149.2 mg/dL. The mean IMT was 1.308 mm. There were no statistically significant differences in any of these baseline characteristics among the three treatment groups (probucol, pravastatin, and diet alone).

Baseline characteristics (including lipids) for the two subgroups of interest (patients ≥75 years old and patients <75 years old) are shown in the Table 1. In general, the three treatment groups were well matched for age and sex at baseline. The elderly subgroup (≥75 years old) included a higher proportion of women, and more patients had cerebrovascular disease compared with the younger subgroup (<75 years old) ($p<0.01$ for probucol, $p<0.01$ for pravastatin, and $p<0.01$ for diet alone; chi-square test). The potential importance of chance differences in baseline characteristics between any of the four subpopulations receiving either of the two active treatments was evaluated by assessing the relationship of all listed baseline variables to total mortality or major coronary events.

Drug Treatment and Serum Lipids

The percent changes of serum lipids after 2 years of treatment are displayed in Fig. 1. Mean between-group differences (intention-

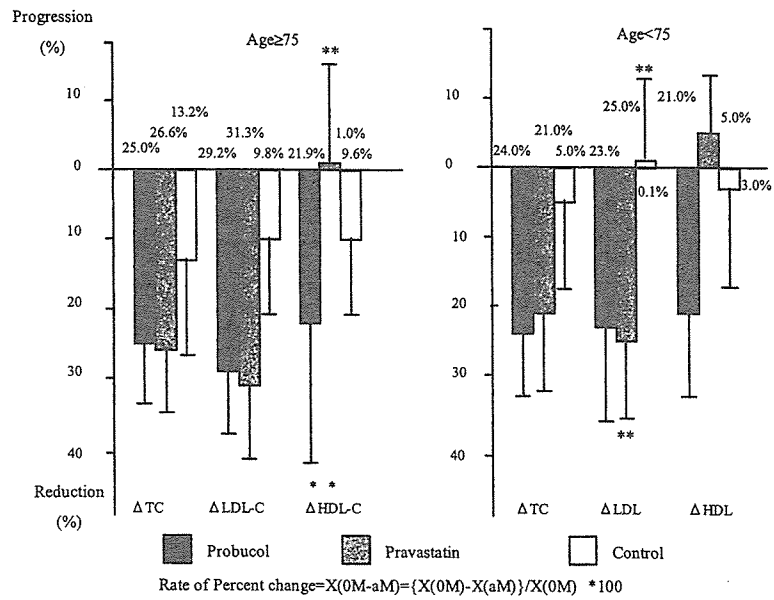

Fig. 1 Percent changes of serum lipids after 2 years. Among patients ≥75 years old, there was a significant decrease of serum total cholesterol in each of the three subgroups (by 25.0%, 26.6%, and 13.2% compared with baseline, respectively). There was also a significant decrease of serum LDL-cholesterol by 29.2%, 31.3%, and 9.8%, respectively, while the HDL-cholesterol levels of the probucol and control groups were significantly lower after 2 years. Patients <75 years old from the probucol and pravastatin groups showed a significant decrease of serum total and LDL-cholesterol levels (by 23.6% and 21.0% or 23.3% and 25.5% compared with baseline, respectively). In the probucol group, HDL-cholesterol was significantly reduced after 2 years (21.9%, $p<0.01$).

to-treat) of the percent change from baseline over the full duration of the trial are shown for total cholesterol, LDL cholesterol, and HDL cholesterol.

Patients ≥ 75 Years Old

After 2 years of treatment, there was a decrease of serum total cholesterol in each of the three groups, which showed a significant reduction of 25.0%, 26.6%, and 13.2% compared with baseline, respectively. After 2 years, there was also a significant decrease of serum LDL-cholesterol in the three groups, with the reduction being 29.2%, 31.3%, 9.8%, respectively. The serum HDL-cholesterol level of the pravastatin group was increased by 1.0% after 2 years, but this change was not significant. On the other hand, the HDL-cholesterol level showed a significant decrease in the probucol and control groups by 21.9% and 9.6%, respectively (Mann-Whitney U test). Triglyceride levels showed no significant changes throughout the study.

Patients < 75 Years Old

After 2 years of treatment, there was a significant decrease of serum total cholesterol and LDL-cholesterol levels in the probucol and pravastatin groups, with a reduction of 23.6% and 21.0% versus 23.3% and 25.5% compared with baseline, respectively. In the control group, total cholesterol and LDL-cholesterol levels were also lower at the end of the study, but the changes were not significant. The HDL-cholesterol level of the probucol group was significantly reduced after 2 years (21.9%, $p<0.01$). In the pravastatin group and the control group, however, HDL-cholesterol showed no significant changes throughout the study. Triglyceride levels also showed no significant changes throughout the study in any of the groups.

Intima-Media Thickness

The percent change of carotid IMT after 2 years is shown in Fig. 2. The decrease of IMT in patients ≥75 years old from the

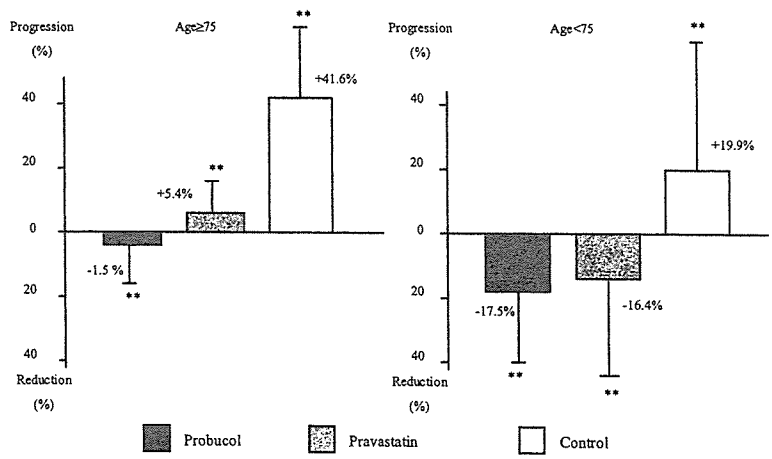

Fig. 2 Percent changes of carotid IMT after 2 years. Among patients ≥75 years old from the probucol and pravastatin groups, IMT showed a significantly greater decrease compared with that in patients <75 years old (both $p<0.01$). In the control group, IMT increased significantly by 19.9% ($p<0.05$). The change of IMT was significantly different in the treated groups compared with the control group (both $p<0.001$).

probucol and pravastatin groups was significant compared with that in patients <75 years old (both $p<0.01$; Mann-Whitney U test). In the control group, however, IMT showed a significant increase of 19.9% after 2 years ($p<0.05$; Mann-Whitney U test). The changes of IMT in the probucol and pravastatin groups were significantly different compared with that in the control group (both $p<0.001$; Mann-Whitney U test), while there was no significant difference in the change of IMT between the probucol and pravastatin groups at 24 weeks after the completion of treatment. There was no significant increase of IMT in the probucol group after 2 years of treatment.

Total and CHD Mortality

Among the 82 patients in the probucol group, two suffered a major cardiovascular event (2 deaths from coronary heart disease). Major events occurred in 4 of the 83 patients from the pravastatin group (3 deaths from coronary heart disease and 1 nonfatal myocardial infarction) and 11 of the 81 patients from the control group (8 deaths from coronary heart disease and 3 nonfatal myocardial infarctions). Of the 16 deaths that occurred during this study, two were in the probucol group, 5 were in the pravastatin group, and 9 were in the control group. Among these 16 patients, 13 deaths were from cardiovascular causes, while the others were due to gastrointestinal bleeding and infection. Total mortality and CHD mortality in the patients ≥75 years old are shown in Table 2.

