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Review

Statins: Beneficial or Adverse for Glucose Metabolism

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Large-scale clinical trials have established that statin use for lowering blood cholesterol is beneficial in reducing atherosclerotic cardiovascular diseases in different populations. However, the general reputation of statins seems to be clouded by a potential adverse effect of a class of statins on glucose metabolism. This paper reviewed clinical data of statins regarding the effects on diabetes mellitus and glucose metabolism. At least five randomized controlled studies, primarily investigating the protective effect of statins on the risk of cardiovascular diseases, have addressed the effect of statins on glucose metabolism in Western countries. One study showed that pravastatin (40 mg/day) was protective against the development of diabetes mellitus. Two studies of atorvastatin (10 mg/day) and one study of simvastatin (40 mg/day) showed no measurable effect of these regimens on the risk of diabetes mellitus or the clinical course of diabetes mellitus. One study of atorvastatin (80 mg/day) versus pravastatin (40 mg/day) suggested a deterioration of glucose metabolism associated with a high dose of atorvastatin. In Japan, a few case reports have noted a potential adverse effect of atorvastatin on glycemic control in patients with diabetes mellitus; however, seven clinical trials have showed no such effect of atorvastatin although these studies were relatively small in size and short in follow-up. Only one of the two observational studies suggested a possible adverse effect of atorvastatin on glycemic control. Evidence is extremely limited regarding atorvastatin use and deterioration in glycemic control, and further studies are needed to draw a conclusion on this issue.

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Key words; Pravastatin, Atorvastatin, Diabetes mellitus, Insulin, Blood glucose

Introduction

Statins, HMG-CoA reductase inhibitors, enhance the expression of low-density lipoprotein (LDL) receptors in the liver and consequently lower blood LDL cholesterol levels through inhibiting cholesterol synthesis in the liver[1]. From large-scale clinical trials in different populations[2-4], it has been established that statin use substantially reduces the risk of cardiovascular diseases. In addition to lowering LDL cholesterol levels, statins are known to suppress the progression of atherosclerosis by their pleiotropic effects including the improvement of thrombus formation, antioxidant effect, improvement of vascular endothelial cell damage, anti-inflammatory action, and stabilization of plaques[5]. Evidence from clinical trials has given statins the general reputation as very effective and safe cholesterol-lowering drugs, although adverse effects of statins, such as elevation of liver enzymes and rhabdomyolysis, are recognized. However, an incident of fatal rhabdomyolysis associated with cerivastatin raised a concern that the clinical efficacy and safety of statins may differ by the class of statins[6]. Differences in the structural and physical properties of statins might result in the variation in pharmacokinetics, pleiotropic effects, and drug interactions[5].

It has been a matter of recent concern whether atorvastatin deteriorates diabetes mellitus or glycemic control. In 2003, immediately after the introduction of atorvastatin, two independent groups each reported two cases of diabetes mellitus showing deterioration in

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Table 1. Atorvastatin use and deterioration of blood glucose status in patients with diabetes mellitus: case reports presented at recent meetings in Japan

Authors	Main findings	Reference
Nunoi, et al.	Deteriorated HbA1c with ATR for 2 months (2 cases).	J Jpn Diab Soc, 46: 202, 2003
Murakami, et al.	Deteriorated FBS with ATR 5 mg for 3 M and 10 mg for 2 months (2 cases).	J Cardiol, 42 (Suppl 1): S455, 2003
Katoh, et al.	Deteriorated HbA1c with ATR 5 mg for 1 month and with ATR 10 mg for 4 months (2 cases).	J Jpn Diab Soc, 48: 71, 2005
Kodera, et al.	Deteriorated non-fasting BS/HbA1c with ATR for 3-4 months (2 cases)	J Jpn Diab Soc, 48: 392, 2005
Seguchi, et al.	Deteriorated FBS/HbA1c with ATR for 3-6 months (3 cases)	J Jpn Diab Soc, 48: 392, 2005
Fukuniwa, et al.	Deteriorated HbA1c with ATR 5 mg for 2 months and then with PRV 10 mg for 2 months (1 case)	J Jpn Diab Soc, 48: 451, 2005

ATR: atorvastatin, BS: blood sugar, FBS: fasting blood sugar, PRV: pravastatin.
Based on the Japan Medical Abstracts Society web version 3 with a combination of key words (HMG-CoA reductase inhibitors, diabetes mellitus, proceedings, and human).

glycemic control during treatment with atorvastatin, and at least eight such cases were reported subsequently at meetings in Japan (**Table 1**). Very recently, a case of type 2 diabetes mellitus occurring after atorvastatin treatment was published[7]. In this case, however, hyperglycemia, which was resolved with insulin therapy and discontinuation of atorvastatin, recurred with pravastatin use. As discussed in detail below, a substudy of a multicenter randomized controlled trial, which was presented at the 2004 meeting of the American Heart Association (AHA), suggested that a high dose of atorvastatin (80 mg/day) might deteriorate glycemic control[8]. In this paper, we review clinical data concerning the effects of statins on glucose metabolism, especially from the safety aspect, and discuss the possible mechanisms of these effects. For this task, we searched for relevant articles in PubMed with the combination of "Hydroxymethylglutaryl-CoA Reductase Inhibitors"[MeSH], "Clinical Trials"[MeSH] and "Diabetes Mellitus"[MeSH], and also the Japan Medical Abstracts Society web version 3 with a combination of key words (HMG-CoA reductase inhibitors, diabetes mellitus, original article/proceedings, and human). The search was limited to publications in the year 2000 and thereafter, and was done on January 10, 2006. Related articles were also searched for by scanning the references quoted in the articles at hand.

Randomized Controlled Trials in Western Countries

The effects of statins on the risk of diabetes mellitus or glycemic control have been directly addressed in at least five randomized controlled trials with the event of cardiovascular diseases as the primary endpoint. The West of Scotland Coronary Prevention Study[9] was the first clinical trial which investigated the risk of diabetes mellitus associated with statin treatment. Originally, it was a double-blind trial in which 6,595 men aged 45-64 years with hypercholesterolemia but no evidence of cardiovascular disease were randomized to receive either pravastatin (40 mg/day) or placebo treatment[3]. The subjects in the substudy were 5,974 men who had two or more post-randomization measurements of blood glucose and had neither self-reported diabetes nor fasting blood glucose of ≥ 7.0 mmol/L at baseline. The incidence of diabetes mellitus was defined as two glucose measurements of ≥ 7.0 mmol/L and at least one measurement of ≥ 2.0 mmol/L above the baseline level or newly started prescription of hypoglycemic drugs. During the follow-up period of 3.5-6.1 years, 139 became diabetic. After adjustment for body mass index, triglyceride, blood glucose, and other characteristics at baseline, the patients assigned to pravastatin therapy had a hazard ratio of 0.70 (95% confidence interval, 0.50-0.98) for transition to diabetes mellitus[9].

