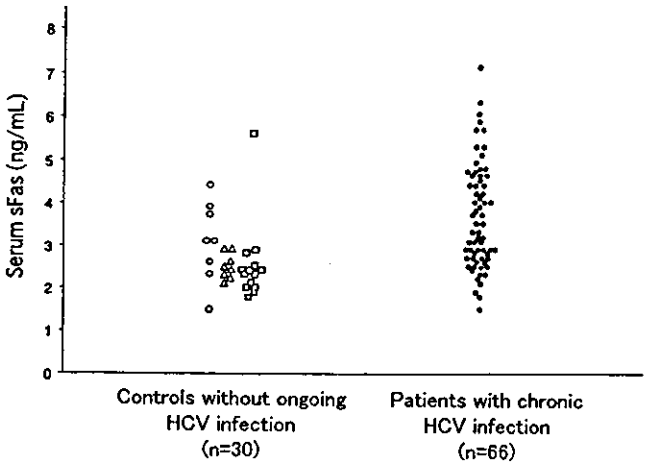

Fig. 1. Serum sFas levels in 66 patients with chronic hepatitis C virus (HCV) infection were compared with those in 30 controls without ongoing HCV infection (3.8 ± 1.3 vs 2.7 ± 0.8 ng/ml; $P < 0.001$). The controls consisted of 8 healthy individuals without serum HCV RNA and HCV antibody (*open circles*), 8 serum HCV RNA-negative but HCV antibody-positive healthy individuals without interferon (IFN) therapy (*open triangles*), and 14 patients who had remained negative for serum HCV RNA for 0.7–8.0 years (median, 4.0 years) after the end of IFN therapy for chronic hepatitis C (*open squares*). The levels of serum soluble Fas-Receptor (sFas) were 3.1 ± 0.9, 2.5 ± 0.3, and 2.5 ± 0.9 ng/ml for these three control groups, respectively, with no significant difference

The elevation of circulating sFas levels in chronic HCV infection was investigated in relation to virological, biochemical, and histological characteristics. In the 66 chronic HCV carriers, sFas levels had no relation to HCV replicative levels, assessed by serum HCV RNA titers (Fig. 2). This was also the case when the correlation was analyzed for each HCV genotype. sFas levels were 3.6 ± 1.2 ng/ml for the 19 patients infected with genotype 1, 3.6 ± 1.1 ng/ml for the 22 patients with genotype 2, and 3.8 ± 1.4 ng/ml for the remaining 25 patients with undetermined genotype(s), with no significant differences among the values. On the other hand, a significant correlation was found between serum sFas levels and ALT activity ($r = 0.440$; $P < 0.001$; Fig. 3). In 35 chronic HCV carriers, the circulating sFas levels were also correlated with liver histological findings (Fig. 4). A significant correlation was revealed between sFas levels and the total grading score ($r = 0.403$; $P = 0.019$), whereas no correlation was evident between sFas levels and the staging score ($r = 0.321$; $P = 0.057$). As for correlations with necroinflammatory scores, sFas levels showed a correlation with piecemeal necrosis ($r = 0.556$; $P = 0.001$), but not with lobular necrosis and inflammation ($r = 0.224$; $P = 0.192$) or portal inflammation ($r = 0.316$; $P = 0.066$).

Changes in serum sFas levels during 8 weeks of enhanced induction treatment with type 1 IFNs were

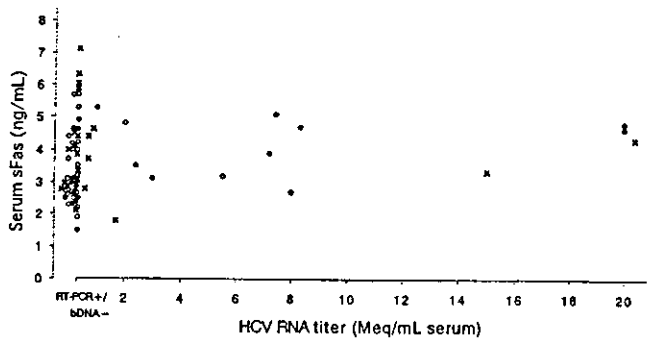

Fig. 2. Relation of serum sFas levels with serum HCV RNA titers and HCV genotypes in the 66 patients with chronic HCV infection. Serum sFas levels had no relation to serum HCV RNA titers and HCV genotypes. *Solid circles*, patients with genotype 1; *open circles*, patients with genotype 2; *crosses*, patients with undetermined genotype

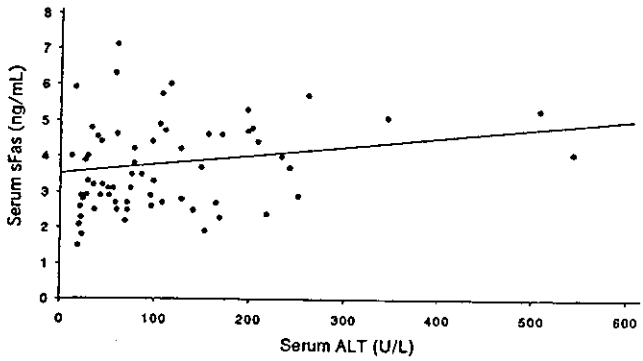

Fig. 3. Relationship between serum sFas levels and serum alanine aminotransferase (*ALT*) levels in the 66 patients with chronic HCV infection ($r = 0.440$; $P < 0.001$)

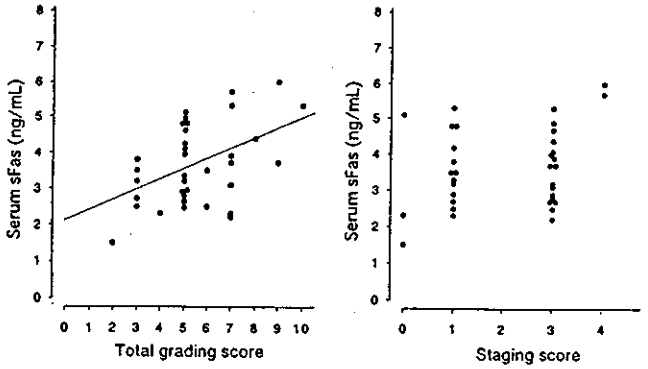

Fig. 4. Relationship between serum sFas levels and histological findings in 35 patients with chronic HCV infection. Serum sFas levels showed a correlation with the total grading score ($r = 0.403$; $P = 0.019$), but not with the staging score ($r = 0.321$; $P = 0.057$)

investigated in 15 chronic hepatitis C patients (Fig. 5). The pretreatment viral load ranged from less than 0.5 to 20.0 Meq/ml (median, 3.0 Meq/ml). Genotypes 1 and 2 were found in 9 and 6 patients, respectively. Of the 8 patients treated with IFN-α2a (group A), serum HCV RNA was cleared during the treatment in 7 patients. In these virological responders, no significant change was seen in serum sFas levels at weeks 1, 2, 4, and 8 as compared with the baseline level (3.7 ± 0.9 ng/ml). Thus, HCV clearance was not accompanied by a decrease in serum sFas levels. Serum ALT activity was normalized in 4 virological responders, but remained elevated in the other 3. In group B, the 7 patients received enhanced induction treatment with IFN-β. A virological response was achieved during the treatment by 6 patients. Despite HCV clearance, the virological responders showed an increase in sFas levels after the initial intensive treatment (3 MU/twice a day for 4 weeks; 4.5 ± 0.9 at baseline vs 7.1 ± 1.3 ng/ml at week 4; $P = 0.018$). The levels returned to the baseline after the subsequent treatment (6 MU/thrice weekly for 4 weeks). In parallel with the elevation of sFas levels, serum ALT activity was also elevated in the absence of HCV viremia in 5 of the 6 virological responders.

A sustained virological response, defined as serum HCV RNA clearance at 6 months posttreatment, was achieved by five patients (four patients in group A and one patient in group B). Table 1 shows the clinical and virological characteristics of the sustained virological responders and those of the patients showing no sustained virological response. A significant difference was observed only for the pretreatment viral load. All sustained virological responders had a low pretreatment viral load of less than 0.5 Meq/ml, whereas the levels ranged from less than 0.5 to 20.0 Meq/ml (median, 7.3 Meq/ml) in patients with no sustained virological response ($P = 0.014$). Pretreatment serum sFas levels were the same for patients with a sustained virological response and those without (4.5 ± 0.6 vs 3.8 ± 1.0 ng/ml; $P = 0.089$). Multivariate analysis also showed only the pretreatment viral load to be predictive of a sustained virological response ($P = 0.038$).

