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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, $P < 0.05$)

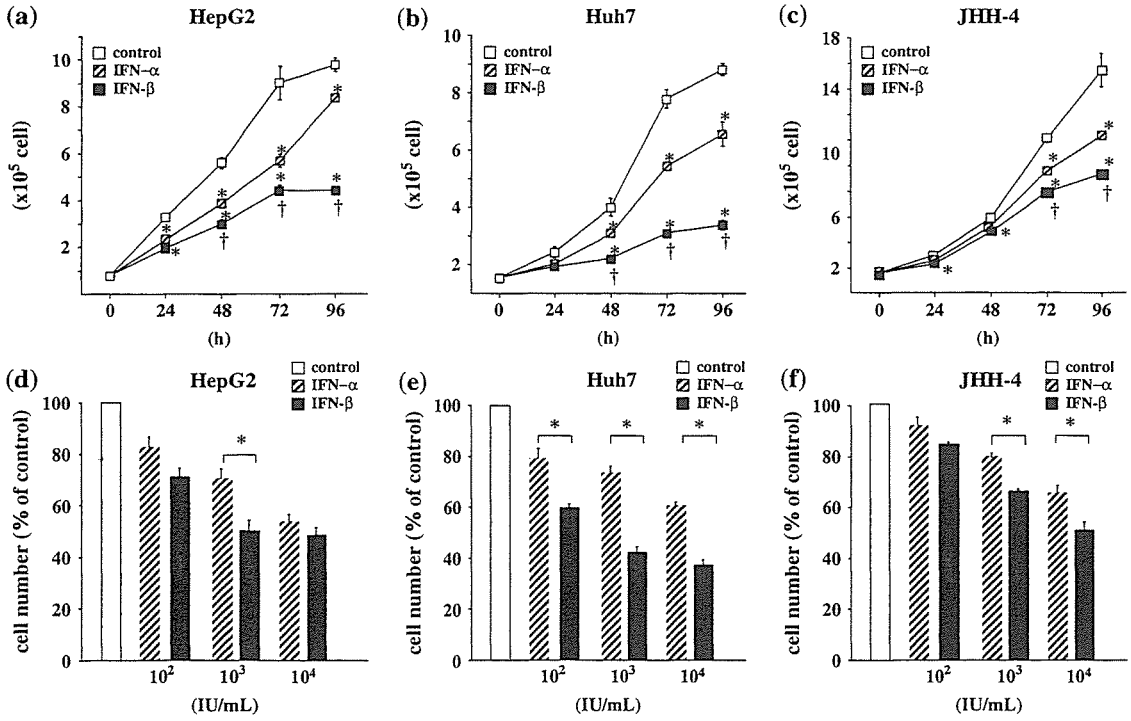

Fig. 1. Effect of IFN-α and β on the cell proliferation of HCC cell lines. Time course of HepG2 (a), Huh7 (b) and JHH4 (c) cell numbers. Dose effect of HepG2 (d), Huh7 (e) and JHH4 (f) cell numbers. Three HCC cell lines were cultured with medium alone as a control, 10^3 IU/mL IFN-α and 10^3 IU/mL IFN-β. 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