In the MRC/BHF Heart Protection Study[10], 20,536 subjects aged 40 to 80 years with and without diabetes mellitus were randomized to receive either simvastatin (40 mg/day) or placebo. The mean duration of follow-up was 4.8 years for participants with diabetes mellitus at entry and 5.0 years for those without. Among the 14,573 subjects without known diabetes mellitus at baseline, there was no difference in the incidence of diabetes mellitus defined as the initiation of oral hypoglycemic or insulin treatment or a specific report of new diabetes mellitus (4.6% in the simvastatin group and 4.0% in the placebo group, $p=0.10$)[11]. Furthermore, among a random sample of 1,087 patients with diabetes mellitus at baseline, HbA1c levels slightly increased in both simvastatin (0.15%) and placebo (0.12%) groups during the study period, with no measurable difference between the

two ($p=0.8$)[11].

In the Anglo-Scandinavian Cardiac Outcomes Trial[12], 19,342 hypertensive patients aged 40 to 79 years were randomized to either of two antihypertensive regimens and 10,305 with non-fasting total cholesterol concentrations of 6.5 mmol/L or less were further randomized to either atorvastatin (10 mg/day) or placebo treatment. The median follow-up was 3.3 years. The occurrence of diabetes mellitus was prespecified as a tertiary endpoint. There was no difference in the development of diabetes mellitus between the atorvastatin and placebo treatments; the hazard ratio for atorvastatin versus placebo was 1.15 (95% confidence interval, 0.91 to 1.44).

In a substudy of the Pravastatin or Atorvastatin Evaluation in Myocardial Infarction (PROVE-IT) presented at the 2004 AHA meeting[8], the effects of the two statins on glycemic control were evaluated. PROVE-IT was the first large-scale clinical study comparing two statins[13]. In this study, 4,162 patients were randomized to receive intensive lipid-lowering therapy with atorvastatin (80 mg/day) or standard lipid-lowering therapy with pravastatin (40 mg/day) immediately after the occurrence of acute coronary syndrome. As compared with patients treated with pravastatin, those with atorvastain had a higher risk of developing HbA1c>6.0% among those with baseline HbA1c ≤ 6.0% regardless of diabetes mellitus; the pooled hazard ratio was estimated to be 1.84 (95% confidence interval 1.52-2.22). This finding does not necessarily indicate that atorvastatin increases the risk of deterioration in glycemic control because the comparison was made against pravastatin treatment.

The Collaborative Atorvastatin Diabetes Study investigated the protective effect of atorvastatin (10 mg/day) versus placebo specifically on cardiovascular disease in 2,838 patients with type 2 diabetes mellitus[14]. No difference was noted between the two regimens with respect to changes in HbA1c levels and the therapeutic modality for diabetes mellitus. The mean HbA1c levels at the baseline were 7.9% in the atorvastatin group and 7.8% in the placebo group. The corresponding values after 4 years of follow-up were 8.3% and 8.1%, respectively. At the baseline, insulin was used in 19.7% of patients in the atorvastatin group and 18.9% of patients in the placebo group. These proportions had not changed significantly at the end of the follow-up period (atorvastatin 20.5% and placebo 18.2%).

In summary, one study showed that pravastatin (40 mg/day) was protective against the development of diabetes mellitus. Two studies of atorvastatin (10 mg/day) and one study of simvastatin (40 mg/day) showed no measurable effect of these regimens on the risk of diabetes mellitus or the clinical course of diabetes mellitus. One study of atorvastatin (80 mg/day) versus pravastatin (40 mg/day) suggested a deterioration of glucose metabolism associated with a high dose of atorvastatin. It should be noted that the onset or deterioration of diabetes mellitus was defined differently in the studies, however.

Clinical Trials and Observational Studies in Japan

None of the reported clinical trials regarding statins and cardiovascular diseases has been extended to examine the effects of statins on the risk of diabetes mellitus or glucose metabolism[4, 15]. With hindsight, a possible adverse effect of atorvastatin on glucose metabolism was noted in a long-term one-arm trial of 287 patients with total cholesterol of ≥ 220 mg/dL. The primary purpose of this trial was to investigate the efficacy of atorvastatin 5-10 mg/day on serum lipids[16]. The majority (81%) of the patients received atorvastatin 10 mg/day throughout the study period. The prescribed dose was changed from 10 mg/day to 20 mg/day in 7% of the patients, from 10 mg/day to 5 mg/day in 5%, and from 5 mg/day to 10 mg/day in 4%. The episode of a pre-specified abnormal elevation of fasting blood glucose was fairly frequently observed during the one-year period, as shown in **Table 2**. Furthermore, the grade of abnormal elevation was more severe for blood glucose than for other laboratory measurements. Sixteen laboratory tests were evaluated in terms of severity. The majority (82%) of the episodes of abnormal change in laboratory tests other than glucose were classified as grade 1 (slight deterioration), but 15 of the 21 episodes of abnormal elevation of blood glucose were classified as grade 2 (moderate deterioration) or grade 3 (severe deterioration). The abnormal elevation of HbA1c was also commonly seen during the study period. It should be noted that the abnormal elevation of blood glucose or HbA1c was evaluated in terms of the number of episodes rather than cumulative incident cases.

We identified 11 published studies examining changes in fasting blood glucose and/or HbA1c after treatment with a specific statin in diabetes patients (**Table 3**). Of these, three were randomized trials[17-19], six were one-arm trials[20-25], and two were retrospective, observational studies[26, 27]. Except for three studies[22, 23, 26], these studies were very small in size with fewer than 100 patients, and a relatively short follow-up period. None of the seven trials found any measurable adverse effect of atorvastatin on glycemic con-

Table 2. Episodes of abnormal laboratory tests occurring in hypercholesterolemic patients treated with atorvastatin 5-20 mg/day for one year

Abnormal laboratory test	No. of patients	No. of episodes
Elevation of gamma-glutamyltransferase	287	50 (17.4%)
Elevation of alanine aminotrasferase	287	34 (11.8%)
Elevation of aspartate aminotrasferase	287	26 (9.1%)
Elevation of fasting blood glucose	281	21 (7.5%)
Decreased testosterone	274	20 (7.3%)
Elevation of creatinine phosphokinase	287	19 (6.6%)
Elevation of choline esterase	287	16 (5.6%)
Elevation of HbA1c	282	15 (5.3%)

Derived from reference (15)

Table 3. Clinical trials and observational studies concerning statins and glycemic control in patients with diabetes mellitus in Japan

Authors (ref.)	Type of study	No. of patients	Statin	Dose (mg/day)	Period	Main findings
Tanaka, et al.[17]	RCT	40	Atorvastatin Placebo	10 –	12 weeks	No change in HbA1c for each group.
Endo, et al.[18]	RCT	47	Atorvastatin Pravastatin	10 20	4 months	No change in HbA1c for each statin.
Kameda, et al.[19]	RCT	14	Atorvastatin Bezafibrate	10 400	9 months	No change in FBS/HbA1c for each drug.
Sato and Miyachi[20]	One-arm trial	26	Atorvastatin	10-20	8 weeks on average	No change in HbA1c.
Hamano[21]	One-arm trial	35	Atorvastatin	10	12 months	No change in FBS/HbA1c.
Sasamoto[22]	One-arm trial	180	Atorvastatin	5-40	3-15 months	No change in FBS/HbA1c.
Suzuki[23]	One-arm trial	160	Atorvastatin	10	3 months to 3 years	No change in HbA1c
Yamada, et al.[24]	One-arm trial	27	Pitavastatin	2	8 weeks	HbA1c increased by 0.17% (95% CI 0.01, 0.33).
Yamada[25]	One-arm trial	57	Pitavastatin	1-2	30 months on average	No change in FBS
Seino, et al.[26]	Observational study	809	Pravastatin Simvastatin Fluvastatin Atorvastatin	5-20 2.5-10 20-60 5-10	3.9 years 3.4 1.9 0.9	No change in FBS/HbA1c for each statin.
Osaki, et al.[27]	Observational study	67	Atorvastatin Pravastatin	10 10	2-3 months	Deteriorated HbA1c (≥ 10% relatively) was more frequent for atorvastatin (7/25, 28%) than pravastatin (3/42, 7%).