Total cardiovascular events were significantly reduced in patients ≥75 years old from the probucol group compared with the control group (relative risk: 0.12; $p<0.05$). The reduction of relative risk was slightly greater than that observed for patients <75 years old (relative risk: 0.20; p=N.S.), but there were overlapping 95% confidence intervals. The relative risk of total death was similarly reduced by probucol in both age groups (86% reduction for patients ≥75 years old), and this decrease was statistically significant. Although the relative risk of total death was also reduced by pravastatin in both age groups (43% reduction for patients ≥75 years old), the change was not significant. The total cardiovascular event rate and total death rate over the duration of the study were more than three times higher in control group patients ≥75 years old (27.3% and 22.7%, respectively) compared with patients <75 years old (8.5% and 6.8%, respectively). Conse-

Table 2 Effect of probucol and pravastatin on clinical events in hypercholesteromoic patients

	Patients, n (%)													
	Probucol n=82		Pravastatin n=83		Control n=81		Probucol				Pravastatin			
							Hazards ratio	95%C.I.		p	Hazards ratio	95%C.I.		p
Age≥75	n=72		n=28		n=22									
All cardiovascular events	1	(3.7)	4	(14.3)	6	(27.3)	0.1247	0.0150	1.0358	0.0184	0.476	0.1343	1.6875	0.2439
Fatal MI	1	(3.7)	3	(10.7)	5	(22.7)	0.1509	0.0176	1.2923	0.0416	0.4317	0.1031	1.8072	0.2403
Non-fatal MI	0	(0.0)	1	(3.6)	1	(4.5)					0.6899	0.0431	11.0317	0.7936
PTCA/CABG	0	(0.0)	1	(3.6)	0	(0.0)								
All cerebrovascular events	0	(0.0)	0	(0.0)	0	(0.0)								
All other events	0	(0.0)	1	(3.6)	0	(0.0)								
All deaths	1	(3.7)	4	(14.3)	5	(22.7)	0.1498	0.0175	1.2828	0.0407	0.5713	0.1533	2.1287	0.4023
Age<75	n=55		n=55		n=59									
All cardiovascular events	1	(1.8)	0	(0.0)	5	(8.5)	0.2080	0.0243	1.7804	0.0968				
Fatal MI	1	(1.8)	0	(0.0)	3	(5.1)	0.3430	0.0357	3.2978	0.3197				
Non-fatal MI	0	(0.0)	0	(0.0)	2	(3.4)								
PTCA/CABG	0	(0.0)	0	(0.0)	0	(0.0)								
All cerebrovascular events	0	(0.0)	0	(0.0)	0	(0.0)								
All other events	0	(0.0)	1	(1.8)	1	(1.7)					1.3237	0.0823	21.2987	0.8434
All deaths	1	(1.8)	1	(1.8)	4	(6.8)	0.2557	0.0286	2.2879	0.1726	0.2990	0.0334	2.6794	0.2322

quently, the absolute risk reduction for patients ≥75 years old was more than three times that for patients <75 years old in the case of both total cardiovascular events and total deaths.

DISCUSSION

FAST was the first clinical trial to clearly demonstrate the benefit of probucol for elderly hypercholesterolemic patients and to also demonstrate an effect of probucol on the incidence of cardiovascular events. FAST showed that probucol therapy could achieve a significant reduction in the risk of major coronary events in patients ≥75 or < 75 years old, as well as significant improvement of all the tertiary CHD and atherosclerosis-related study end-points that were positive in the entire FAST cohort. The magnitude of the observed risk reduction in these subgroups was very similar to that reported for the entire study cohort and for other clinically relevant subgroups that have been analyzed. Although FAST was not specifically designed to assess changes of mortality in elderly subjects, high event rates combined with the substantial percentage of patients in this subgroup allowed us to detect a significant reduction of both all-cause mortality and CHD mortality. Safety and tolerability showed no important differences between the two age groups and were largely consistent with the findings for the entire study cohort[2].

In the subjects ≥75 versus <75 years old, LDL cholesterol showed similar changes (26% vs. 22%). This finding is consistent with other data suggesting that the cholesterol-lowering effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors is enhanced as patients become older[5]. Baseline total cholesterol, LDL-cholesterol, and HDL-cholesterol levels showed no significant relationship with the response to treatment (reduction in relative risk) in any of the subpopulations examined (data not shown), as was also the case for the entire study cohort[2]. The reduction of LDL cholesterol was more significant in the pravastatin group than in the probucol or control groups. Although the control group showed a significant reduction of LDL cholesterol with diet alone, an increase of carotid IMT still occurred, unlike the outcome in the active treatment groups. After 2 years of therapy, there was a significant decrease of serum LDL-cholesterol in all three groups compared with baseline. It was interesting that probucol had an antiatherogenic effect and caused a reduction of CHD events in patients ≥75 years old.

Lipid peroxidation of LDL has been demonstrated to be an important risk factor for the development of atherosclerosis[6][7]. There are several possible reasons, including the increased susceptibility of LDL to oxidation with aging[8], which can be partly explained by modification of its fatty acid composition and a decrease of the antioxidant (vitamin E) content[8]. Recently, Napoli et al. reported that resistance of LDL to peroxidative modification was lower in elderly men than in young men[9]. Furthermore, age was correlated with the extent of lipid peroxidation, supporting the hypothesis that LDL contributes to the increment of plasma lipid peroxides with aging[10][11]. Since oxidation of LDL is considered to be a key event in atherogenesis, it could be an additional reason why atherosclerosis is related to aging.

FAST showed that probucol therapy could delay the increase of IMT independently of its LDL or HDL cholesterol-lowering effect, and a reduction of IMT occurred

earlier with probucol than with pravastatin[2]. In the present study, patients ≥75 years old showed a significantly smaller change of IMT after probucol therapy compared with patients < 75 years old irrespective of the cholesterol-lowering effect. However, it was clearly demonstrated that probucol could reduce the risk of all-cause mortality and major coronary events in CHD patients ≥75 years old. The above findings suggest that there may be another mechanism involved in the effect of probucol. Other investigators have shown that suppression of atherogenesis by probucol is independent of its cholesterol-lowering action and is presumably due to an antioxidant effect on lipids[12)13)]. Because mortality and CHD events increase with age[14)], the absolute reduction of the death rate and event rate was substantially greater for patients ≥75 years old compared with those <75 years old. The relationship between serum cholesterol and the development of CHD has been observed in various epidemiological studies, but is reported to be weaker in elderly persons compared with middle-aged subjects[15)~19)], so the above findings may be unexpected. However, limited data are available about the predictive value of cholesterol in elderly patients with established CHD. Taken together with the results of the present study, the above findings may indicate the importance of ancillary effects of probucol other than cholesterol lowering for reducing the incidence of cardiovascular events. In fact, our data suggest that probucol may have multiple actions, but further studies are needed to investigate the relative contribution of each effect of this drug.

A difference between the effect of probucol and pravastatin on the IMT was not demonstrated by the present study, perhaps because the sample size was small. A large-scale investigation would be necessary to determine whether probucol and pravastatin therapy have a different influence on the IMT. Lack of a placebo control group was another limitation of our study. However, the use of quantitative B-mode ultrasound allowed us to obtain unbiased data. Although FAST was not specifically designed to assess the influence of lipid-lowering therapy on mortality in the elderly, high event rates combined with the substantial percentage of elderly patients in the study population provided the power to demonstrate a significant reduction of both all-cause mortality and CHD mortality among elderly patients receiving probucol. Safety and tolerability showed no important differences related to age or sex, and were generally consistent with the results for the entire study cohort[2)].

In conclusion, the present findings suggest that hypercholesterolemia in the elderly is a morbid state requiring treatment and that probucol is a useful drug for reducing the incidence of cardiovascular disease in hypercholesterolemic elderly persons.

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(和文抄録)

高齢者の高コレステロール血症に対するProbucolの効果

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【目的】高齢者（75歳以上）の高コレステロール血症患者に対して，積極的な脂質低下療法が頚動脈硬化の進展抑制および主要冠動脈イベントリスク低下が認められるか否かについて検討した．
【方法】FASTの対象患者（246例）のうち，75歳以上（76例）と75歳未満（168例）について，脂質低下療法（Probucol Pravastain）および食事療法により，その有効性について頚動脈エコーを用いて評価した．総頚動脈の内膜中膜複合体厚(IMT)を測定し，左右6点のIMTの平均値をIMT値とした．1次エンドポイントは2年間のIMT値の変化率とし，2次エンドポイントは主要冠動脈イベントとした．
【結果】Probucol群及びPravastain群では，年齢に関係なく，高齢者においても動脈硬化の進展抑制を認めた．Probucol群における高齢者のControl群に対する各臨床イベントの相対リスク（95%信頼期間）は総死亡が0.15（0.02-1.28），総冠動脈イベント0.12（0.02-1.04）と有意な進展を認めた．一方，Pravastain群との間では，各臨床イベントの相対リスクに有意差は認められなかった．Probucol群とPravastain群との間では，各臨床イベントの相対リスクに有意差は認められなかった．
【結論】75歳以上の高齢者に対してもProbucolは，頚動脈硬化の進展抑制効果が認められ，さらに主要冠動脈イベントの相対リスクの低下作用を認められる可能性が示唆された．

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A comparison of the antitumor effects of interferon-α and β on human hepatocellular carcinoma cell lines

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Abstract

The antiviral, antiproliferative and immunomodulatory effects of type I interferons (IFNs) are well documented, however, few studies have been published concerning differences in the antitumor effects of IFN-α and β. In the present study, differences in antitumor effect, including the antiproliferative effect, cell cycle change, apoptosis, and the IFN-stimulated gene (ISG) were examined by flow cytometry between IFN-α and β on three human hepatocellular carcinoma (HCC) cell lines (HepG2, Huh7 and JHH4). The antiproliferative effect of both IFNs on the HCC cell lines was time- and dose-dependent, and IFN-β was significantly stronger than IFN-α. The cell cycle effect by both IFNs was an S-phase accumulation, with IFN-β having a tendency to increase the S-phase ratio more strongly than IFN-α, especially in Huh7. Apoptosis marker expression, Fas antigen and intracellular active caspase-3, was increased after the addition of IFNs, especially of IFN-β. The expression of human leukocyte antigen-class I molecules, ISG-encoded protein, was increased after the addition of IFNs, especially of IFN-β. These data suggest that IFN-β has a greater antitumor effect than IFN-α on HCC of a very early stage in patients with chronic hepatitis C.