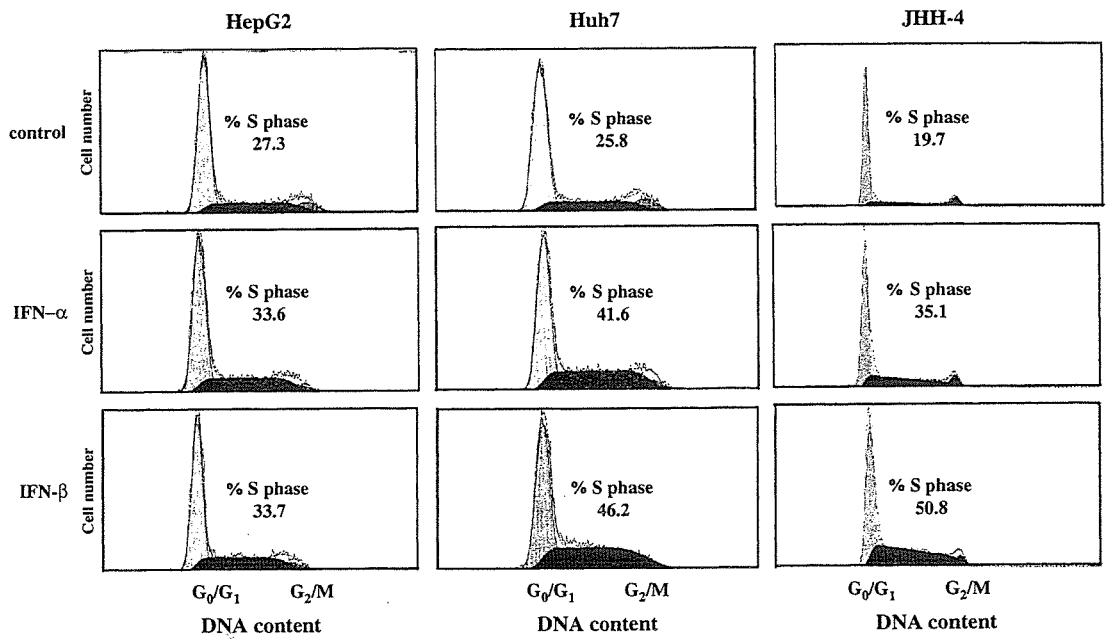

Fig. 2. Effect of IFN-α and β on the cell cycle distribution of HCC cell lines. HepG2, Huh7 and JHH4 were cultured with medium alone as a control, 10^3 IU/mL IFN-α and 10^3 IU/mL IFN-β for 24 h. The cell cycle phase distribution of both HCC cell lines were analyzed after harvest by flow cytometry using 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].

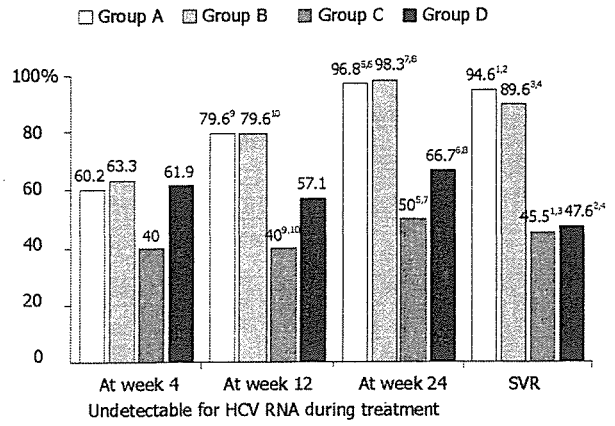

Figure 1 The sustained virological response (SVR) rate and undetectable hepatitis C virus (HCV) RNA rates during the treatment of 173 patients, classified by continuation and discontinuation of interferon and ribavirin combination treatment. Group A patients (n=93) who well tolerated the 24-week treatment 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)		All patients (n = 173)
	Group A (n = 93)	Group B (n = 48)	Group C (n = 11)	Group D (n = 21)	
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)		All patients (n = 173)
	Group A (n = 93)	Group B (n = 48)	Group C (n = 11)	Group D (n = 21)	
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)	(12.1-153)	(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

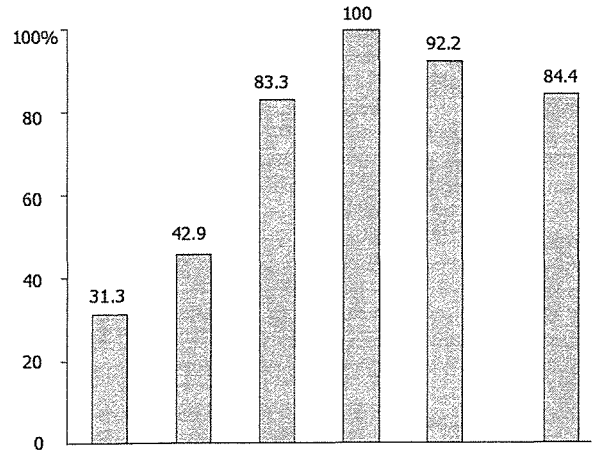

Figure 2 Relationship between the sustained virological response rates and the length of ribavirin treatment period of the 173 studied patients.