RCT: randomized controlled trial, FBS: fasting blood sugar.

trol[17-23]. Only one observational study reported that deterioration of HbA1c was statistically significantly more frequent for atorvastatin than pravastatin[27], whereas the other observational study found no measurable change in fasting blood glucose or HbA1c in relation to atorvastatin and other statins[26]. On the other hand, one of the two studies concerning pitavastatin showed a statistically significant increase in HbA1c after 8-week treatment[24]. Findings from case reports may often signal an alarming adverse effect of a newly introduced drug, but they may sometimes be an extreme of random variation. One study graphically presented HbA1c values of 26 subjects before and after atorvastatin treatment[20]. HbA1c increased markedly in a few individuals, and also decreased substantially in an almost equal number of subjects. Amelioration may not have been taken as seriously as deterioration in the routine clinical practice.

In summary, although the case reports suggested a potential adverse effect of atorvastatin in patients

with diabetes mellitus, none of the seven clinical trials provided supporting evidence. Only one of the two observational studies reported a more frequent deterioration of HbA1c in treatment with atorvastatin than with pravastatin. Observational findings in clinical practice require caution in their interpretation because they may have been ascribed to other concurrent factors associated with the deterioration of diabetes mellitus. Thus, evidence showing that atorvastatin at a dose commonly used in Japan deteriorates glycemic control in patients with diabetes mellitus is extremely limited.

Mechanisms of the Effects of Statins on Glucose Metabolism

Evidence is very limited as regards the mechanisms by which statins exert any influence on glucose metabolism. Statins may improve insulin resistance and be protective against glucose intolerance through their anti-inflammatory effects[28, 29]. Inflammatory markers have been related to an increased risk of diabetes mellitus in adults[30, 31], and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α are implicated as being linked with insulin resistance through their influence on insulin receptor[32, 33]. On the other hand, statins can deteriorate glycemic control by decreasing various metabolites, such as isoprenoid, farnesyl pyrophosphate, geranylgeranyl pyrophosphate, and ubiquinone (CoQ_{10}), which are normally produced during the process of cholesterol synthesis from acetyl CoA via mevalonic acid. Isoprenoid is known to enhance glucose uptake by upregulating the membrane transporter protein glucose transporter 4 (Glut 4), which plays a role in glucose uptake in adipocytes[34]. Suppressed biosynthesis of ubiquinone (CoQ_{10}), an essential factor in the electron-transfer system in mitochondria, may result in delayed ATP production in pancreatic β cells and thereby impair insulin release. It was recently shown that atorvastatin treatment resulted in a reduction of serum CoQ_{10} levels, which was positively correlated with LDL cholesterol levels[35].

These mechanisms may differ by the property of statins. Water-soluble statins, such as pravastatin, are hepatocyte-specific and are not readily taken up by pancreatic cells and adipocytes. Lipid-soluble statins, such as simvastatin and atorvastatin, enter extrahepatic cells easily and may inhibit isoprenoid protein synthesis, consequently attenuating insulin action. Lovastatin, a lipid-soluble statin, was shown to down-regulate insulin-responsive Glut 4 and up-regulate Glut 1 in 3T3-L1 adipocytes leading to marked inhibition of insulin-stimulated glucose transport[34]. Another lipid-soluble statin, simvastatin, inhibited glucose-induced increase in intracellular Ca^{2+} of pancreatic β cells, leading to the inhibition of insulin secretion in a dose-dependent manner, while water-soluble pravastatin had absolutely no effect even at a high concentration of 100 μg/mL[36]. The inhibitory potency of HMG-CoA reductase and lipophilicity of statins may be related to different effects on glucose metabolism, although further studies are needed.

Conclusion

A few clinical studies have suggested that atorvastatin, especially at a high dose, may deteriorate glucose metabolism while pravastatin might improve glucose metabolism; however, evidence is extremely limited, and further studies are needed to draw a conclusion on this issue. The mechanisms by which these statins affect glucose metabolism also need to be studied further. The effect of statins on glucose metabolism, if any, seems particularly important in Japan. Japanese are more prone to developing diabetes mellitus than Caucasians[37], and coronary risk is lower in Japan as compared with Western countries. A decreased risk of coronary artery disease conferred by statins well surpasses any adverse effect of intensive statin therapy in Western countries; however, it is uncertain whether such intensive statin therapy is also applicable in Japan.

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Gender Difference in Coronary Events in Relation to Risk Factors in Japanese Hypercholesterolemic Patients Treated With Low-Dose Simvastatin

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Background Gender differences between the risk factors for coronary heart disease and coronary events were examined in the Japan Lipid Intervention Trial, a 6-year observational study.

Methods and Results Men (12,575) and women (27,013) were analyzed for risk of coronary events (acute myocardial infarction and sudden cardiac death). Simvastatin reduced serum low-density lipoprotein cholesterol (LDL-C) by 27% in both genders, and increased serum high-density lipoprotein cholesterol (HDL-C) in men (5%) and women (4%). The incidence of coronary events was lower in women (0.64/1,000 patient-years) than in men (1.57/1,000 patient-years). The risk of coronary events increased by 18% in men and 21% in women with each 10 mg/dl elevation of LDL-C, and decreased by 39% in men and 33% in women with each 10 mg/dl elevation of HDL-C. The risk increased proportionally with aging in women, but not in men. Diabetes mellitus (DM) was more strongly related to the risk of coronary events for women (relative risk 3.07) than for men (relative risk 1.58).

