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ORIGINAL ARTICLE

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Interferon-α/β upregulate IL-15 expression in vitro and in vivo: analysis in human hepatocellular carcinoma cell lines and in chronic hepatitis C patients during interferon-α/β treatment

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Abstract Type I interferon (IFN) possesses antiviral and antitumor activities and also having an immune regulatory effect, activating cellular immune response and upregulating several cytokines. Recent study has shown that type I IFN upregurates the dendritic cell production of IL-15 capable of activating natural killer cells and $CD8^+$ memory T lymphocytes. However, it is still unknown if type I IFN induces IL-15 production in non-immune cells and if type I IFN affects IL-15 production in vivo. The present study investigated the effect of type I IFNs on IL-15 expression in hepatocellular carcinoma (HCC) cell lines in vitro and in patients with chronic hepatitis C in vivo. When three HCC cell lines, Huh7, HepG2, and JHH4 were cultured in vitro, IFN upregulation of IL-15 expression was observed at both the mRNA and protein levels. In experiments using Huh7 cells, upregulation of IL-15 expression occurred within 24 h of the start of IFN stimulation, and both IFN-α and -β dose-dependently increased IL-15 production in the range from 100 U/ml to 10,000 U/ml of concentration. IFN-β showed stronger activity in IL-15 production induction in vitro than IFN-α. For in vivo examination, sera were obtained from 21 chronic hepatitis C patients treated with IFN and 29 healthy individuals, and the serum IL-15 level was quantified by ELISA. The serum IL-15 level of chronic hepatitis C patients before IFN treatment was similar to that of the healthy controls and significantly increased only during the IFN administration period. These results confirm that IFN-α/β induce IL-15 production and also suggest that IL-15 may be associated with type I IFN-induced immune response.

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Introduction

Interferon (IFN)-α and -β are categorized as type I IFN and possess antiviral activity useful for the treatment of chronic hepatitis C. The hepatitis C virus (HCV), the pathogen of hepatitis C, causes a persistent infection in 80% of patients exposed and leads to chronic hepatitis and hepatic fibrosis that progresses in some patients to liver cirrhosis and hepatocellular carcinoma (HCC) [1–5]. A sustained elimination of serum HCV RNA is observed in 30–40% of patients administered IFN-α or -β [6–11]. Type I IFNs also have antitumor activity and are used for the treatment of chronic myelogenous leukemia and renal cell carcinoma. Moreover, IFN treatment decreases the HCC carcinogenesis rate of chronic hepatitis C patients [12–14].

Type I IFN directly affects cells to induce the antiviral proteins 2′–5′ oligo adenyl synthetase, Mx protein and PKR protein kinase [15], and also affects tumor cells to elicit a cell-cycle arrest or an apoptosis [16–18]. Recently, it has been reported that both type I IFN-induced antiviral defense and tumor suppression are related to p53 gene expression [19]. It has also been shown that type I IFNs activate cytolytic T lymphocytes (CTL) and natural killer cells (NK) [20–22], and that they upregulate production of several T cell-derived cytokines, such as IL-1β, IL-6 and TNF-α [11, 23, 24]. This suggests that the antiviral and antitumor activity of type I IFN is associated with the immune system in vivo. However, the immunological mechanisms of type I IFN are still largely unknown.

It has been reported that type I IFN upregurates IL-15 expression from dendritic cells in vitro [25]. IL-15 is a

four-helix bundle cytokine related to IL-2, and its receptor consists of a unique α-chain and shared IL-2 receptor β- and γ-chains. In contrast to IL-2, which is mainly expressed in activated T cells, IL-15 is produced by various cells such as monocytes/macrophages, epitherial and fibroblast cells, placenta, skeletal muscle, heart, lung, kidney, and liver, but not by normal resting or activated T cells [26]. It is considered that IL-15 is essential for NK and NK-T cell development [27–29] and that it is capable of promoting proliferation, long-term survival and activation of CD8 memory T cells [30–32], suggesting that IL-15 plays a pivotal role in protective immune response. Thus, it is possible that IL-15 may be involved in type I IFN-induced immune response, but the relationship remains to be clarified. To better understand the immunobiological function of type I IFNs and to develop new therapeutic methods, it is important to investigate implications of how type I IFNs affect IL-15 production.

In this study, we attempted to determine if and how IFN-α and -β upregulate IL-15 production in human HCC cell lines. These results confirmed that IFN-α/β induce IL-15 production and show the first evidence of IFN-α/β induced IL-15 production in non-immune cells. Our study also indicated that serum IL-15 levels increase in chronic hepatitis C patients during the IFN-α or -β administration period. These data suggest the clinical significance of IL-15 in type I IFN-induced immune response.

Materials and methods

Cell culture and IFNs

HCC cell lines Huh7, HepG2 and JHH4 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS and antibiotic agents (100 U/ml penicillin G and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. In all experiments, 2×10^5 cells were seeded in a 6-well cell-culture plate and cultured for 24 h to allow the cells to stick to the culture plate and enter their logarithmic growth phase. After 24 h of preincubation, the medium was changed to fresh medium with or without IFNs. Natural human IFN-α (Sumiferon) and natural human IFN-β (Feron) were kindly provided by Sumitomo Pharmaceutical (Japan) and Dai-Ichi Pharmaceutical (Japan), respectively.

Patients with chronic HCV infection and controls

Twenty-one Japanese patients (12 men and 9 women; age range, 31–76 years; mean, 52.2 years) with chronic hepatitis C were studied. All patients were positive for serum HCV RNA and had elevated serum alanine aminotransferase (ALT). No patient was positive for hepatitis B surface antigen (HBsAg) or anti-human immunodeficiency virus (HIV) antibody. Twenty-nine healthy volunteers (16 men and 13 women; age range, 42–74 years; mean, 58.1 years) negative for serum HCV RNA, HBsAg, and anti-HIV antibody and without a clinical history or symptoms of liver disease were recruited as controls. Before any treatment was given, a liver biopsy was done for chronic hepatitis C patients and histological changes were evaluated. Quantification of serum HCV RNA was done for all patients before treatment by competitive polymerase chain reaction (PCR), as described previously [33]. Eleven chronic hepatitis C patients were given 6 million units of natural IFN-α (Sumiferon, Sumitomo Pharmaceutical Co., Japan) by intramuscular injection daily for 14 days, then three times weekly for 22 weeks. Another ten chronic hepatitis C patients were given 6 million units of natural IFN-β (Feron, Dai-ichi Pharmaceutical Co., Japan) by intravascular drip infusion daily for 56 days. The serum ALT level of the chronic hepatitis C patients treated with IFN was tested monthly during the observation period. A qualitative HCV-RNA examination of the serum from the chronic hepatitis C patients was done 6-months after the cessation of IFN treatment. Response to IFN treatment was classified as follows: virological responders were defined as patients in whom serum HCV-RNA was negative at 6 months after the cessation of IFN treatment and biochemical responders were defined as patients in whom serum ALT was continuously normal for 6 months after the cessation of IFN treatment. Informed consent was obtained from all patients and healthy volunteers.

RNA extraction and cDNA synthesis

Total RNA was extracted from cultured cells and liver biopsy samples using the RNeasy Mini kit (QIAGEN, Germany) and treated with RNase-Free DNase Set (QIAGEN, Germany) to remove contaminated DNA, according to the manufacturer's instruction. A concentration of isolated RNA was measured by spectrophotometer, and 200 ng of total RNA was applied to reverse transcription using Superscript II RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with 50 ng of random hexamers according to the manufacturer's instructions. The complementary DNA (cDNA) solution obtained was used for the subsequent PCR.

Preparation of DNA standards

To prepare DNA standards for real-time PCR, IL-15 and β-actin genes were amplified from cDNA by PCR. The forward and reverse primers for human IL-15 were hIL-15F2: 5′-GCAGGGCTTCCTAAAACAGA-3′and hIL-15R2: 5′-GTTGTTTGCTAGGATGATCAG-3′, and those for human β-actin were hβ-actinF1: 5′-GGTCACCCACACTGTGCCCAT-3′ and hβ-actinR1: 5′-GGATGCCACAGGACTCCATGC-3′. For the PCR

of the IL-15 gene, 0.5 μmol of Tris-HCl (pH 8.4), 1.25 μmol of KCl, 37.5 nmol of $MgCl_2$, 5 nmol of dNTPs, 10 pmol each of the forward and reverse primers, 0.5 U of Platinum Taq DNA (Invitrogen, Carlsbad, USA), and 1 μl of cDNA solution were mixed with distilled water to a 50 μl final volume in a 0.5 ml tube. The mixture was incubated in a thermal cycler at 94°C for 3 min, followed by 36 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for a final 3 min. The PCR for β-actin was performed using the same mixture condition as the PCR for IL-15, except for the primer sets. Thermocycling conditions for the β-actin PCR consisted of an initial 94°C for 3 min, followed by 20 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and an additional 72°C for 3 min. PCR products were applied to electrophoresis on 1% agarose gel. Specific amplification was verified according to the predicted size of each amplicon (IL-15 PCR product, 240 bp; β-actin PCR product, 350 bp). IL-15 and β-actin PCR products were then extracted from the gel using the MinElute Gel Extraction Kit (QIAGEN, Germany), according to the manufacturer's instruction. The concentration of the gel-extracted PCR products was measured by spectrophotometer. Finally, the gel-extracted IL-15 and β-actin PCR products were diluted with 0.1 × Tris-EDTA buffer.

Real-time PCR

Human IL-15 and β-actin mRNAs were quantified by real-time PCR. The primer sets for real-time PCR of the IL-15 and β-actin genes were the same used in normal PCR for a standard preparation, as described above. PCR was done using the Light Cycler (Roche, Mannheim, Germany) with LightCycler-FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The PCR condition for IL-15 was as follows: after an initial denaturing at 95°C for 10 min, the amplification was done by 40 cycles of denaturing at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 10 s. The PCR condition for β-actin was as follows: after an initial denaturing at 95°C for 10 min, the amplification was done by 40 cycles of denaturing at 95°C for 10 s, annealing at 57°C for 10 s, and extension at 72°C for 10 s. The amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I that binds to the double-strand DNA amplified by PCR. The copy number of mRNA in the cDNA samples was calculated using standard amplification curves.