Keywords: Antitumor effects; Hepatocellular carcinoma; Interferon-α; Interferon-β

1. Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide. The hepatitis C (HCV), and hepatitis B viruses (HBV) have been directly linked to the development of HCC, especially in patients who have chronic active hepatitis with cirrhosis [1,2]. In Japan, the HCC incidence has increased, resulting in it becoming the third leading cause of death due to cancer.

Interferons (IFNs) are a family of cytokines that elicit a pleiotropic biological effect. IFNs have antiviral, antiproliferative and immunomodulatory effects, and are classified as type I (IFN-α, β and ω) and type II (IFN-γ) [3,4]. IFNs mediate their effects by binding to cell surface receptors (IFN receptors) and activating Janus kinases (JAK), resulting in the phosphorylation of the signal transducers and activators of transcription (STAT). STAT proteins homo- or heterodimerize and form complexes with other transcription factors to activate transcription of IFN-stimulated genes (ISGs) [3]. IFN actions are largely mediated by the proteins encoded by ISGs [5–7]. A number of IFN-related proteins, such as dsRNA-dependent protein kinase (PKR), the 2-5A system, human leukocyte antigen (HLA)-class I molecules and Mx proteins, mediate the antiviral actions of IFNs [3], and IFN-α and β are effective for the treatment of chronic hepatitis C [8,9].

In oncology, IFN-α and β are used for the treatment of a number of solid tumors and hematological malignancies,

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such as malignant melanoma, renal cell carcinoma, and chronic myelogeneous leukemia [4]. Recent reports showed that IFN-α treatment reduced the risk for HCC in patients with chronic hepatitis C [10,11]. We previously reported that IFN-β treatment also reduced the risk of HCC in such patients [12]. Moreover, this study revealed that a reduction in HCC occurrence was independent of virological or biochemical responses of IFN-β [12]. Although the antitumor effect of IFN-α on HCC cell lines has been reported in vitro [13,14], few studies have been published concerning differences in the antitumor effects of IFN-α and β on HCC cell lines [15,16], as was done in this study.

2. Materials and methods

2.1. Cell lines and reagents

The three human HCC cell lines, HepG2, Huh7 and JHH4, were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HepG2 was established by Aden et al. [17] from a liver tumor biopsy obtained from a 15-yr-old Caucasian male. The morphological characteristics and epithelial cell shape were compatible with that of liver parenchymal cells. Histology of the liver biopsy revealed well differentiated hepatocellular carcinoma with a trabecular pattern. Huh7 was established by Nakabayashi et al. [18] from a hepatoma tissue of a 57-yr-old Japanese male with well differentiated hepatocellular carcinoma. JHH4 was established by Homma [19] from a liver tumor biopsy obtained from a 51-yr-old Japanese male with hepatocellular carcinoma. These cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 25 mM HEPES, 50 units (U)/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (FCS) at 37 °C in a humidified incubator with 5% CO_2 in air.

Human natural lymphoblast IFN-α (Sumiferon), with a specific activity of 2.25×10^8 IU/mg, was kindly supplied by Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). Human natural fibroblast IFN-β (FERON), with a specific activity of 3.08×10^8 IU/mg, was kindly provided by Daiichi Pharmaceuticals Co., Ltd. (Tokyo, Japan). Since these natural IFN-α and β were of high purity, they did not contain other cytokines, that might have modulating effects such as tumor necrosis factor.

Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human Fas antigen monoclonal antibodies (mAbs) and HLA-class I molecule mAbs were purchased from Beckman Coulter (Miami, FL). Phycoerythrin (PE)-conjugated polyclonal rabbit anti-active caspase-3 antibodies were purchased from BD Biosciences (San Jose, CA). DMEN, FCS, trypsin/EDTA, and penicillin/streptomycin were purchased from Gibco BRL (Life Technologies, Inc., Gaithersburg, MD).

2.2. Antiproliferative effect of IFN-α and β

The antiproliferative effect of the IFNs was analyzed for three HCC cell lines, HepG2, Huh7 and JHH4. Cells (1×10^5/well) were added in triplicate to a 6-well culture plate (Becton Dickinson). The medium was replaced 24 h later by 1.5 mL of fresh medium containing IFN-α and β. Concentrations of IFN-α and β were 1×10^3 IU/mL and cell lines cultured in medium alone were used as a control. Proliferation of HCC cell lines was determined over a period of 96 h after IFN addition. After the culture, the adhering cells were washed with PBS and detached using 0.25% trypsin/EDTA. The resulting single-cell suspension was washed in washing buffer (PBS containing bovine serum albumin and sodium azide), and the number of viable cells was counted by flow cytometer, CYTORON ABSOLUTE with ImmunoCount 2 software (Ortho Diagnostic Systems). In some experiments, the concentrations of IFN-α and β ranged from 10^2 to 10^4 IU/mL. Cell viability was determined using the trypan blue dye exclusion method and exceeded 95% in all experiments. All assays were analyzed in at least three independent experiments.

2.3. Cell cycle

The effect of the IFNs on the cell cycle phase distribution of the HCC cell lines was analyzed by flow cytometry using the CycleTEST™ PLUS DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA) according to the manufacturer's instructions. Briefly, cells (1×10^5/well) were added in triplicate to a 6-well culture plate, and the medium was replaced 24 h later by 1.5 mL of fresh medium containing 10^3 IU/mL IFN-α or 10^3 IU/mL IFN-β. Cell lines cultured in medium alone were used as a control. The cultured cells were detached 24 h later using 0.25% trypsin/EDTA after washing with PBS. Cells were washed twice with PBS, 250 μL of Solution A (trypsin buffer) was added and the cells were incubated for 10 min at room temperature, followed by the addition of 200 μL of Solution B (trypsin inhibitor and RNase buffer) and incubation for a further period of 10 min at room temperature. Finally, 200 μL of cold Solution C (propidium iodide stain solution) was added and the cells were incubated on ice for 10 min in the dark. The samples were filtered through a 44-μm nylon mesh, and analyzed by flow cytometer, EPICS XL with EXPO32 software (Beckman Coulter).

2.4. Apoptosis-related markers

The expression of surface Fas antigen on the HCC cell lines was analyzed by flow cytometry. Cells (1×10^5/well) were cultured with medium alone as a control, 10^3 IU/mL IFN-α, or 10^3 IU/mL IFN-β. Twenty-four hours after the addition of IFNs, the cells were washed with PBS and detached using 0.25% trypsin/EDTA. Washed cells were incubated at 4 °C for 30 min in 10 μL of FITC-conjugated mouse anti-human Fas antigen mAbs. The samples were then washed with the washing buffer and analyzed by flow cytometer, EPICS XL with EXPO32 software.

Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which are derived from the 32 kDa proenzyme. Caspase-3 is a key

protease that is activated during the early stages of apoptosis [20]. In this study, intracellular active caspase-3 in the HCC cell lines was analyzed. Cells (1×10^5/well) were cultured with medium alone as a control, 10^3 IU/mL IFN-α, or 10^3 IU/mL IFN-β. The cells were washed with PBS 24 h after the addition of IFNs and detached using 0.25% trypsin/EDTA. Washed cells were fixed and permeabilized using Cytofix/Cytoperm kit (PharMingen) according to the manufacturer's instructions, and were incubated in the dark at 4 °C for 30 min in 10 μL of PE-conjugated anti-active caspase-3 antibodies. The samples were then washed with a washing buffer and analyzed by flow cytometer, EPICS XL with EXPO32 software.

2.5. Expression of HLA-class I molecules on HCC cell lines

Cells (1×10^5/well) were cultured with medium alone as a control, 10^3 IU/mL IFN-α, or 10^3 IU/mL IFN-β. The cells were washed with PBS 24 h after the addition of IFNs and detached using 0.25% trypsin/EDTA. Washed cells were incubated in the dark at 4 °C for 30 min in 10 μL of FITC-conjugated mouse anti-human HLA-class I molecule mAbs. The samples were then washed with the washing buffer and analyzed by flow cytometer, CYTORON ABSOLUTE with ImmunoCount 2 software.

