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Congestive Heart Failure

Low-dose carvedilol improves left ventricular function and reduces cardiovascular hospitalization in Japanese patients with chronic heart failure: The Multicenter Carvedilol Heart Failure Dose Assessment (MUCHA) trial

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Background The efficacy and optimum dose of β-blockers have not been established in Japanese patients with chronic heart failure (CHF). The efficacy and safety of two doses of carvedilol, a β-blocker with vasodilator and antioxidant actions, were investigated in Japanese patients with CHF.

Methods After screening and a carvedilol challenge phase, 174 patients with mild to moderate CHF were randomly assigned (double-blinded) to placebo, 2.5 mg of carvedilol twice daily, or 10 mg of carvedilol twice daily. After a 2- to 4-week uptitration phase, maintenance treatment was continued for 24 to 48 weeks. The primary end point was improvement of the global assessment of CHF by the attending physician. Secondary end points were death or hospitalization for cardiovascular disease, cardiovascular hospitalization, hospitalization for heart failure, change of left ventricular ejection fraction, and change in New York Heart Association class.

Results Carvedilol therapy achieved dose-dependent improvement of all end points (*P* for linear trend, range .002 to <.001). Both carvedilol groups showed marked risk reduction (71% to 91%) for cardiovascular and CHF hospitalization and for death or cardiovascular hospitalization (*P* range, .024 to <.001 for pairwise comparisons with placebo). No significant differences were observed for noncardiovascular hospitalization or adverse events.

Conclusions In Japanese patients with mild or moderate CHF, carvedilol achieved dose-related improvement of CHF and left ventricular ejection fraction; cardiovascular hospitalization was markedly reduced. At 5 mg/d, carvedilol conferred an important patient benefit, less than at 20 mg/d. (Am Heart J 2004;147:324–30.)

See related Editorial on page 200.

A number of double-blind, placebo-controlled, randomized studies performed in the United States and Europe have shown a beneficial effect of β-blockers on mortality and morbidity in patients with chronic heart failure (CHF).[1–5] Carvedilol is a third-generation β-blocker with vasodilatory and antioxidant actions, which has been established as an effective drug for mild to severe CHF. However, a placebo-controlled randomized study of β-blocker therapy has not yet been performed in Japanese patients with CHF.

In Western countries, the recommended initial dose of carvedilol for the treatment of CHF is 6.25 mg/d,

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The MUCHA committee members are listed in the Appendix.
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Table I. Baseline characteristics of the 3 randomized groups

Parameter	Placebo group (n = 49)	5 mg Group (n = 47)	20 mg Group (n = 77)	P
Age (y)	62 ± 12	59 ± 9	60 ± 12	.581*
Sex (male/female) (%)	82/18	77/23	74/26	.613†
Etiology of heart failure				
Nonischemic/ischemic (%)	76/24	75/25	71/29	.864†
NYHA class, II/III (%)	80/20	81/19	75/25	.735†
LVEF (%)	29 ± 7	30 ± 8	30 ± 7	.734*
Systolic BP (mm Hg)	121 ± 17	117 ± 16	119 ± 14	.418*
Diastolic BP (mm Hg)	72 ± 11	72 ± 11	73 ± 10	.660*
Heart rate (beats/min)	81 ± 14	74 ± 11	78 ± 17	.097*
Body weight (kg)	60 ± 10	60 ± 11	62 ± 14	.349*
Other medications				
ACE inhibitors (%)	80	81	70	.304†
Diuretics (%)	84	85	88	.743†
Digitalis (%)	59	70	65	.527†

Values presented as mean ± SD.
*Regression analysis.
†χ^2 Test.

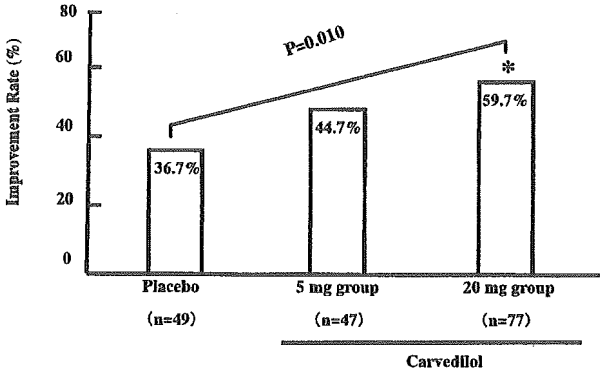
followed by uptitration to 50 mg/d for maintenance treatment if tolerated.[6] In Japan, the dose of carvedilol for the treatment of hypertension and angina pectoris is 10 to 20 mg/d,[7,8] which is less than half of that used in Western countries. However, the effectiveness of such a low-dose carvedilol regimen for Japanese patients with CHF remains unclear. The present study was designed to test the efficacy and safety of low-dose carvedilol regimens in Japanese patients with CHF.

Methods

Study design

The Multicenter Carvedilol Heart Failure Dose Assessment (MUCHA) trial was a randomized, multicenter, placebo-controlled, double-blinded study conducted in 5 phases: (1) screening, (2) challenge, (3) uptitration, (4) maintenance, and (5) downtitration. During the challenge phase after screening, eligible patients received open-label carvedilol (1.25 mg twice daily) for 1 to 2 weeks, and the dose was increased to 2.5 mg twice daily if tolerated. Patients were entered into the double-blinded uptitration phase after tolerating a dose of 2.5 mg twice daily for at least 2 weeks and were randomly assigned to placebo, carvedilol at 2.5 mg twice daily (5 mg group), or carvedilol at 10 mg twice daily (20 mg group) in the proportion of 1:1:2 by the dynamic allocation method. In the 20 mg group, the carvedilol dose was increased stepwise in a double-blinded fashion at 1- or 2-week intervals to reach 10 mg twice daily or the maximum tolerated dose of <10 mg twice daily. Patients then received placebo or carvedilol at a fixed dose for 24 to 48 weeks during the maintenance phase. When the last entered patient had completed 24 weeks of maintenance therapy, all patients were shifted into the downtitration phase.

Figure 1



Improvement rate in each group. Improvement rate is the percentage of patients with moderate or marked improvement of signs and symptoms of heart failure as assessed by the attending physician at the end of the maintenance phase in comparison with baseline. Improvement achieved with carvedilol treatment was evaluated by the Cochrane-Armitage test to assess the dose-response relation; pairwise comparisons with placebo were performed by means of χ^2 test. *$P < .05$ vs placebo.

Inclusion and exclusion criteria

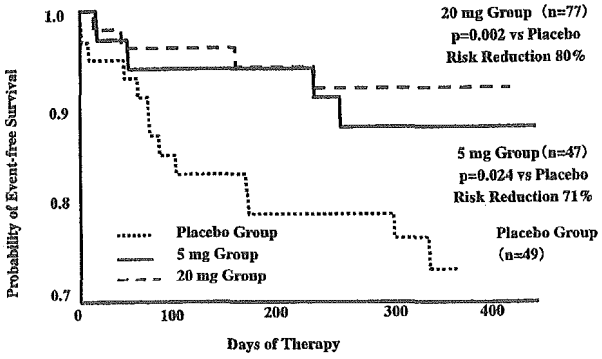
Patients who had ischemic or nonischemic cardiomyopathy with stable symptoms (New York Heart Association functional class [NYHA class] II or III) were eligible for enrollment if their left ventricular ejection fraction (LVEF) was ≤40% (measured by M-mode echocardiography or radionuclide ventriculography at the qualifying examination) and

Table II. Death and CVD hospitalization

Parameter	Placebo group (n = 49)	5 mg Group (n = 47)	20 mg Group (n = 77)	Placebo vs 5 mg Hazard ratio [95% CI]	Placebo vs 5 mg P*	Placebo vs 20 mg Hazard ratio [95% CI]	Placebo vs 20 mg P	P for linear trend†
Death or CVD hospitalization (%)	12 (24.5)	4 (8.5)	4 (5.2)	0.29 [0.10–0.91]	.024	0.20 [0.06–0.60]	.002	.002
CVD hospitalization (%)	12 (24.5)	2 (4.3)	3 (3.9)	0.14 [0.03–0.65]	.003	0.15 [0.04–0.52]	<.001	<.001
Worsening CHF	10 (20.4)	1 (2.1)	2 (2.6)	0.09 [0.01–0.69]	.004	0.12 [0.03–0.54]	<.001	<.001
Other CDV reasons	3 (6.1)	1 (2.1)	1 (1.3)	0.29 [0.03–2.75]	.229	0.20 [0.021–1.88]	.116	.111

*Log-rank test.
†Linear trend test using the Cox proportional hazards regression model.

Figure 2



Kaplan-Meier analysis of the probability of survival without death or CVD hospitalization in patients randomly assigned to placebo (*dotted line*), 5 mg/d carvedilol (*solid line*), or 20 mg/d carvedilol (*broken line*). Graph shows time to first event for each group. Dose-response relation was analyzed by means of Cox proportional hazards regression model; log-rank test was used to compare each carvedilol dose with placebo.

their age was between 20 and 79 years. Patients with the following conditions were excluded: valvular heart disease, hypertrophic obstructive cardiomyopathy, cardiogenic shock, systolic blood pressure <90 mm Hg, bradycardia (<60/min), grade II or III atrioventricular block, life-threatening arrhythmia, unstable angina, resting angina, cor pulmonale, asthma, Raynaud phenomenon, and intermittent claudication. Patients were also excluded if myocardial infarction or coronary artery bypass grafting had occurred within the preceding 3 months.