Conclusions The incidence of coronary events is lower in women. Serum LDL-C is related to an increased risk of coronary events to the same extent in both genders. DM seems to be a more important risk factor in women, trading off the lower risk of coronary events among them. (*Circ J* 2006; **70:** 810–814)

Key Words: Coronary events; Hyperlipidemia; Risk factors; Serum cholesterol; Sex differences

Coronary heart disease (CHD), including myocardial infarction and cardiac sudden death, is one of the leading causes of death in Japan[1] The risk of developing CHD is known to be markedly different between men and women:[2,3] CHD incidence is 2 to 5 times higher among middle-aged men than women. In the Japan Lipid Intervention Trial (J-LIT),[4–7] we previously reported that serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations were positively and serum high-density lipoprotein cholesterol (HDL-C) concentration was inversely related to CHD or cerebrovascular disease risk in patients under treatment for hypercholesterolemia. The role of coronary risk factors in the development of CHD has been studied extensively in men,[8–10] but relatively few studies have investigated women.[2,11]

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This study aimed to assess gender differences in the association of risk factors with CHD in the J-LIT data. The J-LIT is a nationwide cohort study of 52,421 hypercholesterolemic patients treated with open-labeled low-dose simvastatin (5–10 mg/day).[4,5] The J-LIT included a large number of female patients, and we were able to investigate the gender difference in the role of risk factors in the occurrence of coronary events.

Methods

Study Design

The design of the J-LIT study has been previously described.[12] Briefly, study patients with serum TC concentration ≥220 mg/dl, men aged 35–70 years and postmenopausal women aged 70 years or less, were treated with 5–10 mg/day of simvastatin. Body weight, serum lipid concentrations (TC, LDL-C, HDL-C, and triglyceride (TG)) were measured at baseline, and patients were interviewed as regards family history of CHD, number of cigarettes smoked, and the amount of alcohol ingestion. Serum lipid concentrations and CHD-related events (acute myocardial infarction and cardiac sudden death) were monitored every 6 months for 6 years in all patients, including those who discontinued simvastatin. Serum lipid concentrations were determined in each study institution, and the serum LDL-C concentration was calculated using the Friedewald formula for patients with TG concentration ≤400 mg/dl.[13] Study physicians recommended dietary and exercise-therapy for hyperlipidemia to all patients. Additional lipid-lowering

agents were allowed only when an adequate response in serum TC concentration was not gained by simvastatin monotherapy. Each patient was informed of the purpose and method of the study, drug efficacy and the need for long-term treatment and they gave verbal, not written, informed consent.

Subjects

Patients who had been previously treated with a lipid-lowering agent were screened for eligibility after a washout period of at least 4 weeks. For patients previously treated with probucol, the washout period was at least 12 weeks. The exclusion criteria were the occurrence of acute myocardial infarction or stroke within the past month, concurrent uncontrolled diabetes mellitus (DM), serious hepatic or renal disease, secondary hypercholesterolemia, cancer or any other illness with potentially poor survival.

Of the 52,421 patients enrolled, 5,127 were excluded because of a history of CHD, 4,934 for lack of follow-up data, and 2,772 for missing data of the covariates. Therefore, data from 39,588 patients (12,575 men, 27,013 women) were used in the present study.

Endpoints

The primary endpoints were major coronary events, defined as nonfatal and fatal myocardial infarction and sudden cardiac death. Incidence of myocardial infarction or death was counted once for each patient during the treatment, and the follow-up data thereafter were excluded from the analysis. The events were reviewed and determined by the Endpoint Classification Committee.

Statistical Analysis

The mean lipid concentrations were calculated using data available at the follow-up points in time during the treatment period. The data of lipid concentrations after the onset of events were excluded. Data during the treatment period after discontinuation of simvastatin were also included for analysis. Mean values for serum lipid concentrations and age were tested with unpaired t-test, and the prevalence of baseline characteristics were tested with the chi-square test for comparison between men and women. Patients in each sex were categorized into 5–6 groups according to the mean lipid concentrations of treatment period for TC, TG, LDL-C and HDL-C with intervals of 20, 50, 20, 10 mg/dl, respectively, and for the LDL-C/HDL-C ratio with an interval of 0.5. The reference category for the relative risk was set on the group with the lowest lipid concentrations and the lowest value of LDL-C/HDL-C ratio. Relative risks and the 95% confidence intervals (CI) were calculated using the Cox proportional hazards model with adjustment for baseline characteristics such as sex, age, hypertension, DM, body mass index (BMI), ECG abnormality, family history of CHD, alcohol ingestion and cigarette smoking. Heterogeneity between men and women was evaluated by the likelihood ratio test. Two-sided p-value <0.05 was considered statistically significant. All the statistical calculations were performed using SAS software (version 8.02, SAS Institute, Inc, Cary, NC, USA).

Results

Serum Lipids and Other Risk Factors

There were no significant difference as regards the prevalence of obesity (BMI ≥25.0 kg/m^2), hypertension, ECG abnormality, and family history of CHD between men and women (Table 1). In men, the prevalence of DM was higher ($p<0.001$), and cigarette smoking and alcohol ingestion were much more frequent ($p<0.001$).

Table 1 Baseline Characteristics of the Subjects

	Men (n=12,575)	*Women (n=27,013)*
Age (years)	*54.0 (9.1)*	*59.5 (6.5)*
Obesity (%)[a)]	*36.7*	*32.2*
Hypertension (%)[b)]	*45.4*	*46.3*
Diabetes mellitus (%)[c)]	*20.0*	*13.9*
ECG abnormality (%)[d)]	*13.4*	*12.9*
Family history of CHD (%)[e)]	*5.1*	*4.8*
Cigarette smoking (%)[e)]	*43.8*	*4.1*
Alcohol use (%)[e)]	*73.4*	*8.7*
Lipid profiles		
Baseline (mg/dl)		
TC	*268 (41)*	*271 (31)*
LDL-C	*178 (34)*	*184 (33)*
TG	*250 (241)*	*169 (111)*
HDL-C	*49 (15)*	*55 (15)*
During the treatment (mg/dl)		
TC	*218 (31)*	*221 (29)*
LDL-C	*130 (31)*	*135 (28)*
TG	*198 (133)*	*148 (77)*
HDL-C	*51 (13)*	*57 (14)*

Figs are mean±SD unless otherwise specified.
CHD, coronary heart disease; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.
a) Body mass index ≥25 kg/m^2. b) Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. c) Fasting plasma glucose ≥140 mg/dl or medication. d) Study physician's diagnosis. e) Self-reported information.

Lipid profiles at baseline and during the treatment period are shown for men and women in Table 1. Men had higher concentrations of serum TG and lower concentrations of serum HDL-C at baseline and during the treatment in comparison with women. Mean percent changes in the TC, LDL-C, TG, and HDL-C concentrations from baseline to during the treatment in men were –18.8% ($p<0.001$), –27.2% ($p<0.001$), –20.9% ($p<0.001$), and +4.7% ($p<0.001$), respectively, and the corresponding values in women were –18.2% ($p<0.001$), –26.6% ($p<0.001$), –12.8% ($p<0.001$) and +4.4% ($p<0.001$), respectively.

Incidence of Coronary Events

The incidence of coronary events was greater (105/12,575) in men than in women (93/27,013) during the treatment period. Incidence rates of coronary events per 1,000 patient-years were 1.57 in men and 0.64 in women. The age-adjusted relative risk of coronary events for men vs women was 2.81 (95% CI 2.10–3.76, $p<0.001$).