Assay for IL-15 concentration

The IL-15 concentration of supernatants collected from the cell cultures and serum samples was determined by use of a human IL-15 ELISA kit (Genzyme, Cambridge, MA, USA), according to the manufacturer's instructions. The absorbance at 450 nm (reference at 540 nm) was measured. The assay was done in duplicate.

Statistical analysis

Statistical analysis was done using the StatView software package (SAS Institute Inc., Cary, NC, USA). Unpaired Student's *t*-test was used to assess the statistical significance of differences in pre-treatment serum IL-15 levels between sera from controls and chronic hepatitis C patients. Paired Student's *t*-test was used to compare the serially assayed serum IL-15 of chronic hepatitis C patients. The χ^2 test was used for gender comparison of the controls and chronic hepatitis C patients. Ages differences between the controls and chronic hepatitis C patients were compared by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results

IFN-α and -β upregulation of IL-15 production in Huh7 cells

To determine if IFN-α and -β upregulate IL-15 transcription in a HCC cell line, IL-15 mRNA expression level was quantified by RT-PCR in Huh7 cells cultured for 72 h with various concentrations of IFN-α or -β. The β-actin mRNA expression level was also determined for use in adjusting the IL-15 mRNA expression level (Fig. 1a). In comparison with the controls, the IL-15 mRNA level increased in Huh7 cells cultured with IFN-α or -β. This IL-15 increase in the Huh7 cells cultured with both IFNs was dose-dependent in the range from 100 U/ml to 10,000 U/ml of IFN concentration. The IL-15 transcription induction activity was higher in IFN-β than in IFN-α, when compared at the same concentration. These data suggest that IFN-α/β upregulated IL-15 gene transcription in this Huh7 cell line.

To verify IL-15 upreguration of type I IFNs at the protein level, IL-15 concentration in the supernatant of the Huh7 culture was determined by ELISA. Huh7 cells were cultured with or without IFNs at various concentrations. After a 72 h-culture, the IL-15 concentration in the supernatant was examined by ELISA. Figure 1b shows the IL-15 concentration in the culture supernatant of each condition. As expected from the results of IL-15 mRNA quantification, the IL-15 concentration increased in comparison with the controls in the supernatants of the Huh7 cells cultured with IFN-α or -β. The IL-15 concentration dose-dependently increased in both the IFN-α and -β cultures, and the concentration was higher in the IFN-β than in the IFN-α culture.

The Huh7 cell number was determined by flow cytometry after a 72-h culture period to eliminate the possibility that the increase in IL-15 concentration in

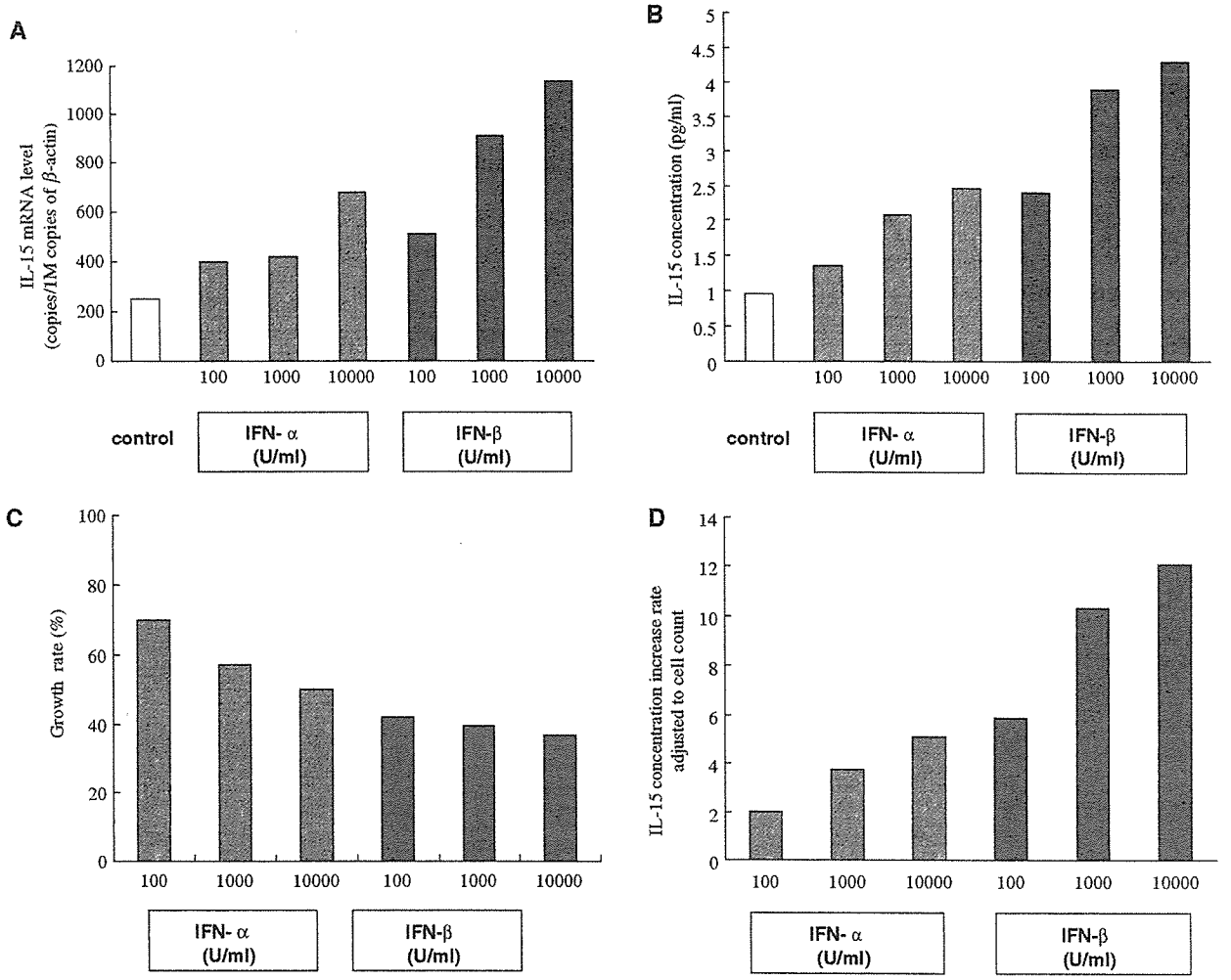

Fig. 1 The effects on IL-15 expression in Huh7 cells of IFN-α and -β at various concentrations. Huh7 cells were cultured for 72 h with or without IFN-α/β at consentrations of 100, 1,000, or 10,000 U/ml. **a** The IL-15 Expression level and the β-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression level. **b** The IL-15 concentration in the culture supernatant was quantified by ELISA. **c** Cell numbers were counted by flow cytometry after detachment with trypsinization, and the cell growth rate of IFN-treated cells in comparison with the controls was calculated. **d** The supernatant IL-15 concentration was adjusted to the cell number, and the IL-15 production increase rate of IFN-treated cells in comparison with the controls was calculated. All experiments were repeated three times and representative results are depicted

Huh7 culture with IFN-α/β might have been caused by an increase in cell number. The cell count data showed that type I IFNs suppressed Huh7 proliferation in a dose dependent manner and that the suppression was stronger in Huh7 cultured with IFN-β than with IFN-α (Fig. 1c). Moreover, the IL-15 concentration of the culture supernatant was adjusted to the cell number of the corresponding culture, and the ratio of IFN-treated conditions to controls was calculated (Fig. 1d). Because the adjustment reflects the IL-15 production level of each cell, it could be shown conclusively that IFNs promote IL-15 production from Huh7 cells. Thus, these results confirmed that IFN-α/β upregulate IL-15 production from Huh7 cells.

We also examined the IL-15 mRNA expression level of Huh7 cells cultured with IFN at different time points. Huh7 cells were cultured with or without 1,000 U/ml of IFN-α/β, the cells were harvested at 24, 48 and 72 h, and the IL-15 mRNA expression level was determined (Fig. 2a). A control culture without IFN showed almost the same IL-15 mRNA expression level throughout the period of observation. In the cells cultured with IFN-α, the IL-15 mRNA level increased at 24 h, and maintained this level to 72 h In the cells cultured with IFN-β, the IL-15 mRNA level also increased at 24 h. However, an even higher level was noted at 72 h. These data suggest that upregulation of IL-15 transcription occurs within 24 h after the start of stimulation by either IFN-α or -β, but that the manner of IL-15 induction may differ between IFN-α and -β.

The IL-15 concentration of the supernatant was determined, by ELISA, in a Huh7 culture with or