2.6. Statistical analyses

Statistical analysis was by the Stat View J-5.0 program (SAS Institute Inc., Cary, NC). Statistical differences between the control and IFN treatment groups were calculated by unpaired student's *t*-test and considered significant at $P < 0.05$.

3. Results

3.1. Antiproliferative effect of IFN-α and β

As shown in Fig. 1, panels (a)–(c), the IFNs showed a significant time-dependent antiproliferative effect on HepG2 (control, IFN-α, IFN-β; $9.8 \times 10^5 \pm 0.5 \times 10^5$ cells, $8.4 \times 10^5 \pm 0.3 \times 10^5$ cells, $4.4 \times 10^5 \pm 0.2 \times 10^5$ cells, respectively), Huh7 (control, IFN-α, IFN-β; $8.8 \times 10^5 \pm 0.4 \times 10^5$ cells, $6.6 \times 10^5 \pm 0.7 \times 10^5$ cells, $3.3 \times 10^5 \pm 0.3 \times 10^5$ cells, respectively) and JHH4 (control, IFN-α, IFN-β; $17 \times 10^5 \pm 2.2 \times 10^5$ cells, $12 \times 10^5 \pm 0.4 \times 10^5$ cells, $9 \times 10^5 \pm 0.6 \times 10^5$ cells, respectively) compared with the control at 96 h after the addition of the IFNs ($P < 0.05$). Furthermore, IFN-β was significantly stronger than IFN-α in time-dependent antiproliferative effect, with the first significant effect observed at 48 h in both HepG2 (IFN-α; $3.9 \times 10^5 \pm 0.3 \times 10^5$ cells, IFN-β; $3.0 \times 10^5 \pm 0.3 \times 10^5$ cells, $P < 0.05$) and Huh7 (IFN-α; $3.1 \times 10^5 \pm 0.1 \times 10^5$ cells, IFN-β; $2.2 \times 10^5 \pm 0.2 \times 10^5$ cells, $P < 0.05$)

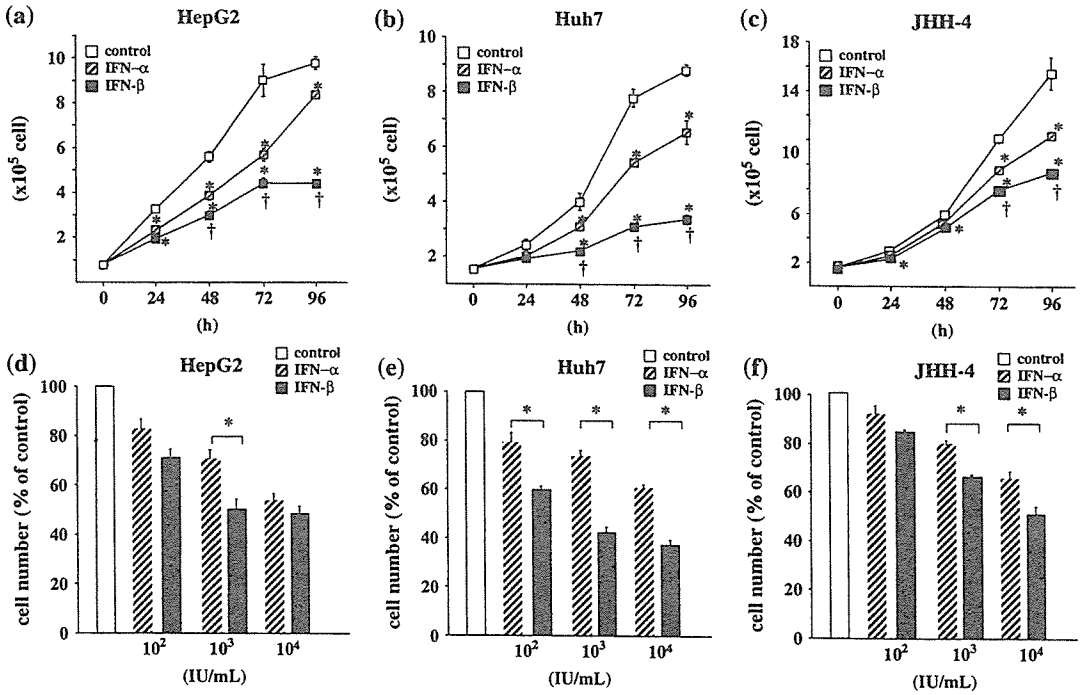

Fig. 1. Effect of IFN-α and β on the cell proliferation of HCC cell lines. Time course of HepG2 (a), Huh7 (b) and JHH4 (c) cell numbers. Dose effect of HepG2 (d), Huh7 (e) and JHH4 (f) cell numbers. Three HCC cell lines were cultured with medium alone as a control, 10^3 IU/mL IFN-α and 10^3 IU/mL IFN-β. Cell numbers were measured by flow cytometry after harvest up to 96 h as described in the "Section 2". Alternatively, three HCC cell lines were cultured with medium alone as, IFN-α (10^2, 10^3 and 10^4 IU/mL) and IFN-β (10^2, 10^3 and 10^4 IU/mL) for 72 h, and cell numbers were measured by flow cytometry after harvest. Values are the mean ± SD. Representative results from three independent experiments, each carried out in triplicate are shown. (a)–(c): * And † indicate statistically significant differences ($P < 0.05$) between the indicated experimental groups (* vs. control, † vs. IFN-α). (d)–(f): The proportion of viable cells cultured with medium alone as a control was considered 100%. * Indicates a statistically significant difference between the indicated experimental groups ($P < 0.05$).

and at 72 h in JHH4 (IFN-α; $9.6 \times 10^5 \pm 0.3 \times 10^5$ cells, IFN-β; $7.9 \times 10^5 \pm 0.1 \times 10^5$ cells, $P < 0.05$).

As shown in Fig. 1, panels (d)–(f), IFNs showed a dose-dependent antiproliferative effect on HepG2 (control, 10^2 U/mL IFN-α and β, 10^3 U/mL IFN-α and β, 10^4 U/mL IFN-α and β; 100%, 83 ± 6.5% and 71 ± 6.0% of control, 70 ± 6.5% and 50 ± 6.9% of control, 54 ± 4.7% and 48 ± 2.1% of control, respectively), Huh7 (control, 10^2 U/mL IFN-α and β, 10^3 U/mL IFN-α and β, 10^4 U/mL IFN-α and β; 100%, 79 ± 7.2% and 60 ± 2.1% of control, 74 ± 4.0% and 42 ± 3.5% of control, 61 ± 2.1% and 37 ± 3.2% of control, respectively) and JHH4 (control, 10^2 U/mL IFN-α and β, 10^3 U/mL IFN-α and β, 10^4 U/mL IFN-α and β; 100%, 91 ± 5.8% and 84 ± 1.7% of control, 79 ± 2.6% and 66 ± 1.5% of control, 65 ± 5.0% and 51 ± 5.9% of control, respectively) at 72 h after the addition of the IFNs. The antiproliferative effect of IFN-β was especially notable in Huh7, since the cell number in the culture with 10^2 IU/mL IFN-β was almost equal to 10^4 IU/mL of IFN-α.

3.2. Effect of IFN-α and β on the cell cycle distribution of HCC cell lines

We next analyzed the mechanism of the antiproliferative effect on HCC cell lines after the addition of IFNs. As shown in Fig. 2 and Table 1, at 24 h, the addition of IFNs significantly increased the S-phase ratio and slightly decreased the G_2/M phase ratio compared with the controls. Furthermore, the increase of the S-phase ratio induced by IFN-β was significantly stronger than that induced by IFN-α in three HCC cell lines. These results suggest that the difference in effect on the cell cycle distribution is a mechanism contributing to the IFN-related antiproliferative effect.

3.3. Effect of IFN-α and β on the expression pattern of apoptosis-related markers of HCC cell lines

Apoptosis is thought to be related to another mechanism of IFN-related antiproliferative effect [3]. To examine the effect of IFN-α and β on apoptosis in HCC cell lines, the expression of surface Fas antigen, a protein encoded by ISGs, and intracellular active caspase-3 were analyzed by flow cytometry. As shown in Table 2, IFNs increased the mean fluorescence intensity (MFI) of Fas antigen on the three HCC lines. IFN-β significantly increased the cell surface expression of Fas antigen on HepG2 and Huh7 in comparison with IFN-α. Furthermore, both IFNs increased the MFI of intracellular active caspase-3 in the three HCC cell lines, and all had a tendency to be more strongly induced by IFN-β than IFN-α. These results suggest that apoptosis is another mechanism contributing to the antiproliferative effect of IFN-β as well as IFN-α.