Discussion

Currently available information suggests that apoptosis-inducing death ligands and corresponding receptors mediate cell death in human liver diseases. Thus, knowledge regarding how this system functions may help in developing rational therapeutic strategies to ameliorate clinical liver diseases. Apoptosis-inducing death receptors belong to the tumor necrosis factor/nerve growth factor receptor superfamily. Fas-Receptor (APO-1/CD95), one of the death receptors, has a single membrane-spanning domain and is expressed in hepatocytes. The particular importance of the Fas-Receptor in chronic hepatitis C has been amply documented.[1,2] Hepatic Fas-Receptor expression has been shown to be upregulated in HCV infection, with a close correlation to the necroinflammatory grade. The Fas-Receptor-mediated apoptotic pathway may also play a significant role in the pathogenesis of other forms of liver injury. An alternatively spliced soluble form of the Fas-Receptor without the transmembrane-spanning domain is produced by Fas-expressing hepatocytes and is considered to serve to bind Fas ligand on cytotoxic T cells, thereby minimizing liver injury.[7-9] However, its clinical relevance has not been well explored.

The present study demonstrated that patients with chronic HCV infection had higher levels of circulating sFas than healthy individuals without previous HCV exposure and patients who had a remote history of HCV infection but had been cleared of HCV spontaneously or following IFN therapy. sFas levels in chronic HCV infection had no relation to HCV replication

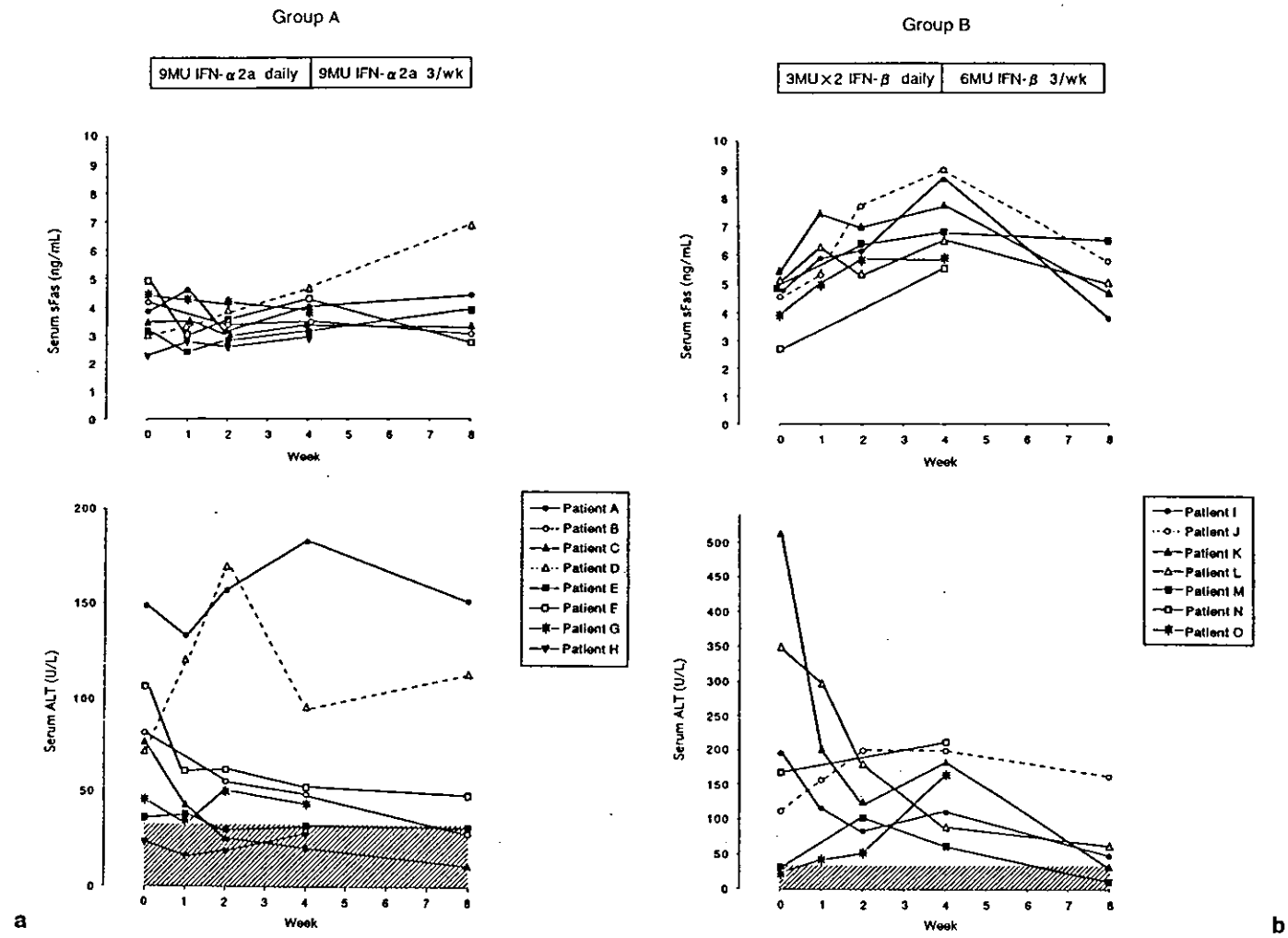

Fig. 5a,b. Changes in serum sFas and ALT levels during 8 weeks of enhanced IFN therapy for chronic hepatitis C. **a** Eight patients (patients A–H) received 9 megaunits (MU) of IFN-α2a daily for 4 weeks, followed by the same dose thrice a week for the subsequent 4 weeks (*group A*). **b** The other patients (patients I–O) received 3 MU of IFN-β twice a day continuously for 4 weeks, and then 6 MU of IFN-β thrice a week for 4 weeks (*group B*). The *Solid* and *dashed lines* in **a** and **b** indicate patients who were negative and positive for serum HCV RNA, respectively, at the end of treatment. In the end-of-treatment virological responders, serum sFas levels showed no significant change during IFN-α2a therapy in group A, while the levels increased following the initial 4-week intensive IFN-β therapy in group B (4.5 ± 0.9 before treatment vs 7.1 ± 1.3 ng/ml at 4 weeks; $P = 0.018$) and returned to the baseline levels at 8 weeks. Serum sFas elevation at 4 weeks was associated with serum ALT elevation observed during IFN-β therapy. *Shaded areas* indicate the normal range of serum ALT activity

and genotypes, but were correlated with biochemical and histological disease activity. Serum sFas levels measured by the assay we used have been shown to correlate with hepatic Fas expression.[10] Hepatic Fas expression in chronic hepatitis C is particularly prominent at the advancing edge of piecemeal necrosis.[1] sFas levels were also correlated with the severity of necroinflammatory reactions, especially that of piecemeal necrosis. Collectively, the data obtained suggest the upregulation of hepatic Fas expression and the involvement of the Fas-mediated pathway in HCV-related liver injury. In contrast with experimental studies, sFas in our study failed to protect hepatocytes against Fas-mediated liver injury. This discrepancy may be attributable to the amount of circulating sFas protein. The inhibition of Fas-Receptor-mediated liver injury by sFas has been reported in mice which were given very large amounts of sFas.[8] Serum sFas levels in mice could have been much higher than those observed in humans, and the levels in chronic hepatitis C patients may not be enough to inhibit the apoptosis caused by Fas ligand on cytotoxic T lymphocytes and its soluble form. Further studies are necessary to clarify the impact of sFas on the Fas-mediated pathway in humans. Thus far, elevation of sFas levels in chronic HCV infection has also been reported,[10] but controversy remains.[11] The

Table 1. Factors predictive of a sustained virological response to IFN therapy with 4-week enhanced induction treatment for chronic hepatitis C

Characteristics	Sustained response (n = 5)	No sustained response (n = 10)	P
Age (years)	50 ± 12 52 (37–63)	47 ± 9 45 (33–58)	0.540
Sex (M/F)	4/1	6/4	0.600
Serum sFas (ng/ml)	4.5 ± 0.6 4.4 (3.7–5.3)	3.8 ± 1.0 3.7 (2.3–5.1)	0.178
HCV genotype (1/2)	1/4	8/2	0.089
Serum HCV RNA titer (Meq/ml)	<0.5 (<0.5)	7.3 (<0.5 to 20.0)	0.014
Serum ALT (U/l)	177 ± 189 105 (43–508)	108 ± 103 75 (21–345)	0.327
Histological grading score	7.4 ± 2.3 8 (5–10)	5.0 ± 1.5 5 (3–7)	0.072
Histological staging score	2.2 ± 1.1 3 (1–3)	1.7 ± 1.3 1 (0–3)	0.463
Total IFN dose (MU)	863 ± 302 891 (384–1206)	611 ± 271 597 (276–990)	0.111

Values are given as means ± SD and medians (ranges)

different findings may be attributable to differences in the necroinflammatory grade of the study populations and the different sFas assays used.