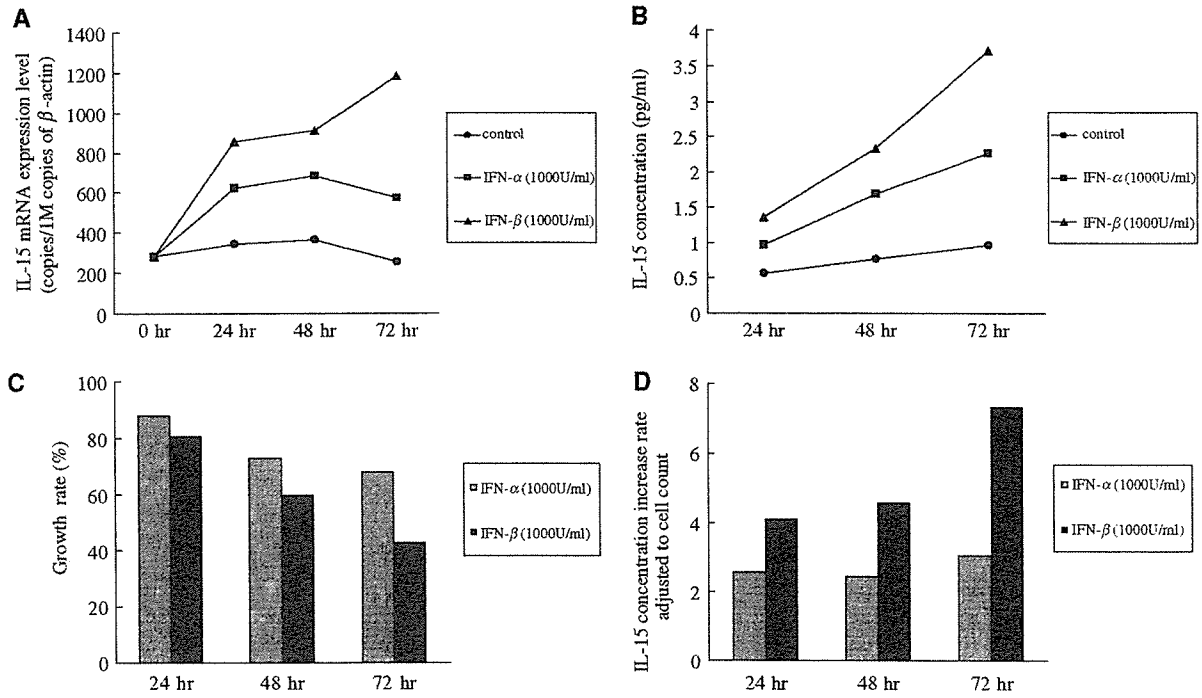

Fig. 2 The effects of IFN-α and -β on IL-15 expression in Huh7 cells at various time points. Huh7 cells were cultured with or without 1,000 U/ml IFN-α/β for 24, 48, or 72 h. **a** IL-15 and β-actin gene expression levels were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression levels. **b** The IL-15 concentration in the culture supernatant was quantified by ELISA. **c** Cell numbers were counted by flow cytometry after detachment with trypsinization, and the cell growth rate of IFN-treated cells in comparison with the controls was calculated. **d** The supernatant IL-15 concentration was adjusted to the cell number, and the IL-15 production increase rate of IFN-treated cells in comparison with the controls was calculated. All experiments were repeated three times and representative results are depicted

without 1,000 U/ml of IFN at 24, 48, and 72 h. At each time point, the IL-15 concentration was higher in Huh7 cultured with IFN than in the control (Fig. 2b). The Huh7 cell-growth rate was determined by cell count, and the data showed that Huh7 cell-growth was time-dependently suppressed by IFNs (Fig. 2c). To compare the IL-15 production level in each cell among different culture conditions, we adjusted the IL-15 concentration of the culture supernatant to the cell number. Figure 2d shows the IL-15 production increase rate of IFN-treated cells in comparison with the controls. At each time point, an increase in the IL-15 level was observed, even after adjustment to the cell number, and the value was higher in the culture with IFN-β than in that with IFN-α. As observed in mRNA quantification, the increase in IL-15 production from cells cultured with IFN-β continued at 72 h. These results confirm that both IFN-α and -β increase IL-15 production from Huh7 cells within 24 h and that the IFN-β activity is stronger than that of IFN-α.

IFN-α and -β upregulattion of IL-15 production in other HCC cell lines

To clarify if type I IFNs upregulate IL-15 expression in other HCC cell lines, we quantified IL-15 mRNA expressed in HepG2 and JHH4 cells cultured with or without type I IFNs. The cells were cultured with or without IFN-α or -β at a concentration of 1,000 U/ml and, after a 72-h culture, the IL-15 mRNA expression level was determined by RT-PCR. Figure 3a shows the IL-15 mRNA levels of HepG2 and JHH4 cells after culture with or without type I IFNs. In both types of cells, the IL-15 mRNA expression level was increased by type I IFNs and, at same concentration, IFN-β showed stronger IL-15 mRNA expression induction activity than IFN-α.

The IL-15 concentration of the supernatant and the number of cells were determined by ELISA and flow cytometry, respectively, in the experiments on HepG2 and JHH4 cultured for 72 h with or without type I IFNs at a concentration of 1,000 U/ml (Fig. 3b, c). The IL-15 concentration in the supernatant of both types of cells cultured with type I IFNs, increased in comparison with their respective controls and was higher in cells cultured with IFN-β than IFN-α. A suppression of cell growth was observed in both types of cells cultured with type I IFNs, and the suppression activity of IFN-β was stronger than that of IFN-α. Figure 3d shows the cell count-adjusted IL-15 level, which reflects IL-15 production from each cell. The adjusted IL-15 data clearly showed that HepG2 and JHH4 cells cultured with IFN-β produced more IL-15 than those cultured with IFN-α. These results indicate that type I IFNs upregulate IL-15

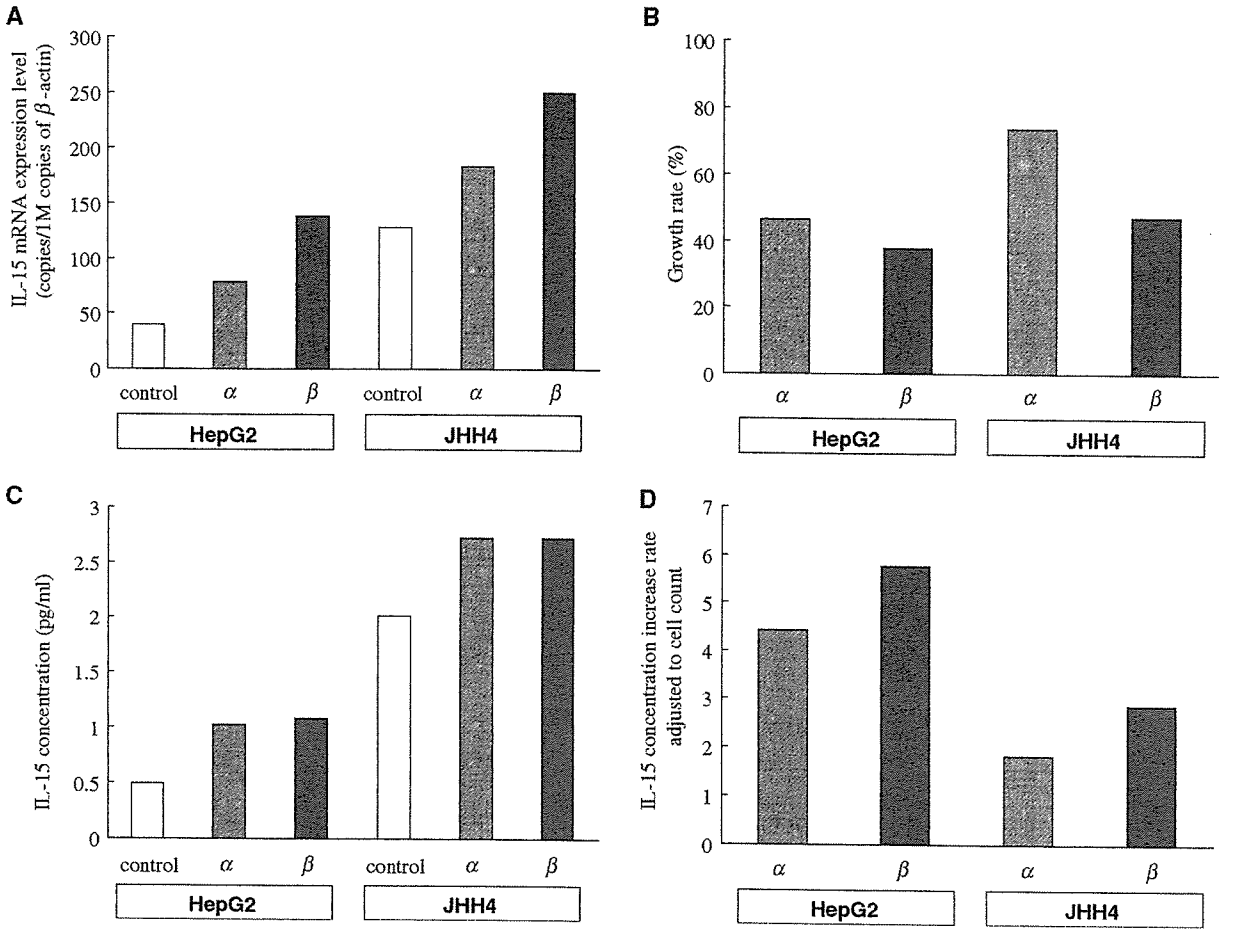

Fig. 3 The effects of IFN-α and -β on IL-15 expression in HepG2 and JHH4 cells. HepG2 cells and JHH4 cells were cultured with or without 1,000 U/ml IFN-α/β for 72 h. **a** Expression levels of IL-15 and β-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression level. **b** Cell numbers were counted by flow cytometry after detachment with trypsinization, and the cell growth rate of IFN-treated cells in comparison with the controls was calculated. **c** The IL-15 concentration in culture supernatant was quantified by ELISA. **d** The supernatant IL-15 concentration was adjusted to the cell number, and the IL-15 production increase rate of IFN-treated cells in comparison with the controls was calculated. All experiments were repeated three times and representative results are depicted

production, not only in Huh7 but also in other HCC cell lines such as HepG2 and JHH4.

IL-15 mRNA expression in liver biopsy samples from chronic hepatitis C patients

To investigate if IL-15 is expressed in human liver, we examined IL-15 mRNA expression in liver biopsy samples obtained from six chronic hepatitis C patients. The amplification of IL-15 transcript by PCR showed that IL-15 mRNA was expressed in all the liver biopsy samples examined (Fig. 4a). We also quantified IL-15 transcripts in liver samples, and the data adjusted by β-actin mRNA expression levels is shown in Fig. 4b. The IL-15 mRNA expression level in the liver showed little valiation between the samples, but generally, it was similar to that of Huh7 cells. These results suggest that IL-15 is expressed in the human liver.