Diuretics, digitalis, angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, vasodilators, and antiarrhythmic agents could be used concomitantly. Drugs prohibited during the study were other β-blockers, αβ-blockers, α-blockers, inotropic agents other than digitalis, and intravenous diltiazem hydrochloride or verapamil hydrochloride.

The institutional review board of each participating hospital approved the study, and each subject gave written informed consent.

Parameters assessed

The primary end point of the study was improvement of the global assessment of CHF (signs and symptoms) by the attending physician. The secondary end points were as follows: all-cause death or hospitalization for cardiovascular disease (CVD), CVD hospitalization, hospitalization for worsening CHF, changes of LVEF, and changes of NYHA class.

Global assessment of heart failure was performed by entering CHF symptoms and objective findings at baseline and follow-up into a study form. Differences between the assessment at baseline and at last follow-up during maintenance treatment were rated according to the protocol and were assigned one of the following 6 grades: markedly improved, moderately improved, mildly improved, no change, worsened, or unassessable. The improvement rate (the primary end point) for each treatment group was defined as the proportion assigned a rating of "moderately improved" or "markedly improved." Data concerning CVD hospitalizations and deaths were reported prospectively by the investigators and were reviewed and classified by the End Point Committee.

The end point of changes in LVEF was defined as the difference between M-mode echocardiography measurements at baseline and last follow-up during the maintenance phase, with the left ventricular volumes being calculated by the method of Teichholz. The Data and Safety Monitoring Board prospectively monitored all serious adverse events.

Statistical analysis

On the basis of data from a Japanese pilot study and the US Carvedilol Heart Failure Trials Program,[9-12] it was projected that a sample size of 160 patients (40 for the placebo group, 40 for the 5 mg group, and 80 for the 20 mg group) would provide 80% power at the $P = .05$ level of significance to detect a dose-response effect among the three groups as well as assessing the efficacy of each carvedilol dose.

Data on the improvement rate from the physicians' global assessments were analyzed by the Cochrane-Armitage test to

Table III. Death or CVD hospitalization rates according to baseline characteristics

Subgroup	Event rate (%)				Hazard ratio (*P*, Log-rank test)	
	Placebo group (P)	5 mg Group (L)	20 mg Group (H)	*P*, linear trend test*	P vs L	P vs H
Sex						
Male (n = 133)	25.0	8.3	7.0	.013	0.27 (.036)	0.26 (.015)
Female (n = 40)	22.2	9.1	0.0	.034	0.39 (.419)	−(.023)
Age (y)						
<65 (n = 99)	24.0	0.0	4.4	.014	−(.005)	0.21 (.019)
≥65 (n = 74)	25.0	22.2	6.3	.041	0.81 (.745)	0.21 (.032)
Etiology						
Nonischemic (n = 127)	24.3	2.9	3.6	.002	0.10 (.007)	0.15 (.005)
Ischemic (n = 46)	25.0	25.0	9.1	.150	0.84 (.829)	0.29 (.156)
NYHA class						
II (n = 135)	28.2	7.9	6.9	.005	0.23 (.013)	0.23 (.006)
III (n = 38)	10.0	11.1	0.0	.215	1.05 (.970)	−(.180)
LVEF (%)						
<30 (n = 76)	33.3	10.0	2.9	.002	0.25 (.059)	0.08 (.003)
≥30 (n = 95)	18.5	7.4	7.3	.140	0.35 (.192)	0.34 (.127)
Systolic BP (mm Hg)						
<120 (n = 84)	15.0	14.8	10.8	.638	0.95 (.950)	0.70 (.633)
≥120 (n = 89)	31.0	0.0	0.0	<.001	−(.004)	−(.001)
Heart rate (beats/min)						
<75 (n = 78)	29.4	11.1	8.8	.102	0.35 (.135)	0.31 (.089)
≥75 (n = 95)	21.9	5.0	2.3	.004	0.18 (.073)	0.09 (.005)

*Linear trend test using the Cox proportional hazards regression model.

Table IV. Death or CVD hospitalization rates according to presence of concomitant disease at baseline examination

Subgroup	Event rate (%)				Hazard ratio (*P*, Log-rank test)	
	Placebo group (P)	5 mg Group (L)	20 mg Group (H)	*P*, linear trend test*	P vs L	P vs H
Hypertension						
Nonhypertensive (n = 132)	22.9	10.3	5.2	.014	0.38 (.104)	0.22 (.013)
Hypertensive (n = 41)	28.6	0.0	5.3	.041	−(.104)	0.15 (.052)
Diabetes						
Nondiabetic (n = 118)	24.2	6.1	7.7	.035	0.21 (.031)	0.30 (.035)
Diabetic (n = 55)	25.0	14.3	0.0	.011	0.49 (.401)	−(.010)
Hyperlipidemia						
Normolipidemic (n = 136)	26.8	8.6	6.7	.007	0.28 (.037)	0.24 (.008)
Hyperlipidemic (n = 37)	12.5	8.3	0.0	.140	0.53 (.648)	−(.145)
Cardiac rhythm						
Sinus rhythm (n = 132)	21.4	11.4	1.8	.002	0.46 (.188)	0.08 (.002)
Atrial fibrillation (n = 41)	42.9	0.0	13.6	.214	−(.014)	0.29 (.106)

*Linear trend test using the Cox proportional hazards regression model.

assess the dose-response relation. When a significant difference was found, pairwise comparisons with placebo were performed with the χ^2 test.

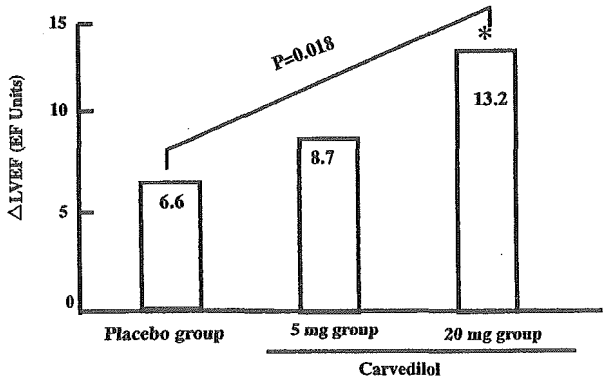
For death or CVD hospitalization, CVD hospitalization, and hospitalization for CHF, Kaplan-Meier curves were constructed as time-to-first event plots for each group, and data were analyzed to detect a dose-response relation by means of the Cox proportional hazards regression model. Then event-free survival curves were compared among the placebo group and carvedilol groups by means of the log-rank

Table V. Change of NYHA class from baseline to the end of the maintenance phase

Group	Improved, n (%)	No change, n (%)	Worsened, n (%)	*P*, Wilcoxon rank sum test	*P*, linear trend test*
Placebo	23 (48.9)	19 (40.4)	5 (10.6)		
5 mg	38 (80.9)	8 (17.0)	1 (2.1)	.037†	.046
20 mg	51 (70.8)	20 (27.8)	1 (1.4)	.051†	

*Linear trend test using the Cochrane-Mantel-Haenszel test.
†Versus placebo.

Figure 3



Changes of LVEF from baseline to end of maintenance phase. Dose-response relation was assessed by means of regression analysis; pairwise comparisons with placebo were done by Student *t* test. Change of LVEF was defined as the difference between baseline value and value at end of maintenance phase and is expressed in ejection fraction (EF) units.

test. All analyses were performed on the full analysis set,[13] and the level of significance was set at $P < .05$ (2-tailed).

Results

Open-label challenge phase

Treatment was started on October 28, 1996, and the trial was completed on March 17, 2000. One hundred ninety patients commenced the challenge phase and 174 patients (91.6%) were randomly assigned to the 3 treatment groups.

Baseline patient profile

Of 174 patients randomly assigned to the 3 treatment groups, one patient in the 20 mg group who received no study medication was excluded from the full analysis set. There were no significant differences in baseline characteristics among the three treatment groups (Table I). Seventy-four percent of patients in the 20 mg group and 100% in the 5 mg group achieved the target dose; the mean dose was 17.2 mg/d in the 20 mg group.

Global assessment of changes in CHF (primary end point)

The percentage of each treatment group with moderate or marked improvement in signs and symptoms of heart failure (improvement rate) ascertained by attending physicians showed a significant dose-response relation to carvedilol ($P = .010$), and in pairwise comparison, the improvement rate was significantly higher in the 20 mg group than in the placebo group ($P = .012$) (Figure 1).

Death or CVD hospitalization

The incidence of death or CVD hospitalization showed a significant dose-response relation ($P = .002$) and was significantly lower in both the 5 mg group ($P = .024$) and the 20 mg group ($P < .002$) on pairwise comparison with the placebo group (Table II and Figure 2). Risk reduction was 71% in the 5 mg group and 80% in the 20 mg group. Carvedilol treatment was associated with a highly significant decrease of the CVD hospitalization rate in the 5 mg group ($P = .003$) and the 20 mg group ($P < .001$), as well as for the linear trend ($P < .001$). Compared with placebo, the reduction in risk of CVD hospitalization was 86% in the 5 mg group and 85% in the 20 mg group (Table II). This included a risk reduction for hospitalization resulting from worsening heart failure of 91% for the 5 mg group and 88% for the 20 mg group.

Subgroup analysis showed that the risk of death or CVD hospitalization was lower in the carvedilol-treated groups regardless of age, sex, underlying cause of CHF, severity of CHF, LVEF, systolic blood pressure, or heart rate (Table III). The risk of death or CVD hospitalization in high-risk patients with hypertension, diabetes mellitus, hyperlipidemia, and atrial fibrillation was also lower in the carvedilol-treated groups (Table IV).