Serum Lipid Concentrations During the Treatment Period and Risk of Coronary Events

The risk of coronary events in relation to serum lipid concentrations is shown in Table 2. Increased risk for coronary events was evident at TC ≥240 mg/dl and LDL-C ≥160 mg/dl in both men and women. An increased risk of CHD associated with elevated concentration of TG (≥250 mg/dl) was noted in women but not in men. In men, the relationship between TG and CHD risk was not measurable. A lower risk of coronary events associated with elevation in HDL-C was seen in both sexes, but the protec-

Table 2 Relative Risk of Coronary Events According to Serum Lipid Concentrations During Treatment[a)]

	Men					Women				
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*
TC (mg/dl)										
<200	*3,442*	*24*	*1.00*	*(Referent)*		*5,833*	*22*	*1.00*	*(Referent)*	
200–219	*3,643*	*23*	*0.99*	*(0.56–1.77)*	*0.984*	*8,194*	*14*	*0.52*	*(0.27–1.02)*	*0.057*
220–239	*3,029*	*25*	*1.46*	*(0.83–2.56)*	*0.192*	*7,070*	*18*	*0.88*	*(0.47–1.64)*	*0.687*
240–259	*1,431*	*15*	*2.01*	*(1.05–3.88)*	*0.036*	*3,668*	*22*	*2.19*	*(1.21–3.98)*	*0.010*
260–	*1,030*	*18*	*3.48*	*(1.86–6.52)*	*<0.001*	*2,248*	*17*	*2.82*	*(1.48–5.36)*	*0.002*
LDL-C (mg/dl)										
<120	*4,680*	*27*	*1.00*	*(Referent)*		*8,050*	*22*	*1.00*	*(Referent)*	
120–139	*3,542*	*23*	*1.24*	*(0.71–2.16)*	*0.456*	*8,418*	*17*	*0.83*	*(0.44–1.57)*	*0.566*
140–159	*2,406*	*21*	*1.84*	*(1.03–3.26)*	*0.038*	*6,185*	*19*	*1.42*	*(0.77–2.64)*	*0.263*
160–179	*1,057*	*12*	*2.60*	*(1.31–5.17)*	*0.006*	*2,673*	*17*	*3.29*	*(1.74–6.23)*	*<0.001*
180–	*648*	*17*	*6.58*	*(3.53–12.25)*	*<0.001*	*1,564*	*17*	*5.78*	*(3.03–11.00)*	*<0.001*
TG (mg/dl)										
<100	*1,521*	*11*	*1.00*	*(Referent)*		*6,337*	*18*	*1.00*	*(Referent)*	
100–149	*3,663*	*22*	*0.84*	*(0.41–1.74)*	*0.634*	*10,444*	*32*	*0.98*	*(0.55–1.76)*	*0.946*
150–199	*3,127*	*33*	*1.51*	*(0.76–3.02)*	*0.243*	*5,861*	*17*	*0.87*	*(0.44–1.71)*	*0.684*
200–249	*1,768*	*18*	*1.46*	*(0.68–3.15)*	*0.330*	*2,429*	*9*	*1.12*	*(0.50–2.53)*	*0.783*
250–	*2,494*	*21*	*1.24*	*(0.58–2.65)*	*0.572*	*1,921*	*17*	*2.62*	*(1.32–5.21)*	*0.006*
HDL-C (mg/dl)										
<40	*2,198*	*36*	*1.00*	*(Referent)*		*1,758*	*10*	*1.00*	*(Referent)*	
40–44	*2,133*	*23*	*0.64*	*(0.38–1.09)*	*0.099*	*2,794*	*17*	*1.12*	*(0.51–2.45)*	*0.776*
45–49	*2,207*	*17*	*0.44*	*(0.25–0.80)*	*0.006*	*4,101*	*24*	*1.09*	*(0.52–2.28)*	*0.819*
50–54	*1,956*	*13*	*0.39*	*(0.21–0.74)*	*0.004*	*4,440*	*13*	*0.57*	*(0.25–1.30)*	*0.179*
55–59	*1,402*	*8*	*0.33*	*(0.15–0.72)*	*0.005*	*4,053*	*13*	*0.66*	*(0.29–1.51)*	*0.324*
60–	*2,679*	*8*	*0.17*	*(0.08–0.36)*	*<0.001*	*9,867*	*16*	*0.33*	*(0.15–0.73)*	*0.006*
LDL-C/HDL-C										
<2.0	*2,851*	*11*	*1.00*	*(Referent)*		*7,426*	*11*	*1.00*	*(Referent)*	
2.0–2.4	*2,719*	*11*	*1.10*	*(0.48–2.55)*	*0.817*	*6,909*	*19*	*1.95*	*(0.92–4.10)*	*0.080*
2.5–2.9	*2,598*	*17*	*1.91*	*(0.89–4.10)*	*0.095*	*5,884*	*14*	*1.68*	*(0.76–3.72)*	*0.199*
3.0–3.4	*1,889*	*20*	*3.21*	*(1.53–6.74)*	*0.002*	*3,545*	*21*	*4.57*	*(2.19–9.54)*	*<0.001*
3.5–4.0	*1,082*	*13*	*3.87*	*(1.72–8.72)*	*0.001*	*1,728*	*12*	*5.04*	*(2.21–11.49)*	*<0.001*
4.0–	*1,194*	*28*	*8.06*	*(3.95–16.44)*	*<0.001*	*1,398*	*15*	*8.56*	*(3.88–18.88)*	*<0.001*

RR, relative risk; CI, confidence interval. Other abbreviations see in Table 1.
a) Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

Table 3 Relative Risk of Coronary Events and Baseline Characteristics[a)]

	Men					Women					*Heterogeneity p value[b)]*
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*	
Age (years)											
<55	*6,281*	*49*	*1.00*	*(Referent)*		*6,137*	*8*	*1.00*	*(Referent)*		*0.008*
55–59	*2,182*	*14*	*0.74*	*(0.41–1.34)*	*0.320*	*6,488*	*15*	*1.82*	*(0.77–4.29)*	*0.174*	
60–64	*2,164*	*17*	*0.87*	*(0.50–1.53)*	*0.627*	*7,112*	*29*	*3.02*	*(1.38–6.62)*	*0.006*	
≥65	*1,948*	*25*	*1.42*	*(0.86–2.34)*	*0.168*	*7,276*	*41*	*4.11*	*(1.92–8.82)*	*<0.001*	
Obesity[c)]	*4,621*	*40*	*0.99*	*(0.66–1.48)*	*0.956*	*8,700*	*32*	*0.91*	*(0.59–1.40)*	*0.663*	*0.676*
Hypertension[d)]	*5,705*	*68*	*2.15*	*(1.42–3.26)*	*<0.001*	*12,511*	*62*	*2.05*	*(1.32–3.18)*	*0.001*	*0.864*
Diabetes mellitus[e)]	*2,513*	*29*	*1.58*	*(1.03–2.43)*	*0.037*	*3,747*	*31*	*3.07*	*(1.99–4.74)*	*<0.001*	*0.019*
ECG abnormality[f)]	*1,681*	*26*	*1.86*	*(1.18–2.91)*	*0.007*	*3,473*	*23*	*1.67*	*(1.04–2.70)*	*0.035*	*0.972*
Family history of CHD[g)]	*637*	*10*	*2.00*	*(1.04–3.84)*	*0.038*	*1,289*	*13*	*3.34*	*(1.85–6.04)*	*<0.001*	*0.317*
Cigarette smoking[g)]	*5,506*	*52*	*1.46*	*(0.98–2.17)*	*0.063*	*1,105*	*9*	*2.94*	*(1.43–6.02)*	*0.003*	*0.148*
Alcohol use[g)]	*9,224*	*70*	*0.63*	*(0.41–0.96)*	*0.031*	*2,337*	*6*	*0.61*	*(0.26–1.45)*	*0.266*	*0.933*