Increased serum IL-15 level in chronic hepatitis C patients during Type I IFN treatment

To investigate the induction of IL-15 production by type I IFNs in vivo, we examined alterations of the serum IL-15 level in 21 chronic hepatitis C patients during treatment with IFN-α or -β. Table 1 shows the clinical characteristics of 11 patients treated with IFN-α, 10 patients treated with IFN-β, and 29 control subjects. No significant difference in sex or age was found between the control subjects and chronic hepatitis C patients. No significant pretreatment difference in IL-15 level (Fig. 5) was found between the samples from controls and the chronic hepatitis C patients. In the chronic hepatitis C patients, the IL-15 level significantly increased during the IFN treatment period in comparison with pretreatment (week 2; $P < 0.0001$, week 4; $P < 0.0001$, end of treatment; $P < 0.0001$). At 6 months after cessation of

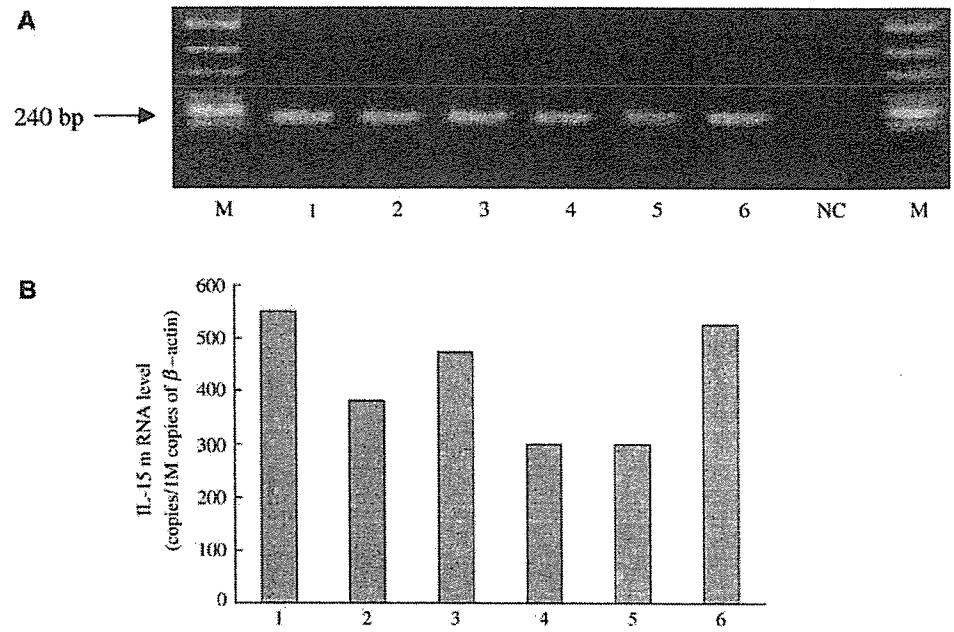

Fig. 4 Interleukin-15 mRNA expression in liver biopsy samples of chronic hepatitis C patients. **a** The IL-15 transcripts were amplified from the liver biopsy samples of six patients by reverse-transcription PCR. DEPC-treated water was used as the negative control. lanes 1–6; liver biopsy samples, lane 7; negative control, M; DNA marker. **b** Expression levels of IL-15 and the β-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level was adjusted to the β-actin mRNA expression level

treatment, the serum IL-15 had returned to a level similar to the pretreatment. No significant differences in the serum IL-15 level of patients by type of IFN received or by virological or biochemical response were found during the observation period (data not shown). These results suggest that both IFN-α and -β increased the serum IL-15 level of the chronic hepatitis C patients during the administration period.

Discussion

Interleukin-15 is considered a key factor for both innate and adaptive immune response. IL-15 expression has been implicated in clinical studies to be associated with graft rejection in transplantations[34–36] and with synovial T cell activation in rheumatoid arthritis[37, 38], suggesting that IL-15 plays an important role in local immune reaction. It has also been reported that IL-15 enhances antitumor immune response by NK cells and CTLs [39–42], and it is expected that IL-15 can be applied to antitumor immunotherapy in the future [43, 44]. The present study revealed that IFN-α and -β upregulate IL-15 production from HCC cell lines in vitro. The finding suggests a possibility that type I IFNs may induce IL-15 production in HCC cells in vivo leading to proliferation and activation of surrounding immune cells, such as NK and CTL. It has been demonstrated that some cancer cells can regulate immune response and lead to inhibition of antitumor immunity [45]. Type I IFNs may elicit the opposite immune regulatory effect, i.e. activation of antitumor immunity through IL-15 production from cancer cells. This may be one of the mechanisms contributing to the antitumor effect of IFN-α and -β.

The serum IL-15 levels of the chronic hepatitis C patients in this study significantly increased during the IFN administration period, indicating that IFN-α and -β stimulate IL-15 production in humans in vivo. In the present study, IL-15 production was increased in human HCC cell lines by IFN-α/β, and a comparable level of IL-15 mRNA expression was observed in liver biopsy samples. It has been reported that multiple STAT signals are activated by IFN-α in hepatocytes [46]. The IL-15 promotor includes two enhancer elements, the binding sites of NF-κB and IRF-1 which are transcription factors in the downstream of type I IFN signals [47]. These findings led us to believe that IFN-α and -β upregulate IL-15 production in hepatocytes in vivo, which in turn contributes to the elevation of serum IL-15 in chronic hepatitis C patients during IFN administration. It is also possible that IL-15 production is increased in immune cells, although a recent report suggested that type I IFN-mediated production of IL-15 was impaired in monocyte-derived dendritic cells of chronic hepatitis C patients[48]. Which cells or tissues were involved in type I IFN-mediated production of IL-15 in vivo remains unknown. The present study, however, demonstrated that type I IFNs upregulate IL-15 production in vivo, and the results suggest that IL-15 is associated with type I IFN-induced immune response.

In the present study, in vitro IFN-β showed a stronger activity than IFN-α in IL-15 upregulation of HCC cell lines. IFN-β also inhibited the growth of HCC cell lines more efficiently than IFN-α, as reported previously [49]. It is unclear what causes these differences between IFN-α and -β, since IFN-α and -β bind to the same receptor. The receptor, type I IFN-R, is composed of at least two subunits; α chain, IFNAR1 and β subunit, IFNAR2 which has short (β_S) and long (β_L) forms. In a previous report,

Table 1 Clinical characteristics of 21 patients treated with IFN-α or -β and 29 control subjects

characteristics	controls (n = 29)	HCV-infected patients (n = 21)	*p* value
sex, n (%)			> 0.999 †
male	16 (55.2)	13 (61.9)	
female	13 (44.8)	8 (38.1)	
age, years§	58.1 ± 10.8	52.2 ± 10.7	0.0632 ‡
HCV viral load¶, n (%)			
10^3	-	2 (6.9)	
10^4	-	2 (6.9)	
10^5	-	4 (13.8)	
10^6	-	10 (34.5)	
10^7	-	3 (10.3)	
HCV genotype, n (%)			
1b	-	13 (44.8)	
2a	-	7 (24.1)	
2b	-	1 (3.4)	
histology, n (%)			
CPH	-	5 (17.2)	
CAH	-	8 (27.6)	
LC	-	5 (17.2)	
NT	29 (100)	3 (10.3)	
virological responder, n (%)	-	8 (27.6)	
biochemical responder, n (%)	-	9 (31.0)	

§ Mean value ± SD
¶ The unit of HCV viral load is "copies per 50 μl of serum"
† Comparison by χ^2 test with Yate's compensation
‡ Comparison by unpaired student's *t*-test
CPH: chronic persistent hepatitis
CAH: chronic active hepatitis
LC: liver cirrhosis
NT: not tested

it was shown that IFN-α2 and IFN-β require a distinct intracytoplasmic region of the β_L subunit for their antiviral response, suggesting activation of a distinct signaling pathway by IFN-β [50]. In another report, it was shown that expression of CXCL11 (alias β-R1, I-TAC), a CXC chemokine ligand for CXCR3, was selectively induced by IFN-β, but not by IFN-α in a fibrosarcoma cell line [51, 52]. These findings suggested differences in biological activity between IFN-α and -β, and that it may be associated with the differences in the level of activity in IL-15 upregulation. We have also reported differences in clinical outcome between patients treated with IFN-α and -β. Sustained virological response was predicted by disappearance of HCV viremia early in the course of IFN administration in patients treated with IFN-α [53], but not in patients treated with IFN-β [11]. Moreover, by IFN-α treatment, the risk of HCC was significantly reduced in chronic hepatitis C patients with a biochemical response. In contrast, in patients treated with IFN-β, there was no difference in the incidence of HCC between patients with and without biochemical response, and there was a tendency for the risk to be reduced irrespective of the biochemical response of patients treated with IFN-β [14]. Thus, differences in biological activity between IFN-α and -β have been suggested by both basic and clinical studies, and these differences may be associated with the differences in the level of activity in IL-15 upregulation.

There was no significant difference in the serum IL-15 level of the patients treated with IFN-α and -β during the administration period. One possible explanation is that the administration routes are different for IFN-α and -β, i.e. intramuscular and intravenous injection, respectively. This difference would affect the drug concentration in tissues including liver. Another possibility is varied response to type I IFN among different cell types,

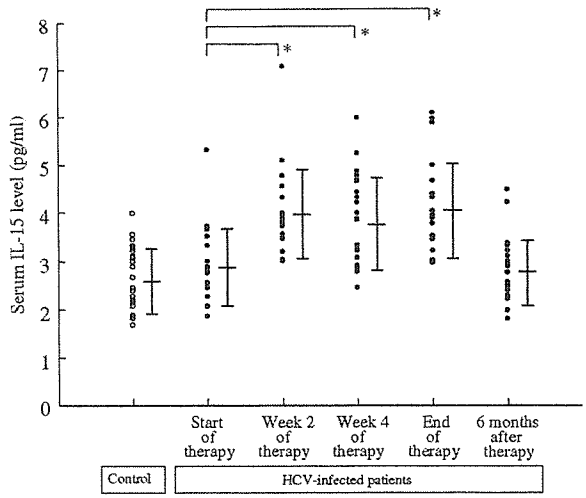

Fig. 5 The serum IL-15 level of IFN-treated patients with type C chronic liver disease and controls. The IL-15 level was quantified by ELISA. Unpaired Student's *t*-test was used to assess the statistical significance of differences in serum IL-15 levels between sera from controls and HCV-infected patients before treatment. Paired Student's *t*-test was used to compare serially assayed serum IL-15 of the HCV-infected patients. *Circles* indicate the individual serum IL-15 levels of the controls (*open circle*) and HCV-infected patients (*closed circle*). *Horizontal bars* show mean ± SD in each sample group. $*P < 0.0001$

or that only some cells or organs show differences in IL-15 expression response as between IFN-α and -β, resulting in the differences being overshadowed by other aspects of systemic response in vivo.