Left ventricular function

There was a significant dose-related increase of LVEF with carvedilol treatment ($P = .018$), and there was a significantly greater increase of LVEF in the 20 mg group compared with the placebo group ($P = .022$) (Figure 3).

NYHA class and adverse events

A significant dose-dependent improvement of NYHA class was observed with carvedilol treatment (Table V).

The incidence of all serious and nonserious adverse events was lower (but not significantly) in the carvedilol-treated groups (63.3%, 51.1%, and 59.7% in the placebo, 5 mg, and 20 mg groups, respectively). Adverse events with a higher incidence (P = NS) in the 20 mg group were dizziness, upper respiratory tract infections, worsening of diabetes, palpitations, headache, and hypotension.

Discussion

The MUCHA trial demonstrated a dose-related improvement in signs and symptoms of heart failure assessed by the attending physicians and improvement of LVEF in Japanese patients with CHF who received long-term carvedilol therapy at low doses of 5 or 20 mg/d. These low doses achieved a remarkable reduction in the risk of death or CVD hospitalization; surprisingly, the 5 mg/d dose achieved a reduction (71%) that was nearly as great as that for the 20 mg/d dose (80%).

In the current study, a dramatic reduction in the risk of hospitalization for CHF or CVD was observed with both carvedilol regimens. Although the majority of the patients were in NYHA class II, this was probably not the reason for the marked reduction of CVD hospitalization, because previous trials performed in the United States and Australasia[1-3] have shown that risk reduction is not related to the NYHA class.

In the current study, carvedilol reduced the risk of death or CVD hospitalization irrespective of the presence of hypertension, diabetes, hyperlipidemia, and atrial fibrillation. In the CIBIS-II study, the β_1-selective blocking agent bisoprolol decreased mortality and hospitalization rates in patients with sinus rhythm but not in patients with atrial fibrillation.[14] In contrast, we found that carvedilol reduced the risk of death or cardiovascular hospitalization irrespective of the presence of atrial fibrillation, consistent with the results of the US Carvedilol Heart Failure Trials Program.[15]

In previous studies of carvedilol therapy for patients with CHF, improvement of LVEF was accompanied by a reduction of rates for death or CVD hospitalization.[10] At a dose of 5 mg/d, however, a marked reduction in the risk of CVD hospitalization in the current study was observed without a statistically significant improvement of LVEF. In patients with CHF, Quaife et al[16] reported that carvedilol improved the right ventricular ejection fraction, with a decrease of the right ventricular end-diastolic and end-systolic volumes and a decrease of the pulmonary arterial pressure. They also suggested that the improvement of right ventricular systolic function was not secondary to the improvement of left ventricular function but was due to the direct effect of carvedilol.[16] Our results may be consistent with a direct effect of low-dose carvedilol on right ventricular function.

The recommended dose of carvedilol shown by the US MOCHA trial was 12.5 to 50 mg/d and that shown by the MUCHA trial in Japan was 5 to 20 mg/d. Since the pharmacokinetics of carvedilol are similar in healthy Japanese and American adults (unpublished observation), whereas β_1-receptor sensitivity is higher in Chinese than in white or black Americans,[17] the difference in the effective dose between patients in Japan and Western countries may depend on a difference in β_1-receptor sensitivity.

The current findings regarding the global improvement of heart failure, LVEF, and morbidity strongly suggest that the recommended dose of carvedilol for Japanese patients with CHF should range from 5 to 20 mg/d. In addition, our finding of reduced morbidity at a low dose of carvedilol (5 mg/d) suggested that low-dose therapy may have a beneficial effect in patients who cannot tolerate a standard regimen. A future large-scale study will be necessary to evaluate the impact of low-dose, long-term carvedilol therapy on survival in Japanese patients with CHF.

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Appendix

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Ursodeoxycholic acid inhibits endothelin-1 production in human vascular endothelial cells

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Abstract

Endothelin-1 is known to be implicated in the pathogenesis of hepatobiliary diseases such as cirrhosis, especially in portal hypertension. This study aimed to investigate the effects of ursodeoxycholic acid on endothelin-1 production in human endothelial cells. The effects of ursodeoxycholic acid and its conjugates (tauroursodeoxycholic and glycoursodeoxycholic acids) on endothelin-1 production as well as nitric oxide (NO) in human umbilical vein endothelial cells (HUVECs) were examined. The production of endothelin-1 and nitric oxide in culture medium was measured using enzyme-linked immunosorbent assay (ELISA) and the Griess method, respectively. Endothelin-1 and endothelial nitric oxide synthase (eNOS) mRNA expression were investigated by real-time quantitative reverse transcriptase/polymerase chain reaction (RT-PCR). Ursodeoxycholic acid (30–1000 μM) inhibited endothelin-1 production in a concentration-dependent manner, and ursodeoxycholic acid at concentrations higher than 300 μM increased nitric oxide production in culture medium. The conjugates of ursodeoxycholic acid also increased nitric oxide production and decreased endothelin-1 production, which was less effective than ursodeoxycholic acid. *N*-nitro-L-arginine-mythel-ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, suppressed the ursodeoxycholic acid-induced nitric oxide production, but it did not antagonize the inhibitory effects of ursodeoxycholic acid on endothelin-1 production. Ursodeoxycholic acid also induced a concentration-dependent decrease in endothelin-1 mRNA expression without significant changes in eNOS mRNA expression. These results provide novel evidence that ursodeoxycholic acid inhibits endothelin-1 production in human endothelial cells, but nitric oxide is not responsible for the inhibitory effect of ursodeoxycholic acid on endothelin-1. Thus, ursodeoxycholic acid therapy may prevent the development of several pathogenesis such as portal hypertension observed in patients with cirrhosis due to the improvement of endothelial function.

Keywords: Ursodeoxycholic acid; Human endothelial cell; Endothelin-1; Nitric oxide; Real-time RT-PCR

1. Introduction

Endothelin-1, a 21-amino-acid peptide (Yanagisawa et al., 1988), is synthesized by various cells including hepatic and endothelial cells. It has been known to be a potent vasoconstrictor and mitogen for vascular smooth muscle cells in the liver (Gandhi et al., 1990; Serradeil-Le Gal et al., 1991), which is implicated in the pathogenesis of a variety of diseases such as hepatobiliary diseases (Tsai et al., 1995; Alam et al., 2000; Shah, 2001). Endothelin-1 is overexpressed in cirrhotic tissues (Pinzani et al., 1996; Kuddus et al., 2000; Tieche et al., 2001), and endothelin-1 level is elevated in patients or animal models with cirrhosis, especially in portal hypertension (Moller et al., 1995; Bernardi et al., 1996; Bruno et al., 2000). The increased expression of endothelin-1 mRNA and the subsequent increase in production has been

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also reported in the experimental models of liver injury and cirrhotic rats (Leivas et al., 1995; Rockey et al., 1998).

On the other hand, nitric oxide (NO), derived from endothelium or other cells, also plays a vital role in regulating vascular tone (Moncada et al., 1991; Nathan, 1992), and it is another important physiological and pathophysiological mediator in hepatobiliary diseases. Nitric oxide is synthesized from L-arginine by isoforms of nitric oxide synthase (NOS). Nitric oxide released from endothelial nitric oxide synthase (eNOS) is a physiologically potent vasodilator and an inhibitor of vascular smooth muscle cell proliferation, which is considered to have a beneficial role. In fact, diminution in eNOS-derived nitric oxide production in liver has been reported to be involved in development of portal hypertension by increasing intrahepatic resistance (Shah, 2001). And 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester (NCX-1000), a nitric oxide-releasing derivative of ursodeoxycholic acid, selectively divers nitric oxide to the liver and protects against development of portal hypertension (Fiorucci et al., 2001).

Ursodeoxycholic acid, a hydrophilic tertiary bile acid, has been wildly used to treat patients with chronic cholestatic liver diseases (Luketic and Sanyal, 1994) and has benefits for the treatment in patients with various liver diseases such as primary biliary cirrhosis and chronic viral hepatitis (Cirillo and Zwas, 1994; Makino and Tanaka, 1998). Ursodeoxycholic acid also inhibits progression of chronic hepatic disorders with special reference to increases in blood flow and limits the development of portal hypertension induced by bile duct ligation (Poo et al., 1992; Poo et al., 1995). The basic mechanism has not yet been identified, but a wide range of cellular actions of ursodeoxycholic acid, i.e., anti-inflammatory and immunomodulating effects (Makino and Tanaka, 1998; Ma et al., 2003), has been proposed. It also protects hepatocytes against oxidant injury via induction of antioxidants (Mitsuyoshi et al., 1999). In human vascular endothelial cells, we have reported that bile acids such as chenodeoxycholic acids increase nitric oxide production by increasing intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Nakajima et al., 2000; Chisaki et al., 2001), but the effects of ursodeoxycholic acid on endothelin-1 production have not been investigated.

In the present study, the effects of ursodeoxycholic acid on endothelin-1 production as well as nitric oxide in human endothelial cells were investigated. Here, we provided novel evidence that ursodeoxycholic acid inhibits endothelin-1 production in human umbilical vein endothelial cells (HUVECs), but nitric oxide is not responsible for ursodeoxycholic acid effects on endothelin-1.