In the present study, changes in sFas levels following type 1 IFN administration were investigated. IFN-α and IFN-β, members of the type 1 IFN family, are currently used to treat a number of infectious diseases and cancers. A growing number of chronic hepatitis C patients have been treated with type 1 IFNs, but the effectiveness of these agents is often limited due to adverse side effects. The pathophysiological mechanisms have not been well defined. Type 1 IFN-induced hepatotoxicity is one of such adverse effects and causes serum ALT elevation in patients with a virological response. Recently, several studies showed that the antiviral effects were more pronounced in patients treated twice daily with IFN-β compared with once-daily dosing, but that IFN-induced liver injury was more common with the twice-daily treatment protocol.[15,16] The present study showed that virological response to high-dose type 1 IFN therapy was not accompanied by a reduction of sFas levels and that serum ALT levels were likely to remain elevated, despite the clearance of circulating HCV RNA. Interestingly, sFas levels in virological responders increased during intravenous twice-daily treatment with IFN-β, and the increase was accompanied by an exacerbation of biochemical disease activity.

Acute hepatopathy with elevated serum aminotransferase levels has also been reported in sheep treated with twice-daily administration of IFN tau, a type 1 IFN, and apoptosis was shown to play a critical role in the IFN tau-induced hepatotoxicity.[4] Type 1 IFNs can affect the expression of various genes and induce apoptosis, but the mechanism of the type 1 IFN-induced apoptosis remains to be explored. Thus far, regulatable expression of the IFN-induced double-stranded RNA-dependent protein kinase, PKR, has been shown to trigger apoptosis, possibly through upregulation of the Fas-Receptor.[17,18] The data obtained in our study support this hypothesis. Elevation of sFas levels and serum ALT activity in virological responders to IFN indicates IFN-induced upregulation of hepatic Fas-Receptor expression and its involvement in type 1 IFN-induced hepatotoxicity. Again, we found that sFas exerted no apparent inhibition of Fas-mediated liver injury during IFN therapy, thus raising further questions as to whether the Fas-mediated pathway is controlled by sFas in humans. Recently, it has been reported that upregulation of sFas can be induced in the very early phase of IFN therapy.[11] Further studies are necessary to work out sFas dynamics in more detail during various treatment regimens.

At present, it remains to be worked out whether sFas levels in chronic hepatitis C are relevant to the therapeutic efficacy of IFN. In the present study, the majority of the patients achieved an end-of-treatment virological response after the enhanced IFN treatment, irrespective of the pretreatment patient characteristics. However, a sustained virological response was related to only the pretreatment viral load. Thus, it was shown that pretreatment circulating sFas levels have little impact on the final therapeutic outcome when patients are subjected to enhanced IFN therapy. Finally, it is likely that upregulation of the Fas-mediated pathway also occurs in other types of liver injury and is accompanied by changes in serum sFas levels. More studies are necessary to address this issue and to further our understanding of sFas.

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Immunohistochemical metallothionein expression in hepatocellular carcinoma: relation to tumor progression and chemoresistance to platinum agents

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Background. Metallothionein (MT), which is known to detoxify heavy metal ions, is considered to serve as a mechanism of resistance to platinum complex compounds. In the present study, MT expression in hepatocellular carcinoma (HCC) was immunohistologically investigated to clarify its relationship to clinical background factors and responsiveness to anticancer drugs. ***Methods.*** Specimens from 117 patients with HCC were immunohistologically studied, using a monoclonal anti-MT antibody. the percentage of MT-positive HCC (MT ratio) cells was determined, to evaluate the extent of staining with anti-MT antibody. Staining with an MT ratio of more than 50% was categorized as diffusely positive; an MT ratio of 5% to less than 50% was focally positive; and an MT ratio of less than 5% was negative. Twenty-two patients received repeated arterial infusion chemotherapy with carboplatin (CBDCA), a platinum-containing compound, and the MT expression was analyzed in relation to their chemotherapeutic response. ***Results.*** The ratio of MT-positive cells in HCC decreased with the degree of histological differentiation and also decreased with higher tumor stage. In patients treated with CBDCA, the ratio of MT-positive cells in responders was significantly lower than that in non-responders. ***Conclusions.*** MT expression decreases with the degree of histological differentiation and decreases with increasing tumor stage in HCC. In addition, MT expression may lower the antitumor effect of CBDCA.

Key words: metallothionein, immunohistochemistry, hepatocellular carcinoma, chemotherapy, platinum compounds

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Introduction

Metallothionein (MT) is a low-molecular-weight intracellular protein rich in cysteinyl residues and with a high affinity for heavy metal ions. It is found in the liver and kidney in high concentrations and is inducible by a variety of metals, hormones, and inflammatory cytokines. It stores essential trace metals, such as zinc and copper, detoxifies heavy metals, such as cadmium and mercury, and scavenges reactive oxygen species showing a protective action in the body.[1–3] Recently, it has been shown that MT expression is related to different stages of development and progression of various tumors in humans.[4–8]

The high affinity of MT for metal ions is related to resistance to anticancer agents, especially resistance to metal-containing chemotherapeutic compounds. MT protects cells from the cytotoxicity of cis-diammine dichloroplatinum II (CDDP) in various cultured cell lines.[9–13] However, no prior studies have examined the relationship between MT expression and drug resistance in hepatocellular carcinoma (HCC).

In the present study, we investigated immunohistochemical MT expression in HCC and evaluated its relation to clinical background factors. Furthermore, we evaluated MT expression in specimens from patients receiving chemotherapy with carboplatin (CBDCA), a platinum-containing compound, to determine the relation of MT expression to the observed chemotherapeutic effects.

Subjects, materials, and methods

Formalin-fixed paraffin-embedded materials from 117 patients with HCC were investigated in this study (Table 1). Of the 117 specimens, 35 were obtained from surgical resections conducted between 1989 and 1997 at the Second Department of Surgery, Chiba University

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Wild-type Precore and Core Promoter Sequences in Patients with Acute Self-limited or Chronic Hepatitis B

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Background: Mutations in the precore region and core promoter were compared between patients with acute and chronic hepatitis B. **Methods:** There were 69 patients with acute self-limited hepatitis B and 210 with chronic hepatitis B who had been followed for >15 years. The hepatitis B virus (HBV) of genotypes A, B and C was detected in 14 (23%), 8 (13%) and 28 (45%) of the patients with acute self-limited hepatitis, respectively, in contrast to 11 (5%), 25 (12%) and 167 (80%) of those with chronic hepatitis. **Results:** At presentation, hepatitis B e antigen (HBeAg) in serum was the more common (82% versus 65%, $P < 0.05$), and the wild-type sequences of the precore region (100% versus 74%, $P < 0.001$) and core promoter (88% versus 36%, $P < 0.00001$) were more frequent in the 50 patients with acute self-limited hepatitis than the 203 patients with chronic hepatitis B who were infected with HBV of genotype A, B or C. Wild-types of both the precore region and core promoter persisted in acute self-limited hepatitis, while they decreased from 28% to 10% in chronic hepatitis over the course of >15 years. **Conclusion:** HBV with the wild-type sequences of the precore region and core promoter prevails in patients with acute self-limited hepatitis, unlike in patients with chronic hepatitis.

Key words: Hepatitis B e antigen; hepatitis B surface antigen; hepatitis B virus; hepatocellular carcinoma; genotypes

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Of two billion people in the world exposed to the hepatitis B virus (HBV), it is estimated that 350 million are infected with it persistently (1). In regions highly endemic for HBV, such as Asia and Africa, the persistent carrier state is established by perinatal transmission or early in infancy. Carriers serve as the reservoir of HBV in the community, and can spread the infection to susceptible individuals. Before hepatitis B surface antigen (HBsAg) was identified as the marker of HBV infection in the early 1970s, transmission in adulthood occurred through transfusions and inappropriate medical practices. Transmission now takes place through promiscuous sexual contact and through the sharing of needles and syringes for injection of illicit intravenous drugs.

The wild-type HBV can direct the synthesis of hepatitis B e antigen (HBeAg) in hepatocytes. The production of HBeAg is abolished by mutations in the precore region that prohibit the translation of HBeAg precursor (2, 3) and is reduced by mutations in the core promoter that down-regulate the transcription of precore RNA for the HBeAg precursor (4, 5). The ability of HBV to encode HBeAg is crucial in persistence of infection as well as pathogenesis of chronic hepatitis B.