The present study provided the first evidence that type I IFN induces IL-15 production in vitro in HCC cell lines and also suggests a mechanism by which IL-15 production from non-immune cells mediates IFN-α/β-induced immune response. Our study also demonstrated that IFN-α and -β induce IL-15 production in human in vivo. These findings may help us better understand the immune regulatory mechanism of IFN-α/β and its implication in IL-15 expression. However, the precise roles of IFN-α/β are still unclear and further study is needed to clarify these issues. Such study will be useful for the development of selective and effective therapies for viral infection and cancer.

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Long-term lamivudine treatment for chronic hepatitis B in Japanese patients: A project of Kyushu University Liver Disease Study

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Abstract

AIM: To determine the efficacy of long-term lamivudine treatment of a large number of Japanese patients with chronic hepatitis B.

METHODS: In this retrospective, multi-center trial, 318 Japanese patients with chronic hepatitis B received 100 mg of lamivudine daily for up to 36 (median 21) mo. Virological response was a decline to a serum HBV DNA level less than 3.7 log copies/mL. Virological breakthrough was defined as the reappearance of a serum HBV DNA level to more than 10-fold the minimum during treatment.

RESULTS: Lamivudine produced virological response in 86.8% of the 318 patients at 6 mo, in 80.2% of 252 patients at 12 mo, in 69.2% of 133 patients at 24 mo, and in 53.6% of 28 patients at 36 mo. Forward stepwise logistic regression analysis showed an HBV DNA level less than 6.8 log copies/mL ($P<0.0001$), HBeAg negativity ($P<0.0001$), a platelet count of 100×10^9/L or more ($P=0.0162$) at baseline, and a decline of the HBV DNA level of more than 3.2 log copies/mL as compared with the baseline level at 3 mo after the start of treatment ($P=0.0003$) to be significantly associated with virological response. Among patients with a virological response, virological breakthrough was seen in 5.3% of 19 patients who responded virologically at 1 mo, in 20.7% of 203 patients at 3 mo, in 27.5% of 51 patients at 6 mo, in 33.3% of 12 patients at 9 mo, and in 100% of 3 patients at ≥15 mo. A virological breakthrough was found significantly more often in patients with delayed virological response.

CONCLUSION: Lamivudine treatment could suppress serum HBV DNA in most of the tested Japanese patients. Long-term efficacy might be seen in patients without HBeAg at baseline, in the absence of cirrhosis, and in patients with a decline in HBV DNA level soon after the start of treatment.

Key words: Hepatitis B virus; Lamivudine; HBeAg; Cirrhosis

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects 400 million people worldwide, three-quarters of whom reside in Asia[1]. The morbidity and mortality of chronic HBV infection are a major public health concern. Vertical transmission of HBV is the main cause of chronic HBV infection in the endemic areas of Asia. In Japan which is endemic for HBV infection, transmission can be easily prevented by vaccination of at risk-infants. The prevalence of hepatitis B surface antigen (HBsAg) carriage in the general population was reported to be less than 2%[2]. However, in areas of high HBV endemicity, persons with HBV-related cirrhosis have an approximately threefold higher risk of hepatocellular carcinoma (HCC) than those with chronic hepatitis but without cirrhosis and a 16-fold higher risk of HCC than the carriers in whom the virus is inactive[3]. Interferon-alpha (IFN-α) has been approved for the treatment of chronic hepatitis B[4], but it is poorly tolerated and effective in only 20%-30% of patients. Since the late 1990s, several studies have demonstrated the effectiveness of the initially available antiviral medicine, lamivudine, for patients with chronic hepatitis B: HBV DNA suppression, normalization of alanine aminotransferase (ALT), loss of hepatitis B e antigen (HBeAg). Moreover, studies have demonstrated the improvement of hepatic histology by the administration of lamivudine compared to placebo[5-8].

Lamivudine, an oral cytosine nucleoside analog, is the (-) - beta-enantiomer of 2',3'-dideoxy-3'-thiacytidine. HBV replicates through a pregenomic RNA intermediate. Lamivudine interferes with HBV reverse transcriptase (DNA polymerase) activity and causes chain termination of nascent viral DNA, leading to the inhibition of HBV replication[9]. Long-term treatment with lamivudine is not an option because it leads to drug resistance in most cases[10,11]. Lamivudine treatment, especially for chronic HBV-infected patients with cirrhosis, may also act as a bridge to more definitive treatments, such as liver transplantation. However, in several countries, including Japan, liver transplantation is not easily available because of insufficiency of donors, and even in other countries, many patients have to wait for long periods for transplantation. Although several non-Asian studies, from North America and Europe, have shown the efficacy of long-term use of lamivudine[12,13], few studies have assessed the efficacy of long-term lamivudine treatment of a large number of Japanese patients with chronic hepatitis B.

To acquire more data on these issues, 37 Japanese liver units involved in the management of HBV-related chronic liver diseases in Kyushu, Japan cooperated in this study. The objective of the present study was to analyze the results of long-term lamivudine administration for the suppression of HBV replication and the clinical outcomes of a large number of Japanese patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients

This retrospective analysis encompassed 318 Japanese chronic hepatitis B patients (231 males and 87 females, mean age 47.8 years) on lamivudine monotherapy for up to 36 (median 21, range 9-36) mo. Clinical features from 403 HBsAg-positive patients with chronic liver diseases, who started lamivudine treatment between December 2000 and March 2004 in 37 Japanese liver units in Kyushu, were recorded in a centralized database. All patients were determined to be serum HBV DNA-positive via polymerase chain reaction (PCR) assay prior to treatment. The diagnosis of chronic hepatitis and cirrhosis was based on a liver biopsy in most patients, if unavailable, on clinical laboratory, and ultrasound data. Eighty five patients were excluded from the present analysis because of one or more of the following reasons: age below 18 years; positive for antibody to hepatitis C virus or human immunodeficiency virus type 1; diagnosis of HCC within 3 mo after enrolment; time of lamivudine treatment within 9 mo; or treatment with anticancer drugs or corticosteroid drugs for other malignancies, such as leukemia, lymphoma or autoimmune diseases. Because this was a retrospective analysis of treated patients, there were no predefined criteria for treatment withdrawal or combination treatments. Criteria for withdrawal and combination treatments after the start of the treatment were dependent upon the strategy used by the physician at each center. In the present study, follow-up was stopped for patients who discontinued lamivudine treatment or started receiving a combination treatment with IFN and lamivudine, or with adefovir dipivoxil and lamivudine.

Therapeutic protocol

The patients received lamivudine (Zeffix®, Glaxo Smith Kline, UK) orally in a single daily dose of 100 mg. Data concerning age, sex, history of prior IFN treatment, Child-Turcotte-Pugh (CTP) score, series of serum laboratory testing of ALT, total bilirubin, albumin, HBeAg, and HBV DNA level were collected. Also, we analyzed virological (time of virological response and virological breakthrough) and biological events (time of ALT normalization, ALT breakthrough, and hepatitis flare) during the observation period. The clinical events recorded were hepatic decompensation (ascites, portal hypertensive bleeding, and hepatic encephalopathy) and liver-related death during the study period.

Biochemical and virological measurement

Quantification of serum HBV DNA was performed at each center using one of the following commercial assays according to local availability: quantitative PCR assay (Amplicor HBV Monitor, Roche Diagnostics, Mannheim, Germany), over a detection range from 2.6 (corresponding to 400 copies/mL) to 7.5 log copies/mL; or transcription-mediated amplification and hybridization protect assay (TMA-HPA, Chugai Diagnostics, Tokyo, Japan), over a detection range from 3.7 log genome equivalents (LGE)/mL (corresponding to 5000 copies/mL) to 8.7 LGE/mL. A decline of the serum HBV DNA level to less than 3.7 log copies/mL during treatment was considered as a virological response. Virological breakthrough was defined as the reappearance of a serum HBV DNA level to more

than 10-fold the minimum during treatment. We analyzed whether or not an early decline of the HBV DNA level at 3 and 6 mo after the start of the treatment was related to virological response and breakthrough.

The serum ALT, bilirubin, and albumin levels were serially determined using the standard method every month before treatment and during the treatment. The upper normal limits for the ALT level were slightly different in each facility, ranging between 30 and 40 IU/mL. Normalization with an ALT level 667 or below during the treatment was considered as a biological response. A deterioration of ALT to an abnormal level after normalization during the treatment was considered as an ALT breakthrough. A deterioration of the ALT level more than 10 times the upper limit of normal (ULN) was considered as a hepatitis flare.

Statistical analysis

Categorical variables were analyzed using χ^2 test or Fisher's exact test. The Mann-Whitney *U*-test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. The Cochran-Armitage's trend test was used to determine the relationship between the increases or decreases in the virological breakthrough rates of patients with virological response. Independent factors associated with responders were studied using forward stepwise logistic regression analysis of the following variables: age at the start of treatment, sex, history of prior IFN treatment, histological staging and grading, pretreatment laboratory data, serum pretreatment HBV DNA level, and the median declines of HBV DNA level at 3 and 6 mo after the start of the treatment. Forward stepwise logistic regression analysis was performed using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The BMDP program LR was used to evaluate the relationship between the clinical features and SVR. Using this method, the most significant associated variable was entered into the model. After adjusting for that variable, the next most significant variable was added to the model. Two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Baseline assessment

The mean age and percentage over 35 years were significantly higher in patients with cirrhosis than in those without cirrhosis, while the mean ALT level, albumin, and platelet counts were significantly higher in patients without cirrhosis than in those with cirrhosis. No significant differences in sex distribution, total bilirubin, positivity of HBeAg, or HBV DNA level were observed between these groups (Table 1). This study consisted of 173 HBeAg-positive and 145 HBeAg-negative patients with a mean pretreatment HBV DNA level of 6.8±1.2 (median 7.0) log copies/mL. Concerning the relationship between HBeAg and HBV DNA level, the mean HBV DNA level was significantly higher in HBeAg-positive patients with (7.3±1.1 log copies/mL) and without cirrhosis (7.2±1.0 log copies/mL) as compared with HBeAg-negative patients with (6.3±1.2 log copies/mL) and without cirrhosis (6.2±1.1 log copies/mL) (both $P<0.0001$). No significant difference in HBV DNA level, even classified by HBeAg status, was observed between patients with and without cirrhosis.