2. Materials and methods

2.1. Materials

HUVECs were purchased from BioWhittaker and cultured in endothelial growth medium (EGM) supplemented with 0.1% human epithelial growth factor, 0.1% hydrocortisone, 0.1% Gentamicin sulfate Amphotericin (GA1000), 0.4% bovine brain extract, 2.0% fetal bovine serum in an atmosphere of 5% CO_2, and 95% air at 37 °C in 25-cm^2 flasks. At confluence, cells were split 1:3 after they were detached using 0.25% trypsin in 0.02% EDTA. Media were changed twice weekly. The confluent cells were used within 3 weeks of establishing primary cultures and at the third to fifth passage.

Ursodeoxycholic acid (Na salt), tauroursodeoxycholic acid (Na salt), and glycoursodeoxycholic acid (Na salt) were kindly provided by Mitsubishi Pharma (Osaka, Japan). *N*-nitro-L-arginine-mythel-ester (L-NAME) was purchased from Sigma (St. Louis, MO).

2.2. Determination of nitric oxide

Nitric oxide released from HUVECs was determined by measuring the concentration of NO_2^-, a stable metabolite of nitric oxide, in culture medium, using the Griess method, as described previously (Nakajima et al., 2000; Ma et al., 2003). Confluent monolayers cultured in 35-mm dishes were washed twice with phosphate buffered saline (pH 7.4), and then, 2 ml of the EGM supplemented with or without various concentrations of ursodeoxycholic acid or the conjugated bile acids (tauroursodeoxycholic and glycoursodeoxycholic acids) were added. Two hours later, 1.5-ml aliquots of cultured medium were collected and centrifuged for 2 min at 12,000 $\times g$. One milliliter of Griess reagent (0.5% naphthylethylenediamine dihydrochloride and 5% sulphanilamide in 25% H_3PO_4) was added to 1 ml supernatant, and the mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was measured in a Beckman DU-70 spectrophotometer (Fullerton, CA). The concentration of NO_2^- was calculated by comparison with the absorbance at 540 nm of standard solutions of 0–150 μM $NaNO_2$ prepared in the EGM.

2.3. Measurement of endothelin-1 concentration

The amount of endothelin-1 produced by HUVECs was determined by measuring the concentration of endothelin-1 in cultured medium, using enzyme-linked immunosorbent assay (ELISA) Kit (TECHNE, Minneapolis, MN). Diluted conjugate (100 μl; antibody to endothelin-1 conjugated to horseradish peroxidase) was added to each endothelin-1 antibody-precoated well. Then 100 μl supernatant (above-mentioned in nitric oxide determination) or standard solution was added to each well with sufficient force to ensure mixing and was incubated at room temperature for 30 min. Afterwards, the content from each well was aspirated and washed with wash buffer (buffered surfactant). Substrate (100 μl; tetramethylbenzidine) was added to each well and was incubated at room temperature for 30 min. Then, 100 μl stop solution (acid solution) was added to each well. The optical density of each well was determined using

a biolumin 960 microplate reader (Molecular Dynamics Japan, Tokyo) set at 450 nm, with the correction wavelength set at 620 nm. The concentration of endothelin-1 was calculated by comparison with standard solutions containing 0–150 pg/ml endothelin-1 prepared in the EGM.

2.4. Protein assay

After HUVECs proliferated to confluence in 35-mm dishes, the cells were washed three times in a HEPES buffered saline solution and then lysed in 0.5 M NaOH. The protein content of the cytolysate of the total cells was measured by the Bradford protein assay.

2.5. RNA extraction and real-time quantitative reverse transcriptase/polymerase chain reaction (RT-PCR)

Total RNAs were isolated as described above (Jo et al., 2004) and then treated with DNase I. They were then converted to cDNAs, using a Super Script first-strand synthesis system (Invitrogen). Quantitative RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (model 7700, Applied Biosystems, Foster City, CA). Gene-specific primers and Taq-Man probes (endothelin-1, accession no. NM 001955; eNOS, accession no. NM 000603) were used to analyze the transcript abundance. The 18 S ribosomal RNA was analyzed as an internal control and was used to normalize the values for the transcript abundance.

2.6. Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance and Dunnett's test. Data are expressed as the mean±S.E.M., and values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Effects of ursodeoxycholic acid on endothelin-1 and nitric oxide production

Fig. 1 shows the effects of ursodeoxycholic acid on the production of endothelin-1. The amount of endothelin-1 (pg/ml) released from HUVECs for 2 h in the culture medium under the control medium and in the presence of various concentrations of ursodeoxycholic acid (30–1000 μM) is shown in Fig. 1. Compared with the control solution, ursodeoxycholic acid decreased the production of endothelin-1 in the culture medium in a concentration-dependent manner. Significant inhibitory effects of ursodeoxycholic acid were observed at concentrations above 30 μM. The basal endothelin-1 release for 2 h was 52±4 (n=6) and 245±24 pg/mg (n=6). The addition of ursodeoxycholic acid in the culture medium decreased the endothelin-1 production (46±2 and 214±20 pg/mg ($p<0.05$, n=6) at 100 μM; and 41±3 and 196±11 pg/mg ($p<0.01$, n=6) at 300 μM; and 35±4.7 and 181±16 pg/mg ($p<0.01$, n=6) at 500 μM).

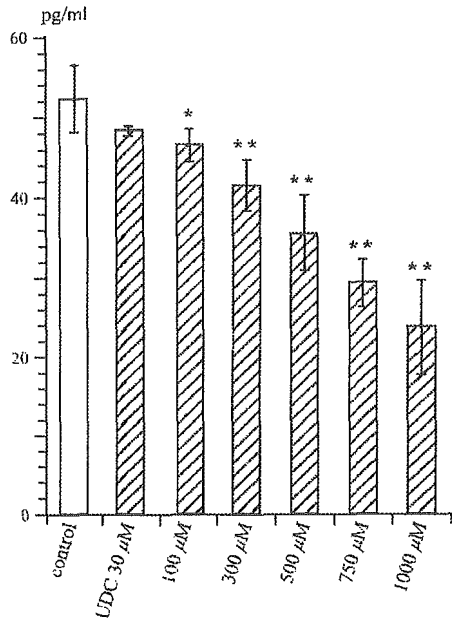

Fig. 1. Effects of ursodeoxycholic acid on the production of endothelin-1 in human umbilical vein endothelial cells (HUVECs). The cells were treated with or without various concentrations of ursodeoxycholic acid (UDC) for 2 h. The amount of endothelin-1 (pg/ml) in the culture medium released from HUVECs was measured and plotted against each concentration of UDC (30–1000 μM). Note that UDC inhibits endothelin-1 production in a concentration-dependent manner. The data represent mean±S.E.M of six different experiments. *$p<0.05$, **$p<0.01$ vs. control.

Fig. 2 illustrates the effects of ursodeoxycholic acid for 2 h on nitric oxide production. The concentration of NO_2^- in the culture medium was measured in the control and the presence of various concentrations of ursodeoxycholic acid (30–1000 μM). Ursodeoxycholic acid at concentrations more than 300 μM significantly increased nitric oxide production. The production of NO_2^- under the control medium for 2 h was 0.55±0.06 μM (n=6) and 2.5±0.3 μM/mg (n=6). The addition of ursodeoxycholic acid in the culture medium increased the NO_2^- production (0.67±0.03 μM, 3.23±0.2 μM/mg ($p<0.05$, n=6) at 300 μM, and 0.82±0.05 μM, 4.01±0.4 μM/mg (n=6, $p<0.01$) at 500 μM).

Figs. 3 and 4 compared the effects of ursodeoxycholic acid and the conjugated bile acids on endothelin-1 (Fig. 3) and nitric oxide (Fig. 4) production. Tauroursodeoxycholic and glycoursodeoxycholic acids as well as ursodeoxycholic acid (500 and 750 μM) increased nitric oxide production in a similar manner (Fig. 4), but they inhibited endothelin-1 production less than ursodeoxycholic acid (Fig. 3).

3.2. Effects of ursodeoxycholic acid on endothelin-1 and eNOS mRNA expression

Fig. 5 shows the effects of ursodeoxycholic acid on endothelin-1 (Fig. 5A) and eNOS (Fig. 5B) mRNA

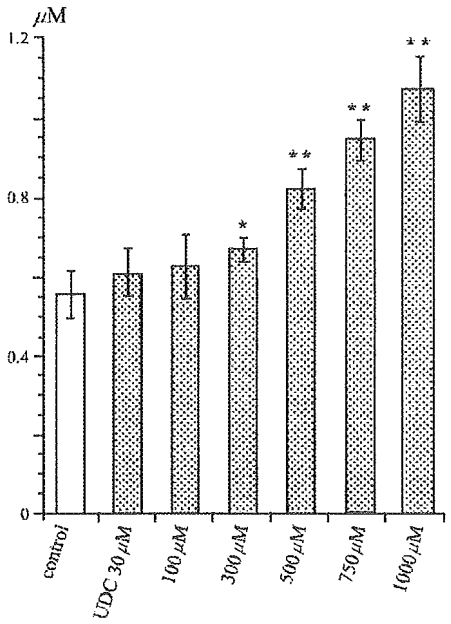

Fig. 2. Effects of ursodeoxycholic acid on the production of nitric oxide in HUVECs. The cells were treated with or without various concentrations of ursodeoxycholic acid (UDC) for 2 h. The concentration of NO_2^- (μM) in the culture medium released from HUVECs was plotted against each concentration of UDC (30–1000 μM). Note that UDC at concentrations higher than 300 μM increases the nitric oxide production. Each column represents the mean±S.E.M of six different experiments. *p<0.05, **p<0.01 vs. control.