Abbreviations see in Tables 1,2.
a) Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use. b) Heterogeneity between men and women, based on the likelihood ratio test. c) Body mass index ≥25 kg/m². d) Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. e) Fasting plasma glucose ≥140 mg/dl or medication. f) Study physician's diagnosis. g) Self-reported information.

tive association was more evident in men. The relative risk for coronary events was substantially increased in patients with LDL-C/HDL-C ≥3.0 in both men and women.

The increase in the risk of coronary events for each 10 mg/dl elevation of LDL-C concentration during the treatment period was 18% (95% CI 12–24%) in men and 21% (95% CI 15–27%) in women, and the decrease in CHD risk associated with each 10 mg/dl elevation of HDL-C concentration was 39% in men and 33% in women. The relationships of coronary events with baseline LDL-C and HDL-C concentrations were also examined, but were much weaker than those observed during the treatment period. With each 10 mg/dl elevation of LDL-C concentration at baseline, the increase in the relative risk was 7% for men

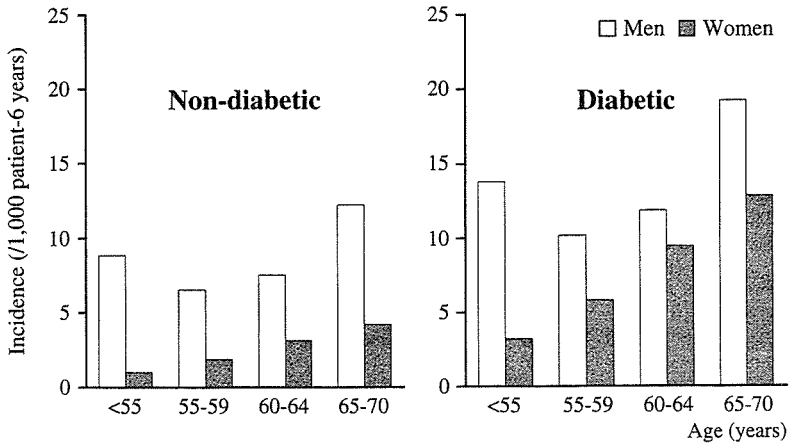

Fig 1. Estimated rates of coronary events according to age in men and women with and without diabetes mellitus (DM). Incidence rates were calculated from coronary heart disease (CHD) relative risks and the proportion of patients in each age category, for men and women separately, using Cox proportional hazards model, in which adjustment was made for age, hypertension, DM, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

and 9% for women and the decrease in risk with each 10 mg/dl elevation of HDL-C at baseline was 20% in both men and women.

Patient Baseline Characteristics and Risk of Coronary Events

The effect of age on the risk of coronary events was seen in women, but not in men (Table 3). Hypertension, DM, ECG abnormalities and a family history of CHD were also risk factors for coronary events in both men and women, but increased risks associated with DM and a family history of CHD were more marked for women than for men; the relative risk with DM was 1.58 in men and 3.07 in women, and the corresponding values for a family history of CHD were 2.00 in men and 3.34 in women. Obesity was unrelated to coronary events in either men or women. Although alcohol ingestion was protective in both men and women to the same extent, cigarette smoking was more strongly related to an increased risk of coronary events in women.

Discussion

This report addresses the gender differences in the relationship of serum lipid concentrations and other risk factors to CHD risk in Japanese patients under long-term treatment for hypercholesterolemia. Although serum TC and LDL-C concentrations were very similarly related to CHD risk in men and women, there was a difference between men and women in the relationship to serum TG and HDL-C concentrations. An inverse relationship of HDL-C to CHD risk was seen in men and women, but the HDL-C concentration showing a decreased risk of CHD differed by sex. The risk was significantly decreased at HDL-C ≥45 mg/dl in men and at HDL-C ≥60 mg/dl in women. The findings agree with observations published in the United States and Europe[2,3] and further indicate that the criterion of "low HDL-C" must be differential for men and women. An increased risk was observed only in women with an extremely high concentration of TG (≥250 mg/dl). Interpretation of this finding is difficult, and we do not have a clear idea about the implication of the present finding on serum TG.

In the present study, men did not show a clear increase in the risk of coronary events with increasing age, whereas there was a progressive increase in the risk with advancing age in women. The latter finding could be a reflection of the increase in serum TC and LDL-C concentrations with increasing age after menopause. The lack of an increasing trend in the association between age and coronary events in men is an unexpected finding, and may have been due to unknown characteristics of the male participants in the present study.

Whereas DM was related to increased CHD risk in both men and women, the increased risk was much greater in women, as indicated by a statistically significant interaction (p=0.019). These results did not change when further adjusted for TC or LDL-C. However, the risk difference between men and women for DM was not unique to the J-LIT patients. In a meta-analysis of 10 prospective studies, Lee et al showed that the effect of DM on the CHD risk was greater in women than in men[14] They showed that the relative risk of coronary death for DM patients vs non-DM patients was 2.58 (95% CI 2.05–3.26) in women and 1.85 (95% CI 1.47–2.33) in men (interaction p=0.045)[14] It was further noted in a later study that DM diminished the female advantage for lower CHD incidence[15] That DM is a stronger CHD risk factor in women may be related to the lower concentrations of HDL-C. Walden suggested that lower HDL-C concentrations in diabetic women as compared with men might be relevant to a stronger association between DM and CHD in women[16] In the present study, mean HDL-C concentrations in female diabetic patients were lower than those of non-diabetic patients (55.5 vs 57.5 mg/dl, p<0.001), but there was no difference in the HDL-C concentrations between the 2 groups in men (50.8 vs 51.3 mg/dl, p=0.09). The relative risk for DM was unchanged with adjustment for HDL-C. When the predicted rates of CHD incidence according to age were examined in men and women with and without DM (Fig 1), the increase in CHD incidence with aging was augmented in the presence of DM. Notably, DM diminished the women's advantage of having a lower CHD incidence in older patients.