Table 1 Baseline characteristics of 318 patients with chronic HBV infection treated with lamivudine (mean± SD)

Characteristics	Cirrhosis		*P*
	No *n* = 216	Yes *n* = 102	
Number of men (%)	154 (71.3)	77 (75.5)	0.5168
Age (yr)	45.0±11.1	53.7±9.7	<0.0001
Number with 35 yr old and over (%)	173 (80.1)	98 (96.1)	0.0003
ALT (IU/L)	320.9±503.3	101.5±95.4	<0.0001
Total bilirubin (mg/dL)	1.2±1.3	1.4±1.3	0.1880
Albumin (g/dL)	4.0±0.4	3.5±0.6	<0.0001
Platelet count (mean ± SD) ($x10^4/\mu L$)	16.2±5.3	9.8±4.4	<0.0001
Number of HBeAg positivity (%)	119 (55.1)	54 (52.9)	0.8112
HBV-DNA[1]	6.8±1.3	6.6±1.2	0.1344
Lamivudine treatment (mo)	20.2±8.9	21.8±9.7	0.1429

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; SD, standard deviation [1]Logarithmic transformed copies/mL.

Virological and biological efficacy during lamivudine treatment period

In analyses of an early decline of HBV DNA level by lamivudine, the mean declines of all the studied patients were 3.2±1.2 (median 3.2) log copies/mL at 3 mo and 3.6±1.1 (median 3.8) log copies/mL at 6 mo after the start of treatment. During the treatment period of up to 36 (median 21) mo, a virological response was found in 90.6% (288/318) patients and ALT normalization was found in 86.2% (274/318) patients. Of the 288 with virological response, 255 (88.5%) had ALT normalization. Of the remaining 30 without virological response, 19 (63.3%), who had achieved virological suppression with a low HBV DNA level of 3.7-4.0 log copies/mL by lamivudine, had ALT normalization, but 11 (36.7%) had no ALT normalization and an HBV DNA level of more than 4.0 log copies/mL.

The mean pretreatment HBV DNA level was significantly lower in patients with virological response (6.6±1.2 log copies/mL) than those without virological response (7.7±0.7 log copies/mL) ($P<0.0001$). The frequency of pretreatment HBeAg positivity was significantly lower in patients with virological response (51.0%, 147/288) than those without virological response (86.7%, 26/30) ($P=0.0004$). No significant differences in sex distribution, age, ALT level, platelet count, presence of cirrhosis, or CTP score were found between the patients with and without virological response (Table 2).

Lamivudine suppressed serum HBV DNA to less than 3.7 log copies/mL in 69.2% patients at 3 mo, in 86.8% patients at 6 mo, in 80.2% patients at 12 mo, in 69.2% patients at 24 mo, and in 53.6% patients at 36 mo. The efficacy rate of virological response decreased with the length of the treatment period of patients who received lamivudine for over 6 mo (Figure 1).

Of the 288 patients with virological response, 224

Table 2 Virological response of 318 patients with chronic HBV infection treated with lamivudine (mean± SD)

Characteristics	Virological response		*P*
	No *n* = 288	Yes *n* = 30	
Number of men (%)	207 (71.9)	24 (80.0)	0.4624
Age (yr)	47.9±11.4	46.9 (22-73)	0.6453
Number of cirrhosis (%)	90 (31.2)	12 (40.0)	0.4403
Baseline laboratory data			
Total bilirubin (mg/dL)	1.3±1.5	1.1±0.6	0.6895
Albumin (g/dL)	3.9±0.6	3.8±0.6	0.2381
Platelet count ($\times 10^4/\mu L$)	14.3±5.9	13.2±5.8	0.2624
Number of HBeAg positivity (%)	147 (51.0)	26 (86.7)	0.0004
HBV-DNA[1]	6.6±1.2	7.7±0.7	<0.0001

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; SD, standard deviation [1]Logarithmic transformed copies/mL

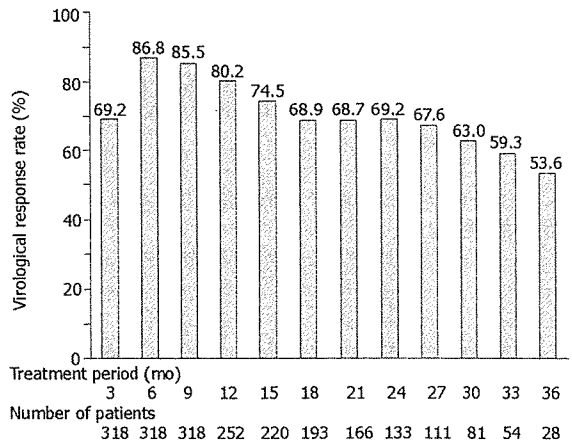

Figure 1 Treatment period and virological response rates to lamivudine treatment of Japanese patients with chronic hepatitis B virus infection.

(77.8%) had a sustained virological response, and 64 (22.2%) had a virological breakthrough. The median follow-up time was significantly shorter for patients with sustained virological response (18 mo) than those with virological breakthrough (27 mo). The frequencies of pretreatment HBeAg positivity [65.6% (42/64) *vs* 46.8% (105/224); $P=0.0123$] and cirrhosis [43.8% (28/64) *vs* 27.7% (62/224); $P=0.0218$] were significantly higher for patients with virological breakthrough than those without a breakthrough (Figure 2). No significant differences in sex distribution, age, or pretreatment HBV DNA level were observed between these groups.

HBeAg status during lamivudine treatment

Of the 318 patients, 173 (54.4%) were detected to have HBeAg in their sera at baseline. Of the 173 HBeAg-positive patients, 82 (47.4%) had clearance of HBeAg and 91 (52.6%) continued to have HBeAg during treatment. Lamivudine led to HBeAg clearance by 6.9% of the patients at 1 mo, by 24.9% of the patients at 3 mo, by 32.9% at 6 mo, by 35.8% at 9 mo, by 37.6% at 12 mo, by 45.1% at 18 mo, and by 47.4% at 36 mo, suggesting

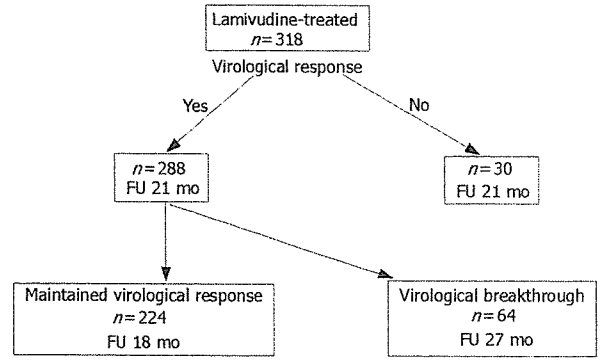

Figure 2 Virological events in all patients during lamivudine treatment period. FU: Follow-up period.

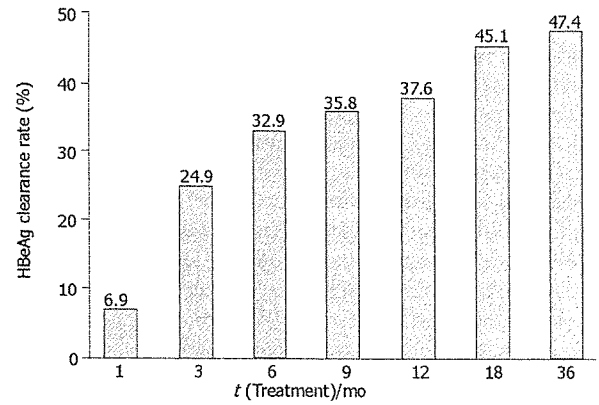

Figure 3 Relationship between treatment period and HBeAg clearance rate during lamivudine treatment of Japanese patients with chronic HBV infection.

that HBeAg clearance rates increased with the duration of lamivudine treatment (Figure 3). HBeAg clearance always occurred after virological response in all the 82 who cleared HBeAg. No significant differences in sex distribution, age, ALT level, platelet count, presence of cirrhosis, or CTP score were found between the patients with and without HBeAg clearance. Of the 145 patients with HBeAg negative at baseline, no patient reversed to HBeAg positive. We observed that the patients who cleared HBeAg (79/82, 96.3%) and the patients with HBeAg negative at baseline (141/145, 97.2%) had a significantly higher virological response rate than those without HBeAg clearance (68/91, 74.8%, $P=0.0002$, $P<0.0001$, respectively).

ALT breakthrough and hepatitis flare during lamivudine treatment

Of the 274 patients with ALT normalization by lamivudine, 231 (84.3%) had sustained ALT normalization, and 43 (15.7%) had an ALT breakthrough. Of the 43 patients with an ALT breakthrough, 4 (9.3%) had a hepatitis flare: 4 males, 3 with cirrhosis and 1 without cirrhosis, and 4 with HBeAg. However, no patient with hepatic decompensation, who had marked hyperbilirubinemia, or had a liver-related death, was observed in this study. The

Table 3 Forward stepwise logistic regression analysis for all independent factors contributing to virological response

Factors	Odds ratio	95% CI	*P*
(At baseline)			
HBV DNA less than 6.8[1]	434.7	104.1-2000	< 0.0001
HBeAg negativity	7.142	2.136-238.0	<0.0001
Platelet count more than 100×10^9/L	4.625	1.242-17.22	0.0224
(During treatment)			
Decline of HBV-DNA more than 3.2[1] within 3 mo of the start of treatment	51.13	11.21-233.0	<0.0001

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CI, confidence interval of odds ratios [1]Logarithmic transformed copies/mL.

Table 4 Forward stepwise logistic regression analysis for all independent factors contributing to virological breakthrough

Factors	Odds ratio	95% CI	*P*
(At baseline)			
Cirrhosis	3.527	1.687-7.371	0.0008
HBeAg positivity	2.512	1.265-4.989	0.0085
Platelet counts less than 100×10^9/L	2.386	1.003-5.676	0.0491
(During treatment)			
Decline of HBV-DNA less than 3.9[1] within 6 mo of the start of treatment	2.358	1.246-4.464	0.0084

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CI, confidence interval of odds ratios [1]Logarithmic transformed copies/mL.

time of ALT changes always depended on the time of virological change: ALT deterioration after normalization followed an increase in the HBV DNA level in all cases. The frequency of HBeAg positivity at baseline was significantly higher in patients with a breakthrough than those without a breakthrough [72.1% (37/43) *vs* 51.5% (119/231); $P<0.0001$]. No significant differences in sex distribution, age, pretreatment HBV DNA level, presence of cirrhosis or CTP score were observed between these groups.