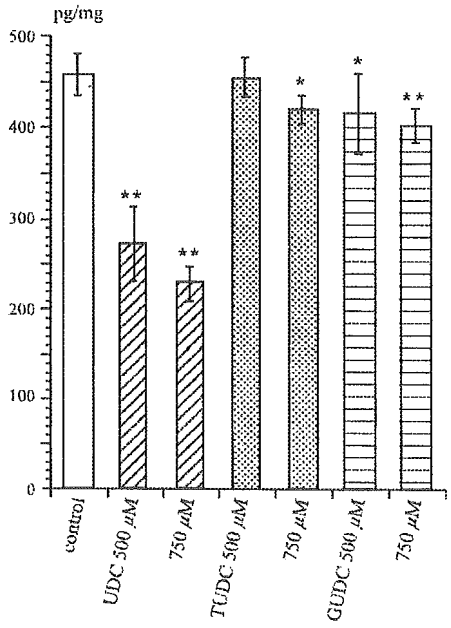

Fig. 3. Effects of ursodeoxycholic acid and the conjugated bile acids on endothelin-1 production. The cells were treated with ursodeoxycholic acid (UDC) or the conjugated bile acids [tauroursodeoxycholic acid (TUDC) and glycoursodeoxycholic acid (GUDC)] for 2 h, and the amount of endothelin-1 released from the cells (pg/mg) was measured and plotted against each concentration of these agents. Each column represents the mean±S.E.M of six different experiments. *p<0.05, **p<0.01 vs. control.

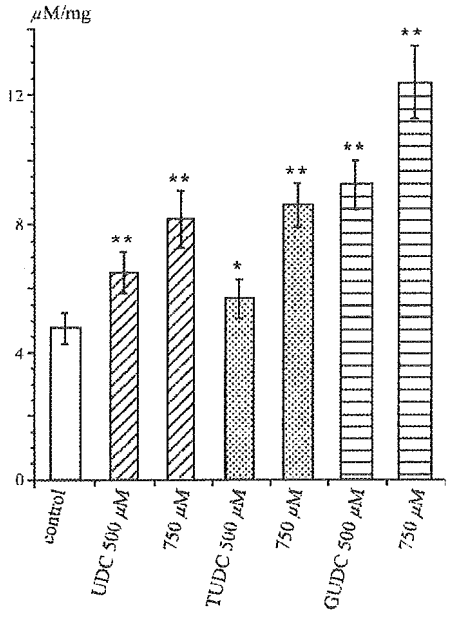

Fig. 4. Effects of ursodeoxycholic acid and the conjugated bile acids on nitric oxide production. The cells were treated with ursodeoxycholic acid (UDC) or the conjugated bile acids [tauroursodeoxycholic acid (TUDC) and glycoursodeoxycholic acid (GUDC)] for 2 h, and the amount of nitric oxide production under these conditions is plotted against each concentration of these drugs. Each column represents the mean±S.E.M of six different experiments. *p<0.05, **p<0.01 vs. control.

expression. The expression of mRNA was compared in cells treated with or without ursodeoxycholic acid (100–750 μM) for 4 h. Ursodeoxycholic acid induced a significant decrease in endothelin-1 mRNA expression. The mean % decrease was 32% of the control at 100 μM, 53% at 300 μM, 91% at 500 μM, and 91% at 750 μM, respectively. On the other hand, ursodeoxycholic acid (100–750 μM) did not modify the iNOS mRNA expression significantly. Similarly, treatment of cells with 500 μM ursodeoxycholic acid for 2 h decreased endothelin-1 mRNA expression to approximately 20% of the control.

3.3. Effects of N-nitro-L-arginine-mythel-ester (L-NAME) on the inhibitory effects of ursodeoxycholic acid on endothelin-1 production

Fig. 6A shows the effects of L-NAME, an inhibitor of nitric oxide synthase, on endothelin-1. The endothelin-1 production was 318±61 pg/mg (n=6) in control, and it was 158±44.7 pg/mg (n=6, p<0.01) at ursodeoxycholic acid (500 μM). The inhibitory effect of ursodeoxycholic acid on endothelin-1 production was not blocked by L-NAME (Fig. 6A, 1 and 2 mM, n=6). The statistical significance was not observed in between ursodeoxycholic acid in the absence of L-NAME and ursodeoxycholic acid in the presence of L-NAME (1 and 2 mM, n=6).

Fig. 6B shows the effects of L-NAME on nitric oxide production. Under the control medium, L-NAME

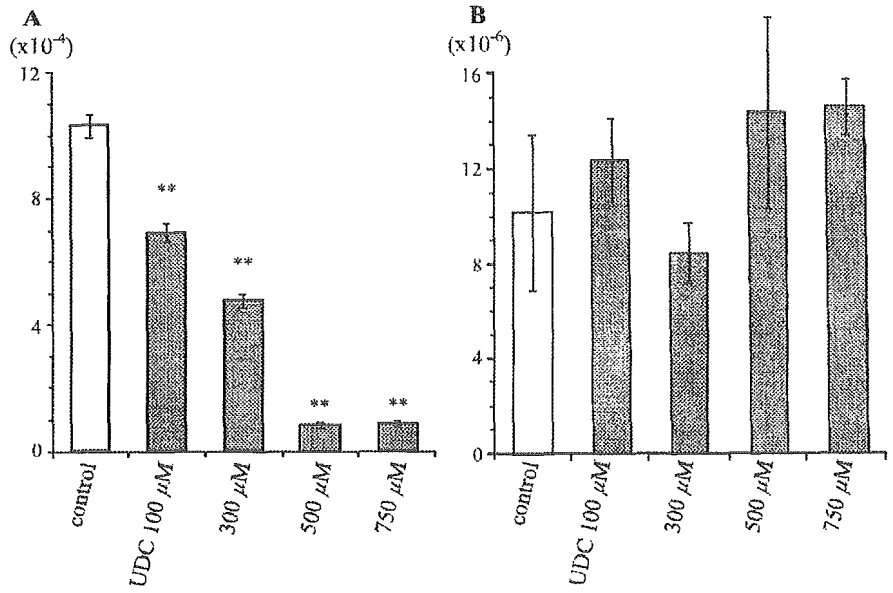

Fig. 5. Effects of ursodeoxycholic acid on endothelin-1 and eNOS mRNA expression. The cells were treated with various concentrations of ursodeoxycholic acid (UDC) for 4 h, and the total RNA was isolated from cells. The expression levels of endothelin-1 (A) and eNOS (B) mRNA were normalized to those of the 18 S ribosomal RNA levels. Data are means±S.E.M from six different samples. $^{**}p<0.01$ vs. control.

decreased nitric oxide production from 3.06±0.5 μM/mg (n=6) in the control to 1.90±0.26 μM/mg (n=6, $p<0.01$) at 1 mM L-NAME, and 2.2±0.17 μM/mg (n=6, $p<0.01$) at 2 mM L-NAME. L-NAME also significantly inhibited the production of NO_2^- induced by ursodeoxycholic acid (Fig. 6B).

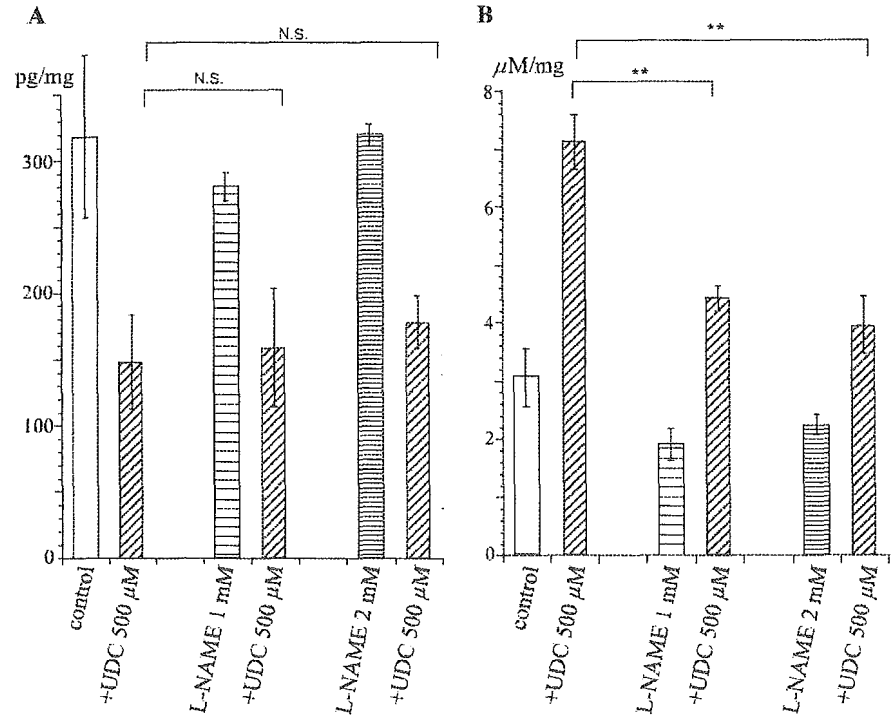

Fig. 6. Effects of L-NAME on the inhibitory effects of ursodeoxycholic acid on endothelin-1 production and the stimulatory effects of ursodeoxycholic acid on nitric oxide production. The cells were treated with or without ursodeoxycholic acid (UDC, 500 μM) in the absence or presence of L-NAME (1 and 2 mM). The amount of endothelin-1 released from the cells for 2 h (pg/mg) were measured and plotted in panel (A). In panel (B), the amount of nitric oxide production under these conditions is plotted. Note that L-NAME inhibits nitric oxide production induced by UDC, but it fails to suppress the inhibitory effects of UDC on endothelin-1 production. Each column represents the mean±S.E.M of six different experiments. $^{**}p<0.01$ vs. UDC in the absence of l-NAME.