3.4. Effect of IFN-α and β on the expression pattern of HLA-class I molecules of HCC cell lines

Ligation of IFNs with IFN receptors results in the upregulation of ISGs [5]. We compared the capacity of IFN-α and β to induce HLA-class I molecules, a protein also encoded by ISGs. As shown in Table 2, the expression of HLA-class I molecules on the three HCC cell lines was significantly increased by both IFNs compared with controls. The increase

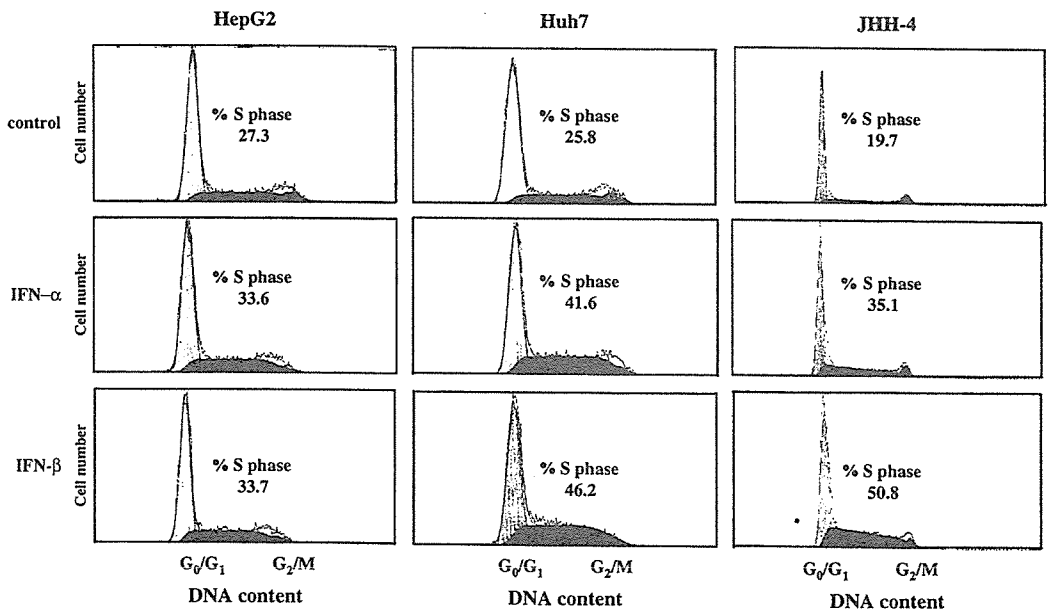

Fig. 2. Effect of IFN-α and β on the cell cycle distribution of HCC cell lines. HepG2, Huh7 and JHH4 were cultured with medium alone as a control, 10^3 IU/mL IFN-α and 10^3 IU/mL IFN-β for 24 h. The cell cycle phase distribution of both HCC cell lines were analyzed after harvest by flow cytometry using CycleTEST™ PLUS DNA Reagent Kit according to the manufacturer's instructions as described in the "Section 2". The x axis indicates DNA content, and the y axis indicates cell number. Results show representative data of three independent experiments.

Table 1
Effect of IFN-α and β on the cell cycle distribution of HCC cell lines

	HepG2			Huh7			JHH-4		
	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
Control	65 (64 ± 1.5)	29 (29 ± 0.8)	7.3 (7.5 ± 1.1)	66 (68 ± 1.8)	25 (25 ± 0.6)	8.5 (7.6 ± 1.9)	70 (72 ± 1.7)	22 (21 ± 0.8)	7.8 (7.0 ± 0.9)
IFN-α (10^3 IU/mL)	62 (61 ± 0.6)	33* (33 ± 0.9)	4.6 (6.1 ± 1.5)	53* (56 ± 2.3)	42* (41 ± 2.0)	2.5 (3.4 ± 0.9)	58* (58 ± 0.5)	35* (36 ± 0.5)	6.6 (6.5 ± 0.1)
IFN-β (10^3 IU/mL)	57* (56 ± 2.9)	34*,† (38 ± 4.3)	6.3 (5.9 ± 1.8)	53* (49 ± 4.4)	47*,† (51 ± 4.4)	0*,† (0)	46*,† (47 ± 0.6)	49*,† (49 ± 0.8)	4.5† (3.9 ± 1.4)

Variables were expressed as median (mean ± S.E.) in three independent experiments.
* Significant difference ($P < 0.05$) compared with control.
† Significant difference ($P < 0.05$) compared with IFN-α.

Table 2
Effect of IFN-α and β on the expression pattern of apoptosis-related markers and HLA-class I molecules of HCC cell lines

	HepG2			Huh7			JHH-4		
	Fas	Caspase-3	HLA-class I	Fas	Caspase-3	HLA-class I	Fas	Caspase-3	HLA-class I
Control	220 (216 ± 5.10).	170 (176 ± 4.00)	140 (137 ± 4.87)	170 (168 ± 6.29)	165 (163 ± 4.97)	110 (111 ± 3.60)	270 (270 ± 20.8)	130 (137 ± 6.67)	60 (58.8 ± 3.15)
IFN-α (10^3 IU/mL)	283* (283 ± 19.1)	210* (233 ± 24.6)	280* (255 ± 36.9)	195 (188 ± 9.47)	210* (268 ± 64.9)	564* (508 ± 120)	370 (355 ± 34.8)	190* (193 ± 8.82)	150* (147 ± 13.6)
IFN-β (10^3 IU/mL)	374*,† (367 ± 27.3)	280* (295 ± 24.6)	500* (445 ± 73.1)	225*,† (230 ± 12.2)	325* (373 ± 52.7)	1678* (1589 ± 512)	455* (438 ± 42.5)	190* (200 ± 10.0)	190* (185 ± 12.3)

Variables indicated mean fluorescence intensity, and were expressed as median (mean ± S.E.) in three independent experiments.
* Significant difference ($P < 0.05$) compared with control.
† Significant difference ($P < 0.05$) compared with IFN-α.

of the MFI of three HCC cell lines tended to be more strongly induced by IFN-β than IFN-α.

4. Discussion

There is accumulating evidence that IFN-β has a preferential antiproliferative effect on glioma, melanoma, and breast carcinoma cells, and that this effect is stronger than that by IFN-α [21–24]. The present study showed that IFN-β also had a superior antiproliferative effect on HCC cell lines than IFN-α. Type I IFNs exert their effects through the type I IFN receptor, which is composed of two major subunits, IFN-α receptor 1 (IFNAR-1) and 2c (IFNAR-2c) [3], which are potentially expressed in HCC cells [13]. IFN-α and β probably utilize a common receptor complex. Natural IFN-α (Sumiferon) was composed of approximately 20 subtypes, in which that contained α 2 subtype of 25%. IFN-α 2 is the subtype that is used as an antitumor and antiviral agent in the clinical setting, including chronic myelogenous leukemia, hairy cell leukemia, renal cell carcinoma and hepatitis C virus (HCV). Natural IFN-β (FERON) was composed of only one subtype, and has found clinical applications in several malignancies and viral diseases such as glioblastoma, melanoma, medulloblastoma and HCV. It was possible that the difference of component between natural IFN-α and β affected the antitumor effects on HCC cell lines in the present study. Previous reports showed that IFN-β had a greater antitumor effects on several cancer cell lines, such as melanoma cell, squamous cervical carcinoma cell, breast cancer cell compared with recombinant IFN-α 2 [21–25]. Because the α 2 subtype is the major subtype of which natural IFN-α is composed, the difference in the antitumor effects between both IFNs used may be involved with that of biological activity, rather than component, in the present study. Although it is still unknown why IFN-α and β have different biological effects, it is possible that IFN-α and β have different signaling events at the receptor level. IFN-β, but not IFN-α, formed a strong complex with IFNAR-1 and IFNAR-2c [26] and, alternatively, an IFN-β specific signaling domain within the cytoplasmic regions of the IFNAR chain was found in IFNAR-2c [27]. These reports suggest that the specific assembly of type I IFNAR leads to the differing biological responses to IFN-α and β. The present study showed that ISGs were more induced strongly by IFN-β than IFN-α since Fas antigen and HLA-class I molecules, proteins also encoded by ISGs, were more effectively upregulated by IFN-β. These results suggest that there are some differences in receptor interaction between IFN-α and β in HCC.

The present study showed that the antiproliferative effect of both IFNs on the HCC cell lines was time- and dose-dependent, and that IFN-β was significantly stronger than IFN-α. IFN-β showed a significantly stronger antiproliferative effect on Huh7 at any concentration examined than IFN-α, after 72 h of incubation, as shown in Fig. 1, panel (e). On other two cell lines, the antiproliferative effect at low (10^2 units/mL) as well as high (10^4 units/mL) concentrations had a tendency to be more strongly induced by IFN-β than IFN-α, although that was not statistically significant, as shown in Fig. 1, panels (d) and (f). It is possible that HCC cell lines differed in their sensitivity to IFNs, but we suppose that IFN-β has a stronger antiproliferative effect on HCC cell lines compared with IFN-α.