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

16 wk, even when taking into account of the dose reductions necessary for patients with a dangerous decrease of hemoglobin caused by ribavirin, as often seen in genotype 2 patients with a low hemoglobin level at pretreatment.

In conclusion, the 24-week IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR in Japanese HCV patients with genotype 2 from the retrospective study of ours. The most significant predictor was continuation of the ribavirin treatment for up to 16 wk. These findings are not pertinent to the other different genotypes.

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Association between fast-migrating low-density lipoprotein subfraction as characterized by capillary isotachophoresis and intima-media thickness of carotid artery

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Abstract

Background: A mildly modified LDL subfraction that is characterized by an increased negative charge exists in plasma. This electronegative LDL separated by ion-exchange chromatography has been shown to be inflammatory and its proportion is increased in patients with hyperlipidemia and diabetes mellitus. The present study examined the association between the level of fast (f)-migrating LDL subfraction characterized by capillary isotachophoresis (cITP) and carotid-artery intima-media thickness (CA-IMT).

Methods and results: This study included 469 subjects who underwent a physical examination. CA-IMT was determined by high-resolution B-model ultrasonoraphy. Levels of charge-based LDL subfractions were measured by cITP on a Beckman P/ACE MDQ system. An increased serum LDL-C level and cITP fLDL level were associated with increased CA-IMT after adjusting for age. The extent of the associations between cITP fLDL and CA-IMT and between LDL-C and CA-IMT were similar as assessed by a receiver-operating characteristic curve analysis. LDL-C, triglyceride, and remnant-like particle cholesterol levels were independently correlated with cITP fLDL, and the LDL-C level had the strongest correlation with cITP fLDL. The association between the cITP fLDL level and CA-IMT was significant in the high LDL-C stratum but not in the low stratum, indicating that it is modified by the LDL-C level. The high-LDL-C-high-fLDL group had the highest relative risk for a high CA-IMT among the groups with each combination of LDL-C and cITP fLDL level.

Conclusion: The cITP fLDL level was associated with CA-IMT and its combination with the LDL-C level is a stronger indicator for a high CA-IMT.

Keywords: Low density lipoprotein (LDL) cholesterol; Intima-media thickness; Carotid atherosclerosis; Capillary isotachophoresis; Fast-migrating LDL subfraction

1. Introduction

Serum level of low-density lipoprotein cholesterol (LDL-C) is an established risk factor of coronary artery disease (CAD), and a reduction in LDL-C levels has been shown to be associated with reduced death rates caused by CAD. LDL is composed of heterogeneous particles that differ in size, composition, and electric charge. Qualitatively modified forms of LDL have been shown to exist in human plasma, including small dense LDL, oxidative modified LDL, glycated LDL, and diasylated LDL [1–5], and they are

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all atherogenic. These forms of modified LDL all have an increased negative charge. The electronegative subfraction of LDL [LDL(−)] has been separated from plasma by anion-exchange chromatography techniques [6,7]. LDL(−) in plasma can also be generated by other processes including enrichment with nonesterified fatty acids or enzymatic modification by phospholipase A2 or cholesteryl esterase/trypsin [7].

Although the origins of LDL(−) are complex and not fully understood, LDL(−) has been shown to have proinflammatory activity on endothelial cells [8] and its proportion is increased in patients with hypertriglyceridemia [9], familial hypercholesterolemia (FH) [9], and diabetes mellitus (DM) [10], patients on hemodialysis [11], and patients with anigographically documented CAD [12].

However, there is still little information available on whether or not the LDL(−) subfraction level is a marker for atherosclerosis and whether or not its association with atherosclerosis is independent of the LDL-C level, partly because of the lack of a routine analytical technique for this modified LDL subfraction.