4. Discussion

The present study showed novel evidence that ursodeoxycholic acid significantly decreased endothelin-1 mRNA expression and endothelin-1 production in human endothelial cells. Ursodeoxycholic acid at concentrations higher than 300 μM increased nitric oxide production, but the inhibitory effects of ursodeoxycholic acid on endothelin-1 production was not inhibited by a nitric oxide synthase inhibitor, L-NAME. These results suggest that ursodeoxycholic acid inhibits endothelin-1 production in human endothelial cells, but nitric oxide is not responsible for the inhibitory effect of ursodeoxycholic acid on endothelin-1 production.

Several mechanisms may be proposed in the inhibitory effects of ursodeoxycholic acid on endothelin-1 production. The bile acids are known to induce cell damage because of their detergent property. However, as reported previously in human endothelial cells (Garner et al., 1991), the concentration of ursodeoxycholic acid used in the present study did not affect the morphology of HUVECs, and the viability measured by trypan blue excursion was not different in between control cells and cells treated with ursodeoxycholic acid. Thus, it is unlikely that the detergent effects are involved in ursodeoxycholic acid effects on endothelin-1. In addition, we have reported that bile acids such as chenodeoxycholic acid increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$; Nakajima et al., 2000). But, ursodeoxycholic acid at concentrations below 300 μM did not significantly increase $[Ca^{2+}]_i$ (Chisaki et al., 2001). Therefore, it also seems unlikely that $[Ca^{2+}]_i$ is involved in the inhibitory effects of ursodeoxycholic acid on endothelin-1 production.

It has been reported that nitric oxide reduces endothelin-1 production in endothelial cells mediated by cGMP-dependent pathway (Cao et al., 1994; Mitsutomi et al., 1999). Bile acids such as chenodeoxycholic acid increase nitric oxide production in HUVECs by increasing $[Ca^{2+}]_i$ (Nakajima et al., 2000). In the present study, ursodeoxycholic acid at concentrations higher than 300 μM increased nitric oxide production without significant changes in eNOS mRNA expression (Fig. 4). High concentrations of ursodeoxycholic acid increase $[Ca^{2+}]_i$ (Chisaki et al., 2001), which may be involved in ursodeoxycholic acid-induced nitric oxide increase. But the inhibitory effects of ursodeoxycholic acid on endothelin-1 were observed at the lower concentration, suggesting that the basic mechanism underlying these effects is different. In addition, taurourodeoxycholic and glycoursodeoxycholic acids as well as ursodeoxycholic acid increased nitric oxide production in a similar manner, while the effects of these conjugates on endothelin-1 were less potent than those of ursodeoxycholic acid. Furthermore, to investigate the role of nitric oxide on ursodeoxycholic acid actions, the effects of L-NAME were investigated. L-NAME significantly inhibited the production of nitric oxide but failed to suppress the inhibitory effects of ursodeoxycholic acid on endothelin-1 production. These findings suggest that ursodeoxycholic acid inhibits endothelin-1 production in HUVECs via nitric oxide-independent mechanism.

In endothelial cells and aortic smooth muscle cells, the half-life of endothelin-1 mRNA is very short (approximately 20 min; Horie et al., 1991; Marsden and Brenner, 1992; Hu et al., 1992). This short lifespan of mRNA has been attributed to the presence of three AUUA sequence in the 3′ -untransated region that is thought to mediate selective mRNA (Inoue et al., 1989). Endothelin-1 mRNA is short lived and endothelin-1 is not stored in secretary granule, suggesting that endothelin-1 biosynthesis is mainly regulated at the levels of transcription (Rubanyi and Polokoff, 1994). Therefore, it is likely that the inhibitory effects of ursodeoxycholic acid on endothelin-1 production are due to the inhibition of the transcriptional level. In fact, the present study clearly showed that ursodeoxycholic acid significantly inhibited endothelin-1 mRNA expression.

The inhibitory effects of ursodeoxycholic acid on endothelin-1 production were observed at concentrations above 30 μM in the present study, and the significant inhibition was evident at 100 μM. The serum ursodeoxycholic acid concentration in patients with primary biliary cirrhosis receiving ursodeoxycholic acid therapy has been reported to vary between 10 and 100 μM (Stiehl et al., 1990; Kita et al., 1999), and it reached to approximately 50–100 μM in patients with liver cirrhosis, receiving ursodeoxycholic acid therapy (Chisaki et al., 2001), suggesting that physiological concentrations of ursodeoxycholic acid inhibit endothelin-1 production in human endothelial cells.

Endothelin-1 has been proposed to be involved in augmenting intrahepatic vascular resistance and subsequently contributing to portal hypertension in patients with hepatobiliary diseases (Rockey and Weisiger, 1996; Sogni et al., 1998; Reichen et al., 1998; Shah, 2001). Taken into account that hepatic endothelin-1 is derived from sinusoidal endothelial and stellate cells and modulates an intrahepatic vascular resistance in a paracrine or autocrine manner, endothelin-1 overproduction in the injured liver may increase portal pressure. Actually, endogenous endothelin-1 increases portal pressure in vivo as well as isolated perfused liver (Isales et al., 1993). A number of studies showed that plasma and hepatic endothelin-1 concentration were elevated in human and experimental liver cirrhosis and obstructive jaundice (Moller et al., 1995; Bernardi et al., 1996; Bruno et al., 2000). Thus, it is very likely that endothelin-1 may play a key role in various pathophysiological conditions such as cirrhotic portal hypertension. It has been reported that endothelin-1 receptor antagonists decrease portal pressure in the experimental cirrhotic rat (Rockey and Weisiger, 1996; Sogni et al., 1998; Reichen et al., 1998). Our findings showing that ursodeoxycholic acid inhibits production of endothelin-1 in human endothelial cells suggest that ursodeoxycholic acid therapy may prevent the development of several pathogenesis such as portal hypertension observed in patients with cirrhosis. In fact, ursodeoxycholic acid has

been reported to limit liver histological and portal hypertension induced by bile duct ligation in the rat (Poo et al., 1992). In addition, the increased circulating plasma endothelin-1 also contributes in part to renal dysfunction in patients with cirrhosis (Tsai et al., 1995), suggesting that ursodeoxycholic acid may provide the additional protective effects by inhibiting endothelin-1 production.

In conclusion, the present study provides novel evidence that ursodeoxycholic acid inhibits endothelin-1 production in human endothelial cells, but nitric oxide is not responsible for ursodeoxycholic acid effects on endothelin-1. Ursodeoxycholic acid therapy may prevent the development of several pathogenesis such as portal hypertension observed in patients with cirrhosis due to the improvement of endothelial function.

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RNAi induction and activation in mammalian muscle cells where *Dicer* and *eIF2C* translation initiation factors are barely expressed

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Abstract

Dicer plays an important role in the course of RNA interference (RNAi), i.e., it digests long double-stranded RNAs into 21–25 nucleotide small-interfering RNA (siRNA) duplexes functioning as sequence-specific RNAi mediators. In this study, we investigated the expression levels of *Dicer* and *eIF2C1~4*, which, like *Dicer*, appear to participate in mammalian RNAi, in various mouse tissues. Results indicate that the levels of *eIF2C1~4* as well as *Dicer* are lower in skeletal muscle and heart than in other tissues. To see if RNAi could occur under such a condition with low levels of expression of *Dicer* and *eIF2C1~4*, we examined RNAi activity in mouse skeletal muscle fibers. The results indicate that RNAi can be induced by synthetic siRNA duplexes in muscle fibers. We further examined RNAi activity during myogenic differentiation of mouse C2C12 cells. The data indicate that although the expression levels of *Dicer* and *eIF2C1~4* decrease during the differentiation, RNAi can be induced in the cells. Altogether, the data presented here suggest that muscle cells retain the ability to induce RNAi, although *Dicer* and *eIF2C1~4* appear to be barely expressed in them.

Keywords: RNA interference; Dicer; eIF2C translation initiation factors; Muscle; C2C12 cell

RNA interference (RNAi) is the process of a sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes. This intriguing gene silencing has been found in various species including flies, worms, protozoa, vertebrates, and higher plants (reviewed in [1–4]). DsRNAs introduced or generated in cells are digested by an RNase III enzyme, Dicer, into 21–25 nucleotide (nt) RNA duplexes [5–8] and the resultant duplexes, referred to as small-interfering RNA (siRNA) duplexes, function as essential sequence-specific RNAi mediators in the RNA-induced silencing complexes (RISCs) [5,7]. Thus, Dicer appears to play an important role in the process of RNAi induction.

In mammalian cells except for a part of undifferentiated cells [9–12], long dsRNAs (>30 bp) can trigger a rapid and non-specific RNA degradation involving the sequence-non-specific RNase, RNase L [13], and a rapid translation inhibition involving the interferon-inducible, dsRNA-activated protein kinase, PKR, instead of induction of RNAi [14]. In contrast, chemically synthesised siRNA duplexes can induce the sequence-specific RNAi activity in mammalian cells without triggering the rapid and non-specific RNA degradation and translation inhibition [15]. Together, it is likely that RNAi activity induced by the long dsRNAs could be masked by those rapid responses to the long dsRNAs in most of mammalian cells.