Relationship between early virological response and virological breakthrough

Among the 288 with virological response, virological breakthrough was seen in 1 (5.3%) of 19 who had virological response at one month, in 42 (20.7%) of 203 at 3 mo, in 14 (27.5%) of 51 at 6 mo, in 4 (33.3%) of 12 at 9 mo, and in 3 (100%) of 3 at ≥15 mo. Cochran-Armitage's trend test revealed that virological breakthrough was significantly more prevalent in patients with delayed virological response ($P<0.0001$) (Figure 4).

Factors contributing to virological response and breakthrough

At baseline, an HBV DNA level less than 6.8 log copies/mL ($P<0.0001$), HBeAg negativity ($P<0.0001$), and platelets count of 100×10^9/L or more ($P=0.0224$) were significantly associated with virological response in the 318 studied patients (Table 3). Of the treatment factors, an early decline of 3.2 or more log copies/mL of HBV DNA at 3 mo after the start of the treatment was significantly associated with the response ($P<0.0001$).

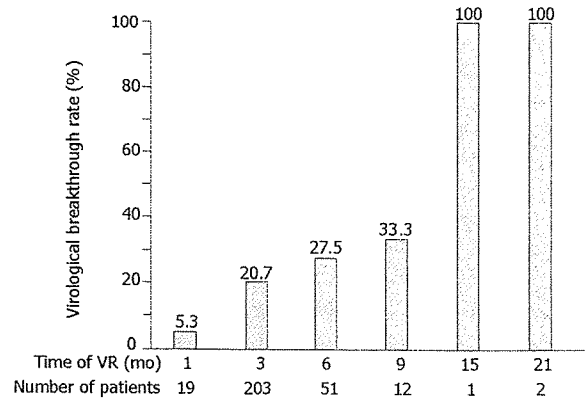

Figure 4 Relationship between time of virological response and virological breakthrough rate during lamivudine treatment of Japanese patients with chronic HBV infection. VR: Virological response.

At baseline, cirrhosis ($P=0.008$), HBeAg positivity ($P=0.0085$), and platelets count less than 100×10^9/L ($P=0.0491$) were significantly associated with a virological breakthrough in the 288 patients with virological response (Table 4). Of the treatment factors, an early decline of 3.8 or less log copies/mL of HBV DNA at 6 mo after the start of the treatment was significantly associated with the breakthrough ($P=0.0084$).

DISCUSSION

To our best knowledge, no such large-scale studies as this of lamivudine have been carried out for Japanese chronic hepatitis B patients. In this retrospective study, good virological and biological efficacy for up to 36 mo of lamivudine treatment was seen in Japanese patients with chronic hepatitis B, with no relation to sex, age, or ALT level at baseline. The effect was sustained for the patients with HBeAg-negative before treatment, absence of cirrhosis, and with an early decline of the HBV DNA level after the start of the treatment. During the treatment, very few patients with a hepatitis flare were seen and none with hepatic decompensation, marked hyperbilirubinemia, or liver-related death were seen in this study. The aims of treatment for chronic hepatitis B are to achieve sustained suppression of HBV replication and remission of inflammation in the liver. The antiviral responses for chronic hepatitis B are categorized as biochemical (ALT normalization), virological (decrease of HBV DNA to less than 5 log copies/mL and loss of HBeAg), and histological, and as on-therapy or sustained off-therapy[14]. Treatment for chronic hepatitis B patients seems to be necessary when the HBV DNA level exceeds 5 log copies/mL, independent of ALT activity[11]. Lamivudine well inhibited HBV DNA replication in Japanese chronic hepatitis B patients.

HBeAg clearance usually predicts long-lasting suppression of HBV, reduced infectivity and an improved clinical prognosis[15]. In this study, 47.4% of patients with HBeAg at baseline had HBeAg eliminated from their sera. Follow-up reports of the multicenter Asian study for Chinese patients showed that HBeAg clearance rates increased with the duration of lamivudine treatment, from 17% to 22% at

12 mo, 27 to 29% at 24 mo, and 33 to 40% at 36 mo[12,16,17]. The results of our study were consistent with those of these non-Japanese patient, although the HBeAg clearance rates within 24 mo were relatively high in our study. Lamivudine was effective in terms of HBeAg clearance in Japanese chronic hepatitis B patients. Patients successfully treated for chronic hepatitis B are less likely to develop cirrhosis, liver failure, and HCC in comparison with those who do not respond to treatment[18]. A randomized controlled trial of lamivudine for chronic hepatitis B patients demonstrated that HCC incidence was reduced by lamivudine antiviral therapy, showing an incidence of 3.9% in lamivudine-treated patients and 7.4% in a placebo control group, with a hazard ratio of 0.49 (95%CI=0.25-0.99)[19]. For chronic hepatitis B patients, antiviral therapy with lamivudine that results in sustained suppression of HBV DNA replication and hepatic necroinflammation may reduce the incidence of HCC.

It has been reported that resistance to lamivudine often develops after 6 mo of treatment[10]. The present study was limited in its value because we detected viruses resistant to lamivudine. However, in our study, the emergence of resistant viruses could be defined by the virological breakthrough (reappearance of serum HBV DNA levels more than 10-fold increase from the minimum). A serious drawback of long-term lamivudine treatment is the development of resistant HBV mutants, i.e., the mutations in a tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase gene, associated with increase in serum HBV DNA and the ALT level[19]. The present study showed that the HBV DNA suppression rates by lamivudine decreased with the duration of treatment, but that a relapse of biochemical response, ALT breakthrough was found only in 15.7% of patients during these treatment periods. Lamivudine treatment withdrawal can cause HBV DNA to revert to pretreatment levels, with the relapse of clinical hepatitis[20]. With the excellent safety and tolerability of lamivudine, continuous therapy is suggested as beneficial[4]. After the start of phylogenetic analyses, based on inter group divergence of 8% or more over the complete HBV nucleotide sequence, seven different genotypes, arbitrarily designed A-G, have been recognized[21,22]. Several reports have shown geographical distribution of the genotypes, with genotypes A and D predominant in Western Europe, B and C in South Asia and the Far East, and F in South America[21-26]. Due to the geographical distribution pattern, HBV genotypes B and C are commonly observed in Japan[24-27]. Moreover, Japan is apparently at a geographical boundary for genotypes B and C, forming a south to north gradient in which genotype C is more frequent in the south of Kyushu, and genotype B is more frequent in the north of Tohoku. Interestingly, however, genotype B is more frequent in Okinawa, the southern-most area of Japan[27]. Our previous epidemiological study of the Japanese HBV genotype distribution showed that 95% of the patients studied had genotype C[24]. Genotype C has been reported to cause more severe liver damage and to have lower rates of HBeAg clearance, which usually indicates cessation of HBV replication and represents a later stage of chronic HBV infection, than genotype B in Japanese patients[24-26]. Accordingly, our results were equivalent in the response to lamivudine to Japanese HBV genotype C patients, although we did not determine the genotyping of our patients.

Another noteworthy finding of our study was that predictive marker of the efficacy to lamivudine and its durability were HBeAg negativity and a low HBV DNA level at baseline. HBV DNA reappears in serum after cessation of lamivudine treatment because HBV replication within the HBV-infected hepatocytes originates primarily from the covalently closed circular DNA (cccDNA) of HBV in the liver. Lamivudine appears to have no effect on the level of cccDNA[28]. Liver injury seems to be particularly severe and rapidly progressive in HBeAg-negative patients, but clinically significant HBV replication persists in them[24]. Most HBeAg-negative chronic hepatitis B patients who are HBV DNA-positive harbor HBV variants with mutations in the precore or core promotor region, which can suppress synthesis of HBeAg[11,26]. The clearance of HBeAg is perhaps a reflection of a loss of the cccDNA pool of HBV in the liver[29]. The great concern of clinicians is that HBeAg negativity and a low HBV DNA level at baseline are significant predictive markers for lamivudine treatment in Japanese patients.

A previous report on Japanese patients showed that the emergence rate of lamivudine-resistant viruses in patients with cirrhosis was higher than those without cirrhosis[28], suggesting that a virological breakthrough appears more frequently in patients with cirrhosis than those without cirrhosis. The present study showed that lamivudine treatment was not so effective or durable in patients with cirrhosis and low platelet counts. Clinicians should always do close monitoring or use other antiviral drugs because hepatitis flare was occasionally severe, especially in patients with cirrhosis. The present study also showed that an early virological response to lamivudine was predictive of both efficacy and durability, but a lack of an early virological response was found to predict a virological breakthrough. A high HBV DNA level reflects a greater pool of virus and a higher rate of virus replication, thereby increasing the likelihood that drug-resistant mutations will be selected. Such an early decrease of viral load after the start of lamivudine might be associated with the lack of viral resistance.

In conclusion, the present study suggests a long-term lamivudine treatment to be safe and to result in the reduction of serum HBV DNA in most Japanese patients with chronic hepatitis B. The efficacy is sustained in patients with HBeAg-negative at baseline, absence of cirrhosis, and a reduction of the HBV DNA level soon after the start of the treatment.

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Original Article

Effect of Probucol on Elderly Hypercholesterolemic Patients in the FAST Study

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Abstract The present study involved a detailed post hoc comparison of the efficacy and safety of lipid-lowering therapy in elderly hypercholesterolemic patients from the Fukuoka Atherosclerosis Trial (FAST). The FAST cohort of 246 hypercholesterolemic patients included 76 patients who were (75 years old. Patients were randomized to receive probucol (500 mg/day) or pravastatin (10 mg/day) therapy, or to a control group (diet alone), and then were followed for 2 years. In patients ≥75 years old, either probucol or pravastatin achieved a significant reduction of carotid intima-media thickness (IMT). In patients <75 years old, lipid-lowering therapy also achieved a significant reduction of IMT. In patients ≥75 years old receiving probucol, the relative risk (95% confidence interval) of all-cause mortality was 0.15 (0.02 to 1.28) and that for major coronary events was 0.12 (0.02 to 1.04). In conclusion, probucol reduced the incidence of cardiovascular disease in elderly hypercholesterolemic patients as well as younger patients.