The current ion-exchange chromatography method for measuring the LDL(−) subfraction gives the proportion of LDL(−) protein content in total LDL separated by ultracentrifugation [6]. Therefore, it is disadvantageous for routine analysis in that it is time-consuming and requires a relatively large amount of samples, and the absolute level of LDL(−) in plasma cannot be determined.

Capillary isotachophoresis (cITP) is another technique that separates and quantifies LDL subfractions according to electric charge. It was originally developed by the research group of Schmitz [13,14]. Fast-migrating LDL (fLDL) carries more negative charge than slow-migrating LDL (sLDL) [13,14]. Since lipoproteins are pre-stained with a fluorescent lipophilic dye, LDL subfractions can be measured directly in plasma and with high sensitivity (only several microliters of sample are necessary). Separation and on-line detection can both be performed within just a few minutes. Therefore, analytical cITP technique may be useful for the routine analysis of lipoprotein profiles. We previously showed that the absolute levels of lipoprotein subfractions can be determined as a peak area relative to an internal marker and the levels of cITP fLDL and sLDL were proportional to the protein content of LDL [15–17].

Measurement of the thickness of the intima and media of carotid arteries by high-resolution B-mode carotid ultrasonography has been used as a non-invasive method for detecting early carotid atherosclerosis [18,19]. Carotid-artery intima-media thickness (CA-IMT) is associated with the prevalence of cardiovascular disease and with cardiovascular risk factors [20].

We investigated the hypothesis that the cITP fLDL subfraction level is associated with CA-IMT. We also hypothesized that the cITP fLDL level contributes to the ability of LDL-C to predict the risk of CA-IMT after controlling for conventional cardiovascular risk factors.

2. Methods

2.1. Subjects

This study included 469 male subjects (aged between 21 and 88 years) who participated in a health examination. This study was approved by the Ethics Committees of Kyushu University Hospital, and samples were collected only after the participants had given their informed consent.

The prevalence of hypertension, diabetes mellitus, and smoker in the study subjects was 39.7% ($n=186$), 13.9% ($n=65$), and 32.6% ($n=153$), respectively. Twelve subjects (2.6%) had a history of stroke, and 12 (2.6%) had a history of coronary heart disease. Hypertension was defined as systolic blood pressure $\geq$140 mmHg, diastolic pressure $\geq$90 mmHg, or treatment with antihypertensive medications. Diabetes mellitus was defined as a self-reported history of diabetes, a fasting plasma glucose concentration $\geq$126 mg/dl, or the use of anti-diabetic drugs. Smokers were defined as those who had smoked past or who were present smokers. Subjects who refused ultrasound examination or who had a fasting blood glucose concentration $\geq$400 mg/dl or triglyceride (TG) level $\geq$400 mg/dl were excluded from the study.

Blood was drawn between 9 and 12 a.m. after an overnight fast and stored at −80 °C until analysis. Storage of samples at −80 °C for up to 5 months does not apparently affect measurements for cITP LDL subfractions [17].

2.2. Ultrasonographic measurement

Common carotid-artery lesions were assessed by high-resolution B-mode ultrasonography with a 7.5 MHz mechanical sector transducer on an Aloka SSD-2000 (Aloka Co. Ltd., Tokyo, Japan), as described previously [21,22]. All assessment of carotid arteries was performed by three specially trained technicians who were unaware of the clinical history or risk factor profile. IMT was measured at points 20, 25, 30 mm proximal to the flow divider on the far wall of the right and left common carotid arteries at the end of the diastolic phase. Using this information, mean CA-IMT was determined for each individual.

2.3. Measurement of serum lipids and lipoproteins

Serum levels of total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C) were measured by enzymatic methods. Serum LDL-C levels were calculated indirectly using the Friedewald formula.

Remnant-like particle cholesterol (RLP-C) levels were measured by an RLP-Cholesterol Immunoseparation Assay using a commercially available kit (JIMRO-II, Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan) [23,24]. Briefly, the RLP immunoseparation gel was washed before use three times with RLP buffer by low-speed centrifugation and suspended by repeatedly inverting the container. After 150 μl of the suspended gel was aliquoted into Hitachi

microsample cups, 5 μl serum samples were added and the mixture was stirred using a steel bead for 2 h at room temperature on an RLP Mixer J-100 (Otsuka Electrics Co., Ltd, Tokyo, Japan). After the gel had settled for 15 min, the cholesterol level in the supernatant was measured with cholesterol reagents included in the assay kit using an auto-analyzer (Hitachi 7600-020S).