It may be of interest to examine the role of Dicer in differentiated mammalian cells possessing the rapid responses to long dsRNAs. Mammalian dicer has been identified and found to be a large multi-domain

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polypeptide (~215 kDa) characterised by containing a putative DExH/DEAH RNA helicase/ATPase domain, a PAZ domain, two RNase domains, and a dsRNA-binding domain [16–20]. The expression of *Dicer* appears to be ubiquitous, but the level of its expression varies among tissues. Of the tissues examined previously, skeletal muscle appeared to express *Dicer* at a low level, i.e., the *Dicer* transcript appeared to be barely detectable at least using RT-PCR [16,17].

In this study, we investigated not only RNAi activity but also the expression levels of *Dicer* and *eIF2C1~4*, which, like *Dicer*, appear to participate in mammalian RNAi [21,22], in mouse skeletal muscle fibers, and muscle cells that differentiated from mouse C2C12 cells. The results indicate that RNAi can be induced by synthetic siRNA duplexes in those cells although the expression levels of *Dicer* and *eIF2C1~4* are lower than those in other tissues and undifferentiated C2C12 cells.

Materials and methods

Preparation and culture of muscle fibers isolated from extensor digitorum longus in mice. Isolation of muscle fibers from mice was carried out as described previously [23]. Briefly, extensor digitorum longus (EDL) was isolated from mice (ICR mouse strain), treated with 0.5% type 1 collagenase (Washington biochemical) in Dulbecco's modified Eagle's medium (DMEM) (Sigma), and incubated at 37 °C for 90 min. After incubation, the EDL was dissociated into single muscle fibers by gently pipetting, and dissociated single fibers were plated on matrigel-coated 24-well culture plates (approximately 100 fibers/well). The muscle fibers were cultured at 37 °C in DMEM supplemented with 10% horse serum (Invitrogen) in a 5% CO_2-humidified chamber. Two–three hours after starting culture, transfection was carried out.

Cell culture. C2C12 cells were grown at 37 °C in DMEM supplemented with 15% fetal calf serum (Sigma), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) in a 5% CO_2-humidified chamber. For induction of myogenic differentiation, cells were cultured at 37 °C in DMEM supplemented with 5% horse serum (Invitrogen) in a 5% CO_2-humidified chamber [24]. The medium was changed everyday.

Synthetic oligonucleotides. RNA and DNA synthetic oligonucleotides were obtained from PROLIGO and SIGMAGENOSIS, respectively. The La2 siRNA duplex described previously was used in this study, and preparation of RNA duplexes was performed as described previously [25].

Transfection and luciferase assay. Reporter plasmids and siRNA duplexes were cotransfected into isolated single muscle fibers and undifferentiated and differentiated C2C12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. When undifferentiated C2C12 cells were used, the day before transfection, the cells were trypsinised, diluted with the fresh medium without

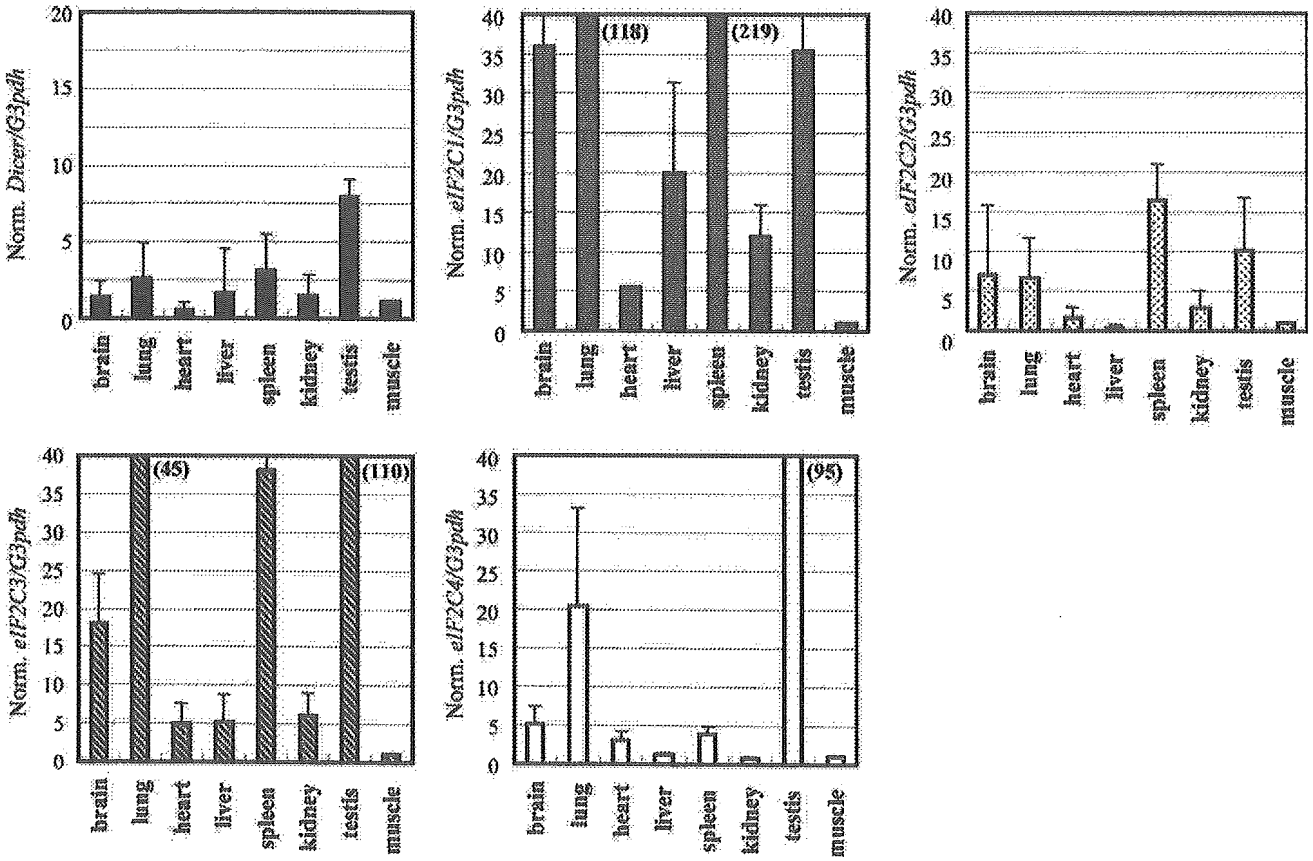

Fig. 1. Expression profiles of *Dicer* and *eIF2C1~4* in various mouse tissues. Total RNA was extracted from indicated tissues and subjected to cDNA synthesis with oligo(dT) primer and a reverse transcriptase. The expression levels of *Dicer* and *eIF2C1~4* were examined by means of a real-time PCR using the synthesised cDNAs as templates. The expression levels of the genes are normalised to that of the *G3pdh* gene examined as a control, and plotted when the expression level of either *Dicer* or *eIF2C1~4* in skeletal muscle is given as 1. Figures in parentheses indicate the averaged expression levels which are over the plotted areas. Data are averages of three independent experiments. Error bars represent standard deviations.

antibiotics, and seeded into 24-well culture plates (approximately 5×10^4 cells/well). Before the transfection, the culture medium was replaced with 0.5 ml OPTI-MEM I (Invitrogen), and to each well, 0.25 μg pGL3-control plasmid (Promega), 0.05 μg pRL-SV40 plasmid (Promega), and 0.2 μg siRNAs were applied. After 4-h incubation, 0.5 ml of the fresh culture medium without antibiotics was added, and further incubation at 37 °C was carried out. In the case of transfection into the isolated muscle fibers, the transfection mixture was directly applied into wells, and further incubation at 37 °C was carried out. When a short-hairpin expression plasmid, pRNA-U6.1/Neo/siRNA (GenScript), was used instead of synthetic siRNAs, 0.1 μg pGL3-control and 0.05 μg phRL-TK (Promega) together with various amounts of pRNA-U6.1/Neo/siRNA were introduced into C2C12 cells. The expression of luciferase was examined using a Dual-Luciferase reporter assay system (Promega) according to the directions provided by the manufacturer.

RT-PCR. Total RNA was extracted from the cultured cells and various mouse tissues using Trizol reagent (Invitrogen). Reverse-transcription (RT) for synthesizing the first-strand cDNAs was carried out using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and the resultant cDNAs were examined by real-time PCR using the ABI PRISM 7000 sequence detection system (Applied Biosystems) with a SYBER Green PCR Master Mix or a TaqMan Universal PCR Master Mix together with Assays-on-Demand Gene Expression products (Applied Biosystems) according to the manufacturer's instructions. For plotting a standard curve, the 1, 5, 25, 125, and 625-fold diluted brain cDNA samples, which were prepared from a brain tissue (total RNA) and designated as standards, were used in every real-time PCR. Expression levels of the genes examined were normalised to that of the control *G3pdh* gene. The PCR primers used in the real-time PCR were as follows:

G3pdh-F; 5′-TCTTCACCACCATGGAGAAG-3′
G3pdh-R; 5′-TCATGGATGACCTTGGCCAG-3′
Dicer-F; 5′-GCAGGCTTTTACACACGCCT-3′
Dicer-R; 5′-GGGTCTTCATAAAGGTGCTT-3′
eIF2C2-F; 5′-AGATGAAGAGGAAGTACCGT-3′
eIF2C2-R; 5′-CAGAACCAGCTTGTGCCTGT-3′

The Assays-on-Demand Gene Expression products used (the Assay ID numbers) were as follows:

eIF2C1; Mm00462977m1, *eIF2C3*; Mm00462959m1, *eIF2C4*; Mm00462659m1.