Both cigarette smoking and family history of CHD were related to a greater increase in the risk of coronary events in women than in men. These differential increases in men and women may have been caused by random variation, as indicated by the lack of statistical significance for the interaction. As regards the effect of cigarette smoking, some studies suggest that smoking is a stronger risk factor in women than in men[2,17] but others have failed to find such a finding[18]

Finally, the present study results indicated that hypertension was an important risk factor in men and women equally, and that alcohol ingestion was protective in both sexes. These findings are in agreement with observations reported elsewhere[19–21]

In conclusion, the incidence of coronary events was 60% lower in women than in men among the J-LIT participants. Although the relationship of serum TC and LDL-C concentrations to coronary events was similar in men and women, the HDL-C concentration associated with a decreased risk of coronary events was slightly higher in women. DM was a stronger risk factor in women, and traded off the women's advantage of having a lower risk of coronary events, especially in aged patients.

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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

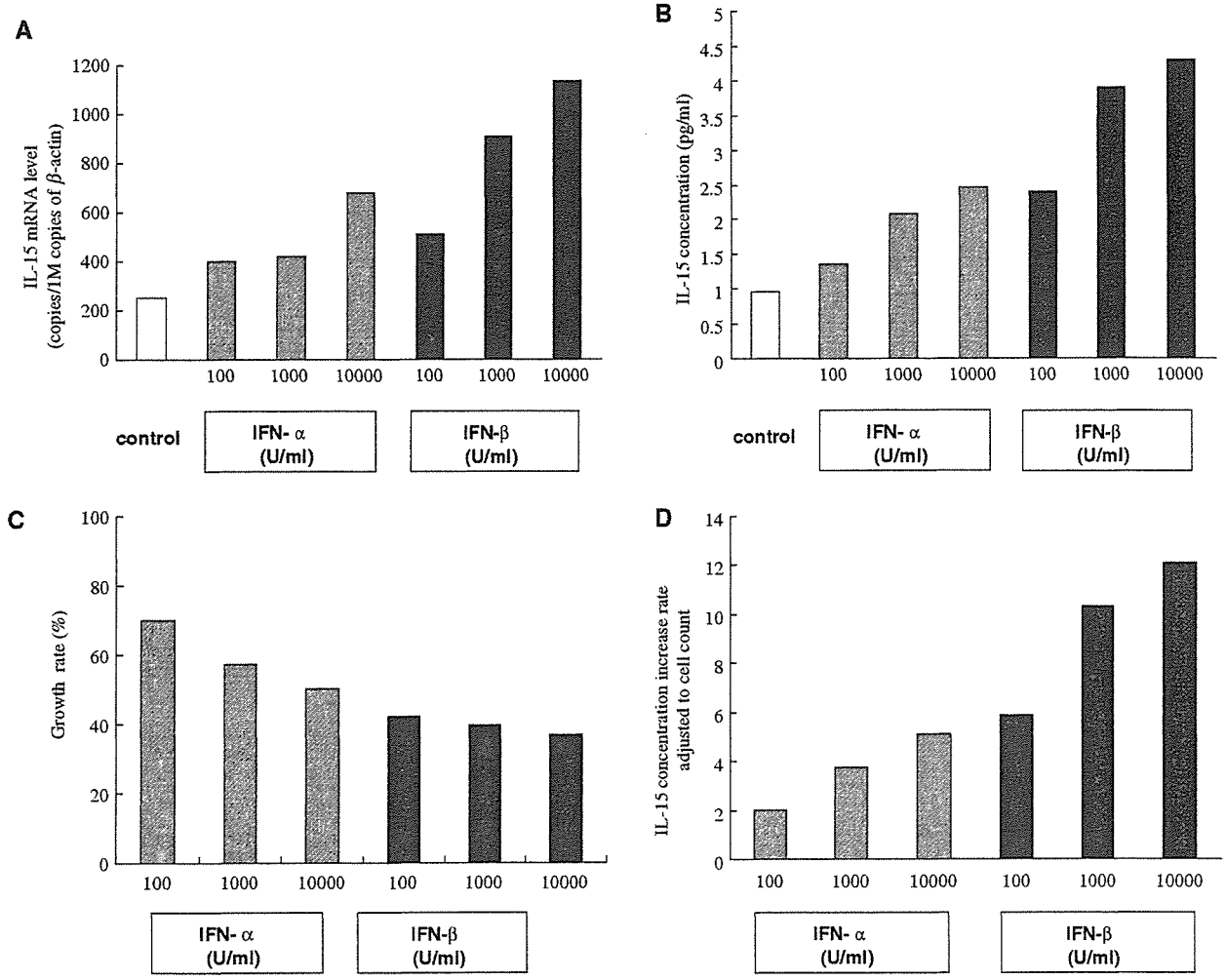

Fig. 1 The effects on IL-15 expression in Huh7 cells of IFN-α and -β at various concentrations. Huh7 cells were cultured for 72 h with or without IFN-α/β at consentrations of 100, 1,000, or 10,000 U/ml. **a** The IL-15 Expression level and the β-actin gene were quantified by reverse-transcription real-time 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

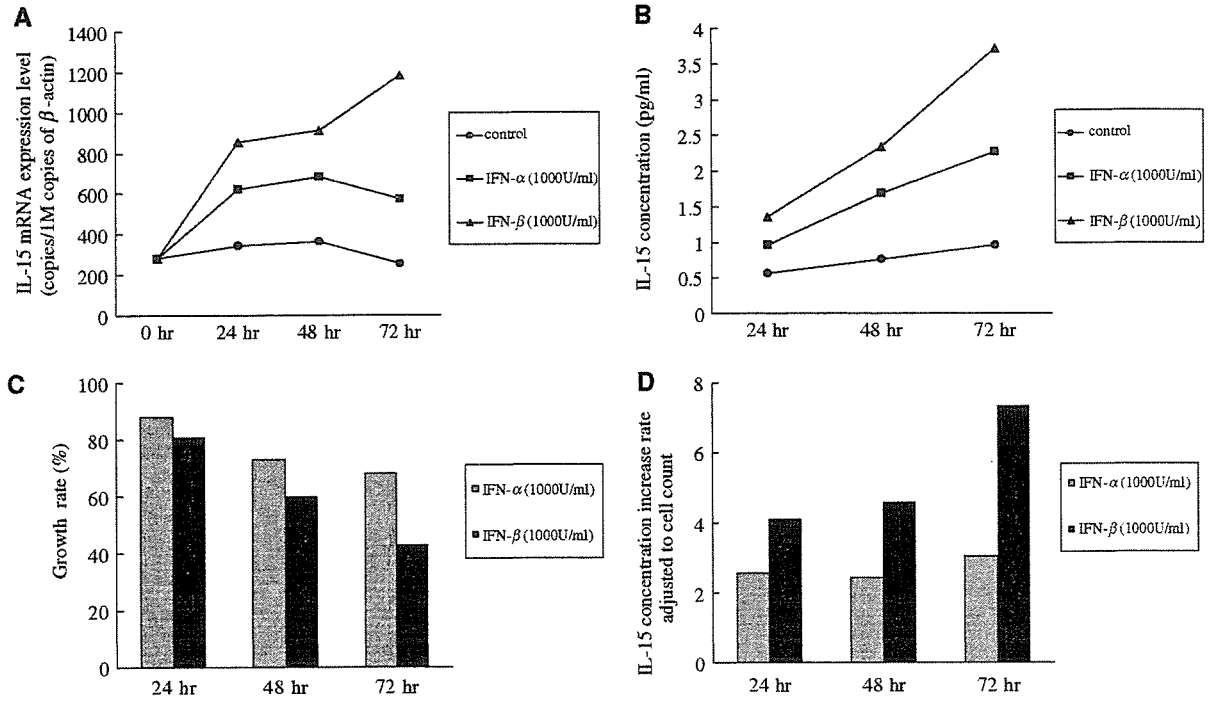

Fig. 2 The effects of IFN-α and -β on IL-15 expression in Huh7 cells at various time points. Huh7 cells were cultured with or without 1,000 U/ml IFN-α/β for 24, 48, or 72 h. **a** IL-15 and β-actin gene expression levels were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression 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