2.4. *Quantification of lipoprotein subfractions by cITP*

Capillary isotachophoresis of serum lipoproteins was performed on a Beckman P/ACE MDQ system (Beckman-Coulter Inc., Tokyo, Japan) according to the method of Bottcher et al. [13] with some modifications, as previously described [15–17,25,26]. Briefly, 6 μl of serum was diluted with 14 μl of leading buffer consisting of 10 mM HCl and 18 mM ammediol (2-amino-2-methyl-1,3-propanediol) (pH 8.8), prestained with 10 μl 0.1 mg/ml NBD C6-ceramide (Molecular Probe Inc., OR, USA) for 5 min at room temperature, and mixed with 50 μl of the mixture containing leading buffer with 0.35% hydroxypropylmethylcellulose (HPMC), spacers, and 5-carboxy-fluorescein as an internal marker. The spacers were *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), D-glucuronic acid, 1-octanesulfonic acid sodium salt, 3-(*N*-tris[hydroxymethyl]methylamino)-2-hydroxypropanesulfonic acid (TAPSO), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), L-serine, L-glutamine, L-methionine, and glycine. The terminating buffer contained 24 mM β-alanine and 13 mM ammediol, and was adjusted to pH 10.5 with saturated barium hydroxide solution. A dimethylpolysiloxane-modified fused silica capillary (AT^{TM}-1) was purchased from Alltech Japan Inc. (Tokyo, Japan). The sample was injected at 20 psi for 18 s into a 30-cm long capillary (i.d. 180 μm), and separation was performed at a constant 30 μA for 1 min and 10 kV for 7 min. The separated zones were monitored with argon-laser-induced fluorescence detection (excitation, 488 nm; emission, 520 nm). Each peak was identified and the peak area in relative fluorescence units was analyzed using 32 Karat Software version 5.0 (Beckman-Coulter Inc., Tokyo, Japan). Levels of cITP lipoprotein subfractions were expressed as the peak area relative to the internal marker.

2.5. *Statistical analysis*

All of the statistical analysis was performed using the SAS (Statistical Analysis System) Software Package (Version 9.1, SAS Institute, CA, USA) at the Fukuoka University. The distribution of variables were examined by the Shapiro–Wilk test [27]. The 33.3th and 66.7th percentiles were used to produce tertiles of CA-IMT. Linear trends of risk factors across tertiles of CA-IMT after adjusting for age were examined by an analysis of covariance (ANCOVA) using a general linear model. Correlation between variables was examined by Spearman correlation. Log-transformed values of TG and RLP-C were used in the data analysis. Low and high LDL-C strata were defined as $<$ and $\geq$ the median value of LDL-C (118 mg/dl), respectively, and low and high CA-IMT were defined as $<$ and $\geq$ the median value of CA-IMT (0.77 mm). The strength of the associations between the cITP fLDL and LDL-C levels was compared using a receiver operating characteristic (ROC) curve analysis. An ROC-curve (plot of sensitivity versus 1-specificity) analysis is a powerful tool for assessing the ability of a continuous variable to discriminate between two groups of subjects, and does not depend on the cutoff value selected. The area under the ROC curve represents the probability for a randomly chosen low CA-IMT subject to exhibit a value lower than the level observed among randomly chosen high CA-IMT subjects. A value of 0.5 means that the distributions of the values in the two groups are similar; conversely, a value of 1.0 means that the distributions of the values in the two groups do not overlap. We determined the area under the ROC curve by the trapezoidal rule and evaluated its significance by the Wald chi-square test, as described previously [28]. Stepwise multiple regression analysis was used to examine the independent variables that are related to cITP fLDL. The significance of the association between the combination of LDL-C and cITP fLDL and CA-IMT after controlling for age and other related variables was examined by a multivariate logistic regression analysis using dummy variables. The odds ratio and 95% confidence interval (CI) were given for each combination of LDL-C and cITP fLDL. All p values are two-tailed. The significance level was considered to be 5% unless indicated otherwise.