5-Bromodeoxyuridine incorporation assay. Cells were metabolically labeled in the culture medium containing 10 μM of 5-bromodeoxyuridine (BrdU) (Sigma) for 20 h, and rinsed with phosphate-buffered saline solution (PBS) followed by fixation with 70% ethanol containing 0.5 M HCl at −20 °C for 1 h. The resultant cells were incubated with anti-BrdU antibody (Oxford biotechnology) at 4 °C overnight. The BrdU-antibody complexes were visualised with Alexa488 conjugated secondary antibody (Invitrogen) and examined using a ZEISS (Axiovert) microscope.

Results and discussion

Expression profiles of Dicer and eIF2C1~4 in various mouse tissues

Previous studies suggested that *Dicer* and *eIF2C* translation initiation factors (*eIF2C1~4*) homologous to the *Ago* genes in *Drosophila* [26,27] contributed to mammalian RNAi [21,22]. *Dicer* appears to be expressed ubiquitously, but its expression level varies among tissues [16,17]. Since little is known about the expression levels of *eIF2C1~4* among tissues, we first examined the levels of expression of *eIF2C1~4* and *Dicer* in various tissues. Total RNA was extracted from mouse tissues and subjected to cDNA synthesis with oligo(dT) primer and reverse transcriptase. The resultant cDNAs were examined by a real-time PCR. The results are shown in Fig. 1. The expression level of *Dicer* in either skeletal muscle or heart appears to be lower than those in other tissues, which agrees with the previous observations [16,17]. It should be noted that the expression levels of *eIF2C1~4* in either skeletal muscle or heart, like the expression profile of *Dicer*, are also significantly lower than those in the other tissues examined. Consequently, the observations suggest that

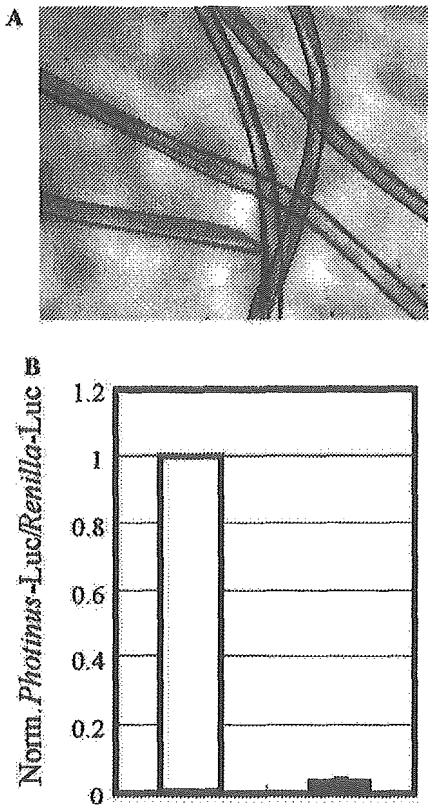

Fig. 2. RNAi induction by synthetic siRNA duplexes in muscle fibers prepared from mouse extensor digitorum longus. (A) Photograph of isolated muscle fibers. Isolation of muscle fibers from mouse extensor digitorum longus was carried out as described in Materials and methods. (B) RNAi activity in isolated muscle fibers. The La2 siRNA duplex against the *Photinus* luciferase gene [25] or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and pRL-SV40 plasmids carrying *Photinus* and *Renilla* luciferase reporter genes, respectively, were cotransfected into the isolated muscle fibers. Twenty-four hours after transfection, cell lysate was prepared and dual luciferase assay was carried out. Ratios of normalised target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the La2 siRNA duplex are normalised to the ratios obtained in the presence of the non-silencing siRNA duplex. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

skeletal and cardiac muscle cells express either *Dicer* or *eIF2C1~4* at a low level.

RNAi activity in muscle fibers isolated from mouse extensor digitorum longus

The observations described above raised the question whether RNAi could occur in muscle, i.e., whether RNAi could be induced under a condition with a low level of expression of either *Dicer* or *eIF2C1~4*. In order to address the question, we isolated mouse muscle fibers from extensor digitorum longus of ICR mice (Fig. 2A), and introduced synthetic 21-nt siRNA duplex targeting the exogenous reporter gene, *Photinus* luciferase, together with a pGL3-control plasmid carrying the *Photinus luciferase* gene and a pRL-SV40 plasmid carrying the *Renilla luciferase* gene as a control into the isolated muscle fibers. For realizing an efficient RNAi induction, we used the La2 siRNA duplex having the potential for inducing a strong RNAi activity in cultured mammalian cells [25]. As shown in Fig. 2B, the results indicate that the La2 siRNA duplex can induce a strong gene silencing of the *Photinus luciferase* gene in the muscle fibers. This result suggests that RNAi can be induced by synthetic siRNA duplexes in skeletal muscle which barely expresses either *Dicer* or *eIF2C1~4*.

RNAi activity during myogenic differentiation of mouse C2C12 cells

To further examine the properties of RNAi in muscle cells and during myogenic differentiation, we investigated RNAi activity in C2C12 cells, a mouse myoblast cell line, which can be induced by changing culture conditions (detailed in Materials and methods) to differentiate into contractile myotubes [24]. First, we

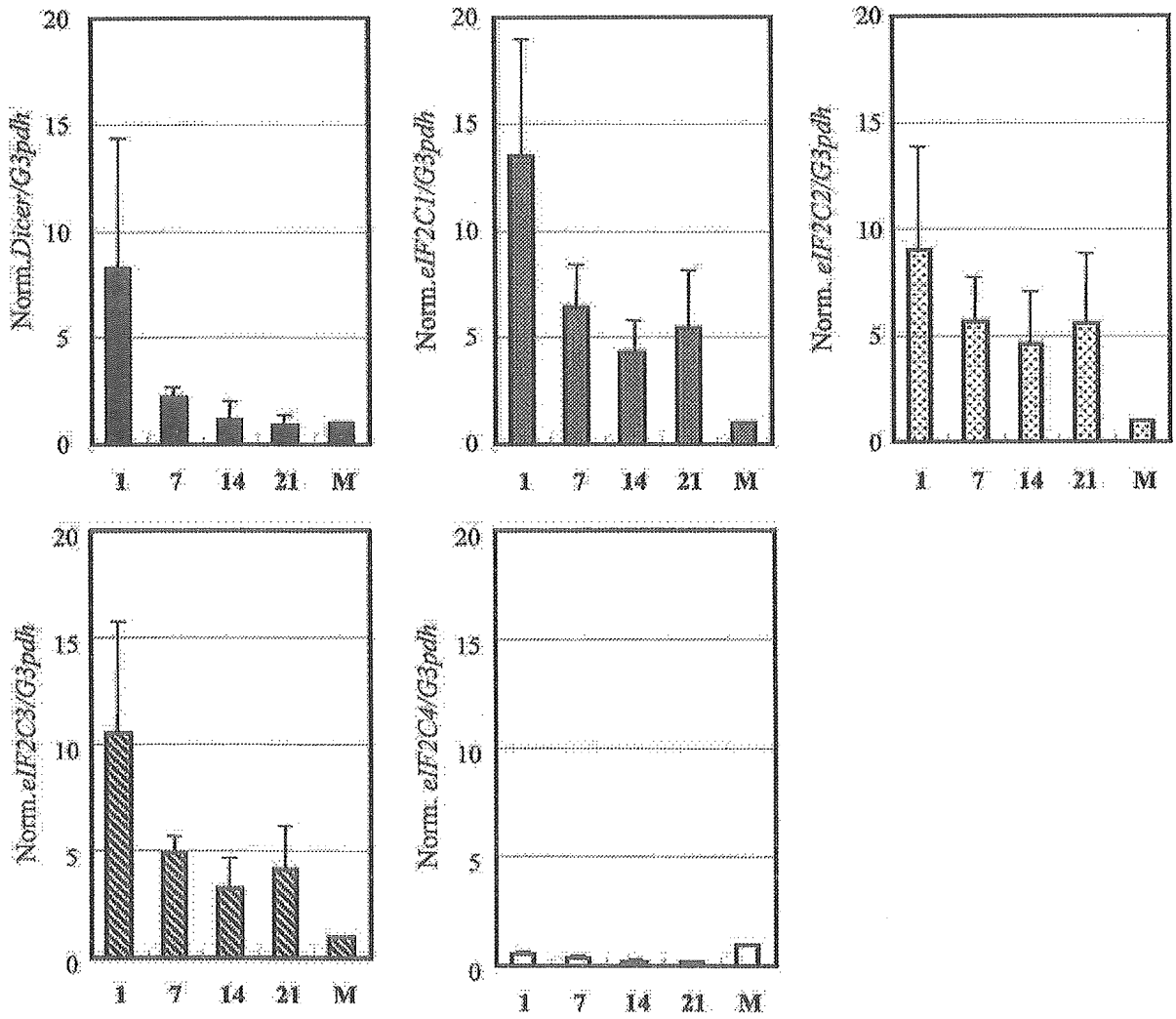

Fig. 3. Expression profiles of *Dicer* and *eIF2C1~4* during myogenic differentiation of mouse C2C12 cells. Total RNA was extracted from C2C12 cells at various days (indicated) after induction of myogenic differentiation of the cells (day 1 indicates undifferentiated C2C12 cells), and subjected to RT-PCR to examine the expression levels of *Dicer* and *eIF2C1~4* as in Fig. 1. The expression levels of the genes are normalised and plotted as in Fig. 1. M indicates skeletal muscle. Data are averages of three independent experiments. Error bars represent standard deviations.