Mutations in the precore region and core promoter were compared between 50 patients with acute self-limited hepatitis B and 203 patients with chronic hepatitis B, all of whom were infected with HBV genotype A, B or C, and their clinical course was followed in a city hospital in Tokyo. The results highlighted markedly different virological characters of HBV, in terms of its ability to encode HBeAg, between acute self-limited and chronic hepatitis B.

Patients and Methods

Patients

In the period from 1971 to 1986, 69 patients with acute self-limited hepatitis B (62 M, 7 F; median age 32 years (range 19–63 years)) visited the Dept. of Gastroenterology at Toranomon Hospital in Metropolitan Tokyo. They had serum HBsAg and antibody to hepatitis B core antigen (anti-HBc) of IgM class in high titres for the diagnosis of acute hepatitis B. HBV infection resolved with the loss of HBsAg from serum in

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62 (90%) within 6 months of admission. There were 210 patients with chronic hepatitis B who visited the same hospital during the 15 years (1971–86) and had been followed for 15 years or longer. Serum samples were collected from patients at regular intervals from the day of admission and were stored at −80 °C until virological tests. Fifty of the 69 (72%) patients with acute self-limited hepatitis and the 203 of the 210 (97%) with chronic hepatitis, who were infected with HBV genotype of A, B or C, were compared for serum HBeAg as well as mutations in precore region and core promoter in HBV DNA samples recovered from their sera. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of Toranomon Hospital. Each patient gave written informed consent on the purpose of this study.

Serum markers of HBV infection

HBsAg was determined with commercial kits by haemagglutination (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan) and radioimmunoassay (AUSRIA II-125, Dinabot, Tokyo, Japan), and IgM anti-HBc was tested by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBc-antiM RIA; Dinabot). HBeAg was determined by ELISA (ELISA, F-HBe; Kokusai Diagnostic, Kobe, Japan). Serotypes of HBsAg were determined by ELISA with commercial kits (HBs Antigen Subtype EIA, Institute of Immunology). The six major genotypes of HBV (A–E) were determined by ELISA with commercial kits (HBV Genotype EIA, Institute of Immunology) after the method of Usuda et al. (6, 7). This involves the expression of preS2 epitopes, detected by monoclonal antibodies, the combination of which is specific for each of the six HBV genotypes. Genotype G, which was discovered recently (8), was determined by the combination of preS2 serotype for genotype D and HBsAg subtype of *adw*; it is specific for this genotype (9). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per millilitre. The lower detection limit of this assay is 3.7 LGE/mL, which is equivalent to 5,000 copies/mL.

Nucleotide sequences of the precore region and core promoter

Nucleic acids were extracted from serum with a Smitest EX & R kit (Genome Science, Tokyo, Japan). For determination of the wild-type or mutants in the precore region, they were amplified by polymerase chain reaction (PCR) with nested primers. The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ (nucleotides (nt) 1580–1599)) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ (nt 2295–2276)) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAC CAC CGT GAA C-3′ (nt 1606–1625)) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ (nt 2014–1995)) for 35 cycles each (94 °C, 1 min (5 min in the first cycle); 53 °C, 2 min; and 72 °C, 3 min (7 min in the last cycle)). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, Calif., USA). Amplification products were isolated with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and then sequenced in ABI Prism 310 Genetic Analyzer (Applied Biosystems). Mutations interfering with the translation or transcription of HBeAg were sought in the precore region and core promoter. These included a G-to-A mutation at nt 1896 in the precore region (A1896) and the double mutation in the core promoter converting nt 1762 from A to T as well as nt 1764 from G to A (T1762/A1764). Also examined was nt 1858 of T or C in the precore region sequence.

Statistical analyses

Categorical data were compared between groups using the chi-squared test as well as the Fisher exact test, and non-categorical data using the Mann-Whitney *U* test. Data analysis was performed using the SAS computer program (10), and *P* values less than 0.05 were considered significant.

Results

Patients with acute self-limited and chronic hepatitis B

Among the 69 patients with acute self-limited hepatitis B, HBV genotypes were A in 14 (23%), B in 8 (13%) and C in 28 (45%). Of the 50 (72% of the total) patients infected with HBV of genotype A, B or C (median age 33 years), 48 were men (96%). Extramarital sexual contacts were reported by 27 (54%). Family members with liver diseases were recognized in 3 (6%), all of whom were wives. Serum HBeAg was detected in 41 (82%) and 4 (8%) had lost HBsAg from serum already at presentation.

The baseline characteristics of patients with acute self-limited hepatitis B who were infected with HBV genotype A, B or C are compared in Table I. HBsAg subtypes were mostly *adw* in patients infected with HBV genotype A or B in contrast to *adr* frequent in those with HBV genotype C. There were no significant differences in demographic and virological characteristics among the patients infected with HBV genotypes A, B and C.

Of the 210 patients with chronic hepatitis B who visited the same hospital and had been followed for >15 years, 203 (97%) were infected with HBV of genotypes A (11 (5%)), B (25 (12%)) or C (167 (82%)). The frequency of genotype A was lower in chronic than in acute self-limited hepatitis B (5% versus 23%, $P < 0.001$). The 203 patients had a median age of 35 years (range 12–66), and there were 166 (82%) males. At least one family member with liver disease was found in 125 in a frequency significantly higher than that in patients with acute self-limited hepatitis B (62% versus 6%, $P < 0.0001$). HBeAg was detected in sera from 131 of them at presentation, significantly less frequently than in patients with acute self-

Table I. Baseline characteristics of patients with acute self-limited hepatitis B

Features	Genotype A ($n = 14$)	Genotype B ($n = 8$)	Genotype C ($n = 28$)
Age (years)*	32 (25–56)	35 (24–55)	32 (19–63)
Male	14 (100%)	8 (100%)	21 (75%)
Infection routes			
Heterosexual	9 (64%)	3 (37%)	18 (64%)
Homosexual	2 (14%)	0	0
Needlestick	0	1 (13%)	1 (4%)
Unknown	3 (22%)	4 (50%)	9 (32%)
Bilirubin (mg/dl)*	7.7 (0.8–17.3)	9.6 (1.2–26.3)	7.7 (0.6–15.9)
ALT (IU/l)*	1896 (968–3300)	1185 (1026–4484)	1983 (670–4056)
HBeAg	13 (93%)	6 (75%)	22 (79%)
HBsAg subtypes			
adw	13 (93%)	7 (88%)	5 (18%)
adr	0	0	22 (79%)
adwr	0	0	1 (4%)
Untypeable	1 (7%)	1 (13%)	0
HBV DNA (LGE†/ml)*	8.5 (<3.7–>8.7)	2.4 (<3.7–5.0)	3.5 (<3.7–>8.7)

*Median values are given with the range in parentheses.
†Log geometric equivalents determined by transcription-mediated amplification and hybridization-protection assay.

limited hepatitis B who were infected with HBV of genotype A, B or C (65% versus 82%, $P < 0.05$).

Baseline characteristics are compared among patients with chronic hepatitis who were infected with HBV genotype A, B or C (Table II). HBeAg was detected at presentation in patients infected with HBV genotype B less frequently than in patients with genotype A or C ($P = 0.018$). HBsAg genotype was predominantly *adw* in patients infected with HBV genotype A or B, while it was frequently *adr* in those with genotype C. HBV DNA levels at presentation were significantly lower in patients infected with HBV genotype B than in those with genotype A or C ($P = 0.001$).

Wild-type sequences of the precore region and core promoter at the presentation of patients with acute self-limited or chronic hepatitis B

Nucleotide sequences of the precore region and core promoter were determined on HBV DNA samples from patients at presentation. Wild-type sequences of the precore region and core promoter that regulate the synthesis of HBeAg were examined and compared between patients with acute self-limited and chronic hepatitis B.

Wild-type sequences of the precore region (43/43 or 100% versus 150/203 or 74%, $P < 0.001$) and core promoter (38/43 88% versus 73/203 or 36%, $P < 0.0001$) were detected significantly more frequently in patients with acute self-limited than chronic hepatitis B, in agreement with HBeAg, which was more frequent in the former than in the latter (Fig. 1). There was no appreciable influence of HBV genotypes on the wild-type sequence of the precore region or core promoter in patients with acute self-limited hepatitis B (Table III). The wild-type sequences of both the precore region and core promoter had persisted until they resolved HBV infection with the loss of HBsAg.