3. Results

Table 1 shows the mean levels of conventional risk factors of CAD, serum levels of lipids and lipoproteins, and RLP-C levels according to tertiles of CA-IMT. Increased age was associated with increased CA-IMT (tertile III versus tertile II versus tertile I: 64.4 ± 0.9 year versus 59.7 ± 0.9 year versus 48.9 ± 1.0 year, $p < 0.05$, by an analysis of variance). The prevalence of DM and serum levels of TC and LDL-C were positively and significantly associated with CA-IMT after adjusting for age, as assessed by an analysis of covariance (Table 1). Body mass index (BMI), prevalence of HT and smoking, and serum levels of TG, HDL-C, and RLP-C were not significantly associated with CA-IMT after adjusting for age (Table 1).

Fig. 1 shows the typical cITP lipoprotein profiles of subjects with low (0.54 mm) and high CA-IMT (1.17 mm). As shown, capillary isotachophoresis clearly separated lipoproteins into eight fractions within 8 min. Peaks 6 and 7 are the two LDL subfractions with fast and slow electrophoretic mobility. Subject with high CA-IMT had apparently higher levels of both fLDL and sLDL than that with low CA-IMT (Fig. 1).

Table 2 shows that age-adjusted mean levels of intermediate-migrating HDL decreased and cITP fLDL and sLDL increased across tertiles of CA-IMT. This result indi-

Table 1
Age-adjusted mean levels of risk factors according to tertiles of carotid-artery intimal-media thickness (CA-IMT)

	Tertiles of CA-IMT			
	Low (<0.67 mm)	Middle (0.67–0.83 mm)	High (≥0.83 mm)	
No. of subjects	142	159	168	
Age (year)	48.9 ± 1.0	59.7 ± 0.9	64.4 ± 0.9	<0.05
Body mass index (kg/m^2)	22.6 ± 0.3	23.9 ± 0.2	23.5 ± 0.2	n.s.
Hypertension (%)	23	40	53	n.s.
Diabetes mellituss (%)	5	12	22	<0.05
Smoking (%)	37	31	30	n.s.
TC (mg/dl)	191 ± 3	199 ± 2	204 ± 3	<0.05
log(TG)	4.7 ± 0.0	4.8 ± 0.0	4.8 ± 0.0	n.s.
HDL-C (mg/dl)	53 ± 1	54 ± 1	52 ± 1	n.s.
LDL-C (mg/dl)	113 ± 3	118 ± 2	123 ± 2	<0.05
log(RLP-C)	2.7 ± 0.0	2.8 ± 0.0	2.9 ± 0.0	n.s.

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RLP-C, remnant-like particle cholesterol. The units of TG and RLP-C were mg/dl.

[a] Assessed by an analysis of covariance or logistic regression analysis after adjusting for age. Continuous variables were adjusted for age by means of linear regression, and categorial variables were adjusted for age by means of logistic regression.