Type I IFNs are known to modify the cell cycle [3]. Although previous studies demonstrated that IFNs induced an inhibitory effect on G_1–S phase transition [28,29], it was recently demonstrated that the S phase of HCC cell lines was delayed by IFN-α [13,14]. We showed a greater increase in the S phase population of HCC cell lines treated with IFN-β than with IFN-α. Qin et al. [30] has reported that IFN-β preferentially induced S phase accumulation in human transformed cells by losing or inactivating the normal G_1 checkpoint conferred by the retinoblastoma protein, which acts as a cell cycle inhibitor. It is possible that IFN-β influences the normal G_1 checkpoint of HCC cell lines.

Induction of apoptosis is a highly attractive mechanism of the antitumor effect of IFNs. Apoptosis plays a critical role in the elimination of cells that sustain DNA damage or undergo uncontrolled cellular proliferation [7,31], and probably occurs as an independent cell cycle arrest [32]. The mechanism of apoptosis has been shown to occur through the ligation of death receptors on the cell surface, such as Fas or tumor necrosis factor-related apoptosis inducing ligand (TRAIL). This leads to the activation of an adaptor protein, Fas associated death domain (FADD) and to the subsequent activation of caspase-8. Activated caspase-8 cleaves additional downstream caspases, including caspase-3, a major effector caspase, and elicits the morphological hallmarks of apoptosis [7,32]. While IFN-α has been shown to induce apoptosis in HCC cell lines [13,14], the present study demonstrated that IFN-β does the same. Previous studies reported that IFN-β preferentially induced apoptosis in non-HCC cell lines, which was correlated with a stronger induction of TRAIL by IFN-β [25,33,34]. The difference in the induction of apoptosis by IFN-β seen in the present study may be related to the more effective induction of ISGs with an apoptotic function, such as Fas and TRAIL.

Tatsumi et al. [35] reported that IFN-α increased the expression of HLA-class I molecules on HCC cell lines. We also showed that HLA-class I molecules were more effectively upregulated by IFN-β. The immunomodulatory effects of type I IFNs occurred by enhancing the expression of HLA-class I molecules, activating $CD8^+$ cytotoxic T lymphocytes, natural killer cells and dendritic cells [3]. These data suggest a more effective antitumor immune response against HCC by IFN-β than by IFN-α.

It is still disputable if the prevention of HCC in patients with chronic hepatitis C treated with IFN-α and β is due to the direct antitumor effect on cancer cells. Several studies showed that the prevention of HCC would be associated with the virological or biochemical responses of IFNs [10,11]. Furthermore, our previous study [12] revealed that a reduction in the HCC development was independent of the biochemical response in natural IFN-β treated patients with chronic hepatitis C, but not in natural IFN-α treated patients, although similar rates of the HCC development were found

in patients with chronic HCV viremia treated with either IFN-α or β. Thus, IFN-β, rather than IFN-α, may directly inhibit HCC growth at a very early stage in patients with chronic hepatitis C, as suggested in the present study, although the results obtained from this study have been done by in vitro model.

In conclusion, IFN-β had a stronger antiproliferative effect than IFN-α by inducing cell cycle change and apoptosis, and upregulated HLA-class I molecules more strongly than IFN-α in three HCC cell lines, indicating that ISGs would be more strongly induced by IFN-β than by IFN-α. These data suggest that IFN-β has a greater antitumor effect than IFN-α in the early stage of HCC in patients with chronic hepatitis C.

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RAPID COMMUNICATION

Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: A project of the Kyushu University Liver Disease Study Group

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Abstract

AIM: To determine the efficacy of an interferon alpha and ribavirin combination treatment for Japanese patients infected with hepatitis C virus (HCV) of genotype 2, a multi-center study was retrospectively analyzed.

METHODS: In total, 173 patients with HCV genotype 2 started to receive interferon-alpha subcutaneously thrice a week and 600–800 mg of ribavirin daily for 24 wk.

RESULTS: The overall sustained virological response (SVR), defined as undetectable HCV RNA in serum, 24 wk after the end of treatment, was remarkably high by 84.4%, (146/173) by an intention-to-treat analysis. A significant difference in SVR was found between patients with and without the discontinuation of ribavirin (46.9% *vs* 92.9%), but no difference was found between those with and without a dose reduction of ribavirin. A significant difference in SVR was also found between patients with less than 16 wk and patients with 16 or more weeks of ribavirin treatment (34.8% *vs* 92.0%).

CONCLUSION: The 24-wk interferon and ribavirin treatment is highly effective for Japanese patients with HCV genotype 2. The significant predictor of SVR is continuation of the ribavirin treatment for up to 16 weeks.

Key words: Hepatitis C virus; Interferon; Ribavirin; Genotype 2

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INTRODUCTION

The heterogeneity of the hepatitis C virus (HCV) genome has warranted the classification of the virus into different genotypes, with six major genotypes and more than 50 subtypes of HCV having been described till date[1-3]. The different genotypes may be important to the pathogenesis of the disease[4], response to antiviral therapy[5], and the diagnosis[6], as shown by molecular epidemiological studies and research on vaccine development.

A currently popular treatment regimen for the treat-

ment of chronic HCV infection in the world is pegylated interferon (IFN) alpha in combination with ribavirin. However, there was no data of response to such combination treatment for Japanese patients, because the treatment was just approved by the Japanese Minister of Health, Labour and Welfare in December 2004. Treatment with these drugs has resulted in a high rate of sustained virological response (SVR), over 50%[7,8]; however, the treatment duration is long, 48 wk and it causes various side effects, which are sometimes serious. Such a combination treatment is also expensive; a 24-wk treatment course costs approximately $20 000[9]. The efficacy and economic aspects need to be analyzed. Quite recently, a very short duration treatment for acute hepatitis C was shown to be highly effective[10].

The HCV genotype has been reported to be the most important predictor of IFN treatment response[7-13]. Patients infected with genotypes 2 and 3 have achieved about 65% SVR in a trial of 24-wk IFN alpha in combination with ribavirin, in contrast to patients with genotype 1 who had under 30% SVR[14,15]. Recently, multicenter studies in Europe and North America showed that patients with genotypes 2 and 3 were able to achieve a high SVR in a trial of 14-16 wk of pegylated IFN alpha in combination with ribavirin[16,17]. However, their analysis included very few genotype 2 patients: one included 23 genotype 2 patients and the other had 43 patients.

The distribution of HCV genotypes in Japan includes about 70% genotype 1b, with the remaining 30% genotypes 2a and 2b[18]. The SVRs to treatment of even shorter duration have not yet been reported for Japanese patients. Data are needed to define whether or not the duration of treatment with IFN alpha in combination with ribavirin can be reduced from 24 wk without compromising antiviral efficacy in patients chronically infected with HCV of genotype 2. This investigation has assessed the efficacy of a 24-wk combination treatment of IFN alpha and ribavirin for Japanese patients with HCV genotype 2 infection and focussed on the issue of the relationship between the duration of treatment and the efficacy.

MATERIALS AND METHODS

Patients

A retrospective study was done on Japanese patients treated between December 2000 and March 2004 that included 173 patients, 20 years or older, who satisfied the following criteria: (1) chronically infected with HCV genotype 2a or 2b; and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Criteria for exclusion were: (1) clinical or biochemical evidence of hepatic decompensation; (2) hemoglobin level less than 115 g/L, white blood cell count less than 3×10^9/L, and platelet count less than 50×10^9/L; (3) concomitant liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive); (4) alcohol or drug abuse; (5) suspected hepatocellular carcinoma; (6) severe psychiatric disease; and (7) treatment with antiviral or immunosuppressive agents prior to enrolment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 32 affiliated hospitals in the northern Kyushu area of Japan.

Informed consent was obtained from all the patients before enrollment in this study. The study was approved by the institutional Ethics Committees of the hospitals involved and conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

Study design

All patients were treated with 6-10 MU of IFN alpha-2b (Intron A; Schering-Plough, Osaka, Japan) subcutaneously daily for the first 2 wk, then thrice a week for 22 wk. Ribavirin (Rebetol; Schering-Plough) was administrated orally for 24 wk at a daily dose of 600-800 mg based on the body weight (600 mg for patients weighing less than 60 kg and 800 mg for those weighing 60 kg or more). The above duration and dose were approved by the Japanese Minister of Health, Labour and Welfare. The 48-wk combination treatment and the ribavirin dosage of 1 000-1 200 mg recommended by the international guidelines were not permitted under the rules of the Japanese national health insurance system during the period of this study. The dose of ribavirin was reduced by 200 mg if the hemoglobin level fell to 100 g/L. Patients were considered to have ribavirin-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of ribavirin was required. Both IFN alpha-2b and ribavirin were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, and 2.5×10^9/L, respectively. The treatment was also discontinued if severe malaise developed, the continuation of treatment was judged not to be possible by the attending physician, or the patient desired to discontinue treatment.