Table II. Baseline characteristics of patients with chronic hepatitis B

Features	Genotype A ($n = 11$)	Genotype B ($n = 25$)	Genotype C ($n = 167$)
Age (years)*	28 (12–55)	39 (25–56)	35 (15–66)
Men	10 (90%)	21 (84%)	135 (81%)
Liver fibrosis*			
F1	7 (64%)	22 (88%)	105 (63%)
F2	4 (36%)	3 (12%)	62 (37%)
HBeAg	8 (73%)	8 (32%)	114 (66%)
HBsAg subtypes			
adw	11 (100%)	24 (96%)	2 (1%)
adr	0	0	138 (83%)
ayr	0	0	2 (1%)
adwr	0	0	23 (14%)
adyr	0	0	2 (1%)
Untypeable	0	1 (4%)	0
HBV DNA (LGE†/ml)*	8.6 (<3.7–> 8.7)	6.9 (<3.7–> 8.7)	7.5 (<3.7–> 8.7)

*Median values are given with the range in parentheses.
†Log geometric equivalents determined by transcription-mediated amplification and hybridization-protection assay.

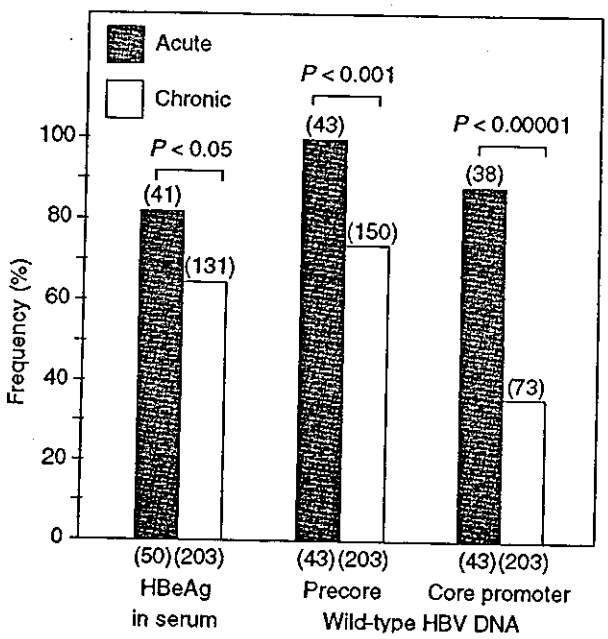

Fig. 1. Prevalence rates of serum HBeAg as well as the wild-type sequences of the precore region and core promoter in HBV DNA samples from patients with acute self-limited or chronic hepatitis B.

In patients with chronic hepatitis B, however, HBV genotypes had an influence on the prevalence of wild-type precore and core promoter sequences (Table IV). At presentation, genotype B infection accompanied the less frequent wild-type precore sequence than genotypes A ($P < 0.01$) or C ($P < 0.05$), as well as the more common wild-type sequence of the core promoter than genotypes A ($P < 0.05$) or C ($P < 0.001$) infection. After >15 years of follow-up, the wild-type precore region was more frequent in patients infected with HBV genotype A than B ($P < 0.001$) or C ($P < 0.001$), and the wild-type core promoter was more common in patients with genotype B than C ($P < 0.001$) infection. The 1858th nucleotide making a pair with nt 1896 of G in the wild-type, or A in precore mutants, was T in all HBV isolates from patients infected with HBV genotype B or C; it was C in all patients infected with HBV genotype A.

Fig. 2 compares the distribution of mutations in the precore region and core promoter between patients with acute self-limited and chronic hepatitis B at presentation. Patients with chronic hepatitis B were classified into two groups by the presence or absence of serum HBeAg at presentation. Wild-type sequences in the precore region and core promoter were detected in 88% of the patients with acute self-limited hepatitis B, significantly more frequently than in 36% of patients with chronic hepatitis B with HBeAg ($P < 0.0001$) or in 13% of those without HBeAg at presentation ($P < 0.0001$). Among the patients with chronic hepatitis B, wild-type sequences in the precore region and core promoter were more frequent (36% versus 13%, $P < 0.01$), and mutations in the precore region and core promoter were more common in those with HBeAg than in those without (51% versus 36%, $P < 0.01$) at presentation.

Precore and core promoter mutations increasing in patients with chronic hepatitis B infected with HBV genotype B or C during the course of >15 years

Nucleotide sequences of the precore region and core promoter were compared in patients with chronic hepatitis B at presentation and after follow-ups >15 years (Fig. 3). Overall, mutations in the precore region increased from 54% to 65% and those in core promoter from 64% to 83% during 15 years or longer. There were marked decreases in prevalence of the wild-type precore region (more than 35%) and core promoter (more than 16%) during >15 years in the patients infected with HBV of genotype B or C (Table III). In

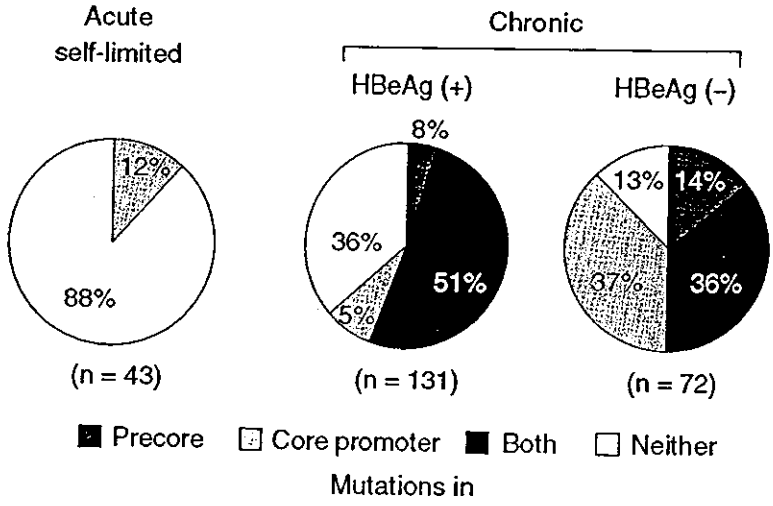

Fig. 2. Distribution of mutations in the precore region and core promoter in HBV DNA samples from patients with acute self-limited or chronic hepatitis B at presentation.

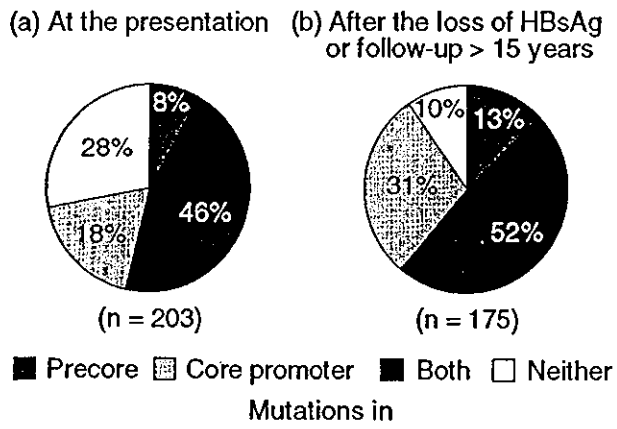

Fig. 3. Changes in mutations of the precore region and core promoter in HBV DNA samples from patients with chronic hepatitis B from presentation until they lost HBsAg or after follow-ups >15

Table III. Wild-type sequences of the precore region and core promoter in patients with acute self-limited hepatitis B who were infected with HBV of genotype A, B or C

Time and genotypes	Wild-type	
	Precore region	Core promoter
At the presentation		
Genotype A ($n = 11$)	11 (100%)	10 (91%)
Genotype B ($n = 5$)	5 (100%)	5 (100%)
Genotype C ($n = 27$)	27 (100%)	23 (85%)
Total ($n = 43$)	43 (100%)	38 (88%)
At the loss of HBsAg		
Genotype A $n = 4$)	4 (100%)	4 (100%)
Genotype B ($n = 1$)	1 (100%)	0 (0%)
Genotype C ($n = 15$)	15 (100%)	13 (87%)
Total ($n = 20$)	20 (100%)	17 (85%)

the 11 patients infected with HBV genotype A, by contrast, precore mutations did not increase at all, while mutations in the core promoter increased by only 9%.

Discussion

In Japan, the HBV carrier state has been established most frequently through perinatal transmission from infected mothers with serum HBeAg to their babies (11, 12). In 1986, the national programme was implemented for protecting babies born to HBeAg-positive mothers with a combined passive and active immunization by hepatitis B immune globulin and vaccine. The indication for immunoprophylaxis was extended to babies born to carrier mothers without serum HBeAg in 1995. Consequently the prevalence of HBsAg in children has decreased from 2% to less than 0.1% in the past 15 years. Transmission of HBV by transfusions has decreased markedly since screening for HBsAg started in 1972 and even further after tests for anti-HBc began in 1986 and for HBV DNA by nucleic acid technology (NAT) in 2000.