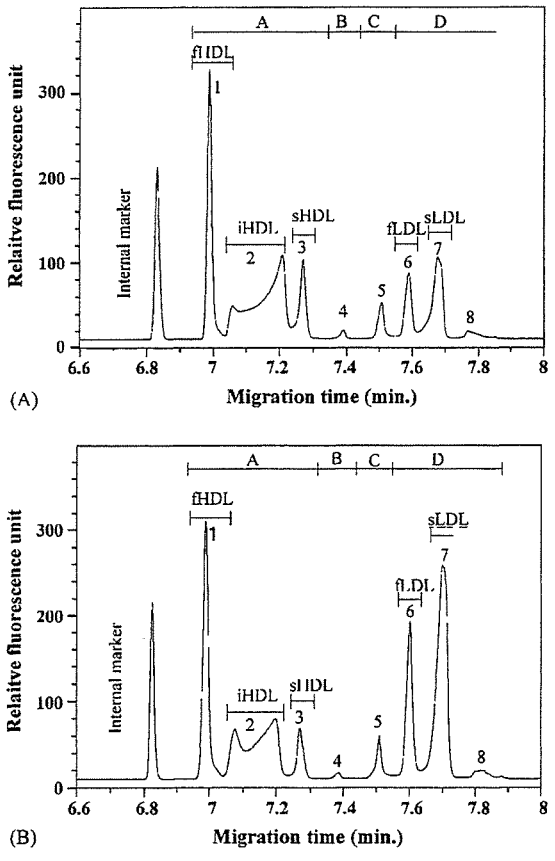

Fig. 1. Lipoprotein profiles as determined by capillary isotachophoresis in serum from subjects with low (A) and high (B) carotid-artery intima-media thickness (CA-IMT: 0.54 and 1.17 mm, respectively). The various lipoprotein subfractions are depicted as follows [13,14]: A, HDL; B, chylomicron/remnants; C, VLDL/IDL; D, LDL. fHDL, fast-migrating HDL; iHDL, intermediate-migrating HDL; sHDL, slow-migrating HDL; fLDL, fast-migrating LDL; sLDL, slow-migrating LDL.

cates that both cITP fLDL and sLDL were positively associated with CA-IMT independent of age. The strength of the associations between cITP fLDL and CA-IMT (two levels) and between LDL-C and CA-IMT were compared by an ROC curve analysis. Fig. 2 shows the plot of sensitivity (true positive) versus 1-specificity (false positive) for the LDL-C level and cITP fLDL level. The area under the ROC curve was similar for cITP fLDL and LDL-C (0.578 and 0.582, respectively).

The cITP fLDL levels were negatively correlated with HDL-C levels ($r=-0.135$, $p<0.01$) and significantly ($p<0.01$) and positively correlated with age, BMI, and serum levels of TC, TG, LDL-C, and RLP-C ($r=0.156$, 0.168, 0.524, 0.208, 0.545, and 0.147, respectively). Stepwise multiple regression analysis selected LDL-C, TG, and RLP-C as independent variables that were related to cITP fLDL (Table 3). The LDL-C level had the strongest correlation with cITP fLDL (Table 3). Fig. 3 shows the correlation between cITP fLDL and LDL-C levels in subjects with low, middle, and high CA-IMT. As shown, cITP fLDL levels were significantly correlated with LDL-C levels in all the three groups of subjects. As also shown in Fig. 3, the regression lines of cITP fLDL versus LDL-C levels in subjects with middle and high CA-IMT (dotted lines) were shifted towards higher cITP fLDL levels as compared with that in subjects with low CA-IMT (solid line). This result indicates that cITP fLDL levels were higher in subjects with middle and high CA-IMT than in subjects with low CA-IMT after controlling for LDL-C levels.

Therefore, LDL-C levels were stratified into low and high strata and the association between cITP LDL and CA-IMT was examined according to LDL-C strata to test its relation to LDL-C levels. As shown in Table 4, the association between cITP fLDL and CA-IMT was significant in the high LDL stratum [odds ratio (95% CI): 2.2 (1.2–3.8)] but not in the low LDL stratum after adjusting for age by a multiple logistic regression analysis. This result indicates that

Table 2
Age-adjusted mean levels of lipoprotein subfractions as measured by capillary isotachophoresis (cITP) according to tertiles of carotid-artery intimal-media thickness (CA-IMT)

	Tertiles of CA-IMT			p^a
	Low (<0.67 mm)	Middle (0.67–0.83 mm)	High (≥0.83 mm)	
cITP fHDL	1.46 ± 0.04	1.47 ± 0.04	1.40 ± 0.04	n.s.
cITP iHDL	2.22 ± 0.04	2.14 ± 0.03	2.10 ± 0.03	<0.05
cITP sHDL	0.41 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	n.s.
cITP VLDL/IDL	0.91 ± 0.04	1.04 ± 0.04	1.01 ± 0.04	n.s.
cITP fLDL	1.09 ± 0.03	1.16 ± 0.02	1.20 ± 0.03	<0.05
cITP sLDL	1.29 ± 0.05	1.36 ± 0.04	1.46 ± 0.04	<0.05

Levels of cITP lipoprotein subfractions are expressed as peak area relative to the internal marker. fHDL, iHDL, and sHDL, fast-intermediate, and slow-migrating high-density lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; fLDL and sLDL, fast- and slow-migrating low-density lipoprotein.