Key words: elderly hypercholesterolemic patients, probucol, pravastatin, intima-media thickness, carotid atherosclerosis, cardiovascular disease

INTRODUCTION

Atherosclerosis is a common disease in the elderly, and atherosclerotic lesions may cause myocardial infarction or stroke. Measurement of the carotid artery intima-media thickness (IMT) by high-resolution B-mode ultrasonography allows noninvasive detection of early carotid atherosclerosis, and the IMT is also a reliable end-point for trials assessing the effect of interventions on disease progression. Furthermore, ultrasonography can directly quantify early atherosclerotic changes and the response to risk factor modification[1)], allowing the use of a smaller patient population to determine the benefits of treatment or accurately assess the presence of early atherosclerosis. The Fukuoka Atherosclerosis Trial (FAST) was the first study to demonstrate the benefit of probucol therapy for patients with hypercholesterolemia and to also reveal an effect of probucol on the incidence of cardiovascular events[2)].

A direct relationship between the serum low-density lipoprotein (LDL)-cholesterol level and the risk of coronary heart disease (CHD) has been most clearly demonstrated in studies on middle-aged men. Although a similar relationship has also been observed

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in middle-aged women and in some older populations (≥65 years), the relationship is reported to be weaker in the elderly and it has been less convincingly established for elderly women compared with elderly men[3]. This may be partly because women and elderly patients have been poorly represented in prior clinical trials of cholesterol-lowering therapy. Consequently, the value of screening the lipid profile and performing cholesterol-lowering therapy in these populations is still unclear[4].

The aim of the present study was to perform a detailed post hoc assessment that compared the effect of lipid-lowering therapy on carotid atherosclerosis in younger and older patient subsets (≥75 and <75 years old) from the FAST population.

METHODS

Patient Selection and Study Protocol

The study design and the baseline characteristics of the patients have been described elsewhere[2]. Briefly, between February 1996 and February 2000, men and women aged 30-89 years with hypercholesterolemia who met the following criteria were eligible to participate in the present study. Exclusion criteria included a serum triglyceride level >350 mg/dl, uncontrolled heart failure, recent myocardial infarction (≤6 months), severe or unstable angina pectoris, hypothyroidism/hyperthyroidism or other endocrine diseases, secondary hyperlipidemia, uncontrolled diabetes mellitus, uncontrolled hypertension, heavy alcohol intake, obese patients on weight reduction programs, diseases that might interfere with drug absorption, any severe illness, and treatment with certain drugs (including corticosteroids, androgens, other lipid-lowering agents, or antiacids containing aluminum salts). Hospital visits for monitoring were scheduled after 2 weeks of therapy and then every 4 weeks thereafter. At each visit, a brief physical examination was performed, and the number of tablets was counted to assess compliance. In both groups, lipids, lipoproteins, and other laboratory parameters (to confirm safety) were also measured at each visit. Written informed consent was obtained from each patient, and this trial was approved by the Ethics Committee of Kyushu University Hospital.

The procedure for measurement of carotid IMT and its reproducibility have been described elsewhere[2]. In brief, ultrasonography was done with the patient in the supine position using an Aloka SSD-2000 (Aloka, Tokyo, Japan) with a 7.5 MHz transducer. The IMT of the far wall of both the right and left common carotid arteries was measured at 2, 2.5, and 3 cm proximal to the carotid bifurcation. The IMT was defined as the distance between two echogenic lines separated by a hypoechoic or anechoic space, with the outer line corresponding to the medial-adventitial border and the inner line representing the luminal-intimal border. The mean IMT was calculated as the average value of the measurements obtained at 6 sites (3 per vessel) in the bilateral carotid arteries. Stenosis was defined as plaque (IMT ≥1.10 mm) occupying more than half of the luminal circumference of the artery on a transverse scan.

Laboratory Tests

Blood samples were collected between 8 and 9 am after a 12-hour fast. Serum cholesterol and triglyceride levels were measured by enzymatic methods. Using the calcium heparin method, high-density lipoprotein (HDL) cholesterol was measured in

the supernatant obtained after precipitation of apolipoprotein B-containing lipoproteins by and LDL cholesterol was calculated using Friedewald's formula. Measurements were done on the day of blood collection, or else the blood was stored at -4 (C for no longer than 3 days until assay.

Statistical Analysis

All data were recorded on standard forms and were entered into a database. Results are expressed as percentages or as the mean (SD. The mean values of numerical variables were compared by the Mann-Whitney U test, while categorical variables were compared by the chi-square test, as appropriate.

The endpoint was the effect of each treatment on the incidence of major atherosclerotic events. The relative risk and 95% confidence interval were calculated with the Cox regression model. In all analyses, $P < 0.05$ was considered to indicate statistical significance. All data were analyzed on an intention-to-treat basis.

RESULTS

Baseline Characteristics

The baseline characteristics of the subjects have been summarized elsewhere[2]. Briefly, the mean age of the patients was 66.1 years and 31.3% were men. The average systolic blood pressure and diastolic blood pressure were 130.8 and 77.1 mm Hg, respectively. Of the 246 patients, 59.3% were recent or former smokers, 42.5% had a history of hypertension, and 22.9% had diabetes mellitus. Baseline serum total cholesterol and LDL-cholesterol levels were 253.0 mg/dL and 166.1 mg/dL, respectively, while the HDL-cholesterol level was 57.0 mg/dL and the serum triglyceride level was

Table 1 Baseline characteristics (including lipids) for the two subgroups of interest (patients ≥75 years old and patients <75 years old)

		Probucol			Pravastatin			Diet alone		
		Age≥75 (n=27)	Age<75 (n=55)	chi-square test	Age≥75 (n=27)	Age<75 (n=55)	chi-square test	Age≥75 (n=22)	Age<75 (n=59)	chi-square test
		No.(%)	No.(%)		No.(%)	No.(%)		No.(%)	No.(%)	
Sex	M	4(14.8)	21(32.0)	0.0568	1(3.6)	21(32.0)	0.0018	6(27.3)	24(40.7)	0.3939
	F	23(85.2)	34(61.8)		27(96.4)	34(61.8)		16(72.7)	35(59.3)	
Smoking	+	12(44.4)	36(65.5)	0.1149	13(46.4)	31(56.4)	0.5321	15(68.2)	39(66.1)	1.0000
	−	15(55.6)	19(34.5)		15(53.6)	24(43.6)		7(31.8)	20(33.9)	
CVD	+	17(63.0)	18(32.7)	0.0181	19(67.9)	15(27.3)	0.0009	14(63.6)	20(33.9)	0.0308
	−	10(37.0)	37(67.3)		9(32.1)	40(72.7)		8(36.4)	39(66.1)	
IHD	+	5(18.5)	4(7.3)	0.2480	8(28.6)	7(12.7)	0.1410	7(31.8)	4(6.8)	0.0104
	−	22(81.5)	51(92.7)		20(71.4)	48(87.3)		15(68.2)	55(93.2)	
HT	+	12(44.4)	25(45.5)	1.0000	16(57.1)	23(41.8)	0.2757	9(40.9)	17(28.8)	0.4415
	−	15(55.6)	30(54.5)		12(42.9)	32(58.2)		13(59.1)	42(71.2)	
DM	+	3(11.1)	12(21.8)	0.3818	5(17.9)	13(23.6)	0.7472	4(18.2)	19(32.2)	0.3331
	−	24(88.9)	43(78.2)		23(82.1)	42(76.4)		18(81.8)	40(67.8)	

	Probucol				p-value Mann Whitney's U-test	Pravastatin				p-value Mann Whitney's U-test	Diet alone				p-value Mann Whitney's U-test
	Age≥75(n=27)		Age<75(n=55)			Age≥75(n=27)		Age<75(n=55)			Age≥75(n=22)		Age<75 (n=59)		
	No.	Mean S.D.	No.	Mean S.D.		No.	Mean S.D.	No.	Mean S.D.		No.	Mean S.D.	No.	Mean S.D.	
sBP	27	73.3±23.1	55	133.3±22.6	0.8434	28	130.2±24.4	55	128.7±22.0	0.5369	22	141.5±24.5	59	127.0±17.0	0.0077
dBP	27	21.6±11.5	55	80.0±13.1	0.0526	28	71.5±10.2	55	77.7±11.3	0.0187	22	78.7±8.2	59	77.4±10.2	0.2531
BMI	27	21.6± 3.3	55	23.9± 4.5	0.0109	28	21.8± 5.7	55	24.4±3.4	0.0118	22	22.3±2.1	59	23.1± 2.9	0.1723
IMT	27	1.5± 0.8	55	1.3± 0.5	0.7556	28	1.4± 0.9	55	1.2±0.4	0.6748	22	1.3±0.5	59	1.3± 0.5	0.8943
TC	27	257.9±25.5	55	249.7±25.5	0.0962	28	258.0±23.5	55	248.2±25.1	0.0349	22	256.0±23.3	59	254.9±30.3	0.3895
LDL-C	27	171.3±31.1	55	163.5±24.7	0.2734	28	161.5±29.2	55	160.2±33.0	0.4325	22	175.2±27.1	59	170.2±36.3	0.4509
HDL-C	27	61.1±19.9	55	56.2±18.7	0.2864	28	62.2±14.3	55	53.9±15.6	0.0218	22	55.1±10.0	59	57.0±16.5	0.8400
TG	27	127.9±56.0	55	150.2±72.3	0.2119	28	171.8±75.0	55	167.1±89.8	0.4880	22	128.5±44.8	59	138.5±63.7	0.6479

CVD: cerevral vascular disease
IHD: ischemic heart disease
HT: hypertension
DM: diabetes mellitus
sBP: systolic blood pressure
dBP: diastolic blood pressure
BMI: body mass index
IMT: intima-media thickness
TC: total cholesterol
LDL: LDL-cholesterol
HDL: HDL-cholesterol
TG: triglyceride