Suspected transmission routes in the 50 patients with acute self-limited hepatitis infected with HBV of genotype A, B or C, disclosed the current inclination of adults who acquire HBV infection in Japan. Extramarital sexual contacts accounted for HBV infection in 54%. Family members with liver disease, by contrast, were less frequent in the case of patients with acute self-limited hepatitis B than in those with chronic hepatitis B (62% versus 6%, $P < 0.0001$). Of the three major genotypes in Japan (A, B and C), genotype A was significantly more frequent in patients with acute self-limited than chronic hepatitis B (28% versus 6%, $P < 0.001$), confirming our previous report (13) and that of others (14). HBV genotype A is rare in Japan, and it would therefore have spread mostly by contacts with professional sex workers from abroad.

The 50 patients with acute self-limited hepatitis and the 203 with chronic hepatitis B, for whom sequencing of HBV DNA was feasible, were compared for serum HBeAg as well as for mutations in the precore region and core promoter; they were all infected with HBV of genotype A, B or C. HBeAg in serum was more frequent in patients with acute self-limited than in those with chronic hepatitis B at presentation (82% versus 65%, $P < 0.05$). The most remarkable virological differences between patients with acute self-limited and chronic hepatitis B were the sequences of the precore region and core promoter. The wild-type precore sequence was detected in HBV DNA samples from all 50 patients with acute self-limited hepatitis B at presentation, and the wild-type core

Table IV. Wild-type sequences of precore region and core promoter in 203 patients with chronic hepatitis B who were infected with HBV of genotype A, B or C

Time and genotypes	Wild-type			
	Precore region	Differences	Core promoter	Differences
At the presentation				
Genotype A ($n = 11$)	11 (100%)	$P < 0.01$	4 (36%)	$P < 0.05$
Genotype B ($n = 25$)	13 (52%)		19 (76%)	
Genotype C ($n = 167$)	126 (75%)	$P < 0.05$	50 (30%)	$P < 0.001$
Total ($n = 203$)	150 (75%)		73 (36%)	
After the loss of HBsAg or follow-up >15 years				
Genotype A ($n = 11$)	11 (100%)	$P < 0.001$	3 (27%)	
Genotype B ($n = 25$)	4 (16%)	$P < 0.001$	13 (52%)	
Genotype C ($n = 167$)	53 (32%)		21 (13%)	$P < 0.001$
Total ($n = 203$)	68 (33%)		37 (18%)	

promoter in 38 (88%) of them. These results have confirmed and extended the report of Ogawa et al., who found the wild-type precore sequence in 23 of 25 (92%) patients and the wild-type core promoter in 16 of 21 (76%) patients with acute hepatitis B from Japan (14). Our findings are also consistent with wild-types of the precore region and core promoter that have been detected much more prevalently in patients with acute self-limited than fulminant hepatitis B (15, 16).

In remarkable contrast, the wild-type precore sequence was detected less often in 150 of the 203 (74%) patients with chronic hepatitis B at presentation ($P < 0.001$) and those in core promoter in only 73 (36%) of them ($P < 0.001$) than in patients with acute self-limited hepatitis B. These results indicate that acute hepatitis B would have been induced by street HBV strains with the wild-type of both the precore region and core promoter. By contrast, chronic hepatitis B would be a consequence of long-standing HBV infection since time of birth in Japan. The wild-type sequences in both the precore region and core promoter were found in only 36% of the patients who presented with chronic hepatitis. Hence the majority would have been seroconverting toward the antibody to HBeAg accompanied by elevated transaminase levels in serum.

The 1858th nucleotide makes a pair with the 1896th nucleotide and affects the mutation from G to A at this position (17–19). It was C in all HBV isolates of genotype A as has been reported. In contrast, nt 1858 was T in all HBV isolates of genotypes B, as well as in those of genotype C, unlike HBV isolates of genotype C from Hong Kong that frequently possess C1858 (20, 21).

There were many differences in the evolution of precore and core promoter mutations between patients with acute self-limited and chronic hepatitis B. The wild-type of the precore region or core promoter persisted in most patients with acute self-limited hepatitis until they resolved HBV infection. It was reasonably delineated in view of a short duration while they had clinical diseases. By contrast, the wild-type of both precore and core promoter decreased in patients with chronic hepatitis B during >15 years of follow-up in a manner dependent on HBV genotypes. Th wild-type precore sequence thus decreased by >35% and the wild-type core promoter by >16% in patients infected with HBV of genotype B or C. These results concur with changes in precore and core promoter mutations in HBV strains infecting 7 Japanese patients followed for 6–11 years (22). In patients infected with HBV of genotype A, however, the wild-type sequences of the precore region persisted during follow-ups >15 years; those of core promoter decreased only from 36% to 27%. These genotype-dependent changes in precore and core promoter sequences may explain, at least in part, the influence of HBV genotypes on the severity and course of chronic hepatitis B (23–26).

In conclusion, acute self-limited hepatitis B is induced by HBV strains of the wild-type sequences in both the precore region and the core promoter. In chronic hepatitis B, mutations in the precore region and core promoter prevail and increase during >15 years of follow-up. HBV genotypes influence the development of precore and core promoter mutations in chronic hepatitis B.

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Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001

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Background. Because genotype A of hepatitis B virus (HBV) is not indigenous, there have been only few data on infection with it in Japan. ***Methods.*** We examined clinical and virological features of the 66 Japanese patients who admitted Toranomon Hospital in Tokyo, Japan, between 1976 and 2001, who were found to have HBV/A infection. HBV genotype A was classified into subtype A (European type) and A′ (South African type) by phylogenetic analysis of the preS1 and preS2 regions, and the S gene sequences. ***Results.*** Of the 66 patients infected with HBV/A, 14 (21%) were asymptomatic carriers, 26 (39%) presented with acute hepatitis, 22 (33%) with chronic hepatitis, and 4 (6%) with liver cirrhosis. HBV/A infection persisted for more than 6 months in 5 of the 26 (19%) patients with acute hepatitis. The frequency of acute hepatitis in patients infected with HBV/A was higher after than before 1991 (2/22 [9%] vs 24/44 [55%]; $P < 0.0001$). The frequency of nucleotide 1858 of T was higher in asymptomatic carriers than in patients with acute hepatitis in whom infection was resolved (5/14 [36%] vs 0/21 [0%]; $P = 0.008$). Of the 57 patients for whom subtypes of genotype A were determined, subtype A was identified in 53 (93%) and subtype A′ in only 4 (7%). All patients infected with subtype A′ were persistently infected with HBV. ***Conclusions.*** HBV/A infection has become more frequent during recent years, predominantly presenting as acute hepatitis, and subtype A′ is uncommon in the Tokyo metropolitan area.

Key words: genotypes, hepatitis B virus, hepatitis B e antigen, subtypes

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Introduction

Hepatitis B virus (HBV) is classified into seven genotypes by a sequence divergence in the entire genome of at least 8%, and named by capital alphabet letters from A to G.[1–3] HBV genotypes have distinct geographic distributions.[4,5] HBV Genotype A is prevalent in Northwestern Europe, North America, and South Africa, and was not reported in any of the Asian HBV genomes isolated until 1990.[1,6]

In 1997, Bowyer et al.[7] identified a unique subgroup of genotype A, based on comparison of the large S gene sequence, and named it A′. Of South African HBV/A isolates, 59% were classified into subgroup A′ and clustered to form a segment discrete from the original genotype A isolates. Amino-acid differences that set A′ isolates apart from the rest of group A tend to cluster in the pre-S2 region (amino acids 7, 10, 32, 35, 47, 48, 53, and 54), with a few changes in the major surface antigen (amino-acid sites 207 and 209). HBV isolates of subgroup A′ were separated from those in Northwestern Europe genotype A by sequence differences ranging from 4.1% to 6.2% in the preS2/S region,[7] as well as by phylogenetic differences in the entire genomic sequence.[8]

To our knowledge, there are no large-scale studies on Japanese patients infected with HBV of genotype A or subgroup A′ with clinical and virological characterizations. In the present study, we examined 68 Japanese patients infected with HBV/A who visited Toranomon Hospital in the Tokyo metropolitan area during 1976 through 2001, and we examined their clinicopathological characteristics and virological features. Further, HBV/A isolates were classified into subtypes A and A′ by phylogenetic analysis. The nucleotide (nt) 1858 of T or C was determined, also, which influences the development of the precore stop-codon mutation at nt from G to A.[9,10]

Patients and methods

Patients

Among the patients who admitted the Department of Gastroenterology, Toranomon Hosital, in metropolitan Tokyo during 1976 through 2001, 2022 were infected with HBV and received liver biopsy. Genotypes of HBV were determined in these patients, and genotype A was detected in 68 (3.4%), B in 239 (11.8%), C in 1649 (81.6%), D in 5 (0.2), and F in 2 (0.1%) of them; HBV isolates from the remaining 59 (2.9%) patients were untypeable. The 68 patients infected with HBV/A were examined medically for the diagnosis of liver disease, and HBV DNA in their sera were studied virologically. The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital, and an informed consent was obtained from each of the patients.