Grouping by continuation or discontinuation of treatment

Patients were divided into the following four categories: Group A, patients who well tolerated the 24-wk combination treatment with IFN and ribavirin without a reduction in the dose of either drug; Group B, patients who received the full 24-wk combination treatment but who needed a reduction of the dose of IFN or ribavirin, or both; Group C, patients who discontinued the ribavirin treatment but continued the 24-wk IFN treatment; and Group D, patients who did not complete the 24 wk of treatment, because of adverse effects or who dropped out.

Determination of HCV RNA and HCV genotype and serotype

The serum HCV RNA level was examined with an Amplicor HCV monitor assay (version 2.0) (Roche, Tokyo, Japan), with a lower limit of quantitation of 500 IU (135 copies/mL) and an outer limit of quantitation of 850 000 IU/mL. Samples with HCV RNA over the limit of 850 000 IU/L were not diluted to determine the levels between 850 000-5 000 000 IU/mL. HCV RNA was also examined with the qualitative Amplicor HCV assay (Roche). HCV genotype was determined by type-specific primer from the core region of the HCV genome. The protocol for genotyping was carried out as described earlier[11,12].

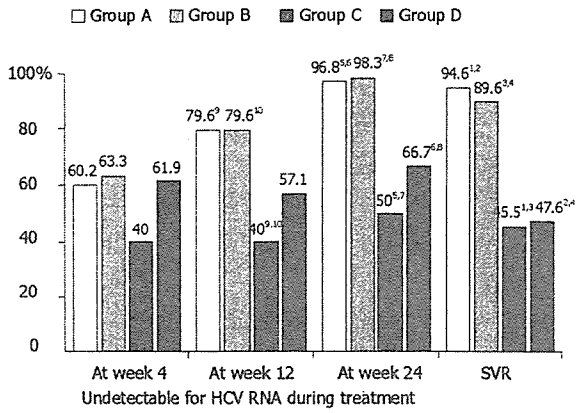

Figure 1 The sustained virological response (SVR) rate and undetectable hepatitis C virus (HCV) RNA rates during the treatment of 173 patients, classified by continuation and discontinuation of interferon and ribavirin combination treatment. Group A patients (n=93) who well tolerated the 24-week treatment with IFN and ribavirin in combination without any reduction in the dose of either drug; Group B patients (n=48) received the 24-week combination treatment, but needed a dose reduction of IFN or ribavirin, or both; Group C patients (n=11) discontinued the ribavirin treatment, but continued the full 24 weeks of IFN treatment; Group D patients (n=21) did not complete the 24 weeks of treatment because of adverse effects (n=17) or dropped out (n=4). [1]P=0.0001; [2]P<0.0001; [3]P=0.0031; [4]P=0.0003; [5]P=0.0001; [6]P=0.0002; [7]P=0.0003; [8]P=0.0002; [9]P=0.124; [10]P=0.0182.

Histological examination

Liver biopsy was done for 117 patients infected with genotype 2 within the 6 months before the start of the treatment. For each specimen, a stage of fibrosis and a grade of activity were established according to the following criteria. Fibrosis was staged on a scale of 0-4: F0=no fibrosis, F1=portal fibrosis without septa, F2=few septa, F3=numerous septa without cirrhosis, F4=cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0=no histological activity, A1=mild activity, A2=moderate activity, A3=severe activity. Liver biopsy was not available from 56 patients who declined to have a biopsy.

Efficacy of treatment

The SVR was defined as undetectable HCV RNA by the qualitative Amplicor HCV assay (Roche) and a normal ALT level (under 40 IU/L) at 6 months after the end or stoppage of the treatment. Patients not achieving a SVR were considered as non-SVR. Patients who had undetectable HCV RNA within 4 wk of the start of treatment were considered to have had an early virological response (EVR).

Statistical analysis

The analysis of SVR was done on an intention-to-treatment basis, including dropouts, who were counted as non-sustained virological responders, and patients who stopped treatment. The χ^2 test or Fisher's exact test was used to examine the association between baseline characteristics and SVR. The Mann-Whitney U test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. Independent factors associated with SVR were studied using forward stepwise logistic regression analysis of the variables. Forward stepwise logistic regression analysis was done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. A P-value of less than 0.05 was considered significant. All P-values were two tailed.

RESULTS

Patient characteristics, dose reduction and discontinuation of treatment regimen

The distribution of Groups A, B, C, and D patients was 93 (53.8%), 48 (27.7%), 11 (6.4%), and 21 (12.1%), respectively. Completing the 24-week ribavirin treatment were 141 patients in Groups A and B. Thirty-two patients of Groups C and D discontinued the ribavirin treatment.

The pretreatment characteristics of these four groups of patients are summarized in Table 1. The median age was significantly younger in Group A (51 years) than in Groups B (56 years) and C (59 years). Significantly more men were in Group A (71.0%) than in Group B (33.3%). The median creatinine clearance was significantly higher in Group A (110 mL/min) than Groups B (92 mL/min) and C (85 mL/min). The median hemoglobin level was significantly higher in Group A (150 g/L) than Groups B (136 g/L), C (134 g/L), and D (134 g/L). The median platelet count was significantly higher in Group A (168 $\times 10^9$/L) than in Group C (127$\times 10^9$/L). No notable differences between the groups were found in body weight, ribavirin dose, HCV RNA level, genotype, or histology.

Virological response

SVR was achieved by 146 (84.4%) of 173 patients. The SVR did not differ between patients with genotypes 2a and 2b (83.1% *vs* 84.6%). The SVRs were 82.4% (14 of 17) (under 100 kIU/mL), 84.2% (16 of 19) (100-199 kIU/mL), 85.7% (24 of 28) (200-299 kIU/mL), 83.3% (15 of 18) (300-399 kIU/mL), 100% (12 of 12) (400-499 kIU/mL), 76.9% (10 of 13) (500-599 kIU/mL), 77.8% (7 of 9) (600-699 kIU/mL), 90.9% (10 of 11) (700-799 kIU/mL), and 82.6% (38 of 46) (800 and over kIU/mL). The SVRs were 76.9-100%. The SVRs of the HCV genotype 2 patients with any level of viremia level did not significantly differ.

Figure 1 shows the SVR and undetectable HCV viremia rate during the treatment of 173 patients, classified by continuation and discontinuation of combination treatment. The SVRs were significantly higher in Groups A (94.6%) and B (89.6%) than in Groups C (45.5%) and D (47.6%). A significant difference of SVR was found between patients with and without discontinuation of ribavirin (46.9%, 15 of 32 of Groups C and D patients *vs* 92.9%, 131 of 141 of Groups A and B patients, P<0.0001). During the treatment period, except for at week 4, the rates of undetectable HCV RNA were also significantly higher in Groups A and B than in Groups C and D.

Figure 2 shows the relationship between SVR and the ribavirin treatment period in all the patients. A significant difference was found between patients with less than 16 wk of treatment period and patients with longer periods

Table 1A Baseline characteristics

Characteristic	Complete Ribavirin treatment (n = 141)		Discontinued Ribavirin treatment (n = 32)		
	Group A (n = 93)	Group B (n = 48)	Group C (n = 11)	Group D (n = 21)	All patients (n = 173)
Median age (yr)	51[1,2]	56[1]	59[2]	50	53
(range)	(20-73)	(25-70)	(53-73)	(29-73)	(20-73)
Male (%)	66 (71.0)[3]	16 (33.3)[3]	5(50.0)	13 (61.9)	100 (57.8)
Body weight					
60 kg or more (%)	60 (64.5)	23 (47.9)	6 (54.5)	12 (57.1)	101 (58.3)
Ribavirin dose by weight					
12 mg/kg or more (%)	24 (25.8)	20 (41.7)	4 (36.4)	9 (42.8)	57 (32.9)
Creatinine clearance (mL/min)	110[4,5]	92[4]	85[5]	101	102
(range)	(53-261)	(46-167)	(60-111)	(41-203)	(41-261)
HCV RNA level					
500 kIU/mL or more (%)	44 (47.3)	22 (45.8)	4 (36.4)	9 (42.8)	79 (45.7)
Genotype 2a (%)	67 (72.0)	28 (58.3)	6 (54.5)	13 (61.9)	114 (65.9)