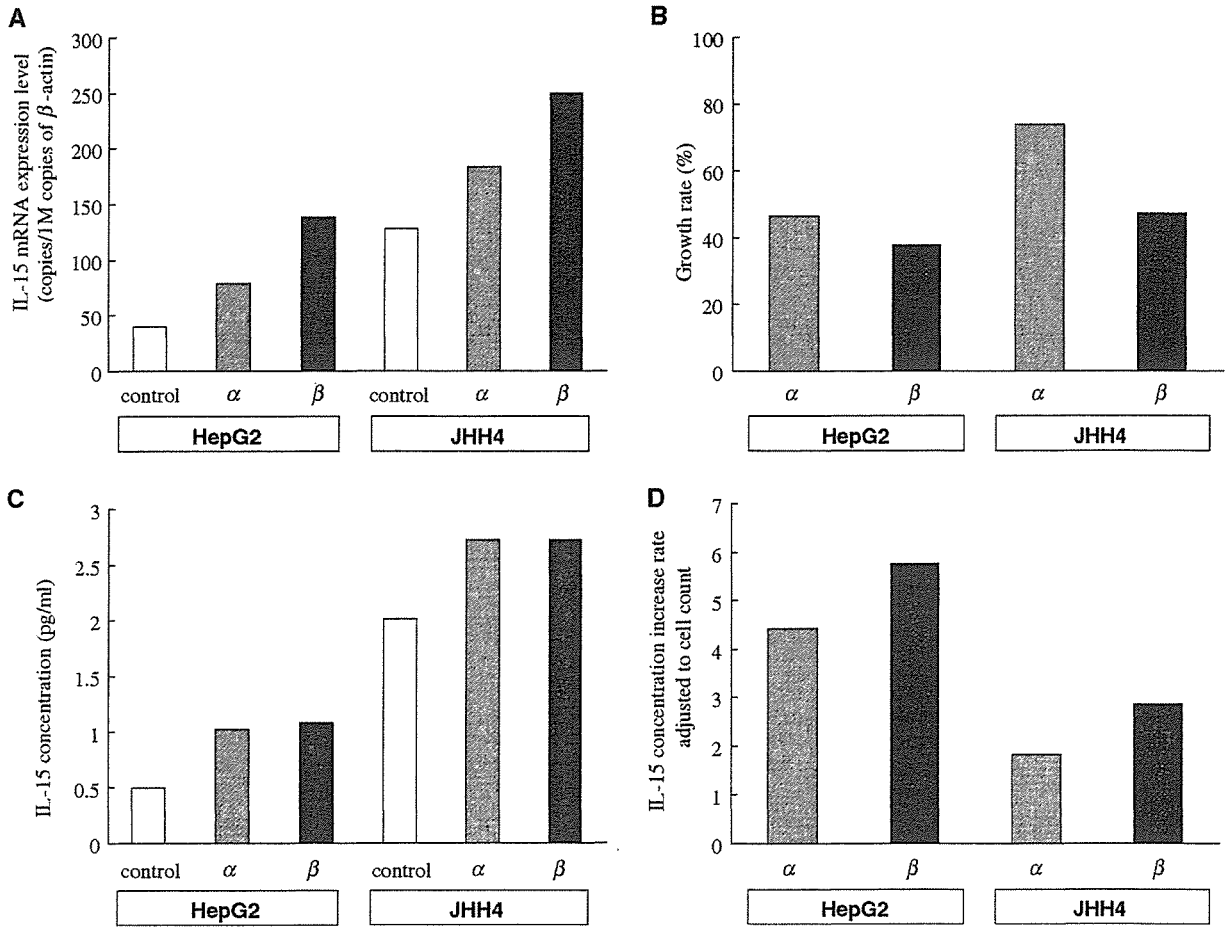

Fig. 3 The effects of IFN-α and -β on IL-15 expression in HepG2 and JHH4 cells. HepG2 cells and JHH4 cells were cultured with or without 1,000 U/ml IFN-α/β for 72 h. **a** Expression levels of IL-15 and β-actin gene were quantified by reverse-transcription real-time PCR. The IL-15 mRNA expression level 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

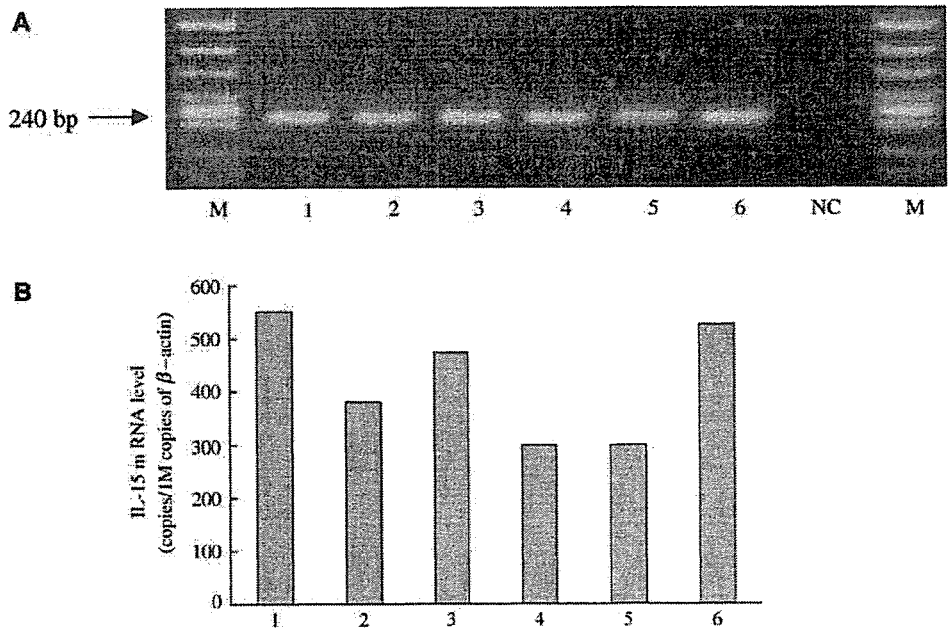

Fig. 4 Interleukin-15 mRNA expression in liver biopsy samples of chronic hepatitis C patients. **a** The IL-15 transcripts were amplified from the liver biopsy samples of six patients by reverse-transcription PCR. DEPC-treated water was used as the negative control. lanes 1–6; liver biopsy samples, lane 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,