These 68 patients infected with HBV/A were classified into four clinical groups. An asymptomatic carrier state was diagnosed in 14 (21%) patients. They had serum alanine aminotransferase (ALT) levels consistently within the normal range (≤50 IU/l) and ultrasonographic findings of a "normal liver" or "fatty liver" at the initial visit. Chronic hepatitis was diagnosed in 22 (32%) patients, based on histopathological examination of liver biopsy specimens obtained under laparoscopy. Four (6%) patients had already developed liver cirrhosis that was diagnosed by findings on laparoscopy and imaging modalities, including ultrasonography and computed tomography.

The remaining 28 (41%) patients were diagnosed with acute hepatitis. They had all contracted HBV infection in adulthood, and possessed antibody to hepatitis B core antigen of IgM class in high titers (2.2–11.7) in serum for the diagnosis of acute hepatitis B. De-novo HBV infection in them was confirmed by the lack of hepatitis B surface antigen (HBsAg) at health checkups they had received before they came down with acute hepatitis.

Serum markers of HBV infection

HBsAg was determined by hemagglutination (MyCell; Institute of Immunology, Tokyo, Japan) and radioimmunoassay (AUSRIA II-125; Dinabot, Tokyo, Japan). Hepatitis B e antigen (HBeAg) and the corresponding antibody (anti-HBe) were determined by radioimmunoassay (AUSRIA-II 125; Dinabot). HBV DNA was determined by transcription-mediated amplification and hybridization assay (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed as log genome equivalents (LGE) per milliliter of serum, with detection limits ranging from 3.7 LGE/ml (corresponding to 5000 copies/ml) to 8.7 LGE/ml. Genotypes of HBV were determined, in sera from the patients at presentation, by commercial enzyme-linked immunosorbent assay (ELISA) kits (HBV Genotype EIA; Institute of Immunology), using monoclonal antibodies against epitopes on the pre-S2-region products.[11,12] Serotypes of HBsAg were determined by ELISA, using commercial kits (HBs Antigen Subtype EIA; Institute of Immunology).

Determination of subtypes A and A′ of genotype A

Nucleic acids were extracted from serum (100 µl), using the Smitest EX & R kit (Genome Science, Tokyo, Japan) and stored at −80°C. Nucleotide sequences spanning the large S gene (preS1 and preS2 regions and the S gene) were amplified in extracted nucleic acids by polymerase chain reaction (PCR) with nested primers. The first-round PCR was performed with BGF1 (sense, 5′-CTG TGG AAG GCT GGC ATT CT-3′ [nt 2757–2776]) and BGR2 (antisense, 5′-GGC AGG ATA GCC GCA TTG TG-3′ [nt 1050–1079]) primers, and the second-round PCR with PLF5Bm (sense, 5′-TGT GGA TCC TGC ACC GAA CAT GGA GAA-3′ [nt 136–162] and BR112 (Antisense, 5′-TTC CGT CGA CAT ATC CCA TGA AGT TAA GGG A-3′ [nt 163–864]), as well as BGF5 (sense, 5′-TGC GGG TCA CCA TAT TCT TG-3′ [nt 2811–2830]) and BGR6 (antisense, 5′-AGA AGT CCA CCA CGA GTC TA-3′ [nt 2831–248]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, Foster City CA, USA). Then, they were purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences of the large S gene in HBV DNA samples from patients were analyzed phylogenetically, along with reference sequences of subtypes A and A′,[7] by six-parameter[13] and neighbor-joining methods.[14]

Mutations in the core promoter and precore region

For determination of the wild-type or mutants in the core promoter and precore region, nucleic acids extracted from serum were amplified by PCR with nested primers. The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ [nt 1580–1599]) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ [nt 2295–2276]) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAC CAC CGT GAA C-3′ [nt 1606–1625]) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ [nt 2014–1995]) for 35 cycles

Table 1. Comparison of 66 patients who were infected with HBV genotype A and presented with various hepatic diseases

Features	ASC (n = 14)	AH (n = 26)	CH (n = 22)	LC (n = 4)	Differences
Male	10 (71%)	26 (100%)	19 (86%)	3 (75%)	NS
Age (years)[a]	28 (18–62)	33 (21–56)	36 (12–55)	43 (22–66)	NS
Follow-up (years)[a]	5 (0.6–15)		9 (3–20)	8 (0.3–21)	NS
Family history[b]	5 (36%)		3 (14%)		NS
Father	3 (21%)				
Mother			1 (5%)		
Siblings	2 (14%)		2 (9%)		
Transfusion	0	0	0	1 (25%)	NS
HCV RNA	0	0	1 (5%)	1 (25%)	NS

ASC, asymptomatic carriers; AH, acute hepatitis; CH, chronic hepatitis; LC, liver cirrhosis
[a]Median values are shown, with ranges in parentheses
[b]Family member with HBV-associated liver disease according to statements of patients

each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis, purified, and sequenced as described above. Mutations interfering with translation or transcription of HBeAg were searched for in the precore region and core promoter. They included a G-to-A mutation at nt 1896 in the precore region (A1896) and a double mutation in the core promoter, converting codon 1762 from A to T, and codon 1764 from G to A (T1762/A1764). Also examined was nt 1858 of T or C in the precore sequence.

Statistical analysis

Nonparametric procedures were used for the analysis, including the Mann-Whitney *U*-test, Fisher's exact test, and Bonferroni test. A P value of less than 0.05 in two-tailed analysis was considered significant.

Results

Characteristics of patients

Table 1 compares demographic features, history of transfusion, and infection with hepatitis C virus (HCV) in the 68 patients who were infected with HBV/A and presented with various liver diseases at the first hospital visit. They were all positive for HBsAg of serotype *adw* in serum. There were no differences in sex, age, duration of follow-up, family members infected with HBV, and history of blood transfusion among them. There was only one mother positive for serum HBsAg in the patients with chronic hepatitis. Her genotype of HBV was B, different from genotype A in her son, who was diagnosed with chronic hepatitis. Homosexual contacts were reported by 11 of the 28 (39%) patients with acute hepatitis. HBV infection persisted in 4 of the 11 (36%) patients who had contracted HBV/A infection through homosexual activity. HCV RNA was detected in 1 patient with chronic hepatitis and 1 with liver cirrhosis.

Of particular note, HBV/A infection persisted for longer than 6 months in 5 of the 26 (19%) patients who presented with acute hepatitis. On liver biopsies undertaken 6–96 months after the estimated time of infection, necroinflammatory changes and fibrosis were detected in all the 5 patients for the diagnosis of chronic hepatitis B.

For the purpose of analyzing trends of liver disease that may change with time, patients were divided into two groups according to the time of the first visit to the Gastroenterology Department at Toranomon Hospital, i.e., before or after 1991, when the serological diagnosis of HCV infection had become nationwide. There were 22 patients in 1976–1990 and 44 patients in 1991–2001. Figure 1 compares the proportions of patients who presented with four categories of clinical diagnosis between the two groups of patients.

There was an apparent increase in the proportion of patients who presented with acute hepatitis in the period 1991–2001 compared to 1976–1990 (2/22 [9%] vs 24/44 [55%]; $P < 0.0001$). By contrast, the proportions of asymptomatic carriers, as well as those of patients with chronic hepatitis and liver cirrhosis, decreased after 1991 (27% vs 17%, 55% vs 22%, and 9% vs 4%, respectively; $P < 0.0001$ for all).

Many patients diagnosed as asymptomatic carriers and those diagnosed with chronic hepatitis or liver cirrhosis in 1976–1990 were less than 40 years of age. However, in 1991–2001, there were many patients diagnosed with chronic hepatitis or liver cirrhosis who were 40 years or older (Fig. 2). Thus, of asymptomatic carriers, those aged less than 40 years significantly increased in frequency from 1976–1990 to 1991–2001. In a mirror image, the frequency of patients younger than 40 years significantly decreased in those with chronic hepatitis or liver cirrhosis. Patients with acute hepatitis were excluded from the comparison, because only three (11%) of them were 40 years or older at presentation.