[1]P=0.0401; [2]P=0.0044; [3]P<0.0001; [4]P=0.0002; [5]P=0.0248

Table 1B Baseline characteristics (continued)

Characteristic	Complete Ribavirin treatment (n = 141)		Discontinued Ribavirin treatment (n = 32)		
	Group A (n = 93)	Group B (n = 48)	Group C (n = 11)	Group D (n = 21)	All patients (n = 173)
Histology					
Stage of fibrosis					
F0 - F1 (%)	27 (43.5)	17 (50.0)	4 (50.0)	9 (42.9)	56 (47.9)
F2 - F3 (%)	35 (56.5)	15 (44.1)	4 (50.0)	6 (28.6)	59 (50.4)
F4 (%)	0	2 (5.9)	0	0	2 (1.7)
Not determined	31	14	3	6	54
Grade of activity					
A0 - A1 (%)	27 (43.5)	17 (50.0)	4 (50.0)	9 (42.9)	43 (47.9)
A2 (%)	35 (56.5)	15 (44.1)	4 (50.0)	6 (28.6)	58 (50.4)
A3 (%)	0	2 (5.9)	0	0	16 (1.7)
Not determined	31	14	3	6	54
Median hemoglobin (g/L)	150[6,7,8]	136[6]	134[7]	134[8]	144
(range)	(117-171)	(116-163)	(121-152)	(121-153)	(116-171)
Median platelet count ($\times 10^9$/L)	168[9]	167	127[9]	157	162
(range)	(79-385)	(58-363)	(55-181)	(57-240)	(55-385)

[6]P=0.0003; [7]P=0.0063; [8]P=0.0225; [9]P=0.0120

(34.8%, 8 of 23 *vs* 92.0%, 138 of 150, P<0.0001), showing that 16 wk of ribavirin treatment significantly contributed to a SVR. Of the 173 studied patients, 104 (60.1%) had an EVR, defined as undetectable HCV RNA within 4 wk of the start of treatment. The SVR was 94 (90.4%) of these 104 patients with EVR, which was significantly higher than the non-EVR patients (52 of 69, 75.4%) (P=0.0142). No significant differences were found between patients with and without undetectable HCV RNA at 8 or 12 wk of the start of treatment. Moreover, we analyzed the relationship between SVR and the length of ribavirin treatment in the 104 patients with EVR. A significant difference was found between patients with less than 16 wk of ribavirin treatment and those with a longer treatment period (46.2%, 6 of 13 *vs* 96.7%, 88 of 91, P<0.0001). These findings showed that 16 wk of ribavirin treatment significantly contributed to a SVR, even in patients with EVR.

Factors contributing to SVR

To assess the independent role of the IFN and ribavirin combination treatment on SVR, an adjustment by forward stepwise logistic regression analysis for all other independent risk factors identified was done. The continuation of ribavirin treatment (P<0.0001) was significantly associated with SVR in analysis of all the patients. A higher SVR (odds ratio = 13.15) was found for patients who continued to receive ribavirin treatment than for those who discontinued it. Other factors such as sex, age, HCV genotype, pretreatment-HCV RNA level, histological findings, pretreatment platelet count and creatinine clearance, history

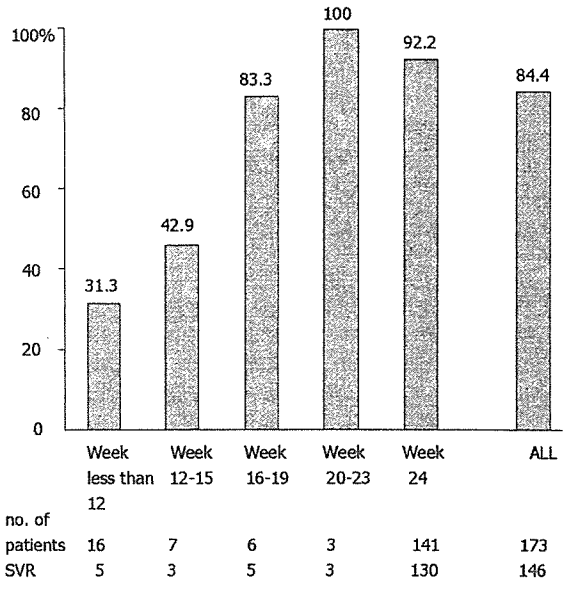

Figure 2 Relationship between the sustained virological response rates and the length of ribavirin treatment period of the 173 studied patients.

of prior IFN, and dose reduction of IFN or ribavirin were not significantly, independently associated with a SVR.

DISCUSSION

The large number of Japanese HCV genotype 2 patients enrolled in this study was sufficient to provide for meaningful statistical analysis, even though it was retrospective. This study demonstrated that a 24-wk IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR (84.4%) in genotype 2 patients, as expected. Importantly, we also showed that dose reductions of ribavirin were not associated with a poor outcome in these patients, only ribavirin discontinuation, and that the addition of ribavirin for up to 16 wk contributed to the high SVR.

In December 2004, pegylated IFN plus ribavirin combination treatment received the official approval in Japan. The combination treatment was not yet approved for clinical use for patients with chronic HCV viremia by the Japanese Ministry of Health, Labour and Welfare at the time of the present study. So far, our most effective and available treatment is the 6-month IFN-alpha plus ribavirin combination.

Remarkably high SVRs were observed for our patients with genotype 2 who took the IFN and ribavirin combination treatment. IFN monotherapy does not result in a satisfactory outcome for patients with chronic hepatitis C, particularly those with genotype 1, which is known to be IFN-resistant, whereas genotype 2 is IFN-sensitive[11-13]. The addition of ribavirin, a synthetic purine nucleoside analog, to IFN enhances the virological response[8,9,13-17]. Our research group, KULDS, also analyzed the data of patients with genotype 1 who were treated with this 24-wk combination treatment: SVR was achieved by 21% of 528 patients with genotype 1 by intention-to-treat analysis (data not published). Differences between genotype 1 and 2 patients still existed following the ribavirin combination treatment. Moreover, a striking finding in our study was that there were no differences among the patients with genotype 2 of any HCV RNA level (76.9-100%). The precise mechanism is unclear, although it possibly originates in different nucleotide sequence of their genome. Further study is needed to clarify the reasons for the differences in antiviral effect, by the use of novel and new tools for the quantification of the HCV replication system[19,20].

How long the ribavirin needs to be administrated to achieve the best efficacy with IFN alpha-treated patients of genotype 2 is unclear. In the present study, SVR after 16 or more weeks of treatment ranged from 83.3% to 100% and was not dependent on the dose reduction of ribavirin treatment but on the discontinuation of IFN or ribavirin treatment. A pilot study from Norway showed that patients with genotype 2 and an EVR obtained a high SVR after 14 weeks of pegylated IFN and ribavirin combination treatment[21]. The Zeuzem group also demonstrated a very high SVR in a 24-week pegylated IFN and ribavirin treatment for genotype 2 patients, and 16-week treatment duration was observed to be a significant independent predictor[17]. In view of the adverse effects, high cost of ribavirin, and the above mentioned findings along with our results, a 16-week ribavirin addition to IFN treatment would seem to produce a high rate of SVR for patients with genotype 2, especially for those with EVR, defined as undetectable HCV RNA within 4 wk of the start of the treatment.

The Davis group attempted to confirm that an EVR in patients with chronic hepatitis C undergoing initial treatment with a combination therapy of pegylated IFN alpha and ribavirin was predictive of SVR[22]. Retrospective analysis of data from other trials[23] has also suggested that patients who do not attain EVR have a nominal chance of SVR with additional weeks of treatment. While the primary goal, or "holy grail", of treatment of chronic hepatitis C is SVR, it must be acknowledged there are other secondary goals that compel physicians to continue treatment without EVR. In fact, patients who do not achieve EVR or SVR may have histological benefit[24], leading to a decreased risk of hepatocellular carcinoma[19]. Thus, it remains to be determined whether or not early discontinuation of treatment would reduce economic costs if a long-term perspective is taken.

Several adverse reactions are associated with ribavirin. One of the most significant reactions is hemolytic problems, especially anemia[15]. Most of our patients who had to have a dose reduction or who discontinued ribavirin were observed to have anemia. It is important to reduce the dose of ribavirin at as early a stage as possible to allow the safe continuation of the combination treatment. The Nomura group pointed out that careful administration is necessary in patients over 60 years, in female patients, and in patients receiving a ribavirin dose by body weight of 12 mg/kg or more[21]. Our forward stepwise logistic regression analysis showed that the continuation of ribavirin treatment was significantly associated with SVR. This combination treatment, which could depend on hemolytic adverse reaction, has a high efficacy, if physicians are able to continue the ribavirin treatment for as short a period as