[a] Assessed by an analysis of covariance. Variables were adjusted for age by means of linear regression.

Table 3
Stepwise multivariable regression analysis of the independent variables related to fast-migrating low-density lipoprotein (fLDL) as determined by capillary isotachophoresis (cITP)

Step	Variable entered	Partial correlation coefficient	F	p
1	LDL-C	0.573	190.1	<0.001
5	log(TG)	0.306	67.3	<0.001
4	log(RLP-C)	0.204	25.6	<0.001

Levels of cITP fLDL are expressed as peak area relative to the internal mark.

the association between cITP fLDL and CA-IMT was modified by the LDL-C level. Fig. 4 shows a three-dimensional plot of the age-adjusted relative risk for a high CA-IMT for each combination of cITP fLDL and LDL-C levels. The high-LDL-C-high-fLDL group had the highest risk for a high CA-IMT among the four groups: the low-LDL-C-low-fLDL group, the low-LDL-C-high-fLDL group, the high-LDL-C-low-fLDL group, and the high-LDL-C-high-fLDL group. Similar results were obtained after additionally adjusting for HT, DM, and smoking (data not shown). These results indicate that the combination of cITP fLDL and LDL-C level was a stronger indicator for a high CA-IMT than either cITP fLDL or LDL-C alone.

4. Discussion

With advances in techniques in lipoprotein analysis, a new LDL subfraction in plasma that is characterized by a greater negative charge than native LDL has attracted considerable attention. The electronegative LDL subfraction separated by ion-exchange chromatography has been shown to contain mildly modified LDL that could be produced from multiple origins [7] and is associated with a pathogenic state that is related to atherosclerosis [9–11]. Therefore, this negatively charged LDL subfraction could be a novel marker for atherosclerosis. However, there is still little evidence to support this point because of the lack of routine analytical techniques for this LDL subfraction. Ion-exchange chromatography is excellent for the separation of LDL(−) and for preparative use [6]. However, since it requires the separation of LDL by ultracentrifugation, the absolute level of LDL(−) in plasma cannot be measured with this technique and routine analysis is also difficult.

Analytical capillary isotachophoresis is a new technique for routine analysis of LDL subfractions according to their electric charges, which was established by the research group of Schmit et al. [13,14]. Several microliters of serum or plasma can be directly analyzed and separation and detection of cITP fast- and slow-migrating LDL can be performed within minutes. However, little attention has been paid to this technique [15–17,25,26], and therefore the clinical significance of the cITP fLDL subfraction is still unclear. We have previously shown that cITP can be used to quantify charge-based LDL subfractions [17] and express the absolute levels of cITP lipoprotein subfractions as the peak area relative to an internal marker [15–17,25,26].

We are the first to report that cITP fLDL and sLDL levels are associated with carotid-artery IMT. This finding is not unexpected because serum levels of LDL-C are associated with CA-IMT and levels of cITP LDL subfractions were correlated with LDL-C levels (Table 3, Fig. 3). We also found using an ROC curve analysis that the ability of cITP fLDL to predict for a high CA-IMT was similar to that of LDL-C (Fig. 2).

Table 4
Multiple logistic regression analysis of the association between fast-migrating LDL determined by cITP and carotid-artery intima-media thickness after adjusting for age in low and high LDL-C strata

	Regression coefficient ± S.E.	Odds ratio (95% confidence interval)	Wald chi-square	p
Low LDL-C	0.12 ± 0.37	1.1 (0.54–2.3)	0.10	n.s.
High LDL-C	0.78 ± 0.29	2.2 (1.2–3.8)	7.36	<0.01

The median value of LDL-C (118 mg/dl) was used to produce low and high LDL-C strata.