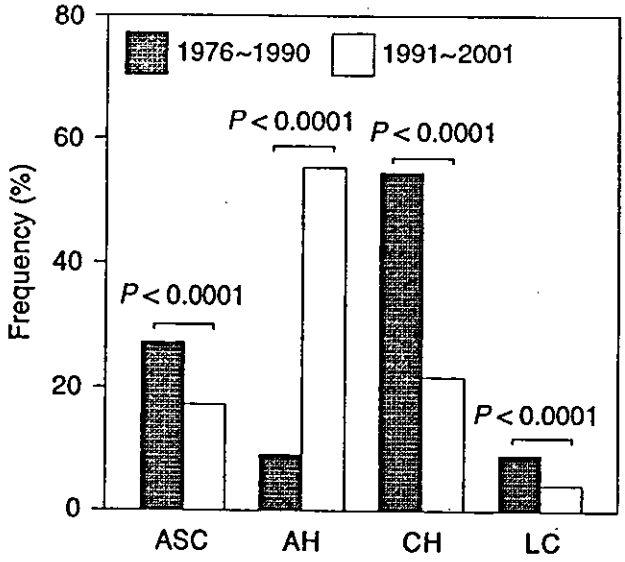

Fig. 1. Comparison of the proportions of liver diseases in patients infected with hepatitis B virus (HBV) genotype A who visited Toranomon Hospital between 1976 and 1990 and between 1991 and 2001. *ASC*, asymptomatic carriers; *AH*, acute hepatitis; *CH*, chronic hepatitis; *LC*, liver cirrhosis

Wild-types in the core promoter and precore region in patients who were infected with HBV genotype A

Table 2 compares the frequency of the wild-type in the core promoter and precore region among patients infected with HBV/A who presented with various liver diseases. The wild-type precore region with G1896 was detected in 61 of the 66 (92%) patients for whom the analysis was possible. All the 14 asymptomatic carriers were negative for HBeAg in serum, and the wild-type core promoter with A1762/G1764 was detected in only 43% of them. The wild-type core promoter was found in all 20 patients with acute hepatitis who had serum HBeAg at presentation and in whom HBV infection was resolved. Of the 5 patients with acute hepatitis who went on to chronicity, all of whom had serum HBeAg, 4 (80%) were infected with HBV/A of the wild-type in the core promoter, a frequency comparable to that in 10 of the 14 (71%) patients with HBeAg-positive chronic hepatitis. Patients with HBeAg-negative chronic hepatitis possessed the wild-type core promoter (38%) and precore region (88%) somewhat less frequently than the others.

The 1858th nt of T or C in patients infected with HBV/A at the time of presentation is shown in Table 3. T1858 was not detected in any patients with acute hepatitis in whom HBV infection was resolved, but it was found in one of the 5 (20%) patients who acquired persistent infection, as well as in 24%–32% of those with the other categories of liver disease. T1858 was

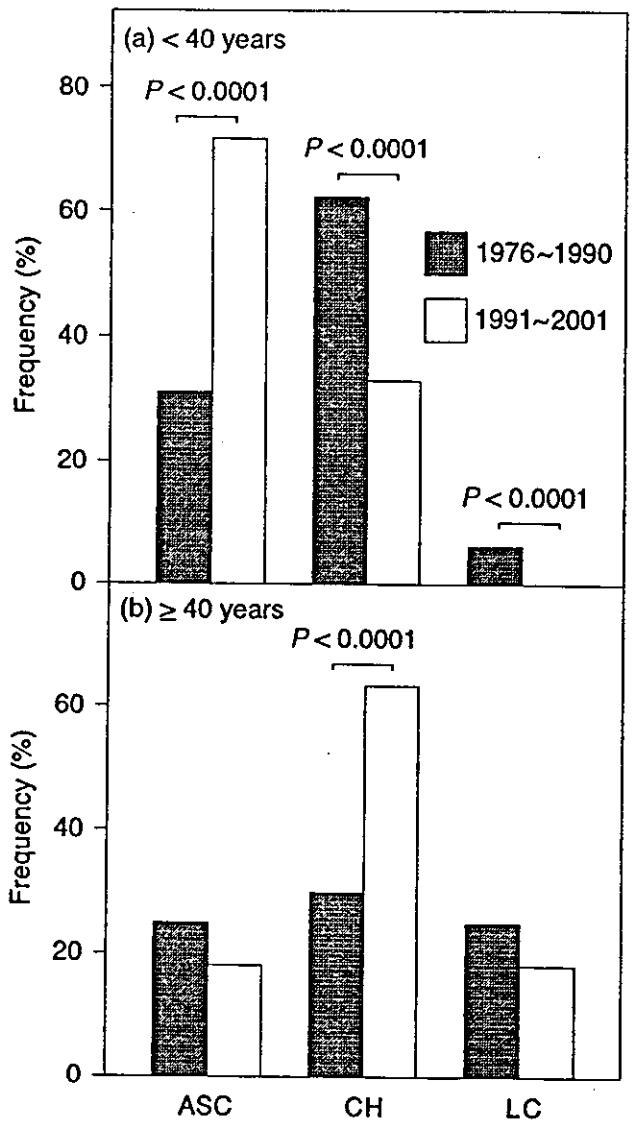

Fig. 2a,b. Comparison of the proportions of patients infected with HBV genotype A aged less than 40 years (**a**) and aged 40 years or older (**b**) who visited Toranomon Hospital between 1976 and 1990 and between 1991 and 2001

detected significantly more frequently in asymptomatic carriers than in patients with acute hepatitis (36% vs 4%; $P = 0.002$), and the difference was even more prominent between asymptomatic carriers and patients with acute hepatitis in whom HBV infection was resolved (36% vs 0%; $P = 0.008$).

Subtypes A and A′ of genotype A

HBV DNA sequences of the large S gene were determined in nucleic acids extracted from 68 patients infected with HBV/A, and subtypes A and A′ were analyzed phylogenetically (Table 4). Subtype A was detected in 53 (80%) patients, and subtype A′ in 4 (6%);

Table 2. Wild-types of core promoter and precore sequence in HBV DNA from patients infected with HBV genotype A at presentation

Liver disease	Wild-type core promoter (A1762/G1764)	Wild-type precore region (G1896)
ASC		
HBeAg (−) (n = 14)	6 (43%)	13 (93%)
AH: Resolved		
HBeAg (+) (n = 20)	20 (100%)	20 (100%)
HBeAg (−) (n = 1)	ND	ND
AH: Persisted for >6 months		
HBeAg (+) (n = 5)	4 (80%)	5 (100%)
CH		
HBeAg (+) (n = 14)	10 (71%)	14 (100%)
HBeAg (−) (n = 8)	3 (38%)	7 (88%)
LC		
HBeAg (−) (n = 2)	1 (50%)	2 (100%)

ND, not detectable

Table 3. Nucleotide 1858 in HBV DNA samples from patients who were infected with HBV genotype A and presented with various liver diseases

Nucleotide at position 1858	ASC (n = 14)	AH		CH (n = 22)	LC (n = 4)	Total (n = 66)
		Resolved (n = 21)	Persisted (n = 5)			
	P = 0.008					
T	5 (36%)	0	1 (20%)	7 (32%)	1 (25%)	14 (21%)
C	6 (43%)	18 (86%)	4 (80%)	15 (68%)	1 (25%)	44 (67%)
Deletion	3 (21%)	0	0	0	0	3 (5%)
Not detectable	0	3 (14%)	0	0	2 (50%)	5 (8%)

Table 4. Subtypes in patients infected with HBV genotype A

Subtypes	ASC (n = 14)	AH		CH (n = 22)	LC (n = 4)	Total (n = 66)
		Resolved (n = 21)	Persisted (n = 5)			
A	11 (79%)	18 (86%)	4 (80%)	18 (82%)	2 (50%)	53 (80%)
A′	1 (7%)	0	0	3 (14%)	0	4 (6%)
Untypeable	2 (14%)	3 (14%)	1 (20%)	1 (4%)	2 (50%)	9 (14%)

distinction between A and A′ was not possible in the remaining 9 (14%) patients. Of the 57 patients in whom subtypes were determined, A was detected in 53 (80%) and A′ in the remaining 4 (6%). The 53 patients infected with subtype A, for whom subtyping was feasible, included 11 of the 13 (85%) asymptomatic carriers, 22 of the 22 (100%) with acute hepatitis, 18 of the 21 (85%) with chronic hepatitis, and both patients with liver cirrhosis. Of the 4 patients infected with subtype A′, 1 presented with the asymptomatic carrier state and the remaining 3 with chronic hepatitis.

Table 5 lists the demographic, histopathological, and virological features of the four patients infected with subtype A′. They comprised two males and two females, and two of them were in their twenties and the remaining two in their forties. Two patient with chronic hepatitis and infected with subtype A′ (patient 3 and 4) cleared HBsAg from serum during about 3 years of follow up.